Microsomal Cytochrome P450 Dependent Oxidation of N-Hydroxyguanidines, Amidoximes, and Ketoximes: Mechanism of the Oxidative Cleavage of Their C═N(OH) Bond with Formation of Nitrogen Oxides

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ABSTRACT: Oxidation by rat liver microsomes of 13 compounds involving a C═N(OH) function (including N-hydroxyguanidines, amidoximes, ketoximes, and aldoximes) was found to occur with the release of nitrogen oxides such as NO, NO_2^-, and NO_3^-. The greatest activities were observed with liver microsomes from dexamethasone-treated rats (up to 8 nmol of NO_2^- nmol of $P450^{-1}$ min^{-1}). A detailed study of the microsomal oxidation of some of these compounds was performed. Oxidation of *N*-(4-chlorophenyl)-N′-hydroxy-guanidine led to the formation of the corresponding urea and cyanamide in addition to NO, NO_2^-, and NO_3^-. Formation of all these products was dependent on NADPH, O_2, and cytochromes P450. Oxidation of two arylamidoximes was found to occur with formation of the corresponding amides and nitriles in addition to nitrogen oxides. Oxidation of 4-(chlorophenyl)methyl ketone oxime gave the corresponding ketone and nitroalkane as well as NO, NO_2^-, and NO_3^-. These reactions were also dependent on cytochromes P450 and required NADPH and O_2. Mechanistic experiments showed that microsomal oxidations of amidoximes to the corresponding nitriles and of ketoximes to the corresponding nitroalkanes are not inhibited by superoxide dismutase (SOD) and are performed by a cytochrome P450 active species, presumably the high-valent P450-iron-oxo complex. On the contrary, microsomal oxidation of N-hydroxyguanidines to the corresponding cyanamides was greatly inhibited by SOD and appeared to be mainly due to $O_2^{\bullet-}$ derived from the oxidase function of cytochromes P450. Similarly, microsomal oxidations of N-hydroxyguanidines and amidoximes to the corresponding ureas and amides were also found to be mainly performed by $O_2^{\bullet-}$, as shown by the great inhibitory effect of SOD (70−100%) and the ability of the xanthine−xanthine oxidase system to give similar oxidation products. However, it is noteworthy that other species, such as the P450 Fe(II)−O_2 complex, are also involved, to a minor extent, in the SOD-insensitive microsomal oxidative cleavages of compounds containing a C═N(OH) bond. Our results suggest a general mechanism for such oxidative cleavages of C═N(OH) bonds with formation of nitrogen oxides by cytochromes P450 and NO-synthases, with the involvement of $O_2^{\bullet-}$ and its Fe(III) complex [(FeIII−O_2^-) or (FeII−O_2)] as main active species.

Generation of nitric oxide (NO) in biological systems has recently been discovered to play a key role in a large variety of physiological functions (1,2).[1] In vivo, NO biosynthesis is dependent on a class of enzymes, called NO synthases (NOSs), which catalyze the oxidation of L-arginine (Arg) by NADPH and O_2 with formation of L-citrulline and NO in two steps. The first step is the monooxygenation of one terminal nitrogen atom of the guanidine function of Arg with formation of N^{ω}-hydroxy-L-arginine (NOHA). This step exhibits all the characteristics of a classical cytochrome P450 dependent monooxygenation reaction (consumption of 1 mol of O_2 and 1 mol of NADPH) (eq 1). The second step is a

three-electron oxidation of NOHA resulting in an oxidative cleavage of the C═NOH bond of NOHA with formation of citrulline and NO (eq 2). This reaction differs from classical monooxygenations as it consumes 1 mol of O_2 and only 0.5 mol of NADPH (3,4).

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[1] Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; Arg, L-arginine; NOHA, N^{ω}-hydroxy-L-arginine; BH_4, tetrahydrobiopterin; 3-MC, 3-methylcholanthrene; PB, phenobarbital; DEX, dexamethasone; P450, cytochrome P450; BHT, 3,5-ditertiobutyl 4-hydroxy toluene; DMSO, dimethyl sulfoxide; TAO, triacetyloleandomycin; TLC, thin-layer chromatography; G6P, glucose 6-phosphate; G6PDH, glucose 6-phosphate dehydrogenase; DLPC, L-α-dilauryl phosphatidylcholine; SOD, superoxide dismutase; X, xanthine; XO, xanthine oxidase.

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NOSs have been identified as heme-thiolate proteins, closely related to cytochromes P450 (*5*−*8*), except that they contain three prosthetic groups in addition to the heme: FAD, FMN, and tetrahydrobipterin (BH_4). NOSs exhibit several UV−vis, EPR, and resonance Raman spectroscopic properties in common with "classical P450s" (*9*−*12*). P450s and NOSs are also comparable in the type of reactions that they catalyze. The first step of NOSs, the N-hydroxylation of Arg has not been found so far to be catalyzed by a P450. However, P450s have been reported to catalyze similar N-hydroxylations of amidines, such as benzamidines (*13*, *14*) and pentamidine (*15*), and guanidines such as debrisoquine (*16*, *17*). The second step of NOSs has been found to be also catalyzed by rat liver cytochromes P450 (*18*, *19*). Finally, both NOSs and P450s are able to act as oxidases as they catalyze the reduction of O_2 by NADPH to O_2^-, H_2O_2, and H_2O. This uncoupling reaction may occur in the absence of substrate (Arg for NOSs) (*20*), or in the presence of compounds that bind to the active site and initiate electron transfer but either cannot undergo oxygen atom transfer or act as bad substrates for the oxygenation step (*21*), a situation well documented in the case of P450s (*22*).

Rat liver microsomes catalyze the oxidative cleavage of the C═N(OH) bond not only of NOHA, but also of some exogenous compounds. For instance, 4-hexyloxybenzamidoxime is transformed to 4-hexyloxybenzamide by a P450-dependent oxidation of its C═N(OH) bond (*23*). Nitrogen oxides are also formed in these reactions, and NO was detected as a mixture of its P450− and P420−Fe(II)-NO complexes by EPR spectroscopy (*18*, *23*). Similarly, the P450-dependent oxidations of two compounds of pharmacological importance have been reported. *N*-hydroxydebrisoquine, a N-hydroxyguanidine, is transformed to the corresponding urea by rabbit liver microsomes and by purified P450 2C3 (*17*). Cytochrome P450 2C3 also catalyzes the N-hydroxylation of one of the amidine functions of the drug pentamidine, with formation of N^{ω}-hydroxypentamidine. This metabolite is further oxidized to the corresponding amide and nitrogen oxides, including NO (*15*). Finally, preliminary results have shown that many compounds containing a C═N−OH function, - i. e., aldoximes, ketoximes, amidoximes, and N-hydroxyguanidines, are oxidized by liver microsomal cytochromes P450 with formation of the corresponding organic products bearing a C═O function and nitrogen oxides (*24*).

This paper describes a detailed study of the oxidation of several compounds containing a C═N−OH function by rat liver microsomes. It shows that the oxidation of N-hydroxyguanidines not only leads to the corresponding ureas but also to the corresponding cyanamides. Similarly, microsomal oxidation of amidoximes leads to the corresponding amides and nitriles. It also shows that these oxidative cleavages of C═N bonds to the corresponding C═O bonds involve a P450-dependent transfer of one oxygen atom from O_2 to the substrate, with concomitant formation of nitrogen oxides. The reaction mechanisms are discussed in relation with that of the second step of NOSs.

MATERIALS AND METHODS

Materials. ^{1}H NMR spectra were recorded with a Brucker ARX spectrometer operating at 250 MHz. The chemical shifts (δ) were reported in parts per million relative to tetramethylsilane. The coupling constants (J) were expressed in hertz with multiplicity of the signals noted: s = singlet, d = doublet, t = triplet, q = quadruplet, and m = multiplet. Infrared spectra were recorded on a Perkin-Elmer 783 spectrophotometer and the characteristic absorption frequencies reported in wave numbers. Mass spectra (MS) were recorded on a Riber-Mag R10−10 C mass spectrometer (Ecole Normale Superieure, Paris, France) operating at 70 eV and using NH_3 in the chemical ionization (CI) or electron impact (EI) mode. EPR spectroscopy was performed at 77 K on a Varian E-109 spectrophotometer using standard conditions as previously described (*18*, *23*). Melting points were determined on a Büchi apparatus using capillary tubes and were uncorrected. HPLC analysis was performed on a Spectra-Physics D2000 multisolvent delivery system and a multiwavelength scanning UV−vis detector Spectra Focus. UV−vis spectroscopy experiments were performed on a Kontron 941 spectrophotometer using 1 cm path length cuvettes. Fluorescence was measured on a Kontron SFM 25 spectrofluorimeter. Thin layer chromatography (TLC) was performed on silicagel on aluminum plates from Merck. All common chemicals and solvents (from Sigma, Janssen, or Aldrich) were of the highest grade commercially available and used without further purification.

Reagents and Biochemicals. NADPH (sodium salt) and NADH (sodium salt) came from Boehringer Mannheim. Superoxide dismutase (SOD) (from bovine erythrocytes, 3000 units/mg), catalase (from bovine liver, 20 000 units/mg), nitrate reductase (from *Aspergillus niger*, 600 units/mg), xanthine oxidase (XO) (from buttermilk, 0.5 units/mg), sulfanilamide, phenobarbital (PB), lactic acid dehydrogenase (from bovine heart, 600 units/mg), sodium pyruvate, and xanthine (X) were obtained from Sigma. Miconazole, 3-methylcholanthrene (3-MC), dexamethasone (DEX), and 1,2-diaminonaphthalene came from Janssen. 4-Chloroaniline, potassium cyanate, 4-chlorobenzamide **2b**, 4-chlorobenzonitrile **2c**, *N*,*N*-dimethyl benzamide **4b,** 4-chloroacetophenone **5b**, 4-(*n*-hexyloxy)benzoyl chloride, 1-(4-chlorophenyl) ethanol, *N*-(1-naphthyl) ethylenediamine dihydrochloride, and hydroxylamine hydrochloride came from Aldrich.

*Synthesis. N-(4-Chlorophenyl)-N′-hydroxyguanidine **1a*** was prepared in 65% yield by reaction of cyanamide **1c** with hydroxylamine hydrochloride in anhydrous ethanol according to the procedure of Schantl and Türk (*25*). Mp 129 °C [lit. (*25*) 129−131 °C]. ^{1}H NMR (d_6-DMSO): 8.42 (s, 1H), 7.76 (s, 1H), 7.30 (d, 2H, J = 8.7), 7.16 (d, 2H, J = 8.7), 5.08 (s, 2H). IR (KBr): 3480, 3340, 1660, 1090, 820.

*4-Chlorophenyl urea **1b*** was obtained in 90% yield by reacting 4-chloro aniline and potassium cyanate in 0.5 M HCl. Mp 210 °C [lit. (*25*) 212 °C]. ^{1}H NMR (d_6-DMSO): 8.65 (s, 1H), 7.41 (d, 2H, J = 8.8), 7.23 (d, 2H, J = 8.8), 5.89 (s, 2H). IR (KBr): 3420, 3310, 1650, 1540, 820. MS (CI) *m*/*z*: 190 (M + NH_4^+), 188 (M + NH_4^+), 173 (M + H^+), 171 (M + H^+, 100).

*4-Chlorophenyl cyanamide **1c*** was obtained in 75% yield after treatment of 4-chloroaniline with cyanogen bromide according to the method of Schantl and Türk (*25*). Mp 97 °C [lit. (*25*) 98−100 °C]. ^{1}H NMR (d_6-DMSO): 10.35 (s, 1H), 7.37 (d, 2H, J = 9.2), 6.96 (d, 2H, J = 9.2). IR (KBr): 3150, 3080, 2230, 1600, 1500, 810. MS (CI): *m*/*z* 170 (M^+ + NH_3), 154, 152 (M^+, 100).

4-Chlorobenzamidoxime ***2a*** *and 4-(n-hexyloxy)benzamidoxime* ***3a*** were obtained in 55 and 45% yields, respectively, by reacting 4-chlorobenzonitrile **2c** and 4-(*n*-hexyloxy)-benzonitrile **3c** with hydroxylamine hydrochloride using a conventional method (*26*).

4-Chlorobenzamidoxime ***2a***: mp 134 °C [lit. (*27*) 135 °C]. ^{1}H NMR ($CDCl_3$): 9.66 (s, 1H), 7.69 (d, 2H, $J = 8.0$), 7.42 (d, 2H, $J = 8.0$), 5.80 (s, 2H). IR (KBr): 3470, 3340, 1920, 1650, 1090, 830.

4-(n-Hexyloxy)benzamidoxime ***3a***: mp 112 °C [lit. (*28*) 110 °C]. ^{1}H NMR ($CDCl_3$): 7.55 (d, 2H, $J = 8.0$), 6.90 (d, 2H, $J = 8.0$), 4.80 (s, 2H), 3.98 (t, 2H, $J = 7.5$), 1.78 (m, 2H), 1.45 and 1.35 (m, 6H), 0.90 (t, 3H, $J = 7.5$). IR (KBr): 3440, 3340, 2920, 1650, 1120, 830.

4-(n-Hexyloxy)benzamide ***3b*** was synthesized in 90% yield by treatment of 4-(*n*-hexyloxy)benzoyl chloride with ammonia. Mp 154 °C [lit. (*23*) 154 °C]. ^{1}H NMR ($CDCl_3$): 7.78 (d, 2H, $J = 8.0$), 6.92 (d, 2H, $J = 8.0$), 5.78 (m, broad, 2H), 4.0 (t, 2H, $J = 7.0$), 1.79 (m, 2H), 1.45 and 1.32 (m, 6H), 0.92 (t, 3H, $J = 7.5$). IR (KBr): 3380, 3170, 2920, 1640, 1610, 1250, 840. MS (CI): *m*/*z* 239 ($M + NH_4^+$), 222 ($M + H^+$, 100).

4-(n-Hexyloxy)benzonitrile ***3c*** was prepared in 83% yield by alkylation of the sodium salt of 4-cyanophenol with *n*-hexyl bromide in anhydrous ethanol. Mp 28 °C. ^{1}H NMR ($CDCl_3$): 7.53 (d, 2H, $J = 8.7$), 6.90 (d, 2H, $J = 8.7$), 3.98 (t, 2H, $J = 6.5$), 1.72 (m, 2H), 1.25 (m, 6H), 0.82 (t, 3H, $J = 6.7$). IR (KBr): 3080, 2920, 2220, 1610, 1260, 830. MS (EI): *m*/*z* 203 (M^+), 119 (*100*), 102.

E-N,N-Dimethyl benzamidoxime ***4a*** was prepared in 54% yield by the addition at 0 °C of a solution of benzhydroxamoyl chloride (*29*) in diethyl ether to a benzene solution of dimethylamine according to a described procedure (*30*). The product was completely converted to the E-isomer by crystallization from toluene as reported (*30*). Mp 130 °C [lit. (*30*) 132 °C]. ^{1}H NMR ($CDCl_3$): 9.05 (s, 1H), 7.53 (broad s, 5H), 2.61 (s, 6H). IR (KBr): 3260, 3000, 2960, 1620, 1370, 950. MS (EI): *m*/*z* 163 (M^+), 147, 104, 77, 51, 44 (*100*).

4-(Chlorophenyl) methyl ketone oxime ***5a*** was obtained in 75% yield after treatment of 4-chloroacetophenone **5b** with hydroxylamine hydrochloride according to a conventional method (*31*). Mp 94 °C [lit. (*31*) 95 °C]. The product was a mixture of Z (95%)- and E- (5%)-isomers as determined by ^{1}H NMR ($CDCl_3$). Z-isomer: 9.38 (s, 1H), 7.54 (d, 2H, $J = 8.0$), 7.34 (d, 2H, $J = 8.0$), 2.25 (s, 3H). E-isomer: 9.35 (s, 1H), 7.62 (d, 2H, $J = 8.0$), 7.42 (d, 2H, $J = 8.0$), 2.36 (s, 3H).

1-(4-Chlorophenyl)-1-nitro ethane ***5c*** was synthesized in 20% overall yield from 1-(4-chlorophenyl)-1-ethanol after bromination with PBr_3 in pyridine (*32*) followed by reaction with silver nitrite in *N,N*-dimethylformamide according to the method of Kornblum and Wade (*33*) and purification by flash chromatography (SiO_2, cyclohexane:ethyl acetate 9/1, r_f, 0.41). ^{1}H NMR ($CDCl_3$): 7.37 (m, 4H), 5.55 (q, 1H, $J = 7.0$), 1.86 (d, 3H, $J = 7.0$). IR (film): 3080, 2990, 1550, 1500, 1100, 840. MS (CI): *m*/*z* 205, 203 ($M^+ + NH_3$), 201, 173, 170, 156, 139 (100).

Compounds **6**−**13** were prepared according to previously described procedures (*24*, *31*).

Preparation of Rat Liver Microsomes. Male Sprague−Dawley rats (175−200 g) were treated i.p. for 4 days either with PB (80 mg/kg/day, in 0.9% saline), 3-MC (20 mg/kg/day, in corn oil), or DEX (50 mg/kg/day, in corn oil). Control rats were treated with corn oil (0.5 mL) only. Microsomes were prepared as previously reported (*34*) and stored at −80 °C until use. Protein concentrations were determined by the method of Lowry (*35*) with bovine serum albumin as standard and cytochrome P450 contents as described previously (*36*).

Binding to rat liver cytochromes P450 was studied by difference visible spectroscopy after addition of increasing amounts of substrates to liver microsomes containing 2 μM P450 in 1 cm path length cuvettes. Difference spectra were recorded between 380 and 500 nm.

The SOD contents in rat liver microsomes were measured using the inhibition by SOD of nitrite formation from hydroxylamine in the presence of a xanthine−xanthine oxidase O_2^- generating system (*37*). Quantitations were done by comparison to standard curves obtained in the presence of increasing amounts of SOD.

Purification of P450 2C3. Preparation of P450 isozymes mixture was obtained from untreated male rabbit liver microsomes and separation of P450 isozymes was performed by preparative HPLC on an anion-exchange column as previously described (*14*).

Enzymatic Incubations. Rat Liver Microsomes. In a typical incubation, substrate (100 μM), liver microsomes (0.5 nmol of P450, about 0.3−0.5 mg of protein), and 0.1 M phosphate buffer, pH 7.4 (500 μL, final volume), were preincubated for 1 min at 37 °C. The reaction was started by addition of 1 mM NADPH and carried out for 10 min at 37 °C. Incubations were stopped either by mixing thoroughly with 500 μL ice-cold acetonitrile containing a known amount of the internal standard for RP-HPLC analysis or by heating for 3 min at 100 °C for NO_3^- (and NO_2^-) assays. Proteins were precipitated by centrifugation (15 min at 10 000 rpm) at 4 °C. Some experiments were performed in the absence of NADPH, upon addition of various amounts of NADH, P450 inhibitors, SOD, or catalase. In some cases, NADPH was substituted by cumyl hydroperoxide (500 μM, final concentration).

Incubations performed under an $^{18}O_2$ atmosphere were carried out as follows. Phosphate buffer (80−100 mL) was boiled for 5 min and degassed with argon for 1 h. Substrates **1a**−**5a** (100−200 μM) were dissolved in the deaerated buffer, and $^{18}O_2$ (99% pure, Matheson) was bubbled through the mixture. Microsomal proteins were added (0.7−1.2 mg/mL), and the mixture was preincubated 5 min at 37 °C before addition of NADPH (1 mM). Reactions were then performed for 20 min at 37 °C and stopped by 5 min of boiling. The mixture was centrifugated 30 min at 3000 rpm, and the supernatant was extracted with methylene chloride. The organic metabolites were purified by TLC using a mixture of ethyl acetate/cyclohexane (9/1, v/v). Spots corresponding to the authentic compounds were scraped, and the silica was extracted with methylene chloride. Metabolites were then further characterized by their HPLC-retention times, UV, ^{1}H NMR, and MS spectra, and comparison to those of authentic samples.

Incubations Using P450 2C3 Based Reconstituted Monoxygenase. Purified P450 2C3 (0.56 nmol mL^{-1}), 0.3 unit of cytochrome P450 reductase, and 40 μM L-α-dilauryl phosphatidylcholine (DLPC) were preincubated 3 min at 37

°C in 50 mM phosphate buffer, pH 7.4. *N*,*N*-Dimethylbenzamidoxime (1 mM), NADPH (0.5 mM), $MgCl_2$ (3.3 mM), and further indicated components were then added to start the reaction (180 μL of total volume). Reaction was stopped by addition of 20 μL of 72% (m/v) trichloroacetic acid, shaking for 5 min, and centrifugation for 10 min at 11 000*g* before HPLC analysis of the supernatant. For the measurement of nitrite ions, the reaction (500 μL of final volume) was stopped by addition of lactic acid dehydrogenase and pyruvate (10 units/mL and 10 mM, respective final concentrations). The mixture was further incubated 10 min at 37 °C, stopped by addition of 100 μL of ice-cold acetonitrile, and centrifugation for 10 min at 11000*g* before measurement of nitrite ions with the Griess reaction.

Xanthine−Xanthine Oxidase System. Incubation mixtures contained 100 μM substrate and 2.5 mM xanthine (X) in 0.1 M phosphate buffer, pH 7.4 (500 μL, final volume). After preincubation for 1 min at 28 °C, xanthine-oxidase (XO) (0.02−0.05 units/mL) was added and the reations were carried out for 10 min. Incubations were stopped and the metabolites were detected as previously described when using the microsomal proteins.

Analytical Procedures. Determination of Nitrite. (1) Fluorimetric Method. Aliquots (250 μL) of supernatant were mixed with 100 μL of 2,3-diaminonaphthalene (0.05 mg/mL in 0.6 N HCl) and 600 μL of water. After 10 min at room temperature, 50 μL of 2.8 N NaOH was added and the fluorescence intensity was measured with excitation at 365 nm and emission at 403 nm according to the method of Misko et al. (*38*).

(2) Griess Reaction. Centrifugated supernatant (500 μL) was mixed for 10 min at room temperature with sulfanilamide (150 μL of 1.8% solution in 1 M HCl) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (100 μL of 0.3% solution in 1 M HCl) before measurement of the absorbance at 543 nm (*39*).

Determination of Nitrate. Nitrate ions were quantitated after reduction to nitrite ions according to a described method (*40*). Aliquots (200 μL) of supernatant were mixed with 100 μL of a mixture containing nitrate reductase (60 milliunits/mL), NADPH (10 μM), G6P (500 μM), and G6PDH (0.1 units/mL), and incubations were carried out 30 min at 28 °C. The total amounts of NO_2^- and NO_3^- ions were determined using the previous fluorimetric assay (*38*), and nitrate levels were obtained by subtracting the previously obtained nitrite values.

Quantitations of NO_2^- and NO_3^- were obtained from standard curves which were run in the same way as the experimental samples.

RP-HPLC Analysis. Separations of metabolites were performed at 25 °C on a 250 × 4.6 mm Nucleosil ODS 5 μ column (SFCC-Shandon, France). Flow rate was 1 mL/min, and the mobile phase was a gradient between solvent A (water containing 5 mM phosphoric acid, pH 2.6) and solvent B (acetonitrile) as indicated.

Analysis of *N*-(4-chlorophenyl)-*N*′-hydroxyguanidine **1a** metabolites was performed using the following solvent program: 0 min, 5% B; 5 min, linear gradient to 40% B in 15 min; 20 min, linear gradient to 100% B in 10 min; 30 min, linear gradient to 5% B in 5 min followed by 15 min reequilibration. The absorbance was monitored at 240 nm. The retention times for N-hydroxyguanidine **1a**, urea **1b**, cyanamide **1c**, and benzamide (internal standard) were 17.3, 25.8, 28.7, and 19.8 min, respectively.

Solvent program for analysis of 4-chlorobenzamidoxime **2a** metabolites was as follows: 0 min, 15% B; 2 min, linear gradient to 60% B in 15 min; 17 min, linear gradient to 100% B in 10 min; 30 min, linear gradient to 15% B in 3 min followed by reequilibration. The absorbance was monitored at 245 nm. The retention times for amidoxime **2a**, amide **2b**, nitrile **2c** and 4-nitrobenzamide (internal standard) were 7.5, 16.3, 22.5, and 14.1 min, respectively.

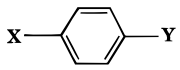
Metabolites from **5a** and **3a** were separated by using the following solvent program: 0 min, 40% B; 2 min, linear gradient to 100% B in 23 min; 30 min, linear gradient to 40% B in 3 min, followed by reequilibration. The absorbance was monitored simultaneously at 225 and 255 nm. The retention times for ketoxime **5a**, ketone **5b**, and nitroethane **5c** were 11.3, 12.7, and 15.0 min while retention times for benzamidoxime **3a**, benzamide **3b**, and benzonitrile **3c** were 7.9, 15.8, and 21.6 min, respectively. Internal standard (4-methylbenzonitrile) had a retention time of 12.1 min.

Metabolites from incubations of *N*,*N*-dimethylbenzamidoxime **4a** were analyzed on a LiChrospher 60RP-select B column (250 × 4.6 mm, 5 μ) using isocratic conditions. The mobile phase was a mixture of acetonitrile/water/phosphoric acid/1-octanesulfonic acid sodium salt (13/87/0.004/0.001 v/v/v/M). Flow rate was 1 mL/min, and detection was performed at 225 nm. The retention times for *N*,*N*-dimethylbenzamide **4b** and the corresponding amidoxime **4a** were 21.5 and 50.7 min, respectively.

RESULTS

*(1) Oxidation of N-(4-Chlorophenyl)-N′-hydroxy Guanidine, **1a**, by Liver Microsomes from Rats Treated with Dexamethasone (DEX).* Incubation of N-hydroxyguanidine **1a** with aerobic liver microsomes from rats pretreated with DEX, in the presence of NADPH, led to two organic products which exhibit HPLC retention times and UV spectra identical to those of authentic samples of *N*-(4-chlorophenyl)urea **1b** and *N*-(4-chlorophenyl)cyanamide **1c** (Figure 1). More complete identification of metabolites **1b** and **1c** was obtained after large-scale incubations (80 mL) followed by extraction and characterization by thin-layer chromatography, ^{1}H NMR, and mass spectrometry. All characteristics of the metabolites were identical to those of authentic samples of **1b** and **1c**, which have been synthesized independently. Formation of NO_2^- and NO_3^-, which was followed by a classical fluorimetric method (see Materials and Methods), appeared to occur at the same time as **1b** and **1c**. In fact, formation of **1b**, **1c**, and nitrite linearly increased with time, for at least 10 min, and with the amount of protein (up to 1.5 mg mL^{-1}) (data not shown).

The corresponding reaction was clearly enzymatic, as boiled microsomes were inactive, and required both NADPH and O_2 (Table 1). NADH could not be used instead of NADPH, and rat liver cytosolic proteins were inactive. These data suggested that microsomal cytochromes P450 were involved in these reactions. Accordingly, a classical inhibitor of those cytochromes P450, miconazole (*22*), led to an almost complete (80−90%) inhibition of the formation of the organic products **1b** and **1c** and of nitrogen oxides



Compound	X	Y	Compound	X	Y
1a	Cl	NH-C(NH_2)=N-OH	**4a**	H	C(=N-OH)N(CH_3)$_2$
1b	Cl	NH-CO-NH_2	**4b**	H	CO-N(CH_3)$_2$
1c	Cl	NH-CN			
2a	Cl	C(NH_2)=N-OH	**5a**	Cl	C(CH_3)=N-OH
2b	Cl	CO-NH_2	**5b**	Cl	CO-CH_3
2c	Cl	CN	**5c**	Cl	CH(CH_3)-NO_2
3a	nC_6H_{13}O	C(NH_2)=N-OH			
3b	nC_6H_{13}O	CO-NH_2			
3c	nC_6H_{13}O	CN			

FIGURE 1: Structure of the substrates used in this study and of their main metabolites.

Table 1: Effects of Incubation Conditions on the Formation of Urea **1b**, Cyanamide **1c**, NO_2^-, and NO_3^- from Oxidation of *N*-(4-Chlorophenyl)-*N*′-hydroxyguanidine **1a** by DEX-Treated Rat Liver Microsomes

incubation conditions[a]	activities[a]			
	1b	**1c**	NO_2^-	NO_3^-
complete system	14.2 ± 2.1	10.3 ± 2.4	22.2 ± 3.5	7.9 ± 2.2
−microsomes	0.2 ± 0.2	0.3 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
boiled microsomes[b]	0.6 ± 0.5	0.9 ± 0.7	0.2 ± 0.2	0.1 ± 0.1
−microsomes + cytosol[c]	0.2 ± 0.2	0.3 ± 0.3	1.7 ± 1.6	0.4 ± 0.3
−NADPH	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1
$-O_2$[d]	1.0 ± 0.1	1.0 ± 0.1	3.2 ± 1.8	nd[g]
−NADPH + NADH[e]	1.5 ± 0.1	1.2 ± 0.2	0.4 ± 0.3	nd[g]
+NADH[e]	12.8 ± 0.9	8.6 ± 1.5	nd[g]	nd[g]
+miconazole (50 μM)	2.2 ± 0.8	2.0 ± 1.0	2.9 ± 1.8	1.0 ± 0.8
+TAO (50 μM)	4.4 ± 1.3	4.6 ± 1.3	2.9 ± 1.6	1.4 ± 1.0
+CO[f]	1.8 ± 0.2	3.9 ± 1.3	8.2 ± 1.5	nd[g]

[a] Results are expressed as nmol of metabolite nmol of $P450^{-1}$ (10 min)$^{-1}$ and are means ± SD from five to eight determinations. The complete system consists in a suspension of DEX-treated rat liver microsomes (0.4 mg of protein mL^{-1}; 1 μM P450) in 0.1 M phosphate buffer, pH 7.4, containing 100 μM **1a** and 1 mM NADPH. [b] Microsomal proteins were heated for 10 min at 90 °C before the experiment. [c] Cytosolic proteins (about 0.5 mg/mL) were used instead of microsomes. [d] Reactions were performed under anaerobic conditions using argon bubbling. [e] NADH concentration 1 mM. [f] Reactions were performed using buffer saturated by a 1/1 (vol/vol) mixture of CO/O_2. [g] nd, not determined.

(Table 1). Another nonspecific inhibitor of cytochromes P450, carbon monoxide, and triacetyloleandomycine (TAO), a specific inhibitor of P450 3As (41), also strongly inhibited the reaction.

*(2) Effects of Classical Inducers of Liver Cytochromes P450 on Microsomal Oxidation of **1a**.* Pretreatment of rats with classical inducers of cytochromes P450, such as 3-methylcholanthrene (3-MC), phenobarbital (PB), or DEX, resulted in a strong increase of the ability of liver microsomes to oxidize **1a** to urea **1b** (by 2-, 8-, and 17-fold, respectively) (Figure 2). It also resulted in an increased activity of liver microsomes to transform **1a** into cyanamide **1c**, although to a lesser extent (by 2-, 5-, and 8-fold) (Figure 2). As a consequence, the **1b**:**1c** ratio varied as a function of the microsomes used, from 0.5 for 3-MC-treated rats to 1.5 for

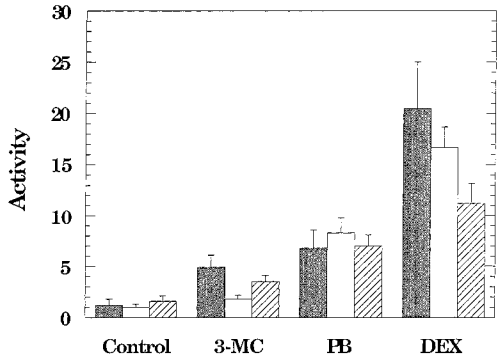


FIGURE 2: Effects of pretreatment of rats by P450 inducers on the oxidation of **1a** by liver microsomes and NADPH. Incubations conditions as in Table 1 using microsomal suspensions containing 1 μM P450 (P450 contents, 0.8, 2.2, 2.4, and 2.1 nmol of P450/mg of protein for microsomes from untreated rats or rats treated with 3-MC, PB, or DEX respectively). Activities [nmol of product (nmol of P450)$^{-1}$ (10 min)$^{-1}$] are means ± SD from three to six experiments. Grey, empty, and hatched bars are for NO_2^-, **1b**, and **1c** formation, respectively.

DEX-treated rats. For all reaction products (**1b**, **1c**, and NO_2^-), the highest activities were obtained with DEX-treated rat liver microsomes. Therefore, most of the following studies on other compounds containing a C═N−OH function have been performed with liver microsomes from DEX-treated rats.

(3) Oxidation of Three Arylamidoximes and a Ketoxime by DEX-Treated Rat Liver Microsomes. Oxidation of 4-chlorobenzamidoxime, **2a**, by aerobic DEX-treated rat liver microsomes and NADPH only led to two organic products, the amide **2b** and nitrile **2c** (Figure 1) as shown by HPLC. NO_2^- and NO_3^- were also formed with rates markedly lower than those previously observed in the case of **1a** (0.7 instead of 3 nmol of [NO_2^- + NO_3^-] nmol of $P450^{-1}$ min^{-1}), under identical conditions (100 μM substrate, 1 μM P450, 1 mM NADPH; compare Tables 1 and 2).

Oxidation of another benzamidoxime, 4-hexyloxy-benzamidoxime, **3a**, by the same microsomes exhibited a very similar behavior, with the formation of the corresponding amide **3b** and nitrile **3c**. Other more polar metabolites could be detected by HPLC, mainly after incubation times longer than 5 min. These products probably arose from the

Table 2: Effects of Incubation Conditions on the Formation of Organic Derived Metabolites and Nitrite and Nitrate from Oxidation of Benzamidoximes **2a**, **3a**, and **4a**, and Ketoxime **5a** by DEX-Treated Rat Liver Microsomes in the Presence of NADPH

		activities[a]			
substrate	incubation conditions	amide (or ketone)	nitrile (or nitroalkane)	NO_2^-	NO_3^-
2a	complete system[a]	4.6 ± 0.9	0.6 ± 0.4	4.8 ± 1.3	2.4 ± 1.0
	−NADPH	0.2 ± 0.1	<0.1	0.5 ± 0.1	<0.1
	+miconazole (100 μM)	<0.1	<0.1	<0.1	<0.1
3a	complete system[a]	16.1 ± 4.4	2.5 ± 0.5	11.4 ± 0.9	4.8 ± 1.5
	−NADPH	<0.1	<0.1	0.3 ± 0.1	<0.1
	+miconazole (100 μM)	<0.1	<0.1	0.2 ± 0.1	<0.1
4a	complete system[b]	26.1 ± 2.5		20.6 ± 1.0	
	−NADPH	<0.1		<0.1	
5a	complete system[a]	5.7 ± 2.0	5.5 ± 1.9	7.4 ± 2.6	2.0 ± 0.8
	−NADPH	<0.1	<0.1	1.1 ± 0.1	<0.1
	+miconazole (50 μM)	0.8 ± 0.4	<0.1	0.5 ± 0.3	0.2 ± 0.2

[a] Complete system as in Table 1. Results are expressed as nmol of product (nmol of P450)$^{-1}$ (10 min)$^{-1}$ and are means ± SD from at least four experiments. [b] Complete system consists in a suspension of DEX-treated rat liver microsomes (0.56 μM P450) in 50 mM phosphate buffer, pH 7.4, containing 1 mM **4a** and 0.5 mM NADPH.

hydroxylation of the *n*-hexyl chain of the substrate; however, no further attempt was made to identify and quantify them.

The N,N-disubstituted benzamidoxime **4a** was oxidized under similar conditions with formation of the corresponding amide **4b** and NO_2^- with rates that were larger than those observed for **2a** and **3a** (Table 2).

Finally, microsomal oxidation of a ketoxime, (4-chlorophenyl)methyl ketone oxime, **5a**, led to the formation of the expected ketone **5b** and of a nitroalkane, 1-(4-chlorophenyl)-1-nitro ethane **5c**, in addition to NO_2^- and NO_3^- (Table 2).

It is noteworthy that the organic metabolites containing a C═O or C≡N function, which are derived from substrates **1a**−**5a**, and nitrogen oxides (NO_2^- + NO_3^-) were always formed in equivalent amounts (Tables 1 and 2).

All the microsomal metabolites of **2a**, **3a**, **4a**, and **5a** were isolated and completely characterized by comparison of their UV, ^{1}H NMR, and mass spectra with those of authentic samples synthesized by nonambiguous methods (see Materials and Methods). As previously observed in the case of **1a** metabolites, their formation could not be detected in the absence of microsomes or in the presence of boiled microsomes (data not shown). Aerobic conditions and the presence of NADPH were absolutely required (Table 2). Finally, 50−100 μM miconazole almost completely inhibited the formation of all these metabolites as well as that of NO_2^- and NO_3^- (Table 2).

The aforementioned data strongly suggested that the oxidation of the C═N−OH function of compounds **1a**−**5a** by liver microsomes, which results in the formation of the corresponding products bearing a C═O function, is catalyzed by cytochromes P450. Interestingly, the microsomal formation of cyanamide **1c** and nitriles **2c** and **3c** from compounds **1a**, **2a**, and **3a**, respectively, as well as the formation of nitroalkane **5c** from **5a**, also appeared to be catalyzed by cytochromes P450.

(4) Generality of the Microsomal Oxidation of Compounds Involving a C═N−OH Function with Formation of Nitrogen Oxides. To test the generality of the oxidative cleavage of C═N−OH bonds by liver microsomes, we have studied the formation of NO_2^- upon oxidation of various aldoximes, ketoximes, amidoximes, and N-hydroxyguanidines by liver microsomes from untreated rats or rats treated by 3-MC, PB, or DEX. Table 3 shows that, for all compounds, DEX-treated rat liver microsomes exhibited the highest activity. On the contrary, microsomes from untreated rats were almost inactive, and 3-MC- and PB-treated rat liver microsomes were only active for some substrates such as **5a** and **8** and several N-hydroxyguanidines, **1a**, **10**, **11**, **12**, and **13**. However, their activities were much lower than those of DEX-treated rat microsomes. In that regard, it is noteworthy that some compounds such as **6**, **3a**, and **9** produced NO_2^- only with the latter microsomes. The level of activity of DEX-treated rat liver microsomes greatly depends on the nature of the oxime function. It is relatively low [3−11 mol of NO_2^- mol of P450^{-1} (10 min)$^{-1}$] with aldoximes, ketoximes, and amidoximes and clearly higher for N-hydroxyguanidines [between 11 and 79 mol of NO_2^- mol of P450^{-1} (10 min)$^{-1}$] (Table 3). Rate of NO_2^- formation from N-hydroxyguanidines seems to depend on the number of alkyl substituents on the nitrogens of the N-hydroxyguanidine function. Thus, N, N- and N, N′-disubstituted compounds **11**, **12**, and **13** led to rates 2−7-fold greater than N-monosubstituted N-hydroxyguanidines, **1a**, **9**, and **10**. This could be related to an increase of the electron richness of the N-hydroxyguanidine function upon increasing the number of electron-donating N-substituents.

Table 3: Formation of NO_2^- during Incubations of Various RR′C═NOH Compounds in the Presence of NADPH and Hepatic Microsomes from Untreated (Control) Rats or from Rats Treated with 3-Methylcholanthrene (3MC), Phenobarbital (PB), or Dexamethasone (DEX)

			activities[a]			
	R	R′	control	3MC	PB	DEX
6	4-Cl-C_6H_4	H	<0.1	>0.1	<0.1	3.0
7	nC_6H_{13}	H	0.2	<0.1	1.0	3.7
5a	4-Cl-C_6H_4	CH_3	0.1	5.0	4.1	7.4
8	nC_5H_{11}	CH_3	<0.1	nd[b]	3.9	7.0
2a	4-Cl-C_6H_4	NH_2	0.7	<0.1	1.0	4.8
3a	4-$C_6H_{13}O$-C_6H_4	NH_2	0.6	0.3	0.5	11.4
1a	4-Cl-C_6H_4-NH	NH_2	0.6	3.6	6.6	22.2
9	4-CF_3-C_6H_4-NH	NH_2	<0.1	<0.1	<0.1	11.0
10	cyclohexyl NH	NH_2	<0.1	<0.1	4.8	28.6
11	piperidyl	NH_2	1.1	7.8	7.5	78.7
12	isopropyl-NH	isopropyl-NH	1.0	8.4	24.5	62.0
13	cyclohexyl-NH	cyclohexyl-NH	<0.1	nd[b]	12.3	58.0

[a] Results are expressed as nmol of NO_2^- (nmol of P450)$^{-1}$ (10 min)$^{-1}$ and are means (±20%) from at least four experiments. Incubations were performed as described in Table 1. [b] nd, not determined.

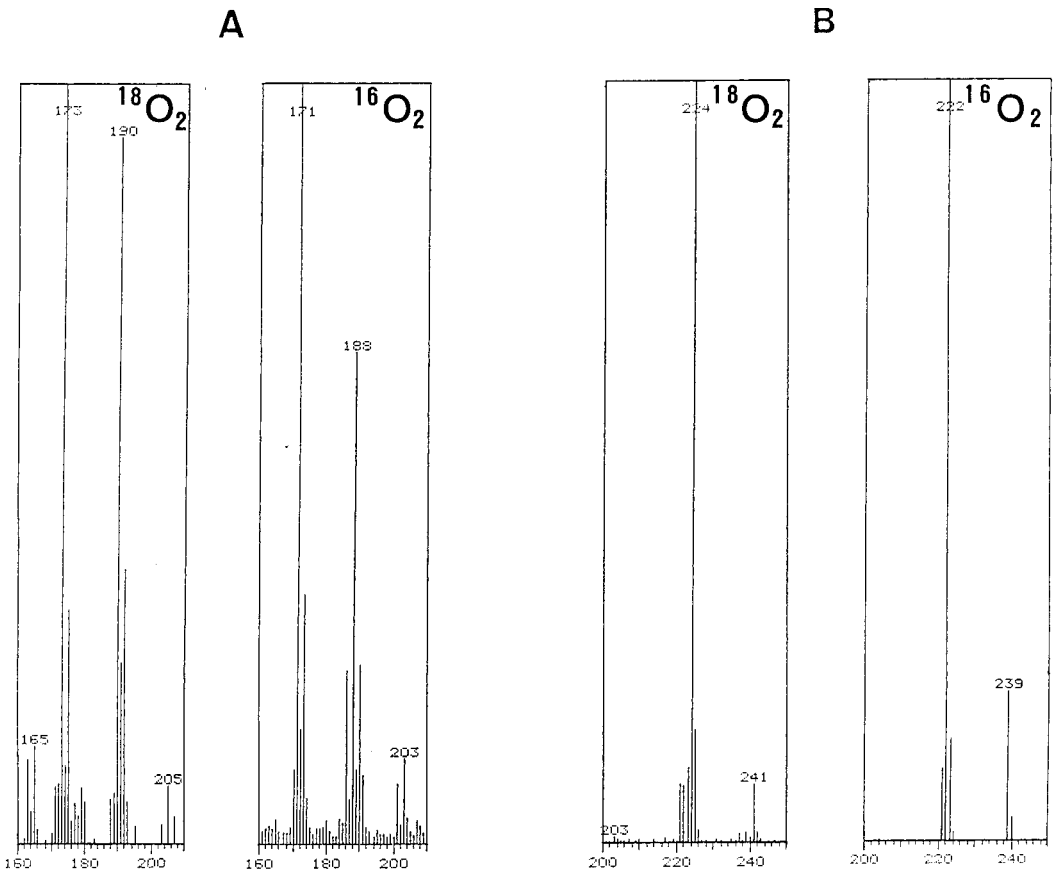


FIGURE 3: Mass spectra of urea **1b** (A) and benzamide **3b** (B) isolated, respectively, from oxidation of **1a** and **3a** by DEX-treated rat liver microsomes and NADPH, either under $^{18}O_2$ and argon or under a normal aerobic atmosphere ($N_2 + {}^{16}O_2$). Spectra obtained in the chemical ionization mode using NH_3. Molecular peaks corresponding to $M + H^+$ and $M + NH_4^+$ are shown.

In the studies described above (paragraphs 1−3), one has followed the formation of NO_2^- and NO_3^- which are the usual products derived from NO in aerobic conditions. Formation of NO itself during these microsomal oxidations of compounds containing a C═N−OH function was also detected by EPR spectroscopy. Thus, EPR spectra of DEX-treated rat liver microsomes incubated with NADPH and either NOHA or amidoxime **3a** showed the signals that are characteristic of the P450 Fe(II)−NO and P420 Fe(II)−NO complexes (*18*, *23*). Similar microsomal incubations of **1a** and **5a** in the presence of NADPH also led to the appearance of the same EPR signals of P450 Fe(II)−NO and P420 Fe(II)−NO (data not shown). These experiments clearly showed that NO itself is formed during those oxidations; however, they did not allow us to quantify its formation. Measurement of steady-state concentrations of NO during oxidation of NOHA, **1a**, **12**, and **13** by DEX-treated rat liver microsomes in electrochemical experiments using a specific electrode (*42*) also led to the conclusion that free NO is formed during those oxidations (*43*).

(5) Mechanistic Studies of Microsomal Oxidations of Oxime-Like Compounds. (a) Experiments using $^{18}O_2$. Experiments were performed in order to determine the origin of the oxygen atom of the C═O function present in ureas and amides which are formed by microsomal oxidation of N-hydroxyguanidines and amidoximes, respectively. For that purpose, large-scale incubations (80 mL) of either **1a** or **3a** in the presence of NADPH and liver microsomes from DEX-treated rats were performed under an argon/$^{18}O_2$ atmosphere (see Materials and Methods). Metabolites **1b**, derived from **1a**, and **3b**, derived from **3a**, were then separated and purified by TLC. The mass spectrum of **1b** obtained from incubation under $^{18}O_2$ showed molecular peaks and several fragments shifted by 2 mass units, when compared to the spectrum of **1b** derived from usual aerobic incubations (under N_2 and $^{16}O_2$). Peaks corresponding to the molecular ion appeared at *m*/*z* 175 and 173 (because of ^{37}Cl and ^{35}Cl) ($M + H^+$, 31 and 100% respectively) instead of 173 and 171 for **1b** prepared from $^{16}O_2$. The intensity of the peak at 171 was less than 5% of that at 173, indicating an almost complete (>95%) incorporation of an oxygen atom from $^{18}O_2$ into the C═O group of **1b** (Figure 3A).

In a very similar manner, incubations of **3a** with DEX-treated rat liver microsomes and NADPH under an argon/$^{18}O_2$ atmosphere, led, after purification by TLC, to a sample of **3b** whose mass spectrum showed a molecular peak at *m*/*z* 224 ($M + H^+$, 100%) shifted by two mass units when compared to that of **3b** derived from incubations under $N_2 + {}^{16}O_2$. The small peak at *m*/*z* 222 (5% of that at 224) indicated that the oxygen atom of the amide group of **3b** mainly comes from $^{18}O_2$ (> 95%) (Figure 3B).

These results showed that microsomal oxidations of **1a** and **3a** into **1b** and **3b**, respectively, are monooxygenation reactions with incorporation of one oxygen atom from O_2. Such microsomal monooxygenations are very often catalyzed by cytochromes P450. The almost complete inhibition that was found with a classical inhibitor of these cytochromes, miconazole (Table 2), showed that cytochromes P450 were mainly involved in the microsomal oxidation of compounds containing a C═N−OH bond. To confirm this key role of

Table 4: Oxidation of **4a** by Cytochrome P450 2C3 and Cytochrome P450 Reductase

	activities[a]	
incubation mixture[a]	**4b**	NO_2^-
complete system	0.87 ± 0.13	1.5 ± 0.04
−NADPH	<0.05	<0.05
−P450 2C3	<0.05	<0.05
−P450 reductase	0.13 ± 0.01	<0.05

[a] Conditions as described in Materials and Methods (0.56 μM P450, 0.3 units of P450 reductase purified from rabbit liver microsomes, 1 mM **4a**, 0.5 mM NADPH). Activities [nmol of product (nmol of P450)$^{-1}$ min^{-1}] are means ± SD from three to seven experiments.

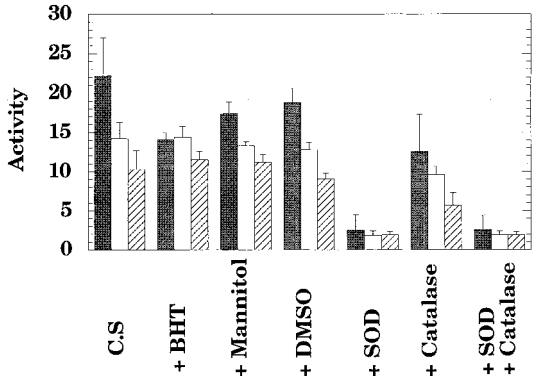


FIGURE 4: Effects of radical scavengers, SOD and catalase on microsomal oxidation of **1a** to NO_2^-, **1b** and **1c**. Conditions as in Table 1, except for the addition of one of the following compounds: 10 μM BHT, 10 mM mannitol, 50 mM DMSO, 50 units mL^{-1} SOD, and 100 units mL^{-1} catalase. Activities [nmol of product (nmol of P450)$^{-1}$ (10 min)$^{-1}$] are means ± SD from three experiments. Black, empty, and hatched bars, respectively, correspond to NO_2^-, **1b**, and **1c**.

cytochromes P450, experiments have been performed by using a reconstituted monooxygenase based on purified cytochrome P450 2C3.

*(b) Oxidation of **4a** by Cytochrome P450 2C3.* Oxidation of the amidoxime **4a** by a monooxygenase reconstituted system involving purified liver P450 2C3, cytochrome P450 reductase, and L-α-dilaurylphosphatidyl choline (DLPC) in the presence of NADPH led to the concomitant formation of amide **4b** and NO_2^- (0.9 ± 0.1 and 1.5 ± 0.1 nmol nmol of P450^{-1} min^{-1}, respectively). This oxidation did not occur if NADPH, P450 2C3, or P450 reductase was omitted (Table 4).

(c) Effects of Inhibitors of Free Radical Oxidations. The oxime functions of compounds **1a**−**13** are chemically very reactive, particularly toward free radical oxidations (*44*). Figure 4 shows that the addition of free radical scavengers such as 3,5-ditertiobutyl 4-hydroxy toluene (BHT) (10 μM), mannitol (10 mM), and DMSO (50 mM) in incubations of **1a** with microsomes had very little effects on the formation of metabolites **1b** and **1c** and of NO_2^-. At the opposite, addition of superoxide dismutase (SOD, 50 units mL^{-1}) strongly reduced (up to 80%) the formation of both the organic metabolites and NO_2^-. Catalase had a weaker inhibitory effect (40% for 100 units mL^{-1}). The results of a more detailed study of the effects of SOD and catalase on microsomal oxidation of **1a** are shown in Figure 5. SOD inhibited the formation of **1b**, **1c**, and NO_2^- in a very efficient manner as less than 3 units/mL of SOD was enough to half-inhibit the reaction (Figure 5A). Catalase was far less

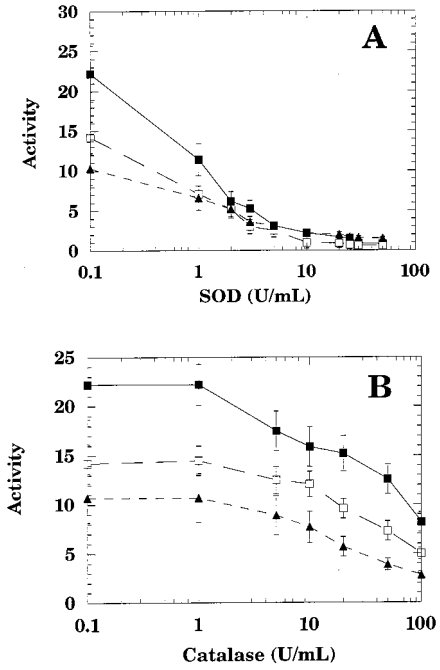


FIGURE 5: Effects of increasing amounts of SOD (A) and catalase (B) on oxidation of **1a** to NO_2^-, **1b** and **1c** by DEX-treated rat liver microsomes. Conditions as in Table 1. Activities [nmol of product nmol of P450)$^{-1}$ (10 min)$^{-1}$] are means ± SD from five experiments. (■) NO_2^-; (□) **1b**; (▲) **1c**.

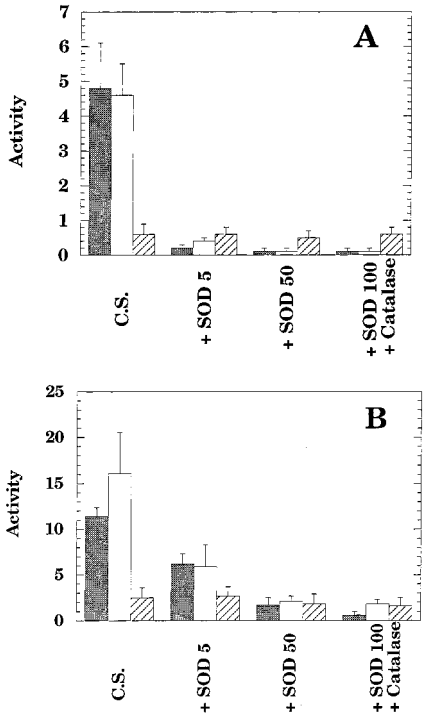


FIGURE 6: Effects of increasing amounts of SOD on oxidation of **2a** (A) and **3a** (B) to NO_2^- (black bars), amides (empty bars), and nitriles (hatched bars) by DEX-treated rat liver microsomes. Conditions as in Table 1. Activities [nmol of product (nmol of P450)$^{-1}$ (10 min)$^{-1}$] are means ± SD from three to five experiments.

efficient, since half inhibition of the reaction was only observed in the presence of more than 30 units/mL of catalase (Figure 5B).

Figures 6 and 7 show the strong inhibitory effects of SOD on microsomal oxidations of compounds **2a**, **3a**, **4a**, and **5a** into the corresponding products containing a C═O function,

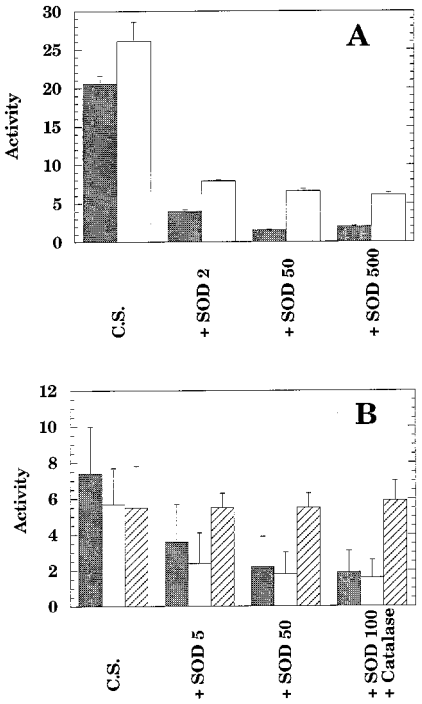


FIGURE 7: Effects of increasing amounts of SOD on oxidation of **4a** (A) and **5a** (B) by DEX-treated rat liver microsomes. Conditions as in Table 1 for **5a** and as in Table 2 for **4a**. (A) Black and empty bars for NO_2^- and **4b**. (B) Black, empty, and hatched bars for NO_2^-, **5b**, and **5c**, respectively.

2b, **3b**, **4b**, and **5b**. The sensitivity of the oxidations to SOD was dependent on the structure of the substrate, as formation of **2b** was almost completely inhibited with 5 units mL^{-1} SOD, whereas those of **3b** and **5b** were only 50−60% inhibited with the same concentration of SOD. Moreover, 0.5 units mL^{-1} SOD already led to a 50% inhibition of the formation of **4b**. It is noteworthy that SOD always inhibited to the same extent the formations of the metabolites containing a keto group and of NO_2^-.

In fact, the maximum level of inhibition observed with a large excess of SOD was also dependent on the substrate structure. It was close to 100% in the case of **1b** and **2b** formation (Figure 5A and 6A), whereas it was smaller for the formation of **3b**, **4b**, and **5b** (85, 80, and 70%, respectively). These residual activities observed with large amounts of SOD were unchanged in the presence of large excess of both SOD (100 units mL^{-1}) and catalase (500 units mL^{-1}) (Figure 6 and 7). It seems that, for some substrates, part of the microsomal conversion of the C═N−OH function to a C═O function is not inhibited even by a very large excess of SOD and catalase. A similar result was observed during NOHA oxidation by DEX-treated rat liver microsomes, 25% of citrulline formation was not inhibited by an excess of SOD and catalase (data not shown). The corresponding reactions thus appear to be mediated not only by $O_2^{\bullet-}$ but also by another oxidizing species.

Although SOD completely inhibited the microsomal transformation of **1a** into cyanamide **1c** (Figure 5A), it did not have any inhibitory effects on microsomal conversions of **2a** and **3a** into benzonitriles **2c** and **3c** (Figure 6). It also failed to inhibit the oxidation of **5a** into the corresponding nitroalkane **5c** (Figure 7B).

As observed in the case of **1a**, catalase was far less efficient than SOD to inhibit the oxidations of **2a**, **3a**, **4a**, and **5a** into the corresponding ketonic products (about 50% inhibition at concentrations up to 100 units mL^{-1}). It was without effects on the formation of nitriles **2c** and **3c** and of the nitroalkane **5c** (data not shown).

Table 5: Oxidation of **1a**, **2a**, and **3a** by Xanthine and Xanthine Oxidase

			activities[a]		
compound	$RC(NH_2)$═N-OH	conditions	NO_2^-	R CO NH_2	RCN
1a	4-Cl-C_6H_4-NH	complete system[a]	25.5	17.5	8.6
		−XO	<0.1	0.7	<0.1
		−X	1.9	0.7	<0.1
		+SOD	6.6	2.1	2.6
2a	4-Cl-C_6H_4	complete system[a]	17.7	17.2	<0.1
		−XO	<0.1	<0.1	<0.1
		−X	5.5	<0.1	<0.1
		+SOD	2.1	<0.1	<0.1
3a	4-n$C_6H_{13}O$-C_6H_4	complete system[a]	7.8	11.2	<0.1
		−XO	<0.1	<0.1	<0.1
		−X	<0.1	<0.1	<0.1
		+SOD	1.7	2.2	<0.1

[a] Complete system, as described in Materials and Methods, contained 100 μM substrate, 2.5 mM xanthine (X) and 0.03 units/mL xanthine oxidase (XO) in 0.1 M phosphate buffer, pH 7.4. In experiments (+SOD), 10 units/mL of SOD were added in the incubate. Activities are expressed as nmol of product $(10\ min)^{-1}$ and 0.03 XO units/mL.

*(d) Oxidation of Compounds **1a**, **2a**, and **3a** by a Xanthine−Xanthine Oxidase System.* The $O_2^{\bullet-}$ generating system using xanthine and xanthine oxidase was found to oxidize **1a** with formation of NO_2^- and **1b** + **1c** in similar amounts (Table 5). Formation of NO_2^-, **1b**, and **1c** was greatly inhibited in the presence of SOD (>70% for 10 units mL^{-1} SOD).

This system also oxidized benzamidoximes **2a** and **3a** to the corresponding amides **2b** and **3b** (Table 5). However, it did not lead to the formation of the corresponding nitriles **2c** and **3c**. Moreover, the xanthine−xanthine oxidase system failed to oxidize ketoxime **5a** into ketone **5b** and nitroalkane **5c** (data not shown).

DISCUSSION

Microsomal Oxidation of N-Hydroxyguanidines. The aforementioned results show that compounds such as **1a** are oxidized to the corresponding urea and cyanamide with concomitant formation of nitrogen oxides (NO and its aerobic products NO_2^- and NO_3^-) (eq 3). The formation of all these

$$\mathrm{ArNH{-}\underset{\underset{NH_2}{|}}{C}{=}N{-}OH \xrightarrow[NADPH + O_2]{P450} ArNH{-}\underset{\underset{NH_2}{|}}{C}{=}O + ArNH{-}CN + NxOy} \qquad (3)$$

products is dependent on NADPH and O_2 and is catalyzed by cytochromes P450. Formation of the urea is a true monooxygenase reaction, its keto oxygen atom coming almost exclusively from dioxygen. Similar results have been recently obtained for microsomal oxidation of *N*-hydroxydebrisoquine, which leads to the corresponding urea and cyanamide, and NO (B. Clement et al., manuscript in preparation).

Formation of all the products derived from **1a** appears to be mediated almost exclusively by $O_2^{\bullet-}$ derived from the oxidase function of cytochromes P450 (complete inhibition

by SOD for **1a**, Figure 5). Accordingly, $O_2^{\bullet-}$ produced in the same buffer by the xanthine−xanthine oxidase system also leads to **1b** and **1c** (Table 5). Moreover, it has been reported that KO_2 in DMSO reacts with **1a** to give **1b** and **1c** (45).

Microsomal Oxidation of Amidoximes. Amidoximes, such as **2a** and **3a**, are oxidized by microsomal cytochromes P450 with formation of nitrogen oxides (NO and its aerobic products NO_2^- and NO_3^-) and the corresponding amides and nitriles (eq 4). Formation of the amides is a monooxygenation reaction, the oxygen atom of their keto group exclusively comes from dioxygen (Figure 3). Microsomal formation of amides from **2a**, **3a**, and **4a** is dependent on NADPH and O_2 and is catalyzed by cytochromes P450. We have checked that oxidation of **4a** to **4b** is catalyzed by a reconstituted monooxygenase system based on a purified cytochrome P450 that was available in the laboratory (P450 2C3).

$$\mathrm{Ar{-}\underset{\displaystyle NH_2}{\underset{|}{C}}{=}N{-}OH \xrightarrow[NADPH + O_2]{P450} Ar{-}\underset{\displaystyle NH_2}{\underset{|}{C}}{=}O + Ar{-}C{\equiv}N + NxOy} \qquad (4)$$

Microsomal amide formation is mainly dependent on $O_2^{\bullet-}$ (75−100%) while that of nitriles is not inhibited by SOD (Figure 6). It thus appears that microsomal formation of nitriles from amidoximes as well as a minor part (∼20%) of the formation of amides from the same substrates is not due to $O_2^{\bullet-}$ but to a cytochrome-P450−iron active species.

Microsomal Oxidation of Ketoximes. Ketoximes, such as **5a**, are oxidized by liver microsomes with formation of the corresponding ketone and nitroalkane as well as of NO, NO_2^-, and NO_3^- (eq 5). These reactions require NADPH and O_2 and are catalyzed by cytochromes P450. Ketone formation is mainly due to $O_2^{\bullet-}$ (about 80%) whereas that of nitroalkane is not.

$$\mathrm{Ar{-}\underset{\displaystyle R}{\underset{|}{C}}{=}N{-}OH \xrightarrow[NADPH + O_2]{P450} Ar{-}\underset{\displaystyle R}{\underset{|}{C}}{=}O + \begin{array}{c} Ar \quad H \\ \diagdown \ \diagup \\ R \quad C \quad NO_2 \end{array} + NxOy} \qquad (5)$$

Generality and Mechanisms of the Microsomal Oxidation of Compounds Containing a C═N−OH Function. The formation of NO and related nitrogen oxides upon microsomal cytochrome P450 dependent oxidation of compounds involving a C═N−OH function has been previously reported in the case of pentamidine (*15*) and amidoxime **3a** (*23*) and for NOHA (*18*, *19*) and some N-hydroxyguanidines (*17*, *24*). The aforementioned results show that this is a quite general reaction occurring on aldoximes, ketoximes, amidoximes, and N-hydroxyguanidines (Table 3). Microsomal cytochromes P450 3A, that are present in DEX-treated rat liver, are particularly efficient catalysts for that reaction (Table 3 and Figure 2). We have also verified that it is catalyzed by a purified cytochrome P450, P450 2C3, in the presence of cytochrome P450 reductase (Table 4). The rate of this reaction greatly depends on the number of nitrogen substituents on the C═N−OH carbon and on the number of electron-donor alkyl substituents on those nitrogen atoms. This leads to the following order of reactivity of C═N−OH-containing substrates: ketoximes < amidoximes < N-substituted N′-hydroxyguanidines < *N*,*N*-disubstituted N′-hydroxyguanidines ≃ N,N′-disubstituted N″-hydroxyguanidines.

Microsomal oxidative cleavage of C═N−OH bonds with formation of the corresponding C═O bonds and nitrogen oxides mainly depends on $O_2^{\bullet-}$ derived from P450-catalyzed reduction of dioxygen by NADPH. Such an uncoupling reaction is well-known for microsomal cytochromes P450 (*22*); it has been found to be responsible for the oxidation of an hydroxylamine, N-hydroxyphentermine, to the corresponding nitrosoalkane (*46*, *47*).

The ability of $O_2^{\bullet-}$ to oxidize N-hydroxyguanidines and amidoximes to the corresponding ureas and amides was shown both by using KO_2 in DMSO (*45*) and the $O_2^{\bullet-}$ generating system based on xanthine and xanthine oxidase in buffer under conditions identical to those used for liver microsomes (this work). KO_2 in DMSO has been reported to be very efficient for the oxidation of amidoximes as it stoichiometrically reacts with amidoximes with selective formation of the corresponding amides and nitrogen oxides in very high yields (*45*). Xanthine and xanthine oxidase also oxidize benzamidoximes **2a** and **3a** with selective formation of amides **2b** and **3b**. Both $O_2^{\bullet-}$ generating systems are less selective in their oxidation of N-hydroxyguanidines, since they lead, as liver microsomes, not only to ureas but also to cyanamides. The urea-to-cyanamide ratio observed with KO_2 in buffers greatly depends on the solvent used and on the pH value (45).

Hydrogen peroxide, which may also be formed by dismutation of $O_2^{\bullet-}$ and by cytochrome P450 dependent reduction of O_2 (*22*), does not seem to be involved to a great extent in the above-described microsomal oxidations, since catalase always exhibited much lower inhibitory effects than SOD (Figure 4 and 5). Moreover, free radicals, such as •OH, possibly formed during microsomal oxidations, do not appear to have an important contribution in oxidations of oxime compounds, as shown by the lack of inhibitory effects of DMSO, mannitol, and BHT (Figure 4).

Another oxidizing species is responsible for the oxidation of amidoximes **2a**, **3a**, and **4a** to amides **2b**, **3b**, and **4b** and ketoxime **5a** to ketone **5b**, which is not inhibited by SOD + catalase even in large excess (20−30% of total oxidation of these substrates, Figure 6 and 7). When considering the classical catalytic cycle of dioxygen activation by cytochromes P450, three oxidizing species appear possible a priori: the iron(II)−dioxygen complex [Fe(II)−O_2 ↔ Fe(III)−O−O•], the iron(III)−peroxide complex [Fe(III)−O−O^-], and the high-valent iron-oxo complex [formally Fe(V)═O]. The first one seems to be responsible for the three-electron oxidation of NOHA to citrulline and NO, by a mechanism which could be that shown in Figure 8 (*48*−*51*). It is thus tempting to propose that this P450 Fe(II)−O_2 complex plays a central role in microsomal oxidations of compounds containing a C═N−OH function. This complex has three possible fates as a function of the positioning of the substrate in P450 active site (Figure 9).

The first one is the dissociation of its Fe−O_2 bond with formation of P450 Fe(III) and $O_2^{\bullet-}$. It is particularly important when the substrate is not well located to react with the Fe(II)−O_2 moiety. This is a frequent situation with microsomal cytochromes P450, and particularly with P450s of the 3A subfamily which catalyze the oxidation of a surprisingly large variety of substrates with an important uncoupling between electron transfer from NADPH and monooxygenation (52). Compounds such as **1a**−**5a** exhibit

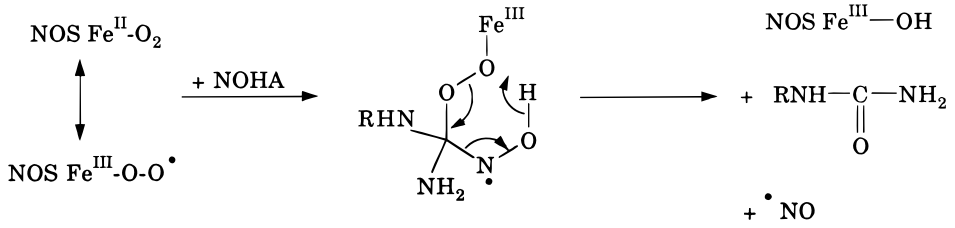


FIGURE 8: Possible mechanism for the second step of NOS (oxidation of NOHA to citrulline and NO).

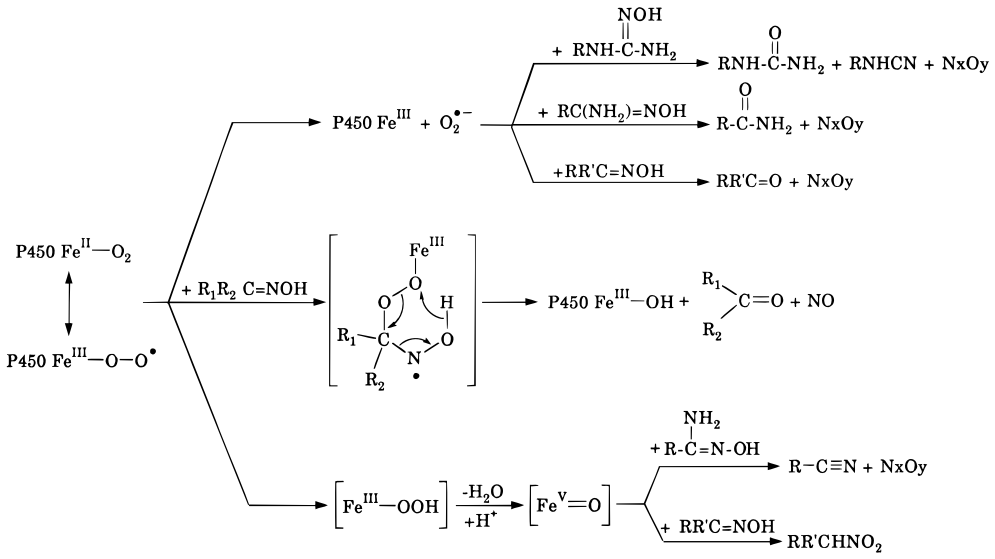


FIGURE 9: Different pathways possibly involved in microsomal P450-dependent oxidations of N-hydroxyguanidines, amidoximes, and ketoximes. R and R′ are alkyl or aryl groups. R_1 and R_2 are alkyl, aryl, RNH, or NH_2 groups. As far as reactions of P450 Fe(V) = O are involved, possible involvement of this species in the formation of ureas, amides and ketones, respectively, from N-hydroxyguanidines, amidoximes, and ketoximes is not mentioned.

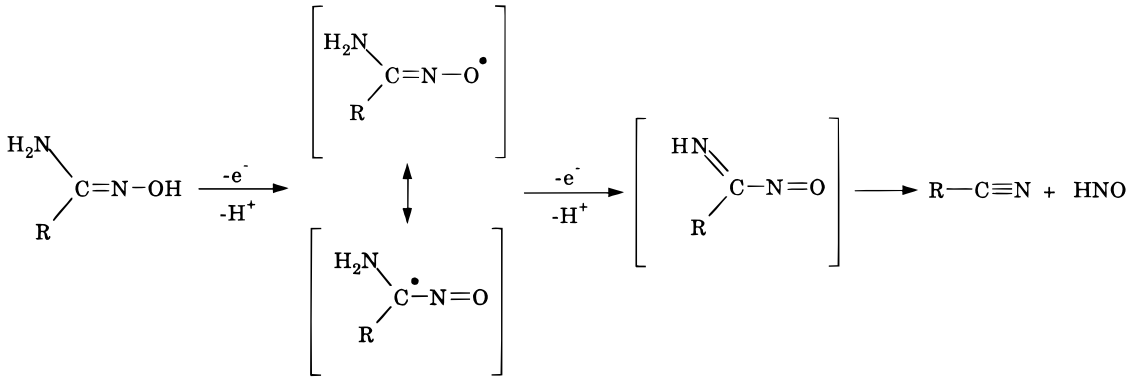


FIGURE 10: Possible mechanism for the oxidation of N-hydroxyguanidines and amidoximes to the corresponding nitriles. The main oxidant in microsomal oxidation of N-hydroxyguanidines (R = NHAr) appears to be $O_2^{\bullet-}$. P450 Fe(V)═O would be the major oxidant in the case of amidoximes.

poor affinities for those cytochromes. They only led to weak spectral changes with microsomes, with appearance of "reverse type I" difference spectra (peak around 420 nm and trough around 390 nm), which could correspond to their binding to P450 Fe(III) via their N-hydroxy oxygen atom; the dissociation constants calculated from these difference spectra were around 500 μM (data not shown). It is thus understandable that these compounds are not well located to react with P450 Fe(II)−O_2 in the P450 active site. Dissociation of the Fe(II)−O_2 bond of the P450−dioxygen complex could thus be faster than its reaction with substrates **1a**−**5a** leading to $O_2^{\bullet-}$ which may eventually react by itself with **1a**−**5a**.

The second possible evolution of P450 Fe(II)−O_2 is a direct addition of this species on the substrate C═N−OH bond. This should be the main reaction involved in NO-synthase-dependent oxidation of NOHA, the natural substrate of NOS which must be very well positioned in the NOS active site. This might also occur, as a minor pathway, during P450-dependent oxidation of some oxime substrates

[20% of citrulline formation from NOHA (data not shown), 20−30% of formation of **3b**, **4b**, and **5b** from **3a**, **4a**, and **5a**, respectively, Figure 6 and 7].

However, one cannot exclude that the oxidation not inhibited by SOD of these substrates is performed by the active oxygen species resulting from the third evolution of P450 Fe(II)−O_2: a one-electron reduction leading to P450 Fe(III)−O−O^- and P450 Fe(V)═O. In fact, oxidation of **1a**, **2a**, and **5a**, either by liver microsomes in the presence of an oxygen atom donor cumyl hydroperoxide or by chemical models of cytochrome P450 based on an iron porphyrin and a peracid, does lead to **1b** and **1c**, **2b**, and **5b**, respectively (data not shown). Moreover, NOSs have been recently reported to catalyze the oxidation of NOHA by H_2O_2 with formation of citrulline and the corresponding cyanamide, N^{δ}-cyano ornithine (*53*). It was proposed that this reaction is performed by a NOS Fe(V)═O species.

Thus, microsomal oxidation of N-hydroxyguanidines to nitrogen oxides and the corresponding ureas and cyanamides is mainly performed by $O_2^{\bullet-}$ derived from P450-catalyzed O_2 reduction by NADPH and, to a minor extent, for some of them (NOHA), by a P450 active oxygen species, either Fe(II) O_2 itself or Fe(V)═O. We have already proposed a mechanism of urea formation, which involves incorporation of one oxygen atom from $O_2^{\bullet-}$ [or Fe(II)-O_2] into the substrate (51); i.e., as shown in Figure 8. The mechanism of formation of cyanamides could involve two successive one-electron oxidations, with intermediate formation of a nitroso compound which is known to rapidly loose HNO (*54*, *55*) (Figure 10). Reports showing that N-hydroxyguanidines are selectively oxidized to corresponding cyanamides by one-electron oxidants such as $Pb(OCOCH_3)_4$ or MnO_2 (*55*) are in agreement with this mechanism. The electron acceptor involved in such microsomal oxidations could be $O_2^{\bullet-}$, P450 Fe(II)-O_2, and P450 Fe(V)═O. Other mechanisms involving the intermediate formation of oxaziridines have been proposed for the oxidation of N-hydroxyguanidines to corresponding cyanamides (*56*).

Microsomal oxidation of amidoximes to amides appears to be mainly performed by $O_2^{\bullet-}$ and, to a minor extent, by P450 Fe(II)−O_2 [or P450 Fe(V)═O]. On the contrary, microsomal oxidation of amidoximes to nitriles are not performed to a great extent by $O_2^{\bullet-}$. Because of the higher redox potentials of amidoximes compared to N-hydroxyguanidines (*26*), it is understandable that amidoximes undergo one-electron oxidations by very potent oxidants such as P450 Fe(V)═O but not by $O_2^{\bullet-}$. Oxidation of amidoximes to nitriles by P450 Fe(V)═O would involve two one-electron oxidations with loss of two protons (and of HNO) without any oxygen atom transfer. Because of its mechanism, this reaction is similar to the dehydrogenation of some substrates by microsomal cytochromes P450 (*9*). As in the case of N-hydroxyguanidines, oxidation of amidoximes to nitriles could involve another mechanism via the intermediate formation of an oxaziridine (*56*).

Finally, microsomal oxidation of ketoxime **5a** to ketone **5b** appears to be mainly performed by $O_2^{\bullet-}$ and, to a minor part, by P450 Fe(II)−O_2 [or P450 Fe(V)═O]. Oxidation of **5a** to nitroalkane **5c** requires a much stronger oxidant, capable of transferring an oxygen atom, which should be P450 Fe(V)═O (*57*).

The above results suggest a general mechanism for microsomal cytochrome P450- and NO-synthase-dependent oxidation of compounds containing a C═N−OH function. The central species in these reactions would be the heme-protein iron−dioxygen complex, which may react either directly in situations where the substrate is well-positioned relative to that species or via $O_2^{\bullet-}$ in less favorable situations. Such reactions always lead to the corresponding C═O function by a monooxygenation process as well as to nitrogen oxides, mainly NO and its aerobic products NO_2^- and NO_3^-. However, it is noteworthy that under in vivo conditions, SOD should inhibit the superoxide-dependent pathway of the reactions. Microsomal oxidations of oximes could have biological and toxicological consequences as they involve the formation of both $O_2^{\bullet-}$ and NO (and other nitrogen oxides).

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