Evidence of Conversion of Estradiol 17-Sulfate to Its 2- and 4-Hydroxylated Catechols by Human Placental Microsomes

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When estradiol 17-sulfate (ES) was incubated with human placental microsomes under an NADPH-generating system, 2- and 4-hydroxyestradiol 17-sulfates (2- and 4-OH-ES) formed as the main products. By kinetic experiments, the apparent $K_m$ values of 44.0 and 360 $\mu$M and apparent $V_{max}$ values of 236 and 140 pmol/mg protein/10 min were obtained for the 2- and 4-hydroxylation, respectively. The results indicate that human placental microsomes have fairly high 2- and 4-hydroxylase activities toward ES. This paper describes the formation of 2- and 4-OH-ES and speculates on their physiological role during pregnancy.

Keywords estradiol 17-sulfate; 2-hydroxyestradiol 17-sulfate; 4-hydroxyestradiol 17-sulfate; placenta

Introduction
We reported previously that estradiol 17-sulfate (ES) is evidently excreted in human urine11 and that the urinary levels during pregnancy increased as the gestation period progressed and increased in late pregnancy to over five-times those in non-pregnant women.2,6
ES was also found in experimental animals such as rats,3 and some of it was converted to 2- and 4-hydroxyestradiol 17-sulfates (2-and 4-OH-ES) by rat liver.4,5 These catechol products showed a strong inhibitory effect, corresponding to o-tocopherol, on the lipid peroxidation in rat liver microsomal systems.6,7 Recently our laboratory reported the presence of ES in pregnancy serum.7,8 Interestingly, the amount of serum ES was negatively correlated to that of serum lipid peroxides in late pregnancy.8,9 These results led us to speculate that ES is required as the precursor to the catechol antioxidant to maintain normal gestation. Because the placenta is recognized as the main production organ of catechol estrogen during pregnancy,9 2- and 4-OH-ES is also expected to be formed by the placenta. This paper describes the evidence for an aromatic hydroxylation of ES by human term placental microsomes, and the physiological role of this biotransformation during pregnancy.

Materials and Methods
Materials ES,11 2-OH-ES,11 4-OH-ES,7 and 2-hydroxyestradiol 3-methyl ether 17-sulfate (2-OH-ES-3-OMe)21 were synthesized in our laboratory, as described previously. Glucose 6-phosphate (G-6-P), G-6-P dehydrogenase (G-6-P DH) and NADP were obtained from Oriental Yeast, Inc. (Osaka, Japan). Sep-Pak C18 cartridges and Column Guards were purchased from Waters, Assoc. (Milford, Mass., U.S.A.) and Millipore Co. (Bedford, Mass., U.S.A.), respectively.

Enzyme Preparation Human placental microsomal fraction was obtained in the usual way.11 Protein concentration was determined by the method of Lowry et al.12

Incubation Procedures Incubation was carried out under the following conditions: Ice-cold reaction vessels contained microsomal protein (0–1.5 mg/ml), an NADPH-generating system (NADP, 0.5 mM; G-6-P, 5 mM; G-6-P DH, 0.6 unit/ml; MgCl2, 5 mM), EDTA (0.1 mM), KCl (90 mM) and substrate (0–400 $\mu$M). The mixture was diluted with Tris–HCl buffer solution (pH 7.4) to 2 ml as a final volume and incubated at 37 °C under aerobic condition for 0–60 min. For control experiments, incubation was performed using boiled microsomes (100 °C for 2 min) with the same procedure as described above.

Work-up Procedure Reactions were stopped by heating reaction vessels in boiling water for 2 min, followed by addition of an exact amount (1 ng) of 2-OH-ES-3-OMe as an internal standard and of ascorbic acid (1 mg) as an antioxidant. The mixture was centrifuged at 1500 × g for 15 min. The supernatant was diluted with distilled water (1 ml), and then passed through the Sep-Pak C18 cartridge. After washing the cartridge with 2 ml of distilled water, the steroid-containing fraction was obtained by elution with methanol (4 ml). The eluate was evaporated under a nitrogen stream at 40 °C to produce the residue, which was dissolved in methanol (200 $\mu$l). The methanolic solution, after being passed once through a Column Guard, was subjected to HPLC.

HPLC HPLC was carried out in a model 803D chromatograph (Tosoh, Tokyo) equipped with an EC-8 electrochemical detector (Tosoh, Tokyo) at 0.5 V vs. the Ag/AgCl reference electrode. A reverse-phase column packed with ODS-120A (250 × 4.6 mm, i.d., 5 $\mu$m, Tosoh) was used and maintained at 40 °C in a column heater. A mixture (60:40, v/v) of 0.5% NH4H2PO4 (pH 3.0) and methanol was used as a mobile phase. Details of the assay procedure for 2- and 4-OH-ES is described elsewhere.13

Recovery Test Known amounts of 2- and 4-OH-ES (500, 100, 50 and 10 ng/vessel) were added to the incubation mixture, which was treated in the way described in the work-up procedure.

Results
ES was incubated with human placental microsomes with an NADPH-generating system. Figure 1 shows a comparison of the chromatograms between the steroidol standards (A) and the incubation products (B). Two peaks (1 and 2) of ES metabolites coincided with those of the authentic specimens, 2- and 4-OH-ES. In addition to these catechols, some peaks considered to be metabolites were

![Fig. 1. High Performance Liquid Chromatograms of Authentic Conjugates (A) and Incubation Products of Estradiol 17-Sulfate by Human Placental Microsomes (B)](image)

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detected (B).

The minor products were estimated as ring B- or D-hydroxylated metabolites, because of their chromatographic behavior, which was similar to that of such metabolites produced by female rat liver microsomes. No peaks except that of ES appeared when the incubation was carried out with boiled microsomes or without microsomes.

In order to confirm the validity of the present method to determine 2- and 4-OH-ES, a recovery test was undertaken using their authentic samples. A known amount of the authentic sulfates was added to the incubation medium, and both sulfates which were recovered throughout the whole clean-up procedure were determined. The result showed a satisfactory rate of recoveries for both catechols as shown in Table I.

Some kinetic parameters of ES 2- and 4-hydroxylations were also measured. The influence of incubation time and that of enzyme amount on the production of two catechol metabolites were demonstrated, and both 2- and 4-hydroxylase activities were linear up to 30 min incubation time and up to 1.5 mg protein/ml. The effect of the substrate concentration on the hydroxylations was investigated at an enzyme concentration of 1.5 mg/ml for 10 min.

The enzyme kinetics of ES 2- and 4-hydroxylations followed classical Michaelis–Menten kinetics producing a Lineweaver–Burk plot. The ES 2- and 4-hydroxylase activities were measured from a series of six separately prepared samples using the same enzyme sources.

Table II shows apparent \( K_m \) and \( V_{\text{max}} \) values of ES 2- and 4-hydroxylations, compared with those of estradiol (E) obtained by Juchau et al. \(^{17} \) The \( K_m \) value of ES 2-hydroxylase was about one-third of that of E. Conversely, the \( K_m \) value of ES 4-hydroxylase was about three-times higher than that of E 4-hydroxylase. The \( V_{\text{max}} \) values of ES 2- and 4-hydroxylase were higher than those of E hydroxylases. The ratios \( (V_{\text{max}}/K_m) \) of each hydroxylation in both substrates were compared, and the ratio of 2-

hydroxylation of ES was found to be fourteen-times higher than that of 4-hydroxylation of ES, and seventy-times higher than that of 2-hydroxylation of E.

### Discussion

HPLC of the incubation product of ES by human placental microsomes showed the production of 2- and 4-OH-ES along with other minor products. The hydroxylation of ES was considered to be enzymatic, because of a negligible amount of chromatographic detection of the products in the case of reactions carried out with boiled microsomes or without microsomes.

The present results suggest that if 2- and 4-OH-ES are detected in pregnancy serum or urine, they might be produced not by sulfoconjugation of 2- and 4-OH-E, but by the hydroxylation of ES, due to the low production activity of maternal 2- and 4-OH-E, \(^{14} \) and to no further metabolism of ES by fetus. \(^{19} \)

Table II shows that ES is a more suitable substrate for 2-hydroxylation by human placental microsomes than E. This results is very interesting in that P450 enzymes generally react more favorably with free steroids than with conjugated ones. Evidently, there was a big difference in the metabolic activities between E and ES using purified P450 enzymes from rat liver microsomes. \(^{20} \)

As for 4-hydroxylation, Table II shows that the affinity of ES to placental estrogen 4-hydroxylase is lower than that of E. However, the \( V_{\text{max}} \) value of ES 4-hydroxylation is much larger than that of E. The so-called metabolic efficiency \( (V_{\text{max}}/K_m) \) of ES 4-hydroxylation is ten-times higher than that of E, suggesting that 4-hydroxylation of ES is no longer ignored in the placental metabolism of ES.

It is evident that preeclampsia is closely related to the lipid peroxides produced by placenta. \(^{21} \) This organ has two contrary physiological activities, the production of lipid peroxide and the decomposition of the peroxides produced. \(^{21} \) Generally, normal pregnancy is maintained through the quantitative balance of such oxidants and antioxidants. \(^{21} \) At present, however, it is unknown of what kind of substance placental antioxidant is composed. Judging from the previous results, \(^{2,26–30} \) and also from the present experiment, it appears that 2- and 4-OH-ES act as endogenous antioxidants during pregnancy.

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