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Oxidation of Quinidine by Human Liver Cytochrome P-450

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SUMMARY

The anti-arrhythmic quinidine has been reported to be a competitive inhibitor of the catalytic activities of human liver P-450$_{DB}$, including sparteine Δ^2-oxidation and bufuralol 1′-hydroxylation, and we confirmed the observation that submicromolar concentrations are strongly inhibitory. Human liver microsomes oxidize quinidine to the 3-hydroxy (K_m 4 μM) and *N*-oxide (K_m 33 μM) products, consonant with *in vivo* observations. Both bufuralol and sparteine inhibited microsomal quinidine 3-hydroxylation. Liver microsomes prepared from DA strain rats showed a relative deficiency in quinidine 3-hydroxylase activity in females compared to males. These observations might suggest that quinidine oxidation is catalyzed by the same P-450 forms that oxidize debrisoquine, bufuralol, and sparteine; i.e., rat P-450$_{UT\text{-}H}$ and P-450$_{DB}$. However, neither of these two purified enzymes catalyzed quinidine 3-hydroxylation, and anti-P-450$_{UT\text{-}H}$, which strongly inhibits human liver microsomal bufuralol 1′-hydroxylation, did not substantially inhibit quinidine 3-hydroxylation or *N*-oxygenation. P-450$_{MP}$, the human *S*-mephenytoin 4-hydroxylase, also does not appear to oxidize quinidine but P-450$_{NF}$, the human nifedipine oxidase, does. Anti-P-450$_{NF}$ inhibited >95% of the 3-hydroxylation and >85% of the *N*-oxygenation of quinidine in several microsomal samples. Quinidine inhibited microsomal nifedipine oxidation and, in a series of human liver samples, rates of nifedipine oxidation were correlated with rates of quinidine oxidation. Thus, quinidine oxidation appears to be catalyzed primarily by P-450$_{NF}$ and not by P-450$_{DB}$. Quinidine binds 2 orders of magnitude more tightly to P-450$_{DB}$, which does not oxidize it, than to P-450$_{NF}$, the major enzyme involved in its oxidation. The substrate specificity of human P-450$_{NF}$ is discussed further in terms of its regioselective oxidations of complex molecules including quinidine, aldrin, benzphetamine, cortisol, testosterone and androstenedione, estradiol, and several 2,6-dimethyl-1,4-dihydropyridines.

Oxidation by P-450 is a common aspect of the overall clearance of drugs as well as other xenobiotics and compounds endogenous to the body. Studies with experimental animal models have provided unequivocal evidence that a number of individual forms of the enzyme exist within a given tissue and that these forms differ in the substrates which they bind and the regio- and stereoselectivity of the oxidations that they catalyze (7). Evidence also exists that humans contain several different forms of P-450 and that these forms play a role in polymorphisms of drug oxidation (8, 9). The first of these to be recognized involved the 4-hydroxylation of debrisoquine (10). Subsequently, the P-450 involved in the reaction (P-450$_{DB}$) has been purified and characterized (2); P-450 forms involved in other polymorphisms have also been characterized (2, 3, 5).[3]

Quinidine has been used as a drug since 1749 (11). It is still used as an anti-arrhythmic, and interactions have been reported with other drugs (12, 13). Recently quinidine has been reported to be a competitive inhibitor of sparteine oxidation and bufuralol hydroxylation, two activities of P-450$_{DB}$ (14, 15). The low K_i value (0.06 μM) suggested that quinidine might be useful in probing structural features of the active site of P-450$_{DB}$, and finding the sites of oxidative attack of P-450$_{DB}$ on quinidine would be of use in this modeling process. We report here that quinidine is not a substrate for P-450$_{DB}$ or its rat ortholog, P-450$_{UT\text{-}H}$; instead another P-450 (P-450$_{NF}$) oxidizes quinidine. Thus, quinidine appears to be bound more tightly to one form of P-450 as an inhibitor than as a substrate to the major enzyme which metabolizes it. The structural features of other substrates for P-450$_{NF}$ are also considered in this report.

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[3] Human liver samples are denoted "HL" with a code number for each individual person. The only other purified P-450 preparation described in the literature comparable to any of the ones described in this paper is "P-450$_{BUF}$" (6) (cf. P-450$_{DB}$), although limited data do not yet permit complete comparison at this time.

ABBREVIATIONS: P-450, liver microsomal cytochrome P-450; HPLC, high performance liquid chromatography; IgG, immunoglobulin G fraction (of sera); P-450$_{UT\text{-}H}$, a rat P-450 with relatively high debrisoquine 4-hydroxylase, bufuralol 1′-hydroxylase, and sparteine Δ^5-oxidase activities which has been purified previously (1); P-450$_{DB}$, a human P-450 with debrisoquine 4-hydroxylase, bufuralol 1′-hydroxylase, and sparteine Δ^5-oxidase activities purified previously (2); P-450$_{MP}$, a human P-450 with *S*-mephenytoin 4-hydroxylase activity purified previously (3, 4); P-450$_{NF}$, a human P-450 with nifedipine oxidase activity which has been purified previously (4, 5); EI MS, electron impact mass spectroscopy; GC, gas chromatography.

Materials and Methods

Chemicals. Quinidine, quinine, and sparteine were purchased from Aldrich Chemical Co. (Milwaukee, WI) as the sulfate salts (hydrates) and used without further purification. (±)-Bufuralol and 1′-hydroxybufuralol were gifts of the Hoffman-LaRoche Co. (Nutley, NJ).

O-Desmethylquinidine (6-hydroxycinchonine) was prepared by slight modification of the method of Small *et al.* (16). It had m.p. 166–170° (dec) (lit. 167–171°) (16), NMR [$(CD_3)_2SO$]; (δ) 1.0–1.5 (m, 2*H*), 1.7–2.4 (m, 3*H*), 2.6 (d, 1*H*), 2.8–3.2 (m, 2*H*), 3.94 (m, 2*H*), 4.9–5.1 (d of d, 1*H*), 5.7 (s, 2*H*), 6.2 (d of d, 2*H*), 7.30–7.50 (m, 2*H*), 7.70 (d, 1*H*), 8.68 (d, 1*H*). EI MS *m/z* 310 (M^+), 136 (C_9H_4N): GC/EI MS (*bis*-trimethylsilyl ether) *m/z* 454 (M^+), 439 ($M-CH_3$).

Quinidine *N*-oxide was synthesized by peracid oxidation. To quinidine (1.0 g, 3.1 mmol) in 25 ml of dry CH_2Cl_2 was added *m*-chloroperbenzoic acid (0.59 g, 3.4 mmol) in portions over 30 min. The reaction mixture was stirred for 30 min at 0°. The solid was filtered, and the residue was evaporated to give a white solid (0.8 g), which was recrystallized from ether/hexane to yield quinidine *N*-oxide (0.74 g, 73%) m.p. 149–151° (lit. 148–150°) (17), NMR (δ) 1.0–1.4 (m, 1*H*), 1.7–2.3 (m, 3*H*), 2.7 (s, 3*H*), 2.8–3.5 (m, 6*H*), 4.8 (m, 1*H*), 5.2 (m, 2*H*), 6.2 (d of d, 1*H*), 7.3–7.5 (m, 4*H*), 7.8 (d, 1*H*), 8.0 (d, 1*H*), 8.7 (s, 1*H*): EI MS *m/z* 340 (M^+), 324 (M-16), 323 (M-OH), 136 ($C_9H_{14}N$).

3-Hydroxyquinidine was prepared by a modification of the method of Carroll *et al.* (18). Quinidine (2.0 g, 62 mmol) was dissolved in concentrated aqueous HBr (12.0 ml, 99 mmol) and heated at 40–50° for 50 hr. The reaction mixture was cooled in an ice-water bath and diluted with 50% aqueous NaOH. The aqueous solution was decanted from the white precipitate. The residual solid was dried under reduced pressure and then recrystallized from aqueous ethanol as white needles to yield a mixture of α- and α'-bromodihydroquinidine: NMR (δ) 1.4–1.95 (m, 8*H*), 2.7–3.1 (m, 4*H*), 3.4 (d of d, 2*H*), 3.95 (s, 3*H*), 4.4–4.7 (m, 2*H*), 5.7 (d, 1*H*), 7.3–7.5 (m, 2*H*), 7.7 (d of d, 1*H*), 7.9 (d, 1*H*), 8.6 (d, 1*H*). The bromodihydroquinidine mixture (1.3 g, 3.2 mmol) was treated with 1,5-diazabicyclo [5.4.0] undec-5-ene (0.88 g, 5.8 mmol) in $(CH_3)_2SO$ (10 ml) at 90° for 2 hr. The reaction mixture was cooled in ice-water and water (30 ml) was added. The white precipitate was filtered and recrystallized from absolute ethanol to give apoquinidine methyl ether: NMR (δ) 1.4–1.95 (m, 8*H*), 2.3–3.2 (m, 4*H*), 3.6–3.8 (d of d, 2*H*), 3.9 (s, 3*H*), 4.2 (t, 1*H*), 5.9 (s, 1*H*), 7.1–7.5 (m, 2*H*), 7.7 (d, 1*H*), 8.8 (d, 1*H*). Apoquinidine methyl ether (1.2 g, 3.7 mmol) was stirred in pyridine (1.9 ml) at room temperature under N_2. Acetic anhydride (3.6 ml, 3.8 mmol), together with a catalytic amount of 4-dimethylaminopyridine, was added and the solution was stirred for 48 hr. The reaction mixture was diluted with water and extracted with CH_2Cl_2. The CH_2Cl_2 layers were dried and evaporated to give the acetate (1.2 g): NMR (δ) 1.4–1.9 (m, 8*H*), 2.1 (s, 3*H*), 2.3–2.9 (m, 3*H*), 3.6–3.8 (m, 3*H*), 4.05 (s, 3*H*), 5.2 (m, 1*H*), 7.3–7.5 (d of d, 3*H*), 8.1 (d, 1*H*), 8.8 (d, 1*H*). The acetate (0.51 g, 1.4 mmol) was stirred in 80% CH_3CO_2H (10 ml) at 0° and $NaIO_4$ (0.9 g, 4.2 mmol) was added. OsO_4 (0.01 g, 0.42 mmol) was then added and the reaction mixture was stirred at 4° for 24 hr, filtered, and evaporated under reduced pressure. The solid was dissolved in water (10 ml); the water was evaporated and the solid residue was redissolved in dilute $NaHCO_3$ (30 ml). The alkaline solution was extracted with CH_2Cl_2; the organic layer was dried and evaporated. The solid residue was chromatographed on silica gel with 5% CH_3OH in CH_2Cl_2 to give 8*R*,9*S*-6-methoxy-3-oxo-9-rubanol acetate (0.28 g): m.p. 161–163° (lit. 162–163°) (18); NMR (δ) 1.8–2.0 (m, 4*H*), 2.1 (s, 3*H*), 2.5–3.0 (m, 3*H*), 3.2–3.7 (m, 3*H*), 4.0 (s, 3*H*), 4.2 (d of d, 1*H*), 6.5 (d, 1*H*), 7.3 (d of d, 2*H*), 8.0 (d, 1*H*), 8.7 (d, 1*H*). The rubanol acetate (0.6 g, 1.7 mmol) was added to a stirred solution of vinyl magnesium bromide (1.1 ml, 8.5 mmol) in tetrahydrofuran (10 ml) at room temperature under N_2. After stirring for 3 hr the reaction was diluted with aqueous NH_4Cl (30 ml) and extracted several times with CH_2Cl_2. The organic extracts were dried and evaporated. The residue was chromatographed on silica gel with 4% CH_3OH in ethyl acetate (1% triethylamine) to give 0.11 g of 3*S*-hydroxyquinidine and 0.10 g of the 3*R*-hydroxy epimer. 3*S*-Hydroxyquinidine had NMR (δ) 1.0–1.3 (m, 2*H*), 1.7–2.3 (m, 3*H*), 2.64 (d, 1*H*), 2.8–3.1 (m, 3*H*), 3.9 (d, 1*H*), 4.0 (s, 3*H*), 5.2 (d of d, 1*H*), 5.6 (d of d, 1*H*), 6.4 (d of d, 1*H*), 7.3–7.50 (m, 2*H*), 7.7 (m, 1*H*), 7.9 (d of d, 1*H*), 8.7 (d, 1*H*); GC/EI MS (trimethylsilyl ether) *m/z* 412 (M^+), 397 ($M-CH_3$), 152 ($C_9H_{14}NO$).

2-Oxoquinidinone was a kind gift of Dr. Dennis Drayer, Cornell University. Examination of the trimethylsilyl ether derivative by GC/EI MS revealed the absence of any other quinidine metabolites. The mass spectrum of the derivative showed *m/z* 385 (M^+), 370 ($M-CH_3$), 136 ($C_9H_{14}N$).

Enzyme preparations. Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN) and used as sources of liver enzymes. Livers from DA strain rats were shipped frozen in dry ice from Dr. A. Küpfer, University of Bern, Switzerland. Human liver samples were obtained from organ donors through the Nashville Regional Organ Procurement Agency, Nashville, TN, and for long-term storage, stored frozen at −70° after rapid freezing in liquid nitrogen (4). Liver microsomes were prepared and stored frozen in glycerol solutions (4). Rat liver P-$450_{UT\text{-}H}$ (1) and human liver P-450_{DB} (2), P-450_{MP} (3), and P-450_{NF} (5) were isolated as described previously and reconstituted with rabbit NADPH-P-450 reductase and L-α-dilauroyl-*sn*-glycero-3-phosphocholine as described in the above references, using the components listed for the assays here. Polyclonal rabbit and goat antibodies were prepared against rat P-$450_{UT\text{-}H}$ (1, 2, 19, 20) and human liver P-450_{MP} (3) and P-450_{NF} (5, 21); criteria for specificity and other properties are described in the indicated references.

Assays. Protein was estimated using Pierce BCA reagent as described by the manufacturer (Pierce Chemical Co., Rockford, IL); samples were heated at 60° for 30 min and the standard curve was prepared with bovine serum albumin ($E^{1\%}_{278}$ = 6.67). Spectral P-450 measurements were made as described by Omura and Sato (22).

Microsomal enzyme incubations were carried out in the following general manner. Microsomes (generally equivalent to about 50 pmol of P-450 unless indicated otherwise) were incubated for 10–15 min at 37° with the substrate and 0.1 M potassium phosphate buffer (pH 7.7) in the presence of an NADPH-generating system (final concentrations of 10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 0.5 IU of yeast glucose 6-phosphate dehydrogenase/ml) in a total volume of 0.50 ml.

Amber vials (7 ml total capacity, Pierce Chemical Co.) were used for quinidine and quinine incubations because of the sensitivity of these compounds to light. In general, the quinidine concentration was 20 μM. Incubations were terminated by the addition of 0.25 ml of cold 0.6 M sodium borate buffer (pH 9.0), and the solution was extracted with 1.0 ml of a CH_2Cl_2-isopropanol mixture (4:1, v/v), with mixing using a vortex device and centrifugation for 10 min at 3000 × *g*; 0.7 ml of the lower phase was transferred to a 3.0-ml capacity amber Reacti-vial (Pierce Chemical Co.) (23). To the remaining material another 1.0 ml of the CH_2Cl_2:isopropanol (4:1, v/v) mixture was added; after mixing and centrifugation, 0.9 ml of the lower phase was added to the Reacti-vial. The resulting (organic phase) samples were reduced to dryness under a nitrogen stream at 30°. Each sample was taken up in 50 μl of CH_3OH, and 20 μl of the solution were analyzed by HPLC. Two basic HPLC systems were utilized. The first was patterned after the method of Guentert *et al.* (23): an Altex cyanopropyl column (4.6 × 250 mm, Beckman Instruments, Palo Alto, CA) was used with a mobile phase consisting of 65% (v/v) 50 mM potassium phosphate buffer (pH 4.75), 30% (v/v) CH_3CN, and 5% (v/v) tetrahydrofuran. The flow rate was 2.5 ml/min. This system was used for routine analysis of 3-hydroxyquinidine. The retention times of 3-hydroxyquinidine and quinidine were 3.0 and 4.75 min, respectively; quinidine *N*-oxide was not resolved from quinidine. The other HPLC system involved a 4.6 × 250 mm Altex octadecylsilyl (C_{18}) column and a mobile phase consisting of 85% (v/v) CH_3OH and 15% (v/v) 20 mM $(NH_4)_2HPO_4$ buffer (pH 9.4) (24). This was used primarily when quinidine *N*-oxide measurements were desired, even though quinidine *N*-oxide (t_R 4.30 min) was not completely separated from 3-hydroxyquinidine (t_R 4.05 min) (quinidine, t_R 13.0 min). In both cases the HPLC unit consisted of a Spectra-Physics 8700 pumping/control system, Spectra-Physics dynamic mixer,

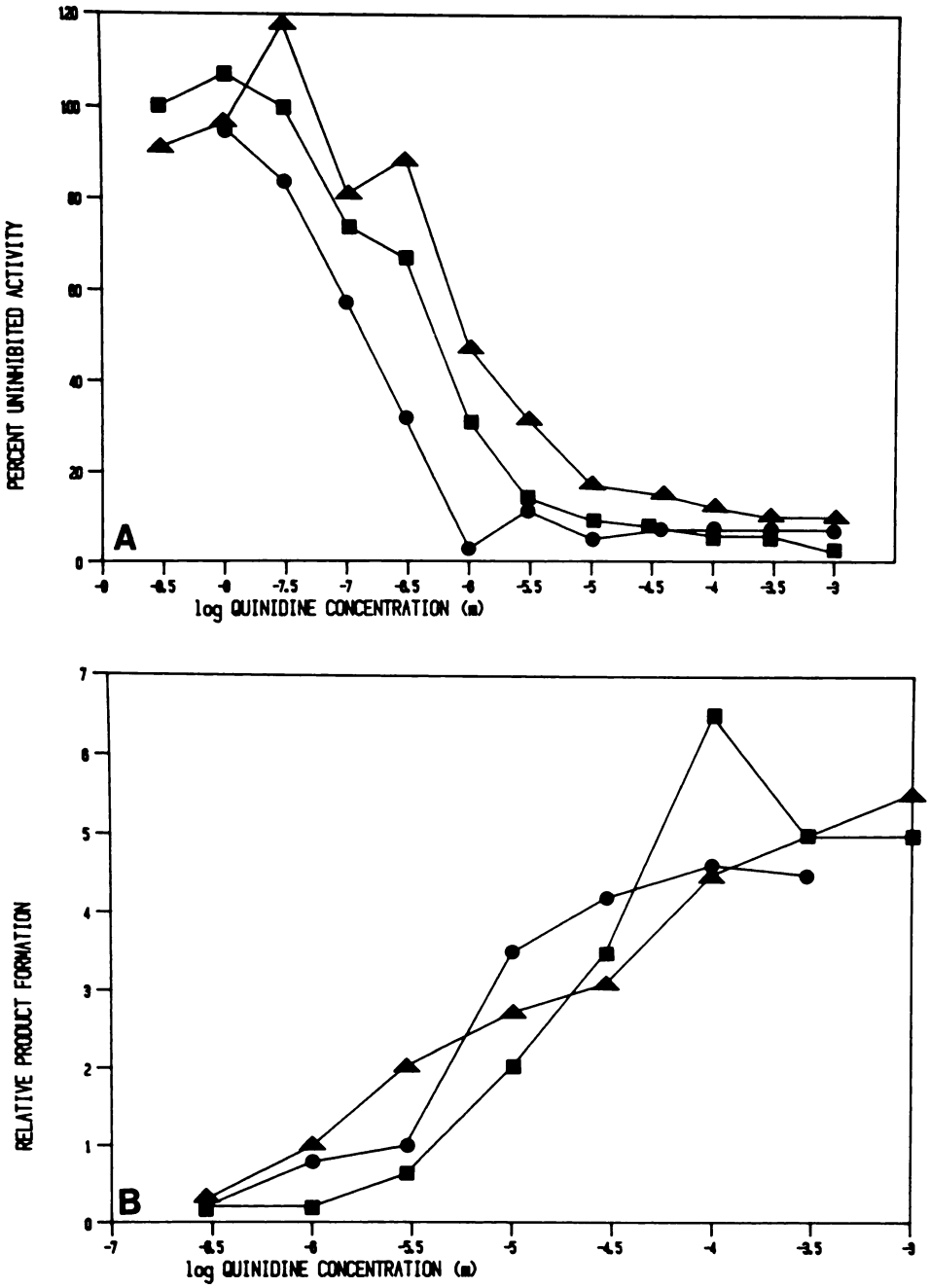

Fig. 1. Inhibition of bufuralol 1′-hydroxylation and formation of a quinidine metabolite. A. Inhibition of bufuralol 1′-hydroxylation. Microsomal protein from liver samples HL 24 (■), HL 39 (▲), or HL 96 (●) equivalent to 500 pmol of P-450 was incubated under the standard conditions for 15 min with 0.5 mM (±)-bufuralol and varying concentrations of quinidine. Rates of 1′-hydroxybufuralol formation were 0.79, 0.87, and 0.37 nmol/min/nmol of P-450 for samples HL 24, HL 39, and HL 96, respectively. B. Formation of an apparent quinidine metabolite. In the analysis of the samples in A, a new peak was observed (t_R 1.7 min) following 1′-hydroxybufuralol (1.1 min) (in this particular study, a 10-cm Rainin Short-one C_{18} octadecylsilyl HPLC column was used with an eluent of 85% (v/v) CH_3OH and 15% (v/v) $(NH_4)_2HPO_4$ (pH 9.2) and a flow rate of 2.0 ml/min). The relative fluorescence of the new peak is plotted as a function of the quinidine concentration.

a 1-cm octadecylsilyl (C_{18}) or cyanopropyl guard column, the HPLC column itself, and a Kratos GM 970 fluorescence detector (excitation at 245 nm, emission at 340 nm; Kratos, Ramsey, NJ), and a Hewlett-Packard 3320A integrator. Quantitations were usually done using external standards.

Bufuralol 1′-hydroxylation was measured as described previously (2, 20, 24) or using the cyanopropyl HPLC system; the fluorescence excitation maximum was 250 nm and the emission wavelength was 300 nm. Quantitations were usually done using external standards; in some cases quinidine was used as an internal standard. Nifedipine oxidation was assayed as described elsewhere (5).

Results

Inhibition of bufuralol 1′-hydroxylation by quinidine. Quinidine has been reported to be a strong competitive inhibitor of the microsomal oxidation of sparteine and bufuralol, two generally accepted substrates for P-450_{DB}; the K_i for both has been reported to be about 0.06 μM (14, 15). We also found that quinidine could inhibit >90% of the 1′-hydroxylation of racemic bufuralol in several different microsomal samples (Fig. 1A). In these experiments the bufuralol concentration was 0.5 mM, which is approximately 50 times the K_m value; 50% inhibition of the activity was observed with quinidine concentrations of 0.1–0.9 μM. No evidence for biphasicity was observed in the inhibition pattern. Meyer *et al.* (15) have suggested that two forms of P-450_{DB} (termed P-450_{BUF} in that report) exist, one which hydroxylates essentially only (+)-bufuralol and is highly sensitive to quinidine inhibition and another which hydroxylates both enantiomers of bufuralol equally well and is 3 orders of magnitude less sensitive to quinidine. In rat and human liver microsomes, the ratio of rates of 1′-hydroxylation

TABLE 1

Comparison of quinidine 3-hydroxylase and bufuralol 1′-hydroxylase activities in rat liver microsomes and purified enzymes

Procedures are described under Materials and Methods. Results obtained with rat liver microsomes [DA or SD (Sprague-Dawley)] are expressed as means of determinations made with three individual animals (±SD); duplicate incubations were done with each preparation and the results were averaged before means were calculated.

Preparation	P-450	Bufuralol 1′-hydroxylase activity	Quinidine 3-hydroxylase activity
	nmol/mg microsomal protein	*nmol/min/ nmol P-450*	*nmol/min/ nmol P-450*
Female DA rat microsomes	0.92 ± 0.14	0.29 ± 0.08	0.11 ± 0.03
Male DA rat microsomes	1.07 ± 0.21	0.86 ± 0.06	0.82 ± 0.14
Female SD rat microsomes	1.06 ± 0.10	2.37 ± 0.15	0.64 ± 0.13
Male SD rat microsomes	0.90 ± 0.05	1.91 ± 0.16	1.42 ± 0.16
Rat P-450$_{UT-H}$		2.70 ± 0.51	0.23 ± 0.24
Human P-450$_{DB}$		0.98 ± 0.12	<0.02

of the (+)- and (−)-isomers is about 2 (2, 6, 15). Thus, if this view were correct, quinidine might be expected to strongly inhibit only about one-third of the microsomal 1′-hydroxylation of (±)-bufuralol. Although only three microsomal samples were examined and other possibilities cannot be ruled out, such results were not observed in the course of these experiments.

The possibility was considered that quinidine generally inhibits electron transport involving P-450$_{DB}$. However, the rate of NADPH oxidation in a reconstituted system containing rabbit NADPH-P-450 reductase and purified human liver P-450$_{DB}$ (18 nmol of NADPH oxidized/min/nmol of P-450$_{DB}$) was unchanged by the addition of 20 μM quinidine.

Oxidation of quinidine. During the course of the microsomal bufuralol 1′-hydroxylase inhibition studies, the appearance of a new fluorescent peak in the HPLC profiles was noted (Fig. 1B). The area of the peak increased as a function of the quinidine concentration (apparent K_m 7–20 μM). Subsequent experiments indicated that the peak was formed from quinidine and not from bufuralol as the result of metabolic switching (i.e., shunting of bufuralol to other products).

Two different HPLC systems were used to separate and quantify the quinidine metabolites, which were identified by comparison to authentic standards (see Materials and Methods). The K_m values estimated for the formation of 3-hydroxyquinidine and quinidine *N*-oxide were about 4 μM and 33 μM, respectively (no evidence for multiphasic behavior was observed), and most of the subsequent experiments were carried out with a quinidine concentration of 20 μM. At these and higher concentrations, the major product is 3-hydroxyquinidine and the only other prominent product is quinidine *N*-oxide, consonant with *in vivo* metabolic studies (23). The formation of these products was absolutely dependent upon the addition of NADPH. No significant amounts of 2-oxoquinidinone or *O*-desmethylquinidine (standards were separated by HPLC) (23) or other fluorescent products were found.

Although quinidine was oxidized with low K_m values, the addition of various concentrations of quinidine to microsomes (range of 0.2–300 μM) did not lead to the production of detectable difference spectra in the Soret region (results not shown).

Since quinidine had been found to be an effective inhibitor of the hydroxylation of bufuralol and sparteine, the ability of these latter two P-450$_{DB}$ substrates to inhibit quinidine 3-hydroxylation was examined. Both compounds did inhibit quinidine oxidation; under the typical quinidine oxidation conditions used, about 0.5 mM (±)-bufuralol or 1 mM sparteine inhibited the reaction by 50%. However, analysis using Dixon and other plots did not suggest that the inhibition was of a competitive nature.

Quinidine 3-hydroxylation by rat liver microsomes and purified P-450$_{UT-H}$ and P-450$_{DB}$. One approach that has been used to gain insight into the roles of the debrisoquine-hydroxylating enzymes P-450$_{UT-H}$ and P-450$_{DB}$ in various metabolic transformations is examination of patterns of oxidation by DA strain rats. Female DA strain rats are deficient in P-450$_{UT-H}$ and its catalytic activities when compared to male

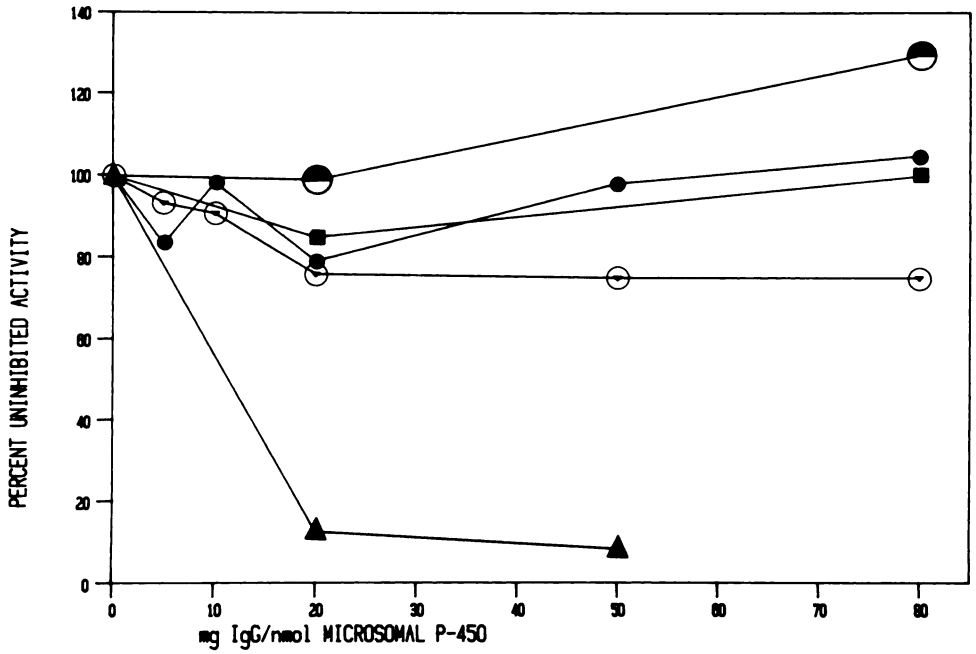

Fig. 2. Effects of antibodies on microsomal bufuralol and quinidine oxidation. Liver microsomal sample HL 96 and goat anti-P-450$_{UT-H}$ were used in the particular study shown; uninhibited rates of bufuralol 1′-hydroxylation (▲), quinidine 3-hydroxylation (●), and quinidine *N*-oxygenation (⊙) were 0.26, 0.82, and 0.19 nmol of product formed/min/nmol of P-450, respectively. Also shown are the effects of a preimmune IgG preparation on quinidine 3-hydroxylation (■) and *N*-oxygenation (◓).

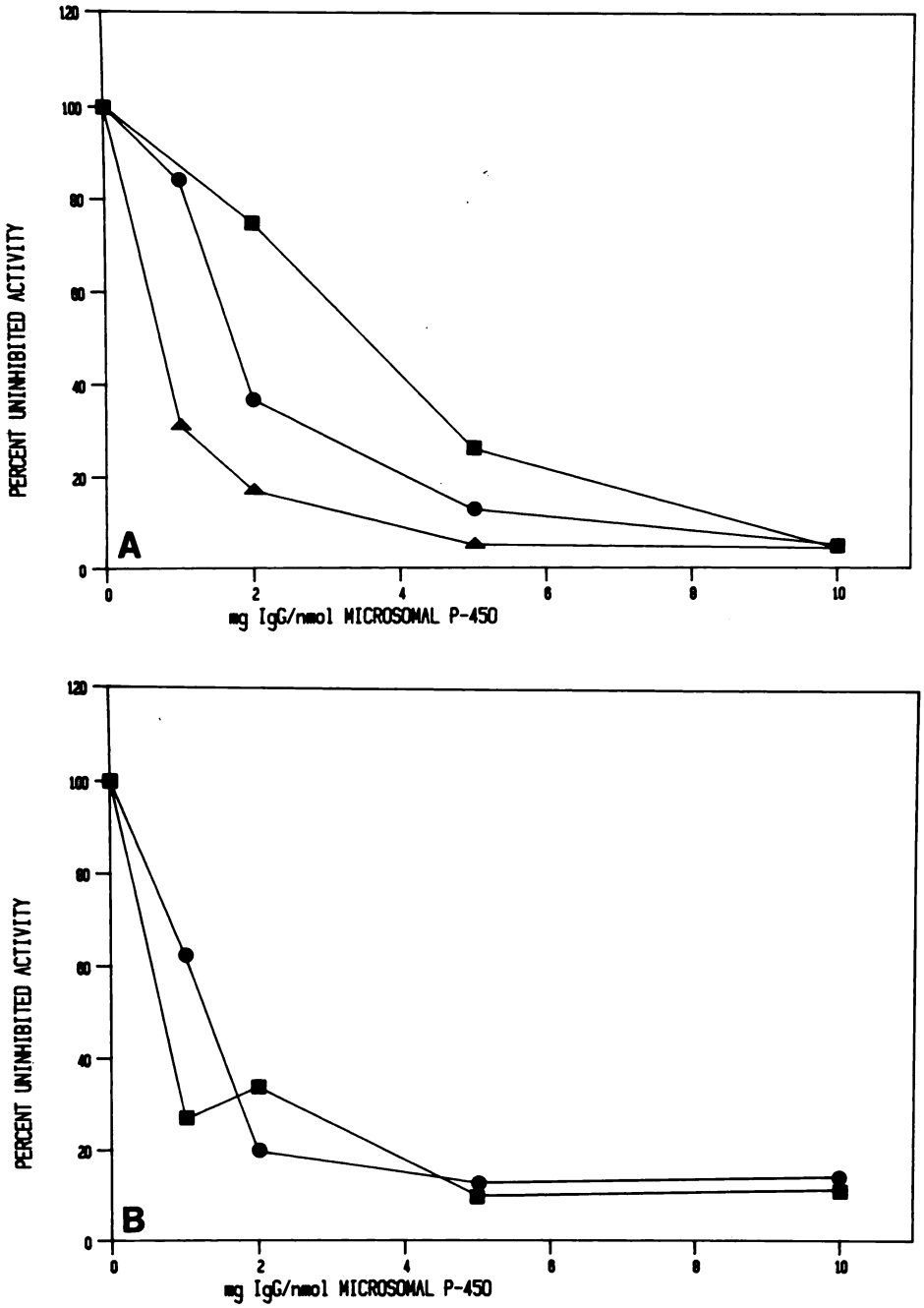

Fig. 3. Effect of anti-P-450$_{NF}$ on microsomal quinidine oxidation. A. Effects of anti-P-450$_{NF}$ on quinidine 3-hydroxylation in liver samples HL 94 (▲), HL 39 (●), and HL 95 (■); the respective uninhibited activities were 1.17, 1.24, and 0.38 nmol of product formed/min/nmol of P-450. B. Effects of anti-P-450$_{NF}$ on quinidine *N*-oxygenation in liver samples HL 39 (●) and HL 95 (■); the uninhibited activity was 0.23 nmol of product formed/min/nmol of P-450 in both cases.

DA rats or rats of other strains (1, 25). We also found that microsomes prepared from DA strain females showed decreased catalytic activity for quinidine 3-hydroxylation when compared to preparations derived from DA strain males or Sprague-Dawley males or females (Table 1). However, the sex difference in quinidine 3-hydroxylation was apparent in Sprague-Dawley as well as DA strain rats. The pattern was not the same as that observed in the case of bufuralol 1′-hydroxylation. The variation among the rats was not due to differences in the total amount of P-450 per mg of microsomal protein.

Table 1 also indicates that preparations of purified P-450$_{UT-H}$ and P-450$_{DB}$ catalyzed bufuralol 1′-hydroxylation but, under the same reconstitution conditions (which were not optimized completely here), did not catalyze quinidine 3-hydroxylation.

Quinidine oxidation activity of human liver P-450 forms. As indicated in Table 1, P-450$_{DB}$ and its rat ortholog P-450$_{UT-H}$ had little catalytic activity toward quinidine. Purified P-450$_{MP}$ did not catalyze quinidine 3-hydroxylation either. Purified P-450$_{NF}$ catalyzed quinidine 3-hydroxylation and *N*-oxygenation, although the rates were low (~0.2 nmol product/min/nmol of P-450) and rather variable. The complexity of reconstitution of P-450$_{NF}$ has been noted before (5) and, in order to circumvent problems in the interpretation of the relative rates of microsomal and purified P-450 forms, we utilized inhibitory antibodies to elucidate the contributions of individual P-450s.

Anti-P-450$_{UT-H}$, which extensively inhibits bufuralol 1′-hydroxylation in human liver microsomes, did not affect quinidine 3-hydroxylation or *N*-oxygenation in human liver microsomes

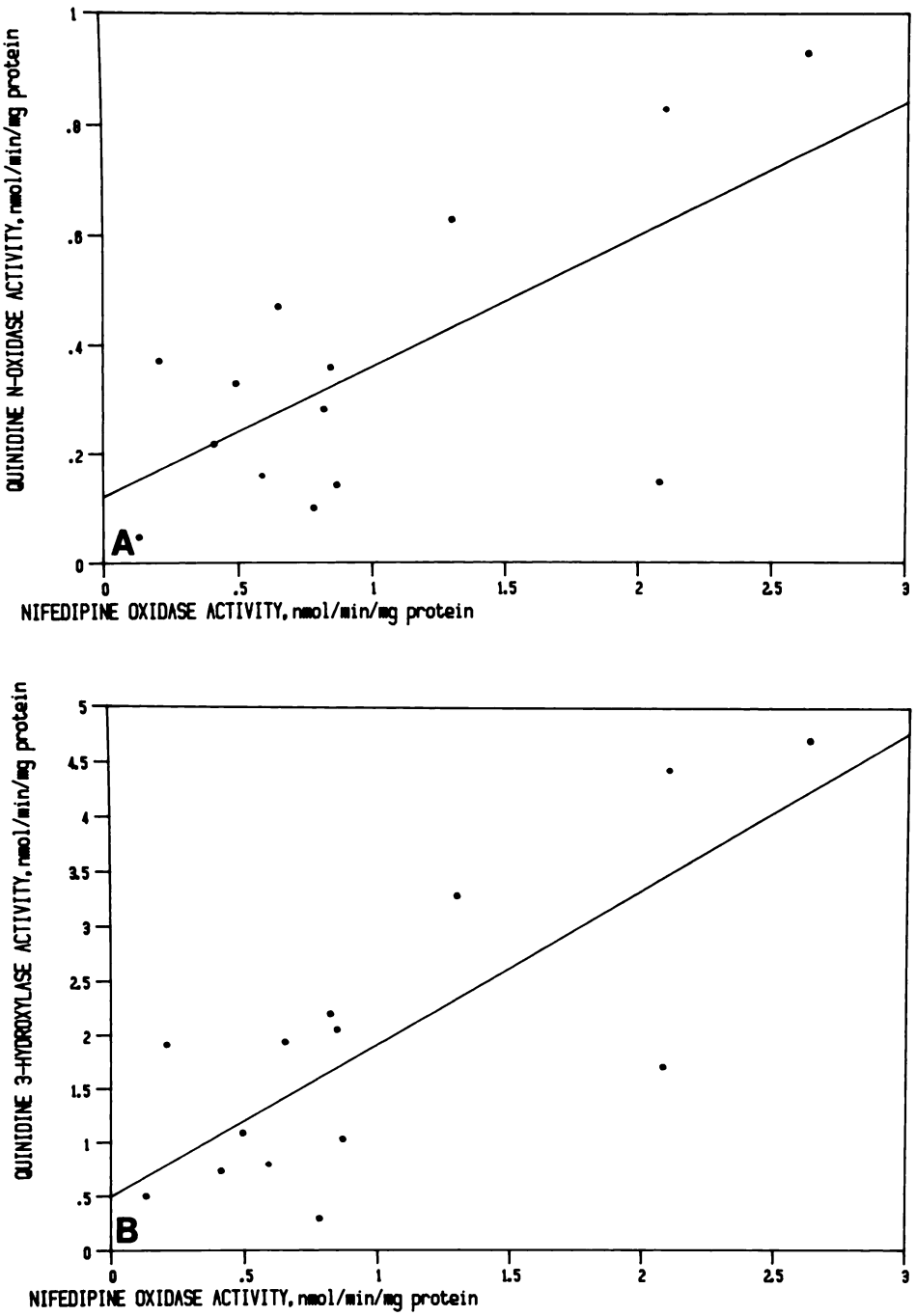

Fig. 4. Correlation of rates of quinidine 3-hydroxylation (A) and quinidine *N*-oxygenation (B) with rates of nifedipine oxidation in 14 human liver microsomal preparations. The lines were drawn using linear regression analysis without weighting.

(Fig. 2). The experiments presented here were carried out with a goat antibody preparation; a similar lack of inhibition was noted with two different antisera raised in rabbits (results not shown). The addition of a particular rabbit anti-P-450$_{MP}$ preparation which completely inhibits human liver microsomal *S*-mephenytoin 4-hydroxylation when added at a ratio of 5 mg of IgG/nmol of P-450 (3) decreased the quinidine 3-hydroxylase activity of this microsomal preparation by <25%. However, the addition of rabbit anti-P-450$_{NF}$ (5) (10 mg of IgG/nmol of P-450) decreased the quinidine 3-hydroxylase activity to 11% of the control value. The anti-P-450$_{NF}$ inhibition studies were extended to other human liver microsomal preparations as well (Fig. 3); extensive inhibition of both quinidine 3-hydroxylation and *N*-oxygenation was found, although some *N*-oxygenation appears to be resistant.

Correlation of quinidine and nifedipine oxidation rates. Since the above work indicated that quinidine is oxidized by P-450$_{NF}$ or a closely related form of P-450, a correlation between oxidation rates of quinidine and nifedipine might be expected. When a set of liver microsomal samples obtained from 14 different individuals were compared, rates of nifedipine oxidation were correlated to rates of quinidine 3-hydroxylation (r = 0.78) and *N*-oxygenation (r = 0.67) (Fig. 4). Rates of quinidine 3-hydroxylation and *N*-oxygenation were also highly correlated to each other (r = 0.91). These correlations are all statistically significant at the $p < 0.005$ level (t test). We had previously shown that rates of nifedipine oxidation were correlated with immunochemically determined levels of microsomal P-450$_{NF}$ (5). Further analysis of the samples used here

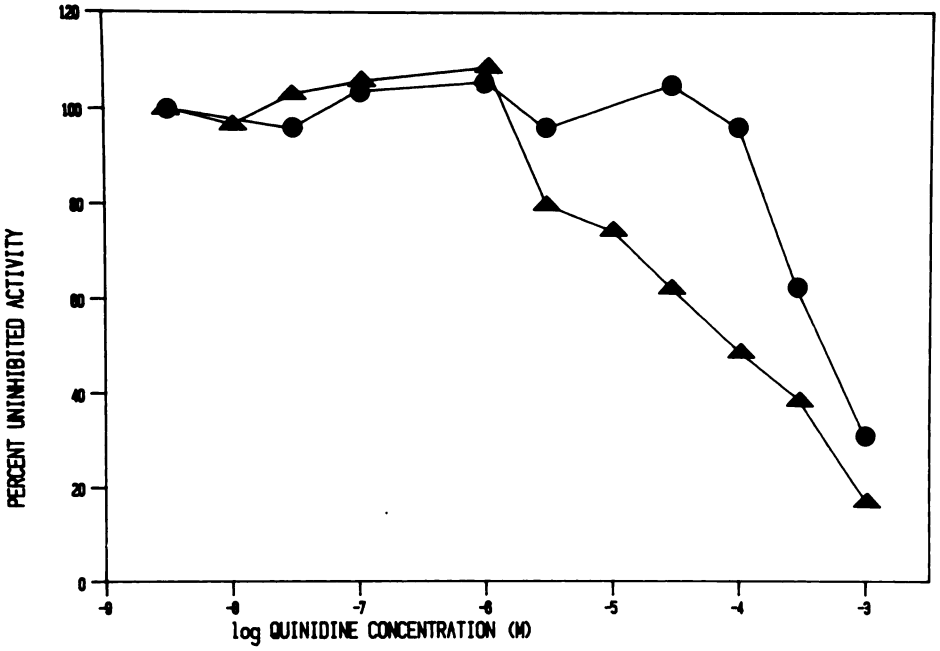

Fig. 5. Inhibition of nifedipine oxidase activity by quinidine. Liver samples HL 24 (●) and HL 95 (▲) were used. The uninhibited rates of nifedipine oxidation were 5.4 (HL 24) and 3.1 (HL 95) nmol of product formed/min/nmol of P-450.

indicated correlation of both quinidine 3-hydroxylation and *N*-oxygenation rates with levels of immunochemically determined P-450$_{NF}$ ($r = 0.82$ in both cases, $p < 0.005$).

Competition of quinidine and nifedipine. Fig. 5 shows the inhibition of human liver microsomal nifedipine oxidation by quinidine. Comparison with Fig. 1A suggests that quinidine is about 200 times more effective in inhibiting bufuralol 1′-hydroxylation than nifedipine oxidation.

Both nifedipine and sparteine concentrations were varied in another competition experiment using a single preparation of human liver microsomes (HL 35). Dixon plots indicated a mixed inhibition pattern, with a tendency toward uncompetitive inhibition at higher (>50 μM) quinidine concentrations. In this set of experiments the apparent K_i value for nifedipine inhibition of quinidine 3-hydroxylation was 14 μM and the apparent K_i value for quinidine inhibition of nifedipine oxidation was 10 μM. These may be compared to respective apparent K_m values of 19 μM and 4 μM for nifedipine oxidation and quinidine 3-hydroxylation.

Discussion

The work presented here indicates that P-450$_{NF}$ is the form of human liver P-450 which is responsible for the oxidation of the anti-arrhythmic agent quinidine to its major products, 3-hydroxyquinidine and quinidine *N*-oxide. The level of P-450$_{NF}$ has been shown to vary in individuals and nifedipine oxidase activity is highly correlated with the amount of the protein (5). Kleinbloesem *et al.* (26) found a segregation of rats of *in vivo* nifedipine clearance and metabolite formation into two groups; this bimodality has not been corroborated elsewhere yet, nor has the biochemical basis been more closely examined. The literature also suggests that quinidine oxidation may be inducible by barbiturates and rifampicin (12, 13), and our assignment of the catalytic activities to P-450$_{NF}$ would be consistent with this observation and with other lines of evidence, such as the increased 2-hydroxylation of estrogens in patients receiving rifampicin, for 2-hydroxylation of estradiol is catalyzed by P-450$_{NF}$ (5). A similar case is found with cortisol 6β-hydroxylation (27). Thus, one might expect quinidine oxidation to show a variation in humans similar to that of nifedipine and to possibly show similar induction patterns.

The finding that P-450$_{NF}$ is the principal catalyst of quinidine oxidation is somewhat surprising in light of the extensive inhibition of P-450$_{DB}$ by the compound. The available kinetic data [K_i of 0.06 μM for inhibition of sparteine and bufuralol 1′-hydroxylation (14, 15), K_m of 4 μM for quinidine 3-hydroxylation, K_m of 33 μM for quinidine *N*-oxygenation, and K_i of 10 μM for inhibition of nifedipine oxidation] suggest tighter binding of quinidine to a site which does not lead to metabolism. This conclusion is supported by the results presented in Figs. 1A and 5. We are unaware of a precedent for tighter binding of a compound to one P-450 form as an inhibitor than to the major form involved in its oxidation. The difference appears to be about 200-fold.

The formation of an *N*-oxide by P-450 is not particularly common, at least in cases where dealkylation (heteroatom release) is possible (28). Generally, when α-hydrogens are available, they are abstracted to result in eventual α-carbon oxidation after initial aminium ion formation. However, Bredt's Rule applies here, and the abstraction of the α-hydrogen is prevented. The possible contribution of liver microsomal flavin-containing monooxygenase (29) to *N*-oxide formation cannot be completely evaluated here, for the enzyme can be labile (29), and our own immunoinhibition studies might not have dealt with the activity if it had been lost prior to preparation of the microsomal samples.

Inhibition of oxidation of certain substrates with other potential substrates has been used in several cases as an approach toward elucidating substrate specificity (14). However, the study described here points out the pitfalls that can be encountered in drawing conclusions from the sole use of this approach. A similar case involved assignment of phenacetin *O*-deethylase activity to P-450$_{DB}$ (30), a conclusion which was supported by correlative analysis and experiments involving DA strain rats (25). However, closer inspection of the steady state kinetics and eventually purification studies led to the

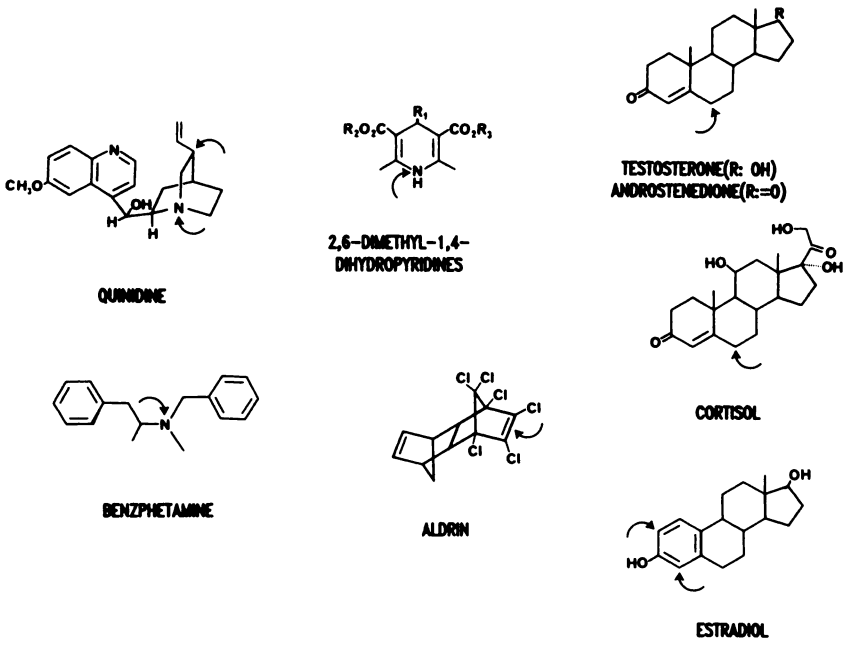

Fig. 6. Structures of substrates for human liver P-450$_{NF}$. *Arrows* indicate primary sites of oxidation. Evidence for the reactions comes from the following sources: quinidine (this work), 2,6-dimethyl-1,4-dihydropyridines (21), aldrin (5), benzphetamine (4, 5), cortisol (27), testosterone and estradiol (5), and androstenedione.[4] In the *N*-dealkylation reactions, the initial site of oxidation is presumed to be the nitrogen (non-bonded electrons) (28). See the text for further discussion.

delineation of P-450$_{DB}$ and P-450$_{PA}$, the high affinity *O*-deethylase (2). In this light, several androgens and estrogens and cortisol have recently been reported to inhibit microsomal mephenytoin 4-hydroxylation (31); most of these steroids now appear to be substrates for P-450$_{NF}$ and not P-450$_{MP}$ (5, 27).

Fig. 6 presents the structures of some of the compounds which are now accepted to be substrates for human P-450$_{NF}$ or closely related P-450s (5), and the sites of oxidation are shown with *arrows*. Two-dimensional inspection of the structures does not indicate any obvious similarities. This is in apparent contrast to the situation with P-450$_{DB}$ substrates, where the basic amine seems to have a role (20). Originally we anticipated that delineation of the sites of oxidation of quinidine might facilitate the characterization of the active sites of P-450$_{DB}$ and P-450$_{UT-H}$. The distance between the basic nitrogen and the 3-position is reasonably appropriate for the working model we have published (20). However, the *lack* of oxidation of quinidine would imply that *none* of the potentially oxidizable sites are positioned near the putative perferryl oxygen (28). Thus, any modeling of the active site of P-450$_{DB}$ must take these points into consideration. Otton *et al.* (14) noted that the removal of a methoxy group from the isoquinoline ring decreased the inhibition of sparteine Δ^2-oxidation by quinidine; we found no evidence for *O*-demethylation at all in microsomes. Otton *et al.* (14) also reported that the stereoisomer quinine was much less effective as an inhibitor (than was quinidine). We found that human liver microsomes catalyzed the NADPH-dependent oxidation of quinine to a polar compound having quinidine fluorescence (i.e., detected with $F_{245/340}$) with a retention time similar to that of 3-hydroxyquinidine in the cyanopropyl HPLC system. The apparent rate of formation appeared to be similar to that of 3-hydroxyquinidine (formed from quinidine), but this product has not been identified, nor is the role of P-450$_{NF}$ in its formation known.

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[4] D. J. Waxman and F. P. Guengerich, unpublished results.

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