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Oxidation of 17α-Ethynylestradiol by Human Liver Cytochrome P-450

F. PETER GUENGERICH

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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SUMMARY

One of the classic examples of adverse drug interactions involves pregnancies in women using the oral contraceptive 17α-ethynylestradiol who also ingest rifampicin or barbiturates or hydantoins. Previous work had demonstrated increased metabolism (2-hydroxylation) of 17α-ethynylestradiol in individuals using rifampicin. In this report evidence is presented for the involvement of a specific form of human cytochrome P-450, termed P-450$_{NF}$, in this phenomenon. Although purified P-450$_{NF}$ has only relatively low catalytic 17α-ethynylestradiol 2-hydroxylase activity, antibodies raised to P-450$_{NF}$ specifically inhibited >90% of the activity in liver microsomes which had either high or low catalytic activity. When different liver samples were compared, rates of microsomal 17α-ethynylestradiol 2-hydroxylation were highly correlated with amounts of immunochemically measured P-450$_{NF}$ or rates of nifedipine oxidation, a characteristic activity of P-450$_{NF}$. Prior incubation of human liver microsomes with NADPH and troleandomycin resulted in decreased 17α-ethynylestradiol 2-hydroxylation. In addition, 17α-ethynylestradiol appears to be a mechanism-based inhibitor in human liver microsomes, as shown by the loss of both spectrally detectable cytochrome P-450 and 17α-ethynylestradiol 2-hydroxylase activity during incubation in the presence of NADPH. Additional experiments did not show any evidence for the involvement of a number of other human cytochrome P-450 enzymes in 17α-ethynylestradiol 2-hydroxylation (i.e., P-450$_{DB}$, P-450$_{PA}$, P-450$_{MP}$, P-450j). These results are consistent with the view that P-450$_{NF}$ is the major enzyme involved in 17α-ethynylestradiol oxidation and that drugs and hormones which influence the expression and activity of this enzyme can influence the efficacy and side effects of this compound.

17α-Ethynylestradiol is a major constituent of oral contraceptive formulations. Obviously the efficacy of the drug is dependent upon its rate of elimination, which is known to vary among individuals (1, 2). A significant fraction of the material found in the plasma exists as the sulfate, but this appears to be a storage form. Some glucuronidation occurs, although not to the extent with natural estrogens. Several pathways of oxidation are known in man, including hydroxylation at the 4, 6α, and 6β sites, formation of the homoannulated product, and loss of the acetylenic moiety (2, 3). The major pathway of metabolism in both rats and man involves initial 2-hydroxylation (1, 2). One of the classic examples of adverse drug interactions in pharmacology is the reported therapeutic ineffectiveness of 17α-ethynylestradiol as a contraceptive in women who are administered rifampicin (4, 5) or diphenylhydantoin (2, 6). In many cases women experienced menstrual bleeding or pregnancy (5, 7). Bolt *et al.* (8) found that rates of *in vitro* 2-hydroxylation of 17β-estradiol and 17α-ethynylestradiol were increased 4-fold in women administered a single 600-mg dose of rifampicin and attributed the rifampicin-induced pregnancies to increased metabolism of 17α-ethynylestradiol. Even though the 2-hydroxy product of 17α-ethynylestradiol may retain some hormonal effectiveness relative to the parent drug, the elimination appears to be more rapid, and, thus, hydroxylation results in an overall decrease in drug efficacy. In addition to considerations about the decreases in therapeutic effectiveness related to 2-hydroxylation of 17α-ethynylestradiol, concern is also given to the binding of the catechol and the *o*-quinone resulting from 2-hydroxylation to protein, as antibodies to such conjugates can be detected in women administered the compound and presenting with vascular disease (9, 10). Such catechols and quinones are also under consideration as candidates for involvement in estrogen-induced tumorigenesis. The mechanism-based inactivation of P-450 during oxidative metabolism of 17α-ethynylestradiol has also been demonstrated in rats and postulated to involve the acetylenic moiety (11).

The mechanism of increased 2-hydroxylation of 17α-ethynylestradiol has been postulated to involve induction of P-450 (8). In rat liver several forms of P-450 catalyze the 2-hydroxylation of 17β-estradiol (12); in humans, P-450$_{NF}$ appears to be the major catalyst of this reaction (13). P-450 enzyme specific-

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ABBREVIATIONS: P-450, liver microsomal cytochrome P-450; HPLC, high performance liquid chromatography; EDTA, ethylenediaminetetraacetate; IgG, immunoglobulin G fraction (of sera).

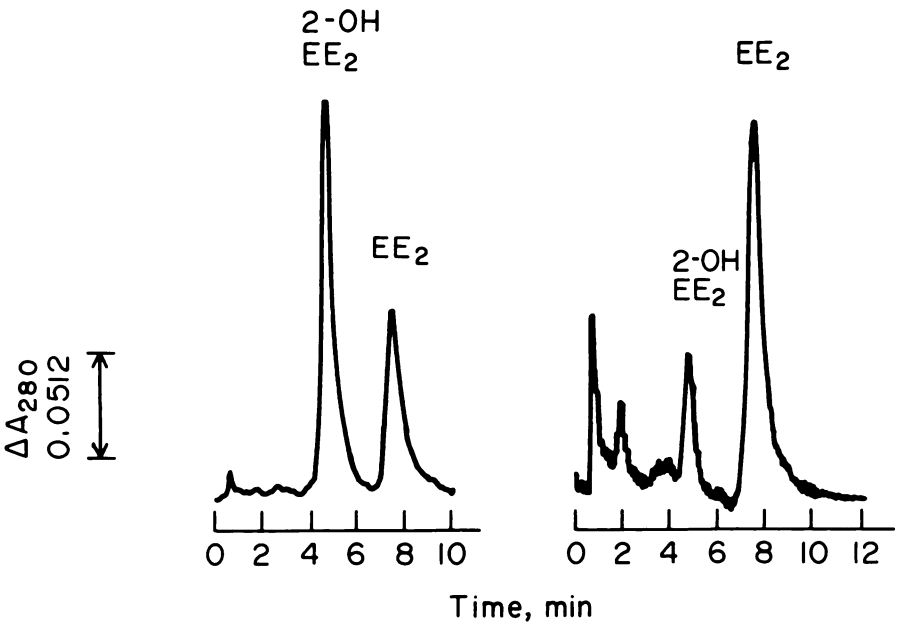

Fig. 1. Separation of oxidation products of 17α-ethynylestradiol. The *left trace* shows the separation of a mixture of authentic 2-hydroxy-17α-ethynylestradiol (*2-OH EE$_2$*) and 17α-ethynylestradiol (*EE$_2$*) on a Du Pont HPLC column under the conditions described in Materials and Methods. The *right trace* is the chromatographic trace obtained when (32% of) an extract of an incubation mixture (4 mg of microsomal protein from sample HL 105, 10 min at 37°, NADPH and ascorbate present) was injected onto the column. See also Fig. 2.

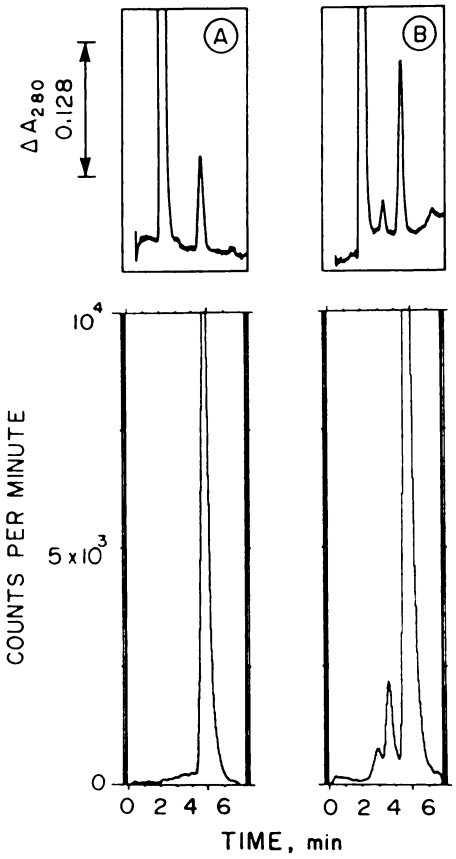

Fig. 2. Microsomal oxidation of 17α-ethynylestradiol. HL 105 microsomes (100 pmol of P-450) were used in these experiments; the general methodology is outlined under Materials and Methods. A. Complete system minus NADPH-generating system. B. Complete system. The *upper* chromatograms show the $A_{280\,nm}$ profiles and the *lower portion* consists of the actual radiochromatographic traces (not redrawn) accompanying the upper traces. A Machery-Nagel column was used in this work under conditions that differ from those present in Fig. 1 (see Materials and Methods).

ities for oxidation of steroids may be markedly altered by changes in the molecules, and although numerous *in vitro* and *in vivo* experiments have been done (8, 14, 15), the human P-450 enzyme involved in 17α-ethynylestradiol 2-hydroxylation has not been identified. Back *et al.* (16) presented evidence that the enzyme is not the same as any of those involved in debrisoquine 4-hydroxylation/sparteine oxidation or antipyrine oxidation.

With some information in hand concerning the catalytic activity of human liver P-450 enzymes toward natural steroids (13), an investigation was initiated to identify the human P-450 responsible for 2-hydroxylation of 17α-ethynylestradiol. Evidence is presented here that P-450$_{NF}$, which has been previously characterized (13, 17), is the major enzyme involved in this reaction.

Materials and Methods

Chemicals. 17α-Ethynylestradiol was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification; solutions were prepared in CH_3OH, and HPLC in the system used for analysis indicated no impurities (as judged by materials absorbing at 280 nm). 17α-Ethynyl-[6,7-^{3}H]estradiol was purchased from New England Nuclear (Boston, MA). The radiochemical purity, as judged by HPLC, was >99% at the time of use. 2-Hydroxy-17α-ethynylestradiol was a gift of Dr. W. Slikker of the National Center for Toxicological Research, Jefferson, AR. Troleandomycin was provided by Dr. D. J. Waxman, Harvard University, Cambridge, MA. The pyridine oxidation product of nifedipine was prepared using a general procedure described in detail elsewhere (18).

Enzymes and antibodies. P-450$_{MP}$ was purified as described (19). P-450$_{NF}$ was purified by a modification of procedures described elsewhere (13). Human liver microsomes (sample HL 105, 2000 mg protein)[1] were solubilized with sodium cholate and applied to an *n*-octylamino Sepharose 4B column (2.5 × 30 cm) as described (13) at a flow rate of 2 ml min^{-1}. The column was washed with 600 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 20%

[1] Human liver samples are denoted "HL" with a code number for each individual. Apparently equivalent forms of some of the P-450 enzymes have now been isolated in other laboratories; for a current list of comparisons see Ref. 20. The P-450$_{NF}$ protein is tentatively classified as a product of the P-450 III A3 gene group (21).

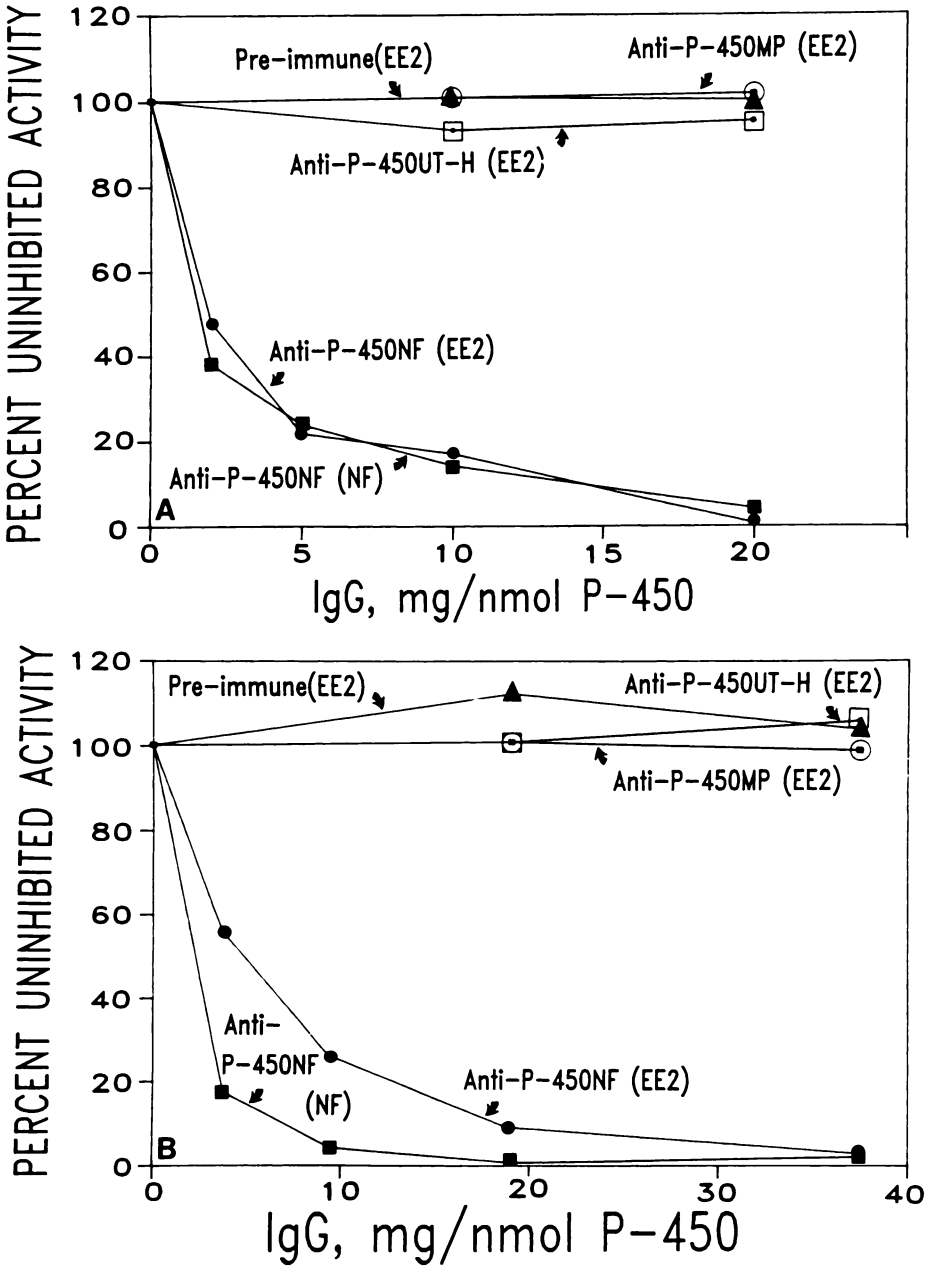

Fig. 3. Inhibition of nifedipine oxidation and 17α-ethynylestradiol 2-hydroxylation in human liver microsomes by anti-P-450$_{NF}$. A. HL 105 microsomes. The uninhibited catalytic activities were 4.55 nmol of nifedipine oxidized min^{-1} (mg protein)$^{-1}$ and 3.19 nmol of 2-hydroxy-17α-ethynylestradiol formed min^{-1} (mg protein)$^{-1}$. B. HL 108 microsomes. The uninhibited catalytic activities were 0.32 nmol of nifedipine oxidized min^{-1} (mg protein)$^{-1}$ and 0.14 nmol of 2-hydroxy-17α-ethynylestradiol formed min^{-1} (mg protein)$^{-1}$. In both parts, 17α-ethynylestradiol 2-hydroxylation was measured in the presence of anti-P-450$_{NF}$ IgG (●), preimmune IgG (○), anti-P-450$_{MP}$ IgG (▲), or anti-P-450$_{UT-H}$ IgG (□). Nifedipine oxidation was measured in the presence of anti-P-450$_{NF}$ (■).

glycerol (v/v), 1 mM dithiothreitol, and 0.5% sodium cholate (w/v). P-450 was eluted with 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.5% sodium cholate, and 0.06% Emulgen 911 (w/v). The red P-450 peak was passed through a 2.5 × 15 cm column of Whatman DE-52 DEAE-cellulose (Whatman Separation Products, Clifton, NJ) connected in-line with the column. The red effluent (void volume) was applied immediately (at a rate of 2 ml min^{-1}) to a 2.5 × 15 cm Hypatite C hydroxylapatite column (Clark Chemical Co., Williamsburg, PA), which was washed with 500 ml of 40 mM potassium phosphate buffer (pH 7.5) containing 0.2% Emulgen 913, 20% glycerol, and 1 mM dithiothreitol. The column was then eluted with 500 ml each of the same buffer containing 70 and 100 mM phosphate. The eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the apparently homogeneous fractions (eluted in the 100 mM phosphate fraction) were pooled. The pooled fraction was dialyzed for 4 hr versus 20 volumes of 20% glycerol and applied to a 2.5 × 5 cm column of Hypatite C hydroxylapatite, which was washed with 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol. When the $A_{280\,nm}$ of the effluent was <0.01, P-450$_{NF}$ was eluted with 0.6 M potassium phosphate (pH 7.5) buffer containing 20% glycerol and 1 mM dithiothreitol, and the pooled fractions were dialyzed for 4 hr versus 20 volumes of a solution composed of 20% glycerol, 1 mM EDTA, and 1 mM dithiothreitol before use in reconstitution assays.

Rabbit anti-P-450$_{NF}$ (13) and anti-P-450$_{MP}$ (19) were prepared against the corresponding antigens and used in immunoblotting and immunoinhibition experiments. Anti-P-450$_{UT-H}$ (22) was raised in rabbits against the rat antigen (orthologous to human P-450$_{DB}$) and used in immunoinhibition studies. Rabbit anti-P-450$_{ISF-G}$, prepared against rat P-450$_{ISF-G}$, recognizes human P-450$_{PA}$ (20) but does not inhibit catalytic activity. Rabbit anti-rat P-450ac, which recognizes human P-450j, was prepared by Drs. J.-S. H. Yoo and C. S. Yang (New Jersey School of Medicine and Dentistry, Newark, NJ) and used in immunoblotting experiments (23).

Assays. Protein was assayed using the Pierce bicinchoninic (BCA) reagent (Pierce Chemical Co., Rockford, IL). Spectral determinations of P-450 were performed according to the method of Omura and Sato (24). Nifedipine oxidation was monitored by HPLC as described else-

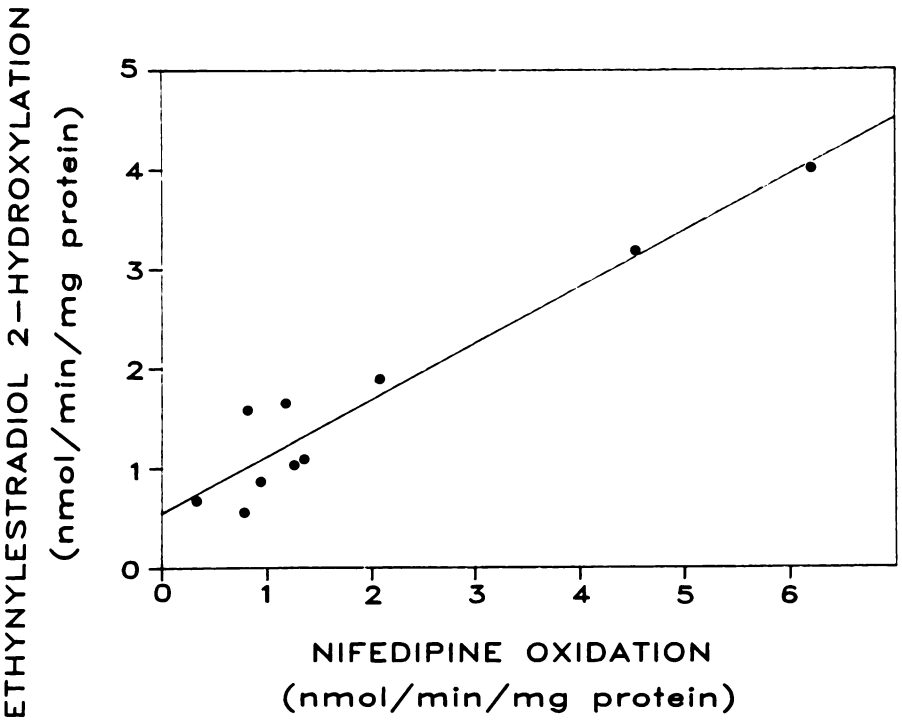

Fig. 4. Correlation of rates of 17α-ethynylestradiol 2-hydroxylation and nifedipine oxidation in various human liver microsomes. The line was drawn using linear regression analysis ($r = 0.95$).

where (13, 18). Immunoblotting experiments were carried out as described previously (25); the dilution of rabbit anti-P-450$_{NF}$ sera was 1/100 in these particular experiments.

17α-Ethynylestradiol 2-hydroxylation was assayed as follows. In typical nonradioactive experiments, human liver microsomes containing 15 nmol of total P-450 were incubated with 0.1 M potassium phosphate buffer (pH 7.4), 1.0 mM L-ascorbic acid (freshly prepared), 50 μM 17α-ethynylestradiol, and an NADPH-generating system consisting of final concentrations of 10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 2 IU glucose-6-phosphate dehydrogenase ml^{-1} in a total volume of 5.0 ml. After incubation (with the NADPH-generating system as the initiating component) for 10 min at 37°, reactions were quenched by the addition of 10 ml of CH_2Cl_2 which had been purged with N_2. The layers were mixed using a vortex device and separated by centrifugation at 2000 × g for 10 min. An aliquot of the organic layer (8.5 ml) was removed and the solvent was evaporated under a stream of N_2 at 23°. The residue, in a conical Reactival (Pierce Chemical Co.), was dissolved in 50 μl of CH_3OH and 20 μl were injected onto an HPLC column [octadecylsilyl (C18), 6.2 × 80 mm, Zorbax 3 μ Silver series, DuPont Instruments, Wilmington, DE). The mobile phase consisted of 0.03% $(NH_4)_3PO_4$ (w/v) in a mixture of 64% CH_3OH/36% H_2O (v/v) (pH 3.0) and the flow rate was 2.0 ml min^{-1} (26). Under these conditions 2-hydroxy-17α-ethynylestradiol eluted at 3.6 min and 17α-ethynylestradiol eluted at 4.7 min (see Figs. 1 and 2, under results). The wavelength of the detector monitoring the effluent was set at 280 nm; external standards were used and amounts of product were estimated by peak height or integration.

In some of the experiments, particularly those involving immunochemical inhibition and reconstitution studies, the size of the reaction was reduced to 0.25 ml, and 100 pmol of P-450 were included. 17α-Ethynyl-[6,7-^{3}H]estradiol was added to a specific radioactivity of 80 μCi μmol^{-1}. After incubation as above, the reaction was quenched with 1.0 ml of CH_2Cl_2. After mixing and centrifugation, 0.75 ml of the organic layer was transferred and taken to dryness under N_2 at 23°. HPLC utilized a 4.6 × 250 nm octadecylsilyl (C18) column (Machery-Nagel, Düren, Federal Republic of Germany) and a mobile (isocratic) phase consisting of 0.03% $(NH_4)_3PO_4$ (w/v) in a mixture of 73% CH_3OH and 27% H_2O (v/v) (pH 3.0); the flow rate was 1.5 ml min^{-1}. The effluent of the HPLC column was passed into a Radiomatic Flo-One scintillation counter and mixed with Flo-Scint III scintillation cocktail, pumped at a flow rate of 6.0 ml min^{-1} (Radiomatic Instruments, Tampa, FL). Typically the counting efficiency was 35% and more than 90% of the radioactivity originally in the substrate was recovered from the column.

Bufuralol 1′-hydroxylation (27), *S*-mephenytoin 4-hydroxylation (19), and nifedipine oxidation (13) were measured as described elsewhere. *N,N*-Dimethylnitrosamine *N*-demethylation was measured by Drs. J.-S. H. Yoo and C. S. Yang using a substrate concentration of 0.1 mM (23). 4-Aminobiphenyl *N*-hydroxylation was measured by M. A. Butler and Dr. F. F. Kadlubar (National Center for Toxicological Research, Jefferson, AR) (28).

Results

2-Hydroxylation of 17α-ethynylestradiol in microsomal and purified preparations. In confirmation of other reports (1, 15, 29), it was found here that the microsomal hydroxylation of 17α-ethynylestradiol occurs primarily at the 2-position (Figs. 1 and 2). Little metabolism occurs when NADPH is omitted. Ascorbic acid was added in these incubations to prevent oxidation of the catechol (15).

Previous studies with humans indicated that anti-P-450$_{NF}$ could inhibit both the 2- and 4-hydroxylation of 17β-estradiol in human microsomes, suggesting that P-450$_{NF}$ may be the major 17β-estradiol 2- and 4-hydroxylase in human liver (13). These observations raised the possibility that P-450$_{NF}$ might be involved in the 2-hydroxylation of 17α-ethynylestradiol.

P-450$_{NF}$ was purified from liver sample HL 105 and reconstituted with NADPH-P-450 reductase and L-α-dilauroylglycero-*sn*-3-phosphorocholine. In the presence of a 3-fold molar excess of human liver cytochrome b_5 (with respect to P-450), the rate of 17α-ethynylestradiol 2-hydroxylation was 0.15 nmol min^{-1} (nmol P-450)$^{-1}$ (without cytochrome b_5, the rate was about one-third of this). Although this rate is not as high as that observed in the microsomal preparation [1.1 nmol of product formed min^{-1} (nmol P-450)$^{-1}$ in this comparison], it should be pointed out that purified preparations of P-450$_{NF}$ and its rat ortholog (P-450$_{PCN-E}$) are notorious for their low activi-

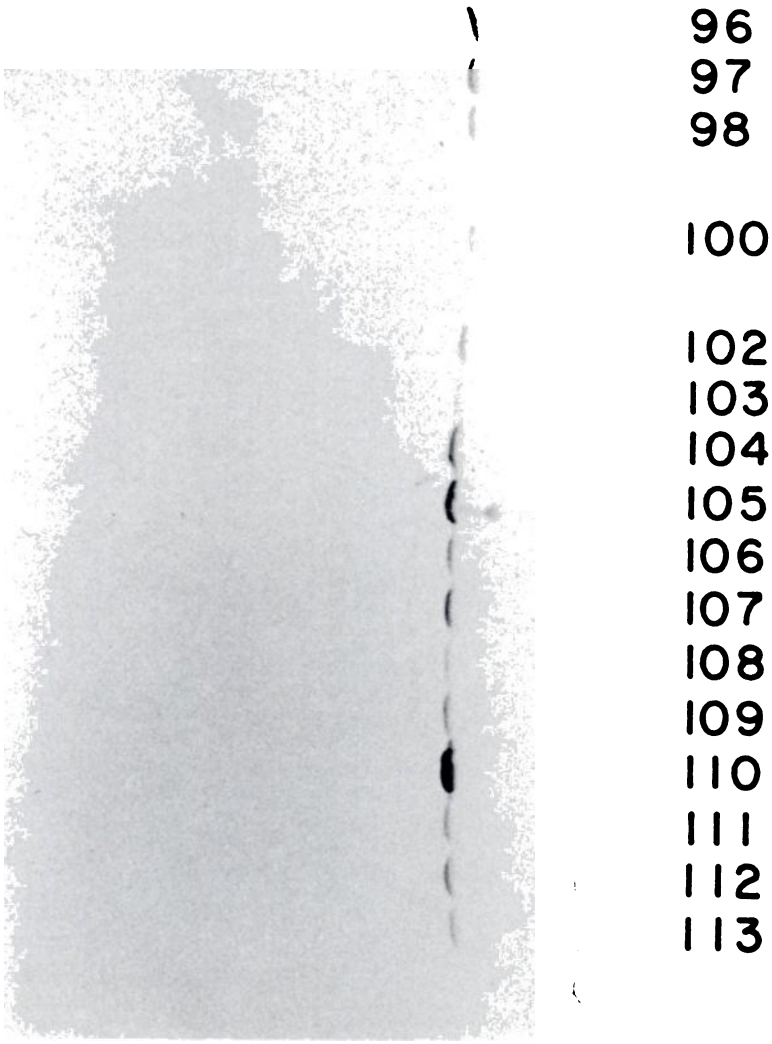

Fig. 5. Immunoblotting of a series of human liver microsomes with anti-P-450$_{NF}$. Each lane included 10 μg of microsomal protein. Liver sample numbers are indicated on the gel.

ties, and the reasons are still unclear (13, 17, 19, 30). Neither P-450$_{MP-1}$ (19) nor a related protein termed P-450$_{MP-3}$ (31)[2] had detectable catalytic activity when reconstituted under the same conditions.

Preliminary studies indicated that anti-P-450$_{NF}$ was very effective in inhibiting 17α-ethynylestradiol 2-hydroxylation. More detailed studies indicated that the inhibition titer was similar for blocking both 17α-ethynylestradiol 2-hydroxylation and nifedipine oxidation, a classic reaction catalyzed by P-450$_{NF}$ (Fig. 3). In both a sample which rapidly hydroxylated 17α-ethynylestradiol (Fig. 3A) and one which was 23-fold lower in activity (Fig. 3B), the extent of inhibition was >90%. Preimmune IgG had no effect on the reaction, nor did anti-P-450$_{MP}$ or anti-P-450$_{UT-H}$, which are known to inhibit reactions catalyzed by P-450$_{MP}$ (19) and P-450$_{DB}$ (22), respectively.

Correlation of 17α-ethynylestradiol 2-hydroxylation with other catalytic activities and P-450 concentrations. Although anti-P-450$_{NF}$ extensively inhibited both nifedipine oxidation and 17α-ethynylestradiol 2-hydroxylation in human liver microsomes (Fig. 3), the possibility must be considered that two (or more) immunochemically related but independently regulated enzymes might be involved in the two reactions. A series of different human liver samples, which had not been selected on the basis of their catalytic activities, was assayed for both activities. The correlation coefficient (r) of 0.95 (Fig. 4) is highly significant. Correlation coefficients determined in such a manner are heavily influenced by points at the ends of a comparison. However, even when the two highest values were dropped, the correlation was still high (r = 0.77).

Immunoblotting experiments with the samples under consideration yielded a single band in all lanes when staining was done with anti-P-450$_{NF}$ (Fig. 5). When the intensity of the individual bands was estimated, a high correlation was found with 17α-ethynylestradiol 2-hydroxylation (Fig. 6) (r = 0.98). A high correlation of blotting with rates of nifedipine oxidation (r = 0.99) was also found, in confirmation of previous work (13).

Other comparisons were made and the correlation coefficients are given in Table 1. Although there was some correlation of 17α-ethynylestradiol 2-hydroxylation with total P-450, this

[2] P-450$_{MP-3}$ (31) appears to be similar or identical to the preparations designated "P-450B" (32) and "HLx" (33) as judged by amino acid sequencing and cDNA sequencing studies (51). The protein does not appear to have S-mephenytoin 4-hydroxylase activity.

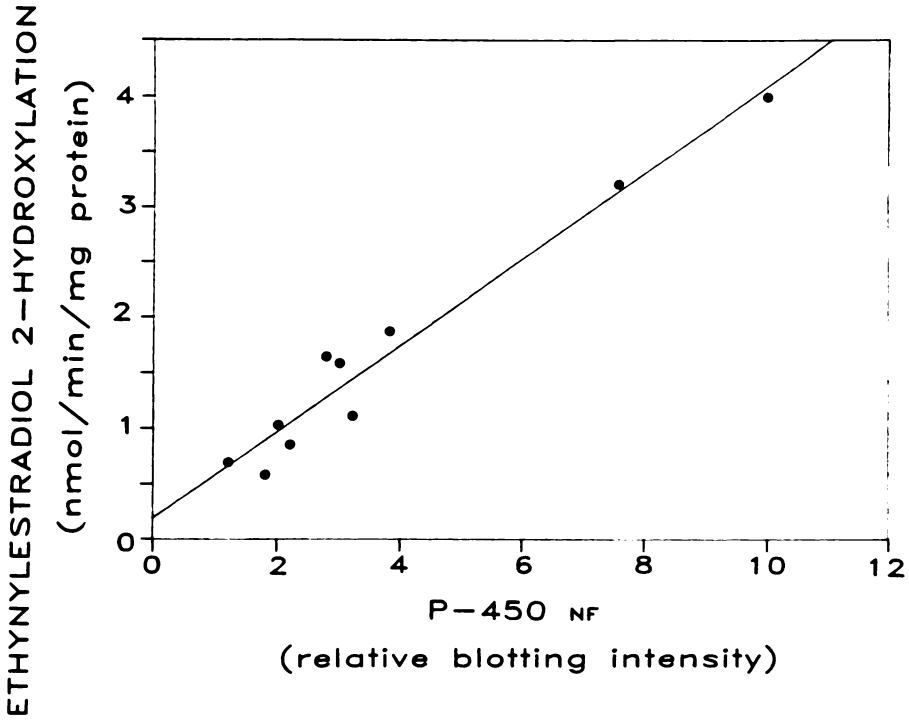

Fig. 6. Correlation of P-450$_{NF}$ immunoblotting intensity with 17α-ethynylestradiol 2-hydroxylation rates in a series of human liver microsomes. The gel used in Fig. 4 was scanned using a densitometer and the areas of the resulting peaks were correlated with 17α-ethynylestradiol 2-hydroxylation rates. The line was drawn using linear regression analysis (r = 0.98).

TABLE 1

Correlation of 17α-ethynylestradiol 2-hydroxylation with other catalytic activities and with immunochemically determined concentrations of P-450 enzymes in human liver microsomes

Catalytic activity	r	P-450	r
		Total P-450	0.76[a]
Nifedipine oxidation	0.95	P-450$_{NF}$	0.98
S-Mephenytoin 4-hydroxylation	−0.05	P-450$_{MP}$	0.23
4-Aminobiphenyl *N*-hydroxylation	−0.91	P-450$_{PA}$	−0.57
N,N-Dimethylnitrosamine *N*-demethylation	−0.18	P-450j	0.42
(±)Bufuralol 1′-hydroxylation	−0.46		

[a] r = 0.76 for n = 11. When the highest value was deleted (n = 10), r = 0.29.

analysis is heavily influenced by a single high value and the correlation coefficient drops sharply (to r = 0.23) when this point is deleted. Mephenytoin 4-hydroxylation is catalyzed by P-450$_{MP}$ (19), P-450$_{DB}$ catalyzes bufuralol 1′-hydroxylation (27), P-450$_{PA}$ catalyzes 4-aminobiphenyl *N*-hydroxylation (28), and P-450j catalyzes *N,N*-dimethylnitrosamine *N*-demethylation (23). In no other case was a significant correlation observed. (The negative correlations observed with P-450$_{PA}$ and 4-aminobiphenyl *N*-hydroxylation have not been examined in further detail as of yet to determine the significance of any possible suppression of the enzyme under conditions which favor P-450$_{NF}$ expression.)

Inactivation of 17α-ethylnylestradiol 2-hydroxylase activity. Another line of evidence that P-450$_{NF}$ is the major catalyst of 17α-ethynylestradiol 2-hydroxylation is that preincubation of human liver microsomes with the antibiotic troleandomycin and NADPH results in the inhibition of catalytic activity toward both 17α-ethynylestradiol and nifedipine (Table 2). Oxidation of troleandomycin by this P-450 and its orthologs is known to produce a derivative which binds tightly to the enzyme and inhibits activity; this inhibition by macrolide antibiotics is highly selective for P-450$_{NF}$ (17), 30a).

Inhibition of 17α-ethynylestradiol 2-hydroxylase and nifedipine oxidase activities was also observed when human liver microsomes oxidized 17α-ethynylestradiol itself (Table 2). The extent of dilution in the experimental design rules out the contribution of competitive inhibition and isotope dilution to the observed results. In addition, a loss of spectrally detectable P-450 was also seen during such incubations (Fig. 7). The extent of P-450 loss is roughly consonant with the amount of P-450$_{NF}$ estimated using immunochemical measurements (13). The destruction appears not to be due to a quinone formed from the *o*-catechol, since the extent of P-450 loss was similar

TABLE 2

Inhibition of microsomal nifedipine oxidase and 17α-ethynylestradiol 2-hydroxylase activities by preincubation with troleandomycin and 17α-ethynylestradiol

Human liver microsomes (sample HL 105, containing 0.25 nmol of P-450) were incubated at 37° for 30 min in the presence of 0.1 M potassium phosphate buffer (pH 7.4) and, as indicated, an NADPH-generating system (see Materials and Methods), 20 μM troleandomycin, and 100 μM 17α-ethynylestradiol, in a total volume of 50 μl. For the subsequent assay of nifedipine oxidase activity, 0.95 ml of a solution composed of 0.1 M potassium phosphate buffer (pH 7.7), 0.20 mM nifedipine, and the NADPH-generating system (see Materials and Methods) was added; reactions proceeded for 10 min at 37° and the formation of the oxidized product was measured as described under Materials and Methods. For the assay of 17α-ethynylestradiol 2-hydroxylase activity, 0.95 ml of a solution composed of 0.1 M potassium phosphate buffer (pH 7.4), 1.0 mM L-ascorbic acid, 50 μM 17α-ethynyl[6,7-^{3}H]estradiol (20 μCi μmol^{-1}), and the NADPH-generating system was added; reactions proceeded for 10 min at 37° and the formation of radioactive 2-hydroxy-17α-ethynylestradiol was measured as described under Materials and Methods. Results are presented as means (±SD) of triplicate experiments (including triplicate preincubations).

Preincubation additions	Catalytic activity[a]	
	Nifedipine oxidation	17α-Ethynylestradiol 2-hydroxylation
None	4.57 ± 0.15 (100)[b]	1.10 ± 0.01 (100)
NADPH	4.93 ± 0.38 (108)	1.10 ± 0.15 (100)
NADPH, troleandomycin	1.03 ± 0.06 (23)	0.28 ± 0.08 (25)
NADPH, 17α-ethynylestradiol	2.83 ± 0.29 (62)	0.47 ± 0.06 (43)

[a] Expressed as nmol of product formed min^{-1} (nmol P-450)$^{-1}$ ± SD.
[b] Numbers in parentheses, percentage control.

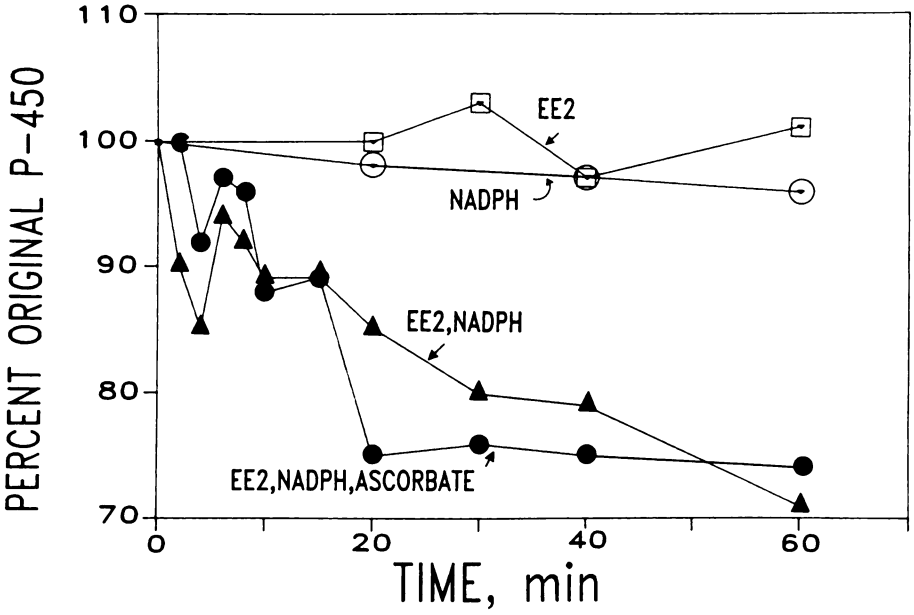

Fig. 7. Loss of spectrally determined P-450 in human liver microsomes during incubation with 17α-ethynylestradiol. Microsomes (sample HL 105) were incubated under typical conditions (see Table 2) with 17α-ethynylestradiol (100 μM) only (EE2; □), NADPH only (NADPH; ○), 17α-ethynylestradiol and NADPH (EE2, NADPH; ▲), or 17α-ethynylestradiol, NADPH, and 1 mM ascorbate (EE2, NADPH, ascorbate; ●).

regardless of whether ascorbate was included or not. When the temporal P-450 loss was fitted to a semilog plot and the apparent first order inactivation rate was compared to the rate of 17α-ethynylestradiol 2-hydroxylation, a partition ratio of 120 was calculated.

Discussion

The therapeutic ineffectiveness of the oral contraceptive 17α-ethynylestradiol in individuals having increased oxidation capability has long been recognized as a classic example of a clinical problem related to alterations of P-450 levels (2, 29). In this paper several lines of evidence argue that the enzyme responsible for this effect in humans is P-450$_{NF}$, the P-450 which appears to be involved in 17α-ethynylestradiol 2-hydroxylation. Evidence for this conclusion includes results of studies using enzyme reconstitution, immunoinhibition, correlation of activities, and inhibitors. The results suggest that this enzyme is the major one involved in the 2-hydroxylation of 17α-ethynylestradiol regardless of the absolute level of catalytic activity in the individual (Figs. 3 and 4).

Some caveats do need to be considered in this assignment, however. Genomic blotting studies suggest that three or more genes may be related to the P-450$_{NF}$ sequence (34), and more recent results indicate that three different cDNA clones can be isolated from a library prepared from a single human liver (31).[3] To date, none of these has been uniquely expressed in a vector system so that catalytic activity can be measured. The N-terminal sequence of P-450 HLFa, isolated by Kitada *et al.* (35), is very similar but differs in some positions from that predicted by any of the clones. In the case of tolbutamide and mephenytoin hydroxylations, extensive *in vitro* evidence for the involvement of a single enzyme in both activities was obtained but *in vivo* measurements may not be consistent with this view (36). Nevertheless, a strong case can be made that either P-450$_{NF}$ or an enzyme which is very closely related (and regulated) is responsible for the 2-hydroxylation of 17α-ethynylestradiol.

Although similar caveats should be applied, the list of steroid hydroxylations catalyzed by P-450$_{NF}$ is now considerable. Hydroxylations include testosterone (6β) (13, 30a), androstenedione (6β), (30a), progesterone (6β), (30a), cortisol (6β) (37), 17β-estradiol (2 and 4) (13), and dihydroepiandrostenedione (16α) (35). Other, nonsteroid substrates include many dihydropyridines (18), quinidine (13), erythromycin and troleandomycin (17), cyclosporin A (38), and (perhaps less definitively) aldrin and *d*-benzphetamine (13).

Although a variety of *in vitro* experiments implicate P-450$_{NF}$ in both cortisol 6β-hydroxylation (37) and 17α-ethynylestradiol 2-hydroxylation (this report), and compounds such as rifampicin enhance the *in vivo* oxidation of both, Park and Maggs (14) allude briefly to *in vitro* experiments (not described) which suggest that "estrogen 2-hydroxylase and cortisol 6β-hydroxylase are distinct enzymes" (14). It is possible that closely related P-450s produced by the multigene family (P-450 III A, Ref. 21) may have independent roles, but further speculation in this area must await more *in vitro* and *in vivo* results.

A major factor in influencing catalytic activity of P-450$_{NF}$-related reactions is the amount of immunochemically detectable protein, as shown by the correlation of immunoreactive protein and catalytic activity for nifedipine oxidation and 17α-ethynylestradiol 2-hydroxylation in this laboratory (Ref. 13; this paper, Fig. 6) and for erythromycin demethylation in another laboratory (17). Thus, one might expect more influences at the regulatory level than in terms of alterations of sequence, etc. The clinical literature indicate that 2-hydroxylation of 17α-ethynylestradiol is enhanced (in humans) by the administration of barbiturates and hydantoins, macrocyclic antibiotics such as erythromycin, and, particularly, rifampicin (2). A liver sample obtained from an individual who was treated with phenobarbital, triacetyloleandomycin, and dexamethasone had unusually high levels of an enzyme which appears to correspond to or is highly related to P-450$_{NF}$ (17). These compounds can include orthologs of P-450$_{NF}$ in experimental animals, although caution is needed in extrapolation of regulatory patterns—for instance,

[3] R. W. Bork, T. Muto, P. H. Beaune, R. S. Lloyd, and F. P. Guengerich, manuscript in preparation.

rabbits do not respond to pregnenolone 16α-carbonitrile (to induce P-450 3c) but rats do (inducing P-450$_{PCN-E}$ or P-450p) (38). However, the working hypothesis that these compounds each can induce P-450$_{NF}$ in humans is presented here.

The mechanism of inhibition by troleandomycin probably involves oxidation of the compound to a nitroso derivative, which forms a tight ligand with the ferrous iron of P-450. This mode of inhibition by troleandomycin (and certain other related macrolide antibiotics) appears to be selective for human P-450$_{NF}$ and its rat and rabbit orthologs in the P-450 III A gene family (17, 39, 40). The mechanism of activation of P-450 by acetylenic steroids has been studied in animal models. The *o*-catechol formed by 2-hydroxylation of 17α-ethynylestradiol can be readily oxidized to the corresponding *o*-quinone, which is capable of binding to protein (9, 10). Such oxidation can be mediated, at least under *in vitro* conditions, by P-450 enzymes, and superoxide anion formation may be a part of the mechanism (41). The quinone does not appear to be responsible for P-450 inactivation, however, since the same degree of loss of P-450 heme was seen in the presence and absence of ascorbate, which readily reduced the quinone (15). Acetylenes (and acetylenic steroids) can become covalently attached to P-450 heme after enzymatic oxidation. The derivatives of a number of compounds have been characterized (11, 42–45) and the formation of the *N*-substituted porphyrins is postulated to involve a metallo-oxetene intermediate or similar species (46). Such a reaction might occur in the inactivation of human P-450 by 17α-ethynylestradiol, since the amount of spectrally detectable P-450 heme lost was considerable (Fig. 7). However, other possibilities cannot be ruled out. Some acetylenic compounds inactive P-450 enzymes without destroying heme, and covalent adducts are formed with the apoprotein (47–49). It is conceivable that such binding could push the heme from the protein. Even in instances where *N*-alkylated pigments are formed, their quantitative input is unclear and, at least *in vitro*, a considerable amount of covalent linking of porphyrin to apoprotein can be observed during the P-450-mediated oxidation of 17α-ethynylestradiol (50).

Although the mechanism of destruction of P-450 cannot be precisely defined from these studies, the work demonstrates that the P-450 heme does disappear and that P-450$_{NF}$ is a major target for apparent mechanism-based inactivation by 17α-ethynylestradiol. Although such suicide inhibition by acetylenic steroids has been shown in animal models, this report shows that the phenomenon can be demonstrated *in vitro*. Other studies suggest that such reactions probably occur *in vivo* in primates and humans as well (3, 45). The studies presented here would suggest that the metabolism of drugs which are oxidized by P-450$_{NF}$ (see above) might be expected to be potentially impaired in individuals consuming acetylenic steroids.

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References

1. Bolt, H. M., H. Kappas, and H. Remmer. Studies on the metabolism of ethynylestradiol *in vitro* and *in vivo*: the significance of 2-hydroxylation and the formation of polar products. *Xenobiotica* **3**:773–785 (1973).
2. Bolt, H. M. Metabolism of estrogens—natural and synthetic. *Pharmacol. Ther.* **4**:155–181 (1979).
3. Schimd, S. E., W. Y. W. Au, D. E. Hill, F. F. Kadlubar, and W. M. Slikker, Jr. Cytochrome P-450-dependent oxidation of the 17α-ethynyl group of synthetic steroids: D-homoannulation or enzyme inactivation. *Drug Metab. Dispos.* **11**:531–536 (1983).
4. Reimers, D., and A. Ježek. Rifampicin und andere Antituberkulotika bei gleichzeitiger oraler Kontrazeption. *Prax. Pneumol.* **25**:255–262 (1971).
5. Nocke-Finck, L., H. Brewer, and D. Reimers. Wirkung von Rifampicin auf den Menstruationszylkus und die Östrogenausscheidung bei Einnahme oraler Kontrazeptive. *Dtsch. Med. Wochenschr.* **98**:1521–1523 (1973).
6. Janz, D., and D. Schmidt. Anti-epileptic drugs and failure of oral contraceptives. *Lancet* **1**:1113 (1974).
7. Breckenridge, A. M., D. J. Back, F. E. Crawford, M. MacIver, M. l'E. Orme, B. K. Park, P. H. Rowe, and E. Smith. Drug interactions with contraceptive steroids, in *The Induction of Drug Metabolism* (R. W. Estabrook and E. Lindenlaub, eds.), Vol. 14, Schattauer Verlag, New York, 607–613 (1978).
8. Bolt, H. M., H. Kappus, and M. Bolt. Effect of rifampicin treatment on the metabolism of oestradiol and 17α-oestradiol by human liver microsomes. *Eur. J. Clin. Pharmacol.* **8**:301–307 (1975).
9. Bolt, H. M., H. Kappus, and R. Kashboher. Metabolism of 17α-ethinylestradiol by human liver microsomes *in vitro*: aromatic hydroxylation and irreversible binding of metabolites. *J. Endocrinol. Metab.* **39**:1072–1080 (1977).
10. Maggs, J. L., P. S. Grabowski, M. E. Rose, and K. B. Park. The biotransformation of 17α-ethinyl[^{3}H]estradiol in the rat: irreversible binding and biliary metabolites. *Xenobiotica* **12**:657–668 (1982).
11. White, I., and U. Muller-Eberhard. Decreased liver cytochrome P-450 in rats caused by norethindrone or ethynylestradiol. *Biochem. J.* **166**:57–64 (1977).
12. Dannan, G. A., D. J. Porabek, S. D. Nelson, D. J. Waxman, and F. P. Guengerich. 17β-Estradiol 2- and 4-hydroxylation catalyzed by rat hepatic cytochrome P-450: roles of individual forms, inductive effects, developmental patterns, and alterations by gonadectomy and hormone replacement. *Endocrinology* **118**:1952–1960 (1986).
13. Guengerich, F. P., M. V. Martin, P. H. Beaune, P. Kremers, T. Wolff, and D. J. Waxman. Characterization of the rat and human microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* **261**:5051–5060 (1986).
14. Park, B. K., and J. L. Maggs. Inter-individual variation in the metabolism of ethynylestradiol, in *Ethnic Differences in Reactions to Drugs and Xenobiotics* (W. Kaslow, H. W. Goedde, and D. P. Agarwal, eds.). Alan R. Liss, Inc., New York, 289–302 (1986).
15. Purba, H. S., J. L. Maggs, M. l'E. Orme, D. J. Back, and B. K. Park. The metabolism of 17α-ethinylestradiol by human liver microsomes: formation of catechol and chemically reactive metabolites. *Br. J. Clin. Pharmacol.* **23**:447–453 (1987).
16. Back, D. J., J. L. Maggs, H. S. Purba, S. Newby, and B. K. Park. 2-Hydroxylation of ethinyloestradiol in relation to the oxidation of sparteine and antipyrine. *Br. J. Clin. Pharmacol.* **18**:603–607 (1984).
17. Watkins, P. B., S. A. Wrighton, P. Maurel, E. G. Schuetz, G. Mendez-Picon, G. A. Parker, and P. S. Guzelian. Identification of an inducible form of cytochrome P-450 in human liver. *Proc. Natl. Acad. Sci. USA* **82**:6310–6314 (1986).
18. Böcker, R. H., and F. P. Guengerich. Oxidation of 4-aryl- and 4-alkyl-substituted 2,6-dimethyl-3,5-bis(alkoxycarbonyl)-1,4-dihydropyridines by human liver microsomes and immunochemical evidence for the involvement of a form of cytochrome P450. *J. Med. Chem.* **29**:1596–1603 (1986).
19. Shimada, T., K. Misono, and F. P. Guengerich. Human liver microsomal cytochrome P-450 mephenytoin 4-hydroxylase, a prototype of genetic polymorphism in oxidative drug metabolism: purification and characterization of two similar forms involved in the reaction. *J. Biol. Chem.* **261**:909–921 (1986).
20. Guengerich, F. P. Cytochrome P-450 enzymes and drug metabolism, in *Progress in Drug Metabolism* (J. W. Bridges, L. F. Chasseaud, and G. G. Gibson, eds.), Vol. 10. Taylor and Francis, London, 1–54 (1987).
21. Nebert, D. W., M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman. The P450 gene superfamily: recommended nomenclature. *DNA* **6**:1–11 (1987).
22. Distlerath, L. M., and F. P. Guengerich. Characterization of a human liver cytochrome P-450 involved in the oxidation of debrisoquine and other drugs by using antibodies raised to the analogous rat enzyme. *Proc. Natl. Acad. Sci. USA* **81**:7348–7352 (1984).
23. Yoo, J.-S. H., F. P. Guengerich, and C. S. Yang. Metabolism of *N*-nitrosodialkylamines in human liver microsomes. *Cancer Res.* **88**:1499–1504.
24. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2385 (1964).
25. Guengerich, F. P., P. Wang, and N. K. Davidson. Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits, and humans using immunochemical staining coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry* **21**:1698–1706 (1982).
26. Slikker, W., Jr., G. W. Lipe, T. J. Sziszak, and J. R. Bailey. Changes in estrogen metabolism after chronic oral contraceptive administration in the rhesus monkey. *Drub Metab. Dispos.* **12**:148–153 (1984).
27. Distlerath, L. M., P. E. B. Reilly, M. V. Martin, G. G. Davis, G. R. Wilkinson, and F. P. Guengerich. Purification and characterization of the human liver cytochromes P-450 involved in debrisoquine 4-hydroxylation and phenacetin *O*-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem.* **260**:9057–9067 (1985).

28. Kadlubar, F. F., M. A. Butler, B. E. Hayes, F. A. Beland, and F. P. Guengerich. Role of microsomal cytochromes P-450 and prostaglandin H synthase in 4-aminobiphenyl-DNA adduct formation, in *Microsomes and Drug Oxidations, Seventh International Symposium* (D. J. Birkett, ed.). Taylor and Francis, London, in press.
29. Breckenridge, A. M., D. J. Back, K. Cross, F. Crawford, M. MacIver, M. l'E. Orme, P. H. Rowe, and E. Smith. Influence of environmental chemicals on drug therapy in humans: studies with contraceptive steroids. *CIBA Found. Symp.* **76**:298–306 (1980).
30. Guengerich, F. P., D. Müller-Enoch, and I. A. Blair. Oxidation of quinidine by human liver cytochrome P-450. *Mol. Pharmacol.* **30**:287–295 (1986).
30a. Waxman, D. J., C. Attisano, F. P. Guengerich, and D. P. Lapenson. *Arch. Biochem. Biophys.*, in press.
31. Guengerich, F. P., D. R. Umbenhauer, C. Ged, M. V. Martin, G. R. Wilkinson, and R. S. Lloyd. Human liver cytochrome P-450 mephenytoin 4-hydroxylase and related proteins, in *Microsomes and Drug Oxidations, Seventh International Symposium* (D. J.. Birkett, ed.). Taylor and Francis, London, in press.
32. Lasker, J. M., J. Raucy, S. Kubota, B. P. Bloswick, M. Black, and C. S. Lieber. Purification and characterization of human liver cytochrome P-450-ALC. *Biochem. Biophys. Res. Commun.* **148**:232–238 (1987).
33. Wrighton, S. A., P. E. Thomas, P. Willis, S. L. Maines, P. B. Watkins, W. Levin, and P. S. Guzelian. Purification of a human liver cytochrome P-450 immunochemically related to several cytochromes P-450 purified from untreated rats. *J. Clin. Invest.* **80**:1017–1022 (1987).
34. Beaune, P. H., D. R. Umbenhauer, R. W. Bork, R. S. Lloyd, and F. P. Guengerich. Isolation and sequence determination of a cDNA clone related to human cytochrome P-450 nifedipine oxidase. *Proc. Natl. Acad. Sci. USA* **83**:8064–8068 (1986).
35. Kitada, M., T. Kamataki, K. Itahashi, T. Rikihisa, and Y. Kanakubo. P-450 HLFa, a form of cytochrome P-450 purified from human fetal livers, is the 16α-hydroxylase of dehydroepiandrosterone 3-sulfate. *J. Biol. Chem.* **262**:13534–13537 (1987).
36. Knodell, R. G., S. D. Hall, G. R. Wilkinson, and F. P. Guengerich. Hepatic metabolism of tolbutamide: *in vitro* characterization of the form of cytochrome P-450 involved in methyl hydroxylation and relationship to *in vivo* disposition. *J. Pharmacol. Exp. Ther.* **241**:1112–1119 (1987).
37. Ged, C., P. H. Beaune, I. Dalet, P. Maurel, and J.-P. Leroux. Cortisol 6β-hydroxylase in human liver microsomes, in *Abstracts, International Symposium on Clinical and Basic Aspects of Enzyme Induction and Inhibition.* Essen, Federal Republic of Germany, July 6–7, 1985.
38. Clair, P., M. Daujat, T. Pineau, C. Dalet, C. Bonfils, and P. Maurel. Regulation of cytochrome P-450 3c, in *Proceedings, International Symposium on Liver Cells and Drugs* (A. Guillouzo, ed.), in press.
39. Mansuy, D., P. Gans, J. C. Chottard, and J. F. Bartoli. Nitrosoalkanes as Fe(II) ligands in the 455-nm-absorbing cytochrome P-450 complexes formed from nitroalkanes in reducing conditions. *Eur. J. Biochem.* **76**:607–615 (1977).
40. Passayre, D., V. Descatoire, M. Konstantinova-Mitcheva, J. C. Wandscheer, B. Cobert, R. Level, J. P. Benhamou, M. Jaouen, and D. Mansuy. Self-induction by triacetyloleandomycin of its own transformation into a metabolite forming a stable 456 nm-absorbing complex with cytochrome P-450. *Biochem. Pharmacol.* **30**:553–558 (1981).
41. Nelson, S. D., J. R. Mitchell, E. Dybing, and H. A. Sasame. Cytochrome P-450-mediated oxidation of 2-hydroyestrogens to reactive intermediates. *Biochem. Biophys. Res. Commun.* **70**:1157–1165 (1976).
42. White, I. N. H. Metabolic activation of acetylenic substituents to derivatives in the rat causing the loss of hepatic cytochrome P-450 and haem. *Biochem. J.* **174**:853–861 (1978).
43. Ortiz de Montellano, R. P., K. L. Kunze, G. S. Yost, and B. A. Mico. Self-catalyzed destruction of cytochrome P-450: covalent binding of ethynyl sterols to prosthetic heme. *Proc. Natl. Acad. Sci. USA* **76**:746–749 (1979).
44. Blakely, D. C., and I. N. H. White. Destruction of cytochrome P-450 and formation of green pigments by contraceptive steroids in rat hepatocyte suspensions. *Biochem. Pharmacol.* **35**:1561–1567 (1986).
45. Ortiz de Montellano, P. R., and M. A. Correia. Suicidal destruction of cytochrome P-450 during oxidative drug metabolism. *Annu. Rev. Pharmacol. Toxicol.* **23**:481–503 (1983).
46. Komives, E. A., and P. R. Ortiz de Montellano. Mechanism of oxidation of π bonds by cytochrome P-450: electronic requirements of the transition state in the turnover of phenylacetylenes. *J. Biol. Chem.* **262**:9793–9802 (1987).
47. Nagahisa, A., R. W. Spencer, and W. H. Orme-Johnson. Acetylenic mechanism-based inhibitors of cholesterol side chain cleavage by cytochrome P-450scc. *J. Biol. Chem.* **258**:6721–6723 (1983).
48. Gan, L.-S. L., A. L. Acebo, and W. L. Alworth. 1-Ethynylpyrene, a suicide inactivator of cytochrome P-450 dependent benzo[*a*]pyrene hydroxylase activity in liver microsomes. *Biochemistry* **23**:3827–3836 (1984).
49. Ortiz de Montellano, P. R., and N. O. Reich. Specific inactivation of hepatic fatty acid hydroxylases by acetylenic fatty acids. *J. Biol. Chem.* **259**:4136–4141 (1984).
50. Guengerich, F. P. Covalent binding to apoprotein is a major fate of heme in a variety of reactions in which cytochrome P-450 is destroyed. *Biochem. Biophys. Res. Commun.* **138**:193–198 (1986).
51. Ged, G., D. R. Umbenhaver, T. M. Bellew, R. W. Bork, P. K. Srivastavan, N. Shinriki, R. S. Lloyd, and F. P. Guengerich. *Biochemistry,* in press.

Send reprint requests to: Dr. F. Peter Guengerich, Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University, School of Medicine, Nashville, TN 37232.