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Modulation of Rabbit and Human Hepatic Cytochrome P-450-Catalyzed Steroid Hydroxylations by α-Naphthoflavone

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SUMMARY

Rifampicin induces cytochrome P-450 3c, progesterone 16α- and 6β-hydroxylation, 17β-estradiol 2-hydroxylation, benzo[*a*]pyrene hydroxylation, and erythromycin *N*-demethylation in rabbit liver microsomes. Kinetic analysis of the 6β-hydroxylation of progesterone as catalyzed by liver microsomes prepared from rifampicin-treated B/J rabbits exhibits a curvilinear double-reciprocal plot, suggestive of substrate activation. Further experimentation demonstrated that α-naphthoflavone could augment the catalytic efficiency [V_{max}/K_m] observed for the 16α- and 6β-hydroxylation of progesterone and the 2-hydroxylation of 17β-estradiol, whereas erythromycin *N*-demethylase activity was partially inhibited. Allosteric activation of these steroid hydroxylases by α-naphthoflavone is also found for human liver microsomes, indicating that the activation of these enzymes is conserved in man and rabbit.

The metabolism of several carcinogens and other foreign compounds by the P-450 monooxygenases in man (1–5), mouse (6), rat (7–9), hamster (5), and rabbit (4, 5, 10) is known to be directly stimulated by certain flavonoids. This reflects direct activation of the enzyme rather than induction of P-450 synthesis, and the effect can be determined directly for the reaction catalyzed *in vitro*. Moreover, the stimulation of P-450 enzymes by flavonoids has been shown to increase the metabolism of xenobiotics *in vivo* as demonstrated by Conney and co-workers (8, 9).

Among a number of purified reconstituted forms of rabbit P-450, benzo[*a*]pyrene metabolism catalyzed by P-450 3c is rather selectively activated by α-naphthoflavone (10, 11). P-450 3c is induced in liver by rifampicin (12, 13), and rifampicin greatly induces the microsomal benzo[*a*]pyrene hydroxylase that is stimulated by α-naphthoflavone (10). The activation of the benzo[*a*]pyrene hydroxylation by α-naphthoflavone is also elevated following the treatment of mice with rifampicin (6), suggesting that such activation of the enzyme induced by rifampicin may be conserved across species.

Rifampicin also induces regiospecific hydroxylations of certain steroids in rabbit, (14) and man (15), as well as erythromycin *N*-demethylation in rabbit (13, 14). We therefore examined whether α-naphthoflavone stimulates hepatic progesterone 6β- and 16α-hydroxylation, 17β-estradiol, 2-hydroxylation, and erythromycin *N*-demethylation induced by rifampicin in B/J rabbits. This particular strain was utilized because it does not express a form of P-450 3b that efficiently catalyzes a major portion of microsomal progesterone 6β- and 16α-hydroxylation, 6β$^+$ (16), and it expresses only low concentrations of P-450 1 (<0.1 nmol/mg), an enzyme that expresses a high efficiency 17β-estradiol 2-hydroxylase (17) and benzo[*a*]pyrene hydroxylase in rabbit liver (10). The combined or individual presence of these enzymes would greatly confound the kinetic analysis of the rifampicin-inducible enzyme in these reaction pathways. Our studies indicate that α-naphthoflavone stimulates the 6β- and 16α-hydroxylation of progesterone as well as the 2-hydroxylation of 17β-estradiol in microsomes prepared from rabbits treated with rifampicin. Conversely, α-naphthoflavone partially inhibits erythromycin *N*-demethylation. An examination of these effects with human liver microsomes containing different concentrations of the human homolog of P-450 3c indicates that these properties of activation by substrate and/or modulation by allosteric effectors are conserved in both man and rabbit.

Materials and Methods

Preparation of microsomes. Rabbits of strains B/J and III/J, coefficient of inbreeding, each 1.0 (18), were allowed free access to standard laboratory chow and water. For induction studies, an aqueous solution of rifampicin (100 mg/ml) was administered, 100 mg/kg intraperitoneally, for 7 days. On the seventh day of rifampicin treatment, food was withdrawn, and the following day the rabbits were killed by a lethal intravenous injection of pentobarbital. Rabbit liver microsomes

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ABBREVIATIONS: P-450, cytochrome P-450; RIF-B/J, rifampicin-treated B/J rabbit.

were prepared as described (19). Human liver microsomes were kindly provided by Dr. P. Guzelian of the Medical College of Virginia (human microsomes #6 and 4), Dr. F. P. Guengerich, Vanderbilt University (human microsomes #FH69, FH39, FH84, and HL15), and Dr. L. Waskell of the Veterans Administration Medical Center, San Francisco, CA (human microsomes #041581 and 41280). One sample containing elevated concentrations of P-450 HLp, human microsomes #4, was prepared from a liver biopsy obtained from a female patient during the removal of a kidney for donation (20). The patient received 80 mg of dexamethasone, 840 mg of pentobarbital, and 300 mg of diphenylhydantoin in divided doses during the 2 days before surgery.

Enzyme assays. Protein concentrations were determined according to the method of Bensadoun and Weinstein (21). P-450 content was assessed as described by Omura and Sato (22). Procedures describing the assay of progesterone metabolism were as reported earlier (23), as were those for 17β-estradiol 2-hydroxylation (17). Erythromycin *N*-demethylation was measured by the production of [^{14}C]formaldehyde from *N*-[*methyl*-^{14}C]erythromycin (New England Nuclear) using a procedure described previously for aminopyrine *N*-demethylation (24).

Immunological procedures. Monoclonal antibodies to P-450 3c were developed by fusing splenocytes prepared from mice immunized with purified P-450 3c with the myeloma line SP 2/0 as described previously for P-450 1 (25). Hybridomas secreting antibody recognizing purified P-450 3c were identified by enzyme-linked immunosorbent assays. Positive cultures were expanded, and the media of each were tested for antibody reactive with the purified antigen as well as liver microsomes prepared from untreated and rifampicin-treated rabbits. Each of the cultures produced antibodies that recognize P-450 3c and that bound more extensively (>5-fold) to microsomes from rifampicin-treated as compared to untreated rabbits. These cell lines were selected for subcloning resulting in the development of 20 independently derived, monoclonal antibody-secreting hybridoma lines. None of these monoclonal antibodies were cross-reactive toward P-450s 1, 2, 3a, 3b, 4, 5, or 6 as judged by enzyme-linked immunosorbent assays.

Each of the cell lines was grown as an ascites culture in mice. The ascites fluid was harvested and tested for its capacity to inhibit the progesterone 6β-hydroxylase activity catalyzed by microsomes prepared from RIF-B/J rabbits. None of the ascites preparations inhibit this activity. Three, which we will designate, α3c.1, α3c.2, and α3c.3, were found to recognize P-450 3c in Western blotting experiments. Of the three monoclonal antibodies, only α3c.2 reacted with a protein antigen in human and rat liver microsomes. The concentration of the latter was elevated following treatment of rats with triacetyloleandomycin, which induced P-450p. For the experiments shown in Fig. 5A (see Results), microsomes (10–20 μg) and P-450 3c (5 pmol) were electrophoresed in the presence of sodium dodecyl sulfate in 10% polyacrylamide gels and transferred to nitrocellulose as described by Towbin *et al.* (26). The monoclonal antibody α3c.2 was labeled with ^{125}I by the chloramine T method (27) and then was used to visualize reactive antigens by means of autoradiography.

Results

The effects of rifampicin treatment on the concentration of P-450 and the progesterone hydroxylase activities of liver microsomes prepared from rabbits of strains B/J and III/J are summarized in Table 1. As shown, the specific P-450 content of liver microsomes prepared from rifampicin-treated III/J rabbits is not significantly increased relative to untreated III/J rabbits, whereas an approximate 2-fold increase in the specific P-450 content of RIF-B/J microsomes is observed relative to the control group ($p < 0.01$). Maurel and co-workers (28, 29) have estimated the concentration of P-450 3c in hepatic microsomes from untreated and triacetyloleandomycin-treated rabbits to be 0.13 and between 0.7 and 1.7 nmol/mg, respectively. Although we have not determined the concentration of P-450 3c in all of the samples summarized in Table 1, the results of Western blotting experiments, such as those shown later, indicate that the concentrations of P-450 3c in several untreated and RIF-B/J rabbits are similar to those reported for untreated and triacetyloleandomycin-treated outbred rabbits.

TABLE 1

Specific P-450 content and rates of the 6β- and 16α-hydroxylation of progesterone (10 μM)

Microsomes[a]	N[b]	P-450[c]	Progesterone[d]	
			6β	16α
III/J-UNT	6	2.17 ± 0.41[e]	1.76 ± 0.55[e]	0.78 ± 0.32[e]
III/J-RIF	4	2.28 ± 0.23[e]	3.91 ± 1.51[f]	1.18 ± 0.47[e,f]
B/J-UNT	10	1.60 ± 0.47	0.50 ± 0.23	0.46 ± 0.15
B/J-RIF	9	2.76 ± 0.80[f]	4.42 ± 1.40[f]	0.69 ± 0.16[f]

[a] UNT represents untreated rabbits and RIF represents rifampicin-treated rabbits.
[b] *N*, number of independent microsomal preparations tested.
[c] Expressed as nmol of P-450/mg of microsomal protein.
[d] Expressed as nmol of product formed/min/mg of microsomal protein.
[e] Interstrain, within treatment groups, $p < 0.05$.
[f] Intrastrain treatment groups, $p < 0.05$.

The result of rifampicin treatment on the observed progesterone 6β-hydroxylase activity is also more pronounced in microsomes prepared from rabbits of strain B/J, giving rise to rates that are up to 10 times greater for the RIF-B/J microsomes relative to their corresponding controls ($p < 0.01$, Table 1). This reflects in part the lower 6β-hydroxylase activity and P-450 concentration exhibited by B/J rabbits as compared to III/J rabbits (16). Unlike rabbits of strain III/J, rabbits of strain B/J do not express the 6β^+ subform of P-450 3b, a high efficiency progesterone 6β- and 16α-hydroxylase (16). The rate of progesterone 16α-hydroxylation catalyzed by microsomes from B/J rabbits is also lower than that catalyzed by microsomes from III/J rabbits ($p < 0.05$). Rifampicin induces about a 1.5-fold increase in this activity ($p < 0.01$). A similar increase, about 0.3 nmol/min/mg, is seen for III/J rabbits, but the effect is less significant for this strain.

17β-Estradiol 2-hydroxylation is also increased by rifampicin treatment in both strains, from ~0.5 to ~1.1 nmol/min/mg, and there appears to be no significant difference in this increase between the two strains. Rabbits of both strains B/J and III/J express extremely low levels of P-450 1, a high efficiency 17β-estradiol 2-hydroxylase (17), and a benzo[*a*]pyrene hydroxylase (10) which is elevated in certain outbred rabbits. The *N*-demethylation of erythromycin was also elevated in RIF-B/J microsomes relative to microsomes from untreated B/J rabbits, 5.7 and 1.4 nmol/min/mg, respectively. The results shown for III/J rabbits are similar to those of Lange *et al.* (14), who showed previously that rifampicin treatment of outbred rabbits results in an increase in both erythromycin *N*-demethylation and progesterone 6β-hydroxylation.

The effect of α-naphthoflavone, which was previously shown to stimulate the benzo[*a*]pyrene hydroxylase induced by rifampicin in rabbit liver microsomes (10), was examined with respect to its effect on the conversion of 17β-estradiol to 2-hydroxyestradiol, progesterone to 6β- and 16α-hydroxyprogesterone and erythromycin to its N-demethylated derivative as catalyzed by microsomes prepared from untreated and RIF-B/J rabbits. When added to incubations containing RIF-B/J microsomes, α-naphthoflavone (5 μM) stimulated the observed rate of progesterone 6β-hydroxylation by a factor of 2- to 3-fold and progesterone 16α-hydroxylation and 17β-estradiol 2-

hydroxylation approximately 2-fold when each substrate was present at an initial concentration of 10 μM. In contrast, the *N*-demethylation of erythromycin (250 μM) as catalyzed by RIF-B/J microsomes was inhibited by approximately 50% by 5 μM α-naphthoflavone. At 25 μM α-naphthoflavone, the stimulation of the 6β- and 16α hydroxylation of progesterone was similar to that seen at 5 μM, whereas no effect is seen at 0.5 μM α-naphthoflavone. When microsomes from untreated B/J rabbits were examined, 5 μM α-naphthoflavone elicited only a 10–20% increase in either progesterone or 17β-estradiol hydroxylation, whereas erythromycin *N*-demethylation activity was not significantly affected. Thus, rifampicin induces steroid hydroxylases which are activated by α-naphthoflavone.

In order to better characterize the enzyme activities responding to rifampicin treatment and the stimulatory effects of α-naphthoflavone, kinetic analyses were undertaken for the dependence of progesterone 6β- and 16α-hydroxylation and 17β-estradiol 2-hydroxylation on substrate concentration. Double reciprocal plots for the dependence of the rate of progesterone 6β-hydroxylation on substrate concentration exhibit a pronounced curvature for microsomes prepared from RIF-B/J rabbits (Fig. 1). In contrast, microsomes prepared from the untreated B/J rabbits display a linear double reciprocal plot characterized by a lower apparent V_{max} and catalytic efficiency, V_{max}/K_m (Table 2). In the presence of α-naphthoflavone, an essentially linear double reciprocal plot is obtained for the RIF-B/J microsomes (Fig. 1). At high substrate concentrations, the velocities obtained for the RIF-B/J microsomes in the absence of α-naphthoflavone approach that obtained in its presence, suggesting that the substrate progesterone activates the enzyme. At low substrate concentrations, the efficiency of the enzyme falls off dramatically in the absence of the effector.

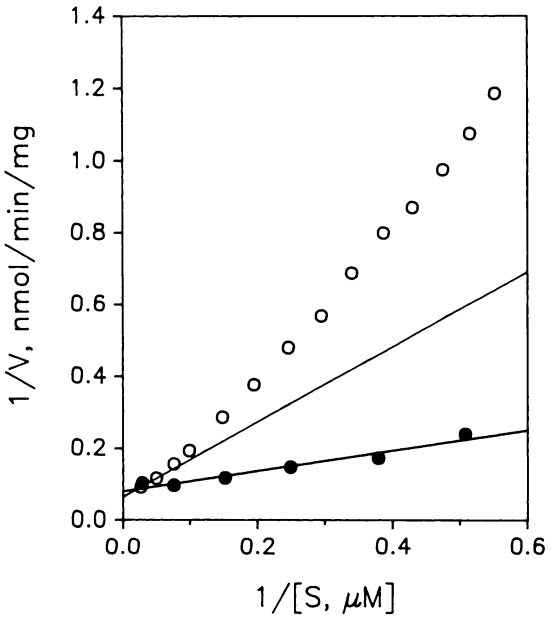

Fig. 1. Lineweaver-Burk analysis of the 6β-hydroxylation of progesterone as catalyzed by microsomes obtained from RIF-B/J rabbits in the absence (○) and presence (●) of 5 μM α-naphthoflavone. The assay conditions are described under Materials and Methods. A linear regression line ($r^2 = 0.95$) is shown for the results determined in the presence of α-naphthoflavone. The line shown for the results obtained with substrate alone has been drawn as a tangent to the points obtained at the highest substrate concentrations in order to illustrate the curvilinearity of the experimental points.

This does not reflect a loss of linearity in the time course of the reaction or on the concentration of microsomal protein.

We have reported previously (23) that double reciprocal plots of progesterone 16α-hydroxylation appear to reflect the contributions of two populations of microsomal enzymes in outbred rabbits with distinctly different kinetic properties leading to biphasic plots. This is also observed for strain B/J. Following treatment with rifampicin, there is an apparent increase of rate at all substrate concentrations. The double reciprocal plot is linear in the presence of the α-naphthoflavone, exhibiting a higher rate at each substrate concentration. It cannot be determined from these results whether the enzyme exhibiting the high K_m is affected by α-naphthoflavone or whether rifampicin induces a third component which is activated by α-naphthoflavone.

The kinetic characteristics of 17β-estradiol 2-hydroxylation were examined in a similar fashion. Microsomes prepared from either untreated or RIF-B/J rabbits exhibit linear double reciprocal plots with the RIF-B/J microsomes exhibiting an approximately 1.5-fold greater apparent V_{max} relative to their corresponding controls (Table 2). In the presence of 5 μM α-naphthoflavone there is a 3-fold increase in catalytic efficiency for the RIF-B/J microsomes (Table 2). This augmentation is due largely to a decrease in the apparent K_m for 17β-estradiol in the presence of α-naphthoflavone (Fig. 2).

Human liver microsomes were characterized in order to assess whether α-naphthoflavone had a significant stimulatory effect on progesterone 6β-hydroxylation and 17β-estradiol 2-hydroxylation in man as well as rabbit. Guzelian and coworkers (20) have described the apparent induction of the human homolog of P-450 3c, P-450 IIIA3,[3] in a patient who voluntarily received dexamethasone prior to a liver biopsy. This sample, designated number 4 in the original study, was shown by these investigators to contain elevated concentrations of a protein which reacts with antibodies to rat P-450 p (IIIA2) and rabbit P-450 3c and to exhibit elevated erythromycin *N*-demethylase activity when compared to hepatic microsomes prepared from biopsy samples obtained from other patients. A cDNA corresponding to this protein was subsequently cloned, and the amino acid sequence predicted for P-450 IIIA3 (30, 31) indicates that it is homologous, exhibiting about 70% sequence similarity, to rat P-450 p (32) and rabbit P-450 3c.[4] This sample, together with another designated as number 6 (20), which contained lower concentrations of P-450 IIIA3, was kindly provided to us by Dr. P. S. Guzelian for characterization in this study.

As was seen for microsomes from the RIF-B/J rabbits, curvature was observed for the double reciprocal plot of progesterone 6β-hydroxylation, as catalyzed by the human liver microsomes #4 (Fig. 3). Addition of 5 μM α-naphthoflavone essentially abolished this curvature and increased the efficiency of this reaction pathway nearly 10-fold (Table 2). In contrast to the results obtained for 16α-hydroxylation of progesterone as

[3] The following correspondence between common names for P-450 forms used in the text and a proposed uniform system of nomenclature (32) is as follows: rabbit P-450 3c, IIIA4; rabbit P-450 3b, IIC3; rabbit P-450 1, IIC5; human P-450_{NF} or P-450 HLp, IIIA3; rat P-450p, IIIA2; rabbit P-450 2, IIB1; rabbit P-450 3a, IIE1; rabbit P-450 4, IA2; rabbit P-450 6, IA1; rat P-450 PB1, IIC6.

[4] The amino acid sequence predicted from a cDNA characterized in our laboratory indicates that P-450 3c is the rabbit homolog of rat P-450 IIIA1 and IIIA2 and of human P-450 IIIA3. C. Potenza, U. Pendurthi, D. Strom, G. Schwab, K. Griffin, and E. Johnson, manuscript in preparation.

TABLE 2

Kinetic parameters of the 6β- and 16α-hydroxylation of progesterone and 2-hydroxylation of 17β-estradiol as catalyzed by liver microsomes prepared from treated and untreated rabbits and humans in the absence and presence of α-naphthoflavone (5 μM)

Microsomes[a]	Progesterone						17β-Estradiol		
	6β			16α					
	K_m[b]	V_{max}[c]	Eff.[d]	K_m	V_{max}	Eff.	K_m	V_{max}	Eff.
B/J-UNT	38.5	2.5	0.06	0.3/24.3[e]	0.07/0.8[e]	0.2/0.03[e]	39.0	2.9	0.07
B/J-RIF	ND[f]	15.5[g]	0.5[g]	0.3/72.5[e]	0.3/3.8[e]	1.0/0.05[e]	42.1	4.5	0.1
B/J-R + ANF	3.6	12.5	3.5	4.7	2.1	0.4	15.0	4.0	0.3
Human #4	ND[f]	3.0[g]	0.13[g]	49.6	1.1	0.02	49.0	2.6	0.05
+ ANF	3.2	3.9	1.2	3.1	0.7	0.2	15.6	3.1	0.2

[a] UNT and RIF denote untreated and rifampicin-treated rabbits, respectively; ANF denotes α-naphthoflavone.
[b] Expressed throughout as μM substrate concentration.
[c] Expressed throughout as nmol of product formed min^{-1} mg $protein^{-1}$.
[d] Catalytic efficiency, V_{max}/K_m, expressed as min^{-1} mg $protein^{-1}$.
[e] The observed dependence on substrate concentration was analyzed as the sum of two enzymes. The kinetic constants shown above were determined by a least squares fit using the Newton-Raphson procedure of velocity determinations at 12 or more concentrations of progesterone ranging from 1 to 40 μM. Estimated values at each substrate concentration are within 1% of each of the observed rates.
[f] Due to the curvilinear nature of these data, a K_m value was not determined.
[g] V_{max} was estimated from the velocity at the highest substrate concentrations, whereas the efficiency was estimated from the first order dependence of the velocity curve at the lowest substrate concentrations. The efficiency at high substrate concentrations approaches that seen in the presence of α-naphthoflavone.

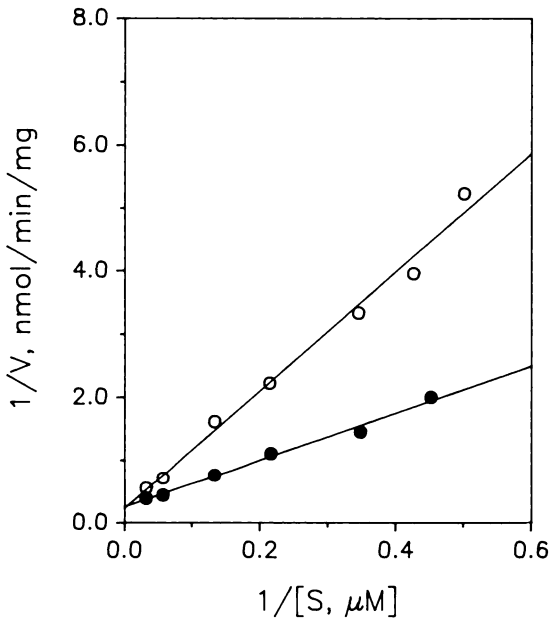

Fig. 2. Lineweaver-Burk analysis of the 2-hydroxylation of 17β-estradiol as catalyzed by microsomes obtained from RIF-B/J rabbits in the absence (○) and presence (●) of 5 μM α-naphthoflavone. The assay conditions are as described under Materials and Methods. In each instance, the linear regression lines are shown ($r^2 > 0.99$).

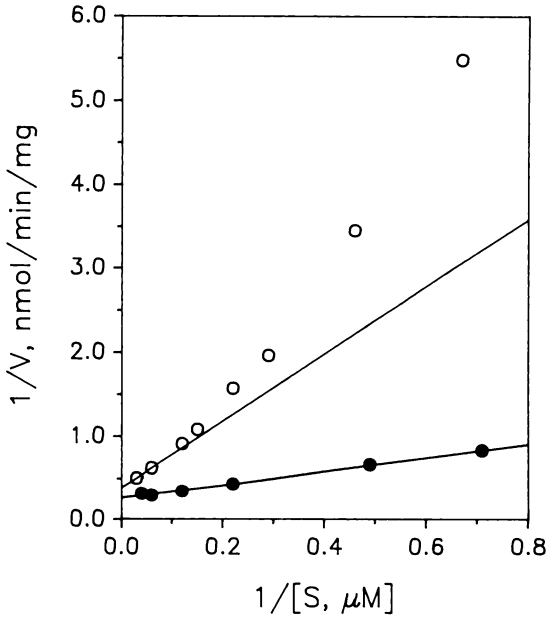

Fig. 3. Lineweaver-Burk anlaysis of the 6β-hydroxylation of progesterone as catalyzed by human microsomes #4 in the absence (○) and presence (●) of 5 μM α-naphthoflavone. The experimental conditions were as described in Fig. 1 as are the fit of the data. A linear regression line is shown for the results obtained in the presence of α-naphthoflavone, ($r^2 > 0.99$).

catalyzed by the RIF-B/J microsomes, the double reciprocal plot for human microsomes #4 was linear. The effect of added α-naphthoflavone was to increase the catalytic efficiency approximately 10-fold (Table 2). As was the case for the RIF-B/J microsomes, there was no evidence for substrate activation of 17β-estradiol 2-hydroxylation catalyzed by the human microsomes #4 (Fig. 4). Inclusion of 5 μM α-naphthoflavone to incubations containing human microsomes #4 resulted in an approximately 4-fold increase of catalytic efficiency and a lower apparent K_m (Table 2). Characterization in detail of microsomes from the second donor, human microsomes #6 (20), revealed that α-naphthoflavone affected the slope of double reciprocal plots in a similar fashion, although the apparent V_{max} was lower, ~30% of that seen for microsomes #4 for each reaction.

The capacity of α-naphthoflavone to stimulate the 6β-hydroxylation of progesterone was observed for other preparations of human liver microsomes, as shown in Fig. 5. The concentration of P-450 IIIA3 was seen to vary among human liver samples characterized by Western blotting using polyclonal antibodies to either rat P-450p or rabbit P-450 3c (20), and this variation was also observed using a monoclonal antibody developed in our own laboratory to P-450 3c (Fig. 5A). As shown in Fig. 5B, there is a correlation between the progesterone 6β-hydroxylase activity determined for these microsomes in the presence or absence of α-naphthoflavone and the intensity of the band revealed by Western blotting ($r^2 > 0.9$ and 0.78, respectively). The range of variation is not as great, however, as the difference in the concentration of P-450 3c elicited by the treatment of rabbits with rifampicin in this study (Fig. 5B).

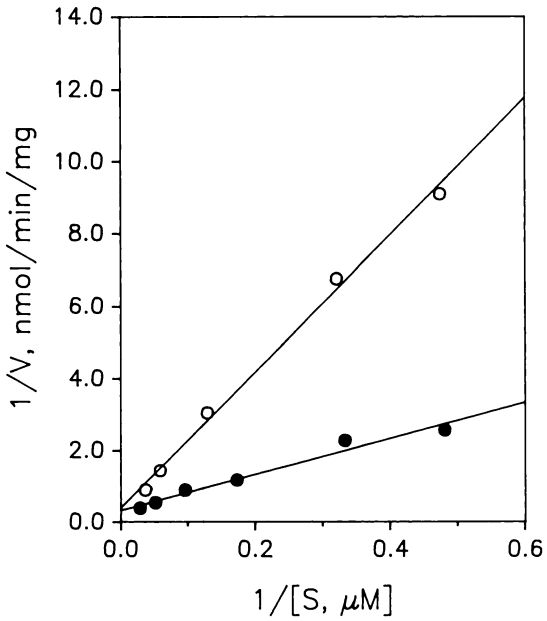

Fig. 4. Lineweaver-Burk analysis of the 2-hydroxylation of 17β-estradiol as catalyzed by human microsomes #4 in the absence (○) and presence (●) of 5 μM α-naphthoflavone. The experimental conditions were as described in the legend to Fig. 2 and linear regression lines are shown ($r^2 > 0.99$ and >0.97, respectively).

Discussion

The results presented here indicate that both a liver progesterone 6β-hydroxylase and a 17β-estradiol 2-hydroxylase are stimulated by α-naphthoflavone in man and rabbit. The effects of α-naphthoflavone on the kinetic properties of these enzymic reactions are very similar for each species. Characterization of the substrate dependence of the progesterone 6β-hydroxylase in B/J rabbits, which exhibit a low basal activity for this pathway in the absence of induction of the enzyme, reveals that α-naphthoflavone increases the apparent efficiency of the rifampicin-induced enzyme as judged by the decrease in slope of the double reciprocal plot. α-Naphthoflavone increases the efficiency of this enzyme in human liver microsomes in the same manner. These effects are similar to that elicited by 5β-pregnane-3β,20α-diol for the 16α-hydroxylation of progesterone catalyzed by one of two forms of rabbit P-450 3b (16) and to that of 4′-benzyloxy-3′-methoxy-7,8-benzoflavone on benzo[a]pyrene hydroxylation catalyzed by male rat liver microsomes (33). Huang *et al.* (5) indicated, however, that α-naphthoflavone and flavone stimulate the metabolism of benzo[a]pyrene-catalyzed liver microsomes prepared from untreated rabbits by altering V_{max} and not K_m. The relation of this enzyme to the one induced by rifampicin (10) is unclear.

The curvature seen in the double reciprocal plot in the absence of α-naphthoflavone suggests that the substrate itself activates both the human and rabbit enzymes. At high substrate concentrations, the activity observed in the absence of α-naphthoflavone appears to be slightly higher than that seen in its presence. This suggests that the apparent V_{max} is lower in the presence of α-naphthoflavone and that the increase in efficiency observed at lower substrate concentrations reflects a greater lowering of the apparent K_m for the substrate. Thus, both K_m and V_{max} appear to be altered by α-naphthoflavone. The effect of α-naphthoflavone on the K_m for 17β-estradiol can be more clearly seen because the double reciprocal plots are linear in the presence or absence of the effector. α-Naphthoflavone effects a roughly 3-fold lowering of the apparent K_m for 17β-estradiol.

It is interesting to note that although both progesterone and 17β-estradiol hydroxylations are stimulated in the RIF-B/J rabbit and in human microsomes by α-naphthoflavone, this flavonoid partially inhibits the *N*-demethylation of erythromycin in the same microsomes. These activities may be catalyzed by distinct enzymes. However, the differential effect on the two reactions could arise because α-naphthoflavone elicits a change in P-450 3c that favors steroid hydroxylations but diminishes erythromycin metabolism. Metyrapone was shown by Waxman and Walsh (34) to either inhibit or stimulate rat P-450 PB1 depending on the substrate employed.

The enzymes that are stimulated by α-naphthoflavone are induced by rifampicin in rabbits and by dexamethasone in man. The correlation between the activity of the enzymes which are stimulated by α-naphthoflavone and the expression of P-450 IIIA3 in man and the induction of P-450 3c in rabbits suggests that these cytochromes are the forms of P-450 that are stimulated by α-naphthoflavone. It is not clear whether these are the only P-450s that are activated by α-naphthoflavone. None of the monoclonal antibodies produced in this study were inhibitory, thus we cannot use them to directly gauge the contributions of these cytochromes to the catalysis of the stimulated reactions. Guengerich *et al.* (35) have shown that a polyclonal

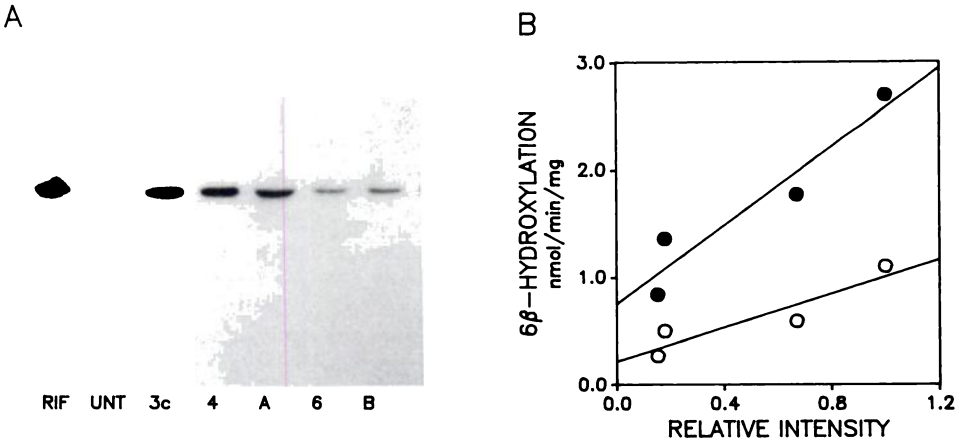

Fig. 5. A. Western blot of untreated B/J liver microsomes (*UNT*), rifampicin-B/J liver microsomes [*RIF*], P-450 3c (*3c*), human liver microsomes #4 (*4*), #FH39 (*A*), #6 (*6*), and #041581 (*B*). Ten μg of each rabbit microsomal sample, 5 pmol of P-450 3c and 20 μg of each human liver microsomal sample were resolved by sodium dodecyl sulfate-polyacrylamide electrophoresis (10% acrylamide concentration), Western blotted and visualized as described under Materials and Methods. B. The rates of progesterone [10 μM] 6β-hydroxylation in the presence (●) or absence (○) of 5 μM α-naphthoflavone are plotted versus the relative intensity determined by densitometry of the Western blot in A. Linear regression lines are also displayed, $r^2 > 0.9$ and 0.78, respectively.

antibody to P-450 IIIA3 extensively inhibits both estradiol 2-hydroxylation and testosterone 6β-hydroxylation catalyzed by human liver microsomes, indicating that the activity seen in the absence of α-naphthoflavone is largely catalyzed by P-450 IIIA3 in man.

The induction by rifampicin of the enzymes that are stimulated by α-naphthoflavone in the rabbit suggests that P-450 3c is the form of P-450 catalyzing this reaction. Based on the ratio of the activity induced by rifampicin (Table 1) to the apparent change in the concentration of P-450 3c, a turnover number of 2–4 nmol of 6β-hydroxyprogesterone/min/nmol of P-450 3c would be predicted for reconstituted P-450 3c at 10 μM progesterone. Lange *et al.* (12) have reported a value of 3.9 nmol of 6β-hydroxyprogesterone/min/nmol for reconstituted P-450 3c at an unspecified concentration of substrate. Our reconstituted preparations are less active. They are characterized by both a poor K_m, 130 μM, and a poor V_{max}, 1 nmol/min/nmol of P-450 3c. The turnover number at 10 μM progesterone is less than 0.1 nmol/min/nmol of P-450 3c. These low rates suggest that we cannot reconstitute this activity with the purified protein. Attempts to reconstitute this activity from other column fractions obtained in the first purification step were also unsuccessful. The activities obtained for the reconstitution of P-450s of family III for other species have generally been low (20, 36). Although the activity of the reconstituted enzyme is low, there is some evidence for activation. In the presence of α-naphthoflavone, a biphasic double reciprocal plot is obtained for purified P-450 3c reconstituted with reductase, suggesting that a portion of the enzyme exhibiting a V_{max} of about 0.1 nmol/min/nmol of P-450 3c is activated and that the activated enzyme exhibits a K_m of around 1 μM. In addition, Ingelman-Sundberg and Johansson (37) have presented evidence that a purified preparation of P-450, subsequently identified as P-450 3c, exhibited cooperativity for 6β-hydroxylation with respect to the concentration of androstenedione when reconstituted with reductase in egg yolk phosphatidylcholine vesicles. These authors also demonstrated that microsomes prepared from outbred, phenobarbital-treated New Zealand White rabbits exhibit positive cooperativity with respect to androstenedione for 6β-hydroxylation. Thus, the implication that P-450 3c and P-450 IIIA3 catalyze the reactions stimulated by α-naphthoflavone is largely derived from the coordinate expression of these activities with each cytochrome.[5]

The selectivity exhibited by different forms of P-450 for positive effectors suggests a selective interaction with the appropriate cytochrome. We cannot determine from these kinetic data whether this interaction occurs at the catalytic site or at a distinct effector site. Moreover, if the interaction occurs at the catalytic site, the activation of the enzyme could reflect either cooperative interaction of monomeric P-450 proteins or a hysteresis effect where initial binding of the allosteric effector elicits a transformation of the enzyme from one form to another which persists following the dissociation of the effector. If such a distinct effector site exists, it is interesting to note that it has been conserved in man and rabbit. Moreover, the potential that steroids can function as positive effectors of these enzymes hints that there may be as yet some unrealized function for this mode of regulation of in endogenous metabolism.

[5] Transient transection of COS-1 cells with the P-450 3c cDNA in an expression vector leads to the expression of a progesterone 6β-hydroxylase which is stimulated by α-naphthoflavone. This provides further evidence for the participation of P-450 3c in these reactions. Potenza *et al.*, manuscript in preparation.

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