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ESTROGEN SULFOTRANSFERASE AND 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITIES IN GUINEA-PIG CHORION THROUGH GESTATION

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Summary—Estrogen sulfotransferase (EST) activity measured under optimal *in vitro* conditions in the 105,000 *g* cytosols (HSS) of homogenized intrauterine tissues (amnion, chorion, endometrium, decidua basalis and placenta) from guinea-pigs at the 50th day of gestation indicated that the highest specific activity occurred in the chorion. EST activity in the chorion increased from day 34 (early gestation) to peak around day 45 (mid-gestation), before significantly decreasing around day 50 and further declining to barely detectable levels beyond day 60 (late gestation, the onset of parturition). 17β-Hydroxysteroid dehydrogenase (17β-HSD) activity in the chorion was almost completely membrane associated. The specific activity of the 17β-HSD reduction reaction in the 105,000 *g* pellet was 2.5-fold higher at mid-gestation than at late gestation, while the specific activity of the 17β-HSD oxidation reaction was 1.7-fold higher at mid-gestation as compared with late gestation. When intact pieces of chorion tissue from mid- and late gestation were incubated with 5 nM [^{3}H]estradiol (E_2), approx. 80% of the recovered free estrogen was E_1 (estrone). Only chorion from animals at the onset of parturition were able to produce detectable amounts of E_2 from 5 nM [^{3}H]E_1. Under the same experimental conditions the ratio of estradiol sulfate (E_2S) to estrone sulfate (E_1S) isolated from the media and methanol washes of late gestation chorion tissue was 3–4 times greater than for the day 45 tissue.

INTRODUCTION

The best defined mammalian model of parturition is that of the sheep. Maturation of the fetal hypothalmic–pituitary–adrenal axis causes increasing secretion of cortisol [1]. This cortisol activates placental 17-hydroxylase/lyase, allowing conversion of progesterone to estrogen [2]. The increasing ratio of estradiol (E_2) to progesterone is reflected in the maternal peripheral plasma [3]. E_2 then appears to act in conjunction with prostaglandins and oxytocin [4] to induce myometrial contractions, possibly by gap junction formation [5].

This exact sequence of events does not seem to occur with the guinea-pig, in that there is not a consistent alteration in peripheral steroid levels at the onset of parturition [6]. Also, progesterone [7] and cortisol [8] do not seem to have any blocking or inducing effect respectively upon the triggering of myometrial contractions. However this does not exclude an intrauterine change in steroid metabolism at term, possibly regulated by enzyme activities of the guinea-pig fetal membranes.

The guinea-pig chorion tissue expresses considerable estrogen sulfotransferase (EST) activity [9]. 17β-Hydroxysteroid dehydrogenase (17β-HSD) is present in a variety of reproductive tissues [10, 11]. The present study examines the activity of EST and 17β-HSD of the guinea-pig chorion at distinct stages of gestation to determine if these enzymes could be altering the local hormonal milieu of the guinea-pig fetus during gestation, thus affecting the initiation of uterine contractions.

EXPERIMENTAL

Chemicals and reagents

[6,7-^{3}H(*N*)]-E_1 and [6,7-^{3}H(*N*)]-E_2 (sp.act. 40–60 Ci/mmol) were purchased from New England Nuclear Corp., Dorval, Quebec and were purified before use by the procedure of Hobkirk *et*

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Abbreviations: E_1 = estrone; E_1S = estrone sulfate; E_2 = estradiol; E_2S = estradiol-3-sulfate; EST = estrogen sulfotransferase; 17β-HSD = 17β-hydroxysteroid dehydrogenase; HSS = high speed supernatant; MTG = monothioglycerol; PAPS = 3′-phosphoadenosine-5′-phosphosulfate.

al. [12]. Unlabeled E_1 and E_2, monothioglycerol (MTG), Trizma base, NAD^+ and NADH were purchased from Sigma Chemical Co., St Louis, Mo. 3′-Phosphoadenosine-5′-phosphosulfate (PAPS), synthesized by the method of Singer [13], was obtained in 1 mM Tris buffer at a pH of 8.0 from the Contracts Office, University of Dayton, Dayton, Ohio. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco Laboratories, Grand Island, N.Y. Water and methanol were of HPLC grade and all other chemicals of ACS grade were purchased from Canlab, Toronto, Ontario, BDH Chemicals, Canada Ltd, Toronto, Ontario or Fisher Scientific Co., Toronto, Ontario.

Animals

Guinea-pigs were of the English Short-hair variety purchased originally from local suppliers or from Charles River, Canada Ltd, St Constant, Quebec. Their diet consisted of Purina chow and water *ad libitum* with occasional vegetable supplementation. Two females and one male were housed per cage in light- and temperature-controlled quarters. Mating was indicated by the appearance of a vaginal plug marking day 0 of gestation. A standard curve relating the fetal weight to the stage of gestation was also used [14].

Tissue preparation

Guinea-pigs were sacrificed by cervical dislocation, as approved over the period of study by the Canadian Council for Animal Care. All manipulation of tissues, including homogenizing and centrifuging was carried out at 0–4°C. Tissues were immediately excised, rinsed thoroughly with 0.9% physiological saline and blotted before weighing. They were minced with scissors, then homogenized by two 15-s bursts at setting 6 with a Brinkmann Polytron (Brinkmann Instruments, Westbury, N.Y.) in 0.25 M sucrose, 10 mM Tris/acetate, 1 mM Na_2EDTA and 1 mM monothioglycerol pH 7.6 (TSM), utilizing 1 g of tissue per 10 ml of buffer. Homogenates were centrifuged at 800 *g* for 15 min in a Sorvall RC2B centrifuge. The supernatants were then centrifuged at 105,000 *g* for 1 h in a Beckman L5-65 ultracentrifuge. The cytosol from the last step (HSS) was stored at −20°C. The 105,000 *g* pellet was washed twice with 1 ml TSM, then manually homogenized with an all glass hand homogenizer in 500 μl of TSM and stored at −20°C.

EST assay

The assay which has been previously published [15], was performed in duplicate. Blanks contained all components except PAPS. After incubation free steroids were extracted with diethyl ether and duplicate aliquots of the aqueous phase were counted with scintillation cocktail. The samples were counted for a sufficient length of time to give a counting error within ±5%. The ^3H counting efficiency was 40% and counts were corrected to dpm by the channels ratio method.

17β-HSD assays in the 105,000 g supernatant and pellet

Interconversion between E_1 and E_2 was assayed using the 105,000 *g* supernatants and pellets of homogenized chorion from 3 individual animals at days 45 and 60+ of gestation by a procedure adopted from Tait *et al.* [16]. In brief, a sample of HSS or pellet was incubated with substrate (10 nmol [^3H]E_1 or 15 nmol [^3H]E_2, 300,000 dpm) and cofactor (0.5 μmol NAD^+ or NADH) and adjusted with buffer to a 0.5 ml incubation volume. Heat-inactivated samples served as blanks for the assay. The ether extracts from the incubations were dried over anhydrous Na_2SO_4 and carrier estrogens (30 μg) were added to aid in visualization of the products after TLC. The ether extracts were dried and applied to TLC plates (Whatman Silica Gel 60 A, Maidstone, England) in 2 × 100 μl of diethyl ether. The plates were developed with dichloromethane/ethyl acetate (4:1, v/v) and after drying were exposed to u.v. (wavelength = 250 nm) to visualize the carrier estrogens. Absorbing zones containing the estrogen products were scraped off and transferred to scintillation vials. A non-absorbing zone of each plate was added to account for background fluorescence. To each vial were added 250 μl methanol and 5 ml scintillation cocktail. Samples were counted and the activity of 17β-HSD was calculated according to the amount of E_1 or E_2 produced. The recovery of [^3H]E_1 and [^3H]E_2 standards (30 μg, 100,000 dpm) was used to monitor the procedural losses from drying, spotting and TLC.

Protein determination

Protein concentration was measured by the method of Bradford [17] using the Bio-Rad Protein Assay Kit with a bovine serum albumin (Bio-Rad) as standard.

Whole tissue incubations with 5nM [^{3}H]E_1 or [^{3}H]E_2

The estrogen substrate for whole tissue incubations was dried under nitrogen and dissolved in 20 μl of methanol. DMEM (pH 8.0) and 10 μl of 100 mM MTG in TS (TSM minus MTG) were added to give a final substrate concentration of 5 nM [^{3}H]E_1 or [^{3}H]E_2 in a 1 ml incubation volume. Approx. 200–300 mg pieces of chorion tissue were added to the substrate tubes, which were then vortexed and gently agitated in a water bath at 37°C under air. The blanks contained heat-inactivated tissues. After 2 h of incubation 3 M acetate buffer pH 4.0 was added and the tissues were transferred to methanol for 48 h at 4°C. Incubation media and tissue methanol washes were treated separately for the identification of metabolites.

Ether extraction of tissue incubation media and methanol extracts

Cold carrier was added to the medium to aid in the recovery of the label. The media were centrifuged at 3000 *g*, then the supernatants were passed through a C_{18} reverse-phase Sep-Pak cartridge (Waters Associates) and concentrated into 5 ml of methanol. The samples were dried and partitioned between 3 M acetate buffer pH 4.0 and ether. The water soluble extracts were concentrated into 3 ml of methanol with a Sep-Pak cartridge and ether extracts were dried and solubilized in methanol for storage at −20°C. The tissue methanol washes were filtered through a 0.45 μm nylon filter, then extracted in the same manner as for the media.

Ether extracted ^{3}H

The ether extracts were chromatographed on Sephadex LH-20 in chloroform:hexane:ethanol (5:5:1, by vol) (Solvent A). Aliquots of the 1 ml fractions collected were counted in 5 ml of scintillation cocktail at an efficiency of 40%. The peak tubes were dried and solubilized in 1 ml of methanol for recrystallization with 20 mg of the appropriate cold carrier (E_1 or E_2). One mg samples of the mother liquor and crystals from the recrystallizations were dissolved in 1 ml of methanol, counted in 10 ml of scintillation fluid and the specific radioactivities of the samples were calculated. Estrogen acetyl derivatives were synthesized by dissolving estrogen crystals in 200 μl of pyridine and 100 μl of acetic anhydride and incubating at room temperature for 15 h. The derivatives were then recrystallized once from methanol:water and their specific radioactivities calculated.

Identification of estrogen sulfates

The water-soluble ^{3}H-labeled fractions from the media and methanol washes of tissue incubations were chromatographed on DEAE Sephadex (A25) in a manner previously described [18]. The peak tubes corresponding to the regions where E_1S and E_2S standards eluted were separately pooled and desalted using a C_{18} reverse-phase Sep-Pak cartridge.

Free steroids were regenerated from their sulfates by the procedure of Loriaux *et al.* [19]. The samples were dried, solubilized with 250 μl dry methanol and incubated for 15 h at 50°C with 1 ml dry ethyl acetate:glacial acetic acid (9:1, v/v). Following evaporation under nitrogen and partitioning between 3 M acetate pH 4.0 and diethyl ether, aliquots of the aqueous and the ether phases were counted to quantify the extent of solvolysis. The ether extracts were chromatographed on Sephadex LH-20 in Solvent A. Peaks corresponding to authentically labeled [^{3}H]E_1 and [^{3}H]E_2 were recrystallized from methanol with the appropriate cold carrier. Acetyl derivates were also synthesized and recrystallized from methanol:water as previously described.

Statistical analyses

Results are expressed as mean values ± SEM for the number of observations indicated in the Results section or figure legends. The significance of difference between any two mean

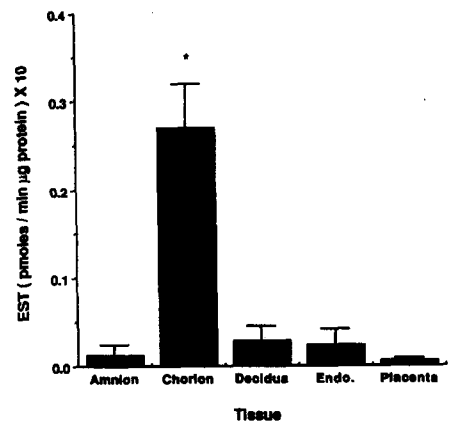

Fig. 1. A comparison of estrogen sulfotransferase activities in guinea-pig intrauterine tissues. The cytosols of tissues from two pregnant animals at the 50th day of gestation were assayed for EST activity with 0.1 μM [^{3}H]E_1 and 50 μM PAPS. The results are mean values ± SEM (*$P < 0.05$ compared to decidua basalis; Endo., endometrium).

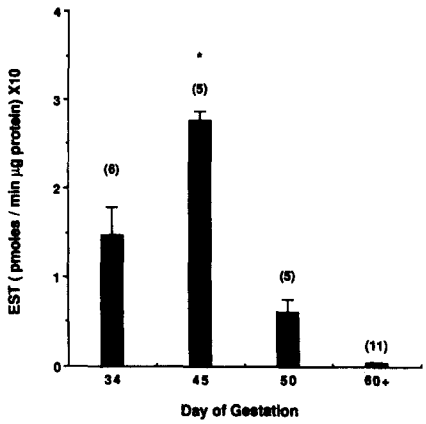

Fig. 2. The gestational profile of EST in the cytosols of homogenized chorion. EST activity was assayed with 0.1 μM [^{3}H]E_1 and 50 μM PAPS. The results are mean values $\pm$ SEM from (n) animals at each stage of gestation (*$P < 0.05$ compared to day 60+ of gestation).

values was determined by Student's unpaired t-test.

RESULTS

EST activity

Figure 1 compares the EST specific activities in the cytosols of homogenized intrauterine tissues from two pregnant guinea-pigs at the 50th day of gestation. The activity was significantly higher in the chorion than in any other tissue assayed ($P < 0.05$). Less than 3% of the incubated ^{3}H was recovered in the aqueous phase of the blanks (minus PAPS).

Variation of chorion EST activity with time of gestation is shown in Fig. 2. The animals were grouped into 4 stages: day 34 (included days 34–35 of gestation, $n = 6$), day 45 ($n = 5$), day 50 (included days 48–51 of gestation, $n = 5$), and day 60+ (the onset of parturition marked by relaxation of the pubic symphysis, $n = 11$). It can be seen that the activity of the enzyme peaked around the 45th day of gestation and decreased by day 50 to 25% of the peak activity. At the onset of parturition the specific activity of EST had decreased to approximately 3% of the peak mid-gestational activity.

17β-HSD activity

Figure 3 shows the values for chorion 17β-HSD activity in the oxidative ($E_2 \rightarrow E_1$) and reductive ($E_1 \rightarrow E_2$) directions when assayed in 105,000 g pellets and supernatants of mid and late gestation. The recovery of E_1 and E_2 standards (30 μg, 100,000 dpm) were 76.4 $\pm$ 1.8% ($\pm$SEM) and 74.7 $\pm$ 1.8% respectively. Apparent steroid conversion by the boiled, day-45 pellet blanks were 7.2 $\pm$ 4.8% ($E_2 \rightarrow E_1$) and 4.5 $\pm$ 0.5% ($E_1 \rightarrow E_2$) respectively, that of the active pellet, while the day-60+ pellet blanks were 2.0 $\pm$ 0.5% and 6.7 $\pm$ 1.2% respectively, that of the active pellet. Activity of the enzyme in the day-45 HSS was 2.8% ($E_2 \rightarrow E_1$) and 4.5% ($E_1 \rightarrow E_2$) respectively, that of the activity in the pellet, while day-60+ HSS expressed activities 4.7% ($E_2 \rightarrow E_1$) and 3.8% ($E_1 \rightarrow E_2$) respectively, that of the pellet. These values were only slightly above blank values. The specific activity of the reduction reaction catalyzed by 17β-HSD was 2.5-fold greater in the day-45 chorion pellet than in the pellet from day-60+ tissue. Also, the specific activity of the oxidative reaction was 1.7-fold greater at mid-gestation than at the onset of parturition.

Whole chorion incubations with 5nM [^{3}H]E_1 and [^{3}H]E_2

The percentage of ^{3}H recovered as either E_1 or E_2 by LH-20 chromatography of the ether extracts of media and methanol washes of days 45 and 60+ tissues which had been incubated with 5 nM [^{3}H]E_1 or [^{3}H]E_2 are listed in Table 1. At both stages of gestation, approx. 80% of the free [^{3}H]-estrogen isolated from the media and tissue after incubation with [^{3}H]E_2 was in the form of E_1. However, E_2 production from E_1 was limited to the chorion from guinea-pigs at the onset of parturition (approx. 8% of the recovered estrogen was E_2).

In order to confirm the identify of the labeled steroids in question, all fractions eluted between

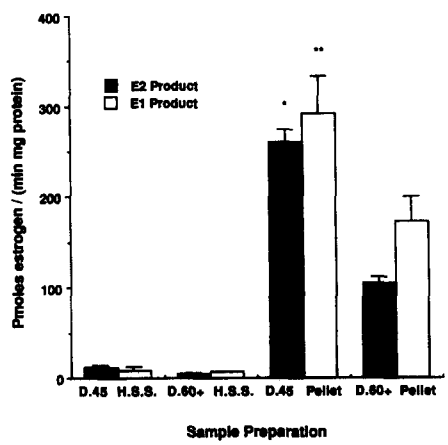

Fig. 3. A comparison of 17β-HSD activities in subcellular fractions of homogenized chorion at days 45 and 60+ of gestation. The results are mean values $\pm$ SEM for chorion from three animals at both stages of gestation assayed in duplicate (*$P < 0.05$ compared to day 60+; **$P < 0.1$ compared to day 60+; note that E2 Product refers to E_2 produced from incubation with [^{3}H]E_1, E1 Product refers to E_1 produced from incubation with [^{3}H]E_2).

Table 1. Percentage of ^3H recovered as [^3H]E_1 and [^3H]E_2 from the ether extracts of the media and methanol washes of days 45 and 60 + gestation chorion tissue incubated with 5 nM [^3H]E_1 or [^3H]E_2 (n.d. = not detectable)

Stage	Steroid recovered	[^3H]E_1 Substrate		[^3H]E_2 Substrate	
		Media	Tissue	Media	Tissue
Day 45	E_1	100%	100%	78.5%	86.1%
	E_2	n.d.	n.d.	21.5%	13.9%
Day 60 +	E_1	91.7%	91.3%	79.8%	80.7%
	E_2	8.3%	8.7%	20.2%	19.3%

13 and 19 ml from LH-20 were pooled as apparent E_1 and those between 30 and 40 ml as apparent E_2 according to the elution of authentic labeled standards. Table 2 summarizes the results of crystallizing E_1 and E_2 with carrier E_1 and E_2 respectively. The specific activities of the mother liquor and crystals after three crystallizations agreed, indicating that the steroids were indeed [^3H]E_1 and [^3H]E_2. Further recrystallization in the acetylated form confirmed this.

When estrogen sulfates produced by the incubation of chorion tissue explants with 5 nM labeled estrogens were separated from unconjugated steroids on DEAE–Sephadex in a 0–0.8 M NaCl gradient, recovery of ^3H varied between 75 and 87% of the total amount loaded. After mild solvolysis, all of this initially water-soluble ^3H was recovered in the ether phase, indicating successful cleavage. The free products thus produced were shown to consist completely of [^3H]E_1 and [^3H]E_2 by LH-20 chromatography followed by crystallization as described above.

Figure 4 compares E_2S/E_1S ratios recovered from days 45 and 60 + media and tissue after incubation with either [^3H]E_1 or [^3H]E_2. Analysis of estrogen sulfate production is a better indicator of 17β-HSD activity in the tissue than is the identification of unconjugated estrogens, since the latter presumably could include substrate not taken up into the tissue and exposed to enzymic activity. The E_2S/E_1S ratio isolated from the media and tissue with either [^3H]E_1 or

Table 2. Crystallization of the estrogens isolated by Sephadex LH-20 chromatography of the ether extracts of the media and methanol washes from days 45 and 60 + gestation chorion tissues incubated with 5 nM [^3H]E_1 or [^3H]E_2 (SA = specific radioactivity in units of dpm/mg; ML = mother liquor)

Crystallization[a]	E_1		E_2	
	Crystal	ML	Crystal	ML
1st	2706	3980	2654	3344
2nd	2556	3021	2589	2786
3rd	2653	2728	2526	2679
Acetyl derivatives				
Calculated SA[b]	2297	—	1930	—
Experimental SA	2142	2222	2006	2041

[a]Initial specific radioactivity = 3000 dpm/mg.
[b]Calculated from experimental specific radioactivity of 3rd crystal taking into account the change in molecular weight upon acetylation.

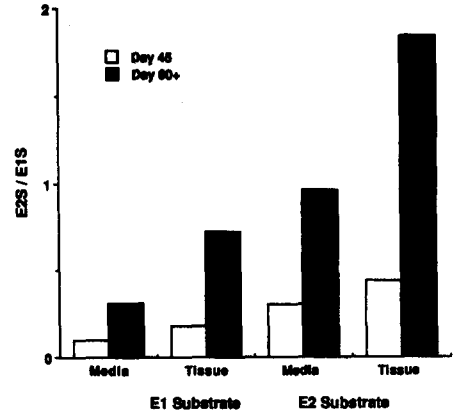

Fig. 4. Relative estrogen sulfate production by guinea-pig chorion tissues from days 45 and 60 + of gestation incubated with 5 nM [^3H]E_1 and [^3H]E_2 for 2 h at 37°C. Results are for extracts pooled from three tissue incubations at each stage of gestation.

[^3H]E_2 as substrate was 3–4 times greater with chorion from the onset of parturition than with tissue from mid-gestation, suggesting that chorion near term retains or produces more estrogen sulfate in the 17-reduced form. The buffer medium exhibited a lower E_2S/E_1S ratio than did the tissue when either substrate was incubated.

DISCUSSION

The present results suggest that EST and 17β-HSD activities of the guinea-pig chorion may alter the steroidal hormone milieu of the fetus, possibly facilitating the initiation of myometrial contractions. The specific activity of EST, as estimated by assay of high speed cytosols, peaks around the 45th day of gestation, decreasing to less than 3% of the peak activity at the onset of parturition. EST activity in the non-pregnant human [20] and porcine [21] endometrium is regulated by progesterone, resulting in the down-regulation of the estrogen receptor [22]. The observation that the non-pregnant guinea-pig uterus exhibits no obvious EST activity [15] suggests that it is not regulated by progesterone, but may be influenced by a factor of fetal origin. A fetal factor (cortisol) is known to activate 17 hydroxylase/lyase activity in the sheep placenta.

17β-HSD activity in the guinea-pig chorion appears to be restricted to the high speed pellet in both the oxidative and reductive directions. The specific activity of 17β-HSD as measured in high speed pellets appears to decrease approx. 50% to term in both the oxidative and reductive directions. Since no attempt was made to purify

17β-HSD from the 105,000 *g* pellet, it is uncertain if the same or different enzymes catalyze the oxidative and reductive reactions. The direction of the reaction may depend upon substrate concentrations or cofactor availability.

Whole chorion tissue from mid and late gestation actively oxidizes E_2 to E_1, however only chorion from guinea-pigs near term is able to produce detectable amounts of E_2 from E_1. This formation of E_2 represents a possible net accumulation as measured at one point in time. Local effective *in vivo* production of E_2 by 17β-HSD in term chorion could actually be much higher due to metabolic clearance of the product and E_2 compartmentalization in the tissue. The tendency for whole chorion at term to slightly alter the E_1/E_2 balance was not reflected in the assays of 17β-HSD at the subcellular level. It should be noted, however, that the 17β-HSD assay with the pellet was designed to measure "optimal" activity in the presence of unlimiting nucleotide cofactor concentration. An example of a discrepancy between *in vivo* [23] and *in vitro* [16] results exists for 17β-HSD activity in breast tumour tissues. *In vitro*, the oxidation reaction, ($E_2 \rightarrow E_1$) is strongly favoured, while *in vivo* the reduction reaction ($E_1 \rightarrow E_2$) is more important, producing E_2 which may influence the growth of the tumour.

Similarly, the amount of estrogen sulfate produced by whole chorion tissue incubated with 5 nM estrogen substrate and endogenous PAPS cannot be directly compared with the results of cytosolic EST assays under "optimal" conditions with 100 nM estrone and excess cofactor. It is clear that even though the cytosolic assays indicated very low EST activity at late as compared with mid-gestation, the whole tissue studies provided evidence for definite sulfation of a substantial percentage of the low concentration of estrogen substrate utilized under experimental conditions which may more closely approximate the physiological state.

A greater proportion of the E_2 produced from tissue incubations of late gestation chorion with [^{3}H]E_1 is isolated in the sulfated rather than the free form, indicating that the estrogen sulfate may be a temporary precursor form available for generation of the active form (E_2) via sulfatase activity. Recent results from our laboratory [24] have shown that chorion estrogen sulfate sulfatase activity increases approx. 6-fold between day 45 of gestation and the onset of parturition. During this same period, the level of endogenous E_2 in the chorion tissue estimated by RIA increases 6-fold. The net effect of EST, estrogen sulfate sulfatase and 17β-HSD activities is for E_1S to be the major estrogen metabolite produced at mid-gestation by chorion, while during late gestation the same tissue may produce relatively more E_2 in both the free and sulfated forms. This trend towards E_2 retention in the chorion late in gestation approximately coincides with the period during which strong chorion–endometrium physical association exists, the onset of which is between the 50th and the 55th days of gestation. This local increase in estradiol may induce the chemical (prostaglandin [25] and oxytocin receptor [26] synthesis) and structural (gap junction formation [27]) events which have been attributed to the action of this steroid near parturition in the sheep and human. It has recently been claimed that E_1S itself may exert biological actions upon guinea pig endometrium which are qualitatively different from those of E_2 [28, 29]. Thus the balance of EST and estrogen sulfatase activities in the chorion may be important not only in furnishing free "active" E_2, but also in providing estrogen sulfate.

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