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Steroid production and excretion by the pregnant mouse, particularly in relation to pregnancies with fetuses deficient in Δ^7-sterol reductase (*Dhcr7*), the enzyme associated with Smith–Lemli–Opitz syndrome

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ABSTRACT

This study has shown that the mouse has a great increase in steroid production during pregnancy in similar fashion to the human. Many steroids were provisionally identified in maternal urine of the wild-type mouse. The major progesterone metabolites appear to be hydroxylated pregnanolones, particularly with hydroxyl groups in the 16α position. Rather than estriol being the major end-product of feto-placental steroid synthesis as in the human, the pregnant mouse produces and excretes large amounts of androgen metabolites, ranging in polarity from androstanetriols to androstanepentols. These steroids have 15α- or 18-hydroxyl groups with additional hydroxylation at uncharacterized positions. From metabolite data the peak of pregnancy progesterone production appears to be between 7.5 and 14.5 gestational days, while for C_{19} metabolites peak excretion is later.

The starting-point of the studies was to study pregnancy steroid production by a mouse model for Smith–Lemli–Opitz syndrome, 7-dehydrosterol reductase (DHCR7) deficiency. In human pregnancies with DHCR7 deficient fetuses large amounts of 7- and 8-dehydrosteroids are excreted, products secondary to high fetal 7- and 8-dehydrocholesterol (DHC) accumulation. This agrees with existing evidence that human feto-placental steroid synthesis utilizes little maternal cholesterol as precursor. In contrast, this study has shown that pregnant mice carrying *dhcr7* deficient fetuses with relatively high DHC production had essentially undetectable maternal excretions of steroids with Δ^7- and Δ^8-unsaturation. As mutant mouse mothers have essentially normal cholesterol production (little or no DHC build-up), this suggests maternal cholesterol is primarily utilized for pregnancy steroid synthesis in the mouse.

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1. Introduction

The increasing role of transgenic mice in the study of human disorders demands studies to determine how the metabolism of these animals differs from the human counterpart. In a recent study we described the urinary corticosteroid metabolic profile of the mouse as a prerequisite to studying animals with mutations affecting corticosteroid metabolism [1]. The current study focuses on the steroid synthesis of the pregnant mouse. The impetus came from our studies of Smith–Lemli–Opitz syndrome (SLOS) where distinct steroid metabolites excreted by a woman with an affected fetus can be used for prenatal diagnosis of the condition. SLOS is a malformation and mental retardation disorder caused by disabling mutations in Δ^7-sterol reductase (DHCR7, EC 1.3.1.21), a critical enzyme in the terminal stages of cholesterol synthesis. Individuals with this disorder produce elevated amounts of the cholesterol precursors 7-dehydrocholesterol and its isomer 8-dehydrocholesterol (7-and 8-DHC*).

A feature of pregnancies carrying an affected fetus is the significant proportion of estrogen and other steroids present in maternal urine that have Δ^7- or Δ^8-unsaturation. In normal human pregnancy the fetus is almost wholly responsible for producing the cholesterol used for synthesizing DHEA-sulfate, the precursor of estriol. Thus, if adrenal 7- or 8-DHC are utilized as precursors in a SLOS affected fetus, 7- or 8-dehydrosteroids (C_{18}, C_{19}, and C_{21}) would be produced and excreted as well as the normal counterparts. Such urinary "dehydrosteroids" have been utilized in the prenatal diagnosis of SLOS [2,3]. Metabolites of *placental* progesterone

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with Δ^7- or Δ^8-unsaturation are minor urinary components. Even though this organ is a fetal tissue it largely uses maternal cholesterol as progesterone precursor.

Dehydrosterol reductase (Dhcr7) deficient mice have been studied for several years [4,5]; null mutant homozygotes do not survive, but null mutant heterozygotes, homozygotes with milder mutations, and compound heterozygotes with one mild allele survive. We have a colony of such mutants engineered to mimic specific mutations observed in humans [5]. We undertook a study on these animals to see if in pregnancies with homozygous or compound heterozygous mutant pups the mother excreted Δ^7- or Δ^8-dehydrosteroids, in similar fashion to the human, to what extent pregnancy urine steroids in the mouse may originate with fetal cholesterol and its fetal synthesized steroid products. If the situation was similar to the human then to some extent maternally excreted steroids should have additional unsaturation. The presence of such steroids in mouse urine would be expected to be less than in the human because it is known that the mouse fetus has greater access to maternal cholesterol than in the human [6–8]. Also, due to Mendelian inheritance, the probability is that only a fraction of a given litter would be affected pups.

There is little information from published studies to suggest close similarities between the human and mouse in pregnancy steroid synthesis. In contrast to the human, it is known that the maternal ovary remains active throughout pregnancy, and that the placenta and fetal liver have active 17-hydroxylase/17,20-lyase, enabling androgen formation in these organs. Also, in contrast to the human the mouse fetal adrenal only has transient 17-hydroxylase activity [9–12] so androgen synthesis by this organ is not available throughout gestation.

However, prior to any other experiments our first goal was to document the previously undescribed urinary steroid excretion of the pregnant mouse, and this we have achieved by GC/MS.

2. Materials and methods

2.1. Wild-type mice

C57BL/6J mice were utilized in this study. All animal procedures were previously approved by the Institutional Animal Care and Use Committees of the participating institutions.

2.2. Mutant mice

Two strains of DHCR7 mutant mice were obtained from F.D. Porter (NIH). A null mutation contained a partial deletion of *Dhcr7* (Δ) [4] and a hypomorphic mutant contained a point mutation (T93M) [5]. We backcrossed both mutant strains to wild-type C57BL/6J (JAX) for multiple generations ($N = 12$ for Δ, $N = 9$ for T93M) to provide the mutations in a defined genetic background. T93M mutants were then maintained by inbreeding, since T93M/T93M homozygotes were viable, but Δ/+ mutants were maintained by continued backcrossing, since homozygous mutants die soon after birth [4]. Compound heterozygotes (Δ/T93M) were routinely generated by mating Δ/+ females with T93M/T93M males. Genotypes of animals were determined by PCR as described previously [4,5].

Samples from a third and independently derived strain of mutant mouse were also investigated in collaboration with a group at the Medical College of Wisconsin (SB Patel, co-author). These mice contained the null mutation, $Dhcr^{Tm1GST}$ ($Dhcr^{-}$), originally described by Fitzky et al. [13]. Homozygous $Dhcr^{-/-}$ mice do not survive more than a day after birth. Mating $Dhcr^{+/-}$ males and females generated pregnant mice carrying $Dhcr^{-/-}$ fetuses. Matched control pregnant mice with no affected fetuses were $Dhcr^{+/-}$ females mated to wild-type C57BL/6 males. Non-pregnant females were also $Dhcr^{+/-}$.

For timed pregnancies animals were mated and females were checked for a copulatory plug each morning. Evidence of a plug was taken as embryonic day 0.5. Table 1 summarizes the genotypes of the breeding pairs studied, the outcome of pregnancies and genotypes of pups if determined.

2.3. Urine collection and analysis

Urine samples from pregnant mutant mice were collected for 1–3 days prior to delivery (16–19 days after evidence of a copulatory plug) unless otherwise indicated. Two wild-type pregnancies had urine collections made for 2 days prior to, and throughout gestation. The length of collections was recorded. Typically a collection was made from 4.5 to 7.5 days (E4.5–E7.5) gestation, 7.5–10.5 days, 10.5–14.5 days, 14.5–17.5 days and 17.5–20.5 days. In the Oakland facility, urine was collected from the animal by absorption onto filter paper underneath a wire grid covering the cage floor. The timing of collection was recorded and the clearly defined urine spots were excised from the paper and stored in a freezer. We estimate that greater than 90% of the urine excreted during a time period was recovered. For steroid recovery, samples were sliced into small pieces which were placed in a test-tube and extracted at least three times with 5 ml of water, or until water remained colorless following extraction. The extraction was undertaken with intermittent vortexing and sonication. Conventional metabolic cages were used by the Wisconsin group for urine collection from the "null-mutant" pregnancies.

Urine analysis was as previously described [1] and included solid-phase extraction (SPE), hydrolysis of steroid conjugates, re-extraction by SPE, adding of an internal standard (stigmasterol) and derivatization to form methyloxime-trimethylsilylethers (MO-TMS). The final derivative was dissolved in 100 μl cyclohexane which was transferred to an autosampler vial for GC/MS analysis.

Table 1
Outcomes of Dhcr7 mutant mouse breeding.

Female	Male	Affected pups genotype	Number pregnancies	Pups in each litter (affected/total)[a]
Δ/T93M	Δ/+	Δ/Δ, Δ/T93M	5	3/8, 0/5, 2/8, 0/4, 0/4
Δ/+	Δ/T93M	Δ/Δ, Δ/T93M	4	1/6, 1/7, 3/7, 3/8
T93M/+	T93M/T93M	T93M/T93M	3	6/9, 5/9, 2/8
T93M/T93M	T93M/+	T93M/T93M	5	4/7, 4/7, 3/5, 5/8, ?/6[b]
Δ/+	T93M/T93M	Δ/T93M	6	3/5, 4/6, 2/6, 3/10, 1/8, (1)/11[c]
$Dhcr7^{+/-}$	$Dhcr7^{+/-}$	$Dhcr7^{-/-}$	3	2/8, 2/9, 3/10

Different crosses of mutant mice were set up to obtain urine from pregnant mice carrying fetuses with different genotypes. For two of the crosses the female itself had an affected genotype so reciprocal crosses were also tested.

[a] By genotype and tissue DHC/C ratio determination.

[b] The six pups had largely been devoured and were not genotyped.

[c] Nine deceased and not genotyped.

2.4. Fetal tissue and amniotic fluid collection

For collection of fetal material, pregnant mice were killed on the 14th day of gestation by cervical dislocation. Amniotic fluid was collected by puncturing individual amniotic sacs and absorbing the fluid (approximately 100 μl) on filter paper. Placenta and fetal tissue (liver and brain) were taken for sterol analysis.The remaining carcass tissue was used for DNA extraction and genotyping by PCR.

2.4.1. Mutant pregnancies

Two were studied in detail with maternal urine collection, pregnancy termination and fetal tissue sterol analysis. These were the result of mating between Δ/+ females and T93M/T93M males. Resulting fetuses were Δ/T93M or +/T93M.

2.5. Gas chromatography/mass spectrometry (GC/MS)

We used an Agilent 5975 instrument (www.agilent.com). The steroids were separated on a DB-1 cross-linked methyl-silicone column, 15 m × 0.25 mm i.d., film thickness 0.25 mm (J&W Scientific, Folson, CA, USA). Helium was the carrier gas at a flow-rate of 1.2 ml/min. A 2 μl aliquot (in cyclohexane) of the final derivatized extract was injected in splitless mode (valve opened 2 min). The GC temperature was ramped as follows: initial 100 °C, held for 3 min, increased to 210 °C at 25 °C/min, thereafter increased to 280 °C at 2.5 °C/min, finally, increased to 300 °C at 15 °C/min and held 4 min. The injector, transfer line, and ions source were kept at 260, 280 and 230 °C, respectively. The mass spectrometer was operated in the electron impact mode. The mass range scanned was from 90 to 800 amu.

As far as possible Individual steroids were identified by interpretation of their mass spectra. The fragmentation patterns of MO-TMS derivatives of steroids containing different functional groups are relatively well known [14] and some authentic reference steroids were available. The degree of substitution of androstanes is readily determined. Androstanediolones will have molecular mass of 479, androstanetriolones masses of 567, androstanetetrols molecular ions at 612, and so forth. Steroids with possible additional unsaturation will have masses 2 Da less. Identification of murine steroids was a particular challenge since they generally contained at least two additional functionalities in addition to the expected 3- and 17-oxy groups (for C_{19} steroids). Identification of one additional group through specific fragmentation was usually straightforward with MO-TMS derivatives, but the position of the second could often not be delineated, although it could usually be established where the group was not. Thus, some of our identified steroids have a hydroxyl group at unknown position. In addition, with a complex metabolome of previously unknown metabolites it is frequently not justifiable on a time, effort and cost basis to identify the configuration of the 3-hydroxyl and 5-hydrogen, although these are most likely largely 3α-hydroxy compounds based on previous documentation of steroid metabolism by rodents [1,15]. Documentation and tabulation of the individual identified steroids was achieved by categorizing them according to their retention times. This was achieved by co-injecting the sample with a series of n-alkanes (C22–C36) which permitted the assignment of a retention index (RI) for each component.

2.6. Quantification

We had only limited ability to quantify excretion rates, even though most collections were timed. The complexity of the mouse steroid metabolome precluded individual steroid quantification through measurement of areas of the TIC (total-ion-current) peaks. Once we had ascertained the basic structures (type and number of functionalities) of excreted metabolites we chose a quantitatively important and structurally significant ion and measured this relative to the *m*/*z* 394 ion (M-90) of our internal standard stigmasterol. Since appropriate reference compounds were not available, relative response factors were not measured so quantitative values reported are approximate, but consistent for a particular compound. Peak areas for individual compounds evaluated from the TIC and SIM chromatograms were similar re-inforcing the validity of our measured excretions.

3. Results

3.1. Steroids identified in urine of pregnant mice

The pregnant mouse urinary steroid metabolome was found to be extremely complex with many very polar steroids. While the first additional hydroxyl group (after the 3 and 17 positions) was relatively easy to place, the site of the next addition proved more challenging. In absence of final placement additional hydroxyl functionalities have been termed X, or Y; we do not imply that all Xs or Ys are the same group.

3.1.1. Androstanediolone and androstanetriol (RI 2692, 2700 and 2771)

Two steroids had identical spectra (Fig. 1a) with molecular ion at *m*/*z* 479 and a strong M-31 ion at *m*/*z* 448 indicating an androstanediolone structure. The spectrum and RI of the first peak was identical to authentic 5β-androstane-3α,15α-ol-17-one (Fig. 1b). The second peak with slightly longer retention time was assumed to be the 3α,5α epimer since 15β-hydroxylation has not been reported in the mouse. The spectrum proved to have fragment ions structurally informative for a 15-hydroxylated 17-oxosteroid. An important initial loss was M-45 giving an ion at *m*/*z* 434; and a major low-mass ion was at *m*/*z* 196. The origin of these fragmentations has not been elucidated.

Corresponding androstanetriols, 5β-androstane-3α,15α,17β-triol and most likely 5α-androstane-3α,15α,17β-triol were also identified. The spectra of authentic and isolated compounds are shown in (Fig. 1c and d). The molecular ions are at *m*/*z* 522 but the spectrum is dominated by ions at *m*/*z* 217 and 191. The "217" ion is formed by fragmentation of the 17–18 and 14–15 bonds, while the *m*/*z* 191 is a re-arrangement ion with the structure $(CH_3)_3Si–O–CH–O–Si(CH_3)_3$.

3.1.2. Androstanetriolones

Many androstanetriolones (AT-ones) were detected. Five prominent ones #1–5 had retention indices 2631, 2710, 2778, 2875 and 2884.

3.1.2.1. AT-one 1 (RI 2631). The molecular ion was at *m*/*z* 567 and prominent ion formed by loss of the oxime at *m*/*z* 536. The fragment at *m*/*z* 521 (M-46) is significant because it indicates the presence of an 11β-hydroxyl group. A 46 Da loss and the ions at *m*/*z* 213 and 182 are shared by 11β-hydroxyandrosterone so it must be presumed that the steroid is an androstane-3α,11β, X-triol-17-one. One original feature of the spectrum is the presence of ions at M-103 (*m*/*z* 464) and *m*/*z* 103. Loss of a primary TMS group from androgens typically represents the presence of an 18-hydroxyl group so 18 must be a likely contender for the final position.

3.1.2.2. AT-ones 2,3,4 (RI 2778, 2875 and 2884). These steroids had spectra (Fig. 2) with molecular ions at *m*/*z* 567 with loss of the 17-oxime giving prominent ions at *m*/*z* 536. Strong evidence of the presence of a 15α-hydroxyl group are the ions at *m*/*z* 133 and 196 and loss of 45 and 147 mass units from the molecular ion distinctive of steroids with derivatized 15- and 17-functions. The position of the additional hydroxyl group was not ascertained but clearly could

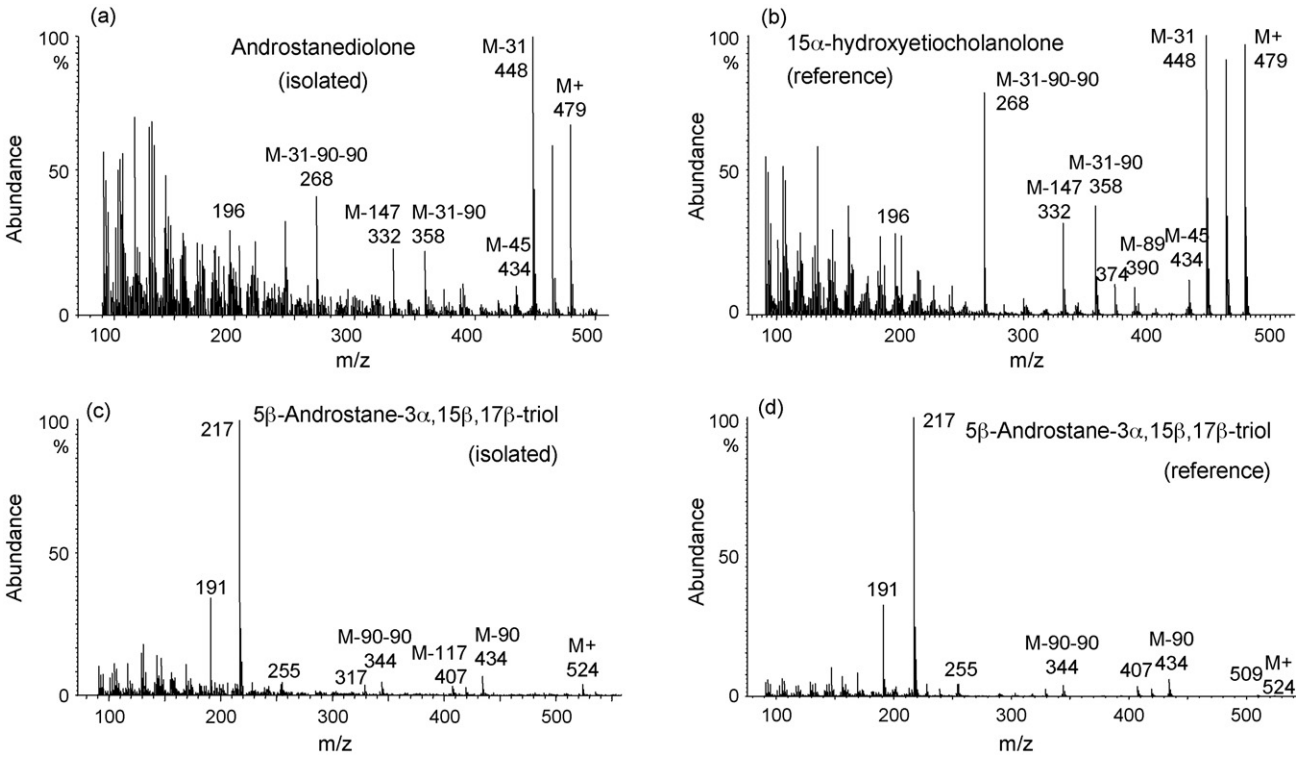

Fig. 1. Mass spectra of andostanediolones and androstanetriol isolated from pregnant mouse urine, and equivalent reference compounds, as methyloxime-trimethyl silyl ethers (MO-TMS).

not be in the D-ring as this would prevent genesis of the specific fragments indicative of 15-hydroxylated structure. Hydroxylation at C18 is excluded because of lack of ions representing loss of a primary trimethylsilyl group. Remaining candidate positions would be 1, 2, 6, 7 and 11.

3.1.3. Androstanetetrols

Five prominent androstanetetrols (Atetrols) were found listed #1–5 with retention indices 2654, 2751, 2784, 2786 and 2829.

3.1.3.1. Atetrols 1 and 4 (RI 2654 and 2784). These two steroids had essentially identical mass spectra (Fig. 3) and must be assumed to be stereoisomers. The molecular ions were at *m*/*z* 612. Prominent ions are formed by loss of trimethylsilanol at *m*/*z* 522, 432 and 342. A *m*/*z* 217 base peak (and associated *m*/*z* 191 ion) are shared with the androstanetriol described above so it must be assumed that the compound contains 15α- and 17β-hydroxyl groups. The spectrum has distinctive ion at *m*/*z* 467, M-145. This loss has been reported in steroids with derivatized hydroxyls at positions 3 and 6. Another important ion is at *m*/*z* 494, M-118 but the genesis of this ion is unknown. Important ions at *m*/*z* 143 and 142 may suggest the presence of vicinyl 2- and 3-hydroxyls. These steroids will likely have the structure 5α(or β)-androstane-3α,X,15α,17β-triol.

3.1.3.2. Atetrol 2 (RI 2751). Base peak at *m*/*z* 217 and second most prominent ion at *m*/*z* 191 indicating a 15α-hydroxylated structure. It shares with #1 and 4 an ion at *m*/*z* 467 (M-145). Ions characteristic for this steroid were present at *m*/*z* 387, 297 and 256. An additional hydroxyl at positions 6 most likely from available evidence.

3.1.3.3. Atetrol 3 (RI 2782). This had very different spectra (Fig. 3) from those described above. The most distinctive fragmentation is through the loss of 103 (*m*/*z* 509) with further losses of trimethylsilanol (*m*/*z* 419, 329 and 239). Clearly the steroid must have a primary hydroxyl which can only be at positions 18 and 19. The latter position is unlikely and has never been found in an androstane metabolite, although may be present in estrogen synthesis intermediates. The spectrum is very similar to that

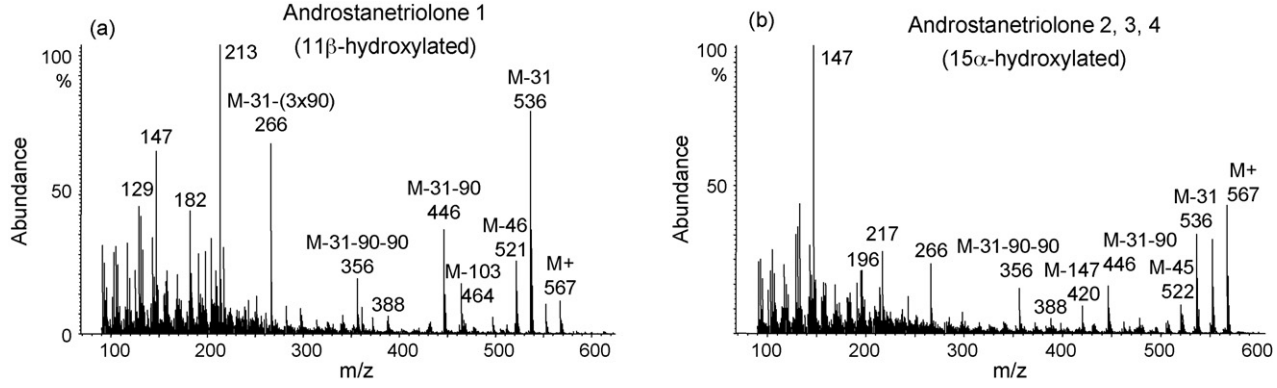

Fig. 2. Mass spectra of two androstanetriolones isolated from mouse urine as MO-TMS ethers.

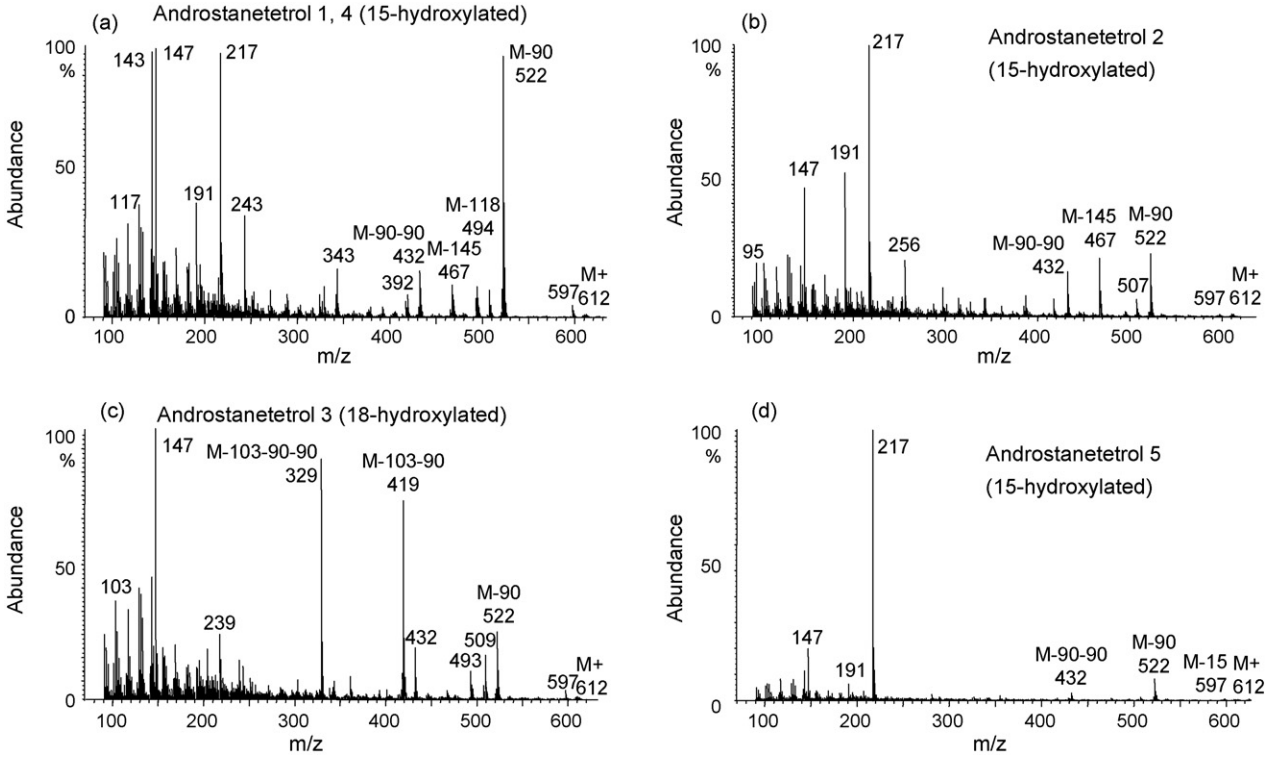

Fig. 3. Mass spectra of four androstanetetrols isolated from mouse urine as TMS ethers.

of 5-androstene-3β,16α,17β,18-tetrol previously identified in the human newborn [16]. This steroid is certainly 18-hydroxylated and we propose the structure androstane-3α,X,17β,18-tetrol. A second minor steroid with RI 2820 had same spectrum and was clearly an isomer.

3.1.3.4. Atetrol 5 (RI 2829). This steroid (Fig. 3) clearly was another 3α,X,15α,17β-tetrol based on the prominence of the *m*/*z* 191 and 217 ions. The position of the final hydroxyl could not be ascertained but certainly not the same as position "X" in androstanetetrols 1 and 4 on the basis of major differences in mass spectra.

3.1.4. Androstanetetrolones and androstanepentols

Several steroids of this type (M^+ 655 and *m*/*z* 700, respectively) were also found but their structures were not determined.

3.1.5. Pregnanetriolone (RI 2925) and Pregnanetetrolones (RI 3096, 3154)

Two major pregnanes were found that had mass spectra inappropriate for corticosterone metabolites so likely are formed from progesterone. Other progesterone metabolites may have been present but not recognized. As shown in Fig. 4 one steroid had a molecular ion at *m*/*z* 595 and base peak *m*/*z* 564 (M-31), the other a molecular ion at 683 and M-31 at *m*/*z* 652. Prominent ions at *m*/*z* 188 showed they could be 16-, 17- or 21-hydroxylated compounds. However, the absence of ions at *m*/*z* 175 excluded hydroxylation at C-21 suggesting that the compounds were 21-deoxy progesterone metabolites. The presence of an ion at *m*/*z* 156 was appropriate for 16- or 17-hydroxylated steroids and the absence of a prominent ion at *m*/*z* 158 mitigates against the presence of a 17-hydroxyl. We believe the steroids to be 16α-hydroxylated with additional hydroxyls not in D-ring or side-chain. In contrast to the androstane series the presence of 15α-hydroxylated metabolites was not confirmed.

3.2. Timecourse of pregnancy steroid production

3.2.1. Individual excretion of steroids in wild-type mice

Fig. 5 shows a composite of excretion of individual steroids and groups of compounds at time points during the two normal pregnancies studied. The results for androstanetriolones and

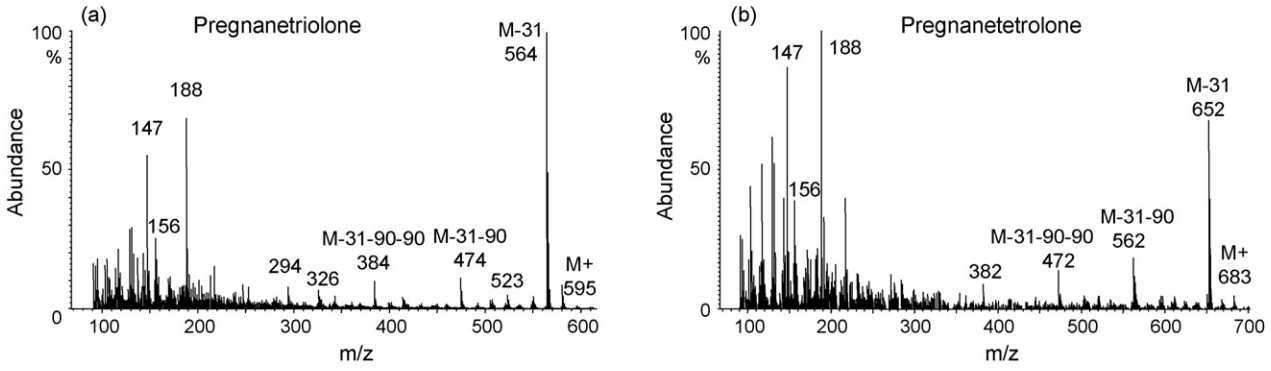

Fig. 4. Mass spectra of a pregnanetriolone and pregnanetetrolone isolated from mouse urine as MO-TMS ethers.

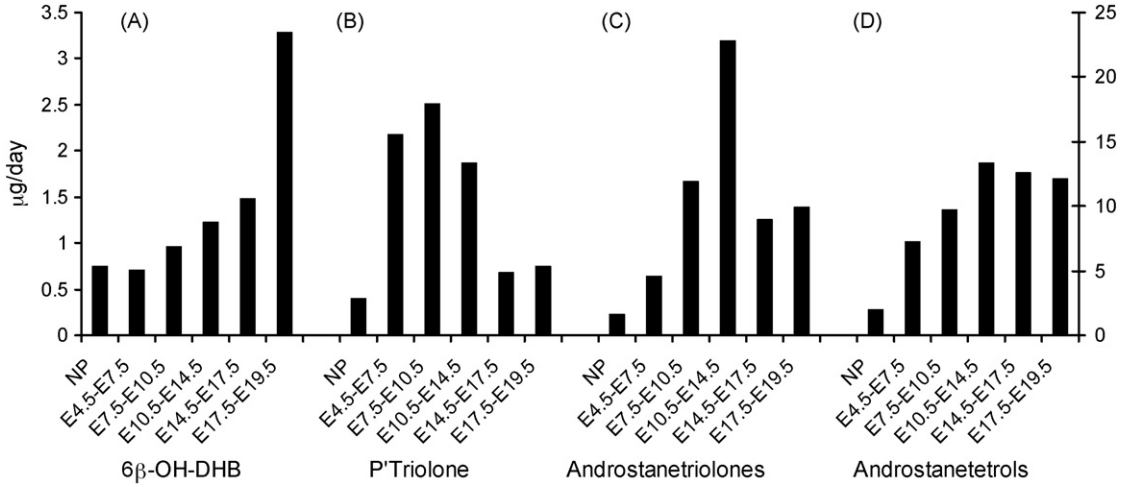

Fig. 5. Time course of steroid excretion of two normal mouse pregnancies (mean). (A) The corticosterone metabolite increases throughout pregnancy; (B) the putative progesterone metabolite peaks early and decreases after E14.5; (C and D) the polyhydroxylated androstanes (summed) increase more slowly but retain high excretion till term. *Abbreviations*: NP, non-pregnant. Urine collection prior to breeding; E4.5–E7.5: urine collected during the days 4.5 and 7.5 of gestation, etc.

androstanetetrols are the sum of all measured. The graph was then constructed from the mean for the two pregnancies.

3.2.1.1. Corticosterone metabolite. Corticosterone largely is a product of the maternal adrenal and its measurement was not a focus of this study. We did measure one metabolite 6β-hydroxy-20α-dihydrocorticosterone (6β-hydroxyDHB) among the many excreted [1] to investigate how the secretion changed during pregnancy. The mean result obtained for two animals is shown in Fig. 5. There was a steady increase during gestation with a large increase at the end. The final excretion being five times non-pregnant (NP) levels.

3.2.1.2. Progesterone metabolites. The excretion of pregnanetriolone, the presumed major progesterone metabolite, reaches high levels by E7.5 and peaks at E10.5–14.5. The excretion then tapers-off reaching near NP levels by E17.5.

3.2.1.3. Androstanes. The excretion of polar androstanes increases dramatically. Androstanetriolones peak at 10.5–14.5 days then decrease to term. Androstanetetrols show a slower increase, maximize at E14.5–17.5 and remain high to term.

While the total production of androstanetetrols increases steadily throughout pregnancy, they do so at different rates. This is illustrated in Fig. 6 which shows that the excretion of one of the 15α-hydroxylated andostanetetrols (#5) increases steadily throughout pregnancy while an 18-hydroxylated tetrol (#3) starts low but at mid-pregnancy becomes dominant.

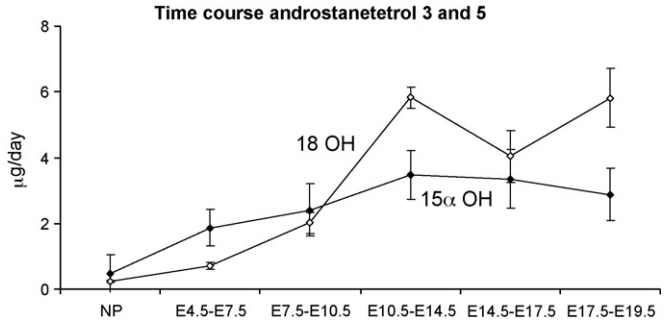

Fig. 6. Different time course of excretion of a 15α- and 18-hydroxylated androstanetetrol.

3.3. Correlation of steroid excretion and fetal sterols in Dhcr7 mutant mice

Two pregnancies with female Δ/+ mated with T93M/T93M male were studied in detail until termination at E14.5. Maternal urine collections were made prior to termination and amniotic fluid was collected at the termination. Pup genotyping and brain and liver sterols were determined. One pregnancy (A) had five pups, three Δ/T93M and two +/T93M. The other (B) had six pups, four Δ/T93M and two +/T93M. Fig. 7 shows the brain and liver sterol levels of the affected pups and the ratio between DHC and cholesterol (C). DHC could not be detected in the unaffected pups so the DHC/C ratio was essentially zero. These results clearly show reduced dehydrosterol reductase levels in the Δ/T93M pups. As previously shown [17] the DHC/C ratio is much higher in brain than in liver.

In spite of only trace amount of amniotic fluid (AF) being collected on filter paper DHC and cholesterol could be measured in most samples. Without knowing the AF volume absolute values of DHC and cholesterol were not measurable. The DHC/C ratio was determined and this proved to be low compared to fetal values (Fig. 7). DHC was not detected in AF from the unaffected fetuses (+/T93M).

3.3.1. Maternal urine from E14.5 termination: seeking 7- or 8-dehydrosteroids

Little if any 7- or 8-dehydrosteroids are produced and excreted. By recording selected ions 2 Da less in mass than characteristic ions for saturated steroids we showed the presence of an androstenetriolone (Fig. 8), andostenetetrols and androstenepentols. However, these steroids could also be detected in the wild-type pregnancies which suggests they are Δ^5 rather than Δ^7 or Δ^8. Potential progesterone metabolites (pregnanetriolone or pregnanetetrolone) with additional unsaturation were sought but not detected even at trace levels.

3.3.2. Steroid production in late-term mutant pregnancies

As shown in Table 1, a number of pregnancies were generated by mating mice of different mutant genotypes. Resulting litters usually contained one or more affected pups, that is, pups with two mutant alleles and abnormally high levels of DHC. However, in one cross, Δ/T93M females by Δ/+ males, 3 of 5 litters were small and had no affected pups suggesting either fetal demise or cannibalism of affected pups after birth. Notable in this cross was that the mother herself had a relatively severe mutant geno-

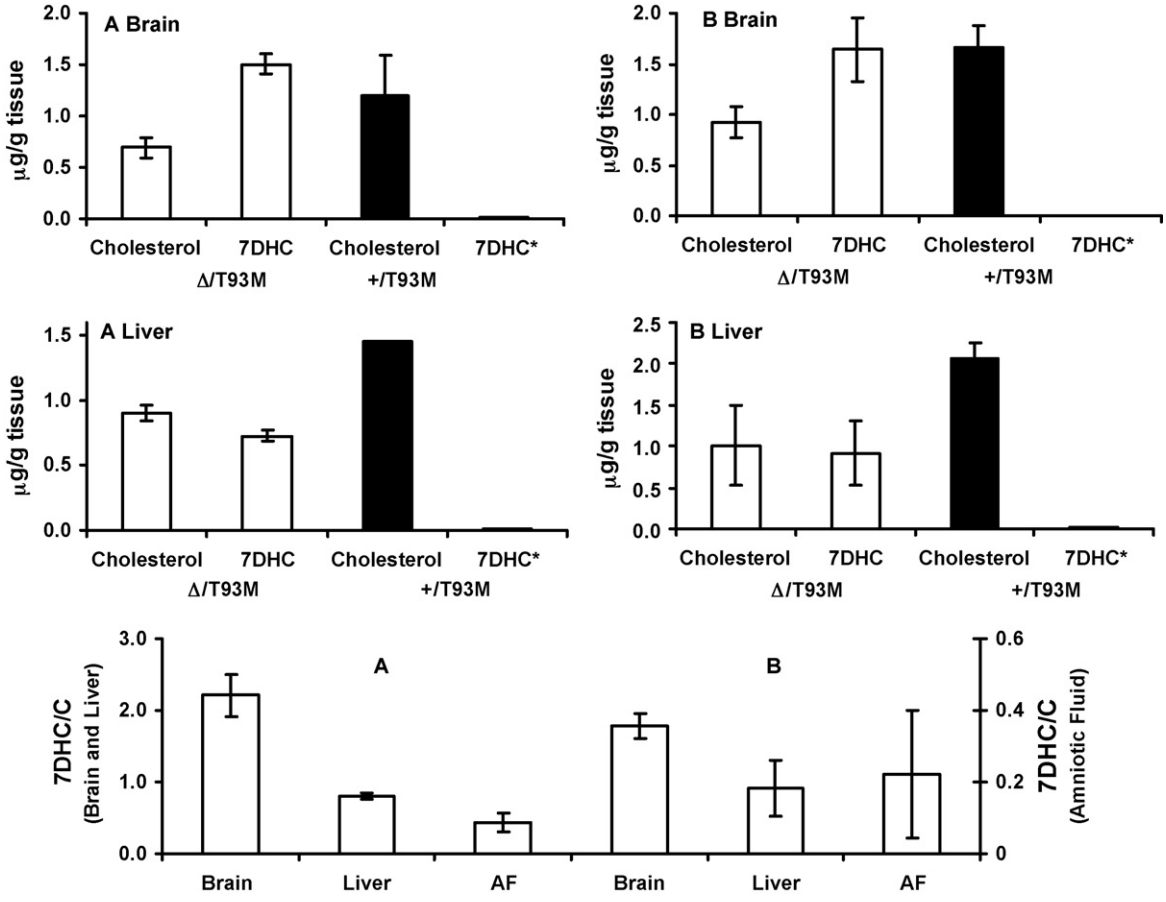

Fig. 7. Tissue and amniotic fluid analysis from two mutant pregnancies terminated at E14.5. Results given are dehydrocholesterol (DHC) and cholesterol concentrations and DHC/cholesterol ratios in fetal brain, liver and amniotic fluid (for latter, ratio only). For pregnancy (A) the values given are the mean from analysis of three Δ/T93M pups and two +/T93M pups. For pregnancy (B) the values are the mean from analysis of four Δ/T93M pups and two +/T93M pups. In the lower (ratio) panel the scale on left represents brain and liver, and on right amniotic fluid.

type. When late term maternal urine was analyzed, pregnancies with Δ/Δ, Δ/T93M and T93M/T93M fetuses had the same urinary steroids as wild-type pregnancies and at similar excretion level. Identical results were found for pregnancies carrying fetuses homozygous for the null mutation, *Dhcr*$^{-/-}$. No steroids with an additional double-bond were detected in urine of these pregnancies that were not also present in urine from wild-type pregnancies. This was true both for the putative progesterone metabolites (pregnanetriolone and pregnanetetrolone) and androstanes. This was also the case even when the mother was T93M homozygous or Δ/T93M.

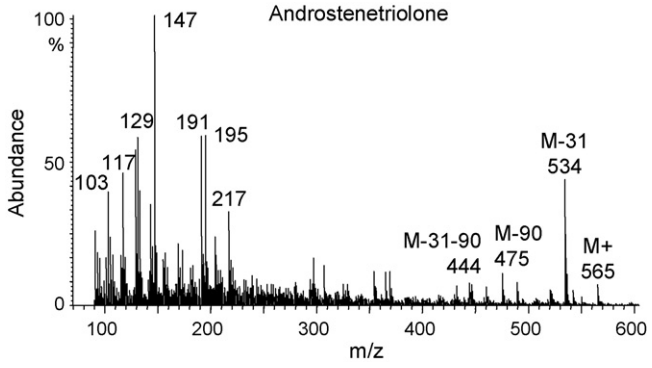

Fig. 8. Mass spectrum of a steroid (as MO-TMS ether) identified as an androstenetriolone.

4. Discussion

The production of steroids during pregnancy in the mouse is poorly understood. According to the data reported here there is huge increase in steroid synthesis over non-pregnancy in similar fashion to the human. The urine steroid metabolome of the pregnant mouse has not been previously described, and very little is known about excretion at other periods of life. We are aware of only one publication on steroid excretion that uses similar methodology, that of Alasandro et al. [18]. They document the excretion of androstenetriols, androstanetriolones and androstanetetrolones as well as many pregnane polyols. Our results for non-pregnancy in many ways agree with theirs. Clearly, the results show that the mouse produces and excretes steroids with greater polarity than the human. One other paper has recently been published on mouse steroid excretion [15] but this addressed analysis of a targeted suite of less-polar steroids (including hormonal androgens), excreted free or as glucuronide conjugates. The few that were found in this study were not dominant excretory products. Similar to the study of Alasandro et al. [18] our goal was to define what steroid analytes were quantitatively major metabolites in the mouse. Our analytical procedure utilized sulfatase as well as beta-glucuronidase hydrolysis, with C18 cartridge extraction, which is known to quantitatively extract steroids of all polarities. Thus, our data set is very different from that of Lootens et al. [15].

While all metabolites characterized were detected in non-pregnant and pregnant animals, many steroids we identified in pregnancy urine seemed to be characteristic of the condition

and were found to be excreted in high amount. These generally either had structures of androstanes with polarity ranging from androstanediolones to androstanepentols, or had the structure of hydroxylated pregnanolones. It appears that the most notable transformation in androstane metabolism is 15α-hydroxylation since our GC/MS data showed that many steroids had that functional group. Hydroxylation in that position by female rodents has been known for decades since the pioneering GC/MS rat urinary studies of Eriksson and co-workers in the 1960s and 1970s [15, and references therein]. In addition, genes for more than one 15α-hydroxylase have been found in the mouse [19,20], and in liver are specific to the female. An andostanetriolone with an 11β-hydroxyl was also detected, both in pregnant and non-pregnant animals and we found evidence for other hydroxylations, particularly at the18-position. We do not know where these metabolic reactions occur but placenta and maternal and fetal liver are candidate organs. There is a bi-modal excretion of steroids. The peak of progesterone metabolites appears early (E4.5–14.5) and then their excretion decreases to near non-pregnancy levels before term. This data is in complete agreement with that of deCatanzaro et al. [21] who measured urinary progesterone throughout gestation in pregnant mice and report the highest excretion on E8. The polar androgens peak later (E14.5–17.5) even though they also decrease prior to parturition. The later production of androstanes may suggest a fetal source, certainly this is the case in human pregnancy with the production of estriol. There was also difference in the excretions between the androstanes with different functional groups. Those with 15α-groups have an increased production as early as E4.5–7.5 while the presumed 18-hydroxylated compound did not significantly change till the E7.5–10.5 period. Estrogens were sought but not found, although other investigators have shown by an immunoassay technique that estradiol excretion peaks at about E8 [21]. The low excretory levels of estradiol and metabolites, relative to the high excretion of the neutral steroids renders them unidentifiable using our current methodology. Thus, it appears that the large amount of C_{19} steroids produced by the pregnant mouse are not aromatized to a significant extent, unless of course there is significant and selective estrogen excretion by the fecal route.

Steroid synthesis in the human feto-placental unit follows two independent routes. Progesterone is a product of the placenta and is metabolized largely to pregnanediol prior to excretion. Androstenes are also important products of the fetus although they are placentally aromatized to estriol prior to excretion. As would be expected there have been major differences reported in pregnancy steroidogenensis between the species. In the first instance the rodent ovary remains active throughout gestation while in the human the placenta replaces ovarian steroidogenic functions during the first trimester. Ovariectomy at any time in murine pregnancy causes termination emphasizing its continual essential role in progesterone and estrogen synthesis.

In contrast to the human, the mouse placenta (and fetal liver) have 17-hydroxylase activities allowing the production of androgens, while in the human the lack of such activity results in reliance on the fetal adrenal for production of androgen and estrogen precursors. Our documented increasing androstane production during pregnancy in the mouse suggests an important role for the feto-placental unit in their synthesis. This would necessitate an extra-placental supply of pregnane precursors as placental progesterone synthesis apparently ceases by the second half of pregnancy. Arensburg et al. [12] found peak levels of P450scc, StAR protein, and a specific dehydrogenase (3βHSD VI) at E9 but enzyme activity is lost after day 11. In rat pregnancy progesterone synthesis in minced placental tissue peaks at day 12 and decreases towards term [12]. Thus, placenta seems not to be involved in *de novo* steroid synthesis in the second half of pregnancy. Interestingly, 17-hydroxylase activity in mouse pregnancy is critical, at least in early days; Cyp17 knockout fetuses die before E7 [22]. In contrast, human CYP17 "knockouts", individuals with the 17-hydroxylase deficiency syndrome form of CAH have normal pregnancies and birth and are rarely diagnosed before puberty exemplifying the fact that this is a non-fatal disorder.

We have shown that large amounts of polar androstanes are produced during mouse pregnancy, increasing steadily from the E4.5–7.5 period. Whether these are metabolites of androgens (or progesterone) essential for maintenance of pregnancy is not known. Current research suggests that in the mouse pregnancy steroidogenesis is at least a result of combined ovarian and placental action, with a role for the fetus possible. In addition the fetal mouse liver, in contrast to the human, has 3βHSD and 17-hydroxylase activities [23] so is capable of making active hormones such as progesterone and C_{19} steroids.

The fetal adrenal is unlikely to have a major role in steroid synthesis through most of murine pregnancy. However, cytochrome P450scc is present after E11 and surprisingly the fetal adrenal has identifiable 17-hydroxylase between E12.5 and E14.5. This activity is lost after E16.5 [9]. Our study has shown that large amounts of C_{19} steroids are produced before E12.5 and after E16.5 which would argue against a major role of the mouse fetal adrenal in late-pregnancy steroidogenesis.

Since there is little current evidence for or against a fetal role in pregnancy steroidogenesis in the mouse, we have attempted to use a human model to answer this question. The human disorder Smith–Lemli–Opitz syndrome is caused by a mutation in the enzyme that converts 7-dehydrocholesterol (7-DHC) to cholesterol. This results in a build-up of 7-DHC and its isomer 8-DHC (formed by a Δ^7–Δ^8 isomerase). In a fetus affected with this disorder fetal adrenal 7- and 8-DHC are used as precursors for steroid synthesis and end-products of this synthesis, C_{18}, C_{19} and C_{21} steroids with Δ^7- or Δ^8-unsaturation, are excreted in maternal urine once the fetal adrenal becomes active at 10-11 weeks gestation [2,3]. We reasoned that if the mouse fetus utilizes fetal cholesterol to some extent for pregnancy steroidogenesis, then in a pregnancy with mutant pups with documented (by tissue analysis) DHC excess, maternal urinary steroids should include some with Δ^7- or Δ^8-unsaturation. We sought such metabolites in urine from pregnancies with such fetuses but failed to detect any steroids that were not in wild-type pregnancies. The apparent paucity of such metabolites was the case even in pregnancies where the mother was homozygous mutant (T93M/T93M) or compound heterozygous (Δ/T93M) when a majority of the pups were homozygous or compound heterozygous mutants. Pregnancies with mutant fetuses ($Dhcr^{-/-}$) gave identical results. The absence of Δ^7 or Δ^8 metabolites in urine of homozygous T93M or compound heterozygous adult mice (pregnant or non-pregnant) is not itself surprising since it has been documented that the abnormal accumulation of DHC in mutant animals largely "self-corrects" with maturity, in contrast to the human [5,17]. The mechanism by which this occurs is not known.

The lack of confirmed Δ^7- or Δ^8-unsaturated steroids in maternal urine from affected pregnancies probably signifies that pregnancy steroidogenesis in the mouse utilizes as precursor cholesterol largely of maternal origin, whether or not certain biosynthetic steps occur in the feto-placental unit. It has been estimated that up to 35% of fetal cholesterol in the mouse originates in the mother [6–8] indicating efficient transplacental transfer. By contrast, in the human little cholesterol is thought to cross the placental barrier [10]. In the human amniotic fluid sterols originate largely in the fetus as DHC/C values of amniotic fluid (AF) and neonatal plasma from the same SLOS affected pregnancy are essentially identical (Dr. Lisa Kratz, personal communication; data from five affected pregnancies). In the mouse we found the AF DHC/C ratios less than 20% of pup liver tissue values indicating a mostly maternal origin of sterol.

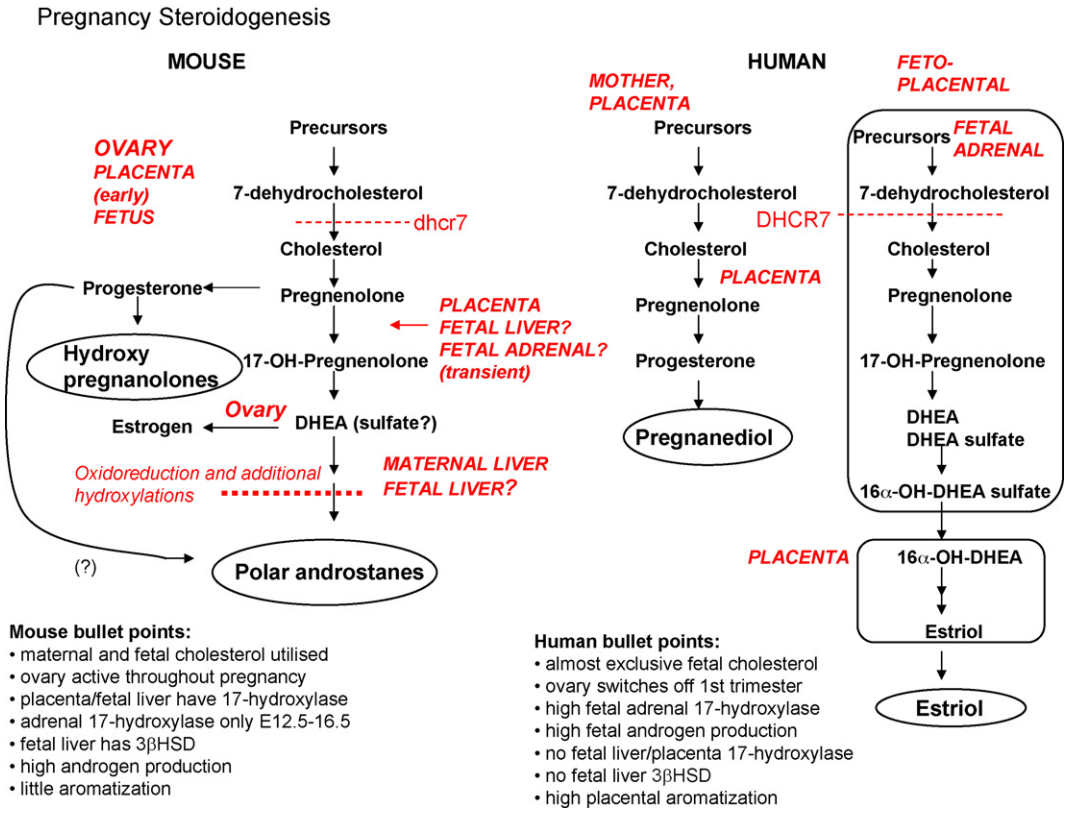

Fig. 9. Summary of steroid synthesis and metabolism by the pregnant mouse and human. Major maternal urinary metabolites enclosed in ovals.

Fig. 9 condenses our discussion of pregnancy steroidogenesis in the human and mouse. Summarizing our studies we have shown that the pregnant mouse produces a large amount of steroids in pregnancy. In early pregnancy these are a mixture of progesterone and C_{19} steroids but later in gestation C_{21} metabolites decrease while androstanes increase. It is not known whether the polar androstane metabolites are also progesterone metabolites or are formed by an independent pathway, as is the case for the human synthesis of estriol. Exactly to what extent the maternal ovaries, placenta and fetal organs are involved in their synthesis is undetermined. Initial investigations on mice with dhcr7 mutations suggests that in contrast to the human, fetal sterols are not important precursors of the quantitatively major pregnancy steroids.

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