J. steroid Biochem. Vol. 22, No. 2, pp. 195–201, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

15- AND 16-HYDROXYLATIONS OF ANDROGENS AND ESTROGENS IN THE HUMAN FETAL LIVER: A CRITICAL STEP IN ESTETROL BIOSYNTHESIS

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(*Received* 9 *March* 1984)

Summary—To elucidate the main metabolic pathways which lead to the foeto–placental biosynthesis of estetrol (**I**), we investigated the 15α- and 16α-hydroxylations of potential precursors of this estrogen in the human fetal liver. We determined the 15α- and 16α-hydroxylation capacity of the fetal liver for each precursor by GC–MS. The results suggest that estetrol is derived only from estradiol sulfate (**II**) and DHEA sulfate (**III**). 15α-Hydroxy-androstenedione (**IV**) can no longer be regarded as a good precursor of estetrol. The phenolic pathway appears to be a more likely route than the neutral pathway, even when derived from DHEA sulfate.

INTRODUCTION

Numerous investigations have shown that estetrol can be considered as a good indicator of fetal well-being [1, 2, 3]. Estetrol is a specific product of fetal metabolism because the 15α-hydroxylation occurs primarily in the fetal liver [4–9]. Although the 15α-hydroxylated steroids have also been detected in non-pregnant women, the feto placental unit is known to be the principal producer of 15α-hydroxylated estrogens [10–14]. Estetrol can be synthesized from phenolic and neutral substrates [4, 5, 15–19]. Nevertheless, the relative importance and final phases of various metabolic pathways which produce estetrol have not yet been clearly defined. Four principal biosynthetic routes are usually postulated:

(1) A phenolic pathway for which estradiol (**V**) is the preferential precursor [6]. The transformation of this estrogen into estetrol has not been well demonstrated. Estriol (**VI**), 15α-hydroxy-estrone (**VII**) and 15α-hydroxy-estradiol (**VIII**) are possible intermediates [15].

(2) A neutral pathway for which DHEA sulfate and 15α-hydroxy-androstenedione are the best precursors [18]. In this case, 15α- and 16α-fetal hydroxylations precede the placental aromatization and the 15α-hydroxylation takes place prior to the 16α-hydroxylation [19].

(3) A "mixed" pathway for which either the 15α- or the 16α-hydroxylation of the same androgens precede the placental aromatization [15].

(4) Another route also exists for DHEA sulfate. By placental aromatization, this substrate joins the phenolic pathway and is converted into estetrol.

To investigate this problem by a different approach than that usually encountered in the literature, we decided to study the biochemical properties of the enzymes involved in the formation of estetrol [20]. In this paper, we report our investigation of the behavior of the fetal liver hydroxylases.

EXPERIMENTAL

Materials

All organic solvents and reagents were of analytical grade quality (Merck, Darmstadt, F.R.G.). Glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were from Boehringer (Mannheim,

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Abbreviations and Trivial names: **I.** Estetrol: 1,3,5(10)-estratriene-3,15,16α,17β-tetrol. **II.** Estradiol sulfate: 1,3,5(10)estratriene-3,17β-diol sulfate. **III.** DHEA sulfate; 17-oxo-5-androsten-3β-ol sulfate. **IV.** 15α-Hydroxy-androstenedione: 15α-hydroxy-4-androstene-3,17-dione. **V.** Estradiol: 1,3,5(10)-estratriene-3,17β-diol. **VI.** Estriol: 1,3,5(10)-estratriene-3,16α,17β-triol. **VII.** 15α-Hydroxy-estrone: 15α-hydroxy-1,3,5(10)-estratriene-3-ol-17-one. **VIII.** 15α-Hydroxy-estradiol: 15α-hydroxy-1,3,5(10)-estratriene-3,17β-diol. **IX.** DHEA: 17-oxo-5-androsten-3β-ol. **X.** 16α-Hydroxy-dehydroepiandrosterone: 5-androsten-3β,16α-diol-17-one. **XI.** 15α,16α-Dihydroxy-dehydroepiandrosterone: 5-androsten-3β,15α,16α-triol-17-one. **XII.** Estrone: 1,3,5(10)-estratriene-3-ol-17-one. **XIII.** Androstenedione: 4-androstene-3,17-dione. **XIV.** Testosterone: 17β-hydroxy-4-androsten-3-one. **XV.** 15α-Hydroxy-testosterone: 15α,17β-dihydroxy-4-androsten-3-one. **XVI.** 16α-Hydroxy-testosterone: 16α,17β-dihydroxy-4-androsten-3-one. **XVII.** Estrone sulfate: 1,3,5(10)-estratriene-3-ol-17-one sulfate. **XVIII.** Estriol sulfate: 1,3,5(10)-estratriene-3,16α,17β-triol sulfate.

F.R.G.). Tween 80 (polyoxyethylene sorbitan monooleate) was purchased from Sigma Chemical Company (St Louis, MO, U.S.A.) Methoxyamine hydrochloride, trimethylchlorosilane, bistrimethylsilylacetamide and dry pyridine to prepare *O*-methoximetrimethylsilyl ethers for GC–MS analyses were from Macherey-Nagel (Düren, F.R.G.). Aryl sulfatase was obtained from Boehringer (Mannheim, F.R.G.).

Steroids and reference compounds were from Steraloids, Inc. or Merck (Darmstadt, F.R.G.), or were a generous gift from Professor D. N. Kirk (M.R.C., London, U.K.).

Methods

Gas–liquid chromatography mass spectrometry.

Analyses were carried out with a computerized LKB 2091 gas chromatograph–mass spectrometer instrument (Bromma, Sweden). A glass capillary column (20 m long, 0.25 mm internal dia) coated with SE-30 and heated at 240°C was used. Helium was the carrier gas at a pressure of 0.6 bar. A solid injector device was used. Substances were chromatographed as TMS or MO–TMS derivatives according to Horning's method [21].

Preparation of human fetal microsomes.

Human fetuses were obtained following abortions performed via hysterectomies. Three anencephalic fetuses at 27, 29 and 38 weeks of pregnancy constituted the basic biological material for this study. Livers were removed immediately after death of the fetuses. The tissues were washed in cold isotonic KCl, frozen and stored at −80°C until use. The microsomal fractions were prepared according to Sano *et al.*[22] and Ingelman-Sundberg *et al.*[23]. The final suspension was stored at −80°C; under these conditions, no alteration in the monooxygenase enzymatic activities of human liver samples was observed [24, 25]. The protein concentration was measured according to the method of Lowry *et al.*[26].

Incubation conditions.

Analysis of the fetal metabolism of various estetrol precursors was performed according to Cantineau *et al.*[20]. Incubation time was 45 min. The samples were derivatized (MO–TMS or TMS) prior to GC–MS analysis. Two techniques were used to hydrolyze the sulfo-conjugated steroids before extraction: (1) Dehydroepiandrosterone sulfate was hydrolyzed at pH 4.7 (2 M sodium acetate buffer) in a shaking water bath at 100°C for 4 h. (2) Sulfo-conjugated estrogens were enzymatically hydrolyzed at 37°C in the presence of 60 mU of aryl sulfatase per ml. The pH of these solutions had been previously adjusted to 4.5 [27].

RESULTS

Androgens, estrogens and their available 15α- or 16α-hydroxylated derivatives were incubated with fetal liver microsomes. The metabolism of these substrates was investigated by mass chromatography.

16α-Hydroxylation of non-conjugated estetrol precursors

The results (Table 1) showed that dehydroepiandrosterone (**IX**) was significantly hydroxylated in the 16α position. The androstenedione 16α-hydroxylation was negligible when compared to the dehydroepiandrosterone 16α-hydroxylation. No 16α-hydroxylated estrogen metabolites were detected. 15α-Hydroxylated androgens were not at all oxidized on the 16α position, except for 15α-hydroxy-androstenedione, for which a clearcut result was impossible to determine. The mass spectrum (Fig. 1) showed a molecular ion (at $m/e = 520$) and a few other characteristic peaks [M-15, M-31, M-90 and M-(90 + 15)]. Unfortunately, reference compounds were unavailable. The mass spectrum did not present a typical fragmentation confirming the presence of a second hydroxyle group in the 16α position.

15α-Hydroxylation of non-conjugated estetrol precursors

No other 15α-hydroxylations were detected, except for the 16α-hydroxy-dehydroepiandrosterone (**X**),

Table 1. Hydroxylation of various estetrol precursors by fetal liver microsomes

Substrates	Hydroxylations		
	16α	15α	On other positions
Dehydroepiandrosterone	+ + +	ND	+
Androstenedione	+	ND	+
Testosterone	ND	ND	+
15α-Hydroxytestosterone (**XV**)	ND		ND
16α-Hydroxytestosterone (**XVI**)		ND	ND
15α-Hydroxyandrostenedione	Possible		ND
16α-Hydroxydehydroepiandrosterone		+	ND
Estrone	ND	ND	ND
Estradiol	ND	ND	ND
Estriol		ND	ND

+ Very low yield. + + + High yield. ND Not detected.
Results were reproduced with five separate incubations: 3 incubations were performed with each liver preparation, and 2 incubations were performed on pooled microsomes.

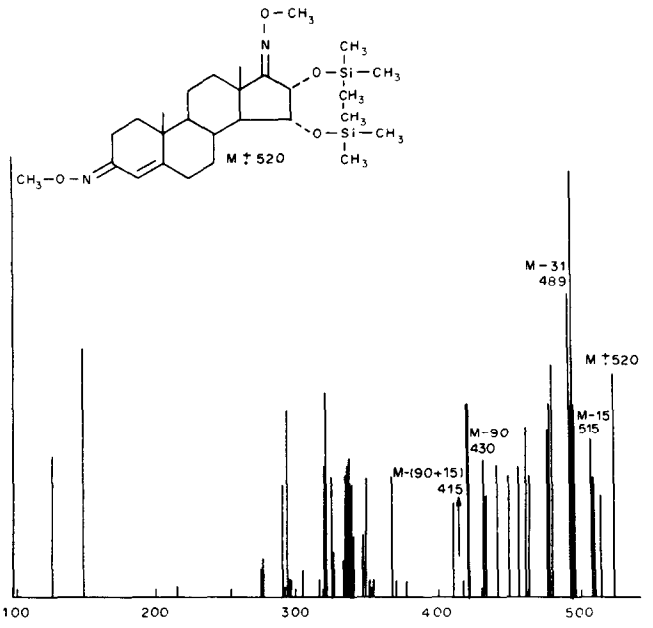

Fig. 1. Mass spectrum of the metabolite obtained after incubation of 15α-hydroxy-androstenedione with fetal liver microsomes.

which could be transformed into 15α,16α-dihydroxy-dehydroepiandrosterone (**XI**). The yield of this 15α-hydroxylation was much lower than the dehydroepiandrosterone 16α-hydroxylation. The mass spectrum of this polyhydroxylated metabolite (Fig. 2) showed a few characteristic ions. The molecular ion and some peaks were typical of a derivatized (MO–TMS) trihydroxy estrogen: M-15, M-31, M-90, M-(90 + 15), M-(90 + 31), M-(2 × 90) and M-(2 × 90 + 15). Three other peaks were also present: *m/e* 217, 191 and 147 which were present in the 15α-hydroxy-estradiol mass spectrum [28]. According to Sloan *et al.*[29], the *m/e* 147 could indicate the presence of two proximate trimethylsilyloxy groups in the steroid skeleton.

Hydroxylation of estetrol precursors on positions other than carbons 15 and 16

Other hydroxylated metabolites of estetrol precursors were observed in fetal liver microsomes by mass

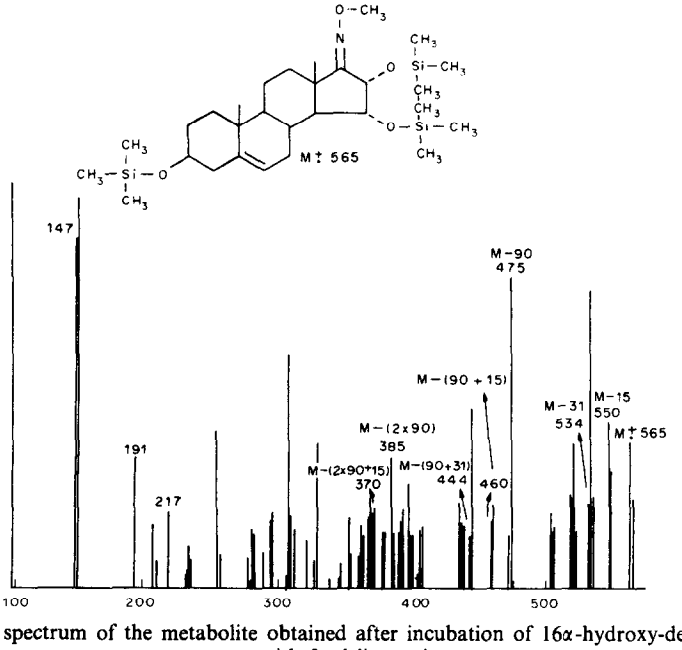

Fig. 2. Mass spectrum of the metabolite obtained after incubation of 16α-hydroxy-dehydroepiandrosterone with fetal liver microsomes.

Table 2. Hydroxylation of sulfo-conjugated estetrol precursors by fetal liver microsomes

Substrates	Hydroxylations		
	15α	16α	On other positions
Dehydroepiandrosterone sulfate	ND	+ + +	ND
Estrone sulfate (**XVII**)	ND	16α + 16β	ND
Estradiol sulfate	+	ND	ND
Estriol sulfate (**XVIII**)	ND		ND

+ Very low yield. + + + High yield. ND Not detected.
Incubations were performed on microsomes pooled from the 3 available livers.

chromatography. As they did not act as potential intermediates in the steps leading to estetrol biosynthesis, these compounds were not formally identified. Nevertheless, only androgens were hydroxylated in positions other than carbons 15 and 16. Estrogens were not at all metabolized. Table 1 summarizes the results obtained.

15α- and 16α-Hydroxylations of sulfo-conjugated estetrol precursors

In 1961, Diczfalusy *et al.*[30] demonstrated that fetal estrogens were usually sulfo-conjugated. Since these sulfo-conjugated steroids were the principal source of our 15α- and 16α-hydroxylated derivatives [6], we decided to study the metabolism of the sulfo-conjugated estetrol precursors in fetal liver microsomes. This analysis was also carried out by mass chromatography. Table 2 illustrates the results obtained.

Contrary to dehydroepiandrosterone which underwent several transformations, we found that its sulfo-conjugated form was hydroxylated only in the 16α position and with a high yield. Sulfo-conjugated estradiol was hydroxylated in the 15α position, and estrone (**XII**) in the 16α and 16β positions. No hydroxylation of estriol was observed. All of these reactions had very low yields as compared to the dehydroepiandrosterone 16α-hydroxylation. The metabolites produced were identified by the comparison of their chromatographic properties and mass spectra with those of authentic reference samples, or on the basis of the data found in the literature [23].

DISCUSSION AND CONCLUSIONS

To complete our *in vitro* study of estetrol biosynthesis, we investigated the 15α- and 16α-steroid hydroxylases in the fetal liver microsomes prepared from fetuses of the same stage of development as the placentas used in our previous publication [20]. Although it is well documented that maternal serum and urinary estriol and estradiol levels are much lower in anencephalic pregnancies, we used microsomal preparations obtained from affected fetuses. This particular phenomenon is related to a lower production of precursors and not to a deficiency in liver enzymes [31]. Moreover, a high level of DHEA-16α-hydroxylase was measured in such liver samples [32]. As certain 15α- and 16α-dihydroxylated estetrol precursors were unavailable, we used the most appropriate and reliable analytical method available for our study, i.e. mass chromatography. This method provided only qualitative data from which we were able to compare the extent of hydroxylation of the various precursors. This comparison was possible for the following reasons: (1) The initial substrates and their corresponding 15α- and 16α-hydroxylated metabolites produced ionic currents of similar intensity when they were equally concentrated in the samples studied. (2) The variations observed between the magnitude of the 15α- and 16α-hydroxylations of the estetrol precursors were great and highly significant.

The analysis of the conclusions of our previous paper [20] and of this present study enabled us to define better the various biosynthetic pathways of estetrol.

A. The phenolic pathway

We confirmed that androstenedione (**XIII**) and testosterone (**XIV**) were the preferential substrates of the placental aromatase [20]. We also demonstrated that estradiol was hydroxylated in the 15α position provided that it was sulfo-conjugated in position 3. Unfortunately, sulfo-conjugated 15α-hydroxy-estrogens were unavailable; hence, we could not demonstrate that these precursors were converted to estetrol. Nevertheless, free or sulfo-conjugated estriol never led to the formation of estetrol in our experiments. Estradiol appeared to be a good precursor for estetrol, and 15α-hydroxylated estrogens as the actual biosynthetic intermediates.

B. The neutral and "mixed" pathways

The 15α-hydroxylation of DHEA was never detected. Its 16α-hydroxylation was significant in the fetal liver but estriol was never converted into estetrol. We never observed a 15α-hydroxylation of androstenedione. Furthermore, our previous study [20] proved that the aromatization of the 15α-hydroxy-androstenedione was a possible pathway for the formation of the 15α hydroxylated estrogens when the supply of this substrate to the placenta was extensive. We also demonstrated that the 15α-hydroxylation was the limiting step in es-

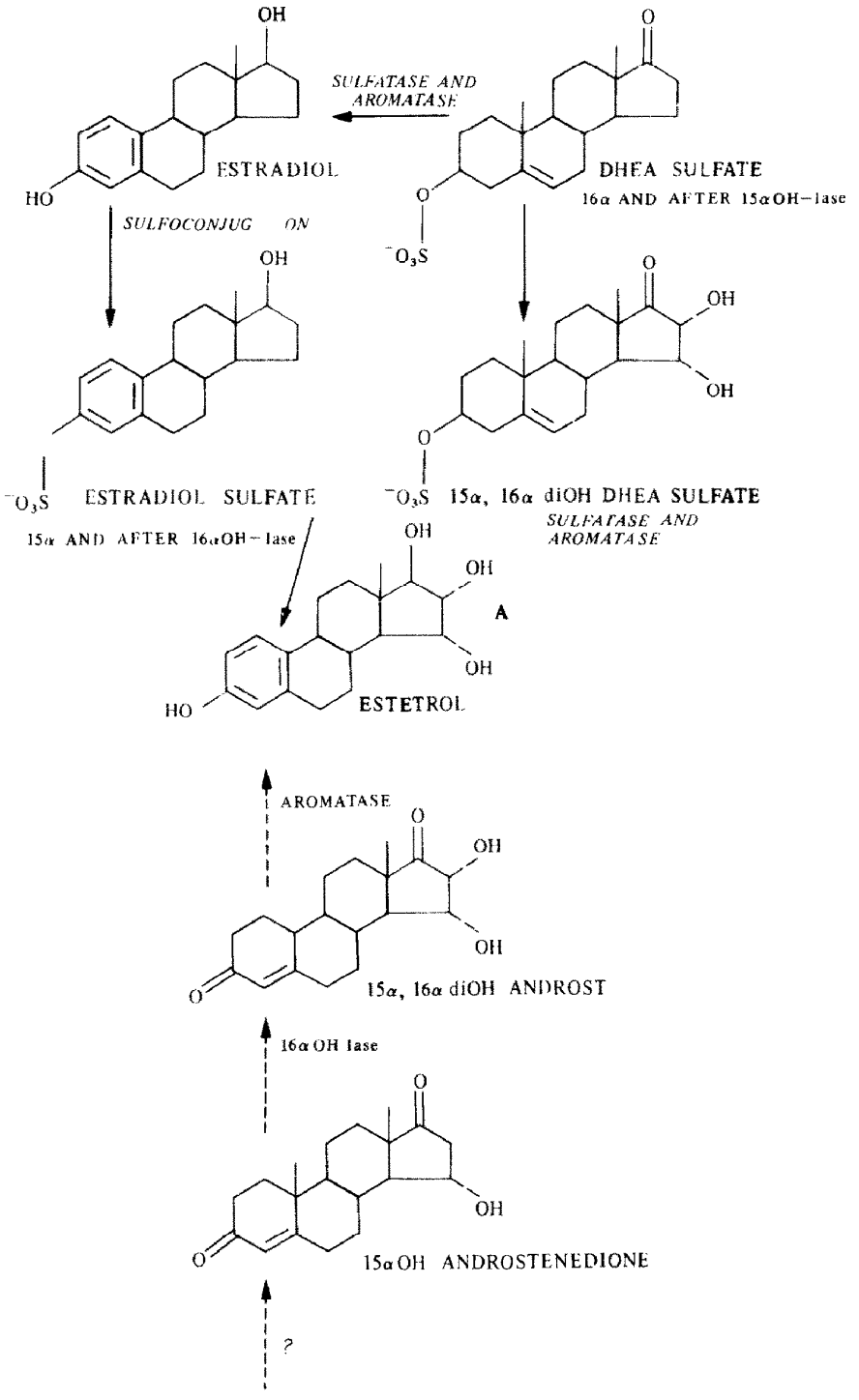

Fig. 3. Possible pathways for estetrol biosynthesis.

tetrol biosynthesis. A small amount of 15α-hydroxy-androstenedione should be formed and the aromatization of this compound would be negligible. Accordingly, the "mixed" pathway (see Introduction 3) arising from neutral precursors could be disregarded. On the contrary, the neutral pathway (see Introduction 2) suggested in the literature was more obvious. DHEA sulfate was significantly metabolized in the 16α position in the fetal liver and our GC–MS data support the hypothesis of a 15α-hydroxylation of the 16α-hydroxy-dehydroepiandrosterone. The 16α-hydroxylation of 15α-hydroxy-androstenedione was possible but could not be conclusively confirmed. Unfortunately, the 15α,16α-dihydroxy-androgens were unavailable and we could not measure their aromatization in the placenta. However, the existence

of this pathway cannot be entirely ruled out, but more likely originates from DHEA sulfate than from 15α-hydroxy-androstenedione.

In conclusion, we suggest that the following pathways may lead to estetrol biosynthesis:

(A) The phenolic pathway is more important. Two precursors are involved. Estradiol and DHEA sulfate. This latter substrate via aromatization in the placenta is converted into estradiol. When estradiol is transformed into estetrol, this precursor must be sulfo-conjugated to undergo the hydroxylations and the 15α-hydroxylation occurs prior to the 16α-hydroxylation.

(B) A neutral and less significant pathway originating from DHEA sulfate is also possible. In this case, the 16α-hydroxylation takes place prior to the 15α-hydroxylation, contrarily to the information found in the literature [19].

Acknowledgements—The authors are grateful to Ms Janice Lynn Delaval for her assistance in rewriting the manuscript. The authors thank Ms Martine Verbys for drawing the figures. This work was financially supported by a grant No. 34522.81 from the Fonds de la Recherche Scientifique Médicale.

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