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CATECHOLOESTROGEN SYNTHESIS AND METABOLISM IN THE RABBIT UTERUS DURING THE PERIIMPLANTATION PERIOD

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Summary—Microsomal oestradiol-2/4-hydroxylase (OE-2/4-H) and cytosolic catechol-O-methyltransferase (COMT) (EC 2.1.1.6) activity in the uteri of pregnant and pseudopregnant rabbits during the periimplantation period were studied. The apparent K_m for the 4-hydroxylation of oestradiol (3.18 μM) was considerably less than for the 2-hydroxylation reaction (13.36 μM), whereas the V_{max} were almost equal. This suggests that 4-hydroxyoestradiol (4-OH-OE_2) is the predominant product of OE-2/4-H in the rabbit uterus. These reactions were inhibited by SKF-525A, indicating the involvement of cytochrome *P*450 dependent monooxygenases. Uterine cytosolic COMT utilized 2-hydroxyestradiol (2-OH-OE_2) as the preferred substrate as compared to 4-hydroxyoestradiol (4-OH-OE_2). Since the rabbit uterus has a considerable capacity to synthesize 4-OH-OE_2 and a lower capacity to metabolize it, it could be suggested that more 4-OH-OE_2 than 2-OH-OE_2 could be available to the uterus for its physiological activities. Furthermore, an increase in OE-2/4-H in Day 6 pseudopregnant and pregnant uteri with a concomitant decrease in COMT suggests the involvement of catecholoestrogens in the implantation process in the rabbit.

INTRODUCTION

Ovarian progesterone and oestrogen are essential for implantation in the mouse and rat. In contrast, ovarian oestrogen is not an absolute requirement for implantation in the rabbit [1]. Therefore, either oestrogen is not required for implantation in this species, or if required, it is provided by the uterus and/or the implanting blastocyst. Indeed, both the endometrium [2, 3] and the blastocyst [4, 5] of this species have the ability to synthesize oestrogen. Furthermore, blastocysts have been shown to influence endometrial oestrogen synthesis [2]. One of the earliest prerequisites for implantation is increased endometrial capillary permeability at the site of the blastocyst [6]. Prostaglandins are thought to play an important role in this event during implantation [7, 8]. Catecholoestrogens are more effective than phenolic oestrogens in stimulating prostaglandin synthesis in the rat and human uterus [9], as well as in the rabbit blastocysts and endometrium [10]. Furthermore, catecholoestrogens have short half-lives and rapid clearance rates. Therefore, they are not likely to function as circulating hormones. If they play any role in implantation, they are likely to be formed locally at the site of implantation where they can function in a paracrine or autocrine fashion. Therefore, in the present study we examined the catecholoestrogen synthetic ability of the rabbit uterine microsomes during the periimplantation period. In addition, as catechol-O-methyl transferase (COMT) (EC 2.1.1.6) is the major enzyme involved in the metabolism of catecholoestrogens, we studied this enzyme in uterine cytosols in order to determine the relationship between the synthesis and metabolism of catecholestrogens by the rabbit uterus.

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EXPERIMENTAL

Chemicals

Two-hydroxyoestradiol (2-OH-OE_2), 4-hydroxyoestradiol (4-OH-OE_2) and 2-methoxyoestradiol (2-Me-OE_2) were purchased from Steraloids (Wilton, N.H.). HPLC-grade methanol and sodium acetate were from Fisher Scientific (St. Louis, Mo.) and glacial acetic acid (HPLC-grade) was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. Radioactive oestradiol, [^{14}C]OE_2 (56.4 mCi/mmol) and adenosyl-L-methionine, *S*-[methyl-^{14}C] (SAM) (46.0 mCi/mmol) were purchased from New England Nuclear (Boston, Mass.). SKF-525A was obtained from Smith-Kline and French Laboratories (Philadelphia, Penn.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Preparation of tissues

New Zealand White rabbits were induced to superovulate [11] and were mated to fertile males (Day 0). Pseudopregnancy in superovulated animals was induced by intravenous injection of 50 i.u. HCG (Day 0). On the appropriate day, animals were killed by intravenous injection of a lethal dose of pentobarbital. Each uterine horn was flushed with 5 ml of TEDG buffer (10 mM Tris–HCl, 1.5 mM EDTA, 1.5 mM DTT and 10% glycerol, pH 7.6) containing 10 μl of 5 mM E-64 and 50 μl of 10 mM phenylmethyl sulphonyl fluoride (PMSF). A portion (2 cm) of each horn was minced and homogenized in 5 vol of TEDG buffer containing 10 μM E-64 and 0.2 mM PMSF in a Polytron P-10 (Brinkman Instruments, Westbury, N.Y.) followed by rehomogenization using a glass/teflon homogenizer, and centrifuged at 10,000 g for 20 min. The 105,000 g supernatant fluid was stored at -70°C for COMT assay. The pellet was resuspended in TEDG buffer and again centrifuged at 144,000 g for 60 min. The final pellet was resuspended in TEDG buffer and stored at -70°C until assayed for OE-2/4-H activity. Protein was measured by the Bradford[12] method using bovine serum albumin as a standard.

OE-2/4-H assay

This enzyme was measured by a product isolation method [13, 14]. Sample of microsomal homogenate (200 μg protein) was incubated at 30°C for 30 min with 100 μl of reaction mixture containing 0.075 μCi [4-^{14}C]OE_2 (10 μM), 2-OH-OE_2 (1 μM), 4-OH-OE_2 (1 μM), ascorbic acid (10 mM) and NADPH (1.5 mM) in Hepes/Tris (0.05 M/0.05 M) buffer at pH 8.0. Reactions were terminated by the addition of 100 μl of ice-cold 1 N HCl. The reaction products and the unreacted substrate were extracted with ethylacetate saturated with ascorbic acid, evaporated to dryness, and redissolved in methanol for HPLC analysis. Blank values were obtained by using samples not containing NADPH. Separation of catecholoestradiols and oestradiol was performed as previously described [13, 14] by using an LC 300 liquid chromatograph equipped with a flow detector (Flow-One Model IC, Radiomatic, Tampa, Fla) and an electrochemical detector. For confirmation, catecholoestrogens formed in some of the reactions were chromatographed on an alumina column [15] before separation by HPLC.

COMT assay

COMT was assayed essentially by the method of Hoffman *et al.*[16]. General conditions for the assay were as follows: samples of uterine cytosol (50 μl containing 100 μg protein) were incubated in duplicate at 37°C for 20 min with the reaction mixture (150 μl) containing 100 mM Tris–HCl buffer (pH 9.0), 50 mM $MgCl_2$, 1 mM DTT, 5 μM unlabeled SAM, 0.002 μCi [^{14}C]SAM and 100 μM of substrate (2-OH-OE_2 or 4-OH-OE_2). Reactions were started by the addition of the catechol substrate. Blanks consisted of the tubes lacking catechol substrate. At the end of reaction, O-methylated catecholoestrogens were extracted into 5 ml of toluene. The organic phase was separated by centrifugation, and the radioactivity in 4 ml of this phase was determined by liquid scintillation counting. Usually, when 2-OH-OE_2 is incubated with COMT in the presence of *S*-adenosylmethionine, both 2- and 3-monomethyl ethers are formed. In this study, the total rate of methylation was measured without separating the two isomeric ethers.

RESULTS

Characteristics of microsomal OE-2/4-H of rabbit uterus

Mitochondrial fractions of uterine homogenates contained no OE-2/4-H activity. Unlike other tissues [13, 17–20], uterine microsomes have the ability to produce either equal or slightly greater amounts of 4-OH-OE_2 than 2-OH-OE_2. These two catecholoestrogens were distinctly separated by our HPLC system (Fig. 1). The retention times of the products exactly matched those of radioinert authentic 4-OH-OE_2 (10.7 min) and 2-OH-OE_2 (11.7 min) (Fig. 1). Formation of the products under our assay conditions was linear up to 210 μg of microsomal protein (data not shown). Enzyme activity was linear with time up to 20 min and then increased at least up to 60 min but not linearly (data not shown). Figure 2 shows the enzyme's substrate dependence. Using Lineweaver–Burk plots, apparent K_m of 3.18 μM and 13.36 μM, and V_{max} of 27.47 pmol/mg/30 min and 38.75 pmol/mg/30 min were obtained for the production of 4-OH-OE_2 and 2-OH-OE_2, respectively. SKF-525A, a non-competitive inhibitor of *P*450, was used to determine whether catecholoestrogen formation by the rabbit uterus is mediated by cytochrome *P*450. This compound inhibited the formation of both 4-OH-OE_2 and 2-OH-OE_2 in a dose-dependent manner (40% inhibition at 25 mM and 87% inhibition at 100 mM).

Characteristics of cytosolic COMT in the rabbit uterus

Formation of 2-methoxyoestradiol under our assay conditions was linear with time for at least 60 min and with increasing protein concentrations at least up to 120 μg (data not shown). The activity increased with increasing pH up to 9.0 (data not shown). The enzyme was dependent on either of the catechol substrates, i.e. 2-OH-OE_2 or 4-OH-OE_2 (Figs 3 and 4). Changes in velocity versus substrate concentrations were plotted as Lineweaver–Burk plots for each substrate. Analysis of the data by least squares regression gave an apparent K_m of 1.35 μM and V_{max} of 10.2 pmol/mg/min for 2-OH-OE_2, and a K_m of 1.89 μM and V_{max} 4.8 pmol/mg/min for 4-OH-OE_2.

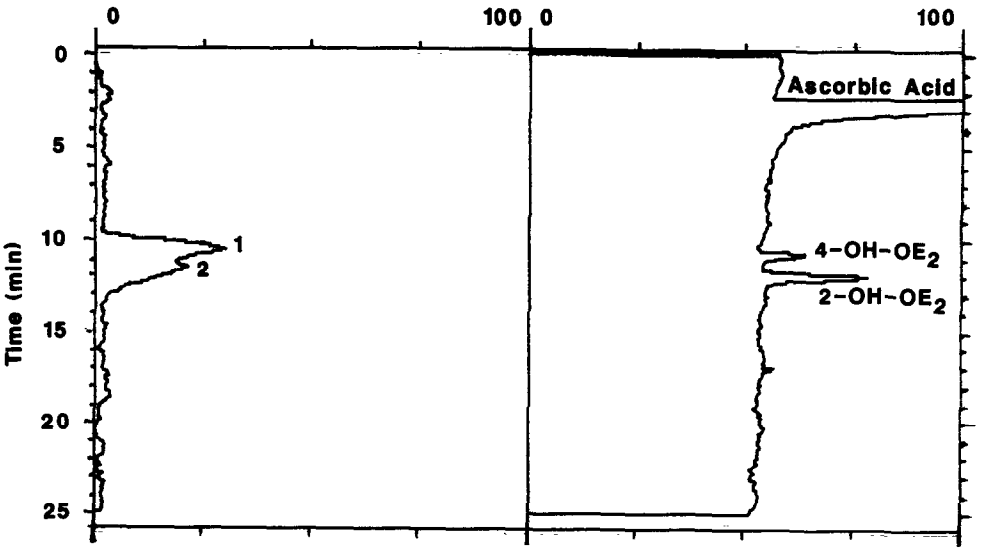

Fig. 1. HPLC tracings (left—radiometric, right—electrochemical [EC] channel) of the catechol products obtained by reacting the microsomal fraction of rabbit uterus (Day 6 of pregnancy) with [^{14}C]OE_2 in presence of NADPH, followed by alumina chromatography. Column: – 5 μM octadecylsilane-econosphere, 4.6 x 250 mm (Altech Assoc. Inc., Ill.). Mobile phase: methanol, 0.1 M sodium acetate in water and glacial acetic acid, 50:42.5:7.5, 1 ml/min. The first peak in the EC channel corresponds to that of ascorbic acid used to prevent oxidation of catechol oestrogens. Peaks 1 and 2 in the radiometric channel correspond to the authentic 4-OH-OE_2 and 2-OH-OE_2 respectively in the EC channel.

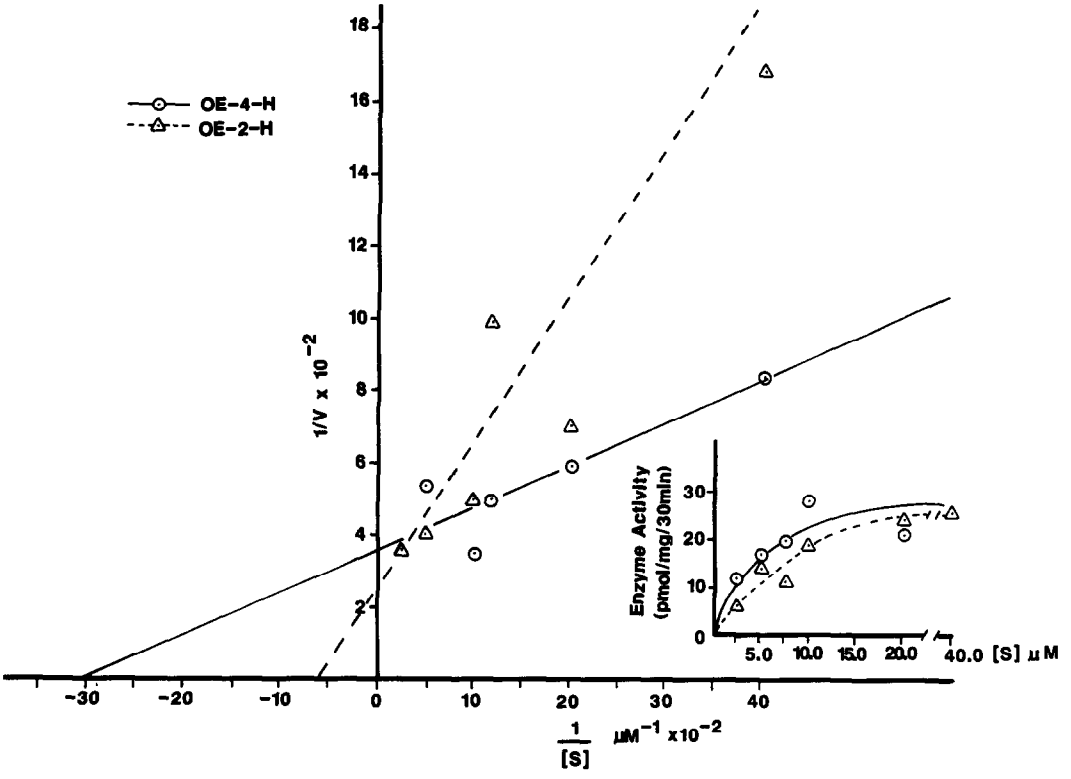

Fig. 2. Dependence of the formation of 2- and 4-OH-OE_2 on the concentration of substrate (S) oestradiol. The formation of 2- and and 4-OH-OE_2 by rabbit uterine microsomes (Day 6 of pregnancy) with 2.5–40 μM oestradiol (OE_2) (containing 50,000 cpm of [^{14}C]OE_2 per assay) and NADPH (1.5 mM) is plotted. *Inset*, formation of 2- and 4-OH-OE_2, expressed as pmol per mg protein/30 min, is plotted directly as a function of the concentration of OE_2.

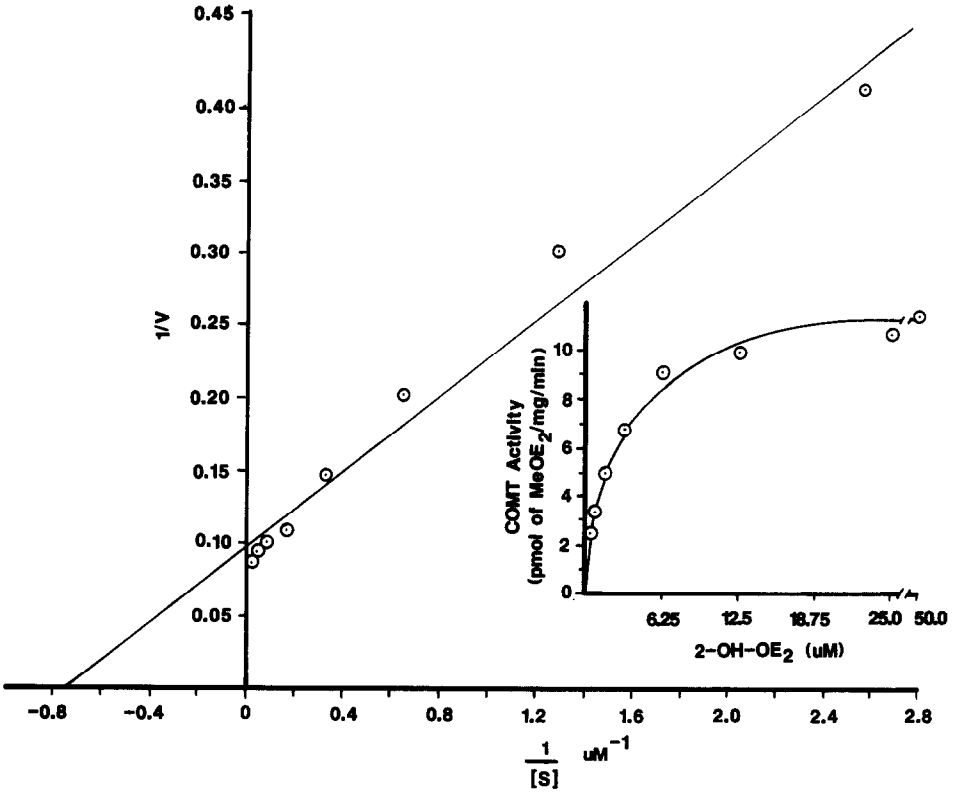

Fig. 3. Dependence of the formation of 2-methoxyoestradiol (2-Me-OE_2) on the concentration of substrate (S), 2-OH-OE_2. The formation of 2-Me-OE_2 by rabbit (Day 6 of pregnancy) uterine cytosols (100 μg of protein) with different concentrations of 2-OH-OE_2 is plotted. Incubations were performed as described in "Experimental" section. *Inset*, formation of 2-Me-OE_2, expressed as pmol/mg protein/min, is plotted directly as a function of the concentration of 2-OH-OE_2.

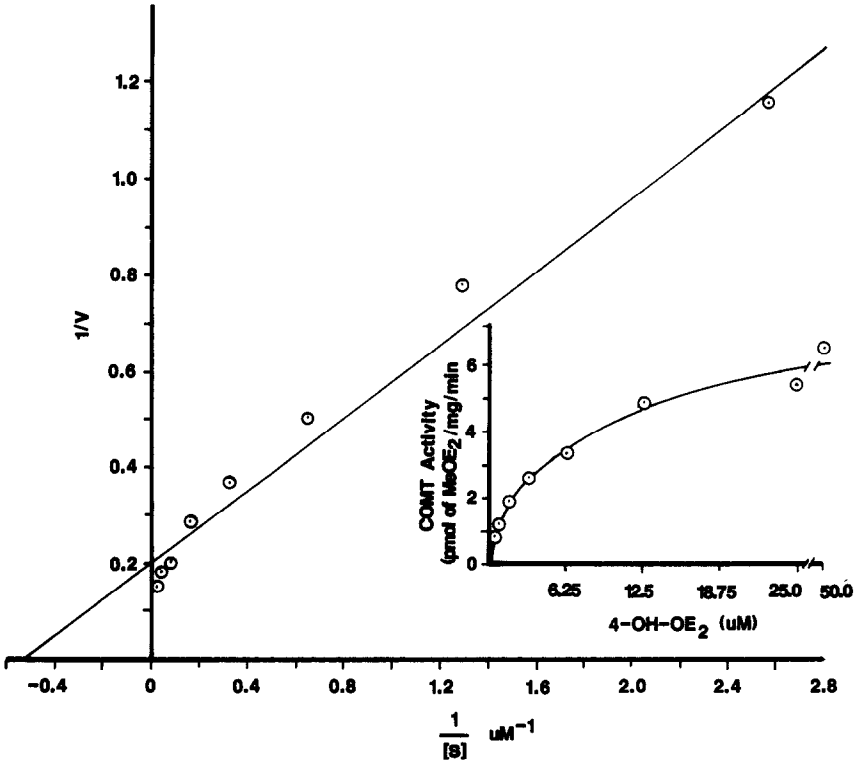

Fig. 4. Dependence of the formation of 4-methoxyoestradiol (4-Me-OE_2) on the concentration of 4-OH-OE_2. Experimental conditions and calculations are similar to those in Fig. 3.

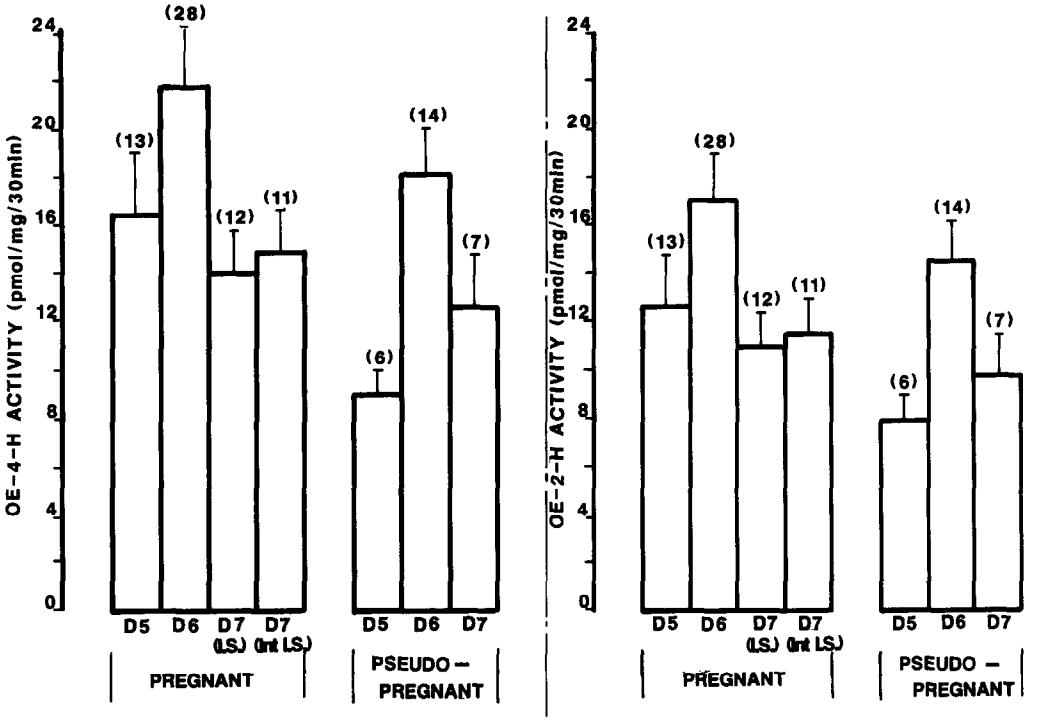

Fig. 5. Oestrogen-4-hydroxylase (OE-4-H) and oestrogen-2-hydroxylase (OE-2-H) activities (mean ± SEM) in the uterine microsomes of rabbits on different days (D) of pregnancy and pseudopregnancy. Numbers in parentheses represent the numbers of uterine horns. I.S., implantation sites; Int.I.S., interimplantation sites.

OE-2/4-H activities on different days of pregnancy and pseudopregnancy

As shown in Fig. 5, uteri of Day 6 pregnant as well as pseudopregnant animals had the highest OE-2/4-H activities. Analysis of the data by one-way analysis of variance (ANOVA) indicates no significant effect of gestational age on the formation of both 4-OH-OE_2 and 2-OH-OE_2. However, the unpaired t-test indicates that the difference between the activities of Day 6 and Day 7 pregnant uteri was significant ($P < 0.05$), whereas that between Day 5 and Day 6 pregnant uteri was not. On the other hand, ANOVA in pseudopregnant animals indicates significant age effect on OE-2/4-H activities ($P = 0.01$). Furthermore, pregnant animals had more OE-2/4-H activities than the pseudopregnant animals only on Day 5 ($P < 0.05$), while no significant difference between pregnant and pseudopregnant animals was observed on any other day. The activity of Day 7 implantation sites was not different from Day 7 interimplantation sites.

COMT activities on different days of pregnancy and pseudopregnancy

When comparisons were made among pregnant animals, the highest COMT activity was observed on Day 5 and the lowest on Day 7 implantation sites. The order of COMT activity in pregnant animals was: Day 5 uterus > Day 6 uterus > Day 7 interimplantation sites > Day 7 implantation sites (Fig. 6). The pattern was similar in pseudopregnancy (Fig. 6) ($P < 0.05$). When ANOVA was performed among Day 5, Day 6 and Day 7 implantation sites, the decrease in activity was significant ($P < 0.01$); among Day 5, Day 6 and Day 7 interimplantation sites, the decrease in activity was also significant ($P < 0.01$). Like OE-2/4-H, significant changes in COMT activities between pregnancy and pseudopregnancy were observed only on Day 5 ($P < 0.01$), but not on other days. Although OE-2/4-H showed no difference, COMT in implantation sites was significantly lower than that in interimplantation sites ($P < 0.05$).

DISCUSSION

Most of the cytochrome $P450$s which catalyze aromatic hydroxylations of oestrogens, both xenobiotic induced [21, 22] as well as native forms present in the liver [23–25], brain [18, 26], ovary [13, 20], kidney [27] and blastocyst [19] exhibit predominantly 2-hydroxylase activity as compared to 4-hydroxylase. In contrast, under our assay conditions, uterine microsomes produced either equal or slightly greater amounts of 4-OH-OE_2 than 2-OH-OE_2. The findings that the enzyme requires NADPH and that its activity is inhibited by SKF-525A suggest the involvement of cytochrome $P450$ dependence monooxygenase in these hydroxylation reactions. Furthermore, the apparently lower K_m value of OE_2 for the

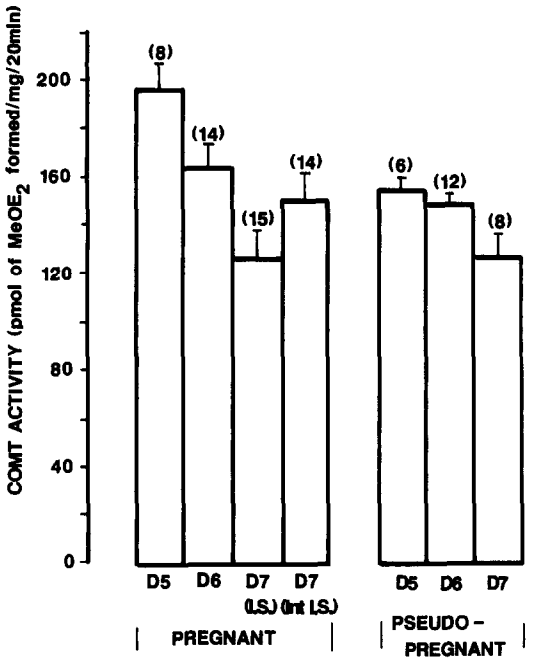

Fig. 6. Soluble COMT activities (mean ± SEM) in the uteri of rabbits on different days (D) of pregnancy and pseudopregnancy. Samples of uterine cytosols were assayed for COMT using 2-OH-OE_2 as catechol substrate as described in "Experimental" section. Numbers in parentheses represent the number of uterine horns. I.S., implantation sites; Int.I.S., interimplantation sites.

4-hydroxylation reaction as compared to that for 2-hydroxylation is further evidence of the dominance of the former reaction in uterine microsomes.

As in the rat liver [28], 2-OH-OE_2, not 4-OH-OE_2, appears to be the preferred substrate for COMT in the rabbit uterus, since the K_m values of both the substrates are almost equal, whereas the V_{max} is more than double when 2-OH-OE_2 was used as substrate. Because the ratio of OE-4-H to OE-2-H in the uterus is greater than that in other tissues and the metabolism of 4-OH-OE_2 is less than that of 2-OH-OE_2, we can speculate that more 4-OH-OE_2 is available to the uterus than 2-OH-OE_2. This observation may have physiological importance because 4-OH-OE_2 is more active than 2-OH-OE_2 in many respects: (i) it has higher affinity for binding with the classical intracellular oestrogen receptors in many tissues including uterus [28]; (ii) it is more potent in initiating implantation in the uteri of mice [29] and rats [30], and in stimulating uterine prostaglandin synthesis [9, 10].

Our next attempt was to find out whether the embryo and/or endocrine factors influence catechol oestrogen production by the rabbit uterus. Wise and Heap[2] observed a significant influence of the embryo on endometrial oestrogen synthesis. They observed that while the gravid uterine horn had much more aromatase and C17,20-lyase activities than did the non-gravid horn, endometrium from animals in oestrus or pseudopregnancy had insignificant activities [2]. However, our present study demonstrates that the embryonic influence on uterine production of catechol oestrogens and their metabolism by COMT are not apparent. Although both OE-2/4-H and COMT activities in the uterus of Day 5 pregnant animals were significantly higher than those of the pseudopregnant animals, with time both the pregnant and pseudopregnant animals showed almost similar activities. Therefore, these enzymes in pregnant and pseudopregnant uteri are likely to be influenced predominantly by endocrine factors. Progesterone could be an important factor and was found in the uterine flushings of both pseudopregnant and pregnant rabbits [31, 32]. The presence of progesterone was positively correlated with the protein (especially uteroglobin) content in the flushings [31, 32]. Our preliminary results (unpublished) also indicate that progesterone induces cytochrome *P*450 catalyzed catecholoestrogen formation in the mouse uterus.

The action of oestrogens to enhance prostaglandin synthesis in the progesterone-primed uterus is not altered by the administration of anti-oestrogens or inhibitors of protein or RNA synthesis [33]. Using actinomycin D and cycloheximide, Finn and Martin[34], Leroy *et al.*[35] and others [36] have shown that oestrogen induced receptive and non-receptive phases to implantation or decidualization in progesterone-primed uterus do not directly involve transcriptional control. Furthermore, 2-fluoroestradiol, a biologically active oestrogen, with a very limited capacity to form catecholoestrogen, is ineffective in inducing implantation in the delayed implanting rat [37], but can inhibit oestradiol-induced implantation in this species [38]. These observations as well as our present findings suggest that oestrogen, either of embryonic or uterine origin, could be converted locally to catecholoestrogens in the target tissues and thus initiates implantation via generation of prostaglandins. In the rabbit, implantation occurs in the evening of Day 6 of pregnancy. The peak uterine OE-2/4-H activity occurs in rabbits on Day 6 of pseudopregnancy and pregnancy, and could be involved in preparing the uterus for implantation. A significant decrease of OE-2/4-H activity on Day 7 corresponds to a similar decrease of aromatase activity [2]. Furthermore, rabbit blastocysts on Day 6 of pregnancy have detectable aromatase activity which increases at the time of implantation [5]. The blastocysts also have the ability to convert OE_2 into catecholoestradiols, however the hydroxylation reactions are not stoichiometric [unpublished data]. This could be due to the presence of some inhibitor of OE-2/4-H or some other factors present in the rabbit blastocyst. As COMT activities in Day 6 and Day 7 pregnant uteri are lower than in Day 5 uteri, we can speculate that more catecholestrogens would be available in the uteri of the animals on Days 6 and 7 of pregnancy. Similarly, lower COMT activity in implantation sites than in interimplantation sites provides further support for the concept that more

catecholoestrogens could be available locally for initiation of blastocyst implantation. However, changes in COMT levels are not only related to changes in catecholoestrogen levels, but also to those of catecholamines. This is evidenced by the fact that the uterus has at least two structurally distinct adrenergic neuronal systems [39] and endogenous epinephrine levels in the uterus rise during pregnancy [40]. Further investigation will be required to clarify this issue.

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