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The *in Vivo* Metabolism of 16α-Hydroxylated C_{19} Steroids in Late Pregnancy*

Edward V. YoungLai and Samuel Solomon

ABSTRACT: Following the intravenous administration of ^{3}H- and ^{14}C-labeled 16α-hydroxylated C_{19} steroids to subjects in the third trimester of pregnancy, labeled 3β,16α-dihydroxyandrost-5-en-17-one, 16α-hydroxyandrost-4-ene-3,17-dione, 3α,16α-dihydroxy-5α-androstan-17-one, 3α,16α-dihydroxy-5β-androstan-17-one, 3α,16α-dihydroxyandrost-5-en-17-one, androst-5-ene-3β,16α,17β-triol, and estra-1,3,5(10)triene-3,16α,17β-triol (estriol) were isolated from the urine in either the sulfate or glucosiduronate fraction or in both. In other to determine the origin of these neutral metabolites and estriol, the following pairs of substrates were injected into pregnant subjects in the third trimester: (a) [7-^{3}H]3β,16α-dihydroxyandrost-5-en-17-one (16D) and [4-^{14}C]16α-hydroxyandrost-4-ene-3,17-dione (16Δ^4), (b) [7-^{3}H]16D and [4-^{14}C]17-oxoandrost-5-ene-3β-yl sulfate (DS), and (c) [7-^{3}H]16D-3 sulfate (16DS) and [4-^{14}C]DS. From the percentage conversion of the injected radioactivity, the specific activities, and the ^{3}H/^{14}C ratios in the urinary metabolites, it was possible to conclude that 16D and 16Δ^4 were equally well converted into estriol (E_3), but no β,γ-unsaturated alcohols were formed from 16Δ^4; DS was converted into 16D and androst-5-ene-3β, 16α,17β-triol (DT) and both 16D and DS gave rise to E_3; 16DS and DS were not efficiently converted into 16Δ^4 or 3α,16α-dihydroxyandrost-5-en-17-one but formed urinary E_3 in good yield. In two studies, [^{14}C]3-hydroxyestra-1,3,5(10)-trien-17-one was isolated while in one study [^{14}C]3β-hydroxyandrost-5-en-17-one and [^{14}C]3α-hydroxy-5α-androstan-17-one were isolated in crystalline form. While all the injected steroids were good precursors of E_3, 16DS and DS seemed to be the most efficient. Moreover, the metabolite possessing the 3α-hydroxy-5-ene structure was derived mainly from 16D. From the specific activities of the isolated E_3 it was evident that all the precursors studied were not the sole source of E_3 in late pregnancy. When labeled 16D was injected intravenously to a normal male and a normal female in the luteal phase of the menstrual cycle, most of the abovementioned neutral urinary metabolites were isolated but no labeled E_3 was found, indicating that E_3 in the normal female is not formed from the aromatization of a 16α-hydroxy neutral steroid.

It has been well documented that in man 16α-hydroxylated C_{19} steroids serve as precursors of estriol[1] during pregnancy (Ryan, 1958, 1959; Magendantz and Ryan, 1964; Siiteri and MacDonald, 1966; Dell'Acqua *et al.*, 1967a,b). The metabolism of these steroids in the maternal and fetal compartments has not, to date, been extensively investigated. Siiteri and MacDonald (1966) reported that 16α-hydroxydehydroisoandrosterone administered to subjects in the third trimester of pregnancy was converted into androst-5-

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[1] The following trivial names of steroids have been employed in the text: 16α-hydroxydehydroisoandrosterone = 3β,16α-dihydroxyandrost-5-en-17-one; deoxycorticosterone = 21-hydroxypregn-4-ene-3,20-dione; 16α-hydroxyetiocholanolone = 3α,16α-dihydroxy-5β-androstan-17-one; 16α-hydroxyandrosterone = 3α,16α-dihydroxy-5α-androstan-17-one; dehydroisoandrosterone = 3β-hydroxyandrost-5-en-17-one; androsterone = 3α-hydroxy-5α-androstan-17-one; androstenedione = androst-4-ene-3,17-dione; 16α-hydroxyandrostenedione = 16α-hydroxyandrost-4-ene-3,17-dione; dehydroisoandrosterone sulfate = 17-oxoandrost-5-ene-3β-yl sulfate; 16α-hydroxydehydroisoandrosterone sulfate = 16α-hydroxy-17-oxoandrost-5-ene-3β-yl sulfate; estrone = 3-hydroxyestra-1,3,5(10)-trien-17-one; estradiol = estra-1,3,5(10)-triene-3,17β-diol; estriol = estra-1,3,5(10)-triene-3,16α,17β-triol.

TABLE I: Urinary Metabolites Isolated after the Intravenous Administration of [7-^{3}H]16α-Hydroxydehydroisoandrosterone and [4-^{14}C]16α-Hydroxyandrostenedione (^{3}H/^{14}C = 11.5) in a Pregnant Female.

Metabolite	Method of Identifcn and Detn of Sp Act.	Sp Act. (dpm of ^{3}H/mg)	^{3}H/^{14}C
16α-Hydroxyandrostenedione	Rid[a]	5.2×10^5	3.2
16α-Hydroxyandrosterone	Irp	6.3×10^5	4.4
16α-Hydroxyetiocholanolone	Irp	7.6×10^5	4.6
3α,16α-Dihydroxyandrost-5-en-17-one	Infrared, Irp	2.2×10^6	∞
16α-Hydroxydehydroisoandrosterone	Crystzn, Rid, infrared, mp	9.1×10^4	∞
Androst-5-ene-3β,16α,17β-triol	Crystzn, infrared, mp, Rid	8.3×10^4	∞
Estriol	Crystzn, infrared, mp	3.8×10^4	10.1

[a] Rid = isotope dilution; Irp = isotope ratio procedure; crystzn = crystallization to constant specific activity; mp = melting point and mixture melting point.

ene-3β,16α,17β-triol and estriol. YoungLai and Solomon (1967b) found that labeled 16α-hydroxydehydroisoandrosterone was metabolized in late pregnancy to urinary 16α-hydroxyandrostenedione, 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone, 3α,16α-dihydroxyandrost-5-en-17-one, androst-5-ene-3β,16α,17β-triol, and estriol.

The aim of the present investigations was to determine the precursor role of 16α-hydroxydehydroisoandrosterone, 16α-hydroxyandrostenedione, 16α-hydroxydehydroisoandrosterone sulfate, and dehydroisoandrosterone sulfate in the formation of estriol and other neutral metabolites in late pregnancy. To accomplish this aim, ^{3}H- and ^{14}C-labeled steroids were injected together into subjects in the third trimester of pregnancy and the ^{3}H/^{14}C ratios as well as the specific activities were determined for each of the neutral and phenolic metabolites isolated from the urine.

Materials and Methods

Except where otherwise stated, the methods used in the isolation and identification of urinary metabolites have previously been described (Ruse and Solomon, 1966; YoungLai and Solomon, 1967b). The [7-^{3}H]16α-hydroxydehydroisoandrosterone used in these studies was prepared in an identical manner as described previously (YoungLai and Solomon, 1967b). It had a specific activity of 3.19×10^8 dpm/mg and was shown to be over 97% pure by the use of the isotope dilution technique. The [4-^{14}C]16α-hydroxyandrostenedione was prepared by incubating 100 μCi of [4-^{14}C]androstenedione mixed with 4.66 mg of carrier using the same conditions previously described by Ruse and Solomon (1966). The methylene chloride extract of the fungal incubation medium was chromatographed on thin-layer plates in the system ethyl acetate–*n*-hexane (2:1) and the area corresponding to 16α-hydroxyandrostenedione was eluted. The material in the eluate was chromatographed on paper in the system isooctane–toluene–methyl alcohol–water (5:5:7:3) to the solvent front and then in the system benzene–cyclohexane (1:1)–propylene glycol for 29 hr. The ultraviolet-positive material was located at 14.8 cm in the latter system, and it was eluted and rechromatographed on a small silica gel column. Elution with 2% ethanol in methylene chloride provided [4-^{14}C]16α-hydroxyandrostenedione which was over 97% pure as judged by the use of the isotope dilution technique and had a specific activity of 4.28×10^7 dpm/mg.

[4-^{14}C]Dehydroisoandrosterone sulfate was prepared by the method of Fieser (1948) using pyridine, *p*-chlorosulfonic acid, and [4-^{14}C]dehydroisoandrosterone (56 mCi/mmole). Radiochemical purity was established by the symmetry of the radioactive peak on a paper chromatogram using the system isopropyl ether–*t*-butyl alcohol–ammonium hydroxide–water (6:4:1:9) for 8 hr, and by isotope dilution with carrier dehydroisoandrosterone sulfate. The [7-^{3}H]16α-hydroxydehydroisoandrosterone sulfate [1.62 Ci/mmole) was the same as that prepared previously (YoungLai and Solomon, 1967a) and was used soon after preparation since it has been observed that this steroid decomposes with prolonged storage.

Two batches of [1-^{14}C]acetic anhydride were used in these studies and these had specific activities of 1.16×10^5 dpm (i) and 2.28×10^4 dpm (ii) per mg of deoxycorticosterone acetate, respectively, while the [^{3}H]acetic anhydride had a specific activity of 2.24×10^5 dpm/mg of deoxycorticosterone acetate.

The subjects who volunteered for these studies were a normal male, a normal female in the luteal phase of the menstrual cycle, and three normal females in the third trimester of pregnancy. After the administration of the labeled steroids, completeness of urinary collection was checked by the determination of creatinine. The urinary conjugates were hydrolyzed first by solvolysis and then by β-glucuronidase (YoungLai and Solomon, 1967b), with the exception of one study which will be described. Extracts thus obtained were processed in a manner identical with that previously described (YoungLai and Solomon, 1967b). In several instances there was sufficient weight in the urinary metabolite to permit identification by infrared analysis and specific activities were determined by direct crystallization. Where carrier steroid was available the isotope dilution technique was used to confirm the identity of the urinary metabolites. In some instances, where minute amounts of labeled metabolites were present, the isotope ratio procedure (YoungLai and Solomon, 1967b) was used for their identification and the determination of specific activities.

The 17-ketosteroids, as well as estrone, were found in the first peak of radioactivity eluted from the initial silica gel columns used to fractionate the crude extracts and they were purified by chromatography on a Celite partition column using the system isooctane–*t*-butyl-alcohol–methyl alcohol–water (10:2:7:3) and on paper in the system Ligroin B–90% methyl alcohol for 12 hr. These metabolites were then further purified by chromatography on small silica gel columns prior to crystallization. The methods used in the identification and proof of radiochemical purity for each metabolite will be indicated in the tables.

Results

In the first study to be described we wanted to compare the precursor role of 16α-hydroxyandrostenedione and 16α-hydroxydehydroisoandrosterone in the formation of estriol and to determine whether a Δ^4-3-ketosteroid could, under favorable circumstances, be converted into a β,γ-unsaturated alcohol. We therefore injected intravenously a mixture of 2.86×10^7 dpm of [7-^{3}H]16α-hydroxydehydroisoandrosterone and 2.50×10^6 dpm of [4-^{14}C]16α-hydroxyandrostenedione (^{3}H/^{14}C = 11.5) in a subject in the third trimester of pregnancy. Each 24-hr urine collection was hydrolyzed with Glusulase (Endo Laboratories, New York) as previously described (Ruse and Solomon, 1966). Table I shows the urinary metabolites isolated, the method used for identification and determination of specific activity, and their ^{3}H/^{14}C ratios.

The next two studies were carried out in order to determine whether dehydroisoandrosterone sulfate could be 16α hydroxylated and serve as a precursor of some of the urinary metabolites isolated. In the first of these studies 2.26×10^7 dpm of [7-^{3}H]16α-hydroxydehydroisoandrosterone and 4.40×10^6 dpm of [4-^{14}C]-dehydroisoandrosterone sulfate (^{3}H/^{14}C = 5.1) were injected intravenously in a subject in the third trimester of pregnancy. The urinary conjugates were hydrolyzed to give a sulfate fraction and a glucosiduronate fraction, each of which was processed separately. Table II shows the urinary metabolites isolated and identified, their ^{3}H/^{14}C ratios, and the method used for the identification and determination of specific activity. In addition to the 16α-hydroxylated neutral metabolites and estriol, dehydroisoandrosterone and androsterone were isolated from the sulfate fraction while androsterone and estrone were isolated from the glucosiduronate fraction. In the second of these studies, 2.72×10^7 dpm of [7-^{3}H]16α-hydroxydehydroisoandrosterone sulfate and 3.85×10^6 dpm of [4-^{14}C]dehydroisoandrosterone sulfate (^{3}H/^{14}C = 7.1) were injected in a subject in late pregnancy. The urinary conjugates were separated into sulfate and glucosiduronate hydrolyzed fractions

TABLE II: Urinary Metabolites Isolated after the Injection of [7-^{3}H]16α-Hydroxydehydroisoandrosterone and [4-^{14}C]Dehydroisoandrosterone Sulfate (^{3}H/^{14}C = 5.1) in a Pregnant Female.

Metabolite	Method of Identifcn and Detn of Sp Act.	Sp Act. in Sulfate Fraction (dpm/mg)	^{3}H/^{14}C	Method of Identifcn and Detn of Sp Act.	Sp Act. in Glucosiduronate fraction (dpm/mg)	^{3}H/^{14}C
16α-Hydroxyandrostenedione		*a*		Rid[b]	$(7.4 \times 10^5)/0$	∞
3α,16α-Dihydroxyandrost-5-en-17-one				Irp	$(1.7 \times 10^6)/0$	∞
16α-Hydroxydehydroisoandrosterone	Crystzn, infrared, mp	$(4.1 \times 10^4)^c/(1.5 \times 10^4)$	2.7	Rid	$(1.6 \times 10^6)/0$	∞
Androst-5-ene-3β,16α,17β-triol	Crystzn, infrared, mp, Rid	$(4.7 \times 10^4)/(1.5 \times 10^4)$	3.2			
Estriol	Rid, crystzn, infrared, mp	$(2.4 \times 10^4)/(7.9 \times 10^3)$	3.0	Crystzn, infrared, mp	$(3.2 \times 10^4)/(4.4 \times 10^3)$	7.3
Dehydroisoandrosterone	Infrared, mp, Rid	$0/(2.2 \times 10^4)$	0			
Androsterone	Infrared, mp, Rid	$0/(1.0 \times 10^4)$	0	Infrared, mp, Rid	$0/(1.4 \times 10^4)$	0
Estrone				Rid, crystzn, infrared, mp	$0/(5.7 \times 10^3)$	0

[a] These metabolites could not be isolated. [b] See footnote *a* in Table I. [c] The values in the numerator of each fraction refer to specific activity in disintegrations per minute of ^{3}H per milligram while the denominator values refer to specific activity in disintegrations per minute of ^{14}C per milligram.

TABLE III: Urinary Metabolites Isolated after the Injection of [7-^{3}H]16α-Hydroxydehydroisoandrosterone Sulfate and [4-^{14}C]Dehydroisoandrosterone Sulfate (^{3}H/^{14}C = 7.1) in a Pregnant Female.

Metabolite	Method of Identifcn and Detn of Sp Act.	Sp Act. in Sulfate Fraction (dpm/mg)	^{3}H/^{14}C	Method of Identifcn and Detn of Sp Act.	Sp Act. in Glucosiduronate Fraction (dpm/mg)	^{3}H/^{14}C
16α-Hydroxyandrostenedione		[a]		Rid[b]	(1.2×10^5)[c]/(1.1×10^4)	10.9
3α,16α-Dihydroxyandrost-5-en-17-one				Irp	$(1.5 \times 10^5)/(1.2 \times 10^4)$	12.2
16α-Hydroxydehydroisoandrosterone	Rid	$(6.0 \times 10^5)/(3.2 \times 10^4)$	18.6			
Androst-5-ene-3β,16α,17β-triol	Crystzn, infrared, mp, Rid	$(3.4 \times 10^5)/(2.4 \times 10^4)$	14.1			
Estriol	Infrared, Rid	$(5.8 \times 10^4)/(9.8 \times 10^3)$	5.9	Crystzn, infrared, mp	$(6.5 \times 10^4)/(8.1 \times 10^3)$	8.0
Estrone				Infrared, Rid	$0/(8.7 \times 10^3)$	0

[a] See footnote *a* in Table II. [b] See footnote *a* in Table I. [c] See footnote *c* in Table II.

and the metabolites were isolated and identified. Their ^{3}H/^{14}C ratios and the method used for their identification and determination of specific activity are shown in Table III. Dehydroisoandrosterone and androsterone were not isolated in sufficient quantity to permit proper identification. In the two studies described 16α-hydroxyandrosterone and 16α-hydroxyetiocholanolone were observed on chromatography of the urinary extracts but they were not isolated and analyzed as it was felt that their contribution was not very significant.

A total of 2.40 × 10^7 dpm of [7-^{3}H]16α-hydroxydehydroisoandrosterone was injected intravenously into a normal male and 2.34 × 10^7 dpm of this steroid was injected intravenously in a normal female in the luteal phase of the menstrual cycle. Four days' urine of the normal male and 5 days' urine of the normal female were processed. The urinary metabolites were isolated and identified, and their specific activities and the percentage conversion from the injected steroid are shown in Table IV. Except for 16α-hydroxydehydroisoandrosterone, which was identified by infrared analysis in the sulfate fraction and its specific activity determined by acetylation with [^{14}C]acetic anhydride in both fractions, all the other metabolites isolated were identified by means of the isotope ratio procedure. Labeled estriol was not isolated in either of these studies.

Discussion

A direct *in vivo* approach to the study of the formation of steroids in the fetus and placenta in late pregnancy is not possible. There are a number of indirect means of conducting such studies. One of these is the injection of labeled precursors into the maternal compartment; a second is to inject labeled precursors into the amniotic fluid or into the abdominal cavity of the fetus at the time of amniocentesis. In the studies reported we chose to inject the labeled precursors into the maternal circulation because it was previously established that the 16α-hydroxylated steroids (Young-Lai and Solomon, 1967b) as well as dehydroisoandrosterone sulfate (Siiteri and MacDonald, 1966) reach the placenta and can be aromatized to form estrogens. There are however a number of difficulties in the interpretation of the data obtained from such studies. It is difficult to determine what proportion of the steroid injected is metabolized in the maternal compartment, and how much of it reaches the placenta and the fetus. In addition it is difficult to determine whether the metabolic conversions observed are occurring uniquely in the fetoplacental unit or in this compartment as well as in the maternal compartment. In spite of the inherent difficulties of interpretation of the data obtained, it is possible to deduce some basic information concerning the precursors of estriol and neutral steroids formed by the placenta and excreted into the maternal urine.

Ward and Engel (1966) have recently demonstrated the conversion of androstenedione into 3β-hydroxyandrost-5-en-17-one by an acetone powder of sheep adrenal microsomes fortified with reduced diphosphopyridine nucleotide. Rosner *et al.* (1965) have reported the analogous reduction of [4-^{14}C]progesterone to 3β-

TABLE IV: Urinary Metabolites Isolated after the Injection of [7-^{3}H]16α-Hydroxydehydroisoandrosterone to Normal Subjects.

	Sp Act. (dpm/μg)				Percentage Conversion of Injected Steroid	
	Sulfate		Glucosiduronate			
Metabolite	Male	Female	Male	Female	Male	Female
16α-Hydroxydehydroisoandrosterone	234[a,b]	169[a,b]		5800[b]	2.7[c]	3.3
3α,16α-Dihydroxyandrost-5-en-17-one			8520	5760	9.8	8.3
16α-Hydroxyandrosterone	1110	663	8570	5560	6.4	6.1
16α-Hydroxyetiocholanolone		558	3150	1560	5.2	4.9

[a] Refers to infrared analysis. [b] Refers to isotope dilution. [c] All other metabolites were identified by the isotope ratio procedure.

hydroxypregn-5-en-20-one by homogenates of rabbit testes but the demonstration was not as unambiguous as the results obtained by Ward and Engel (1966). These findings strengthen the possibility that the 3β-hydroxysteroid dehydrogenase–isomerase system could operate in the reverse direction. Whether such a reversible reaction occurs *in vivo* remained to be determined. Since the 16α-hydroxyl group partially inhibited the Δ^{4}-isomerase enzyme it seemed reasonable to us that the above reaction might be confirmed *in vivo* with 16α-hydroxyandrostenedione. The data shown in Table I are in part the results of such a study. It is apparent from these data that none of the 3β-hydroxy-5-ene steroids contain detectable amounts of ^{14}C, indicating that the 16α-hydroxyandrostenedione was not converted in any appreciable amount into such steroids. Our only reservation concerning this conclusion is that the 16α-hydroxyl may also partially inhibit the isomerase mediating the conversion of an α,β-unsaturated ketone into a β,γ-unsaturated alcohol. Such an inhibition is theoretically possible but has not been described. In the same study the urinary estriol had about the same ^{3}H/^{14}C ratio (10.1) as that of the injected steroids (11.5). It is therefore possible that both of the injected steroids entered the pool of estriol precursors in the feto–placental unit in equal proportions and that all the 16α-hydroxydehydroisoandrosterone therein was converted into 16α-hydroxyandrostenedione prior to aromatization. Support for this concept comes from the data of Dell'Acqua *et al.* (1967b) who found that 16α-hydroxydehydroisoandrosterone and 16α-hydroxyandrostenedione were equally well converted into estriol by the perfused midterm human placenta.

The isolated 16α-hydroxyandrostenedione, 16α-hydroxyetiocholanolone, and 16α-hydroxyandrosterone all had similar specific activities and ^{3}H/^{14}C ratios, indicating that they were all derived from a common precursor. Moreover, it is evident that more 16α-hydroxyandrostenedione is converted into the 5α- and 5β-reduced metabolites than 16α-hydroxydehydroisoandrosterone. The unusual steroid, 3α,16α-dihydroxyandrost-5-en-17-one, had a rather high specific activity and this probably reflects metabolism in the maternal compartment, because if it were formed in the feto–placental unit it would have been diluted with endogenous steroid as is evident for the 3β-hydroxy-5-ene steroids isolated. The low specific activities of 16α-hydroxydehydroisoandrosterone, androst-5-ene-3β,16α,17β-triol, and estriol can be attributed to high production of endogenous steroid in the feto–placental unit. On the other hand, the fact that Glusulase hydrolysis was employed in this study may be important in the interpretation of these specific activities. That this consideration may be important can be deduced from the fact that in the subsequent study the same steroid, 16α-hydroxydehydroisoandrosterone, had a higher specific activity in the glucosiduronate fraction than in the sulfate fraction and steroids conjugated with either glucosiduronic acid or sulfuric acid, or with both, are all hydrolyzed by Glusulase.

Having determined that 16α-hydroxyandrostenedione and 16α-hydroxydehydroisoandrosterone were efficiently converted into estriol, we devised experiments to determine whether dehydroisoandrosterone sulfate could be 16α hydroxylated and serve as a precursor of the urinary metabolites previously isolated. From the data obtained in the first of these studies, shown in Table II, it can be deduced that dehydroisoandrosterone sulfate is extensively 16α hydroxylated, probably in the fetus, and is subsequently converted into estriol by the placenta. The similarity in specific activities with respect to ^{14}C, of 16α-hydroxydehydroisoandrosterone and androst-5-ene-3β,16α,17β-triol, in relation to the 17-keto steroids, dehydroisoandrosterone, and androsterone indicates that they all have a common precursor, while the lower specific activities of the isolated estrone and estriol indicate further dilution by other precursors. The rather high specific activities of 16α-hydroxyandrostenedione, 16α-hydroxydehydroisoandrosterone, and 3α,16α-dihydroxyandrost-5-en-17-one in the glucosiduronate fraction probably reflects metabolism of the injected precursors in the maternal compartment. Siiteri and MacDonald (1966) have reported the results of four experiments in which the same precursors, [7-^{3}H]16α-hydroxydehydroisoandrosterone and

TABLE V: Per Cent of Injected Radioactivity in the Isolated Metabolites.

<table>
<tr><th></th><th></th><th>16α-Hydroxyandrostenedione</th><th colspan="2">16α-Hydroxydehydroisoandrosterone</th><th colspan="2">3α,16α-Dihydroxyandrost-5-en-17-one</th><th>Androst-5-ene-3β,16α,-17β-triol</th><th colspan="2">Estriol</th></tr>
<tr><th>Expt[a]</th><th>Precursor[b]</th><th>G[c]</th><th>S[c]</th><th>G</th><th>S</th><th>G</th><th>S</th><th>S</th><th>G</th></tr>
<tr><td>1</td><td>16D</td><td>0.1</td><td>2.8</td><td>0.7</td><td>0.7</td><td>12.0</td><td>0.3</td><td></td><td></td></tr>
<tr><td>2</td><td>16D</td><td>1.9</td><td>0.2</td><td>1.3</td><td>d</td><td>5.1</td><td>0.9</td><td>0.2</td><td>5.9</td></tr>
<tr><td>3</td><td>16D plus</td><td>2.1</td><td colspan="2">1.3</td><td colspan="2">13.1</td><td>3.1</td><td colspan="2">7.4</td></tr>
<tr><td></td><td>16Δ^4</td><td>6.4</td><td colspan="2">0.0</td><td colspan="2">0.0</td><td>0.0</td><td colspan="2">7.9</td></tr>
<tr><td>4</td><td>16D plus</td><td>2.3</td><td>2.2</td><td>0.1</td><td></td><td>6.0</td><td>1.5</td><td>0.6</td><td>6.2</td></tr>
<tr><td></td><td>DS</td><td>0.1</td><td>3.7</td><td>0.0</td><td></td><td>0.0</td><td>2.5</td><td>1.3</td><td>4.6</td></tr>
<tr><td>5</td><td>16DS plus</td><td>0.2</td><td>5.2</td><td></td><td></td><td>1.0</td><td>3.7</td><td>0.5</td><td>17.9</td></tr>
<tr><td></td><td>DS</td><td>0.02</td><td>2.6</td><td></td><td></td><td>0.6</td><td>1.9</td><td>0.4</td><td>15.9</td></tr>
</table>

[a] The percentages in expt 1 and 2 were obtained from previous data (YoungLai and Solomon, 1967b). In expt 3 the urinary conjugates were hydrolyzed with Glusulase. [b] 16D = [7-^{3}H]16α-hydroxydehydroisoandrosterone; 16Δ^4 = [4-^{14}C]16α-hydroxyandrostenedione; 16DS = [7-^{3}H]16α-hydroxydehydroisoandrosterone sulfate; DS = [4-^{14}C]dehydroisoandrosterone sulfate. [c] S = sulfate fraction; G = glucosiduronate fraction. [d] These metabolites were not isolated.

[4-^{14}C]dehydroisoandrosterone sulfate, were injected simultaneously in pregnant women. They isolated 16α-hydroxydehydroisoandrosterone, androst-5-ene-3β,16α,17β-triol, and estriol but it was not evident from their studies in what form these steroids were excreted. However, the ^{3}H/^{14}C ratios in the isolated metabolites obtained by these authors were analogous to those found in our study, in that the 16α-hydroxydehydroisoandrosterone and androst-5-ene-3β,16α-17β-triol had lower ^{3}H/^{14}C ratios than that of the injected steroids, while the estriol had a higher ^{3}H/^{14}C ratio. The specific activities of the three 16α-hydroxylated steroids isolated from the sulfate fraction of our study provide additional evidence for the formation of estriol *via* 16α-hydroxydehydroisoandrosterone and androst-5-ene-3β,16α,17β-triol. That androst-5-ene-3β,-16α,17β-triol can be converted into estriol was demonstrated both *in vitro* with placental microsomes (Ryan, 1958) and *in vivo* by injecting the labeled steroid into the umbilical vein of the intact feto–placental unit at midpregnancy (Dell'Acqua *et al.*, 1967a).

Because of the rather extensive process of 16α hydroxylation of dehydroisoandrosterone sulfate in late pregnancy, it was of interest to study the metabolism of 16α-hydroxydehydroisoandrosterone sulfate and dehydroisoandrosterone sulfate in the same subject, and the results of such a study are shown in Table III. It is apparent from the data in this table that dehydroisoandrosterone sulfate can give rise to 16α-hydroxyandrostenedione and 3α,16α-dihydroxyandrost-5-en-17-one. This was not observed in the previous study (Table II) where the ^{3}H/^{14}C ratios may have been too high to reveal a small amount of radioactivity due to ^{14}C in these metabolites. This difference may also be attributed to the variability introduced in studying different subjects. The high specific activities of 16α-hydroxydehydroisoandrosterone and androst-5-ene-3β,-16α,17β-triol with respect to ^{3}H may be due to metabolism and excretion of part of the injected steroid prior to mixing. Also the specific activities of the isolated 16α-hydroxydehydroisoandrosterone, androst-5-ene-3β,16α,17β-triol, and estriol suggest that sequence 1 is an important one in late pregnancy, but not the

$$\begin{tikzcd}
\text{dehydroisoandrosterone sulfate} \arrow[r] \arrow[d] & \text{16}\alpha\text{-hydroxydehydroisoandrosterone} \arrow[d] \\
\text{estrone} & \text{16}\alpha\text{-hydroxyandrostenedione} \arrow[d] \\
 & \text{estriol}
\end{tikzcd} \quad (1)$$

only route for the formation of estriol. It is to be noted that the specific activities of estriol are lower than those of 16α-hydroxydehydroisoandrosterone and androst-5-ene-3β,16α,17β-triol. This suggests that other precursors are contributing to the formation of estriol. Further support for this pathway is obtained from the data of Kirschner *et al.* (1966) who found that dehydroisoandrosterone sulfate was a better precursor of estriol than estrone sulfate within the feto–placental compart-

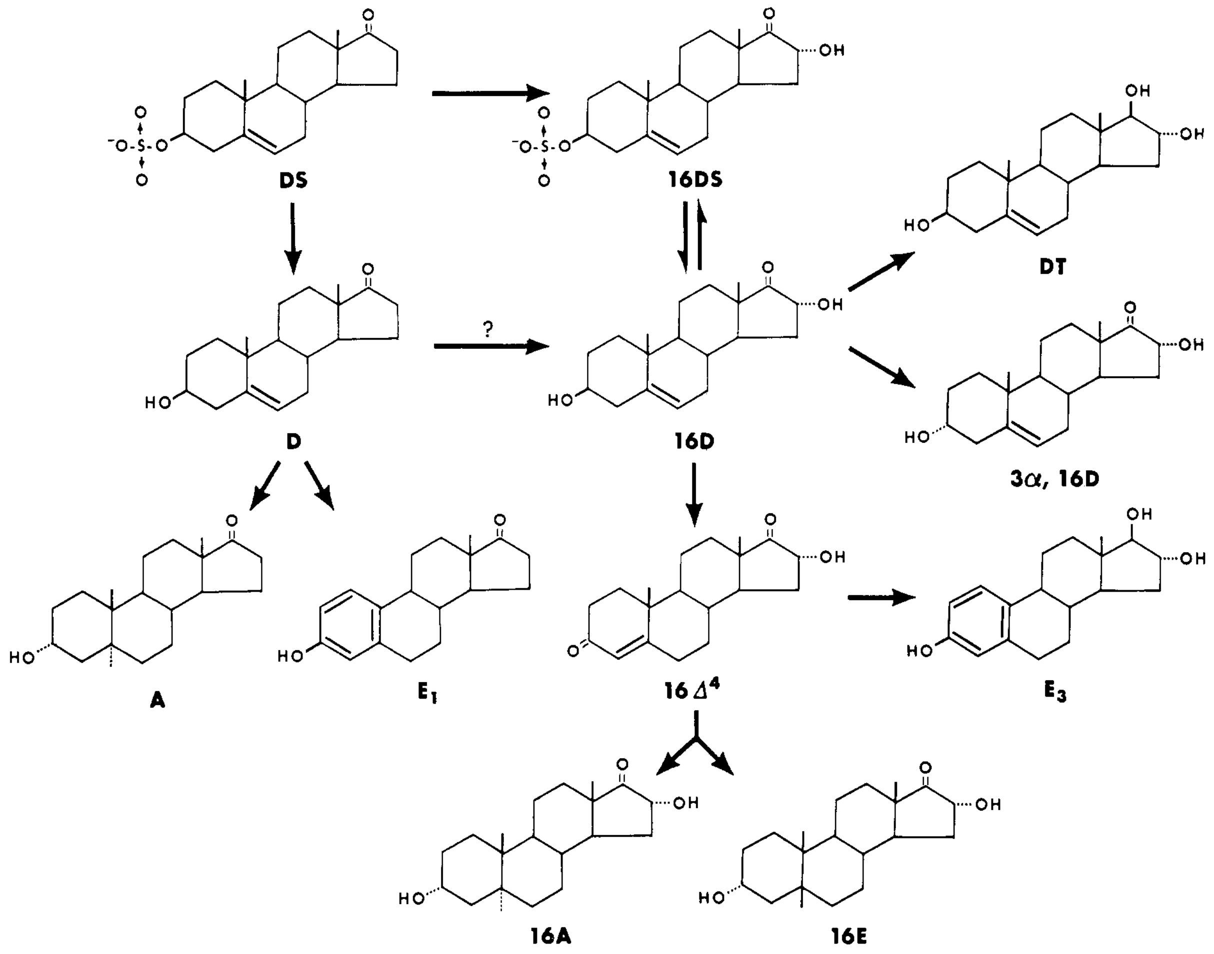

FIGURE 1: Formation and metabolism of 16α-hydroxysteroids in the third trimester of pregnancy. D = dehydroisoandrosterone; DS = dehydroisoandrosterone sulfate; 16 = 16α-hydroxyl group; DT = androst-5-ene-3β,16α,17β-triol; A = androsterone; E = etiocholanolone; E_1 = estrone; 3α,16D = 3α,16α-dihydroxyandrost-5-en-17-one; E_3 = estriol; Δ^4 = androstenedione.

ment whereas dehydroisoandrosterone sulfate from the maternal compartment is converted into estriol mainly *via* a phenolic pathway.

In a previous study (Table II) it was possible to isolate dehydroisoandrosterone and androsterone as well as estrone. There was sufficient weight in the isolated androsterone and dehydroisoandrosterone to permit identification by infrared and melting point. This is in contrast to the reported findings of other workers that the excretion of 17-keto steroids is decreased (Hain, 1939; Dobriner *et al.*, 1948; Patti *et al.*, 1963) during the third trimester of pregnancy, and Dobriner *et al.* (1948) who had reported that no androsterone could be found in late pregnancy. In a later study (Table III), although there was initial evidence for the presence of labeled 17-ketosteroids, there was insufficient radioactivity to permit identification. Moreover, 16α-hydroxydehydroisoandrosterone was not isolated from the glucosiduronate fraction (Table III).

Table V presents the per cent of radioactivity in the isolated metabolites. It is evident from these data that all the labeled steroids injected in subjects in the third trimester of pregnancy are good precursors of estriol. It should be noted that these values are not corrected for losses which may be as high as 50–80% (Siiteri and MacDonald, 1966; Nachtigall *et al.*, 1966). Dehydroisoandrosterone sulfate as well as its 16α-hydroxylated analog seem to be the most efficient precursors of urinary estriol. In all the studies performed in late pregnancy, the conversion of 16α-hydroxydehydroisoandrosterone into 16α-hydroxyandrostenedione was found to be about 2%. When the sulfated steroids were injected only a minute conversion into 16α-hydroxyandrostenedione (<0.5%) could be detected. However, when 16α-hydroxyandrostenedione was administered 6.4% of the steroid was recovered indicating that this portion of the injected steroid was presumably conjugated with glucosiduronic acid and excreted without being further metabolized. A sizable conversion of dehydroisoandrosterone sulfate into 16α-hydroxydehydroisoandrosterone and androst-5-ene-3β,16α,17β-triol was observed. It should be noted that in all of the studies described androst-5-ene-3β,16α,17β-triol was not isolated from the glucosiduronate fraction indicating that this steroid is probably excreted exclusively as the sulfate. Whether one or more of the hydroxyl groups is conjugated to sulfuric acid cannot be determined from this type of study. Although 16α-hydroxydehydroisoandrosterone was isolated from both the sulfate and glucosiduronate fractions, this steroid was present in greater quantities in the sulfate fraction. From the small recovery of 16α-hydroxydehydroisoandrosterone it appears that a major portion of the labeled steroid is metabolized and excreted as urinary products. To date our studies indicate that 3α,16α-dihydroxyandrost-5-en-17-one is the single most abundant urinary metabolite of 16α-hydroxydehydroisoandrosterone, the 16α-hydroxyl group inhibiting the Δ^4-isomerase enzyme. Further evidence for this inhibition was recently provided by Fukushima *et al.* (1967) who isolated urinary pregn-5-ene-3α,16α,20α-triol after the administration of 16α-hydroxypregnenolone to normal subjects.

As a control to the pregnancy studies, the metabolism of [7-^{3}H]16α-hydroxydehydroisoandrosterone was studied in a normal male and a normal female in the luteal phase of the menstrual cycle and the results obtained are shown in Table IV. The specific activities of 16α-hydroxydehydroisoandrosterone isolated from the sulfate fraction of male and female urine were low. This finding may be interpreted as a reflection of an endogenous pool of circulating 16α-hydroxydehydroisoandrosterone sulfate if the *in vivo* metabolism of this steroid parallels that of dehydroisoandrosterone sulfate (Gurpide *et al.*, 1965; MacDonald *et al.*, 1965). It is possible that 16α-hydroxydehydroisoandrosterone and its sulfate are secreted by the adrenal gland but it may also be formed in part by peripheral metabolism, probably in the maternal liver as reported by Baulieu *et al.* (1965), who demonstrated the direct conversion of injected [7-^{3}H,^{35}S]dehydroisoandrosterone sulfate into 16α-hydroxydehydroisoandrosterone sulfate in a normal male.

By now 16α-hydroxydehydroisoandrosterone has been isolated from the urine of various subjects and in addition we have found this metabolite in the urine of the normal female. The other three metabolites isolated, namely, 3α,16α-dihydroxyandrost-5-en-17-one, 16α-hydroxyandrosterone, and 16α-hydroxyetiocholanolone, have hitherto not been reported as constituents of normal male or female urine. The failure to isolate labeled estriol in the normal male and female indicates that in the absence of the placenta 16α-hydroxydehydroisoandrosterone is not aromatized when administered intravenously. Furthermore, results from current investigations (P. Preumont, K. J. Ryan, E. V. YoungLai, and S. Solomon, to be published) reveal that when labeled 16α-hydroxydehydroisoandrosterone was incubated with microsomal fractions and homogenates of normal human corpora luteal tissue, 16α-hydroxyandrostenedione was formed but not estriol. Our inability to isolate labeled estriol from the urine of the normal female in the luteal phase of the menstrual cycle and the presence of this steroid in normal female urine indicate that it is probably formed by the 16α hydroxylation of estrone or estradiol by the maternal liver (for a review of these findings, see Dorfman and Ungar, 1965).

Figure 1 is an attempt to summarize the various metabolic conversions described above, and related findings of other investigators. Estriol occupies a dominant position in this scheme because it is formed from all of the precursors studied. While this is not a complete picture of the events occurring in the third trimester of pregnancy, it is evident that a number of neutral C_{19} steroids play a very important role in the formation of urinary estriol.

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