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# HEPATIC CATECHOLESTROGEN SYNTHASES: DIFFERENTIAL EFFECT OF SEX, INDUCERS OF CYTOCHROMES *P*-450 AND OF ANTIBODY TO THE GLUCOCORTICOID INDUCIBLE CYTOCHROME *P*-450 ON NADPH-DEPENDENT ESTROGEN-2-HYDROXYLASE AND ON ORGANIC HYDROPEROXIDE-DEPENDENT ESTROGEN-2/4-HYDROXYLASE ACTIVITY OF RAT HEPATIC MICROSOMES

QUANG D. BUI, JUDITH WEISZ* and STEVEN A. WRIGHTON[1]

The Milton S. Hershey Medical Center, The Pennsylvania State University, P.O. Box 850, Hershey, PA 17033 and [1]Eli Lilly and Co., Lilly Corporate Center, Indianapolis, IN 46285, U.S.A.

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**Summary**—Formation of catecholestrogens (CE) by rat hepatic microsomes was re-examined because as recently shown; (1) CE formation can be catalyzed by an NADPH-dependent estrogen-4-hydroxylase (E-4-$H_{(NADPH)}$) and by a peroxidatic, organic hydroperoxide-dependent estrogen-2/4-hydroxylase (E-2/4-$H_{(OHP)}$), in addition to the established NADPH-dependent estrogen 2-hydroxylase (E-2-$H_{(NADPH)}$); and (2) the indirect radiometric and the COMT-coupled radioenzymatic assays, used in many previous studies, may fail to provide an accurate measure, in particular, of 4-OH-CE. Using a direct product isolation assay, hepatic microsomes of both male and female rats were shown to express E-2/4-$H_{(OHP)}$ activity with properties similar to those of peroxidatic activity in other tissues. The activities of E-2/4-$H_{(OHP)}$ and E-2-$H_{(NADPH)}$ were affected differently by 5 out of 7 inducers of cytochromes *P*-450 administered *in vivo*. Phenobarbital and dexamethasone caused a 4- and 2–3-fold increase in E-2-$H_{(NADPH)}$ activity, respectively, but only a 38 and 20% increase in E-2/4-$H_{(OHP)}$ activity. Ketoconazol and β-napthoflavone caused a modest increase in E-2-$H_{(NADPH)}$ activity but a decrease in OHP-dependent activity. Clofibrate decreased peroxidatic activity by 50% and NADPH-dependent activity by approximately 20%. Both activities were increased by ethanol but decreased by isoniazide, an agent which induces the same form of cytochromes *P*-450 as ethanol. Polyclonal antibody against $P\text{-}450_p$, a form of *P*-450 induced by glucocorticoids, inhibited E-2-$H_{(NADPH)}$ but not E-2/4-$H_{(OHP)}$ activity of untreated and of dexamethasone- and phenobarbital-treated rats. This study establishes that CE formation may occur in liver via the peroxidatic pathway and indicates that this pathway depends on forms of *P*-450 different from those mediating E-2-$H_{(NADPH)}$ activity. It also confirms and extends previous observations of the involvement of multiple, constitutive and induced forms of cytochrome *P*-450 in NADPH-dependent 2-hydroxylation in liver.

## INTRODUCTION

A major route for the inactivation of primary estrogens, estrone and estradiol ($E_2$), involves their conversion in liver to their 2-hydroxylated catechol metabolites (2-OH-CE) [1]. Several forms of cytochromes *P*-450 have been identified which can mediate formation of catecholestrogens (CE) in liver [2–5]. In the presence of NADPH, these function as estrogen 2-hydroxylases (E-2-$H_{(NADPH)}$). They generate predominantly 2-hydroxylated CE (2-OH-CE) with 4-hydroxylated CE (4-OH-CE) representing only minor reaction products [1, 5, 6].

We have recently identified in extrahepatic tissues two additional pathways for the hydroxylation of ring A of estrogens [7, 8]. One of these, an NADPH-dependent estrogen 4-hydroxylase (E-4-$H_{(NADPH)}$), generates predominantly 4-OH-CE with 2-OH-CE representing the minor reaction products [8]. The second pathway requires an organic hydroperoxide (OHP) as the co-factor, involves a peroxidatic mechanism, and generates 2- and 4-OH-CE in similar amounts [7,8]. This OHP-dependent estrogen-2/4-hydroxylase (E-2/4-$H_{(OHP)}$) activity

*To whom correspondence should be addressed.

appears to be analogous to the peroxidatic, OHP-dependent mechanism for the metabolism of xenobiotics by hepatic microsomes [9–12].

Using pharmacological inhibitors of cytochromes *P*-450 *in vitro*, we have obtained presumptive evidence that microsomal peroxidatic E-2/4-$H_{(OHP)}$ activity, like peroxidatic metabolism of xenobiotics, depends on cytochromes *P*-450 and that the form(s) of *P*-450s catalyzing this activity differ from those mediating E-2-$H_{(NADPH)}$ activity [7].

The properties of 4-OH-CE differ in several respects from those of 2-OH-CE. Thus, whereas estrogenic potency of phenolic estrogens is markedly reduced by 2-hydroxylation, it is preserved and may even be enhanced by 4-hydroxylation [13]. In addition, 2-OH-CE are more effective competitive inhibitors of the *O*-methylation of catecholamines by catechol-*O*- methyltransferase (COMT) than 4-OH-CE [1], and 2-OH-CE can inhibit the *O*-methylation not only of catecholamines but also of 4-OH-CE by COMT [14]. Thus, in estrogen target cells in which both 2- and 4-OH-CE are generated, 4-OH-CE may persist and produce autocrine and/or paracrine effects. Together, these findings suggest that the biological significance of E-4-$H_{(NADPH)}$ and E-2/4-$H_{(OHP)}$ is likely to differ from that of the established E-2-$H_{(NADPH)}$ activity.

The primary aim of this study was to determine whether rat hepatic microsomes express, in addition to the established E-2-$H_{(NADPH)}$ activity, either the peroxidatic E-2/4-$H_{(OHP)}$ or the E-4-$H_{(NADPH)}$ activities. We used a direct product isolation assay which allows for accurate and concurrent measurement of both 2- and 4-OH-CE formation and at the pH optima required for full expression of NADPH- and OHP-dependent activities [7, 8]. The utility of the radiometric assay, which is based on the measurement of the amount of $^3H_2O$ generated from regiospecifically labeled substrates, is limited by the instability of the ortho C–H bonds at pH other than neutral, and a spurious release of $^3H$, especially from substrates labeled at C-4 [15–17]. In contrast, the COMT-coupled radioenzymatic assay [18] may underestimate CE formation, in particular that of 4-OH-CE, because *O*-methylation of CE is incomplete at pH lower than 8.5, the pH optimum for COMT, and because 2-OH-CE inhibit the *O*-methylation of 4-OH-CE by COMT [19].

We report here the finding that rat hepatic microsomes express in addition to E-2-$H_{(NADPH)}$ activity also peroxidatic E-2/4-$H_{(OHP)}$ activity. In addition, we present evidence, based on sex differences in the ratios of E-2-$H_{(NADPH)}$ to E-2/4-$H_{(OHP)}$, on the effects of inducers of cytochromes *P*-450 *in vivo* and antibodies against *P*-$450_p$ *in vitro* on CE formation by the two pathways that they are mediated by different forms or profile of forms of cytochromes *P*-450.

## EXPERIMENTAL

### *Animals*

Male and female Sprague–Dawley rats (175–225 g) were purchased from Flow Labs (Dublin, Va.). The rats were housed in pairs and given unlimited access to food and water. The inducers were administered to female rats by the following regimens: phenobarbital, 80 mg/kg in saline intraperitoneally for 3 days; dexamethasone, 300 mg/kg gavage for 3 days; clofibrate, 400 mg/kg for 3 days; β-napthoflavone, 80 mg/kg for 3 days and; isoniazid (INH), 0.1% (w/v) in drinking water, for 10 days. The inducers were suspended in corn oil unless indicated otherwise. Ethanol was administered to the rats in a liquid diet as described by Lieber and DeCarli [20]. Microsomes from a minimum of 3 rats were used to test each experimented variable. The microsomes were prepared by differential centrifugation as described previously [21] and stored at −70°C. Rat cytochrome *P*-$450_p$ (the form of *P*-450 induced by glucocorticoids) was purified, and goat polyclonal anti-*P*$450_p$ was prepared as described previously [22].

### *Direct product isolation assay*

The chemicals used, the assay procedure and its validation have been described in detail previously [7, 8]. Briefly, the microsomal preparations (approx. 50 μg protein) were incubated in a final volume of 150 μl with [6, 7] $^3H$-$E_2$ substrate and with either 5 mM NADPH in Tris–HCl/Hepes buffer at pH 7.4 when measuring monooxygenase activity or with 50 mM cumene hydroperoxide in Tris–citrate buffer at pH 6.0 when measuring peroxidatic activity. The incubations were carried out at 30°C and in the presence of 10 mM ascorbic acid, strategies previously shown to protect CE from oxidative degradation without affecting significantly enzyme activity [16, 17]. Enzyme activity was arrested after 10 min of incubation by addition of 0.25 M perchloric acid. The CE were absorbed from the aqueous medium onto neutral

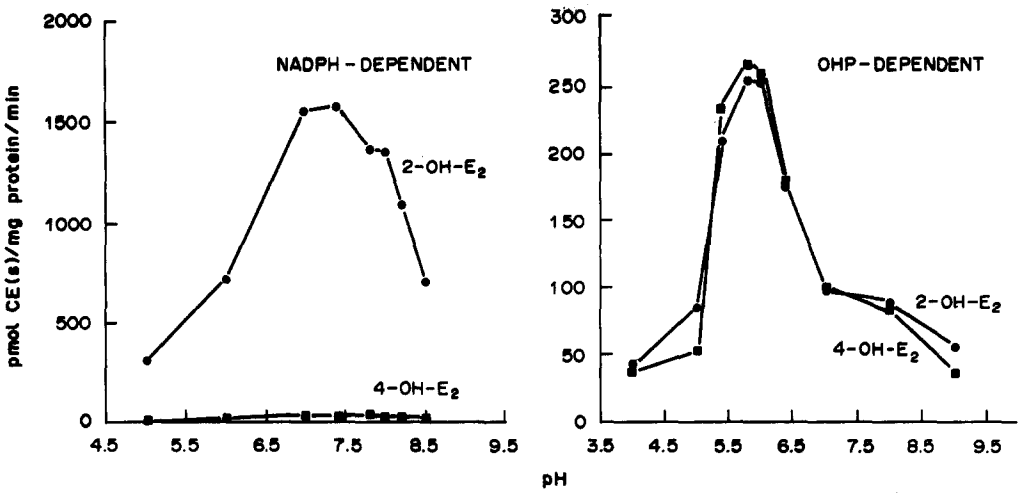



Fig. 1. Conversion of estradiol ($E_2$) to 2- and 4-hydroxyestradiol (2- and 4-OH-$E_2$) by microsomes from rat liver—pH dependence. Microsomes prepared from livers of male rats were incubated with [$^3$H]17$\beta$-estradiol (10 $\mu$M or 50 $\mu$M for NADPH- and OHP-dependent activities, respectively) at the pH indicated on the horizontal axis. Incubations were carried out for 10 min using either NADPH (5 mM or cumene hydroperoxide (25 mM), as the cofactor. For details of the product isolation assay used to quantify CE formation see Methods.

alumina to separate them from residual precursor and any non-catechol metabolites. CE eluted from the neutral alumina with 0.2 M HCl were separated from each other by thin-layer chromatography. Internal $^{14}$C-labeled tracers were used to correct for procedural losses and blank values obtained with heat denatured tissue were subtracted. Protein concentrations were measured according to Lowry *et al.* [23], using bovine serum albumin as standard. Samples were assayed in duplicate. The inter- and intra-assay coefficient of variation for the product isolation assay are less than 10 and 18%, respectively.

To determine the effect of the polyclonal anti-*P*-450$_p$ on NADPH- and OHP-dependent activities, the microsomal preparations were preincubated for 30 min at room temperature with either non-immune goat IgG (approx. 0.93 mg) or with the polyclonal antibody developed against highly purified cytochrome *P*-450$_p$ (approx 1.06 mg) [22]. The reaction was then started by adding substrate and co-factor. In assays for peroxidatic activity the 0.25 M sucrose in the incubation mixture was replaced with 20% glycerol which prevented decline in enzyme activity during the preincubation period.

### *Statistical analysis*

Treatment effects were evaluated using ANOVA, either two-way or multivariate ANOVA, depending on the number of treatments or factors being compared in a given experiment followed by Dunnett's test or by Student's *t*-test as indicated in Results and figure legends.

## RESULTS

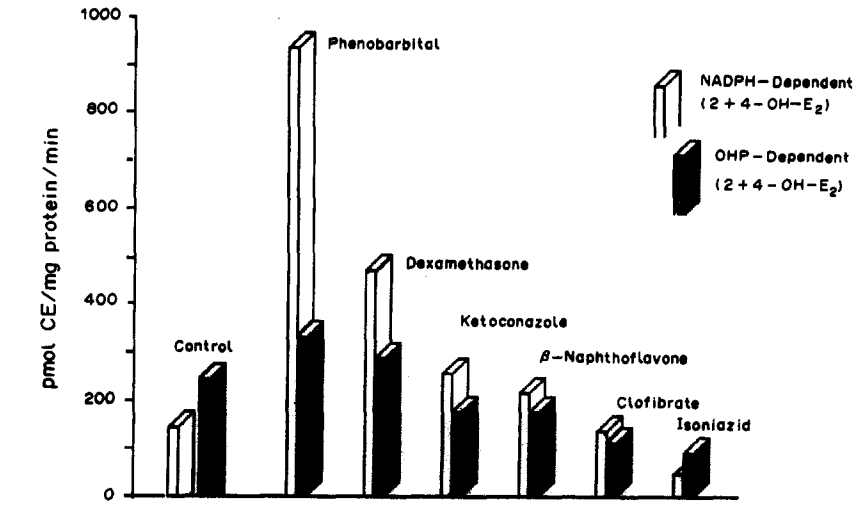
### *Peroxidatic, OHP-dependent CE formation*

In the presence of cumene hydroperoxide and at acidic pH both 2- and 4-OH-CE were formed in similar amounts (Fig. 1, Table 1). The pH optimum of this peroxidatic E-2/4-H$_{(OHP)}$ activity was 6.0 (Fig. 1). There was no significant sex difference in peroxidatic CE formation (Table 1). This is in contrast to the NADPH-dependent activity, which was significantly higher in males than females ($P = <0.0001$) (see below). Consequently, the ratio of NADPH:OHP-dependent activities was higher in males than females.

Table 1. Conversion of estradiol to 2- and 4-hydroxyestradiol (2- and 4-OH-$E_2$) by microsomes from male and female rats.

| | NADPH-dependent (pmol/mg protein/min) | | OHP-dependent (pmol/mg protein/min) | |
|---|---|---|---|---|
| | 2-OH-$E_2$ | 4-OH-$E_2$ | 2-OH-$E_2$ | 4-OH-$E_2$ |
| Male | 456 ± 28 | 7 | 125 ± 10 | 102 ± 15 |
| Female | 193 ± 35 | 7 | 102 ± 12.5 | 103 ± 14 |

Microsomes were incubated for 10 min under conditions optimized for either organic hydroperoxide- (OHP-) or NADPH-dependent catecholestrogen formation with [$^3$H]17$\beta$-estradiol (10 or 50 $\mu$M for NADPH- and OHP-dependent activities, respectively) as described in Methods. Results expressed as pmol/mg protein/min. $N = 5$ males, 5 females. Evaluation of the data by repeated measures ANOVA indicated the following significant effects; (1) sex $P = <0.003$ and (2) enzyme $P = <0.003$ and interaction between sex and enzyme $P = <0.002$. Significant difference between sexes by least squares means *t*-test was $P = <0.005$ for E-2-H$_{(NADPH)}$ and not significant for E-2/4-H$_{(OHD)}$ activity.




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| % CHANGE | NADPH | | +453* | +178* | +52 | +25 | -19* | -72* |
| | OHP | | +38* | +20 | -26 | -27 | -53* | -62* |
| SEM | NADPH | 19 | 30 | 23 | 8 | 23 | 7 | 6 |
| | OHP | 23 | 8 | 32 | 13 | 17 | 12 | 12 |

Fig. 2. Conversion of estradiol ($E_2$) to 2- and 4-hydroxyestradiol (2- and 4-OH-$E_2$) by NADPH- and OHP-dependent mechanism by microsomes from male rat liver—influence of inducers of cytochromes *P*-450. For method of administration of inducers, incubation of microsomes under conditions optimized for NADPH- or OHP-dependent activities and quantification of the amounts of 2- and 4-OH-$E_2$ see Methods. $E_2$ substrate concentration = 10 $\mu$M. Numbers below horizontal axis = percentage difference between values obtained for controls and treated animals in NADPH- and OHP-dependent activities for each of the pharmacological agent (% change) and SEM. *Significantly different from controls $P = <0.05$ determined by multivariate ANOVA followed by Dunnett's test.

*NADPH-dependent CE formation*

The optimum pH for NADPH-supported CE formation was 7.0–7.5 (Fig. 1). This is in contrast to the pH optimum of around 8.0 for NADPH-dependent activities in all extrahepatic tissues studied to date [7, 8, 24, 25]. E-2-$H_{(NADPH)}$ activity of males was higher ($P = <0.0001$) than that of females as reported previously by others (Table 1) [5, 6, 18]. At pH 7.4, 2-OH-CE formation predominated over 4-OH-CE formation under all experimental conditions tested and in both sexes. The amounts of 4-OH-CE generated were less than 3% of the amounts of 2-OH-CE formed except in incubations of microsomes from dexamethasone-treated rats in which it was 9–13% (Fig. 2).

*Effect of inducers of cytochrome P-450 on hepatic microsomal E-2-$H_{(NADPH)}$ and E-2/4-$H_{(OHP)}$ activities of females*

The two pathways of CE formation were affected differentially by 5 out of the 7 pharmacological agents used (Fig. 2). Phenobarbital and dexamethasone increased E-2-$H_{(NADPH)}$ activity by approx. 4- and 2-fold respectively (Fig. 3). E-2/4-H (OHP) activity was also increased in the same microsomal preparations. However, the magnitude of the increase was only about 1/10th the increase in NADPH-dependent activity (Fig. 3). Administration of

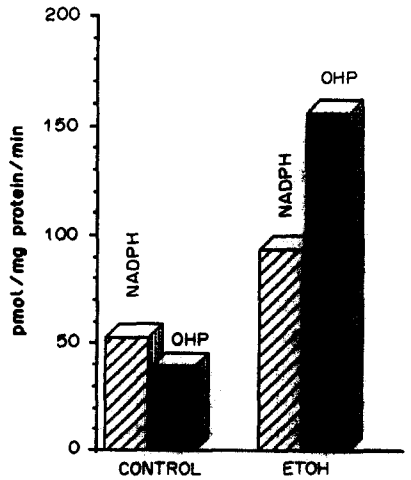



Fig. 3. Conversion of estradiol ($E_2$) to 2- and 4-hydroxyestradiol (2- and 4-OH-$E_2$) by NADPH- and OHP-dependent mechanism by microsomes from rat liver: influence of ethanol. The rats were fed the Lieber and DeCarli liquid diet [20] to which the ethanol was added with controls received isocaloric liquid diet. Microsomes prepared from livers of female rats were incubated with [$^3$H]17$\beta$-$E_2$ (10 $\mu$M) under conditions optimized for either organic hydroperoxide (OHP) or NADPH-dependent activities and the labeled 2- and 4-OH-$E_2$ produced isolated as described in Methods.

ketoconazol and β-napthoflavone was associated with a modest increase in NADPH-dependent activity (52 and 25%, respectively), but a small decrease in OHP-dependent activity (25 and 27%, respectively). In the case of clofibrate an approx. 50% decrease in OHP-dependent activity was associated with an approx. 20% decrease in NADPH-dependent activity.

Subjecting the data to multivariate ANOVA (MANOVA) for interaction between drugs and the two enzymatic activities, the dependent variables treated as repeated measures, revealed significant effects of: (1) enzyme (d.f. = 1 and 14, $F = 122.8$, $P = <0.0001$); (2) drug (d.f. = 6 and 14, $F = 104.4$, $P = <0.0001$); (3) interaction between drugs and the two enzymatic activities (d.f. = 6 and 14, $F = 62.9$, $P = <0.0001$). Significant differences at $P = <0.05$ between control and drug-treated groups were identified by Dunnett's test for NADPH-dependent activity for rats treated with phenobarbital, dexamethasone, clofibrate and isoniazid, and for OHP-dependent activity for rats treated with phenobarbital, clofibrate and isoniazid. In this analysis, the sum of 2- and 4-OH-$E_2$ generated by OHP-dependent activity was used following the demonstration by paired $t$-test that there was no statistically significant difference in the effect of the drugs on the amounts of these two products.

Maintaining the rats on the Lieber and DeCarli liquid diet was associated with low levels of both NADPH- and OHP-dependent activities (Fig. 3). Adding ethanol to this liquid diet resulted in a significant increase in both types of activities $P = <0.05$ by ANOVA (Fig. 3). However, isoniazid, an agent which induces the same form of cytochromes $P$-450 as ethanol [26], caused a significant decrease in both NADPH- and OHP-dependent activities (see above and Fig. 2).

*Effect of polyclonal anti-P-450$_p$ IgG on hepatic microsomal E-2-H$_{(NADPH)}$-dependent activity*

Preincubation of microsomes with IgG did not affect NADPH-dependent enzyme activity. Preincubation with the antibody developed against highly purified $P$-450$_p$ [22] caused a similar absolute decrease in E-2-H$_{(NADPH)}$ activity of hepatic microsomes of both sexes (222 vs 207 pmol/mg protein/min in males and females, respectively) (Fig. 4A). This represented an approx. 30% decrease from control levels in males and a 60% decrease in females.

The effect of the antibody against $P$-450$_p$ on CE formation was tested using hepatic microsomes from dexamethasone- and phenobarbital-treated female rats since this form of $P$-450, which is induced by both these agents, has been implicated in CE formation [5]. The antibody reduced E-2-H$_{(NADPH)}$ activity of hepatic microsomes from dexamethasone-treated rats by approx. 50% (350 pmol/mg protein/min) and by approx. 25% (190 pmol/mg protein/min) of those treated with phenobarbital (Fig. 4B). Residual microsomal E-2-H$_{(NADPH)}$ activity of dexamethasone-treated rats, after incubation with the antibody, was twice that of controls preincubated with the same antibody. The ratio of 2- to 4-OH-CE was not affected by either non-immune IgG or by the immune-IgG.

*Effect of polyclonal anti-P-450$_p$ IgG on microsomal E-2/4-H$_{(OHP)}$ activity*

During a 20 min preincubation at room temperature, in buffer containing 20% glycerol, there was a modest (15–20%) decline in peroxidatic activity (Fig. 5). there was an additional small decline in the presence of non-immune IgG. A further slight decline in enzyme activity occurred when the preincubations were carried out in the presence of anti $P$-450$_p$. Thus, no convincing, specific inhibition of peroxidatic activity was demonstrable.

## DISCUSSION

Hepatic microsomes, like those from human term placentae and rat anterior pituitary [7, 8] when incubated at acidic pH and in the presence of cumene hydroperoxide generated 2- and 4-OH-CE in similar amounts. This indicates the presence in rat liver of a form(s) of cytochrome $P$-450 which can catalyze CE formation by a peroxidatic mechanism.

The finding of a difference between males and females in ratio of the NADPH- and OHP-dependent activities, as well as of the differential effect on CE formation by the two mechanisms, of inducers of cytochromes $P$-450 and of the polyclonal antibody to $P$-450$_p$, the glucocorticoid-induced form of $P$-450, supports the conclusion that the two activities are mediated by different forms of cytochrome $P$-450. The similar level of peroxidatic activity in the two sexes implies that this reaction is not catalyzed by sex specific forms of $P$-450.

There was no evidence for the presence in rat liver of the form(s) of $P$-450 which hydroxylate

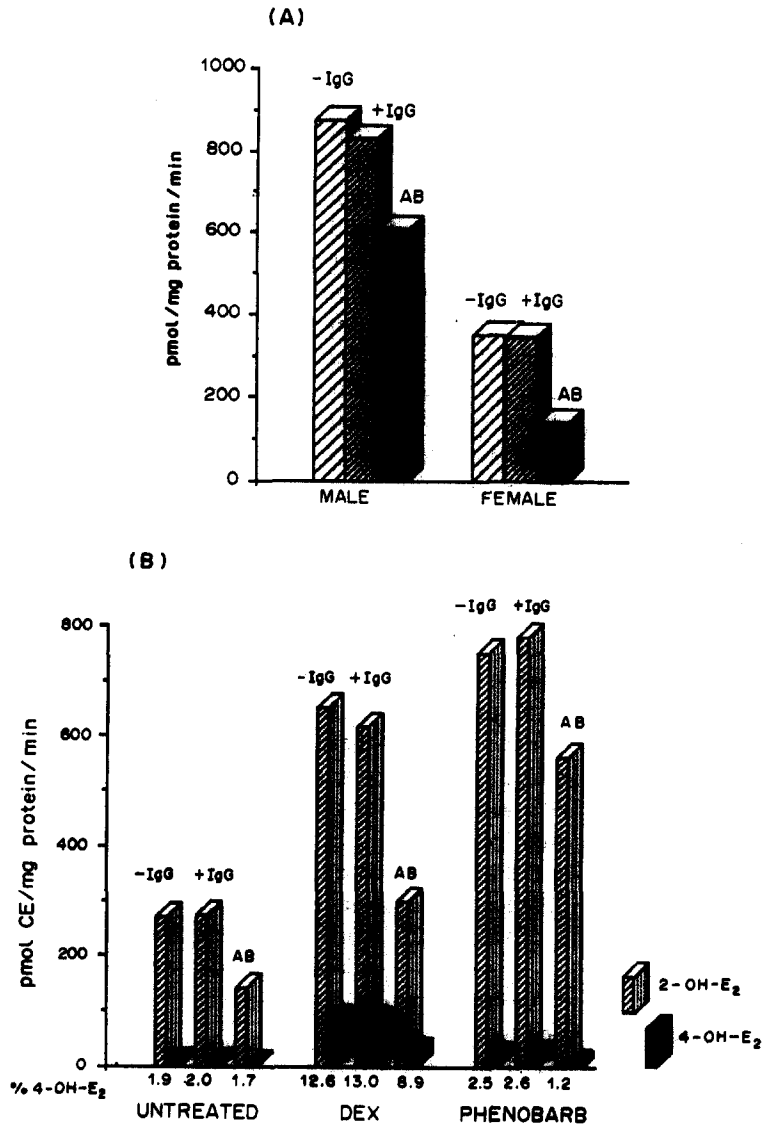



Fig. 4. Conversion of estradiol ($E_2$) to 2- and 4-hydroxyestradiol (2- plus 4-OH-$E_2$) by NADPH-dependent mechanism by microsomes from female rat liver: Influence of a polyclonal antibodies (AB) against cytochrome $P$-450$_p$, the glucocorticoid inducible form of $P$-450. (A) Microsomes from male and female rats; (B) microsomes from female rats treated with either phenobarbital or dexamethasone and from untreated controls. The microsomes were preincubated for 10 min with immunoglobulin (AB) in the absence of substrate or co-factors. Controls were incubated without or with non-immune immunoglobulin (−IgG or +IgG). For preparation of microsomes and details of the assays for measurement of CE formation see Methods. Numbers below horizontal axis in (B) = amount of 4-OH-$E_2$ expressed as percentage of the amount of 2-OH-$E_2$ produced.

estrogens preferentially at C-4 [8]. The amounts of 4-OH-$E_2$ generated by hepatic microsomes under conditions optimized for NADPH-dependent activity were consistently less than 3% of the amounts of 2-OH-$E_2$ generated in the same incubations, irrespective of the sex of the animals or their pretreatment with pharmacological agents. Only dexamethasone caused a relatively greater increase in 4- than 2-OH-$E_2$ formation. However, even in these animals 4-OH-$E_2$ formation was still only about 12% of the amount of 2-OH-$E_2$ generated. Thus, in liver the small amounts of 4-OH-CE produced in the presence of NADPH are likely to represent minor by-products of the cytochromes $P$-450 which catalyze NADPH-dependent 2-hydroxylation. This interpretation is supported by the finding that the polyclonal antibody against $P$-450$_p$ inhibited 2- and 4-hydroxylation to the same extent. This is in contrast to the hamster kidney in which NADPH-dependent 2- and 4-hydroxylation appear to be mediated by different forms of cytochromes $P$-450 [27]. Microsomes from this tissue incubated under

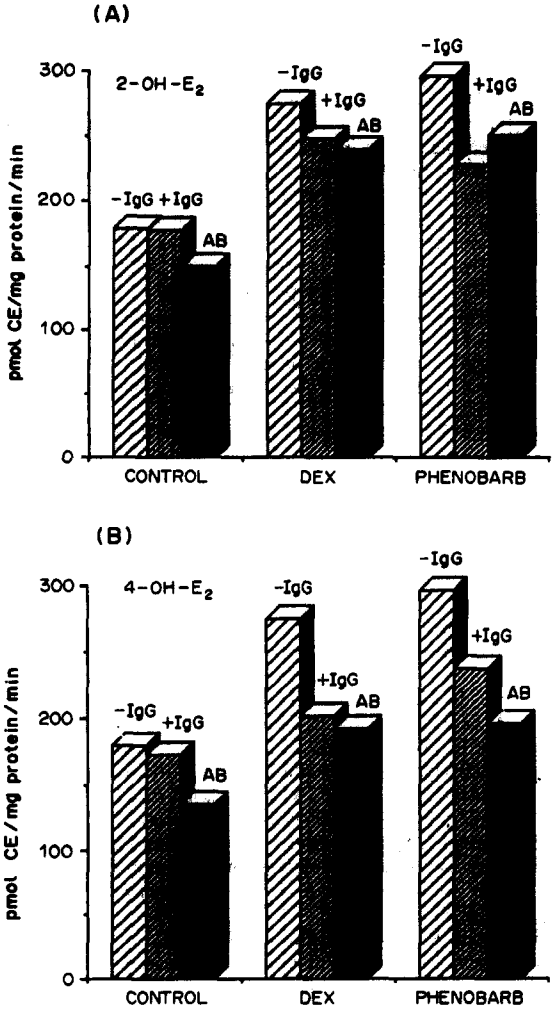



Fig. 5. Conversion of estradiol ($E_2$) to 2- and 4-hydroxyestradiol (2- and 4-OH-$E_2$) by microsomes from female rat liver by peroxidatic, organic hydroperoxide-dependent mechanism: influence of a polyclonal antibody against cytochrome $P$-450$_p$, the glucocorticoid inducible form of $P$-450. The microsomes were preincubated for 20 min with immunoglobulin (AB) in the absence of substrate or cumene hydroperoxide co-factor. Controls were incubated without or with non-immune globulin (−IgG or +IgG). The incubation medium for OHP-dependent activity included 20% glycerol which prevented the decline in peroxidatic activity during the preincubation period. For preparation of microsomes and details of the assay for measuring CE formation, see Methods.

conditions optimized for NADPH-dependent activity generated 2- and 4-OH-CE in similar amounts and the antibody against rat hepatic $P$-450p inhibited only 2-hydroxylation (Weisz, Bui and Wrighton, unpublished).

Our findings on relative amounts of 2- and 4-hydroxylation differ quantitatively from those in the one study in which 4- as well as 2-hydroxylation was examined systematically [5]. In that study, Dannan *et al.*, reported a ratio of 2- to 4-hydroxylation of around 10:1, with the exception of microsomes from dexamethasone-treated female rats in which the ratio was 1:1. The discrepancy is likely to be due to use in the previous study of the radiometric, tritiated water release assay which may be associated with spurious release of tritium, in particular, from C-4 [15].

The induction of E-2-$H_{(NADPH)}$ by dexamethasone and phenobarbital and its inhibition by a polyclonal antibody against $P$-450$_p$ [22] is in agreement with findings by others [5]. Together, these data indicate an important role in NADPH-dependent 2-hydroxylation for the $P$-450 gene family III, the forms of $P$-450 inducible by glucocorticoids and their analogues, and their closely related constitutive form(s) [28]. The study also provides further evidence that several forms of inducible cytochrome $P$-450, besides those in gene family III, may hydroxylate estrogens at C-2. This is suggested by the finding of a larger increase in microsomal E-2-$H_{(NADPH)}$ activity of phenobarbital than dexamethasone-treated animals, a failure of anti-$P$-450$_p$ antibody to inhibit fully the increase in E-2-$H_{(NADPH)}$ activity caused by phenobarbital and dexamethasone as well as by the increase in E-2-$H_{(NADPH)}$ activity caused by ethanol.

The finding of an increase in E-2-$H_{(NADPH)}$ activity caused by ethanol is in contrast to the findings Hoffman *et al.* [29]. These investigators reported a decrease in hepatic microsomal E-2-$H_{(NADPH)}$ activity of males and no change in that of females administered ethanol. However, the study did not control for the change in diet of rats fed ethanol which, as shown, in this study, may itself cause a marked decline in enzyme activity. The form of cytochrome $P$-450 responsible for the increased E-2-$H_{(NADPH)}$ activity in the ethanol-treated rats does not appear to be $P$-450j, since administration of isoniazid resulted in a significant decline rather than an increase in CE formation.

There is reason to suppose that under normal conditions in liver NADPH-dependent activity and, therefore, 2-hydroxylation predominates. Consequently, since the liver is the major source of CE in the body as a whole, 2-hydroxylation is found to predominate when the disposition of estrogens is examined *in vivo* in the whole organism. This was demonstrated in the one study in which the conversion of primary estrogens in *in vivo* to both 2- and 4-OH-CE was measured [30].

Whether and under what physiological or pathological circumstances hepatic E-2/4-$H_{(OHP)}$

activity is expressed remains to be determined. By analogy with NADPH-dependent activity [31], the availability of co-factor is likely to be rate limiting in the expression of peroxidatic activity. One proposed source of co-factors for peroxidatic E-2/4-$H_{(OHP)}$ activity is the OHP(s) generated in the course of prostaglandin synthesis. In addition, OHP(s) generated when cell damage results in lipid peroxidation could provide the oxidizing co-substrates needed for expression of peroxidatic activity. This, in turn, could amplify the damage to liver since CE are substrates for redox cycling [32] and active radicals produced by redox cycling can cause cell damage. Since peroxidatic CE synthase activity generates 2- and 4-OH-CE in similar amounts and 2-OH-CE protect 4-OH-CE from inactivation by COMT [14] when peroxidatic activity is expressed 4-OH-CE could remain available as substrates for redox cycling. In light of the different functional implications of the two pathways of CE formation, it will be of interest to determine whether pathological conditions associated with lipid peroxidation and, thereby, the generation of OHPs can trigger or increase E-2/4-$H_{(OHP)}$ activity in liver.

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