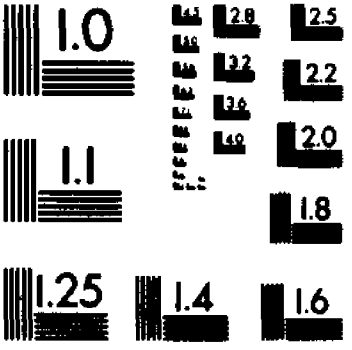

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SYNTHESIS, METABOLISM AND SECRETION OF ESTROGENS IN THE HUMAN TERM PLACENTA

City University of New York PH.D. 1986

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SYNTHESIS, METABOLISM AND SECRETION OF ESTROGENS IN THE HUMAN TERM PLACENTA

by

Seth Guller

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the City University of New York.

1986

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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The City University of New York

Abstract

SYNTHESIS, METABOLISM, AND SECRETION OF
ESTROGENS IN THE HUMAN TERM PLACENTA

by

Seth Guller

Adviser: Dr. Erlio Gurpide

This dissertation describes studies of metabolism and distribution of estrogens in the human placenta, conducted both at the subcellular and the whole organ levels.

Initially, preparations of microvillar membranes (i.e., microvillar vesicles from which cytoplasmic contamination has been removed) were employed to successfully search for plasma membrane associated enzymes involved in steroid metabolism (17β dehydrogenases and sulfatases). Differences in kinetic characteristics for the dehydrogenation at C-17 of testosterone (T) and estradiol (E_2) were found in microvillar, microsomal, cytosolic and mitochondrial fractions. Comparison of pH optima for activity for the cytosolic and microvillar enzymes also revealed significant differences. The molecular weight of microvillar E_2 17β dehydrogenase (E_2DH), estimated by gel filtration on Sephadex G-100 was found to be 137,000, approximately twice that observed for the cytosolic enzyme. However, similar isoelectric points were found for the cytosolic and the solubilized microvillar enzyme. Studies with trypsin and with antibody to purified cytosolic E_2DH revealed that both microvillar sulfatase and E_2DH

enzymes are protected in the microvillar membrane environment. Although placental microvilli were a rich source of steroid metabolizing enzymes, specific binders for E_2 were not found at this site.

In experiments in which labeled estrone (E_1), E_2, and estriol (E_3) were generated within the syncytium during simultaneous maternal and fetal perfusion of isolated term placentas with unconjugated precursors, two major observations were made: (1) the distribution of ^3H-E_1, ^3H-E_2, and ^3H-E_3 between "fetal" and "maternal" perfusates was unexpectedly different for each compound and (2) ethynyl estradiol (EE), a competitor of estrogen binding, provoked a large and selective release of ^3H-E_2 to the "fetal" perfusate in placentas labeled with E_1 and E_2. This phenomenon was repeated using a variety of estrogen precursors and binding competitors as well as different perfusion conditions. These data are consistent with a model which depicts both the binding and metabolism of E_2 at sites within the capillary endothelium.

It was suggested that steroid binding and/or metabolizing activities at the level of the microvillar membrane or capillary endothelium can influence the amount and potency of estrogen delivered to mother and fetus.

Acknowledgements

I wish to express my sincerest gratitude to my thesis advisor, Dr. Gurpide, for his thoughtful guidance during the three and a half years I spent in his laboratory. Under his direction I was able to develop my skills as a scientist in a congenial atmosphere that prompts independent thinking.

To the members of my graduate committee, Drs. Cederbaum and Li: I want to thank them for their constructive criticism as well as for always leaving the office door open.

Leaving this laboratory is not an easy task for me because of the wonderful people I have been exposed to. To the "over 30" crowd, Fred, Chris, Honorée, Beth, Patricia, Leszek, and Achille, I wish only good things and appreciate the input they gave to me both on scientific and personal levels. To the "under 30" crowd of graduate students and ex-graduate student, Mindy, Sharon, Dan, and Roz, I thank you for sharing the trials and tribulations of graduate school. To Mindy, this time I'm going first.

For the technical help I've received during my tenure as a graduate student I want to thank my laboratory roommates, Alex, Carlo, and Grazyna.

Most of all, I would like to thank my wife, Graciela Krikun, whose constant support has made me believe in myself. You have made the successes more wonderful and the failures a little easier to bear.

To my son Benjamin, who didn't help me at all during my graduate student training, "Don't touch the stereo."

Table of Contents

Page

List of Tables

List of Figures

Abbreviations Used in This Text

E_1	estrone
E_2	estradiol
E_3	estriol
EE	ethynyl estradiol
E_1S	estrone sulfate
DHEAS	dehydroepiandrosterone sulfate
A	androstenedione
16αOHA	16α-hydroxyandrostenedione
T	testosterone
P	progesterone
E_2DH	estradiol dehydrogenase
LDH	lactic dehydrogenase
AIKP	alkaline phosphatase
PAGE	polyacrylamide gel electrophoresis
NBT	nitroblue tetrazolium
Membranes	microvillar membranes formed from osmotically disrupted microvillar vesicles
TLC	thin layer chromatography
PBS-G-C	phosphate buffered saline containing 25% glycerol and 0.3% sodium cholate
KRB	Krebs-Ringer bicarbonate

I. Introduction to Thesis Research

In humans, as well as in other species with hemochorionic placentation, maternal blood is in direct contact with the fetal syncytiotroblast and exchange of oxygen and nutrients between mother and fetus occurs by direct transfer through the syncytial plasma membrane (1). The maternal side of the syncytium, i.e., the "placental brush border", is rich in microvillar projections which markedly augment the surface available for feto-maternal exchange. Syncytial microvilli are easily "pinched off" from suspended fragments of term placentas by mild mechanical forces during stirring and can be collected as microvillar vesicles by differential centrifugation (2). The potential utility of these preparations in the study of feto-maternal exchange processes is suggested by the microvillar location.

Initially, this dissertation describes evidence indicating for the first time that estradiol 17 hydrogenase (E_2DH) and steroid sulfatases are associated to microvillar membranes obtained from microvillar vesicles by hypotonic treatment. Analysis of activities of marker enzymes was used to assess the level of contamination of these preparations by other placental subfractions. Special emphasis was placed on the comparison of E_2DH activity in microvillar membranes and the much studied cytosolic enzyme (3-5). Accessibility to inactivation by trypsin and anti-E_2DH antibodies was used to gain information concerning the topography of E_2DH and sulfatase in the microvillar membrane. Partial purification and isoelectric focusing of E_2DH activities from microvillar membranes and cytosol were carried out to further characterize and compare the enzymes isolated from these two sites. A comparison of relative activities towards

testerone (T) and E_2 as substrates was conducted in microvillar membrane, cytosolic, microsomal, and mitochondrial fractions obtained from human term placenta to determine if unique ratios of activities existed in these various placental subfractions.

The presence of steroid metabolizing activities at the syncytial plasma membrane could have a special significance concerning the rate of entry and subsequent transfer of steroids from mother to fetus and the utilization of circulating sulfates that serve as estrogen precursors.

The studies on placental microvillar membranes were prompted by results obtained during placental perfusions, also carried out as part of this thesis research project, aimed to evaluate the intraplacental metabolic fate of estrone (E_1) and E_2 formed in the syncytium from C_{19} steroid precursors.

In vivo isotopic studies in the pregnant Rhesus monkey had revealed an unequal distribution of placental E_1 and E_2 between fetal and maternal circulations (6). Similar results were obtained during extracorporeal perfusion of human placentas with isotopically labeled estrogen precursors (7). In experiments performed as part of this thesis research, the fate of E_1 and E_2 formed from aromatization of labeled androgens was compared with the feto-maternal distribution of estriol (E_3), formed simultaneously from ^{3}H-16α-hydroxyandrostenedione (16αOHA), in the presence or absence of competitors for estrogen binding (ethynyl estradiol, diethylstilbestrol). Findings from these experiments contradict the concept of a simple diffusional release of estrogens from the placenta and called for an examination of extrasyncytial placental metabolism of E_1 and E_2 or

the presence of carriers facilitating the movement of estrogens across syncytial or endothelial membranes, as described in a model that incorporates the results of the research work reported in this Dissertation.

II. General Background

A. Placental morphology and circulation

The placenta develops from the ectodermal layer of the developing blastocyst during pregnancy (8). The human placenta at term (greater or equal to 37 weeks of gestation) is disc-like in appearance (Fig. 1) and is coated by a thin grayish layer of decidua basalis, a maternal tissue of uterine origin (9). Outgrowths of this tissue known as septa divide the placenta into 15-30 cotyledons (8). Each cotyledon is an independent circulatory unit, and together they form the cobblestone-like appearance of the maternal surface. On the other hand, the fetal surface of the placenta is easily recognized by its highly vascular appearance (9).

A macroscopic view through a section of term placenta depicting the major features of maternal and fetal circulation is shown in Figure 2. The maternal and fetal blood systems are in close though not direct contact, and normally there is no gross intermingling of blood (1). The repeating structural unit of placenta is the villus, or finger-like projection, across which passage of maternal nutrients to the fetus takes place (10). The fetal circulation takes the form of two large umbilical arteries which bring deoxygenated blood from the fetus to the placenta. These arteries branch into a large number of small capillaries in the area of the villi (9). Oxygen

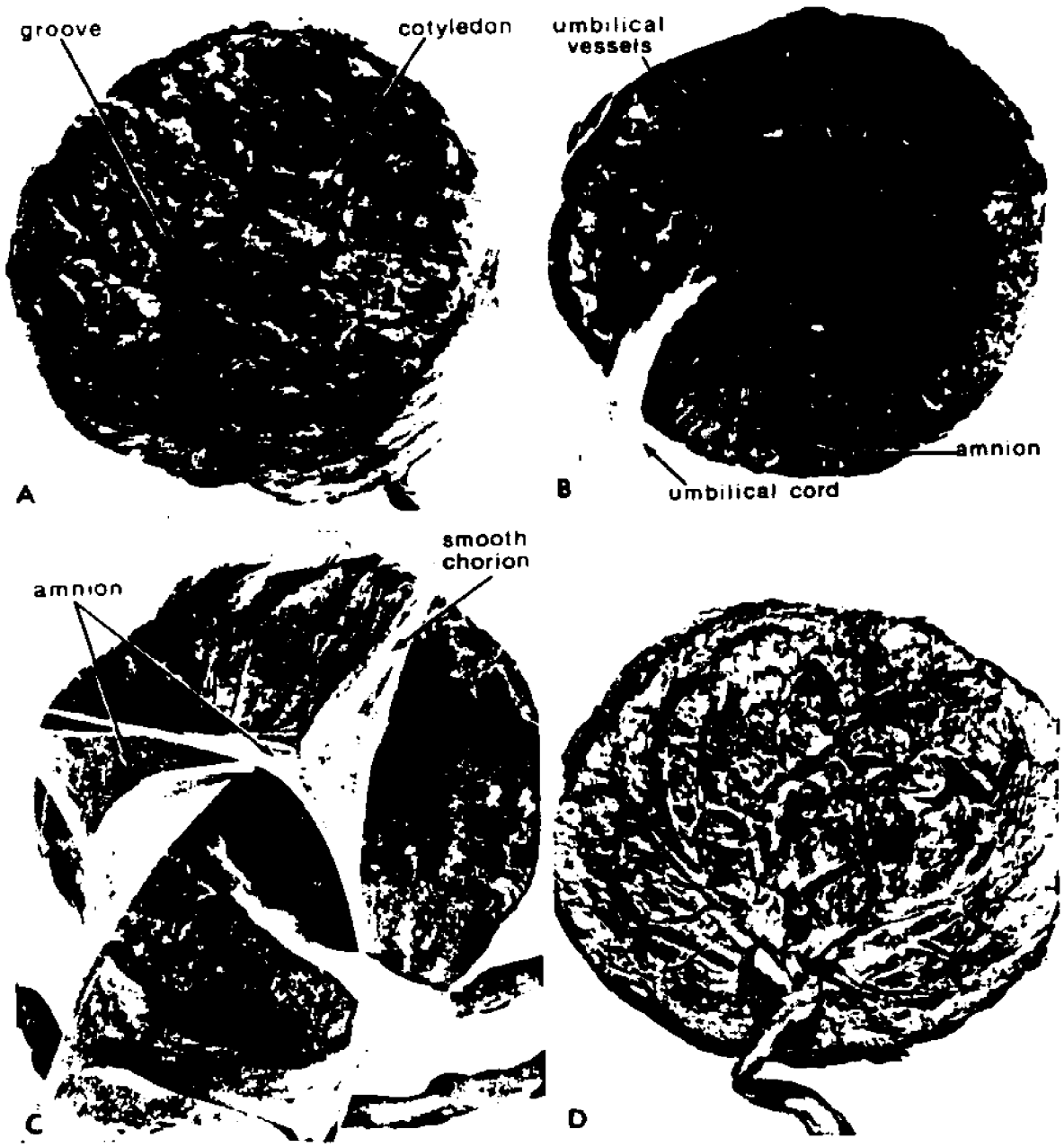

groove
cotyledon
A
umbilical vessels
amnion
umbilical cord
B
amnion
smooth chorion
C
D

Fig. 1 Placental morphology

Photographs of term placentas, about one-third actual size (ref. 9, p. 124), depicting: (A) Maternal surface showing cotyledons (B) Fetal surface showing blood vessels (C) The amnionic and chorionic membranes (D) Placenta with a marginal attachment of the umbilical cord.

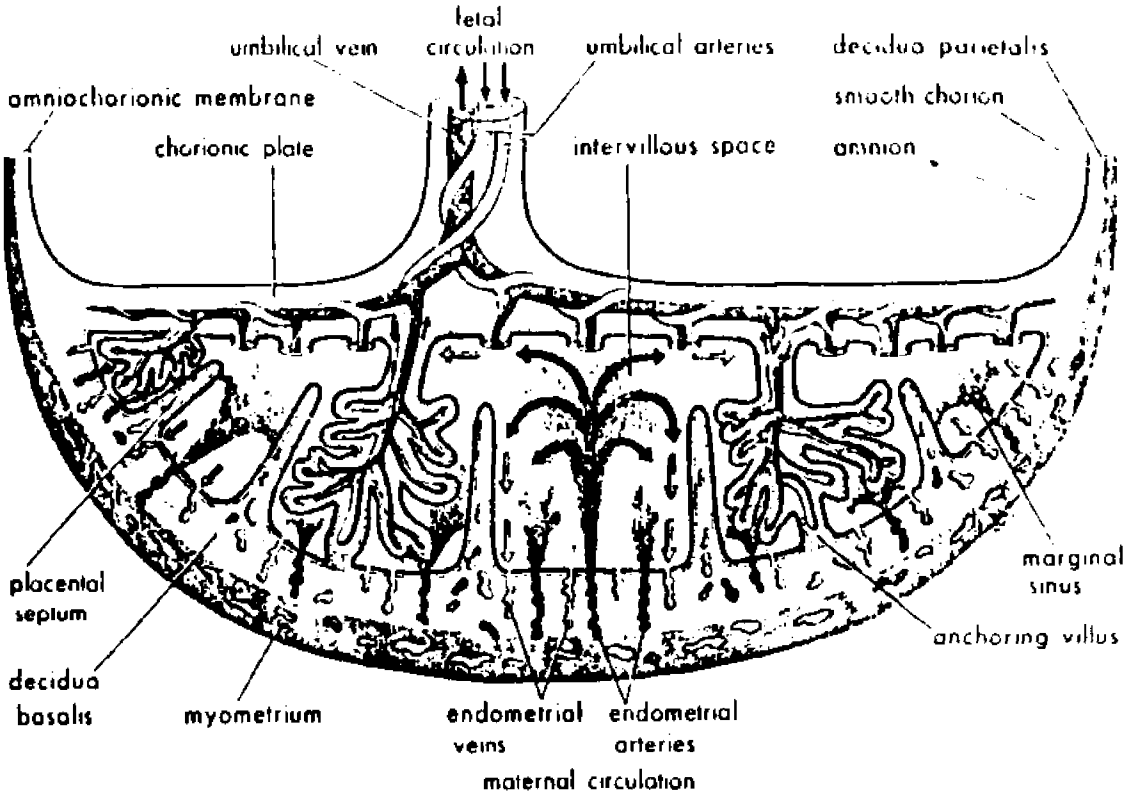

fetal
circulation
umbilical vein
umbilical arteries
decidua parietalis
smooth chorion
amnion
amniochorionic membrane
chorionic plate
intervillous space
placental
septum
decidua
basalis
myometrium
endometrial
veins
endometrial
arteries
maternal circulation
marginal
sinus
anchoring villus

Fig. 2 Placental circulation

Schematic drawing of a section through a term placenta (ref. 9, p. 116), depicting the major features of maternal and fetal circulations. Maternal blood is driven into the intervillous space in funnel-shaped spurts, and exchange occurs with fetal blood as the maternal blood flows around the villi.

and nutrients are transferred from the maternal blood across the placenta and enter thin-walled veins which follow the arteries to the point of attachment of the umbilical chord and merge to form the umbilical vein (9). Thus, oxygenated blood is carried from the umbilical vein to the fetus.

The maternal circulatory input to the placenta, unlike the macroscopically visible fetal vasculature, is composed of 80-100 small spiral endometrial arteries (10). The blood flow in these vessels is pulsatile in nature, and blood is spurted in streams that collect to form a marginal lake which bathes the surface of the villi. Maternal blood leaves the placenta through endometrial veins located in the floor of the intervillous space (10).

Human placentation is classified as villous hemomonochorial: i.e., the villus being the major repeating geometric form in the area of nutrient exchange, and hemomonochorial, in that there is one continuous layer of trophoblast (placental tissue) separating maternal and fetal blood systems (11). The predominant trophoblastic tissue at term is the syncytium (Fig. 3), that is, a multinucleate layer without cell divisions (12). The cytotrophoblast layer though much reduced at term is much more prominant early in pregnancy, and is thought to provide stem cells from which the syncytiotrophoblast develops (13). The syncytium, in addition to possessing a full complement of subcellular organelles, contains the enzymes for steroid and peptide hormone synthesis in placenta (12).

The membrane across which nutrient and gas exchange actually takes place is that of the microvillar membrane (12). These brush border-like membranes project from the surface of the villi (Fig. 3),

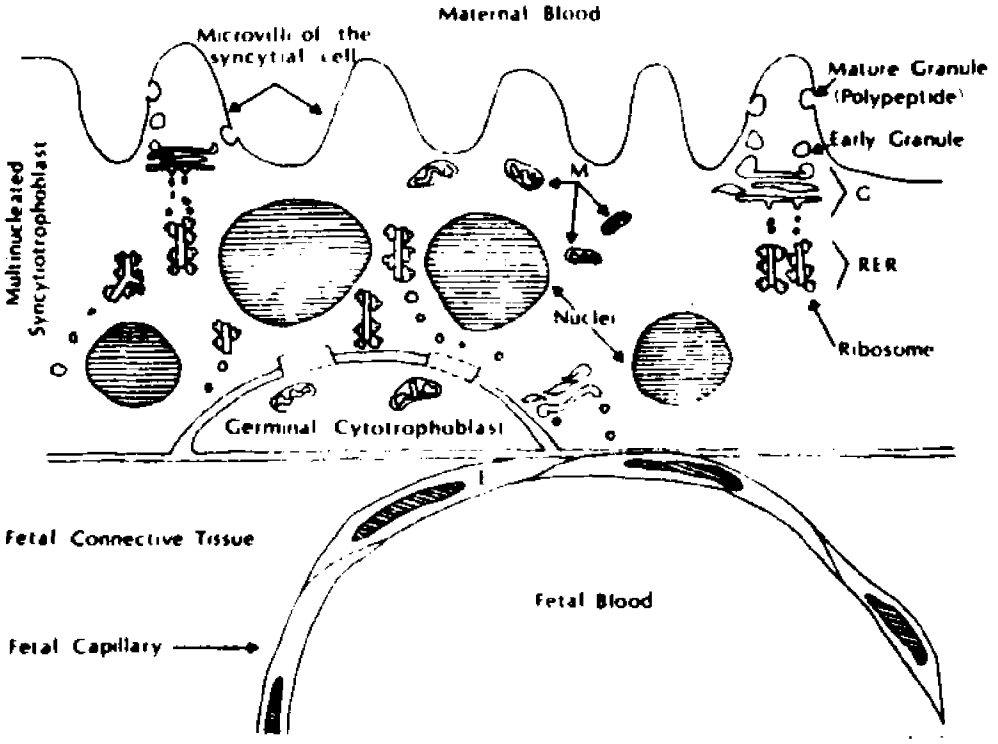

Maternal Blood
Microvilli of the syncytial cell
Mature Granule (Polypeptide)
Early Granule
Multinucleated Syncytiotrophoblast
M
C
RER
Nuclei
Ribosome
Germinal Cytotrophoblast
Fetal Connective Tissue
Fetal Blood
Fetal Capillary

Fig. 3 Microscopic view of placenta

Schematic drawing of the human placenta at term, adapted from a cross-sectional electron micrograph (ref. 12, p. 18). The abbreviations are: E = endothelial cell, G = Golgi complex, M = mitochondria, and RER = rough endoplasmic reticulum.

increasing the surface area available for the transfer of nutrients from maternal blood (11). Due to the unique location of syncytial microvilli during pregnancy, preparations of these microvilli have been extensively used in the biochemical characterization of maternal-fetal exchange processes (14-16).

B. Microvillar vesicles, characteristics and use

In 1974, Smith et al. (2) succeeded in obtaining relatively pure preparations of microvillar vesicles from slices of human term placenta by means of gentle agitation in cold saline followed by differential centrifugation. Since this original work, the procedure has been modified slightly by others (17,18). Microvillar vesicles as prepared by this procedure range in size from 0.1 to 0.3 μm (18). Based on the finding that neuraminadase removes almost all sialic acid residues from microvillous vesicle preparations (19), it has been concluded that they are oriented "right-side-out", that is, that they have the orientation present in the intact trophoblast. This technique takes advantage of the fact that there is no gross homogenization of tissue and as a result little particulate contamination exists in these preparations as judged by marker enzyme analysis (17, 18). Preparations of microvillar vesicles were found to be contaminated by endoplasmic reticulum (between 5 and 10%, ref. 18) whereas mitochondrial contamination was minimal (less than 2% ref. 18). Data on the level of soluble contamination of microvillar vesicle preparations were reported in one study (20), using the soluble marker choline acetyl transerfase in which the level of activity of this enzyme in microvillar vesicles was determined to be approximately 25% of that observed for placental homogenates. Carlson et al. (21)

have prepared microvillar vesicle from human term placentas by means of homogenization and differential centrifugation, but this procedure is more lengthy and is not as well suited for use in binding and transport studies.

Approximately 30 distinct microvillar proteins have been resolved by SDS gel electrophoresis (22), the major protein components being IgG, transferrin, serum albumin, actin, and alkaline phosphatase. The first three proteins mentioned most likely exist in microvillar vesicle preparations as a result of adsorption from maternal blood (23). The presence of high levels of alkaline phosphatase in preparations of microvillar vesicles is not surprising as this activity has been used for many years as a plasma membrane marker, with the highest concentrations of this enzyme having been recorded in brush border membrane preparations from kidney and intestine (24). The lipid component of microvillar vesicles is primarily phosphatidyl choline and phosphatidyl ethanolamine and is rich in cholesterol and sphingomyelin as anticipated for a plasma membrane (23).

Carbohydrate (15,25) and amino acid (16,18) transport in placenta have been extensively studied using microvillar vesicle preparations, with obvious physiological relevance to transfer of maternal nutrients to the fetus. Receptors for acetylcholine (20), insulin (26), and epidermal growth factor (EGF) (27) have been established in these preparations by showing a high degree of specific binding. Since both insulin and EGF receptors are implicated in protein kinase activity (28,29), it is not surprising that this activity has been found in material solubilized from microvillar vesicle preparations (30).

Fant et al. (31) have documented the process of glucocorticoid transport in preparations of placental microvillar vesicles. They

have concluded that transport of glucocorticoid occurred in addition to binding by demonstrating decreased ^{3}H-corticosterone transport in the presence of increased levels of sucrose, a non-permeant solute which at high concentrations reduces intravesicular volume without significantly affecting the accessibility to surface binding sites (19). The uptake of ^{3}H-corticosterone was found to be specific for glucocorticoids as transport was inhibited by corticosterone, cortisol, and dexamethasone, but not by testosterone, progesterone, or estradiol.

Concentrations of β adrenergic receptor (32) and levels of adenylate cyclase activity (33) were found to be higher in human placental plasma membrane preparations from which microvillar membranes had been removed (fetal or basal membranes) than in microvillar vesicle preparations, suggesting that separate transport systems exist at the maternal and fetal plasma membranes. However, due to the multicellular composition of placenta (12), in studies using fetal plasma membranes, the authors must be specific as to the cell origin of plasma membrane to be studied.

Recently, preparations of placental microvillar vesicles have been used as tools to study some very basic immunological concepts. It has been known for many years that some trophoblast can break away from the main body of the placenta during pregnancy and will eventually lodge in maternal lung and remain undisturbed in this site (34), suggesting an immunologic neutrality. Biochemical data have confirmed an apparent absence of human leukocyte type A (HL-A) antigen associated with microvillar vesicle preparations (35) which had been postulated based on immunohistochemical staining (36). A

lack of HL-A antigen on trophoblast has a clear significance regarding maternal acceptance of the fetoplacental unit, which is relevant to tumor immunology and clinical organ transplantation research, areas in which much thought is directed towards understanding possible mechanisms of evasion of immunologic recognition and attack in a foreign host (36).

C. Biosynthesis and conjugation of estrogens during pregnancy

During pregnancy the passage of steroids through the microvillar plasma membrane and fetal capillary membranes is of vital importance in that the placenta must rely upon C_{19} precursors present in both maternal and fetal blood for estrogen biosynthesis (37). The placenta does not possess C_{17-20} lyase activity and this cannot convert progesterone (P) to androstenedione (A) the C_{19} precursor of estrone (E_1) and estradiol (E_2) (38).

By approximately the seventh week of pregnancy the transition from luteal to placental production of estrogens occurs (38). Figure 4 outlines the pathways for the placental synthesis of E_1, E_2, and estriol (E_3), the three major estrogens formed by placenta during pregnancy.

Based on the work of many groups it is known that E_2 and E_1 are synthesized in the placenta from circulating dehydroepiandrosterone sulfate (DHEAS) (39-42). The DHEAS utilized for the conversion was found to be derived from both fetal and maternal blood streams, with each compartment contributing approximately 50% of the DHEAS utilized in the production of E_1 and E_2 (40). The sulfated precursor which was observed to be the major form of C_{19} steroid secreted by the maternal and fetal adrenals (41), is known to be hydrolyzed to

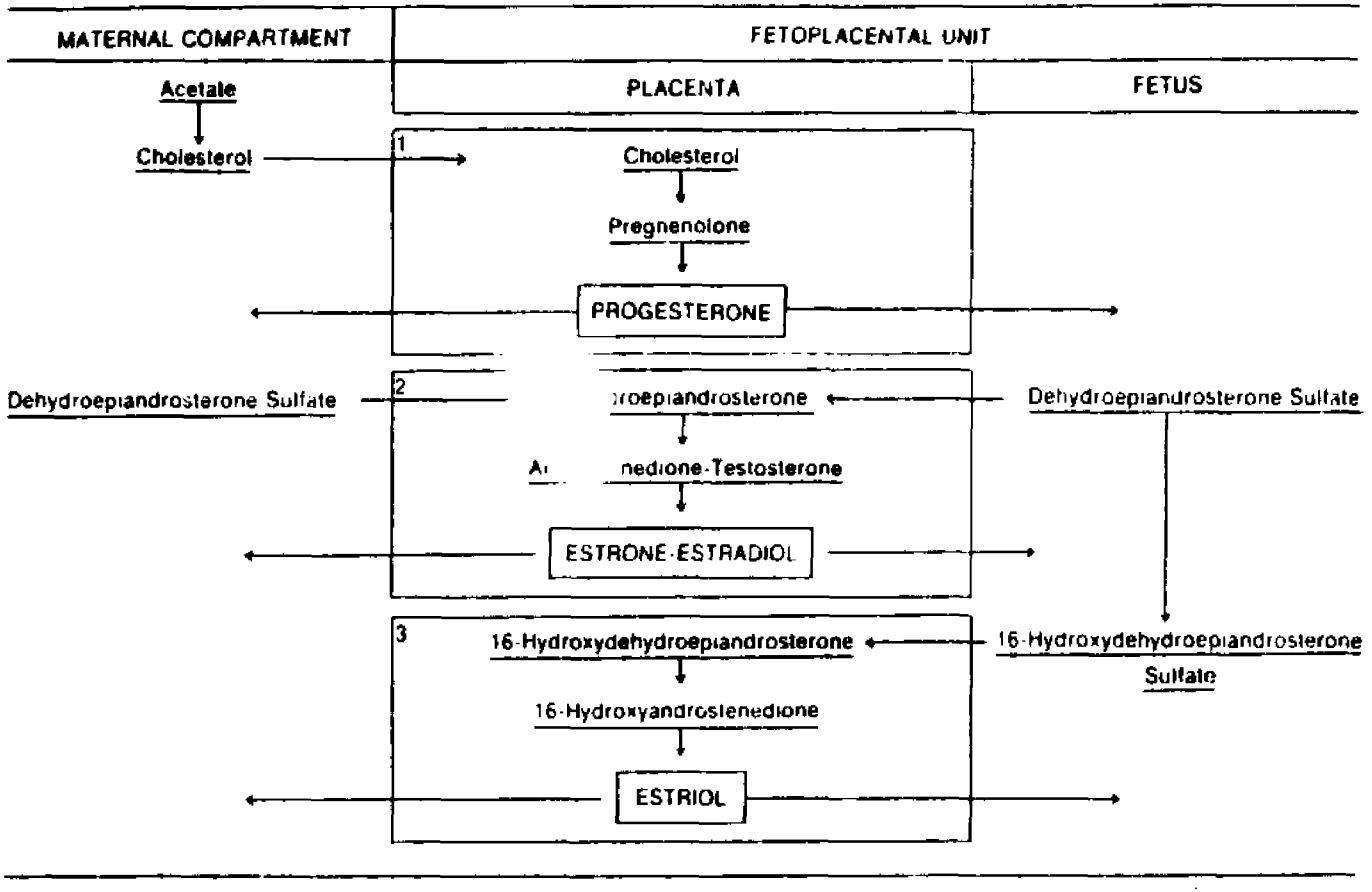

MATERNAL COMPARTMENT
FETOPLACENTAL UNIT
PLACENTA
FETUS
Acetate
Cholesterol
1
Cholesterol
Pregnenolone
PROGESTERONE
Dehydroepiandrosterone Sulfate
2
roepiandrosterone
Dehydroepiandrosterone Sulfate
A
nedione-Testosterone
ESTRONE-ESTRADIOL
3
16-Hydroxydehydroepiandrosterone
16-Hydroxydehydroepiandrosterone Sulfate
16-Hydroxyandrostenedione
ESTRIOL

Fig. 4 Pathways of estrogen synthesis during pregnancy

Outline of the pathways of estrogen synthesis during pregnancy. Note, that the C_{19} precursor for the placental synthesis of estrone and estradiol is supplied by both maternal and fetal circulations, whereas the precursor of estriol is principally derived from the fetal circulation. Taken from ref. 38, P. 4.

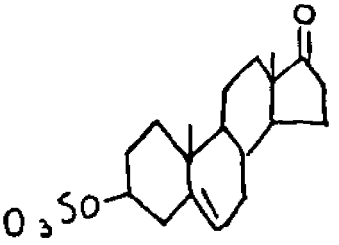
free DHEAS by a very active placental sulfatase (42), the characteristics of which will be discussed in a later section. The structures of the steroid intermediates involved in the formation of E_1 and E_2 during pregnancy are shown in Figure 5.

The conversion of DHEAS to androstenedione (A) was observed to be a function of a placental $^5\Delta/3\beta$ hydroxysteroid dehydrogenase system (43). Work by Meigs and Ryan (44) has documented the fact that aromatization of A to E_1 takes place in placental microsomes and to a lesser extent in mitochondria. Recent work has demonstrated that the process occurs as a concerted series of steps involving repeated hydroxylations of the angular methyl group of C_{19}, followed by the removal of the methyl group and introduction of another double bond into the A ring of the androgen (45). The enzyme(s) involved is of the mixed function oxidase variety requiring molecular oxygen, NADPH, and microsomal P_{450} (45). Thompson and Siiteri have succeeded in obtaining an active form of solubilized P_{450} aromatase (46) but as yet extensive purification has not been achieved. Great discrepancies exist in the values reported as estimates of the Km of aromatization, ranging from nM (47) to μM (48) concentrations.

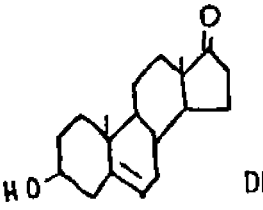
Placental E_1 produced via aromatization of A is reduced to E_2 by 17β hydroxysteroid dehydrogenase (17βOHSD) (3). This enzyme will be discussed in a later section.

Although placental E_1 and E_2 are synthesized from precursors present in both maternal and fetal circulations, E_3 has been determined to be synthesized almost exclusively from fetally derived 16α hydroxydehydroepiandrosterone sulfate (16αOH DHEAS) (49). Fetal liver function is responsible for hydroxylation at the 16α position of DHEAS produced in the fetal adrenal (50). Experiments have been conducted

(A)

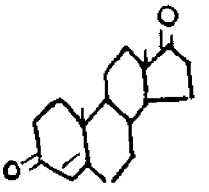


SULFATASE



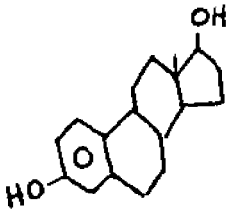
DHEA

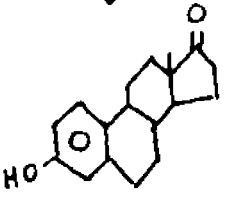
Dehydroepiandrosterone
Sulfate (DHEAS)



Androstenedione (A)

17β E_2DH

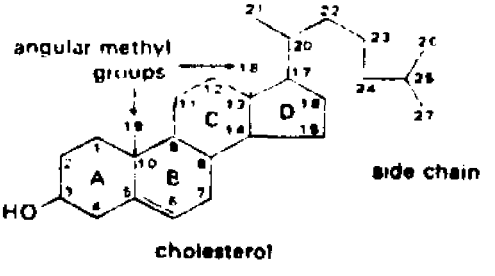




Estradiol (E_2)

Estrone (E_1)

(B)



cholesterol

Fig. 5 Structure of intermediates in estrogen synthesis

Structure of the intermediates in the synthesis of E_1 and E_2 as well as the sites of action of sulfatase and 17β E_2DH, enzymes of prime importance in this dissertation study, are depicted (A). Numbering of the carbon atoms of the cholesterol molecule is also shown (B).

which have shown that when 16αOH DHEAS is presented to the placenta, the sulfate is cleaved as for DHEAS, and free 16αOH DHEA is converted to 16αOH A (49,50). The aromatization of 16αOH A was found to be accomplished through a system similar to that which forms E_1 from A, but in this case the estrogen produced is 16αOH E_1 (48). Engel and Groman have observed that reduction of 16αOH E_1 to 16αOH E_2 (i.e., E_3) was carried out by the action of 17βE_2DH (3). The structure of intermediates in the synthesis of E_3 are identical to those displayed for the synthesis of E_1 and E_2 in Figure 5, with the exception that a 16αOH group is present on the steroid backbone throughout the entire pathway from 16αOH DHEAS to E_3. It should be noted that within placenta the pool of E_1 and E_2 is separate from that of E_3 as the placenta has been shown to lack the ability to hydroxylate steroids at the 16α position (51).

The ratio of E_3 to E_1 plus E_2 in the urine of nonpregnant women is approximately one, whereas in pregnant women this ratio approaches ten or more (52). Since a functioning fetal adrenal is responsible for the elevated levels of E_3 observed during pregnancy, the levels of E_3 in maternal plasma and urine have been used as an index for fetal well being (53). Indeed, extremely low levels of E_3 have been observed in human pregnancies involving anencephalic fetuses (54) and in cases of pregnancy induced toxemia (55).

Estetrol (E_4), the 15α-hydroxy derivative of E_3, is a unique compound in that it is synthesized in the fetal liver by successive hydroxylations at the 15α and 16α positions (56). Thus, more recently, E_4 has also been proposed as an index of fetal health (57) as its production is a direct function of fetal liver enzymatic activity.

The formation of unconjugated placental estrogens from C_{19} pre-

cursors represents the end point of estrogen biosynthesis during pregnancy. However, extensive conjugation of estrogens to soluble metabolites takes place in maternal and fetal compartments.

As estriol is the major estrogen formed by placenta during pregnancy, it is not surprising that approximately 95% of the total estrogen in human maternal urine at term is in the form of E_3 (58). The 16-glucuronide conjugate of E_3 was observed to be the estrogen present in highest concentration in maternal urine, although a significant level of E_3-3-glucuronide (E_3-3-G) was also reported in this study (59). In maternal blood a different pattern exists, as the major estrogen conjugates found to be present were E_1 sulfate (E_1S), E_3 sulfate (E_3S), and E_3G (60). During pregnancy E_1 is present in maternal blood primarily as the E_1-3-sulfate conjugate (61). The majority of E_2 in maternal serum is present in the free form (61). Conjugated estrogens present in maternal serum are thought to arise primarily in the maternal compartment at liver and adrenal sites as the placenta is not considered to be a major site of steroid conjugation (38).

Of the unconjugated estrogenic steroids in maternal serum at term E_2 was found to be present in highest concentration (62), and this compound is known to possess a relatively short half life in maternal blood (63). Unconjugated E_3 is rapidly cleared from blood (63), on the order of 15 min following injection. On the other hand, Ruder et al. (64) noted that E_1S was cleared slowly from maternal plasma, most likely due to its binding to albumin.

Arylsulfurylation is the dominant pathway of estrogen conjugation in virtually every fetal tissue (65). This conclusion was based on data obtained from a wide variety of <u>in vivo</u> experiments:

(1) injection to the amniotic fluid prior to interruption of pregnancy (66) (2) perfusion or injection into a previable fetus (67) (3) injection into the umbilical circulation of the intact fetal-placental unit (68). These studies have demonstrated that E_3-3-sulfate (E_3-3-S) is the principal product of fetal estrogen conjugation, and that the compound is not metabolized further to any extent. Results from measurement of the levels of estriol conjugates in cord blood (69) indicate that E_3-S was present in highest concentration, though significant quantities of glucuronide and sulfoglucuronide conjugates were also found. Thus, sulfoconjugation appears to be the primary pathway of estrogen conjugation in the fetus, while in the adult glucuronidation and sulfoconjugation activities are present at high levels.

D. Characteristics of sulfatase and estradiol 17β dehydrogenase

Steroid sulfatase and estradiol 17β dehydrogenase (E_2DH) are two enzymes which are intimately involved in the production of placental estrogen. By virtue of steroid sulfatase activity, unconjugated C_{19} steroids are made available to the placental aromatase system (s) which functions to convert precursor to estrogen. The type of estrogen that is present within and thus presumably released by placenta to fetal and maternal circulations is influenced by the action of E_2DH. Due to the critical importance of sulfatase and E_2DH activities in relation to placental estrogen synthesis and release, a description of each enzyme follows.

Steroid sulfatase catalyzes the cleavage of the sulfate moiety of 3β hydroxysteroid sulfates (70). During pregnancy placental sul-

fatase, a microsomal enzyme(s) is of particular importance in that the placenta is an "incomplete" endocrine organ in regard to estrogen biosynthesis and must rely upon sulfated C_{19} precursors present in both maternal and fetal blood systems (38). The importance of the hydrolysis within placenta of C_{19} sulfates prior to aromatization of these compounds to estrogens is evident from pregnancies complicated by placental sulfatase deficiency (71). Although not a life-threatening disorder, it results in markedly diminished levels of estrogens in maternal blood and urine, and is associated with occasional impairment of normal parturational mechanisms (72). Also, for a yet undetermined reason only male infants have been delivered from these abnormal pregnancies (73).

Estrone sulfate (E_1S) and dehydroepiandrosterone sulfate (DHEAS) constitute major forms of C_{18} and C_{19} steroids respectively that circulate in maternal and fetal blood during pregnancy (38). On the basis of data obtained by many investigators (74-77), it appears that these compounds are hydrolyzed by different sulfatases. French and Warren (74) have suggested that in microsomes obtained from human placenta p-nitrophenyl sulfate sulfatase, E_1S sulfatase, and DHEAS sulfatase are different enzymes. Dealing with a partially purified microsomal steroid sulfatase from human placenta, Gauthier et al. (75) concluded that DHEAS and cholesterol sulfate were hydrolyzed by two different steroid sulfatases. Partial purification of steroid sulfatase from rat liver and analysis of kinetic and physico-chemical parameters led Iwamori et al. (76) to conclude that E_1S sulfatase and p-nitrophenyl sulfate sulfatase are identical but are different from the other membrane-bound steroid sulfatases. More recently, Prost and

and Adessi (77) presented strong evidence for the existence of two enzymes in the hydrolysis of DHEAS and E_1S in human endometrium on the basis of: (1) noncompetitive inhibition of DHEAS sulfatase activity by E_1S and E_1S sulfatase activity by DHEAS (2) widely varying pH curves (3) different sensitivities to thermal inactivation (4) magnesium chloride inhibition of E_1S, but not DHEAS sulfatase activity. These authors also found that unconjugated steroids inhibited both sulfatase activities, a common feature of all steroid sulfatases studied to date (78).

Unequivocal proof of the existence of two distinct sulfatase enzymes responsible for the hydrolysis of E_1S and DHEAS would be ideally obtained by extensive purification and physical separation of the respective proteins. Generally, there has been a failure to effect extensive purification of membrane-bound sulfatases (74-76), a major problem being the maintenance of active solubilized forms of these enzymes. However, Noël et al. (79) were able to successfully solubilize cholesterol sulfate sulfatase activity from human placental microsomes by Triton X-100 detergent and effect an extensive purification, though no claim of homogeneity was made. A minimum molecular weight of 72,000 was reported in this study. Other values reported for the molecular weight of steroid sulfatase(s) are 300,000 (75) and 1,000,000 (80).

Based on data obtained from perfusions of human term placentas _in situ_ with sulfated and nonsulfated steroids it was suggested that the steroid sulfatase is the rate-limiting step in the conversion of sulfated steroids to estrogens (81). However, results from _in vitro_ studies have indicated that sulfatase activity is greater than that of

3β hydroxysteroid dehydrogenase and aromatase activities, the other enzymes involved in estrogen synthesis (82). Therefore, it has been postulated that inhibition of sulfatase by unconjugated steroids present in the fetal blood may be important in controlling its activity, thereby rendering it the rate-limiting enzyme in estrogen synthesis by the placenta (78). Gibb and Lavoie (83) have recently conducted detailed kinetic studies in microsomes obtained from human term placenta which have shown that the rate-limiting step in estrogen synthesis from DHEAS changed as the concentration of DHEAS was altered during the experiment. Specifically, sulfatase was found to be the rate-limiting enzyme at concentrations of DHEAS less than 1 μM, and aromatase was rate-limiting at concentrations of 2-4 μM. These concentrations are similar to the concentration of DHEAS observed to be present in human pregnancy serum at term (84). These results are somewhat intuitive in nature, as the values reported for the Km of placental sulfatase are in the μM range (77,79,83) and for aromatase mostly in the nM range (47,83,85). Thus, at low concentrations of DHEAS, sulfatase would be expected to be the rate-limiting enzyme.

Following placental aromatization of androstenedione and testosterone there exists a large pool of estrone (E_1) and estradiol (E_2) within placenta. The relative concentration of each estrogen in placenta is controlled through the action of estradiol 17β dehydrogenase (E_2DH).

The interconversion of E_2 and E_1 was first shown in soluble preparations from human term placenta by Ryan and Engel over 30 years ago (86), and this activity was named 17βE_2DH by Langer and Engel

some time later (87). This enzyme functions in the reversible interconversion of E_2 and E_1 utilizing either $NAD^+(H)$ or $NADP^+(H)$ as cofactor, with the bulk of this activity within placenta reported to be localized to the soluble subcellular fraction (3). Since either cofactor can be employed in oxidation or reduction of substrate, a transhydrogenase function has also been assigned to this enzyme (88). The original description of this enzyme by Langer and Engel (87) detailed its rather high degree of specificity for compounds of the C_{18} estrane series, but since this time the soluble enzyme from human term placenta has been shown to possess multifunctional activities and as a result is also called 17β hydroxysteroid dehydrogenase (17βOHSD) (89). Tobias et al. (90), using homogeneous preparations of 17βOHSD, showed by virtue of superimposable patterns of inhibition of $17\beta E_2DH$ ($E_2 \rightarrow E_1$) and 20α hydroxysteroid dehydrogenase (20αOHSD, 20α hydroxyprogesterone (20αOHP)---) that these activities were functions of one enzyme. Earlier work by Purdy et al. (91) had demonstrated that E_2DH and 20αOHSD activities were inseparable during purification. Jarabak has demonstrated using pure preparations of soluble 17βOHSD that testosterone (T) is also a substrate for the enzyme (4). Kinetic studies of this enzyme have revealed that the Km values for E_2 and E_1 as well as those for cofactor are on the order of 10 μM (3). Michaelis constants for the oxidation of T and 20αOHP are approximately 100 and 300 μM respectively (4,90), indicating that the enzyme has a much greater affinity for E_2 than other substrates during dehydrogenation. The enzyme behaves <u>in vitro</u> with a strict stereospecificity: it is a "B" dehydrogenase, that is, it transfers the hydrogen "pro S" of the nicotinamide and leads to "S" alcohols (92).

As stated above, the bulk of 17βOHSD within human term placenta that catalyzes the interconversion of E_2 and E_1 is localized within the soluble subcellular fraction. However, a large portion of the activity within the human term placenta that is responsible for the interconversion of T and androstenedione (A) is present in the microsomal fraction (93). The characteristics of a mitochondrial form of 17βOHSD within human term placenta have also been described (94). Microsomal and mitochondrial forms of 17βOHSD within human term placenta are equally efficient in the oxidation of T or E_2 as substrates (94-96), quite different in this respect than the soluble activity.

Early attempts at purification of soluble 17βE_2DH from human term placenta were limited by its lability until the observation was made that the enzyme was stabilized by its substrate E_2, or by relatively high concentrations of glycerol (87). Using glycerol as a stabilizing agent, Langer and Engel (87) were able to accomplish a 50-fold purification by ammonium sulfate fractionation and adsorption and desorption from calcium phosphate gel.

In 1962 Jarabak et al. (97) were able to achieve in eight steps a 2500-fold purification of E_2DH with an overall yield of 29% by including glycerol throughout the purification procedure and exploiting the thermal stability of the enzyme prior to ion exchange chromatography. Though no claim of purity was proposed, the congruence of activity and protein concentration curves upon electrophoresis in a glycerol density gradient was consistent with this conclusion.

Chromatography using hydroxylapatite for the purification of soluble E_2DH was introduced in 1966 (98). Two years later Descomps et al. (99) shortened the procedure for isolation of pure enzyme and by 1970

homogeneous enzyme was obtained in four steps using hydroxylapatite and DEAE cellulose chromatographies (3).

An important advance was made by Nicolas et al. (100) who developed an affinity column consisting of estrone linked to Sepharose 4B which enabled them to achieve a 100-fold purification of the enzyme in a single step. By use of estriol-sepharose affinity column chromatography during purification Chin et al. (101) were the first to crystallize pure 17βE_2DH from human term placenta with a specific activity of approximately 7 μ moles E_2 oxidized per min per mg protein. In 1985, Mendoza-Hernández et al. (102) reported a one-step 2400-fold purification to homogeneity by using chromatrography on Cibachrome Blue F3G-A coupled to sepharose and selective elution with $NADP^+$.

In 1969 Jarabak and Sack (4) estimated the molecular weight of soluble 17βOHSD from human term placenta to be 62,-65,000 by gel filtration and NADPH titration. Hagerman (103) obtained a value of 62,000 using density gradient centrifugation in sucrose as the method to estimate molecular weight. These values are in close agreement with the values obtained by Burns et al. (104) for the native enzyme as determined by ultracentrifugation in the presence of 20% glycerol or E_2. Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate gave values of 33,500 (105) and 37,000 (106). These data taken in conjunction with the results obtained with enzyme subjected to polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate after cross-linking with diethyl suberimidate (106) lead to the conclusion that the native enzyme has a molecular weight of approximately 68,000 and that it is composed of two polypeptide chains, each possessing a molecular weight of approx-

imately 34,000. Since these original studies these values have been corroborated by other groups (90,92,102).

In addition to the heavily studied soluble 17βOHSD from human placenta discussed above, enzymes with similar function have been reported in testes (107), endometrium (108), kidney (109), liver (110), and skin (111). Soluble 17βOHSD from rabbit liver exhibited higher maximum velocities towards androgens than towards estrogens (112). Similar specificities have been reported for soluble 17βOHSD of guinea pig liver (110) and kidney (109), and particulate 17βOHSD from human endometrium (113). In contrast, estrogens are preferred substrates for the 17βOHSD of chicken liver (114), sheep ovary (115), and human ovary (116).

Heterogeneity appears to be a feature common to mammalian 17βOHSD enzymes including those of guinea pig liver (110), guinea pig kidney (109), pig testes (117), human skin (111), and human testes (118). Most recently, Antoun et al. (112) have demonstrated the existence of three forms of 17βOHSD isolated from rabbit liver cytosol. Interestingly, of the studies in which the authors have reported a molecular weight (110,112,117), their values consistently ranged between 30 and 40,000, and in no case was any evidence of subunit structure indicated. The authors concluded that the source of heterogeneity was therefore not due to differences in quarternary structure but was postulated to be a result of the presence of charge-isomers of 17βOHSD. Engel and Groman (3) have reported that multiple forms do exist for the soluble enzyme from human term placenta, yet no explanation as to the possible source of heterogeneity was offered.

The first tissue in which a regulatory and control function of

17βOHSD activity has been extensively examined is that of human endometrium (113,119). The enzyme has been found to be present in higher concentrations during the secretory phase of the menstrual cycle as compared with the proliferative phase (108). The predominant direction of conversion was observed to be E_2 to E_1, which was obtained from experiments in which superfusion of slices of human endometrium with mixtures of $^{3}H\text{-}E_2$ and $^{14}C\text{-}E_1$ were conducted (120). Thus, by stimulation of endometrial E_2DH activity the conversion of a potent estrogen E_2 to E_1, a compound which is a poor competitor for E_2 binding to nuclear receptors (121) and diffuses out of cells more easily than E_2 (120), is facilitated. Therefore, Tseng and Gurpide (119) proposed that endometrial E_2DH activity would be expected to control both the intracellular concentration of E_2 and the response to circulating estrogens within endometrium. In addition, from *in vitro* and *in vivo* experiments using progesterone and synthetic progestins (113,119), progestational induction of human endometrial E_2DH activity was established. These results suggested that progesterone was responsible for the large increase in endometrial E_2DH activity observed during the secretory phase of the menstrual cycle (108). Since this original report in human endometrium, other investigators have demonstrated that hormonal control of 17βOHSD activity does exist in other tissues (122-123).

The usefulness of the method of *in vitro* superfusion of tissue slices to study *in vivo* phenomenon was established during experiments with rabbit uterus (124). Perfusions of uterus with labeled estrogens were conducted *in situ*, *in vitro*, and in tissue slices. Under all three conditions the rate constant of conversion of E_1 to E_2

was 10-20 times larger than the rate constant for the opposite direction. This directionality of 17βE_2DH activity is opposite to that observed for human endometrium (120) and the authors suggested that species or tissue differences could be due to variations in the affinity of substrates for the enzyme and/or in ratios of oxidized and reduced cofactors.

A functional role of 17βOHSD activity within placenta has not been established. Superfusion of slices of human term placenta with labeled estrogens was conducted (125), and it was concluded that within human placenta the rate constant for the conversion of E_2 to E_1 was equivalent to the rate constant for the opposite direction. Unlike the human uterus, the human placenta does not appear to be a target organ for estradiol action as levels of estrogen receptor in placenta were found to be nonexistent (126) or extremely low (127). However, some groups have sought to establish a relationship between the levels of E_2 in maternal serum and general placental growth (128).

E. Placental secretion and uptake of estrogens

The synchronous function of the cascade of placental enzymes involved in estrogen biosynthesis culminates in the generation of relatively large quantities of estrogen during pregnancy. In fact, the combined value for the placental secretion rates of E_3 into fetal and maternal circulations approximates 50 mg per day (38). The secretion rates for E_1 and E_2 were found to be on the order of 15 mg per day (38).

Most models of estrogen action depict the movement of unconjugated estrogens through biological membranes as a passive process (129). Thus, if only passive diffusion determined the rate of placental secre-

tion of estrogens to maternal and fetal circulations, one would expect to observe similar patterns of distribution for all estrogens studied. However, based on *in vivo* experiments in the Rhesus monkey Walsh and McCarthy concluded that E_2 was preferentially secreted to the maternal circulation whereas E_1 was secreted in substantial quantities into both fetal and maternal circulations (6). These results were obtained from experiments in which labeled E_1 and E_2 were infused into a maternal and umbilical vein and blood samples were obtained from catheterized fetal and maternal vessels. The implication of these results will be discussed in a later section in the context of secretion of other placental products.

Once estrogens are formed by placenta, a question arises as to their ability to reenter placenta. A major determining factor to placental entry appears to be the conjugation status of the estrogen studied (130-132). Results from *in situ* perfusions of human placentas at midpregnancy with labeled mixtures of conjugated and free estrogens by Levitz et al. (130) indicated that the transplacental transfer of free estrogen was much greater than for conjugated forms. Specifically, the rates of placental passage of estriol glucuronide and sulfate were found to be significantly lower than for estriol. Similar results were obtained by Holinka and Gurpide (130) during *in vivo* experiments in which labeled mixtures of ^{3}H-E_1S and ^{14}C-E_1 were injected into the abdominal aorta of rabbits. Shortly after injection analysis of ^{3}H to ^{14}C ratios in uterine tissue revealed that the uptake of E_1 was much larger than that of E_1S. The authors postulated that capillary barriers may hinder the uptake of E_1S by the uterus. Similarly, Mandel et al. (132) found that conjugated estro-

gens administered vaginally in postmenopausal women exert mainly a local effect, indicating that these compounds are not absorbed into the general circulation. In addition, on the basis of data obtained during perfusion of human term placentas, Dancis et al. (133) concluded that binding to serum albumin could influence the rate of placental transfer of estrogens.

F. Placental perfusion

In situ perfusion has been employed at the Karolinska Institute to obtain valuable information concerning the synthesis and release of estrogens by human placenta (131,134). However, since the middle 1960's this type of experimentation has been outlawed, based on ethical grounds. Studies on nonhuman primates are most relevant to the human placenta but this research is costly and interspecies differences exist (135). For these reasons, the use of in vitro perfusion of human placenta has expanded over the last 20 years.

Extracorporeal perfusion of human placenta through fetal vessels has been conducted since the late 1930's (136). Fetal vessels, large and plainly visible, are easily catheterized and perfusions can be conducted for extended periods of time without significant structural damage to the placenta (137). On the other hand, maternal circulatory input to the placenta normally supplied by small endometrial spiral arteries (10) to a large extent cannot be successfully mimicked during in vitro placental perfusion by insertion of needles through the decidual layer on the maternal surface. Krantz et al. (138) provided the first description of an extracorporeal perfusion of human placenta with maternal side considerations. The placenta was placed in an arti-

ficial uterine chamber so that the maternal surface was perforated by a series of polyethylene tubes. Studies employing perfusion of whole organ (139) as well as of a single cotyledon (140) have been conducted. Investigators have chosen to use either unrecycled or recycled perfusion sytems depending on experimental requirements (141). Schneider et al. (142) have described an exceedingly simple chamber for the simultaneous "fetal" and "maternal" perfusion of a single cotyledon which is widely used today.

In vitro perfusion of human placentas has been used to examine the transport of fatty acids (143) and amino acids (144), as well as carbohydrates (145). Transplacental movement of vitamin D has also been investigated during perfusion (146), with obvious relevance to fetal growth during pregnancy.

Studies involving in vitro perfusion of placentas have provided vital information towards our understanding of the now well documented pathways of estrogen biosynthesis during human pregnancy. During perfusion it was noted that the placenta lacks C_{17}-C_{20} lyase activity (139), which, in this case, is the inability to convert P to 17αOHP to DHEA. Thus, the placenta is dependent on C_{19} precursors present in both maternal and fetal blood for estrogen biosynthesis. Many groups have demonstrated that extensive aromatization of labeled C_{19} steroids takes place during perfusion of placentas through fetal vessels (139,140,147,148). It was observed that labeled E_3 was not generated during perfusion of term placentas with labeled E_2 (147), reflecting the absence of 16α hydroxylase activity in placenta.

G. Asymmetric release of placental products

The method of in vitro placental perfusion has also been used to

effectively study the pattern of estrogen release by the human placenta. Gurpide et al. (7) conducted perfusions of a single cotyledon through vessels with a solution of ^{3}H-A or ^{3}H-T. Fractions of unrecycled "fetal" and "maternal" perfusates were collected to estimate the rates of output into each perfusate of labeled E_1 and E_2, formed by aromatization of the perfused androgen. An unequal distribution of the two estrogens was observed, i.e., a significantly larger portion of E_2 than of E_1 was released to the "maternal" perfusate. The pattern of asymmetric estrogen release is consistent with the observation of preferential placental secretion of E_2 to the maternal circulation noted by Walsh and McCarthy (6) during in vivo experiments in the Rhesus monkey. On the basis of these results it was postulated that the asymmetric pattern of placental estrogen release might arise as a result of the action of E_2 specific binding proteins within the syncytium that release E_2 to the maternal circulation, or through extrasyncytial conversion of E_2 to E_1. The suggestion that more E_2 is released to the maternal circulation than to the fetal circulation also came from results obtained from earlier studies in which labeled E_2 was administered into mother and fetus in cases of erythroblastosis fetalis (i.e., hemolytic disease of the fetus and newborn) (149), and by measurement of estrogen levels in umbilical and uterine blood samples (150).

Asymmetric placental secretion of protein synthesized in the human placenta has also been noted (151,152). The protein hormones, human chorionic gonadotropic (hCG) and human placental lactogen (hCS) are known to be present at higher concentrations in maternal blood but at barely detectable levels in fetal blood (151,152). Pregnancy associ-

ated placental proteins (PAPP), a new class of approximately 20 distinct proteins, are also known to be preferentially secreted towards the maternal circulation (153).

Based on the proven utility of in vitro placental perfusion in the study of the pattern of estrogen distribution in the human placenta (7), it is likely that the method could serve as a valuable tool for evaluating factors regulating the secretion and release of other placental products.

III. Metabolism of Steroids by Placental Microvillar Membranes

A. Introduction

This section of dissertation research is comprised of a study in which microvillar estradiol 17β dehydrogenase (E_2DH) and sulfatase enzymes were biochemically characterized and compared with similar enzymes obtained from other placental subfractions. The study is novel in its approach as few investigators have considered the plasma membrane as a potential site of steroid metabolism. In addition, due to the unique location of placental microvilli (i.e., they form the boundary between the syncytium and maternal blood), steroid metabolism at this site could play a role in controlling the type of estrogen released to maternal circulation.

It has been demonstrated that the placenta depends upon sulfated C_{19} precursor, i.e., dehydroepiandrosterone sulfate (DHEAS), present in maternal blood to synthesize estrone (E_1) and E_2 (38), and that conjugation diminishes the rate of passage of steroid through biological membranes (131-133). Therefore, microvillar sulfatase activity might be expected to influence the rate of placental estrogen synthesis by modulating the level of DHEA that enters placenta.

B. Materials and methods

1. Preparation of microvillar vesicles and microvillar plasma membranes

Normal term placentas were transported to the laboratory immediately after delivery and villous tissue was obtained from central portions of the cotyledons, avoiding as much as possible contamination with maternal decidua. In order to obtain microvilli from the syncytium according to the method of Smith et al. (2), as modified by Whitsett and Lessard (17), fragments of placental tissue about 1 cm^3 in size were suspended in PBS buffer (140 mM sodium chloride--8 mM sodium phosphate, pH 7.4) at 4° C for 30 min with constant stirring. The suspension was filtered through a 500 µm opening stainless steel sieve and the filtrate was centrifuged at 3,000 x g for 10 min. The supernatant was subjected to centrifugation at 10,000 x g for 15 min and the new supernatant was recentrifuged at 100,000 x g for 30 min. The pellet was resuspended in PBS by homogenization and the suspension was centrifuged at 10,000 x g for 10 min to separate heavier particles from microvillar vesicles that remained in suspension or could be readily resuspended by gentle swirling. The yield of this preparation was about 15 mg of vesicle protein per 100 g of placental tissue processed.

Microvillar membranes were prepared by suspending microvillar vesicles at a concentration of about 0.4 mg vesicle protein/ml in a hypotonic solution (PBS:H_2O, 1:8) for 30 min at 25° C. The pellet obtained by centrifugation at 100,000 x g for 60 min was resuspended by homogenization in PBS and this suspension is called the membrane

preparation.

Cytosol was prepared by using fragments of trophoblastic tissue thoroughly washed with cold PBS. After tissue disruption in 2.5 volumes of PBS using a Polytron homogenizer (Brinkman Inst., Westbury, NY), the homogenate was centrifuged first at 3,000 x g for 15 min and then at 100,000 for 45 min, always at 4° C, to yield cytosol as a supernatant.

Mitochondria and microsomal fractions were prepared as described by Canick and Ryan (154). One hundred g of placental tissue yielded approximately 140 mg of microsomal and 20 mg of mitochondrial protein.

2. Electron microscopy

Membrane preparations were fixed with 1% glutaraldehyde in 67 mM sodium cacodylate buffer, pH 7.4, and post-fixed in osmium tetroxide and tannic acid. Samples were dehydrated and sections were taken at silver to gold thickness. Staining was carried out with uranyl acetate and lead citrate before examination with a JEOL 100-B electron microscope.

3. Measurement of enzymatic specific activities

Using non-limiting concentrations of substrate and cofactors, lactic dehydrogenase (LDH) activity, a cytosolic marker, was measured spectrophotometrically as described by Cohen et al. (155), alkaline phosphatase (AlkP) activity, a plasma membrane marker, as described by Mattenheimer (156), NADPH-cytochrome c reductase, a microsomal marker, as described by Phillips and Langdon (157), and succinate-cytochrome c reductase, a mitochondrial marker, as described by Brown and Beattie (158). Estradiol 17β dehydrogenase (E_2DH) activity was estimated either spectrophotometrically at 340 nm, by measuring the rate

of formation of NADH at different concentrations of estradiol (E_2) and excess NAD^+ (200 μM) following procedures described by Langer et al. (87), or radiometrically, by measuring the rate of conversion of ^3H-E_2 to ^3H-E_1 or ^3H-testosterone (T) to ^3H-androstenedione (A) as previously described (108). Linearity in the amount of ^3H-E_1 formed as a function of time of reaction (1-10 min) and protein concentration (0.4-1.7 mg/ml) was demonstrated. Since the solubility of E_2 in the assay mixture (about 20 μM) could be of the same order of magnitude as the K_m of cytosolic E_2DH for this substrate, enzymatic activities (V_{max}) were calculated by extrapolation using Lineweaver-Burke plots or from measurements at 10 μM substrate concentrations (v_{10}) estimating V_{max} values from the Michaelis-Menten equation, i.e., $V_{max} = [(K_m + 10)/10]v_{10}$. The validity of these estimates was verified in all experiments in which Lineweaver-Burke plots were obtained.

Sulfatase activities were measured by incubating ^3H-E_1S or ^3H-DS (100 μM) in 50 mM Tris HCl, 10 mM $MgCl_2$, 0.25 mM monothioglycerol buffer, pH 7.4, at 25° C for various time periods. Samples of the reaction mixture were mixed with methanol (1:10 v/v) containing indicators and carriers (E_1 + E_2, 500 μg each, or ^{14}C-D + 200 μg D) and the supernatants were subjected to TLC using a chloroform:ethyl acetate, 4:1 system. Amounts of ^3H-E_1 or ^3H-D formed by hydrolysis of the sulfates were estimated from $^3H/^{14}C$ ratios or specific activities (dpm/u.v absorbance units) of the products. Linearity of the assays using membrane, microsomal, cytoplasmic or vesicular fractions was observed during incubations up to 10 min for ^3H-E_1 and for ^3H-DS. No formation at ^3H-E_2 during incubations of ^3H-E_1S was noted and specific activities of E_1 did not change after a second TLC.

Aromatase activity was measured by incubating 20 μM [6,7-^{3}H]-A in 100 mM NaPi, pH 7.4, plus 3 mM NADPH, at 37° C, at a protein concentration of 0.5 mg/ml. Tritiated E_1 and E_2 were separated from ^{3}H-A by TLC using an ethyl ether:hexane, 3:1 system. Linearity in reaction rate for up to 20 min was observed. All aromatase values were calculated from summed production rates for E_1 and E_2.

Proteins were measured by the colorimetric method of Bradford (159) using a Biorad protein determination kit.

4. Treatment of membranes with salt solutions and detergents

Membranes suspended in diluted PBS (1:8) were centrifuged and the pellet was resuspended in diluted PBS at 25° C. This procedure was repeated, measuring specific activities of AlkP, E_2DH, E_1S and DHEAS sulfatases in each of the pellets. Membranes were similarly treated with 2M NaCl, with Triton X-100 (Sigma, St. Louis, MO) at 0.1 or 0.3% concentrations in diluted PBS, or with a 0.3% solution of sodium cholate in 0.6 mM sodium phosphate-72 mM NaCl buffer, pH 7.4, containing 25% glycerol (PBS-G-C) for stabilization of the solubilized E_2DH activity.

5. Treatment of microvillar vesicles and membranes with trypsin

Cytosol and suspensions of microvillar vesicles or membranes, at concentrations of about 1 mg protein/ml, were incubated in PBS with porcine trypsin (Type 1X, Sigma) at concentrations of either 100 or 300 μg/mg protein for 75 min at 25° C, stopping the reaction by addition of soybean trypsin inhibitor (Type 1S, Sigma) at 400 and 1200 μg/mg protein before measuring E_2DH and sulfatase activities. Control incubations without trypsin were assayed in the presence of trypsin inhibitor.

6. Antiserum to placental estradiol 17β dehydrogenase

Anti-E_2DH serum from rabbits inoculated with purified human placental E_2DH was kindly made available to us by Dr. F. Naftolin, Yale University. The antiserum was diluted 10-fold with PBS, pH 7.4, containing 25% glycerol (PBS-G buffer).

The effects of the antiserum on E_2DH activity were compared in cytosol, in membranes and in the sodium cholate extract of membranes. Cytosol was prepared by homogenizing placental tissue in PBS-G to obtain a final concentration of 1.25 mg protein/ml, membranes were suspended in PBS-G to a concentration of 4.6 mg protain/ml, and a solution of solubilized E_2DH was prepared by extracting membranes with the sodium cholate solution described above (PBS-G-C) to obtain a concentration of 1.35 mg protein/ml. Cytosol (10 µl) was incubated with 50 µl of the diluted antiserum plus either 40 µl of PBS-G or 40 µl of PBS-G-C. Ten µl of the membrane suspension was incubated with 50 µl of the diluted antiserum. Ten µl of the membrane extract was incubated with 10 µl of the diluted antiserum plus 50 µl of the cholate buffer (PBS-G-C). All incubations were carried out at 4° C for 18 h and E_2DH activity was measured radiometrically. Controls were obtained by replacing the diluted antibody solutions in the incubations by the corresponding buffer (PBS-G or PBS-G-C).

7. Characterization of estradiol 17β dehydrogenase

a. Purification of E_2DH by gel filtration

Estradiol 17β oxidoreductase present in cytosol or extracted from membranes was purified by chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ). The cytosolic enzyme precipitated with ammonium sulfate at 50% saturation was redissolved in

PBS-G-C containing 150 µg/ml of human serum albumin and stored at 4° C for more than 6 months without loss of activity. Aliquots of the stored solution (8-10 mg protein) were applied to a Sephadex G-100 column (2 x 50 cm) previously equilibrated with the elution buffer (0.3% sodium enolate in PBS-G-C). The presence of 0.3% sodium cholate detergent in the PBS-G elution buffer did not alter the profile of cytosolic E_2DH activity during Sephadex G-100 fractionation. Elution was carried out at 25° C at a flow rate of 9 ml/(cm^2 x h) collecting 2 ml fractions. Each fraction was assayed spectrometrically at 340 nm for protein content and radiometrically for E_2DH activity. The 100,000 x g supernatant of a PBS-G-C extract of microvillar membranes was applied to the Sephadex G-100 column under the same conditions described above. In this case, however, E_2DH activity was assayed radiometrically.

b. PAGE isoelectric focusing

Fractions eluted from Sephadex containing E_2DH activity were pooled and concentrated by ultrafiltration in a Centricon Microconcentrator (Amicon, Danvers, MA) to 1/50 of the original volume. The concentrate (20-50 µg) was applied to PAGE disc gels (15 cm) containing 5% acrylamide, 0.2% bisacrylamide, 25% glycerol, 1% ampholites pH 3-10 (Biorad, Richmond, CA) and 2% ammonium persulfate. The gels were polymerized with 0.17% TEMED (International Biotechnologies Inc., New Haven, CT). Electrophoresis was conducted at 25° C under a constant voltage of 400 V for a period of 2 h. The solution at the anode was 0.01% phosphoric acid and at the cathode 0.02% NaOH. Following focusing, the gels were either stained with Coomassie Blue (Biorad, 0.25% in 0.1% acetic acid, 10% trichloroacetic acid, and

30% methanol) to localize proteins or preincubated with 200 μM NAD^+ for 10 min at 25° C to eliminate endogenous E_2 associated with E_2DH. After this preincubation, the gels were further incubated in the dark for 18 h at 37° C with the NBT reagent prepared by mixing 1.5 ml of a 1.7 mg/ml solution of nitroblue tetrazolium (Eastman Kodak, Rochester, NY), 0.25 ml of 0.25 mg/ml phenazine methosulfate (Sigma), 0.5 ml of 5 mg/ml NAD^+ and 0.4 ml of ethanol with E_2 (or without it in control runs), as described by Pollow et al. (160). Gels were photographed and the intensity of the color in each band was quantitated on the negatives using a densitometer (E.C. Apparatus Corp., St. Petersburg, FL). Coomassie protein staining patterns in Figure 12 are of cytosolic proteins precipitated with 35-50% $(NH_4)_2SO_4$ and the 100,000 x g supernatant of microvillar membranes treated with 0.3% sodium cholate 67 mM NaCl.

8. Estrogen binding assays

Microvillar membranes obtained from human term placenta were suspended in PBS containing 1 mM dithiothreiotol at a protein concentration of approximately 2 mg/ml. Incubation of membranes (200 μl) with ^{125}I-E_2 or ^{3}H-E_3 was carried out for 3 h at 30° C in the absence (total binding) or the presence (nonspecific binding) of a 100-fold excess of unlabeled E_2. Separation of "free" and "bound" counts was accomplished following incubation with 0.23% Dextran-coated charcoal (30 min, 4 C) and centrifugation at 1500 x g for 10 min. It was noted that "trapping" of membrane protein by the Dextran-coated charcoal reagent did not occur under the conditions of assay. Radioactivity was measured in a 100 μl aliquot of the supernatant using Dimiscint (National Diagnostics, Somerville, NJ) as the scintillant.

C. Results

1. Initial observation of estradiol metabolism in preparations of microvillar vesicles

Incubation of vesicles with $^{3}H\text{-}E_2$ at 4° C resulted in a fast and complete conversion of the labeled substrate to $^{3}H\text{-}E_1$ in the absence of any added cofactor (Fig. 6). No conversion of $^{3}H\text{-}E_1$ to $^{3}H\text{-}E_2$ was noted under similar conditions. In order to determine the distribution of vesicular E_2DH between membrane and trapped cytosol, enzymatic activities were measured in intact and disrupted vesicles.

2. Electron micrographs of microvillar membranes (osmotically treated vesicles)

Electron micrographs of microvilli from term placentas are shown in Figure 7. The preparation of microvillar membranes has the appearance and degree of homogeneity already described in other publications for intact microvillar vesicles (2,17,18).

3. Specific activities of steroid metabolizing and marker enzymes in preparations of microvillar membranes and placental fractions

a. Lactic dehydrogenase, alkaline phosphatase, and estradiol 17β dehydrogenase

Results from measurements of protein content and specific activities of lactic dehydrogenase (LDH), alkaline phosphatase (AlkP), and estradiol 17β dehydrogenase are shown in Table 1. The protein content of intact vesicles was reduced by approximately 50% when vesicles were placed in hypotonic medium, most likely due to the release of trapped syncytial cytoplasm. The complete removal of cytoplasm from the vesicles in hypotonic medium was made evident by the absence of LDH activity, a marker for soluble enzymes, in the pellet of membranes remaining after disruption of the vesicles. It was estimated that LDH ac-

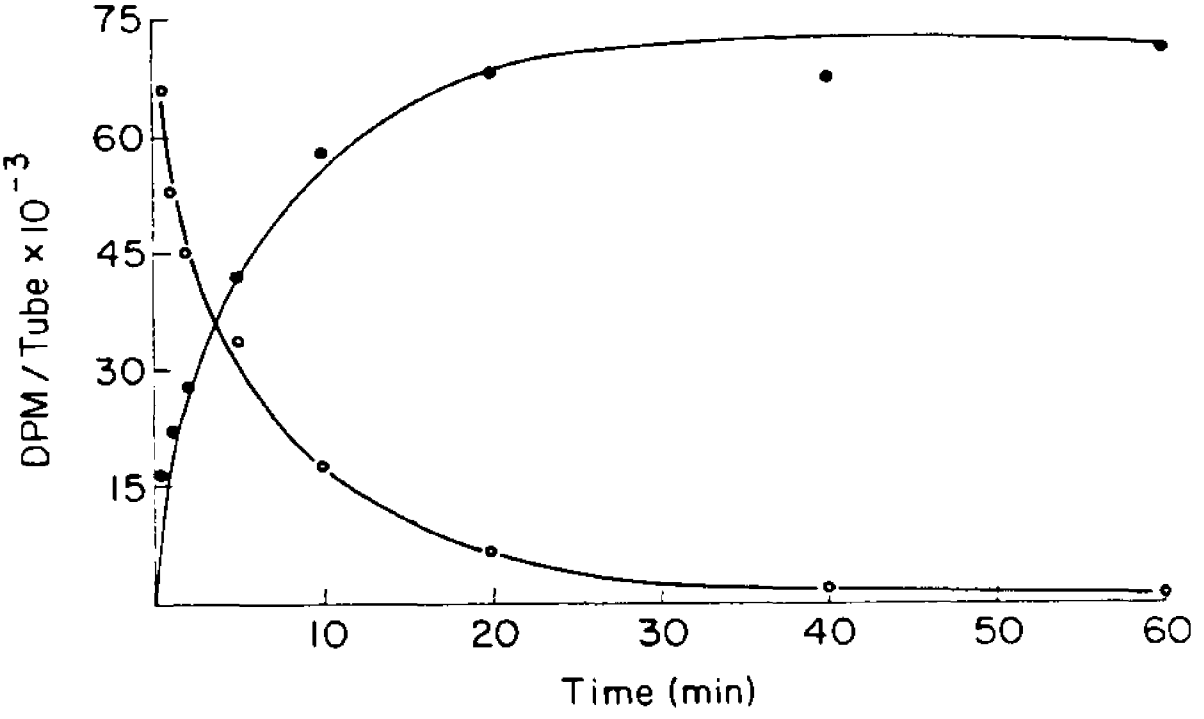

75
60
45
30
15
DPM / Tube x 10^-3
10
20
30
40
50
60
Time (min)

Fig. 6 Metabolism of $^{3}H\text{-}E_2$ by microvillar vesicles

Intact vesicles were incubated with 18 nM $^{3}H\text{-}E_2$ at 4° C at a protein concentration of 3.3 mg/ml without added co-factor. Levels of $^{3}H\text{-}E_1$ (●) and $^{3}H\text{-}E_2$ (o) in vesicles was determined at various times following extraction and TLC.


0.5 µm

Fig. 7 Electron micrograph of microvillar membrane preparations

Osmotically disrupted vesicles (microvillar membranes) isolated from human term placenta (X 42,000). Placental microvillar vesicles were subjected to hypotonic treatment and were fixed in glutaraldehyde prior to preparation for electron microscopy.

Table 1. Specific Activities of LDH, AlkP, E_2DH, and Steroid Sulfatases in Microvillar Vesicles, Microvillar Membranes and Placental Cytosol

Specific activities of E_2DH, E_1S sulfatase, and DHEAS sulfatase are expressed as nmol product formed per min and per mg protein were determined radiometrically. Specific activities of alkaline phosphatase and lactic dehydrogenase are expressed as µmol product formed per min per mg prot. Protein values reflect the relative yield of microvillar vesicle and microvillar membrane material obtained from approximately 130 g of placental tissue.

Parameter	Fractions		
	Cytosol	Vesicles	Membranes
Protein (mg)			
X±SD (n=4)	-	20±2.8	9.1±1.4
Enzymatic sp.act(U/mg prot.)			
X±SD			
LDH (n=4)	0.46±0.09	0.23±0.03	∿0
AlkP (n=4)	0.06±0.003	3.2±1.5	6.5±3.3
E_2DH (n=4)	0.72±0.09	0.52±0.02	0.12±0.02
E_1S sulfatase (n=3)	∿0	0.55±0.12	1.3±0.30
DHEAS sulfatase (n=3)	∿0	0.10±0.02	0.13±0.04

tivity could be demonstrated spectrophotometrically at even less than 1/100 of the amounts present in the vesicle preparation. Alkaline phosphatase, a marker for plasma membranes, was practically all retained by the membranes, as indicated by a doubling in specific activity after vesicle disruption. Only about 10% of the E_2DH activity in the vesicles was retained by the membranes; the rest appeared to have been removed with the trapped cytoplasm since the specific activity of the enzyme lost (enzyme lost/protein lost) was similar to the specific activity of E_2DH in placental cytosol also shown in Table 1.

b. NADPH cytochrome c reductase, aromatase, and sulfatase

Table 1 also illustrates the association of estrone sulfate (E_1S) and dehydroepiandrosterone sulfate (DHEAS) sulfatases to microvillar membranes. Since these sulfatase activities are mainly found in the microsomal fraction the presence of microsomal contamination in the microvillar membrane preparations was evaluated by measuring NADPH-cytochrome c reductase and aromatase activities, usually considered microsomal markers (Table 2). From the data shown in Table 2, it can be estimated that the level of microsomal contamination in microvillar membrane preparations is about 14% as judged from identical results obtained with both microsomal markers and would account for no more than 0.47 of the 1.3 and 0.08 of the 0.13 units found per mg plasma membrane protein of E_1S and DHEAS sulfatase activities, respectively. These findings support our contention that steroid sulfatases are associated with microvillar membranes whereas aromatase is not.

Table 2. Specific Activities of E_1S and DHEAS Sulfatases, NADPH Cytochrome c Reductase, and Aromatase in Microvillar Membranes and Microsomes from Term Placenta

Levels of NADPH cytochrome c reductase, E_1S and DHEAS sulfatases, and aromatase activities were determined in microvillar membranes and microsomes from three different placentas. All specific activities are expressed as nmol product formed per min and per mg protein.

Sample	Specific activities Units/mg protein (X±SD, n=3)			
	NADPH-cyt. c reductase	E_1S sulfatase	DHEAS sulfatase	Aromatase
Microvillar membrane	18±6.1	1.3±0.3	0.13±0.04	0.046±0.009
Microsomal fraction	130±7.2	3.4±0.2	0.57±0.23	0.33±0.05

4. Association of estradiol 17β dehydrogenase and estrone sulfatase enzymes to the microvillar membrane

a. Effects of washing with salt solutions and detergent

In order to obtain some information as to the strength of association to the microvillar membrane of E_2DH and E_1S sulfatase, washing experiments under four different conditions were conducted (Table 3).

Two washes of the isolated membranes with hypotonic buffer (PBS: H_2O, 1:8) resulted in an 18% fall in E_2DH specific activity, whereas two washes with 2 M NaCl decreased the specific activity by 41%. Although this decline in specific activity was considerably higher than the 10% fall in the specific activity of AlkP measured after 2 washes with 2 M NaCl in the same preparations, it indicates significant retention of E_2DH by the membrane in the presence of high salt concentrations, one of the criteria used to determine association of an enzyme to plasma membranes (161). Estrone sulfate sulfatase activity in microvillar membranes was more resistant than AlkP to removal by repeated washings with 2 M NaCl, as reflected by the increased specific activity following this treatment. Treatment of the membrane preparations with Triton X-100 at 0.1 or 0.3% concentrations in diluted PBS reduced the specific activity of E_2DH to 12 or 8% of the original value, respectively. Alkaline phosphatase specific activities were reduced to 67 and 60% of the starting values in the same preparations. Microvillar membrane associated E_1S sulfatase could be completely solubilized by treatment with 0.1% Triton X-100, which is not surprising in light of a recent study reporting success in the solubilization of microsomal steroid sulfatase from human placenta using low levels of this detergent (79).

Table 3. Retention of AlkP, E_2DH, and E_1S Sulfatase Activities in Microvillar Membranes Washed with Salt Solutions and Detergent

The retention of enzymatic activities by microvillar membranes, following various washing procedures, is expressed as a percentage of the original specific activity remaining in the resuspended pellet following each wash.

		% activity retained by membranes		
Procedure	Wash #	AlkP	E_2DH	E_1S sulfatase
1:8 PBS	1	115	104	ND[a]
	2	105	82	ND
2 M NaCl	1	104	83	160
	2	90	59	185
Triton X-100 (0.1%)	1	78	24	36
	2	67	12	1
Triton X-100 (0.3%)	1	48	11	ND
	2	60	8	ND

[a]not determined

b. Effect of trypsin

Experiments were conducted testing the sensitivity of microvillar E_2DH and E_1S sulfatase activities to trypsin in order to obtain information concerning the degree of exposure of these enzymes to the medium (Table 4). Trypsin, used at concentrations of 100 and 300 µg/mg protein affected only slightly the activity of membrane-associated E_2DH, whereas it completely inhibited the activity in cytosol. The large inactivation of E_2DH in vesicles suggests that they are permeable to trypsin, assuming that the bulk of the activity resides in trapped cytoplasm, or that microvillar membranes are permeabilized by tryptic degradation. Sulfatase activity in both microsomal and microvillar membrane fractions was not inhibited at 100 or 300 µg/mg protein concentrations. These results indicate that the placental microvillar membrane affords protection to E_2DH and sulfatase enzymes rendering these activities insensitive to inactivation by trypsin.

c. Effect of anti-estradiol 17β dehydrogenase antibodies

Cytosolic and microvillar membrane fractions of a term placenta were tested for E_2DH activity in the presence or absence of an anti-human placental E_2DH rabbit antiserum. As shown in Table 5, the antiserum effectively inhibited the activity of a cytosol sample but had no effect on a preparation of microvillar membranes. The enzymatic activity solubilized by treatment of the membranes with sodium cholate was inhibited by the antibody.

These results suggest that the membrane-associated enzyme, or at least its antigenic sites, were buried in the membrane and protected from interaction with the antibody.

Table 4. Effects of Trypsin on E_2DH and E_1S Sulfatase Activities in Placental Subcellular Fractions

Incubation of samples with trypsin was carried out for 75 min at 25° C in a shaking water bath. Incubations were terminated by the addition of trypsin inhibitor to both control and treated fractions. Inhibition of 17β E_2DH activity by trypsin is expressed as a percentage of control activity. One milliunit (mU) of E_2DH and E_1S sulfatase activity equals 1 pmol ^{3}H-E_1 formed per min.

Exp. #	Trypsin concentration (μg/mg prot.)	Sample	Enzymatic activity	
			Control (mU/assay)	Inhibition by trypsin (%)
E_2DH				
1	100	Membranes	42	2
		Vesicles	320	93
		Cytosol	300	100
2	300	Membranes	45	27
		Vesicles	350	95
		Cytosol	350	100
E_1S Sulfatase				
3	100	Membranes	52	0
		Microsomes	180	0
4	300	Membranes	52	0
		Microsomes	140	0

Table 5. Effects of an Anti-E_2DH Antiserum on Cytosolic and Microvillar Membrane E_2DH Activity

Polyclonal antibody to purified cytosolic E_2DH was incubated with cytosol, microvillar membranes, and the 100,000 x g supernatant of solubilized microvillar membranes for 18 h at 4° C. Aliquots were removed and assayed radiometrically for 17β E_2DH activity. Inhibition of 17β E_2DH activity is expressed as a percentage of control activity.

Sample	Medium	E_2DH activity (mU/assay)		Inhibition (%)
		Control	+Antibody	
Cytosol	PBS-G	7.6	1.5	80
	PBS-G-C	8.3	0.86	90
Microvillar membranes	PBS-G-C	14	12	14
Solubilized from microvillar membranes	PBS-G-C	2.0	0.07	97

5. Comparison of characteristics of estradiol 17β dehydrogenase activity in microvillar membrane and other placental fractions

a. pH dependence

Figure 8 illustrates the results obtained when E_2DH activities in one preparation of microvillar membranes and one of placental cytosol were determined in various buffer solutions of different pH by measuring the conversion of ^3H-E_2 to ^3H-E_1 (membranes) or the rate of formation of NADH during incubations with 10 μM E_2 and 200 μM NAD^+ (cytosol). While the E_2DH activity of cytosol increased continuously from pH 7.5 to 10 in glycine buffer, the activity of membranes placed in the same buffer showed 2 peaks, one at pH 8.5 and another at pH 9.5, with a sharp fall at pH 10. The influence of buffer composition, in addition to that of pH, was evident from the differences in relative activities at pH 8.5 and 9.5 observed in glycine and carbonate buffers. Significantly higher activities were found in membranes when the assays were conducted at 17 mM than at 100 mM phosphate buffer at pH 7.4. No such dependence on ionic strength was observed in assays performed on cytosol. The differences in patterns of pH dependence of E_2DH activity in cytosol and membranes suggest that the activity of the membrane-associated enzyme is influenced by the lipid-protein environment.

b. Kinetics of dehydrogenation of estradiol and testosterone

Table 6 shows values for Michaelis constants (K_m) and maximal velocities (V_{max}) of 17β hydroxysteroid oxido-reductase activity for E_2 and T in microvillar membranes, microsomes, mitochondria, and cytosol. These values were calculated from Lineweaver-Burke plots such as those in Figure 9 which show data from ra-

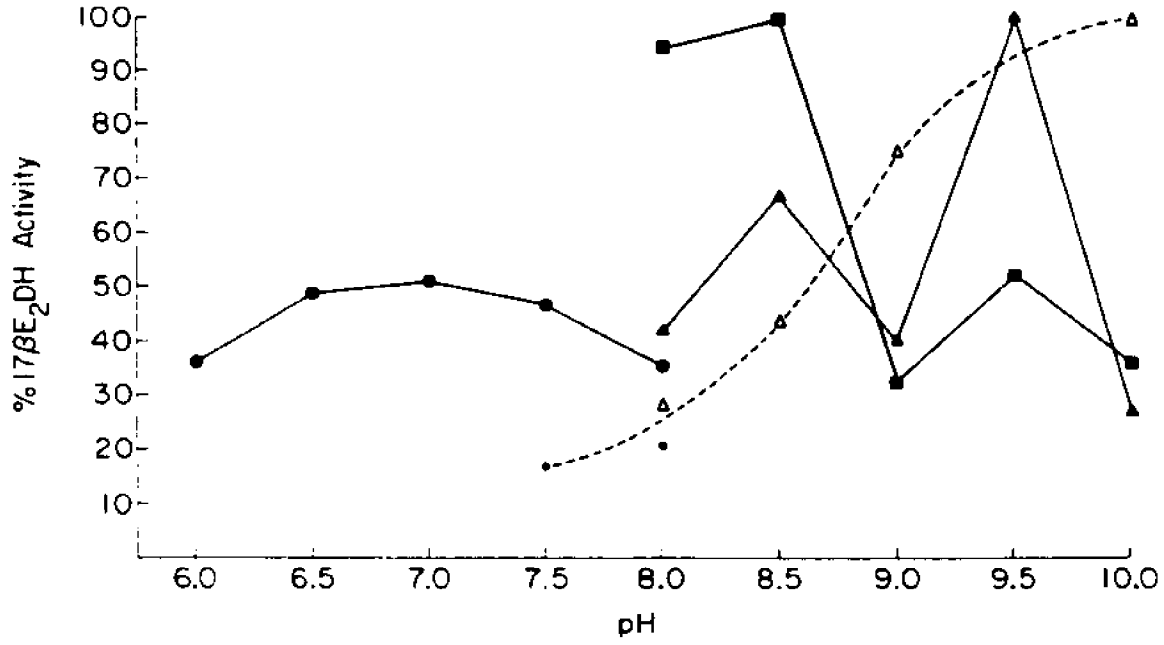

100
90
80
70
60
50
40
30
20
10
% 17βE2DH Activity
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

Fig. 8 pH dependence of E_2DH activity in placental microvillar membranes and cytosol fractions

Effect of pH upon 17β E_2DH activity in microvillar membranes and cytosol were compared in one placenta. Levels of 17β E_2DH activity in membranes were determined radiometrically and are expressed as percentages of the value obtained for 0.1 M glycine pH 9.5. Levels of E_2DH activity in cytosol were determined spectrophotometrically and are expressed as percentages of the value obtained for 0.1 M glycine pH 10.0.

●, ○ NaPi 0.1 M, ▲,△ glycine 0.1 M, ■, □ Na_2CO_3 0.1 M. Filled symbols represent values for microvillar membranes, open symbols for cytosol.

Table 6. Kinetic Parameters of E_2DH in Microvillar Membranes, Cytosol and Other Subcellular Fractions

Kinetic values corresponding to 17β hydroxysteroid dehydrogenase activity were compared in various subcellular fractions from human term placenta. One unit of activity is defined as 1 nmol E_1 or A formed per min. The $V_{max\ E_2}/V_{max\ T}$ ratios depict the relative E_2 and T-metabolizing capabilities of cytosolic and microvillar enzymes within four different placentas.

Samples	K_m (μM)		K_{mE_2}	V_{max} (U/mg prot.)		$V_{max\ E_2}$	$V_{max\ E_2}/V_{max\ T}$
	E_2	T	K_{mT}	E_2	T	$V_{max\ T}$	relative to homologous membranes
Microvillar membranes	1.3	57	0.02	0.17	0.024	7.1	1.0
				0.12	0.060	2.0	1.0
				0.11	0.035	3.1	1.0
				0.12	0.052	2.3	1.0
Mitochondria	0.43	0.37	1.2	0.84	0.36	2.3	-
Microsomes	0.69	0.44	1.6	0.56	0.83	0.7	-
Cytosol	11	170	0.07	1.6	0.04	40	5.6
				1.2	0.20	6.0	3.0
				2.0	0.13	15	4.8
				2.1	0.32	6.6	2.9

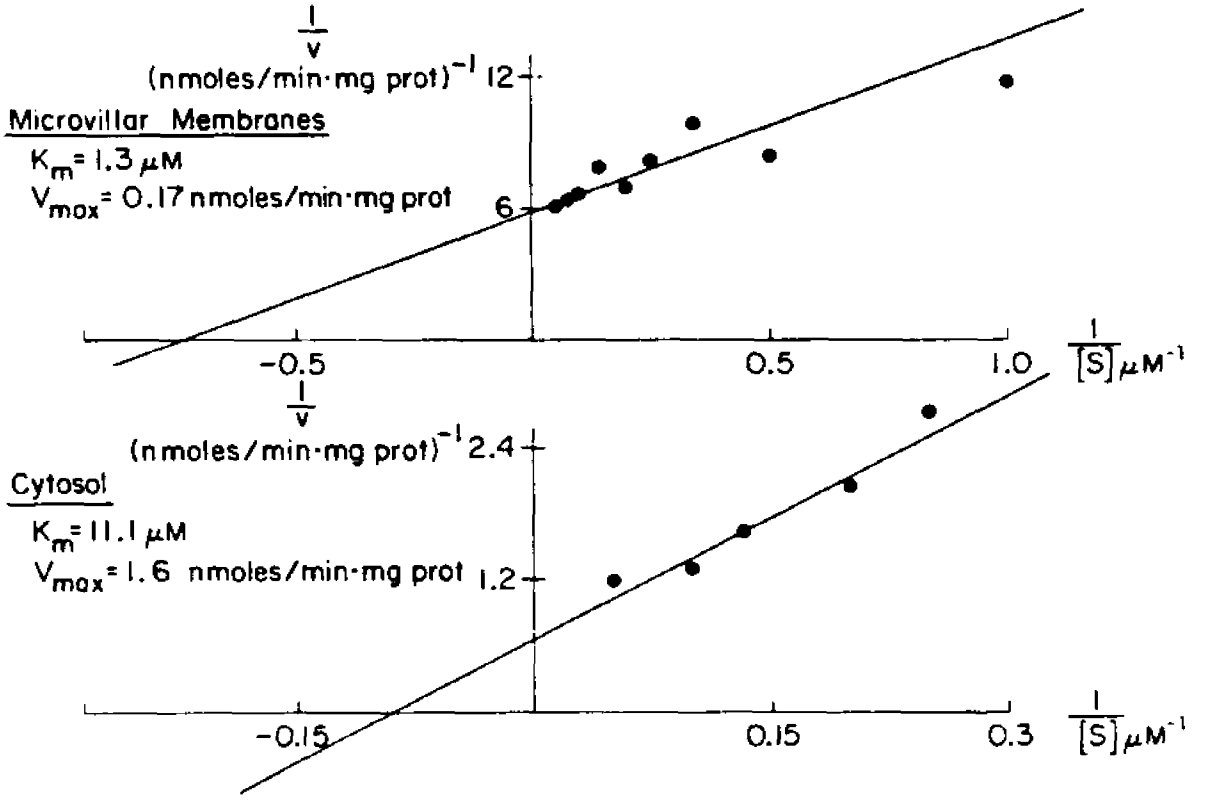

1/v
(nmoles/min·mg prot)⁻¹
12
Microvillar Membranes
K_m= 1.3 μM
V_{max}= 0.17 nmoles/min·mg prot
6
-0.5
0.5
1.0
1/[S] μM⁻¹
1/v
(nmoles/min·mg prot)⁻¹
2.4
Cytosol
K_m= 11.1 μM
V_{max}= 1.6 nmoles/min·mg prot
1.2
-0.15
0.15
0.3
1/[S] μM⁻¹

Fig. 9 Kinetic analysis of E_2 DH activity in microvillar membranes and cytosol

Microvillar membranes were incubated with ^3H-E_2 at concentrations ranging from 1 to 20 µM in 0.1 M NaPi buffer, pH 7.5, containing NAD^+ at a concentration of 200 µM. Conversion to ^3H-E_1 was measured after separation by TLC. Cytosol was incubated under the same conditions except that the concentration of E_2 was varied between 4 and 20 µM and initial rates were determined spectrophotometrically.

diometric evaluation of the oxidation of ^{3}H-E_2 in microvillar membranes (upper panel) and spectrophotometric data on oxidation of E_2 in cytosol (lower panel).

Results obtained in cytosol or microvillar vesicles with both methods showed close agreement. Only the radiometric method, which is more sensitive than the spectrophotometric, was used in all samples when T was the substrate and in microvillar membranes when E_2 was the substrate. Some of the values for V_{max} in Table 4 were estimated from single point measurements at 10 µM substrate concentrations, as described under Experimental Procedures. The validity of these estimates was verified in all experiments from which Lineweaver-Burke plots were obtained. Linearity of the assay with respect to reaction time and protein concentration was demonstrated in cytosol (1-10 min for E_2, 10-40 min for T, 0.4-1.7 mg protein/ml) as well as in membranes (up to 10 min and up to 2 mg protein/ml).

The results shown in Table 6 reveal large differences in the Michaelis constants corresponding to the cytosolic and membrane-associated enzymes. They also indicate differences in the specificity of the 17β hydroxysteroid dehydrogenase of cytosol and membranes, viz. the rate of utilization of T as a substrate, relative to that of E_2, is higher in the microvillar membranes than in cytosol.

6. Isolation and characterization of estradiol 17β dehydrogenase from microvillar membrane and cytosolic fractions

   a. Purification on Sephadex G-100, estimates of molecular weight

Estradiol 17β dehydrogenase associated to microvillar membranes was extracted with sodium cholate containing glycerol (PBS-G-C buf-

fer) to prevent inactivation of the enzymatic activity. The chromatographic behavior of the solubilized enzyme on Sephadex G-100 was compared to that of the cytosolic enzyme concentrated by precipitation with ammonium sulfate. The chromatograms corresponding to these two preparations are shown in Figure 10. Correlation of MW of standards with elution volumes indicated that the MW of the E_2DH solubilized from the microvillar membranes (∿137,000) was about twice as large as that of the cytosolic enzyme (Fig. 11). If a molecular weight of 137,000 is to be assigned to the microvillar enzyme, this suggests the possibility that the membrane associated enzyme is a tetramer since placental cytosolic E_2DH is considered to be a dimer (105,106). On the other hand, the presence of carbohydrate or lipid moieties attached to proteins is a potential source of anomalous behavior observed during gel filtration chromatography.

b. Isoelectric focusing on polyacrylamide gels

Both the cytosolic (fractions 28-32) and the membrane-associated (fractions 20-24) enzymatic activities eluted from Sephadex G-100 were concentrated by ultrafiltration and subjected to isoelectric focusing on polyacrylamide gels, localizing E_2DH activity with the NBT reagent. In order to distinguish non-specific NBT colored bands, which are detected both in the presence or absence of added E_2, from specific NBT colored bands appearing only after addition of E_2, it was necessary to preincubate the gels with NAD^+ to eliminate endogenous E_2 associated with the enzyme. As shown in Figure 12, a single specific E_2DH band, focusing at pH 5.2, was detected in both the cytosolic and the solubilized membrane preparations, visually and densitometrically.

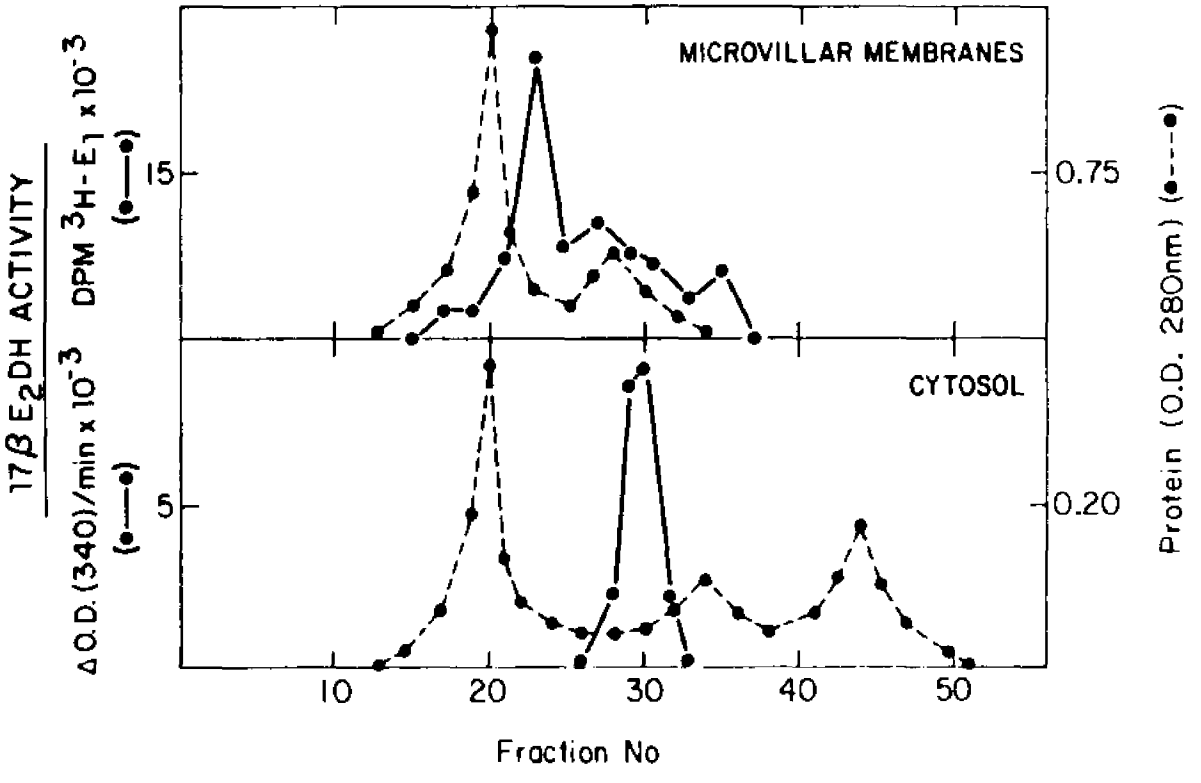

17β E2DH ACTIVITY
Δ O.D. (340)/min x 10-3 (•—•)
DPM 3H-E1 x 10-3 (•—•)
MICROVILLAR MEMBRANES
CYTOSOL
15
5
0.75
0.20
10
20
30
40
50
Fraction No
Protein (O.D. 280nm) (•---•)

Fig. 10 Sephadex G-100 fractionation of E_2DH isolated from microvillar membranes and cytosol

The 100,000 x g supernatant of microvillar membranes treated with 0.3% sodium cholate 67 mM NaCl and cytosolic proteins precipitated with 35-50% $(NH_4)_2 SO_4$ were applied to a Sephadex G-100 column previously equilibrated with PBS-G-C buffer as described under Methods section.

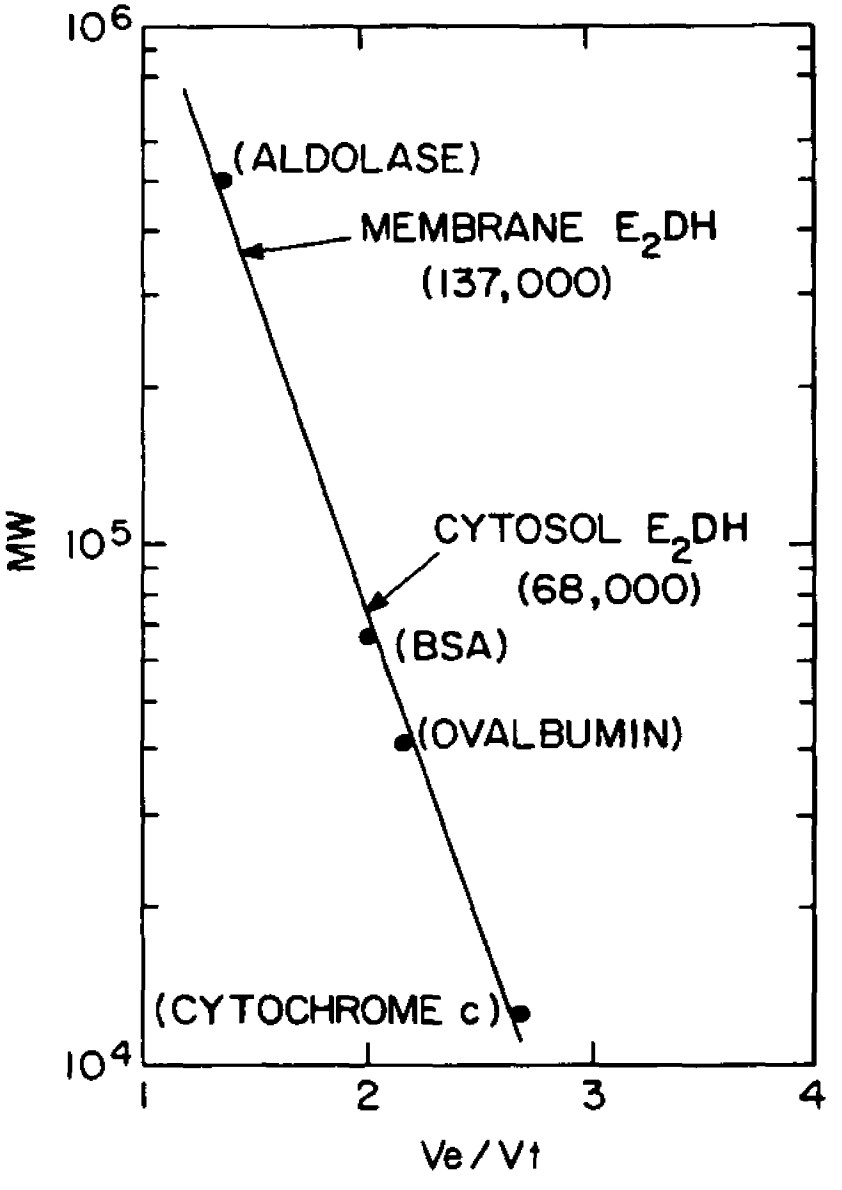

10^6
10^5
10^4
MW
(ALDOLASE)
MEMBRANE E_2DH
(137,000)
CYTOSOL E_2DH
(68,000)
(BSA)
(OVALBUMIN)
(CYTOCHROME c)
1
2
3
4
Ve / Vt

Fig. 11 Estimate of M.W. of E_2DH isolated from microvillar membranes and cytosol following gel filtration on Sephadex G-100

Estimation of molecular weight of E_2DH isolated from microvillar membranes and cytosol was based on profiles of E_2DH activity appearing in Fig. 5.

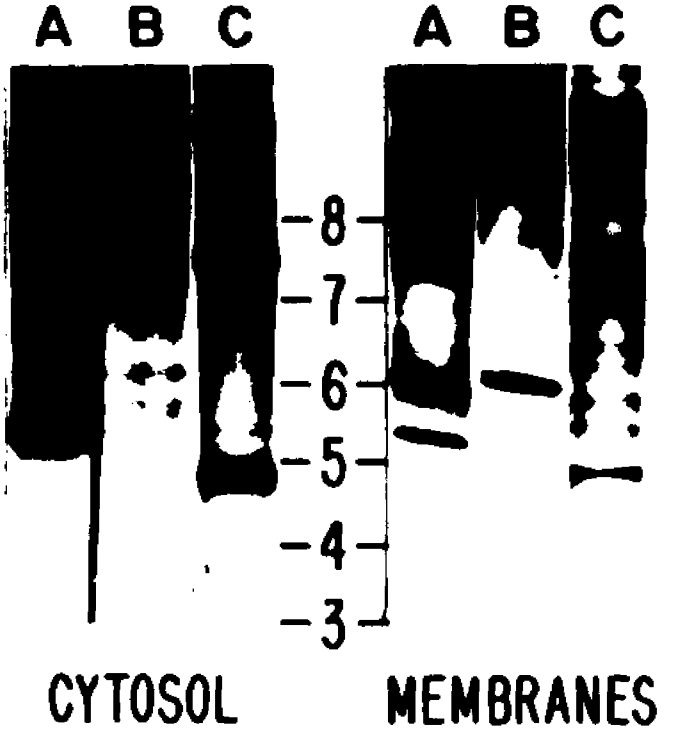

A B C
A B C
8
7
6
5
4
3
CYTOSOL
MEMBRANES

Fig. 12 PAGE isoelectric focusing patterns of E_2DH purified from placental cytosol and microvillar membranes by Sephadex G-100 fractionation

Isoelectric focusing was carried out on polyacrylamide disc gels. All gels to be stained with NBT reagent were subjected to prior treatment with 200 μM NAD^+ for 10 min to eliminate endogenous E_2 associated with E_2DH. (A) NBT staining in the presence of E_2; (B) NBT staining in the absence of E_2; (C) Coomassie protein stain.

7. Tests of estrogen binding to microvillar membranes

Placental microvilli are in direct contact with maternal blood (1). Since it is known that placenta secretes E_2 preferentially towards the maternal circulation (6,7), we investigated the possibility that receptors specific for E_2 exist in placental microvilli. Assays of the level of estrogen receptor in preparations of microvillar membranes were conducted. As can be seen from the results summarized in Table 7 we could not demonstrate high levels of specific binding of estrogen (total minus nonspecific) in preparations of microvillar membranes. The radioligands employed in this study were ^{125}I-E_2 and ^{3}H-E_3; both are compounds which are not significantly metabolized by placenta (162). These results are not surprising in light of the data obtained in other studies (126,127) in which negligible levels of specific binding of E_2 were found in nuclear and cytosolic preparations obtained from human term placenta.

D. Discussion

Microvillar membranes, a convenient source of syncytial plasma membrane for the study of levels and regulation of receptors and metabolic activities, were examined for their steroid metabolizing capability. The purity of these preparations could be evaluated by electron microscopy and by measuring levels of enzymatic markers for soluble cytoplasmic components (LDH), endoplasmic reticulum (NADPH-cytochrome c reductase) and mitochondria (succinate-cytochrome c reductase). The results of marker enzyme analysis revealed that soluble contamination of microvillar vesicles was completely lost by osmotic disruption during conversion to microvillar membranes (Table 1). In addition, the level of microsomal contamination of mi-

Table 7. Tests of Estrogen Binding to Microvillar Membranes

Preparations of microvillar membranes from human term placenta were incubated for 3 h at 30° C with ^{125}I-E_2 or ^{3}H-E_3 in the absence or presence of a 100-fold excess of unlabeled E_2. The levels of specific binding were determined following separation of bound and free estrogen by treatment with Dextran-coated charcoal.

Steroid	Label	Concentration	Conclusions
E_2	^{125}I	1-100 nM	Specific binding < 10-20%; levels of bound/free were identical for all bound values
E_3	^{3}H		

crosomal contamination of microvillar membranes could only account for a portion of microvillar E_1S and DHEAS sulfatase activities (Table 2).

Although only about 10% of the E_2DH activity in microvillar vesicles remained associated with the membranes after hypotonic disruption, the privileged location of this quantitatively minor activity at the site of entry and exit of androgens and estrogens into and out of the syncytium justifies special consideration.

Both microvillar membrane E_1S sulfatase and E_2DH activities were found to be resistant to tryptic inactivation under conditions in which cytosolic E_2DH activity was completely inactivated (Table 4), indicating that these activities are shielded by the microvillar membrane. However, requirement of the membrane-associated E_2DH for soluble cofactors to exert its action suggests that the catalytic portion of the enzyme protrudes from the membrane towards the cytoplasm. Further evidence as to the concealed nature of microvillar E_2DH was also suggested by the lack of inhibition of E_2DH activity in intact microvillar membranes following incubation with polyclonal antibody to purified cytosolic E_2DH from human placenta (Table 5). Once solubilized, the protective effect of the microvillar membrane was lost and E_2DH activity was inhibited. A model which depicts microvillar E_2DH as being associated with the inner surface of the microvillar membrane (i.e., facing the syncytial cytoplasm) could explain the lack of inhibition of microvillar E_2DH by trypsin and anti-E_2DH antibodies as well as fulfill the postulated need of an exposed site for cofactor binding.

An interesting phenomenon was noted during the course of incuba-

tion of the sodium cholate-solubilized microvillar E_2DH with antibody in that there was a tendency to lose a significant portion of its activity independent of its interaction with antibody, even in the presence of 25% glycerol, a known stabilizer of E_2DH activity (163). No such loss of cytosolic E_2DH activity was seen under identical conditions. This indicated that microvillar membrane E_2DH though antigenically indistinguishable from cytosolic E_2DH once solubilized, was not stable following removal from the membrane. Although washing of microvillar membranes with Triton X-100 and sodium chloride were effective means for removal of E_2DH activity from microvillar membranes (Table 3), the solubilized activity was not stable under these conditions. Although a significant loss of microvillar E_2DH activity was observed following solubilization by sodium cholate, the enzyme was obtained in sufficient quantities to carry out preliminary characterization by gel filtration chromatography and isoelectric focusing.

Comparison of pH dependence patterns, kinetic parameters, and molecular weight determinations revealed significant differences between microvillar E_2DH and the much studied cytosolic form of E_2DH.

The pattern of pH dependence of E_2DH activity in microvillar membranes revealed peaks of activity at pH 8.5 and 9.5 (Fig. 8), possibly reflecting ionization of exposed amino acids at the active site. Under identical conditions of study, this pattern was not observed for cytosolic E_2DH activity. The observed influence of buffer composition as well as osmotic strength (data not shown) on microvillar and not on cytosolic E_2DH activities, also indicated that the level of E_2DH activity in microvillar membranes was affected by the lipid-pro-

tein environment.

In the present study we have investigated the relative 17βOHSD activity towards E_2 and T as substrates in microvillar, cytosolic, microsomal, and mitochondrial preparations obtained from human term placenta (Table 6). Michaelis constants (K_m values) for T and E_2 as substrates were found to be much lower than the corresponding constants for the cytosolic activity, though considerably higher than the values determined for mitochondrial and microsomal forms of the enzyme. In addition, each placental subfraction displayed a unique ratio for the affinity of E_2 relative to that of T as substrate $(\frac{K_m\ E_2}{K_m\ T})$. Calculated values for the rate of utilization of E_2 as compared with T as substrate $(\frac{V_{max}\ E_2}{V_{max}\ T})$ were observed to be lower in microvillar membranes than the corresponding values for cytosolic 17βOHSD. One can calculate the efficiency of enzyme catalysis, i.e., $\frac{V_{max}}{K_m}$ (164). In the present study, the relative efficiency of dehydrogenation of E_2 as compared with T is expressed in the following ratio: $(\frac{V_{max}\ E_2}{K_m\ E_2} \Big| \frac{V_{max}\ T}{K_m\ T})$. The values obtained for the ratio of enzymatic activities in preparations of cytosol, microvilli, mitochondria, and microsomes were 200, 100, 2, and 0.4 respectively.

Based on kinetic studies in rabbit liver (112), human endometrium (108), porcine testes (117), and human placenta (3), it can be generally stated that particulate forms displaying 17βOHSD activity manifest similar activities towards a number of substrates (e.g., T, E_2, and 20αOHP) whereas the cytosolic form from human placenta is more narrow in its specificity. Results from the present study are consistent with this observation. Microvillar 17βOHSD activity was found to be relatively more active towards T as substrate as compared to the cytosolic

enzyme, and less active as compared to microsomal and mitochondrial forms of the enzyme.

A methodologic problem confronted in the present kinetic study deals with the fact that although most of the reported methods to measure placental 17βOHSD utilize E_2 as substrate at a nominal 50–100 μM concentration, the solubility of this steroid in aqueous solutions, even in the presence of 2% ethanol and 2 mg/ml of protein, is less than 20 μM (165), a level likely to be insufficient to saturate the enzyme, particularly in the cytoplasmic form (K_m, E_2 ∿ 11 μM, Table 6). This potential assay error was avoided by estimating V_{max} values from Lineweaver-Burke plots or by using single concentration (10 μM E_2) data and estimating V_{max} from known K_m values according to the formula shown under Materials and Methods.

A molecular weight of 137,000 was estimated for the major peak of microvillar E_2DH activity (Fractions 20-24, Fig. 10). The molecular weight of cytosolic E_2DH is known to be 68,000 composed of identical 34,000 molecular weight subunits (105,106). An attractive hypothesis is that the major microvillar activity is a tetramer with the minor peaks of 17βE_2DH activity (Fractions 29 and 34 respectively, Fig. 10), possibly representing dimeric and monomeric states of association of the enzyme. On the other hand, the presence of covalently bound lipid or carbohydrate residues on microvillar E_2DH could significantly alter its behavior on gel filtration columns. Either of these explanations could account for the different behaviors observed for cytosolic and microvillar enzymes during Sephadex G-100 fractionation. It is imperative to stress the fact that gel filtration chromotography of microvillar and cytosolic E_2DH was carried out under identical condi-

tions. The elution buffer was, in each case, PBS containing 25% glycerol and 0.3% sodium cholate detergent. The presence of detergent did not alter the profile of cytosolic E_2DH activity. However, in the absence of detergent solubilized microvillar E_2DH activity was lost (presumably through precipitation on the column) demonstrating the labile nature of solubilized microvillar E_2DH.

Similar isoelectric points were observed for the cytosolic and microvillar forms of E_2DH (Fig. 12). In each case only one specific band of activity at approximately pH 5.2 was revealed following staining with NBT. A complication was created by the presence of three NBT reducing bands both in the presence or absence of exogenous E_2, in the polyacrylamide gels after electrophoresis of purified cytosolic or membrane-associated enzyme. It was expected that some of these 3 bands would correspond to the enzyme bound to endogenous E_2. Consequently, we preincubated the gels with NAD^+ in order to convert bound E_2 to estrone before adding the NBT reagent. This precedure succeeded in revealing the specific E_2DH band (pI 5.2).

The fact that similar isoelectric points were observed for cytosolic and microvillar activities suggests that the membrane associated region of microvillar E_2DH does not significantly alter the overall charge on the enzyme. Perhaps isoelectric focussing over a narrower range of pH would reveal slight differences in isoelectric points.

Heterogeneity in 17βOHSD activity has been reported for soluble forms of the enzyme in guinea pig kidney (109), rabbit liver (112), and human endometrium (160), as well as as a microsomal form from pig testes (117). Antoun et al. (112) have recently reported the existence of three forms of soluble 17βOHSD in rabbit liver each possess-

ing different relative activities towards E_2 and T. In the present study, widely varying kinetic characteristics were observed for the dehydrogenation of E_2 and T in microvillar, cytosolic, microsomal, and mitochondrial fractions, suggesting that in human placenta 17βOHSD is isozymic.

It has been reported that estrogens circulate mainly as conjugates in maternal and fetal blood (166), and that quantitatively the most important estrogen precursors are fetal and maternal DHEAS and 16αOH DHEAS (38). In addition, in experiments in which perfusions of uteri (130) and placentas (131) with mixtures of labeled estrogens in both free and conjugated forms were conducted, it was ascertained from the levels of these compounds present in the tissue following perfusion, that the movement of steroids across cell membranes is diminished by conjugation. Therefore, the presence of microvillar membrane associated steroid sulfatases could be expected to influence the transfer of estrogens across the syncytium. This is particularly relevant in the case of E_1S, a major conjugated estrogen present in maternal blood during pregnancy (166), which has been reported to be cleared from maternal serum at a slow rate (64). In terms of estrogen biosynthesis during pregnancy, the utilization of sulfated C_{19} steroids as precursors for intrasyncytial aromatization would also be expected to be influenced by the presence of membrane-associated steroid sulfatases. Following aromatization, the potency of estrogen released by placenta to mother and fetus might be expected to be affected by the rates of interconversion of E_1 and E_2 by microvillar E_2DH.

IV. Placental Estrogen Synthesis and Release

A. Introduction

It is known that unconjugated estrogens pass through biological membranes quite easily, due to their lipophilic nature (130,131). In fact, most models that are employed to explain the action of estrogens at the tissue level depict the movement of estrogens through cell membranes by passive diffusion (129). With these ideas in mind, it is quite surprising that results from *in vivo* experiments in the Rhesus monkey indicated that the placenta preferentially secretes estradiol (E_2) to the maternal circulation, whereas estrone (E_1) is secreted in substantial quantities into both maternal and fetal circulations. If passive diffusion was the only mechanism involved in establishing the pattern of estrogen release by placenta, one would expect a similar distribution of E_1 and E_2 between fetal and maternal circulations following their intrasyncytial generation from C_{19} precursors.

In situ perfusion of the human placenta for research purposes has been forbidden on ethical grounds. The method of *in vitro* perfusion of human placenta offers an alternative and has been used recently by many investigators to study various aspects of placental function (143-146). Gurpide et al. (7) adopted a simple *in vitro* perfusion method originally described by Schneider et al. (142) to study the distribution of estrogens in the human term placenta. In experiments in which labeled E_1 and E_2 was generated within the syncytium from labeled C_{19} precursors during simultaneous "maternal" and "fetal" perfusions (7), it was reported that quite remarkably, the asymmetric patterns of estrogen secretion observed *in vivo* in the Rhesus monkey (16) was also seen *in vitro* in the human placenta. On the basis of

these findings it was suggested that the asymmetric pattern of placental estrogen secretion might arise through the action of E_2 specific carriers in the syncytiotrophoblast that release E_2 to the maternal circulation or through extrasyncytial interconversion of E_2 and E_1.

In the present study we further characterized the physiologic distribution of estrogens in the human term placenta through the use of in vitro perfusion with labeled estrogens or their precursors, ^{3}H-androstenedione (A) and ^{3}H-16αhydroxyandrostenedione (16αOHA), to simultaneously generate ^{3}H-E_1, ^{3}H-E_2, and ^{3}H-estriol (E_3) within the syncytium.

B. Materials and methods

1. Placental perfusion

Placentas were perfused as previously described (7). Intact placentas were obtained from the delivery room and brought immediately to the laboratory. A vein and an artery of a selected cotyledon were catheterized promptly. The perfused region, identified by the blanching effect of the buffer, was separated from the bulk of the placenta and clamped in a perfusion chamber designed by Schneider et al. (142). The input catheter (medical grade Teflon tubing, I.D. 0.034", Becton-Dickinson, Rutherford, NJ) was connected to a short section of silastic pump tubing (3/16", Dow Corning Corp., Midland, MI) passing through a peristaltic pump (Buchler Duostaltic Pump, Rainin, Ridgefield, NJ). The other end of the silastic tubing was connected to Teflon tubing leading to a reservoir with the buffer containing the labeled steroids for the "fetal" perfusion (i.e., perfusion through the fetal vessels). The tracer or mixture of tracers selected

for the perfusion was dissolved in a small volume of ethanol and added to the oxygenated Krebs-Ringer bicarbonate buffer containing 1 mg/ml glucose (KRBG). A pH of 7.4 was obtained by bubbling carbon dioxide. In some experiments, after approximately 1 h of perfusion, unlabeled EE was mixed with the labeled compounds to a final concentration of 10^{-5} M and the perfusion was continued. For the perfusion data presented in Figure 21 in the Results section, a protocol was adopted in which short term labeling with $^{3}H\text{-}E_2$ was carried out followed by an extended period of perfusion with unlabeled buffer and then unlabeled buffer plus 10^{-5} M EE. At different times during the perfusion, samples were taken from the Teflon tubing leaving the pump in order to correct for absorption of the tracers to the silastic tubing (167). A set of three 19-gauge needles was inserted superficially under the maternal decidua layer in the upper face of the cotyledon to perfuse buffer without labeled steroids ("maternal" perfusion). The needles were connected by silastic tubing, passing through another Buchler Duostaltic pump, to a reservoir with oxygenated KRBG buffer. The "maternal" perfusate leaving the placenta was carried by Teflon tubing from an outlet in the base of the perfusion chamber to the receiving flask. The entire set was placed in an incubation room at 37° C or in a temperature controlled cabinet (Jordon Scientific Products, Philadelphia, PA, Model FT-2TRG-BOD) kept at 37° C. A schematic representation of the perfusion system employed in the present study is shown in Figure 13.

Flow rates were about 5 ml/min for the "fetal" perfusate and about 10 ml/min for the "maternal" perfusate. Perfusions were carried out for 60-180 min and perfusates were collected in several 10-20 minutes fractions.

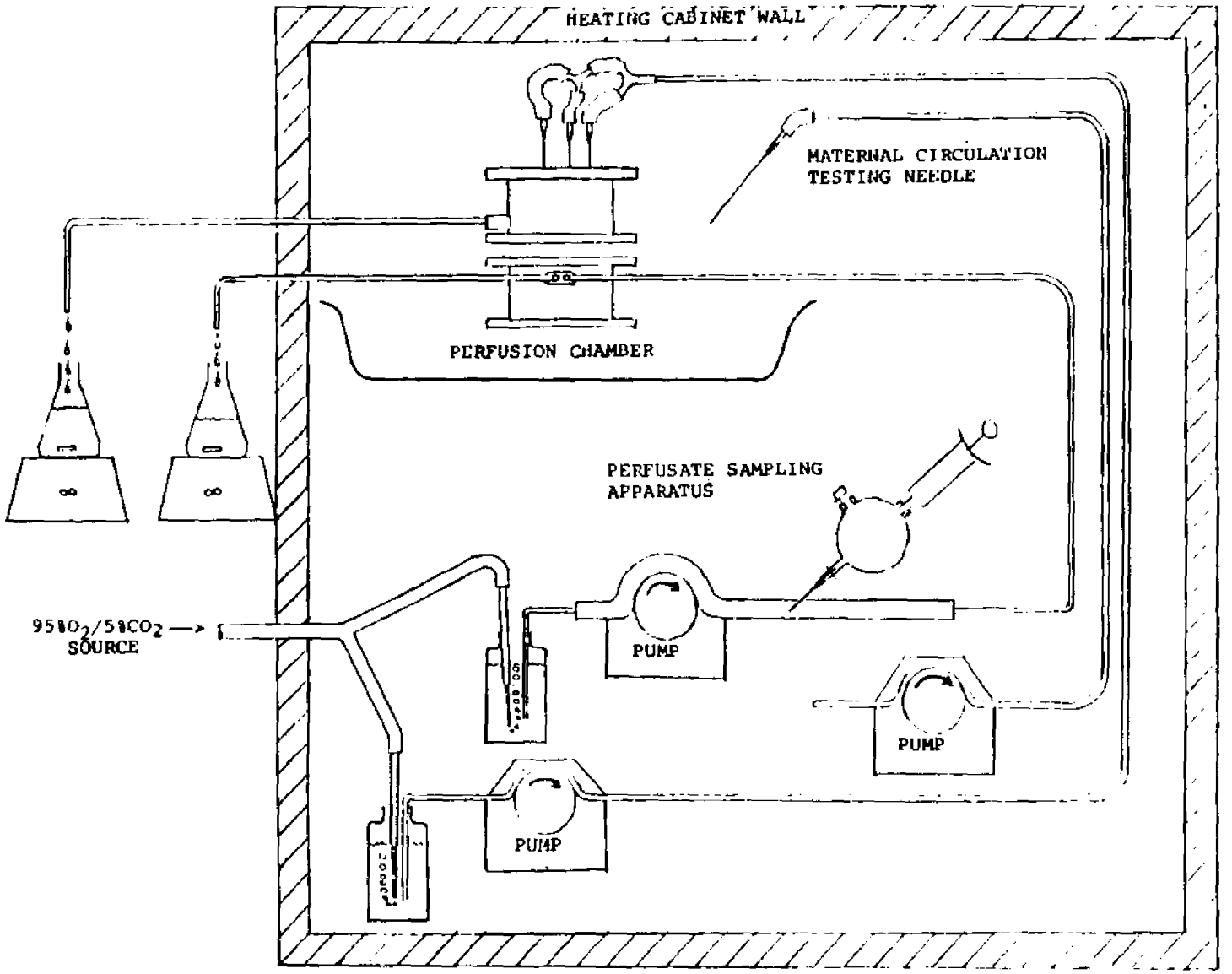

HEATING CABINET WALL
MATERNAL CIRCULATION TESTING NEEDLE
PERFUSION CHAMBER
PERFUSATE SAMPLING APPARATUS
95%O2/5%CO2 —>
SOURCE
PUMP
PUMP
PUMP

Fig. 13 Method of placental perfusion

Schematic diagram of the perfusion system employed in the present study. This type of perfusion was originally described by Schneider et al. (142) and was modified by Gurpide et al. (7). The method of placental perfusion employed in the present study is described in detail on pages 80-81.

2. Analytical procedures for the isolation and quantitation of labeled estrogens

In order to stop the action of enzymes which may leak from the placenta (140), each fraction of "maternal" and "fetal" perfusates was received in bottles containing ethyl acetate and carriers for the compounds to be isolated (200 µg for each androgen and progestin and 500 µg for each estrogen).

The aqueous layer was separated and measured. The ethyl acetate layer was washed with water, dried with anhydrous sodium sulfate, and evaporated to dryness. The residue was chromatographed on silica gel plates using System TCL-1 (Table 8). The bands corresponding to 16αHOA and E_3 were rechromatographed separately using Systems 2 and 3, respectively. Carriers were localized on the plates under u.v. light and losses during purification were estimated by spectrophotometric measurements at 240 or 280 nm (Beckman spectrophotometer, Model DU-7) using Allen corrections (168). Concentrations in the perfusates were corrected for losses and used to calculate output rates, expressed as percentages of the rates of precursor input.

The adequacy of the purification procedures was verified in some experiments by mixing each of the labeled estrogens obtained from the last chromatographic step with 10 mg of the corresponding carrier and crystallizing the mixture from chloroform-n hexane (E_1), ethyl acetate-n hexane (E_2) or methanol-benzene (E_3).

3. Preparation of tritiated 16αhydroxyandrostenedione

[6,7-^{3}H]-A, to be used in the preparation of [6,7-^{3}H]-16αOHA, was supplied by Amersham with a specific activity of 26 Ci/mmol. Its radiochemical purity was ascertained by mixing an appropriate amount of radioactivity with unlabeled A and pure 14-[^{4}C]-A and comparing

Table 8. Solvent Systems for Thin Layer Chromatography

Thin layer chromatography was carried out on Silica Gel GF plates (Analtech Inc., Newark, DE). Steroids were localized on the plates by u.v. light.

System #	Composition	Mobilities	
		Compound	R_F
TLC-1	Chloroform:Ethyl acetate 4:1	E_3	0.03
		16αHOA	0.24
		Testosterone	0.30
		E_2	0.48
		A	0.62
		E_1	0.78
TLC-2	nHexane:Ethyl acetate 1:2	16α-Hydroxy-progesterone	0.31
		16αHOA	0.62
		A	0.78
		Progesterone	0.83
TLC-3	Chloroform:Ethanol 9:1	E_3	0.42
		E_2	0.77
		16αHOA	0.81
		E_1	0.84
		A	0.90

$^{3}H/^{14}C$ ratios in the mixture and in A eluted after thin layer chromatography (System TLC-1, Table 8).

[6,7-^{3}H]-16αHOA was prepared by 16α hydroxylation of [6,7-^{3}H]-A. For this purpose, a culture of Streptomyces rosechromogenes, kindly supplied by Dr. Ross Dixon of Hoffman LaRoche, Nutley, NJ, was incubated with progesterone for 24 h at 25° C to induce 16αhydroxylase activity, and then with [6,7-^{3}H]-A for 8 h at 25° C. Optimal incubation times were determined by analysis of culture samples by thin layer chromatography (System TLC-2 for 16α hydroxyprogesterone and 16αHOA). The incubation mixture was filtered, extracted first with n-hexane and then with ethyl acetate. The ethyl acetate extract was chromatographed using System TLC-2 and the radiochemical purity of the isolated 16αHOA was established by adding carrier and comparing specific activities before and after chromatography. The yield of the preparation was 45%.

C. Results, placental estrogen synthesis and release

1. Distribution of labeled estrone, estradiol and estriol, in "maternal" and "fetal" perfusates during in vitro perfusion with labeled C_{19} precursors

a. Time course for the preparation of tritiated 16α-hydroxyandrostenedione

In light of previous success in this laboratory in employing the method of in vitro placental perfusion as a tool in the study of the patterns of release of estrogens in placenta, this method was adopted to further examine the patterns of estrogen distribution and estrogen binding in placenta not feasible through the use of placental subfractions.

The C_{19} precursor of E_3 in placenta during pregnancy is 16αOHA

which is aromatized to 16OHE$_1$, and reduced to form E$_3$ (38). As labeled 16αOHA is not commercially available, it was necessary to synthesize this compound in the laboratory. Figure 14 illustrates the results obtained for the production of ^{3}H-16OHA from ^{3}H-A using the microbe Streptomyces roseochromegenes. The "induced organism" (see Materials and Methods) was found to optimally convert ^{3}H-A to ^{3}H-16OHA after 8 h of incubation, and this time point was chosen for incubation of 1 mCi of ^{3}H-A from which 0.45 mCi of ^{3}H-16OHA was generated for use in perfusions.

b. Steady state outputs of labeled estrogens

Table 9 shows the results obtained from experiments in which placentas were perfused with mixtures of ^{3}H-A and ^{3}H-16αOHA. The values presented in this Table were calculated from the concentrations obtained during the last period of collection of perfusate, near an isotopic steady state. All values listed under the heading of "output rate" represent the percentage of isotope in the radioactive precursor appearing in each of the three estrogens in "maternal" and "fetal" perfusates.

c. Time course for the release of estrogens

It is evident from the results illustrated in Figure 15, that of the three labeled estrogens generated within the syncytium during perfusion, E$_3$ approached a steady state much faster than E$_1$ or E$_2$.

d. Flow rates during perfusions

From the results displayed in Figure 16, it was demonstrated that perfusion rates could be kept constant during a 2 h period of perfusion. Results from other studies have revealed that perfusion of placenta for as long as 4 h with isotonic buffer does not significantly alter placental function as judged by analysis of structural integrity

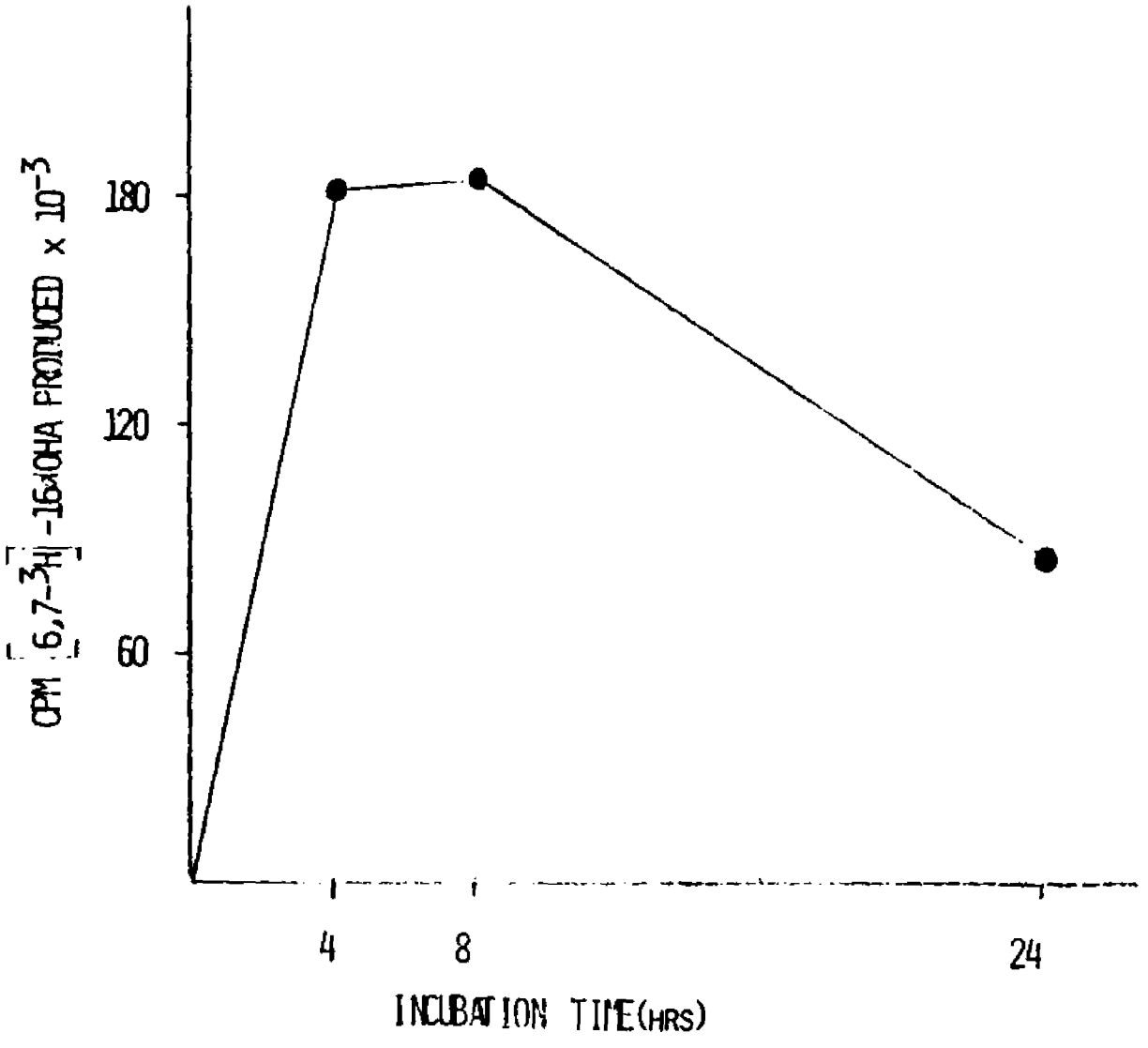

CPM [6,7-3H]-16-OHA PRODUCED x 10-3
180
120
60
4
8
24
INCUBATION TIME(HRS)

Fig. 14 Preparation of tritiated 16α-hydroxyandrostenedione

Tritiated 16αOHA was prepared by incubating ^{3}H-A with the microbe Streptomyces roseochromogenes which had been induced by prior exposure to progesterone. Optimal incubation times were determined following analytical procedures as described in Materials and Methods.

Table 9. Conversion of Perfused C_{19} Precursors to Estrone, Estradiol and Estriol

Labeled estrogen precursors were perfused through fetal vessels as described in Materials and Methods. The rate of output of each labeled estrogen is expressed as a percentage of the input rate of labeled precursor. Output rates listed in Perfusions, "a", correspond to those fractions obtained after a steady state has been achieved (∿60-90 min). Data listed in Perfusions "b" correspond to those fractions obtained 60-90 min following addition of EE to the labeled perfusion medium.

Perf. No.	Duration (min)	Flow rate (ml/min) Fetal	Flow rate (ml/min) Maternal	Labeled Precursor	Addition (10^{-5} M)	Input rate (dpm/min)x10^6	Output rate (% of input rate) Compound	Fetal	Maternal	Total
1	150	4.4	9.2	^{3}H-A	-	0.14	E_1	45	15	60
							E_2^1	2.9	2.9	5.9
				^{3}H-16αHOA	-	0.54	E_3^2	52	13	65
2	180	3.5	10	^{3}H-A	-	0.19	E_1	25	14	39
							E_2^1	1.1	7	8.1
				^{3}H-16αHOA	-	0.63	E_3^2	36	44	80
3a	90	4.8	9.3	^{3}H-A	-	0.12	E_1	26	14	40
							E_2^1	2.3	2.1	4.4
				^{3}H-16αHOA	-	0.86	E_3^2	57	32	89
3b	90			^{3}H-A	EE	0.12	E_1	18	22	40
							E_2^1	27	4.2	31
				^{3}H-16αHOA	EE	0.86	E_3^2	43	37	80
4a	60	5.0	10	^{3}H-16αHOA	-	1.1	E_3	58	44	102
4b	48			^{3}H-16αHOA	EE	1.1	E_3	53	46	99

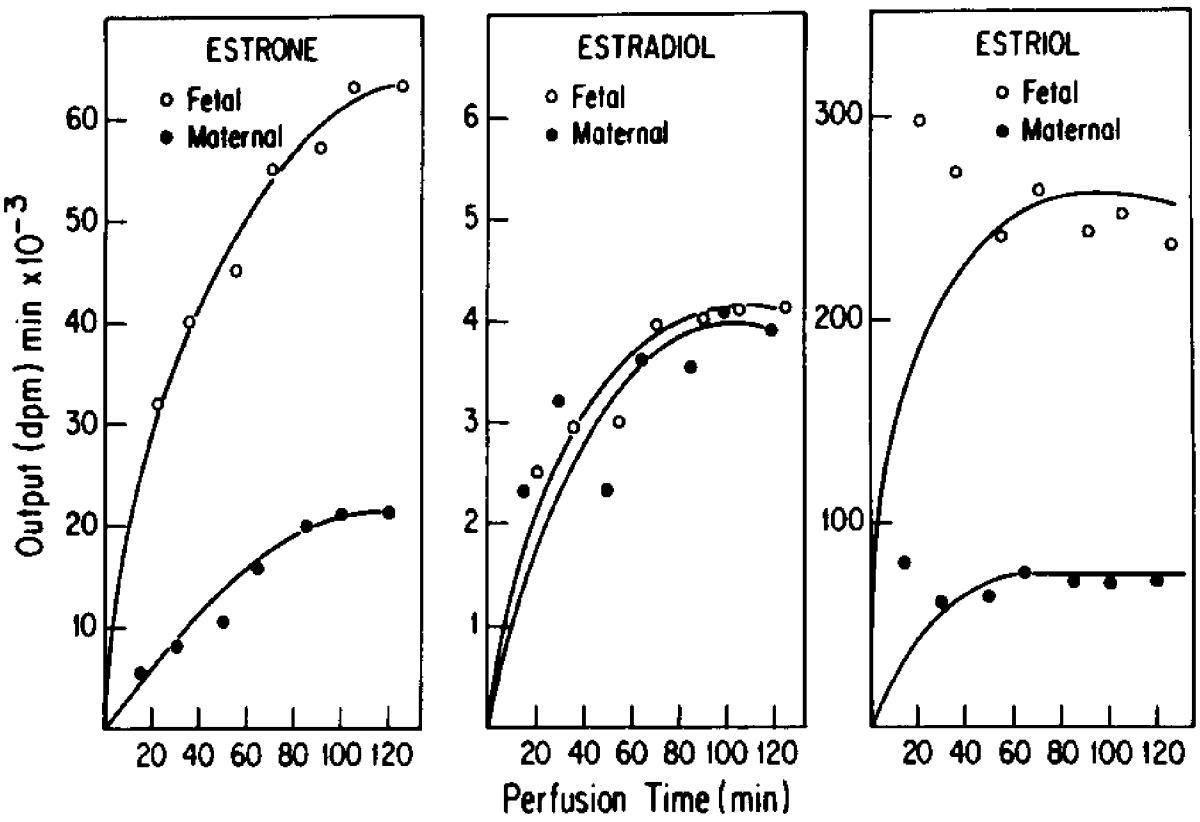

ESTRONE
ESTRADIOL
ESTRIOL
Fetal
Maternal
Output (dpm) min x10⁻³
Perfusion Time (min)
10
20
30
40
50
60
1
2
3
4
5
6
100
200
300
20 40 60 80 100 120

Fig. 15 Time course for the release of estrone, estradiol, and estriol

Concentrations of labeled E_1, E_2 and E_3 in "fetal" and "maternal" perfusates during perfusion of term placenta with mixtures of ^{3}H-A and ^{3}H-16αHOA (perfusion #1).

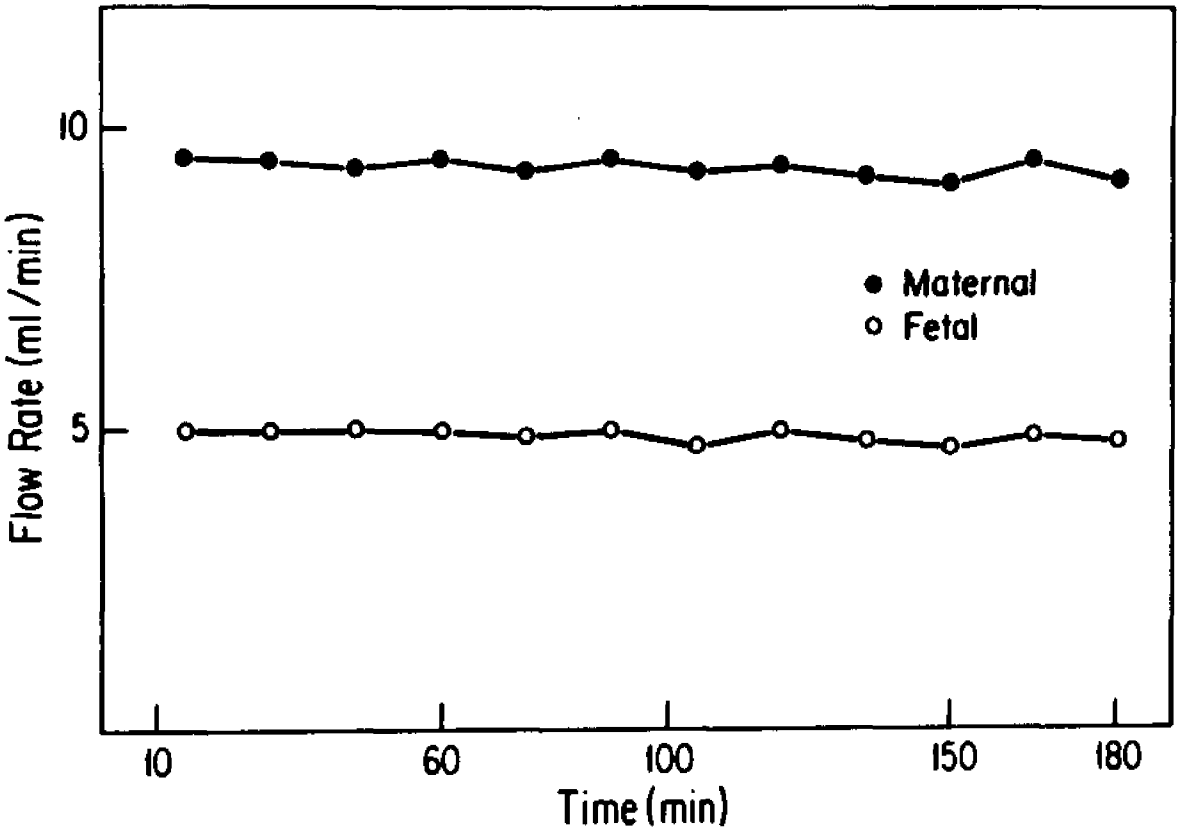

10
5
Flow Rate (ml/min)
Maternal
Fetal
10
60
100
150
180
Time (min)

Fig. 16 Flow rates during perfusion

Rates of flow of "fetal" and "maternal" perfusates during perfusion of term placenta with Krebs-Ringer bicarbonate buffer at 37° C (perfusion #3).

and measurement of enzymatic activities of placenta following perfusion (137,141).

e. Pattern of distribution of labeled estrogens

The distribution of E_1, E_2 and E_3 between "fetal" and "maternal" perfusates, estimated from the data shown in Table 9, are presented in graphic form in Figure 17. Displacement of the horizontal bars with respect to the ordinate axis indicates preferential release of the estrogen represented by the corresponding bar towards the "fetal" (left) or "maternal" (right) perfusates. In confirmation of results reported in a previous publication (7), it is apparent from Figure 17 that E_2 has a greater tendency than E_1 to appear in the "maternal" perfusate. Estriol shows a pattern of distribution closer to that of E_1 and clearly different from that of E_2.

2. Effect of competitors of estrogen binding on release of labeled estrogens generated during <u>in vitro</u> perfusions

a. Effect of ethynyl estradiol on the release of labeled estrone, estradiol and estriol to "maternal" and "fetal" perfusates

Table 9 also presents information on the extent of recovery of perfused precursors as labeled estrogens. The total recovery of perfused A as E_1 and E_2 ranged from 44 to 60% and was increased by the addition of ethynyl estradiol (EE), a competitor of estrogen binding (169), to the perfusion medium containing labeled precursor. This change was mainly due to an increase in the amount of ^3H-E_2 appearing in the "fetal" perfusate. The total recovery of ^3H-16αOHA to ^3H-E_3 ranged from 65 to 100% and was not increased by the addition of EE to the perfusion medium. Figures 18 and 19 illustrate the results obtained in Perfusion #3. The effect of adding EE (10^{-5} M) on the distribution of the estrogens under study is also illustrated in Figure 17.

DISTRIBUTION of E_2, E_1 and E_3 DERIVED from ^{3}H-A and ^{3}H-16α HOA

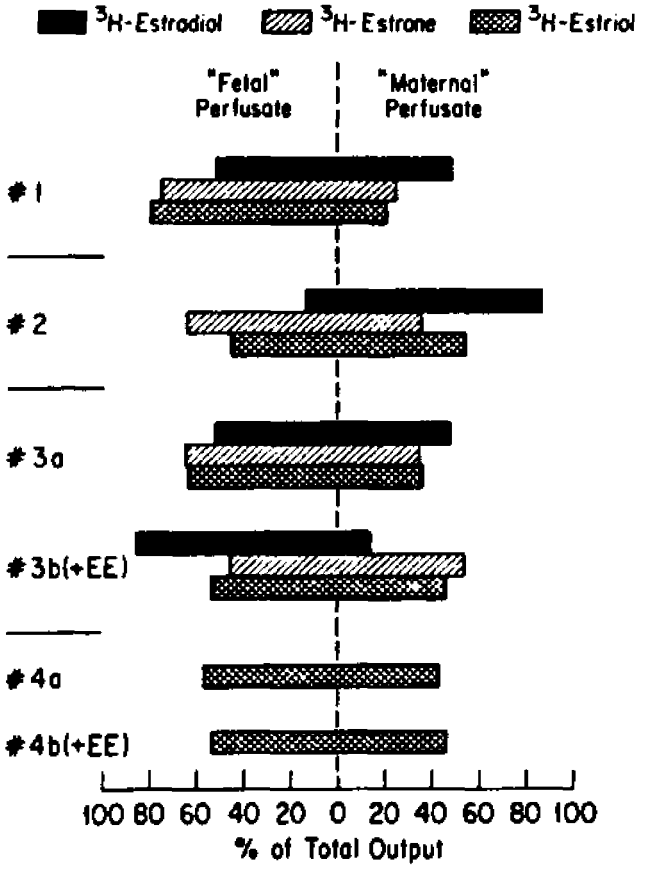


Fig. 17 Pattern of distribution of labeled estrogens

Distribution of labeled E_1, E_2 and E_3 derived from ^{3}H-A or ^{3}H-16αHOA during perfusion of term placentas. Bars indicate the percentages of total steady output of each compound in "fetal" and "maternal" perfusates. Perfusions #3b and #4b present results obtained when 10^{-5} M EE was added to the buffer with the labeled precursors after an initial perfusion period (see Table 9).

Experiment # 3

[6,7 – ^{3}H] 16α Hydroxyandrostenedione Perfusion

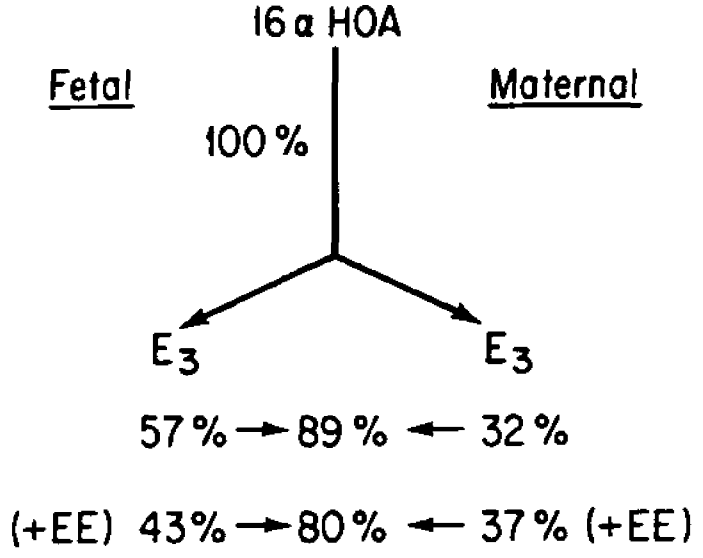


Fig. 18 Percentage of perfused ^{3}H-16α OHA recovered as ^{3}H-E_3 in the "fetal" and "maternal" perfusates at the steady state

This figure depicts the results obtained in Perfusion #3 (see Table 9).

Experiment #3

[6,7 - ^{3}H] Androstenedione Perfusion

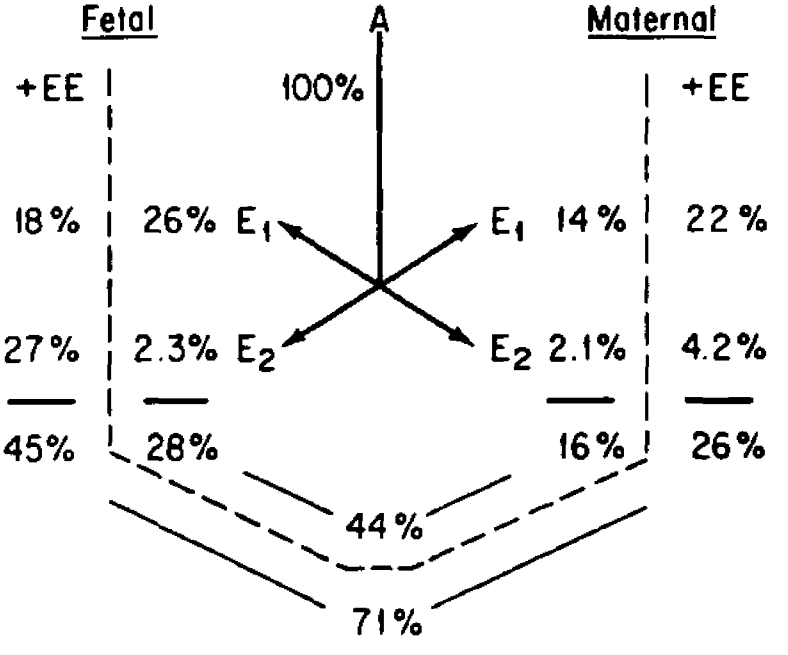


Fig. 19 Percentage of perfused ^{3}H-A recovered as ^{3}H-E_1 and ^{3}H-E_2 in the "fetal" and "maternal" perfusates at the steady state

As in Figure 18, this Figure depicts the results obtained in Perfusion #3 (see Table 9).

The presence of EE provoked a drastic shift of E_2 from the "maternal" to the "fetal" perfusate. In contrast, the effects of EE on the pattern of distribution of E_1 or E_3 were much smaller.

b. Steady state data on the effect of addition of competitor

The response obtained following the addition of EE and other competitors of estrogen binding to the perfusion medium containing labeled steroid was examined in more detail and the results are summarized in Table 10. In these experiments a variety of labeled precursors (as well as labeled E_2) were perfused for approximately 60 min in the absence of competitor (perfusions "a"). At this time 10^{-5} M competitor was added to the labeled perfusion medium and values expressed for perfusions "b" represent outputs observed 30 min following addition of competitor. The data obtained from these perfusions indicate that the primary effect of adding competitor to the perfusion medium containing labeled steroid is to facilitate a large release of ^3H-E_2 to the "fetal" perfusate irrespective of the precursor or the competitor of E_2 binding. This phenomenon is exemplified by the 200-1200% increase observed in the levels of ^3H-E_2 released to the "fetal" perfusate following addition of competitor. Levels of output of ^3H-E_1 into "fetal" and ^3H-E_1 and ^3H-E_2 into "maternal" perfusates were only slightly affected by the addition of competitor.

c. Time course of the release of labeled estradiol to "fetal" perfusate in response to ethynyl estradiol

The release of ^3H-E_2 to the "fetal" perfusate in response to EE is shown in Figure 20. The maximum rate of release of ^3H-E_2 to "fetal" perfusate was obtained 30 min following addition of EE and remained at this elevated level for 1½ h.

Table 10. Effect of Ethynyl Estradiol on the Release of Labeled Estrone and Estradiol, to "maternal" and "fetal" perfusates

Perfusions were conducted as described in the legend of Table 9, with the exception that the values listed in Perfusions "b" correspond to those fractions obtained 30 min following addition of competitor.

Perf. #	Labeled Precursors	Input Rate Dpm/min x 10^{-6}	Addition 10^{-5}M	Output Rate (% Input) Compound	Fetal	Maternal	% Change Fetal	Maternal
5a	^{3}H-A	0.14	-	E_1	22	3.6		
				E_2	1.7	2.1		
5b	^{3}H-A	0.14	EE	E_1	19	4.9	-14	36
				E_2	22	5.3	1200	150
6a	^{3}H-A	0.12	-	E_1	25	13		
				E_2	2.6	2.0		
6b	^{3}H-A	0.12	EE	E_1	19	21	-24	62
				E_2	31	3.0	1100	50
7a	^{3}H-E_2	0.11	-	E_1	31	3.2		
				E_2	1.8	1.8		
7b	^{3}H-E_2	0.11	EE	E_1	25	5.5	-19	72
				E_2	22	3.6	1100	100
8a	^{3}H-T	0.40	-	E_1	18	0.9		
				E_2	1.9	0.5		
8b	^{3}H-T	0.40	EE	E_1	12	2.9	-33	220
				E_2	12	0.3	530	-40
9a	^{3}H-A	0.10	-	E_1	22	2.8		
				E_2	2.9	0.2		
9b	^{3}H-A	0.10	DES	E_1	22	3.6	0	29
				E_2	9.8	0.3	240	50
10a	^{3}H-A	0.09	-	E_1	8.3	1.9		
				E_2	0.7	1.0		
10b	^{3}H-A	0.09	Δ	E_1	9.6	2.0	16	5
				E_2	5.1	2.2	630	120

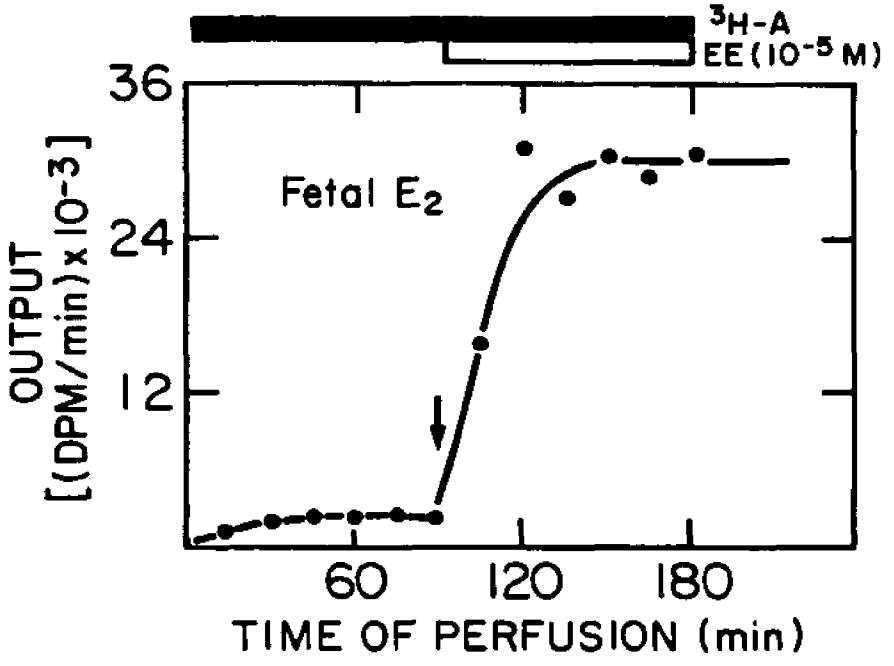

^{3}H-A
EE (10^{-5} M)
36
24
12
OUTPUT
[(DPM/min) x 10-3]
Fetal E_2
60
120
180
TIME OF PERFUSION (min)

Fig. 20 Time course for the release of labeled E_2 to the "fetal" perfusate in response to EE

Perfusion of ^{3}H-A was conducted for 90 min in the absence of EE and for an additional 90 min in the presence of EE (Perfusion #6, Table 10). At 15 min intervals the concentration of ^{3}H-E_2 appearing in the "fetal" perfusate was determined following analytical procedures. A summary of the perfusion protocol is presented in graphic form at the top of the Figure. The arrow indicates the point at which EE was added to the labeled perfusion buffer.

d. Formation of water soluble metabolites

In contrast to the pronounced effect EE had upon the release of $^{3}H\text{-}E_2$ to the "fetal" perfusate, the addition of EE had virtually no effect upon the release of water soluble counts to "fetal" and "maternal" perfusates (Table 11).

e. The fetal capillary as the postulated site of action of ethynyl estradiol

1) Adoption of a modified perfusion protocol

Employment of a perfusion protocol in which labeled steroids and competitors of E_2 binding are present in the perfusion medium is limited in its scope in that under these conditions a saturable binding and a complete release of $^{3}H\text{-}E_2$ to the "fetal" perfusate in response to EE could not be demonstrated. For this reason a perfusion scheme was adopted in which short term labeling with $^{3}H\text{-}E_2$ was carried out followed by an extended period of perfusion with unlabeled buffer and then unlabeled buffer plus 10^{-5} M EE. Under these conditions a large and finite release of $^{3}H\text{-}E_2$ to the "fetal" perfusate was observed in response to EE (Fig. 21). Approximately an 11-fold increase in the rate of output of $^{3}H\text{-}E_2$ into the "fetal" perfusate was noted within 20-30 min after addition of EE, a finding which is almost identical to the results obtained in experiments in which EE was added to the perfusion medium containing $^{3}H\text{-}E_2$ (Table 10, Perf. #7). These results suggest the presence of specific binders of E_2 in or around the region of the fetal capillary (e.g., capillary endothelium) which release bound $^{3}H\text{-}E_2$ in response to the addition of EE. The observed lack of release of $^{3}H\text{-}E_1$ to "fetal" and "maternal" perfusates as well as the slight and delayed release of $^{3}H\text{-}E_2$ to "maternal" perfusate in response to EE (Fig. 21) supports such a hypothesis. However, the

Table 11. Formation of Water Soluble Metabolites from Labeled Precursors in the Absence and Presence of Ethinyl Estradiol

Perfusion numbers refer to the experiments listed in Table 10. Perfusates were washed three to four times with ethyl acetate prior to measurement of radioactivity in a 1 ml aliquot using Dimiscint as the scintillant.

Perfusion #	Output of H_2O Soluble metabolites (% Input)		Total
	Fetal	Maternal	
5 a	8.1	9.2	17.3
b	6.4	8.6	15.0
7 a	12	8.1	20.1
b	10	5.2	15.2

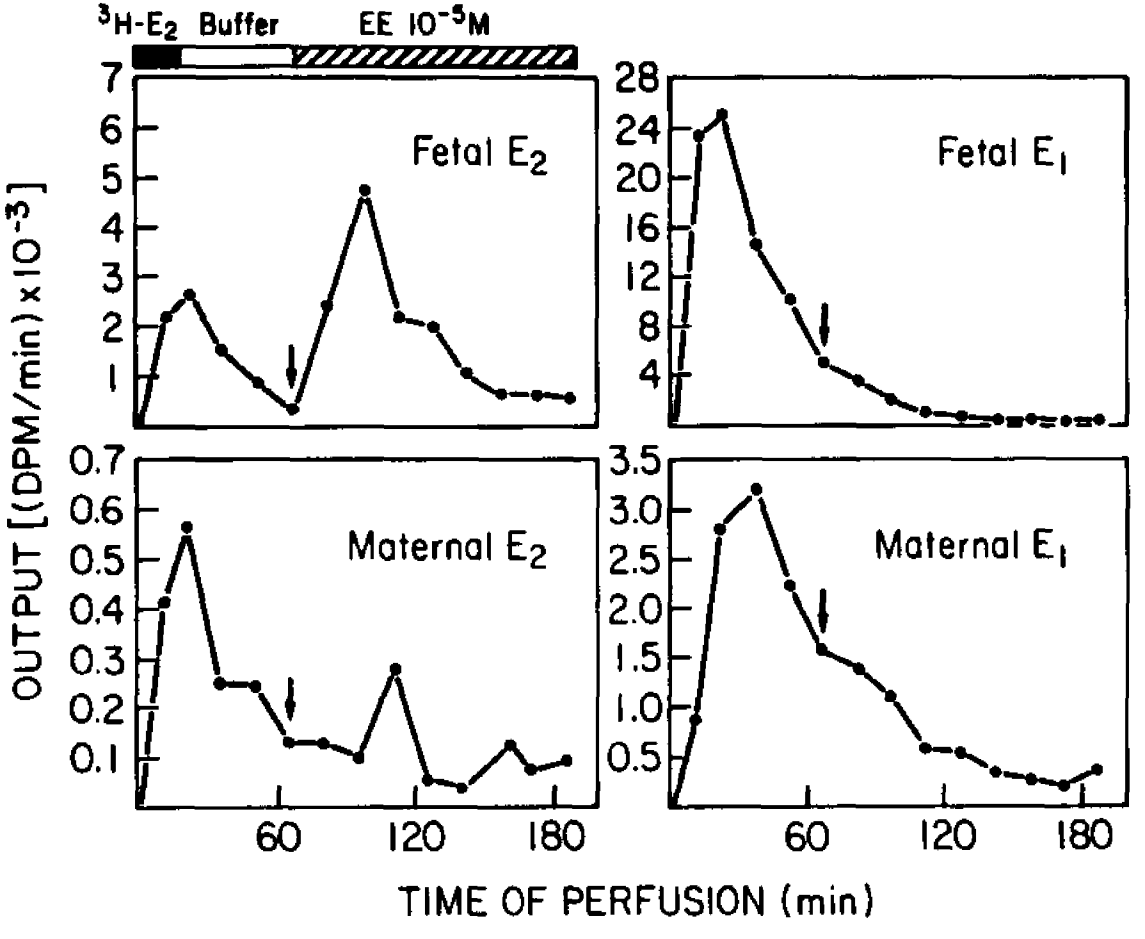

^{3}H-E$_2$ Buffer
EE 10^{-5}M
Fetal E$_2$
Fetal E$_1$
Maternal E$_2$
Maternal E$_1$
OUTPUT [(DPM/min) x 10^{-3}]
TIME OF PERFUSION (min)
7
6
5
4
3
2
1
28
24
20
16
12
8
4
0.7
0.6
0.5
0.4
0.3
0.2
0.1
3.5
3.0
2.5
2.0
1.5
1.0
0.5
60
120
180
60
120
180

Fig. 21 Ethynyl estradiol induced release of labeled E_1 and E_2 to "fetal" and "maternal" perfusates following perfusion with labeled E_2

A different perfusion protocol than previously employed was adopted to examine the release of ^3H-E_2 to the "fetal" perfusate. Short term perfusion (15 min) with ^3H-E_2, followed by a longer period of perfusion with unlabeled buffer (50 min) and then unlabeled buffer containing 10^{-5} M EE, was carried out. The concentration of labeled E_1 and E_2 appearing in "fetal" and "maternal" perfusates was then determined. A summary of the perfusion protocol is presented in graphic form at the top of the Figure. The arrow indicates the point at which EE was added to the unlabeled perfusion buffer.

lack of release of $^{3}H\text{-}E_3$ to "fetal" perfusate in response to EE (Table 9, Fig. 17) suggests that the E_2 binder under consideration is not the typical estrogen receptor found in other tissues (e.g., endometrium) but rather a binding protein with high specificity for E_2.

2. Balance of counts during perfusion

On the basis of the radioactivity appearing as E_1 and E_2 and as water soluble compounds following perfusion with $^{3}H\text{-}E_2$ (Table 12), it was concluded that a complete balance of counts was obtained in this experiment.

D. Discussion/Model of estrogen synthesis, metabolism, and secretion in placenta

In the present study we have perfused placentas with mixtures of ^{3}H-16αOHA and ^{3}H-A in order to compare the pattern of distribution of labeled E_3 with that of E_2 and E_1 (Table 9). The employment of a perfusion protocol which features simultaneous generation of all three estrogens within the syncytium in order to study the pattern of release of estrogens within placenta is novel in its approach. It should be noted that only comparisons of patterns of release of each estrogen are meaningful in these <u>in vitro</u> studies since rates of output into "fetal" and "maternal" perfusates may depend on the efficiency of the "maternal" perfusion, which is expected to differ in each perfusion.

Graphic representation of data obtained when placentas were perfused with mixtures of ^{3}H-16OHA and ^{3}H-A to generate $^{3}H\text{-}E_3$, $^{3}H\text{-}E_2$, and $^{3}H\text{-}E_1$ within the syncytium (Fig. 17) revealed that the distribution of E_3 was similar to that of E_1 but clearly different to that of E_2.

Table 12. Balance of Counts Obtained During Perfusion Featuring Short-Term Labeling with Estradiol

The data displayed in this Table represents the total radioactivity appearing as estrogen and water soluble metabolites in "fetal" and "maternal" perfusates during the experiment described in Fig. 21.

Total DPM $^{3}H\text{-}E_2$ Perfused ($\times 10^{-6}$)	Output (Total DPM $\times 10^{-6}$)			
	Fraction	Fetal	Maternal	Σ
2.00	E_1	1.06	0.21	1.27
	E_2	0.33	0.03	0.36
	H_2O soluble	0.31	0.14	0.45
				2.08

During *in vitro* placental perfusion in which ^{3}H-A or ^{3}H-T were employed (7), it was observed that higher ratios of $^3H\text{-}E_2/^3H\text{-}E_1$ were found in the "maternal" perfusate than in the "fetal" perfusate. This data supported the *in vivo* observation in the Rhesus monkey that the placenta preferentially secretes E_2 towards the maternal circulation and E_1 towards the fetal circulation (6). In the present study in which $^3H\text{-}E_3$ as well as $^3H\text{-}E_2$ and $^3H\text{-}E_1$ were generated $(^3H\text{-}E_2/^3H\text{-}E_1)_{maternal} \Big| (^3H\text{-}E_2/^3H\text{-}E_1)_{fetal}$ cytium the values obtained for the ratio were 3, 11, and 2 in perfusions 1, 2, and 3a respectively (Table 9). This indicated that the previous pattern of asymmetry observed for the distribution of $^3H\text{-}E_1$ and $^3H\text{-}E_2$ (7) still existed under these perfusion conditions. The time course of the approach to steady state for each of the three estrogens labeled estrogens (Fig. 15) showed that the production of $^3H\text{-}E_3$ assumed a steady state distribution much more rapidly than that of E_1 or E_2. The observation of a unique time course for the generation of E_3 is not unexpected as the pool of E_3 within placenta should not be expected to reflect changes in the pool of E_1 and E_2, in that the placenta does not contain the ability to hydroxylate steroids at the 16α position and thus cannot convert E_1 or E_2 to E_3 (139).

The efficiency of conversion of 16αOHA to E_3 under the perfusion conditions is remarkable. The complete conversion obtained in Perf. #4, Table 9, indicates that all of the perfused steroid can leave the fetal capillaries and be exposed to the aromatase system. This is consistent with the observation that the rate of entry of unconjugated steroids into placenta is high in relation to that of steroid sulfates or glucuronides (131).

In the present experiments incomplete conversion of ^{3}H-16OHA to ^{3}H-E_3 or ^{3}H-A to ^{3}H-E_1 or ^{3}H-E_2 might be due to formation of other metabolites, such as catechol estrogens or water soluble estrogen conjugates. In fact, in perfusions in which the placenta was perfused with ^{3}H-A or ^{3}H-E_2 a significant portion (~20%) of the total input appeared as water soluble counts in "fetal" and "maternal" perfusates (Table 11). The identity of specific water soluble metabolites formed during perfusion remains to be elucidated.

It has been suggested that estrogen binding proteins are in some way responsible for the observed pattern of preferential placental secretion of E_2 towards the maternal circulation observed in vivo (6), and can account for the higher ^{3}H-E_2/^{3}H-E_1 ratios found in "maternal" perfusate relative "fetal" perfusate observed during in vitro perfusion (7). If a classical estrogen receptor mechanism is to be implicated in the observed pattern of distribution of this hormone, it follows that it would be expected in the present study that addition of EE, an inhibitor of estrogen binding, to the labeled perfusion medium should significantly affect the distribution of ^{3}H-E_2 between "fetal" and "maternal" perfusates. This anticipated effect was obtained as the addition of EE provoked a very large release of ^{3}H-E_2 to the "fetal" perfusate (Tables 9 and 10, and Fig. 17). If a classical estrogen receptor mechanism were involved in establishing the pattern of estrogen distribution by placenta, it would also be anticipated that EE should affect the release of labeled E_3 during perfusion, as E_3 is known to bind to estrogen receptor (170). Since EE did not change the pattern of distribution of labeled E_3 between "fetal" and "maternal" perfusates (Table 9, Fig. 17), one can hypothe-

size that E_2 specific binding proteins distinct from classical estrogen receptors are involved in establishing the pattern of estrogen distribution by placenta. The distribution of labeled E_1 during perfusion was not affected by the addition of EE (Tables 9 and 10, Fig. 17), which is consistent with the postulation of either classical estrogen receptors or E_2 specific binding proteins. The distribution of water soluble counts during two perfusions (Table 12) was only slightly affected by the addition of EE, possibly reflecting the low affinity of estrogen binders for these compounds.

The effect of adding excess EE to the labeled perfusion buffer and the resulting changes produced in the distribution of ^3H-E_2 and ^3H-E_1 between "fetal" and "maternal" perfusates are summarized in Table 10 (Perfusions 5-10). Irrespective of the labeled precursor that was employed to generate labeled E_1 and E_2 within the syncytium the result was the same; the predominant effect observed was the release of ^3H-E_2 to the "fetal" perfusate following addition of EE. Thus, the particular pathway of syncytial aromatization/oxidation-reduction that leads to that fraction of ^3H-E_2 that can potentially be released to the "fetal" perfusate in response to EE is irrelevant. In addition, a specific release of ^3H-E_2 to the "fetal" perfusate was achieved following addition of diethylstilbestrol (DES) or A to the labeled perfusion buffer (Table 10, Perfusions 9 and 10). In the perfusion system employed, both of these compounds would be expected to compete with ^3H-E_2 for estrogen binding sites; DES by virtue of its known action as a competitor of estrogen binding (169), and A following its aromatization to E_1 and reduction to E_2. For all of the different combinations of estrogen precursors and competitors tested, a

maximum release of $^{3}H\text{-}E_2$ to the "fetal" perfusate was achieved within 30 min following addition of competitor (Fig. 20) and was maintained at an elevated level for the duration of perfusion.

A perfusion protocol was adopted that enabled us to demonstrate a complete and specific release of labeled E_2 to the "fetal" perfusate following addition of EE (Fig. 21). Labeling of postulated placental intrinsic binding sites for E_2 was accomplished through short term perfusion with $^{3}H\text{-}E_2$. A wash with unlabeled buffer was then conducted in the hope that this procedure would remove unbound labeled estrogens. This procedure was followed by addition of excess EE to the unlabeled perfusion buffer. Thus, under these conditions of perfusion any release of label observed in response to EE most likely represents a liberation of "tightly" bound counts. It should be noted that during assays of estrogen receptor extensive washing of receptor-steroid complex has been used in lieu of charcoal adsorption or hydroxylapatite methods for the partition of free and bound steroid (171). As shown in Figure 21 the primary effect of adding EE was to provoke a large and complete release of $^{3}H\text{-}E_2$ to the "fetal" perfusate. The small and delayed release of $^{3}H\text{-}E_2$ to the "maternal" perfusate observed under these conditions could be the result of trans-syncytial diffusion of $^{3}H\text{-}E_2$ from the area of the fetal capillary.

In light of these findings, we postulate that there exist in the region of the fetal capillary, binders for E_2 which release $^{3}H\text{-}E_2$ in response to the addition of EE during perfusion. Since classical estrogen receptors are known to bind E_3 as well as E_2 (169,170), in the present study the lack of release of $^{3}H\text{-}E_3$ to the "fetal" perfusate in response to EE (Table 9 and Fig. 17) indicates that we are

not dealing with a typical estrogen receptor, but perhaps with an E_2 specific binding protein.

The most likely cell type involved in these binding events is that of the capillary endothelium, i.e., a tissue layer which is comprised of polygonal cells which are in direct contact with the capillary lumen (172).

A possible model which could account for the pattern of release of estrogens observed in the present study is depicted in Figure 22. In the model the endothelial and microvillar surfaces are denoted by the abbreviations ES and MS respectively. Labeled syncytial E_2 in transit to the fetal capillary is postulated to combine with an endothelial binding protein (rectangle, Fig. 22). The binding of ^3H-E_2 at this site is hypothesized to facilitate dehydrogenation by 17βE_2DH (triangle, Fig. 22), which is followed by the release of ^3H-E_1 to the "fetal" perfusate. In this manner, the addition of EE would provoke a release of ^3H-E_2 from postulated endothelial receptors to the "fetal perfusate". The postulation of E_2DH activity associated with a binding event is necessitated by previous evidence that the placenta preferentially secretes E_2 towards the maternal circulation whereas E_1 is secreted in substantial quantities to the fetal circulation (6). Thus, high levels of E_1 found in the fetal circulation during in vivo studies would be accounted for in the present study in light of the proposed model by binding and subsequent dehydrogenation of E_2 at the level of the fetal capillary. The linkage between a binding event and enzymatic dehydrogenation is not a novel idea, as it has been postulated to explain binding and transport phenomena of glucocorticoids noted during in vitro studies in human term placenta (31).

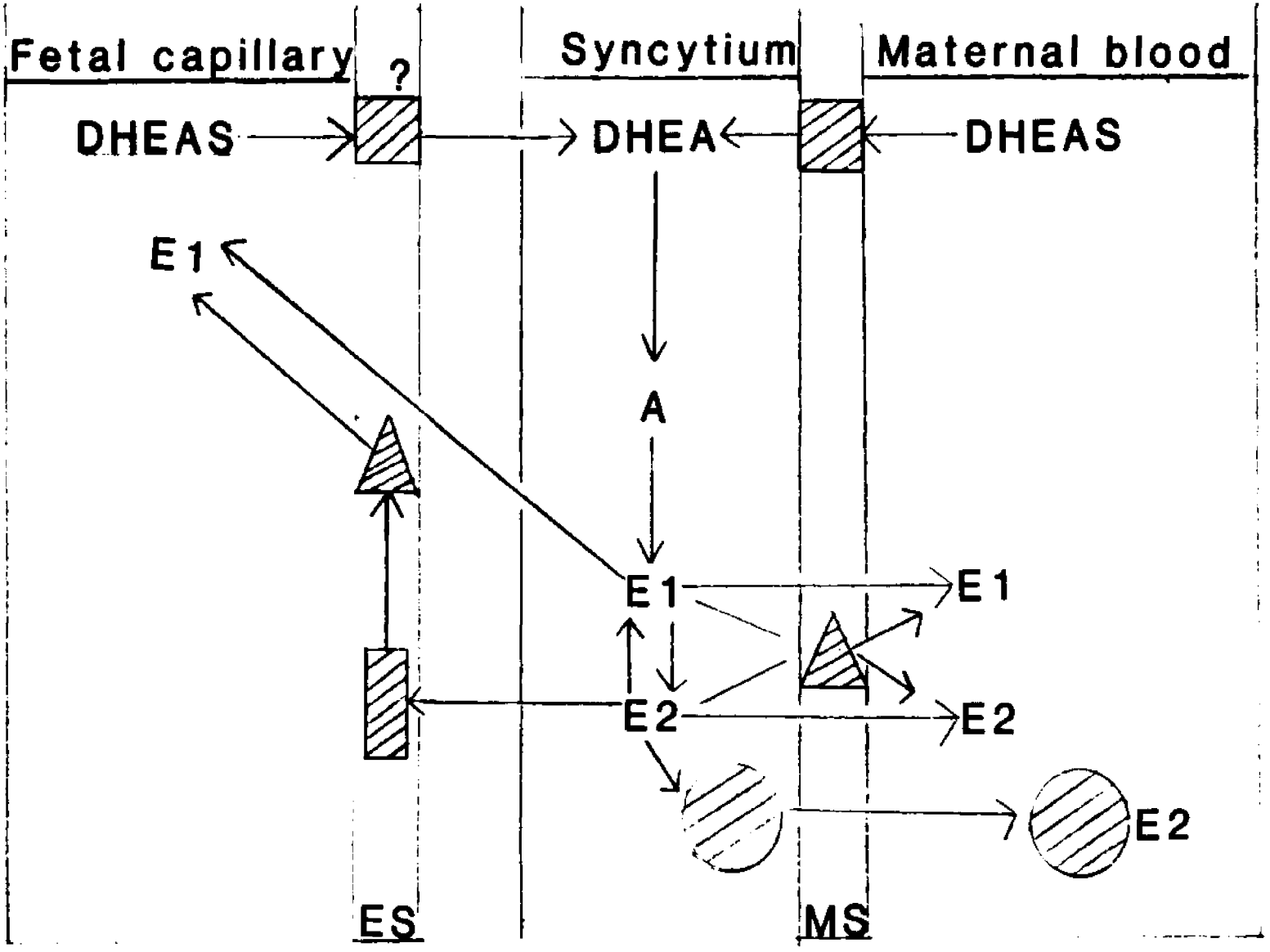

Fetal capillary
?
Syncytium
Maternal blood
DHEAS
DHEA
DHEAS
E1
A
E1
E1
E2
E2
E2
ES
MS

Fig. 22 Model of estrogen synthesis, metabolism, and secretion in placenta

This model incorporates various aspects of human placental estrogen metabolism and binding which are suggested by the results obtained in the present study as well as in other studies. The abbreviations ES and MS found at the bottom of the Figure represent endothelial surface and microvillar surface respectively. In the presented scheme the entry of DHEAS into placenta from maternal blood is postulated to be facilitated by microvillar sulfatase (■), an enzyme documented in the present study. A similar activity could also exist on the endothelial surface (■?). The E_1 formed following aromatization of A could be directly released to fetal and maternal circulations or reduced to E_2 by microvillar E_2DH (▲, documented in the present study) and/or by endothelial E_2DH (postulated in the present study). Syncytial E_2 is hypothesized to combine with an endothelial binding protein specific for E_2 (▮) which facilitates dehydrogenation at this site with subsequent release of E_1 to the fetal circulation. Both of these functions are postulated based on data obtained from perfusions conducted in the present study. Alternatively, E_2 formed within the syncytium is postulated to combine with a syncytial binding protein specific for E_2 (●) which is secreted to the maternal circulation. This possibility was suggested based on the results obtained in other studies (see Discussion).

Microvillar 17βE_2DH and sulfatase activities documented in this dissertation study could also influence the pattern of estrogen release by placenta. The entry of maternal DHEAS into placenta might be influenced by microvillar sulfatase activity (square, Fig. 22), as free esteroids are known to enter placenta at a greater rate than conjugated forms (130, 131). It follows that sulfatase activity at the endothelial surface would influence the rate of syncytial entry of DHEAS present in the fetal blood, but this possibility was not explored in the present study (square with a question mark, Fig. 22).

Walsh and McCarthy (6) and Gurpide et al. (7) have suggested that a syncytial protein that binds E_2 which is subsequently secreted to the maternal blood may be involved in establishing the pattern of preferential secretion of E_2 towards the maternal circulation noted during these studies. This postulated binding protein is denoted by a circle in Figure 22. In the same line of thought, it was suggested in the present study that specific binders for E_2 could exist on the maternal side of the placenta (i.e., in the region of the placental microvilli) which might bind and subsequently release E_2 to the maternal circulation. This possibility was explored by conducting <u>in vitro</u> assays of estrogen receptor using preparations of term placental microvillar membranes. A high degree of specific binding was not found in these preparations (Table 7). This result supports data obtained in other laboratories from which it was concluded that negligible levels of E_2 specific binding exists in both cytosolic and nuclear preparations from human term placenta (126,127).

Although specific binding for E_2 was not found in preparations of placental microvillar membranes future experiments will explore

the possible involvement of E_2DH activity at this site in establishing the pattern of placental estrogen secretion. The type of estrogen that is released by placenta to maternal blood could be influenced by whether microvillar E_2DH functions principally in an oxidative or reductive direction. Thus, "maternal" perfusions could be performed using buffer solutions containing labeled E_1 or E_2 bound to an inert material (e.g., polystyrene beads, ref. 173) small enough to preclude syncytial entry, to determine the predominant direction of conversion of this enzyme.

As methods for the culture of endothelial cells from human umbilical vein are readily available (174,175), future efforts will also be aimed at investigation of E_2 binding and metabolism within endothelial preparations. Milewich et al. (176) have reported that extensive metabolism of androgens occurred in cultures of pulmonary endothelial cells indicating that this cell type does possess steroid metabolizing capability. The use of endothelial cultures in future experiments will allow for an extensive study of E_2 binding and metabolism not feasible through the method of in vitro placental perfusion.

V. Overall Conclusions from the Studies on Microvillar Enzymes and Placental Perfusions

In the initial stages of this project preparations of microvillar membranes obtained from human term placenta were found to be a rich source of steroid metabolizing enzymes. Specifically, E_2DH, E_1S, and DHEAS sulfatase activities were found to be present in microvillar membranes at levels greater than could be attributed to contamination by other subcellular fractions. Topographical analysis of mem-

brane associated activities using trypsin and anti-E_2-DH antibodies revealed that the microvillar membrane does conceal both E_2DH and sulfatase enzymes. Unique kinetic values for microvillar dehydrogenation of T relative to E_2 as substrate were established in relation to mitochondrial, microsomal and cytosolic activities. In addition, lability of the solubilized enzyme, pH *versus* enzymatic activity comparison, and molecular weight determination established unique characteristics of microvillar E_2DH activity as compared with the cytosolic activity. Isoelectric points of microvillar membrane and soluble E_2DH enzymes were found to be similar. A possible *in vivo* relevance of steroid metabolizing activity in placental microvilli was suggested by the unique location of these enzymes in relation to syncytial entry of steroids from the maternal circulation during pregnancy. Although placental microvilli were found to be a source of steroid metabolizing enzyme, high levels of specific binding of E_2 were not found to be present in preparations of microvillar membranes.

Placental perfusions were employed as a tool to study the pattern of estrogen distribution in placenta and did reveal unique distriburions for the release of E_1, E_2, and E_3 to "fetal" and "maternal" perfusates. A very large degree of conversion of ^{3}H-16αOHA to ^{3}H-E_3 was noted during perfusion. The presence of EE, a competitor of estrogen binding, did provoke a large shift in the distribution of ^{3}H-E_2 and not of ^{3}H-E_1 or ^{3}H-E_3, suggesting that unique factors were responsible for the distribution of E_2 in placenta. Under a variety of perfusion conditions it was observed that the primary effect of the addition of competitor of estrogen binding was to provoke a large release of ^{3}H-E_2 to the "fetal" perfusate. A perfusion protocol was

adopted in which it was possible to demonstrate a complete release of $^{3}H\text{-}E_2$ to the "fetal" perfusate in response to addition of EE. Since this release of $^{3}H\text{-}E_2$ occurred to the "fetal" perfusate only, it was suggested that specific binders for E_2 exist in the region of the fetal capillary (i.e., the capillary endothelium) which could be involved in this effect. It was suggested that binding of E_2 at endothelial sites would facilitate reduction to E_1. Documented microvillar and postulated endothelial steroid metabolizing and/or binding activities were incorporated into a model of placental estrogen metabolism and secretion.

In summary, very different processes were found to exist on either side of the syncytial unit. On one hand, extensive steroid metabolism in the absence of specific binding for E_2 was observed to be present on the maternal-facing microvillar surface. Phenomena on the opposite side of the syncytium, that is, in the region of the fetal capillary, were characteristic of a binding event possibly linked to dehydrogenase activity. Observed activities at either or both of these syncytial sites could influence the level of active estrogen (i.e., E_2) secreted to mother and fetus.

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