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COMPARISON OF CYTOCHROME *P*-450 SPECIES WHICH CATALYZE THE HYDROXYLATIONS OF THE AROMATIC RING OF ESTRADIOL AND ESTRADIOL 17-SULFATE

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Summary—For identification of microsomal cytochrome *P*-450 (*P*-450) enzymes which catalyze 2- or 4-hydroxylations of estrogens in the rat liver, estradiol (E_2) and estradiol 17-sulfate (E_2-17-S) were selected as the substrates and incubated with various kinds of purified *P*-450 enzymes: PB-1, PB-2, PB-4 and PB-5 obtained from phenobarbital-treated male rats (Sprague–Dawley); MC-1 and MC-5 from 3-methylcholanthrene-treated male rats; and UT-1, UT-2, UT-4 and UT-5 from untreated animals. The reactions were carried out under the *P*-450-reconstructed system, and the resulting products were determined by HPLC using electrochemical detection. All the enzymes tested were shown to have varying degrees of catalytic activities for 2-hydroxylation of the two substrates; UT-1 and UT-2 had the highest activity. Of the induced *P*-450 enzymes, PB-2 and MC-1 showed fairly high catalytic activity for 4-hydroxylation of E_2. The *P*-450 enzymes obtained from the untreated male rats, especially UT-4, showed the highest catalytic activity for 4-hydroxylation of the two substrates. From these results and also from kinetic experiments, the *P*-450 enzymes which catalyze 2- and 4-hydroxylations of estrogen were considered to be different species. A part of E_2 was converted to such metabolites as estrone and those having a hydroxyl group at positions 6β, 15α or 16α, each production of which was estimated to be catalyzed by single or multiple *P*-450s.

INTRODUCTION

Estradiol 17-sulfate (E_2-17-S) was demonstrated to be endogenous sulfate in the human [1] and in the rat [2], and was found to be metabolized *in vitro* in rats mainly to the catechol metabolites, 2-hydroxyestradiol 17-sulfate (2-OH-E_2-17-S) and 4-hydroxyestradiol 17-sulfate (4-OH-E_2-17-S) [3, 4]. From enzymatic studies of these biotransformations, the enzymes which catalyze the C-2 or C-4 hydroxylation of E_2 were considered to be different molecular species of cytochrome *P*-450 (*P*-450) [5, 6].

It has been well recognized that the major metabolic pathway of E_2 is hydroxylation at C-2 or C-4 to the catechol products 2- and 4-hydroxyestradiol (2- and 4-OH-E_2) [7]. From the induction [8] and inhibition studies [9] on these metabolism, the catechol-forming enzymes, E_2-2- and 4-hydroxylases, were also considered as different *P*-450.

Recently, the presence of multiple species of *P*-450s became evident, and numerous kinds of the enzymes have been separated and characterized [10]. Dannan *et al.* [8] differentiated the 2- and 4-hydroxylation enzymes of E_2 by using immunological methods.

This paper describes the identification of *P*-450 which catalyze C-2 and C-4 hydroxylation of E_2-17-S by using 10 kinds of different forms of hepatic *P*-450s, the UT-type enzymes obtained from untreated male rats, and PB- or MC-type enzymes obtained from animals treated preliminarily with phenobarbital or 3-methylcholanthrene, respectively [11, 12], and on the relation with similar *P*-450 which catalyze 2- and 4-hydroxylation of E_2.

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EXPERIMENTAL

Chemicals

The authentic steroids were prepared by known methods [3, 13–15]. Estrone (E_1), estradiol (E_2), estriol (E_3) and 2-hydroxyestriol (2-OH-E_3) were purchased from Sigma (St Louis, MO, U.S.A.). Glucose-6-phosphate (G-6-P), NADP and G-6-P dehydrogenase (G-6-P DH) were obtained from Oriental Yeast (Osaka, Japan). Dilauryl-L-3-phosphatidylcholine (DLPC) was purchased from Sardaryl Inc. (Ontario, Canada). Sep-Pak C_{18} cartridges and column guards were obtained from Waters Ltd (Milford, MA, U.S.A.) and Millipore Co. (Bedford, MA, U.S.A.), respectively. All other reagents and solvents were of reagent grade.

Enzyme sources

Hepatic microsomes were prepared according to the method described previously [3, 4] from Sprague–Dawley male rats, age 58 ± 5 days and weighing 230–280 g. The protein content of the microsomes was determined by the method of Lowry *et al.* [16] with bovine serum albumin as the standard. The different forms of purified *P*-450 enzymes, UT-1 and UT-4 [12] and UT-2, UT-5, PB-1, PB-4, PB-5, MC-1 and MC-5 [11] were prepared from male Sprague–Dawley rats weighing 200–250 g (Nippon Clear, Kyoto, Japan) by the method described previously. Cytochrome b_5 (Cyt-b_5) and cytochrome *P*-450 reductase (reductase) were prepared by the method of Kamataki *et al.* [17]. The amount of *P*-450 was determined by the method of Omura and Sato [18] by using Hitachi model 556 spectrophotometer.

Incubation

Microsomes. Ice-cold reaction vessels contained microsomal protein (1.0 mg/ml), KCl (90 mM), EDTA (0.1 mM), ascorbic acid (AA, 0.1 mM) an NADPH-generating system (NADP 0.5 mM, G-6-P 5 mM, $MgCl_2$ 5 mM, G-6-P DH (1 U/ml) and the substrate (200 μM). The mixture was diluted with 50 mM Hepes buffer solution (pH 7.4) to 1.0 ml final volume and incubated at 37°C under aerobic conditions for 30 min. Boiled microsomes were used for control experiments.

Purified P-450. Ice-cold reaction vessels contained purified enzymes (20–50 pmol), Cyt-b_5 (25 pmol), reductase (0.5 U/ml), DLPC (5 μg), AA (0.1 mM), an NADPH-generating system (as the microsomal conditions) and substrate (2.5–200 μM). The mixture was diluted with 50 mM Hepes buffer solution (pH 7.4) to 0.5 ml final volume and incubated at 37°C under aerobic conditions for 10 min. As blank tests, incubations were carried out using *P*-450 enzymes heated in boiling water for 2 min.

Work-up procedure

Reactions were stopped by heating the reaction vessels in boiling water for 2 min, followed by the addition of an exact amount (1 μg) of internal standards (6-oxoestradiol [19] and 2-methoxyestradiol 17-sulfate [20], for experiments on E_2 and E_2-17-S, respectively). The reaction mixture (the supernatant fraction obtained by centrifugation in the microsomal incubation) was diluted with 1 ml of distilled water and passed through a Sep-Pak C_{18} cartridge. After washing the cartridges with 2 ml of distilled water, the steroid-containing fraction was obtained by elution with methanol (4 ml). The eluate was passed through a column guard, and the methanolic solution was evaporated under a nitrogen stream at 40°C to give the residue, which was subjected to HPLC as the methanolic solution.

HPLC

HPLC was carried out in a model 803D chromatograph equipped with an EC-8 electrochemical detector (Tosoh, Tokyo) at 0.9 V vs the Ag/AgCl reference electrode. A reversed-phase column packed with ODS-120A (5 μm, Tosoh) in a stainless steel column (250 × 4.6 mm, i.d.) was used and maintained at 40°C in a circulating water bath. Mixtures 50:50 and 60:40 (both, v/v) of 0.5% $NH_4H_2PO_4$ (pH 3.0) and methanol were used as mobile phases for the analyses of free and conjugated steroids, respectively; and at a flow rate of 1.0 and 1.1 ml/min, respectively. The column pressure was set at 120 kg/cm^2 in both analyses. Quantification of the metabolites was done by internal standard method.

Recovery test

A known amount of steroids were added to the incubation medium of purified *P*-450 enzymes, the mixtures were treated in the same way as described.

Measurement of nonenzymatic catechol estrogen formation

Either E_2 or E_2-17-S (each 200 μM) was incubated under the same conditions as described, except in the absence of *P*-450, cyt-b_5 and

reductase, and the mixture was treated in the same way as in the above experiments.

RESULTS

Table 1 shows the relation of 10 different forms of hepatic *P*-450 enzymes obtained in our laboratory from male rats [11, 12] to those obtained by other investigators [8, 21–25].

Before the incubation experiments, the recovery test was carried out using authentic samples. A known amount of steroid was added to the incubation medium and the steroid recovered through a whole clean-up procedure was determined, the results of which are shown in Table 2.

Because catechol estrogen is produced nonenzymatically in parts under the *in vitro* condition of microsomal metabolism of E_2 [26] and of E_2-17-S [27], it becomes necessary to measure the exact amount of a nonenzymatic catechol formation under the present experiments. The results obtained in the absence of the *P*-450-reconstructed system revealed an occurrence of autoxidation and the velocity constants for each catechol formation were obtained (Table 3).

As the experimental conditions were established, either E_2 or E_2-17-S was incubated with microsomal or purified enzymes. Table 4 shows the catalytic activities for production of the important metabolites, such as their turn-over numbers. No product formation was confirmed when boiled enzymes were used.

In the metabolism of E_2, the 2-hydroxylation was catalyzed by every *P*-450 employed, especially by UT-1 and UT-2, which also showed catalytic activity for the 16α-hydroxylation. The formation amount of 4-OH-E_2 was small except by UT-4, which also showed the greatest activity for conversion of E_2 to E_1.

Hydroxylation of E_2 at the 6β position was observed when PB-4, PB-5, UT-1 or UT-4 was used, and the highest activity was found when PB-5 was employed. The metabolites, 6α-, 7α- and 7β-OH-E_2, were produced only when E_2 was incubated with MC-5, UT-4 and UT-5. The formation of the 15α- or 16α-hydroxylated metabolite was observed mainly in the case of UT-type enzymes. The occurrence of the dehydrogenation at C-17 to E_1 was observed in every case.

In the metabolism of E_2-17-S, the 2-hydroxylation occurred in all cases, and the greatest activity was obtained by the use of UT-1 and UT-2. A significant amount of the 4-hydroxylated metabolites was produced in the reaction with UT-4 and UT-5. Except for these catechols, no other metabolites were detected, this agrees well with the results using microsomes [28].

Lastly, kinetic experiments on the 2- and 4-hydroxylation of two substrates were carried out to determine which *P*-450 catalyzes each biotransformation. Either E_2 or E_2-17-S was incubated with 5 selected *P*-450 enzymes which have fairly high catalytic activities for both hydroxylations shown in Table 4. The results obtained are summarized in Table 5.

Metabolism of E_2 by PB-2, MC-1, UT-1, UT-2 and UT-4 was demonstrated in the *P*-450-reconstructed system. Of these, PB-2 exhibited the smallest K_m value for the 2- and 4-hydroxylations, 5.2 and 6.3 μM, respectively, meaning that E_2 has the highest affinity to PB-2 in the aromatic hydroxylation. In contrast to K_m

Table 2. Various estrogens recovered from the incubation medium through the whole clean-up procedure

Estrogen	Added (ng)	Detected (ng)	Recovery (%)[a]
2-OH-E_2	100	102	102 ± 2.0
	1000	973	97.3 ± 3.5
4-OH-E_2	10	10.0	100 ± 4.4
	100	96.6	96.6 ± 2.4
E_3	100	94.8	94.8 ± 3.7
6β-OH-E_2	100	95.8	95.8 ± 3.4
E_1	100	95.8	95.8 ± 2.8
2-OH-E_2-17-S	100	93.4	93.4 ± 4.2
4-OH-E_2-17-S	100	96.7	96.7 ± 6.8

[a]Mean ± SD ($n = 3$).

Table 1. Comparison of different forms of cytochrome *P*-450 species purified in present investigation with those of other laboratories

Present report [11, 12]	Levin *et al.* [21, 22]	Guengerich *et al.* [8, 23]	Schenkman *et al.* [24, 25]
PB-1	—	—	—
PB-2	—	PB-C	—
PB-4	*P*-450b	PB-B	—
PB-5	*P*-450e	PB-D	—
MC-1	*P*-450d	βNF/ISF-G	—
MC-5	*P*-450c	βNF-B	—
UT-1	—	—	RLM5
UT-2	*P*-450h	UT-A	—
UT-4	—	—	RLM2
UT-5	*P*-450g	—	RLM3

Table 3. Nonenzymatic 2- and 4-hydroxylations of E_2 and E_2-17-S[a]

Substrate	Metabolites	Production (pmol/tube[b]/10 min)[c]
E_2	2-OH-E_2	18.7
	4-OH-E_2	22.0
E_2-17-S	2-OH-E_2-17-S	11.2
	4-OH-E_2-17-S	6.3

[a]Incubations were carried out in solution containing: AA (0.1 mM), EDTA (0.1 mM), NADP (0.5 mM), G-6-P (5 mM), $MgCl_2$ (5 mM), G-6-P DH (1 U), Hepes buffer solution (50 mM, pH 7.4) and substrate (200 μM).
[b]1.0 ml.
[c]Means ($n = 3$).

Table 4. Comparison of catalytic activities of purified cytochrome *P*-450 for hydroxylation at different positions of E_2 and its 17-S[a]

	E_2[b]						17-S	
Enzyme	2	4	6β	15α	16α	17[c]	2	4
Microsomes	2.89[d]	0.12[e]	0.14	0.03	1.06	0.72	0.54	0.03
PB-1	0.58	0.04	0.03	ND[f]	0.06	0.46	0.15	ND
PB-2	6.33	0.18	ND	ND	0.65	0.84	0.65	0.08
PB-4	0.71	0.08	0.25	ND	0.06	0.40	0.14	ND
PB-5	2.38	0.07	0.85	ND	0.02	0.03	0.17	ND
MC-1	6.19	0.10	ND	0.04	0.03	0.05	0.19	ND
MC-2	1.24	0.15	0.06	0.06	0.03	0.31	0.15	ND
UT-1	15.6	0.59	0.32	0.10	2.68	1.23	1.39	0.06
UT-2	15.5	0.47	0.07	0.11	2.41	1.25	1.64	0.08
UT-4	3.23	1.01	0.60	0.13	0.24	1.63	0.48	0.23
UT-5	0.95	0.13	0.04	0.44	0.43	1.08	0.17	0.15

[a] Catalytic activities by *P*-450 enzymes are shown as nmol/nmol *P*-450/30 min, and those by microsomes are nmol/mg protein/10 min. Each figure is a mean value ($n = 3$). Incubations using *P*-450s were carried out at the enzyme concentration of 50 pmol, and those using microsomes were 1 mg protein/tube.
[b] The minor metabolites, such as 6α-, 7α- and 7β-OH-E_2, 16α-OH-E_1 and others are not included.
[c] Conversion of E_2 to E_1.
[d] Sum of 2-OH-E_1 and 2-OH-E_2.
[e] Sum of 4-OH-E_1 and 4-OH-E_2.
[f] Not detected.

values, UT-1 and UT-4 gave the greatest V_{max} values for the 2- and 4-hydroxylations, respectively. Thus, UT-1 and UT-4 have the highest turn-over activities for hydroxylation of the aromatic ring.

The kinetic parameters for aromatic hydroxylation of E_2-17-S using PB-1, UT-1, UT-2, UT-4 or UT-5 were measured. Of these, UT-4 had the smallest K_m values for the 2- and 4-hydroxylations. As for V_{max} values for production of these catechols, UT-2 and UT-4 exhibited the highest turn-over activity toward the 2- and 4-hydroxylations, respectively.

DISCUSSION

The molecular forms of hepatic *P*-450 enzymes in rats are different in males and females [29], and the expression of physiological action of sex-specific *P*-450 is controlled by gonadal hormones [30]. In view of these points, it would be desirable to examine *P*-450 enzymes from both sexes to complete the studies. In this report, however, the enzymes used were restricted to only those from male rats for the following reason: a big sex difference of rat hepatic microsomal oxygenase was observed in 2-hydroxylation of E_2, which for hepatic enzymes from female rats did not follow the classical Michaelis–Menten kinetics [31]. Therefore, a kinetic analysis on the reaction by purified *P*-450 enzymes was expected to become complicated.

Table 5. Kinetic parameters for 2- and 4-hydroxylation of E_2 and E_2-17-S by different forms of cytochrome *P*-450[a]

	2-Hydroxylation		4-Hydroxylation	
P-450	K_m[b]	V_{max}[c]	K_m[b]	V_{max}[c]
		E_2		
PB-2	5.2	2.58	6.3	0.12
MC-1	6.9	4.85	11.3	0.20
UT-1	9.6	16.2	15.5	0.90
UT-2	7.4	12.1	21.8	0.86
UT-4	6.5	3.76	9.9	1.57
		E_2-17-S		
PB-2	30.8	0.25	18.0	0.08
UT-1	32.6	1.69	18.3	0.12
UT-2	38.3	2.11	35.2	0.18
UT-4	15.6	0.63	17.6	0.32
UT-5	21.3	0.20	25.0	0.15

[a] Incubations were carried out at the substrate concentration of 2.5–100 μM using cytochrome *P*-450 (20 pmol/tube). Each figure is a mean value ($n = 3$).
[b] μM.
[c] nmol/nmol cytochrome *P*-450/min.

Table 1 shows 10 different kinds of rat hepatic *P*-450 enzymes used in the present studies and the relation of these enzymes purified in our laboratory to that purified in others. Most of these enzymes are major components of the constitutive *P*-450 involved in the rat liver [11, 12].

The present study's purpose is to investigate the characteristics of each enzyme participation in the ring-A hydroxylation of E_2 and E_2-17-S, for which it became necessary to establish an exact quantification method for extremely labile catechol products. The quantification method was established by adding AA as the antioxidant [32, 33] to the reaction mixture, followed by careful treatment.

As was observed in a microsomal incubation of estrogen [26], significant extent of autoxidation of E_2 or E_2-17-S occurred also in the present reaction (Table 3). Such nonenzymatic catechol production is no longer negligible, especially in cases where the catalytic activity of *P*-450 is fairly low. Thus, measurement of

the production rates of nonenzymatic catechol formation became important, and these values (in Table 3) were used as blank values for the autoxidation.

In the hepatic metabolism of E_2 in rats, oxidation of the molecule occurred rather at random [34]. The results in Table 4 led us to speculate that each hydroxylation is catalyzed by the specific *P*-450(s).

In the microsomal metabolism of E_2, the 2-, 4-, 6α-, 6β-, 7α-, 7β-, 15α- and 16α-hydroxylated metabolites and E_1 were produced, i.e. an almost random oxidation. Of the microsomal enzymatic activities, the 2-hydroxylation (the sum of the 2-hydroxylation of E_1 and E_2) was highest, followed by the 16α-hydroxylation and the dehydrogenation at C-17.

In the metabolism of E_2 by purified *P*-450s, on the other hand, each hydroxylation is considered to be catalyzed by particular enzyme(s). UT-1 and UT-2 showed high catalytic activity for the 2-hydroxylation. The enzyme UT-1 in the present studies corresponds to RLM5 [24, 25], and the enzyme UT-2 to *P*-450h [21] or UT-A [8, 23], and these enzymes were reported to catalyze 2-hydroxylation of E_2. Due to these results, and by comparison of the V_{max} and K_m values of each *P*-450s (Table 5), and, also due to the fact that UT-1 and UT-2 are the major constitutive hepatic *P*-450s of rats [11, 12], it was suggested that UT-1 and UT-2 are playing major roles in the *in vivo* 2-hydroxylation of E_2. This is supported by the evidence on the participation of multiple enzymes in the 2-hydroxylation of E_2 [35].

Of the *P*-450 enzymes from untreated rats, UT-1, UT-2 and especially UT-4, had high catalytic activity for 4-hydroxylation of E_2. The enzyme UT-4 corresponds to RLM2, which was reported to catalyze not only the hydroxylation of progesterone at 6α-, 6β-, 15α-, 15β- or 16α [24], but also that of testosterone at 7β-, 15α- or 15β [25]. On the other hand, UT-A (UT-2) was shown to possess high catalytic activity for 4-hydroxylation of E_2 [8]. However, the enzyme that catalyzes the 4-hydroxylation is considered not to be UT-2 but UT-4 (RLM2), because UT-4 showed higher catalytic activity and substrate affinity in the 4-hydroxylation than UT-2, as shown in Table 5.

It may be interesting that the UT-4 enzyme (RLM2) has shown the highest catalytic activity for 6β-hydroxylation of E_2 of the UT-type enzymes tested. Because of the high catalytic activity of RLM5 obtained for 6β-hydroxylation of testosterone as well as progesterone [24, 25], and of the similar capacity by UT-4 for 6β-hydroxylation of E_2 (Table 4), *P*-450 (UT-4 or RLM5) may catalyze a hydroxylation at the allylic position in common with the endogenous steroidal hormones having partial allylic structure.

As for 15α-hydroxylation of E_2, the microsomal enzyme activity was extremely low compared with other hydroxylations. Of the *P*-450 enzymes tested, only UT-5 (corresponding to RLM3 [24, 25]) showed a significantly high catalytic activity for the 15α-hydroxylation. This result corresponds with the report by Cheng and Schenkman [36] that RLM3 had the highest catalytic activity for 15α-hydroxylation of E_2.

Regarding the microsomal experiment on E_2, E_1 was produced significantly next to 2- or 16α-hydroxylated metabolites (Table 4). In the metabolism of E_2 in humans, the conversion to E_1 is the major pathway, followed by the competitive hydroxylations between the C-2 and the C-16α. A part of 16α-hydroxyestrone thus produced is then converted to E_3 [37]. In the hepatic metabolism of E_2 in the male rats, however, the major pathway is not the conversion to E_1, but rather the hydroxylation at C-2 or C-16α [38]. In fact, the amount of 2- and 4-OH-E_1 produced by microsomal experiments was $<2\%$ of each of the total 2- and 4-catechol, and that of 16α-OH-E_1 produced was negligible. Further, only negligible amounts of E_3, 2- and 4-OH-E_1 were produced in the incubations with purified *P*-450. Thus, the following metabolic pathways are not important in the rat liver:

$$E_2 \rightarrow E_1 \rightarrow \text{2- and 4-OH-}E_1$$

and

$$16\alpha\text{-}E_1 \rightarrow \text{2- and 4-OH-}E_2 \text{ and } E_3.$$

These three final metabolites should be produced by the direct hydroxylation of E_2.

Due to the great degree of catalytic activity present in E_3 formation, however, UT-1 and UT-2 are thought to catalyze a hydroxylation not only at C-2 but also at C-16α. This agrees well with the recent report by Sugita *et al.* [39] who discovered that the rat hepatic *P*-450 species, $P\text{-}450_{C\text{-}M/F}$, catalyzed the hydroxylation of E_2 at C-16α as well as at C-2.

The evidence that UT-1 and UT-2 are the major enzymes among the *P*-450s of the male rat liver and that their chromatographic behaviors resemble each other [11, 12], led us to consider the possibility of cooperative action of

the two enzymes in the *in vivo* estrogen metabolism at rings -A and -D, judging from the results in Table 4. The catalytic activity for E_3 formation by microsomes is a significantly high value, 1.06; whereas the values for such formation by UT-1 and UT-2 are only 2.68 and 2.41, respectively. No significant increases of the specific activities are observed in spite of the multiple-fold purifications of the enzyme. Because of such a low catalytic activity, UT-1 and UT-2 are not important enzymes for E_3 formation, and *P*-450(s), other than those listed in Table 1, may be present to act for the 16α-hydroxylation.

Application of similar considerations for UT-4 is, however, impossible, even though the catalytic activity for E_1 formation by UT-4 is 1.63, i.e. only about 2 times higher (0.72) than that by microsomes. Because UT-4 is a rather minor constitutive *P*-450 enzyme [11, 12], the microsomal activity for the dehydrogenation at C-17 of E_2 appears low. Essentially, a 17β-hydroxysteroid dehydrogenase may be identified as UT-4.

In contrast with E_2, microsomal metabolism of E_2-17-S was very poor: 2-hydroxylation was the only major metabolism, followed by minor 4-hydroxylation. With respect to the 2-hydroxylation by purified *P*-450s, UT-1 and UT-2 showed high catalytic activity compared to other enzymes (Table 5), but their affinity to the sulfate was lower than that of UT-4 or UT-5. Conversely, the greatest turn-over number for this hydroxylation was observed when UT-2 was used. Because UT-2 and UT-1 are the major *P*-450s in the rat liver [11, 12], both enzymes might be the leading *P*-450s which catalyze a 2-hydroxylation of E_2-17-S. Regarding 4-hydroxylation of E_2-17-S, the highest Michaelis content and V_{max} value were obtained when UT-4 enzyme was used.

From these results, it may be concluded that 2-hydroxylation of E_2 and E_2-17-S is catalyzed mainly by UT-1 and UT-2, and that the 4-hydroxylation is by UT-4. Table 5 shows such a tendency, as the induced *P*-450 enzymes PB-2 and MC-1 have a rather higher substrate-enzyme affinity to E_2, but a rather lower affinity to E_2-17-S than UT-type enzymes. Complex formation between the substrate and the enzyme may become difficult when the substrate changes from a free steroid to its 17-sulfate. This may be shown by the comparison of the reciprocal K_m values for 2- and 4-hydroxylation of the two substrates and their turn-over numbers as shown in Table 6.

Table 6. Ratios of the affinity of substrates to *P*-450 enzymes, and of the turn-over numbers for 2- and 4-hydroxylation of E_2 and its 17-S

	2-Hydroxylation		4-Hydroxylation	
P-450	Affinity[a]	Turn-over[b]	Affinity[a]	Turn-over[b]
PB-2	5.9	10.3	2.9	1.5
UT-1	3.4	9.6	1.2	7.5
UT-2	5.2	5.7	1.6	4.8
UT-4	2.4	6.0	1.8	4.9

[a]Affinity: $(1/K_m$ of $E_2)/(1/K_m$ of E_2-17-S).
[b]Turn-over: $(V_{max}$ of $E_2)/(V_{max}$ of E_2-17-S).

As the reciprocal of the Michaelis constant is defined as affinity of the substrate to the enzyme, we can compare the ability for complex formation of the enzyme with E_2 or with its 17-sulfate. The complex formation between E_2 and PB-2 for the 2- and 4-hydroxylation is about 6 and 3 times higher, respectively, than that between E_2-17-S and the same enzyme. Induced *P*-450 enzyme thus appears to have low affinity to E_2-17-S. Contrary to the induced enzymes, the UT-type ones have rather high affinity values, meaning that the natural constitutive enzymes have high affinity even though the hydrophilic steroids like sulfate. This tendency is significant in the case of 4-hydroxylation, because the ratios for UT-enzymes are all twice as small.

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