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CYTOCHROME P450 DEPENDENT N-HYDROXYLATION OF A GUANIDINE (DEBRISOQUINE), MICROSOMAL CATALYSED REDUCTION AND FURTHER OXIDATION OF THE *N*-HYDROXY-GUANIDINE METABOLITE TO THE UREA DERIVATIVE

SIMILARITY WITH THE OXIDATION OF ARGININE TO CITRULLINE AND NITRIC OXIDE

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Abstract—The microsomal N-hydroxylation of the strongly basic guanidinium group (debrisoquine) to *N*-hydroxyguanidine (*N*-hydroxydebrisoquine) and the retroreduction of the *N*-hydroxyguanidine are demonstrated for the first time. The reduction of the *N*-hydroxyguanidine by liver homogenates and hepatocytes is catalysed by a microsomal NADH-dependent system that is strongly inhibited by hydroxylamine or *N*-methylhydroxylamine. In the presence of these alternate substrates for the reductase the microsomal catalysed N-hydroxylation of debrisoquine is readily characterized. The oxidation was inhibited by antibodies against NADPH cytochrome P450 reductase and the role of the P450 monooxygenase was further verified by studies with partially purified and purified P450 2C3 reconstituted systems. The transformation of *N*-hydroxydebrisoquine to the corresponding urea derivative was also detected in *in vitro* experiments with microsomal fractions and enriched P450 fractions as well as with flavin-containing monooxygenase (FMO). Experiments with catalase, superoxide dismutase and H_2O_2 have shown that the H_2O_2 or O_2^-, respectively, formed from the respective enzyme and the substrate, apparently participated in the reaction. Whereas the N-hydroxylation of the guanidine involves the usual monooxygenase activity of cytochrome P450 the resultant *N*-hydroxyguanidine decouples monooxygenases (cytochrome P450, FMO) and the H_2O_2 and, above all, O_2^- thus formed transform the *N*-hydroxyguanidine further to the corresponding urea derivative. The possibility for the N-hydroxylation of non-physiological guanidines to *N*-hydroxyguanidines and subsequent oxidative conversion to the respective urea is comparable to the physiological transformation of arginine to citrulline via *N*-hydroxyarginine with the liberation of nitric oxide (endothelial derived relaxing factor) and could, therefore, contribute to the efficacy of drugs containing guanidine and similar functional groups.

Numerous drugs such as antihypertensive agents, antidiabetic agents, and antiseptic agents contain a guanidine functional group [1, 2]. While oxidative biotransformations of substances belonging to the strongly basic amidine class of compounds have been investigated in detail [3, 4], an analogous reaction with the guanidine class of compounds has only been detected in the case of the non-basic, perfluorinated *N,N*-diphenylguanidine [5]. The metabolic N-hydroxylation of the typically strongly basic guanidines in which none of the nitrogen atoms are components of an aromatic system or carry aromatic groups as substituents has not been previously reported for xenobiotic agents. Thus, initial biotransformation studies [6] on variously substituted guanidines did not provide any evidence for a microsomal N-oxygenation. Competitive N-dealkylations of substrates containing α-hydrogen atoms have been proposed to account for the lack of this reactivity [7–9].

Since in previous metabolic studies [6] the N-oxygenation of guanidines in connection with a subsequent occurring retroreduction of the primarily formed N-oxygenated metabolites was not investigated, this process could be responsible for the difficulty in detecting N-hyroxylations of guanidines. In the present work, this aspect has been investigated in more detail using debrisoquine (Fig. 1). Debrisoquine is used in many countries as an antihypertensive agent. The genetic polymorphism of the ring hydroxylation by cytochrome P450 giving rise to 4-hydroxydebrisoquine has been described in numerous publications [10]. In addition, ring cleaved carboxylic acids are known [8] in which the primary metabolic step probably involves a hydroxylation at the 1 or 3 position of the tetrahydroisoquinoline ring. This results in a metabolic ring cleavage producing the aldehyde (N-dealkylation), which is finally oxidized to give the respective carboxylic acid. Traces of the phenolic metabolites 5-, 6-, 7- and 8-hydroxydebrisoquine were also identified [8].

The objective of the present work was a specific search for a microsomal N-hydroxylation by means of *in vitro* studies (Fig. 1). *N*-Hydroxydebrisoquine was synthesized for comparative purposes [11]. The

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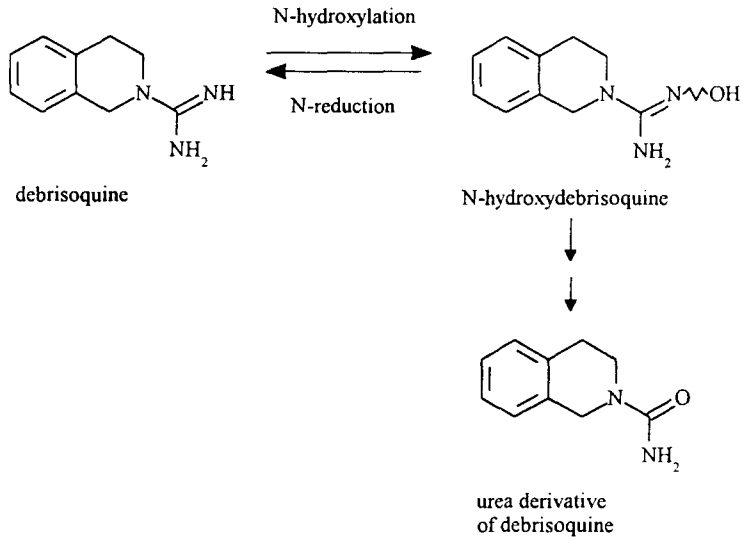

Fig. 1. N-Hydroxylation of debrisoquine, N-reduction of *N*-hydroxydebrisoquine and transformation to its urea derivative.

retroreduction of this N-hydroxylated metabolite to debrisoquine was investigated in the same manner in a search for conditions which would permit the possible detection of the reduction of an *N*-hydroxyguanidine as well as providing the first evidence for the N-hydroxylation of a strongly basic guanidine by cytochrome P450. Furthermore, the example of *N*-hydroxydebrisoquine was examined to determine whether or not non-physiological *N*-hydroxyguanidines could be oxidatively converted to the corresponding ureas by cytochrome P450 (Fig. 1). If nitric oxide (NO) or R-NO were released in the process, this might explain the pharmacological effects of non-physiological guanidines. The formation of NO or a labile species able to release NO [endothelial derived relaxing factor (EDRF†)] is catalysed by NO synthases using arginine as a precursor [12, 13], and probably proceeds by way of *N*-hydroxyarginine [14, 15] with the final result that arginine is converted to the corresponding urea (citrulline) [12–15].

MATERIALS AND METHODS

Reagents and biochemicals

Debrisoquine sulfate was kindly supplied by Hoffmann La Roche and Co. (Basle, Switzerland). L-α-Dilauryl phosphatidylcholine (DLPC), 5β-pregnane-3β,20α-diol (5βPD), ethylene glycol bis-(aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), superoxide dismutase, catalase and Trypan blue were obtained from the Sigma Chemical Co. (Deisenhofen, F.R.G.). Collagenase H was supplied by Boehringer Mannheim (Mannheim, F.R.G.) and (*N*-2-hydroxyethyl)piperazine-*N'*-(2(ethanesulfonic acid) (HEPES) by Fluka (Buchs, Switzerland). Narcoren® was obtained from Rhone Merieux (Laupheim, F.R.G.). *N*-Methylhydroxylammonium chloride, hydroxylammonium sulfate, NADPH (tetrasodium salt) and NADH (disodium salt) as well as all other chemicals and solvents were from Merck (Darmstadt, F.R.G.) unless otherwise stated.

Synthesis

N-Hydroxydebrisoquine was prepared from the corresponding cyanamide by reaction with hydroxylamine according to the procedure of Bailey *et al.* [11]. The urea derivative of debrisoquine was obtained from tetrahydroisoquinoline and potassium cyanate [16]. The products were characterized in the usual manner. Questions on the configuration, *E/Z*-isomerism, and tautomerism of *N*-hydroxyguanidines were clarified by [^{15}N]NMR investigations [17].

Preparation of liver homogenates

Supernatants of 9000 g *and* 100,000 g. The livers of untreated rabbits (3.0–4.5 kg), pigs (slaughter house), and rats (Wistar, 300–400 g) of either sex were used. All subsequent steps were performed as described in previous publications [7]. Cytosol (100,000 *g* supernatant) was obtained by centrifugation at 100,000 *g* for 45 min.

Microsome preparations by fractional acidic precipitation. A 20 mM phosphate buffer solution (pH 7.4) with the addition of 0.25 sucrose was used for the preparation of the homogenates from minced livers. The crude rabbit liver homogenate obtained using a meat mincer was homogenized twice in a homogenizer as described in Ref. 18. The supernatant obtained after centrifugation (12,000 *g*) was adjusted

† Abbreviations: EDRF, endothelial derived relaxing factor; DLPC, L-α-dilauryl phosphatidylcholine; 5-βPD, 5β-pregnane-3β,20α-diol; P450 reductase, NADPH cytochrome P450 reductase; FMO, flavin-containing monooxygenase; HEPES, (*N* - 2 - hydroxyethyl) piperazine - *N* ' - (2 - ethanesulfonic acid); EGTA, ethylene glycol bis(aminoethyl ether)*N,N,N',N'*-tetraacetic acid.

to pH 6.3 by addition of dilute acetic acid. After renewed centrifugation at 12,000 *g* for 20 min, the supernatant was carefully passed through gauze and its volume made up to the original amount by addition of phosphate buffer. The supernatant was discarded, the precipitate (microsome pellet) was resuspended in an appropriate volume of phosphate buffer solution (pH 7.4). The pH was adjusted to 5.5 and the suspension again centrifuged at 12,000 *g* for 20 min. The supernatant was discarded, the microsome pellet was resuspended in buffer solution, and the pH adjusted to 7.4 by addition of sodium hydroxide solution.

Purification of the components of the cytochrome P450 enzyme system and preparation of antibodies

Partial purification of cytochrome P450 and isolation of NADPH cytochrome P450 reductase (P450 reductase). The objective of the enzyme purification was to obtain an enriched cytochrome P450 preparation containing as little detergent as possible while still retaining, in catalytically active form, all of the constitutive cytochrome P450 isoenzymes. A modification of the procedure described by Kling *et al.* [19] for the enrichment of cytochrome P450 from mouse liver by hydrophobic interaction chromatography (HIC) on octyl-Sepharose CL-4B was used to obtain the cytochrome P450 preparation and has been described in more detail elsewhere [20]. P450 reductase from rabbit liver was purified to homogeneity as described by Yasukochi and Masters [21] with slight modifications [20]. Polyclonal antibodies against the reductase were prepared by immunization of goats as reported previously [4, 22, 23].

Purification of cytochrome P450 2C3. The separation of a P450 preparation into isoenzymes and the isolation of P450 2C3 from rabbit livers has already been reported [20]. The unequivocal characterization was based on molecular mass determinations, UV/VIS spectroscopy, the CO difference spectrum, and inhibition experiments with monoclonal antibodies against P450 2C3 [20].

Preparation of hepatocytes

Male Wistar rats (220–280 g, fed *ad lib.*) were used. The hepatocytes were isolated by a modification of the two-step *in situ* collagenase perfusion technique [24] with the following changes. The rats were anesthetized by intraperitoneal injection of Narcoren® (1 mL/kg). The livers were first rinsed with buffer A [pH 7.4, (mM) NaCl 145, KCl 5.36, $MgSO_4 \cdot 7H_2O$ 0.77, $MgCl_2$ 0.93, Na_2HPO_4 0.34, KH_2PO_4 0.44, EGTA 0.2 and HEPES 10] for 15 min at 37° and a flow rate of 30 mL/min. The second step was performed with buffer B (composition as for A except without EGTA but with 0.06% collagenase and 5 mM $CaCl_2$). The liver capsule was carefully dissected and the cells suspended in an ice-cooled washing buffer [pH 7.4, (mM) NaCl 137, KCl 5.36, $MgSO_4 \cdot 7H_2O$ 1.17, Na_2HPO_4 0.79, KH_2PO_4 0.15, glucose 5.6, $CaCl_2$ 1.0 and HEPES 10]. After filtration the cells were centrifuged at 50 *g* with washing buffer twice for 2 min each. After subsequent percoll centrifugation [25] and resuspension in modified Hank's salt solution [(mM) NaCl 137, KCl 5.36, $MgSO_4 \cdot 7H_2O$ 0.77, $MgCl_2$ 0.93, Na_2HPO_4 0.24, KH_2PO_4 0.44, HEPES 20 and glucose 5.55] the cell viability, as determined by Trypan blue staining, was always higher than 90% and often higher than 95%.

Purification of flavin-containing monooxygenase (FMO)

FMO was purified from pig liver essentially as described by Ziegler and Poulsen [26].

Analytical procedures

The cytochrome P450 content was analysed using the method of Omura and Sato [27]. The activity of P450 reductase was determined by the procedure of Williams and Kamin [28] in which the reduction of cytochrome *c* (type 4 horse heart, from Sigma) is monitored (extinction coefficient $\varepsilon = 21\ mM^{-1}\ cm^{-1}$ for the reduced form of cytochrome *c*, 1 mU = 1 nmol of reduced cytochrome *c*/min). The protein contents at the stages of the microsomes and solubilized microsomes were determined by the method of Gornall *et al.* [29] (reagent kit, Merck, Darmstadt, F.R.G.) and at all other stages by a bicinchonic acid method [30] (reagent kit, Pierce, Rochford, SD, U.S.A.). Bovine serum albumin was used as the standard in both methods. All photometric measurements were performed with a Kontron Uvikon 930 spectrophotometer. The SDS–PAGE analyses were performed following the method of Lämmli [31] with a 3% stacking gel and an 8% separation gel (1.5 mm thickness). Staining was achieved with Coomassie brilliant blue (Serva, Heidelberg, F.R.G.).

Incubations

N-Reduction of N*-hydroxydebrisoquine with* 9000 g *supernatant, microsomes, or* 100,000 g *supernatant.* Incubations were carried out in a shaking water bath at 37° in the presence of oxygen using 1.5-mL reaction vessels. The standard incubation mixture (usually 0.3 mL) contained the following components: phosphate buffer (pH 6.3; 40 mM KH_2PO_4 adjusted with dilute NaOH solution at 37°), 1.2 mM *N*-hydroxydebrisoquine, 0.4 mM NADH and 0.5 mg protein of the enzyme source/0.3 mL. After preincubation for 1 min at 37°, the reactions were started by addition of NADH. Incubation time was 20 min. Incubations were terminated by addition of 0.5 mL diethyl ether and cooling the samples in an ice/salt mixture. The excess *N*-hydroxydebrisoquine was removed by four extractions (vibration mixer IKA Vibrofix VF2, Janke and Kunkel, F.R.G.) each with 0.5 mL of diethyl ether, subsequent centrifugation at 11,000 *g* (microliter centrifuge, Hettich, F.R.G.), and careful removal of the organic phase using an air-tight syringe (250 μL, Hamilton, F.R.G.). Methanol (1.0 mL) was added and the incubation mixture was allowed to stand for 2 hr at −20° for protein precipitation. Thereafter, the incubation mixtures were immediately centrifuged at 11,000 *g* for 10 min and 10-μL aliquots taken for the HPLC analysis.

N-Hydroxylation of debrisoquine with 9000 g *supernatant, microsomes, or 100,000* g *supernatant.* Incubations were carried out in a shaking water bath

at 37° in the presence of oxygen using 1.5-mL reaction vessels. The standard incubation mixture (volume 0.3 mL) contained the following components: phosphate buffer (pH 7.4; 8.7 mM KH_2PO_4, 30.4 mM Na_2HPO_4), 1.0 mM debrisoquine sulfate, 0.5 mM NADPH, 3.3 mM $MgCl_2$, 6.0 mM *N*-methylhydroxylammonium chloride, and 0.1 mg protein from the enzyme source/0.3 mL. After a preincubation at 37° for 1 min, the reactions were started by addition of NADPH. The incubations were terminated after 45 min by cooling the mixtures in an ice/salt bath. The pH of the incubation mixture was then immediately adjusted to 8.0 by addition of dilute NaOH solution and 0.5 mL ice-cooled, freshly distilled diethyl ether were added. The mixture was extracted five times with 0.5-mL portions of cooled ether and centrifuged at 11,000 *g* for 5 min. The ether phases were combined using an air-tight syringe and the ether was carefully removed under reduced pressure. The residue was treated with 0.1 mL of the HPLC eluent, the reaction vessel was shaken for 3 min, and finally centrifuged at 11,000 *g* for 10 min. Aliquots of 10 μL of the supernatant were taken for HPLC analysis.

N-Hydroxylation of debrisoquine by reconstituted cytochrome P450 monooxygenase systems and incubations with FMO. The incubations were carried out in a shaking water bath at 37° in the presence of oxygen using 1.5-mL reaction vessels. The incubation mixtures (volume: 0.6 mL) usually contained the following components: phosphate buffer (pH 7.4; 8.7 mM KH_2PO_4, 30.4 mM Na_2HPO_4), 1.6 mM debrisoquine sulfate, 0.5 mM NADPH, 40 μM DLPC, 0.9 U NADPH cytochrome P450 reductase, and 0.3 nmol cytochrome P450. The mixture was preincubated for 3 min in the presence of phosphate buffer, DLPC, reductase and cytochrome P450. Debrisoquine sulfate was then added and the reaction started by addition of NADPH. After 20 min the incubation was terminated by cooling the reaction vessel in an ice/salt bath. Work-up was as described for the experiments with microsomal fractions. The incubations with purified FMO were performed analogously but with the omission of DLPC. In place of the components of the cytochrome P450 enzyme system, up to 1 mg of FMO/incubation mixture (approx. 70 mU) was used.

Oxidative conversion of N*-hydroxydebrisoquine to the urea derivative of debrisoquine by* 9000 g *supernatant, microsomes, or* 100,000 g *supernatant.* The incubations were performed in a shaking water bath at 37° in the presence of oxygen using 1.5-mL reaction vessels. The standard incubation mixture (volume: 0.2 mL) contained the following components: phosphate buffer (pH 7.4), 1.0 mM *N*-hydroxydebrisoquine, 0.5 mM NADPH, 3.3 mM $MgCl_2$, 0.5 mg protein of the enzyme source/0.2 mL. After a preincubation of 37° for 1 min, the reaction was started by addition of NADPH and terminated after 20 min by addition of 20 μL of 72% trichloroacetic acid. The samples were immediately shaken and centrifuged at 11,000 *g* for 10 min. Aliquots of 10 μL of the supernatant were taken for HPLC analysis.

Oxidative conversion of N*-hydroxydebrisoquine to the urea derivative of debrisoquine by reconstituted cytochrome P450 oxidase systems and incubations with FMO.* The incubations were carried out as described for the N-hydroxylation of debrisoquine but with the following changes. The incubation mixture (volume: 0.2 mL) comprised phosphate buffer (pH 7.4), 1.0 mM *N*-hydroxydebrisoquine, 0.5 mM NADPH, 40 μM DLPC, 25 pmol cytochrome P450 and 0.15 U P450 reductase. After 20 min the reactions were terminated and the mixtures worked-up as described for experiments with microsomal fractions. Incubations with purified FMO were performed analogously. In place of the components of the cytochrome P450 enzyme system, up to 0.3 mg FMO/incubation were employed.

Anaerobic incubations. Incubations were carried out as described above with the following modifications. All solutions were degassed at 0–4° under reduced pressure and treated with nitrogen. The solutions were pipetted under nitrogen into the microtest tubes which were then stoppered.

Incubations with hepatocytes. The incubations were carried out in a shaking water bath under aerobic conditions. The substrates were dissolved in modified Hank's buffer solution (final concentration 20–200 μM) and, after 5 min preincubation, added to the hepatocyte suspension. The cell quantity amounted to 10^6 cells/mL and the final volume of the incubation mixture was 2.0 mL. In the case of debrisoquine after 10 min incubation, 200 μL of the incubation mixture were treated with 200 μL methanol, deep-frozen for 2 hr, subsequently centrifuged at 11,000 *g* for 10 min, and aliquots of 10 μL taken for HPLC analysis.

In the case of *N*-hydroxydebrisoquine after 60 min incubation, 300 μL of incubation mixture to which *N*-methylhydroxylammonium chloride had been added (final concentration 80 μM) were worked up as described under "N-hydroxylation of debrisoquine with microsomes".

For the urea derivative of debrisoquine, 200 μL of incubation mixture were treated with 20 μL of 72% trichloroacetic acid solution, shaken and centrifuged at 11,000 *g* for 10 min. Aliquots of 10 μL were taken for HPLC analysis.

Thin layer chromatography

The thin layer chromatographic detection of metabolite debrisoquine formed after N-reduction of *N*-hydroxydebrisoquine was carried out after incubations in 25-mL Erlenmeyer flasks with an incubation volume of 6.0 mL. In contrast to the incubation mixtures for HPLC analysis *N*-hydroxydebrisoquine was employed at a concentration of 1.7 mM and 20 mg microsomal protein were used in each incubation experiment. For the qualitative analysis, six such incubation mixtures were freeze-dried and the lyophilisate extracted twice with 2-mL portions of methanol after 45-min incubation. The organic phases were combined and evaporated to a volume of about 0.5 mL under reduced pressure. The precipitated protein was separated by centrifugation at 4000 *g* for 10 min. The methanol phase was applied in bands to the TLC plates (Si 60, 20 cm × 20 cm × 0.25 mm) and developed in a saturated chamber with the solvent system toluene/methanol/triethylamine (8:1:1, by

vol.) for 45 min. The silica gel of the starting zone was scratched off and extracted with about 4 mL methanol. The silica gel was removed by filtration, the organic phase was evaporated almost to dryness, and the residue applied as spots to another TLC plate (see above). A mixture of *n*-butanol, glacial acetic acid and water (60:15:25, by vol.) was used as mobile phase and the development time was 1 hr. At the same R_f value (0.45) as the parallel migrating debrisoquine standard, a fluorescence extinction was detected under UV light (256 nm). For the preparation of the spray reagent, 0.3 g α-naphthol was dissolved in 30 mL ethanol and treated with 1.0 g sodium (solution 1). Solution 2 contained 0.2 mL diacetyl reagent (2,3-butanedione) in 20 mL ethanol. After spraying with solution 1 and drying with solution 2, the region of fluorescence extinction of the sample under analysis and the reference took on a pink color, while traces of the starting material *N*-hydroxydebrisoquine at an R_f value of 0.6 were colored blue.

HPLC

Debrisoquine. The resulting supernatant was analysed using a high performance liquid chromatograph (Waters 510, Millford, CT, U.S.A.) equipped with a variable wavelength UV detector (Waters 486) set at 208 nm, and an autosampler (Merck AS-200). The areas under the peaks were integrated with a chromatointegrator (Waters 746). Separation and quantification were performed at room temperature on a prepacked, reverse phase column (125 × 4 mm i.d., particle size 5 μm; Lichrospher® RP-select B, Merck). The mobile phase was acetonitrile/KH_2PO_4 buffer (0.05 M, pH 4.0) (8:92, v/v) and the flow rate through the column was 1.0 mL/min. Solvents used in the analysis were filtered through a membrane filter CRC-255 (Schleicher and Schuell, F.R.G.) and degassed by bubbling with helium or sonication. Standard curves (peak area) at the levels of 0.3, 0.6, 1.6, 4.0, 8.0, 16.0, 24.0, 40.0, 48.0 and 64.0 μM debrisoquine were constructed by introducing known amounts of debrisoquine into the usual incubation mixtures which were then incubated and treated in the same manner as the experimental samples. The standard curves were linear over this range with correlation coefficients > 0.9997. The levels of debrisoquine in unknown incubation mixtures were determined directly from these standard curves which were obtained from measurements run in parallel with the experimental samples. The recovery of debrisoquine from incubation mixtures was 100.1 ± 2.9% (N = 44) of that obtained using samples which contained the same amount of debrisoquine dissolved in phosphate buffer. The detection limit for debrisoquine in one incubation mixture was 0.3 μM, which corresponds to a rate of N-reduction of 9.7 pmol debrisoquine/min/mg protein. The retention times were 15.0 ± 0.5 min for debrisoquine and 11.3 ± 0.5 min for *N*-hydroxydebrisoquine.

N-Hydroxydebrisoquine. Separation and quantification were performed as described above for debrisoquine. Standard curves at the levels of 0.025, 0.05, 0.13, 0.32, 0.63, 1.27, 1.90, 2.53 and 3.80 μM *N*-hydroxydebrisoquine were constructed and found to be linear over this range with correlation coefficients > 0.9998. The recovery of *N*-hydroxydebrisoquine from incubation mixtures was 91.6 ± 2.1% (N = 36) of that obtained using samples which contained the same amount of *N*-hydroxydebrisoquine dissolved in phosphate buffer. The detection limit of *N*-hydroxydebrisoquine in one incubation mixture was 0.025 μM which corresponds to a rate of N-oxygenation of 1.7 pmol *N*-hydroxydebrisoquine/min/mg protein.

Using reconstituted cytochrome P450 monooxygenase systems, quantification of *N*-hydroxydebrisoquine was performed with standard curves at the levels of 0.77, 0.65, 0.39, 0.26, 0.13, 0.06 and 0.03 μM *N*-hydroxydebrisoquine. The standard curves were linear over this range with correlation coefficients > 0.9979. The recovery of *N*-hydroxydebrisoquine from incubation mixtures was 80.8 ± 5.0% (N = 36) of that obtained using samples which contained the same amount of *N*-hydroxydebrisoquine dissolved in phosphate buffer. The detection limit of *N*-hydroxydebrisoquine was 0.026 μM which corresponds to a rate of N-oxygenation of 2.6 pmol *N*-hydroxydebrisoquine/min/nmol P450.

Urea derivative of debrisoquine. Separation and quantification were performed essentially as described above for debrisoquine. The mobile phase consisted of acetonitrile/KH_2PO_4 buffer (0.01 M, pH 3.0) (15:85, v/v). Standard curves were constructed with 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 25 μM solutions of the urea derivative of debrisoquine and were found to be linear over this range with correlation coefficients > 0.9998. The recovery rate amounted to 79.7 ± 4.2% (N = 54). It was possible in this manner to detect a concentration of 0.05 μM of the urea derivative which corresponds to a rate of formation of 1.1 pmol/min/mg protein. The retention time was 13.5 ± 0.5 min.

Preparative enrichment of the metabolite N-*hydroxydebrisoquine for UV and mass spectroscopic analysis*

The incubations with microsomal fractions were carried out in 25-mL Erlenmeyer flasks with an incubation volume of 6.0 mL. In contrast to the concentrations of the components used in the experiments on the N-oxygenation of debrisoquine, the following changes were made: 20 mg protein/6.0 mL, 6 mM *N*-methylhydroxylammonium chloride, 40 mM tris(hydroxymethyl)-aminomethane hydrochloride buffer (pH 7.4). The incubation time was 35 min. After transfer of the incubation mixture to a centrifuge tube, the solution was treated with ammonium sulfate to 40% saturation and the protein separated by centrifugation. The pH of the clear supernatant was adjusted to approx. 8.0 with 0.1 M NaOH solution and the mixture extracted four times with four 5-mL portions of diethyl ether each for 15 min. After centrifugation at 4000 *g* for 10 min, the ether phases were removed using a Pasteur pipette, combined, dried over sodium sulfate, and evaporated to dryness under reduced pressure. In order to obtain a sufficient amount of the metabolite,

eight such experiments were run in parallel and the ether phases combined.

Preparative enrichment of the urea derivative of debrisoquine for UV spectroscopy

For enrichment of the urea derivative of debrisoquine, six of the usual experiments with microsomal fractions were run and shaken five times each with 300 μL dichloromethane. The combined organic phases were evaporated to dryness, the residue was taken up in 100 μL of the HPLC eluent, centrifuged, and 20-μL aliquots of the supernatant were taken.

UV spectra of the metabolites

N-*Hydroxydebrisoquine.* The residue obtained from eight combined microsomal incubation experiments was dissolved in 0.2 mL acetonitrile/KH_2PO_4 buffer (0.1 M, pH 4.0) (13:87, v/v) and the mixture centrifuged at 6000 *g* for 5 min. Aliquots of 20 or 40 μL of the clear supernatant were analysed on a high performance chromatograph (Waters 600 multisolvent delivery system, Waters Associated, Eschborn, F.R.G.) coupled to a diode array detector (Waters 9919). In contrast to the analytical conditions described above, a Lichrosorb® RP-Select B column (Hibar Fertigsäule, 250 × 4 mm i.d., Merck) and acetonitrile/KH_2PO_4 (0.01 M, pH 4.0) as eluent at a flow rate of 0.7 mL/min were used. The UV spectrum of the metabolite with a retention time of 13.1 min was identical to the spectrum of *N*-hydroxydebrisoquine recorded under identical conditions by the diode array detector.

Debrisoquine. The same HPLC conditions as described above for the UV spectrum of *N*-hydroxydebrisoquine were used. Neither the combination of several microsomal incubation mixtures nor a prior enrichment was necessary. One incubation experiment with hepatocytes was also sufficient. The UV spectrum of the metabolite with a retention time of 15.0 min was identical with that of debrisoquine recorded under the same conditions by the diode array detector.

Urea derivative of debrisoquine. Six enriched microsomal incubation mixtures (see above) were analysed as described above for the HPLC analysis. The metabolite with a retention time of 13.5 min exhibited a UV spectrum identical to that of the synthesized urea derivative of debrisoquine.

Mass spectrum of the metabolite N-*hydroxydebrisoquine by LC–MS coupling*

For the mass spectroscopic analysis, the ether residue was taken up in 0.3 mL of the mobile phase (see below), the mixture was centrifuged at 6000 g for 3 min, and 50 or 100-μL aliquots of the clear supernatant were subjected to HPLC analysis.

Chromatographic conditions were as follows: mobile phase comprising acetonitrile/ammonium acetate buffer (0.05 M, pH 4.3) (25:75, v/v) at a flow rate of 0.8 mL/min, and an RP-Select B column (see above). The interface to the mass spectrometer (TSQ 700, Finnigan MAT, Bremen, F.R.G.) was via a thermospray system. The data of the mass spectrum and the daughter spectrum of the MH^+ ion of the metabolite with a retention time of 15.4 min agreed with those of *N*-hydroxydebrisoquine recorded under identical conditions.

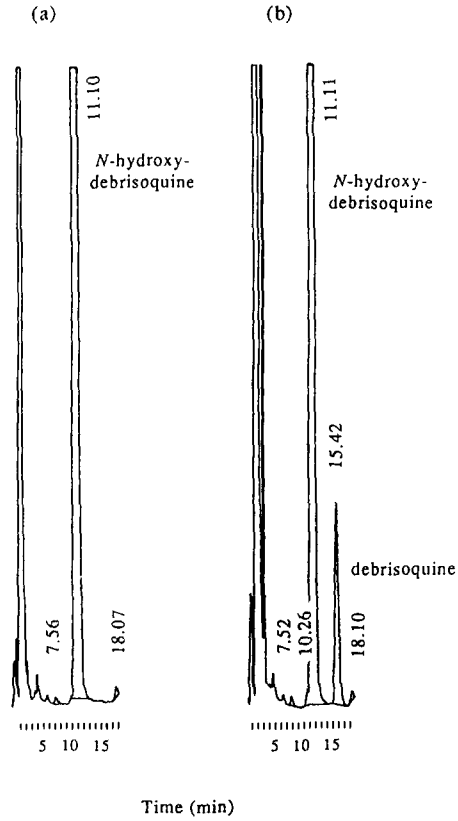

Fig. 2. Representative HPLC chromatogram of *N*-hydroxydebrisoquine metabolism by 9000 *g* supernatant fractions of rat and rabbit liver homogenates. See Materials and Methods for details of reaction mixture content, incubation, sampling and analysis. Key: (a) omission of cofactor; (b) complete system.

RESULTS

Qualitative and quantitative analyses of debrisoquine formed by microsomal reduction of N-*hydroxydebrisoquine*

Qualitative and quantitative analyses. Unequivocal evidence for the formation of debrisoquine from *N*-hydroxydebrisoquine by *in vitro* incubation with microsomal fractions was obtained by TLC and HPLC methods. After 2-fold TLC development with a basic and an acidic mobile phase, fluorescence extinction was detected at the same R_f value as that of the reference substance. Both reference substance and metabolite were colored red by the spray reagent α-naphthol/diacetyl. No debrisoquine was formed in incubation experiments which did not contain either protein or cofactor.

For the qualitative detection by high performance chromatography, the excess of *N*-hydroxydebrisoquine was removed by extracting the incubation mixtures with diethyl ether. After addition of methanol and separation of the protein by

Table 1. Rates of the N-reduction of *N*-hydroxydebrisoquine by microsomal enzymes of rabbit and rat liver

Species	Cofactor	nmol debrisoquine/ min/mg protein
Rabbit	—	<0.0006
	NADH	1.256 ± 0.050
	NADPH	0.420 ± 0.049
Rat	—	<0.0006
	NADH	1.094 ± 0.037
	NADPH	0.294 ± 0.034

Rates ± SD were calculated from three determinations with microsomes isolated from liver homogenates of 10 rabbits or 15 rats; reaction mixture contained the components described in Materials and Methods, except for the omission of cofactors.

centrifugation, the clear supernatant was subjected directly to HPLC analysis. A peak with the same retention time as debrisoquine was observed. Addition of debrisoquine resulted in an intensification of the signal. This result was confirmed by several repetitions of the experiment. The retention time of the analyte also agreed with that of the synthesized reference compound when other eluent systems were used. Figure 2 shows a typical chromatogram from the HPLC analysis of a reaction with microsomal fractions.

A complete UV spectrum of the metabolite was recorded by using a diode array detector. It was identical to that of the reference substance recorded under the same conditions. The correspondence of the chromatographic behavior and UV spectrum of the metabolite with the data of the reference compound allowed its unambiguous characterization. Hence, a mass spectrum was not considered to be necessary. The quantitative characterization of the N-reduction of *N*-hydroxydebrisoquine was achieved by the same HPLC method which was developed on the basis of the method of Moncrieff [32].

Subcellular distribution, different species and cofactor requirements. The reaction rates of the N-reduction of *N*-hydroxydebrisoquine by microsomal enzymes obtained from rabbit and rat livers under differing incubation conditions are listed in Table 1. Of the investigated enzyme preparations, only the 9000 *g* supernatant and the microsomes exhibited marked enzyme activity. The reaction required either NADH or NADPH as cofactor and considerably higher reaction rates were observed in the presence of NADH.

The reaction was found to be proportional to the protein concentration up to a concentration of 0.5 mg microsomal protein/0.3 mL incubation mixture. This concentration proved to be optimal with regard to sample work-up and analysis. Investigations on the dependence of the reaction rate on cofactor concentration at this amount of microsomal protein revealed that concentrations of as little as 0.3 mM NADH effected the maximum reaction rates while higher concentrations did not significantly change the rate of formation of the metabolite. Thus, the chosen concentration of 0.4 mM NADH/incubation experiment could be discounted as a limiting factor for the reaction.

Anaerobic conditions. The incubations were carried out in the presence of oxygen since no significant differences in the reaction rates were observed under anaerobic conditions (data not shown).

Effect of inhibitors. It was assumed that the

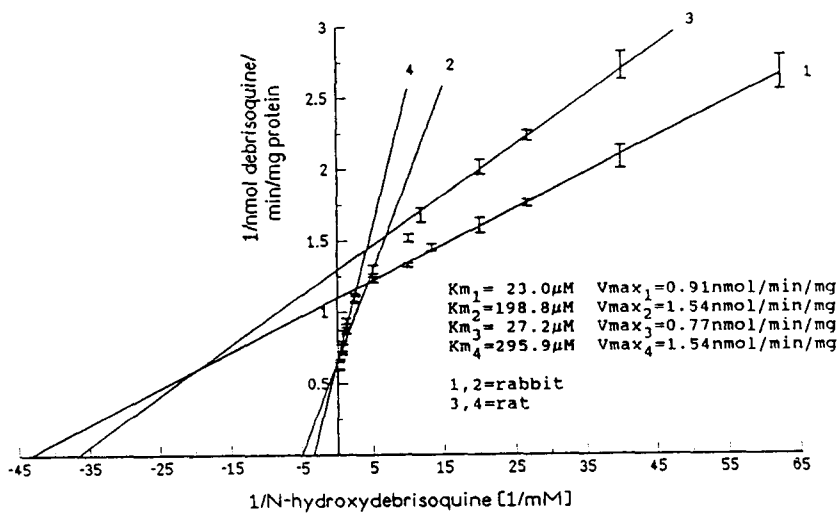

Fig. 3. Lineweaver–Burk plot of the N-reduction of *N*-hydroxydebrisoquine by rabbit liver (1, 2) and rat liver microsomes (3, 4) measured by debrisoquine formation in the incubation mixture. Each point is the mean of three different experiments (three different incubations with an enzyme preparation from 10 rabbit livers or 15 rat livers, two determinations each) ± SD. Incubations were carried out as described in Materials and Methods.

Table 2. Microsomal N-reduction of *N*-hydroxydebrisoquine and N-oxygenation of debrisoquine as a function of pH

pH	Rate of N-reduction of *N*-hydroxydebrisoquine (nmol debrisoquine/ min/mg protein)	Rate of N-oxygenation of debrisoquine (nmol *N*-hydroxydebrisoquine/ min/mg protein)
5.5	0.96 ± 0.04	0.008 ± 0.001
5.7	0.98 ± 0.08	0.016 ± 0.001
5.9	1.04 ± 0.03	0.024 ± 0.005
6.1	1.31 ± 0.03	0.052 ± 0.002
6.3	1.34 ± 0.03	0.069 ± 0.003
6.5	1.26 ± 0.08	0.087 ± 0.005
6.7	1.12 ± 0.08	0.127 ± 0.001
6.9	0.98 ± 0.02	0.126 ± 0.002
7.1	0.71 ± 0.04	0.123 ± 0.001
7.4	0.53 ± 0.02	0.093 ± 0.002
7.8	0.24 ± 0.01	0.066 ± 0.001

Rates ± SD were calculated from two different determinations with microsomes isolated from liver homogenates of 10 rabbits; incubations were carried out as described in Materials and Methods.

enzyme system described by Ziegler and co-workers [33, 34], which reduced hydroxylamines under analogous conditions, was also responsible for the reduction of *N*-hydroxydebrisoquine. Quantitative inhibition studies revealed that, at *N*-hydroxydebrisoquine concentrations of 0.1 mM or 1.16 mM, respectively, equivalent concentrations of hydroxylammonium sulfate, decrease of the reaction rate to approximately 30% of the uninhibited rate occurred. Similar results were obtained using *N*-methylhydroxylammonium chloride instead of hydroxylammonium sulfate.

Kinetics of the N-reduction of N-*hydroxydebrisoquine.* The reaction rate of the enzymatic formation of debrisoquine was linear up to an incubation time of 30 min. For quantitative measurements, the samples were incubated for 20 min in order to obtain a sufficient amount of the metabolite. The N-reduction of *N*-hydroxydebrisoquine during incubations with microsomal fractions from rabbit and rat livers in the presence of NADH showed a biphasic course in the Lineweaver–Burk plot. The kinetic parameters thus obtained can be taken from Fig. 3.

Influence of pH. The effects of pH values between 5.5 and 7.8 on the N-reduction by rabbit microsomes are shown in Table 2. The optimum pH value was 6.3 ± 0.2. These incubations were performed in phosphate buffer (50 mM).

Qualitative and quantitative analyses of debrisoquine formed by reduction of N-*hydroxydebrisoquine using rat hepatocytes*

Conclusive evidence for the formation of

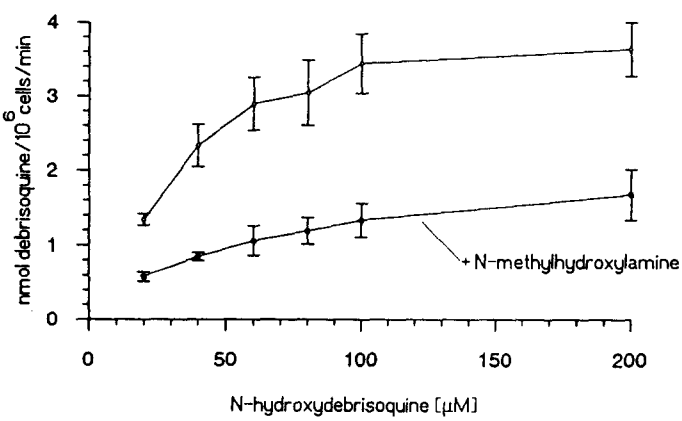

Fig. 4. Effect of *N*-methylhydroxylammonium chloride on the rate of *N*-hydroxydebrisoquine reduction by rat hepatocytes. Incubations were carried out as described in Materials and Methods, except for the addition of *N*-methylhydroxylammonium chloride (80 μM). Each point is the mean of experiments with three hepatocyte preparations (three different incubations with one hepatocyte preparation, two determinations each) ± SD.

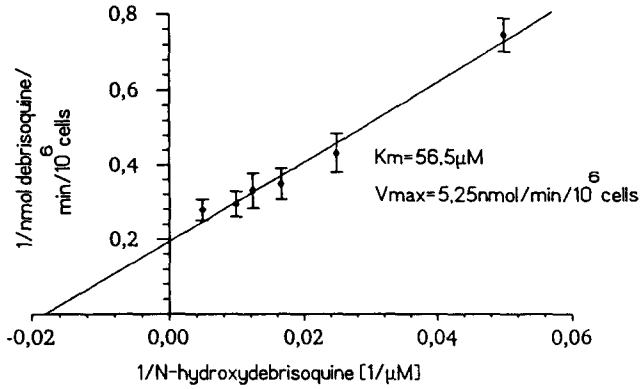

Fig. 5. Lineweaver–Burk plot of the N-reduction of *N*-hydroxydebrisoquine by rat hepatocytes measured by debrisoquine formation in the incubation mixture. Each point is the mean of experiments with three hepatocyte preparations (three different incubations with one hepatocyte preparation, two determinations each) ± SD. Incubations were carried out as described in Materials and Methods.

debrisoquine from *N*-hydroxydebrisoquine in *in vitro* incubations with rat hepatocytes was obtained as described for the incubations with microsomal preparations.

Effect of inhibitors. Quantitative inhibition experiments with hydroxylamine also revealed an appreciable diminution of the N-reduction by hepatocytes. Hydroxylamine concentrations of 100 µM or more, however, gave rise to toxic reactions so that the maximum hydroxylamine concentration used was 80 µM. The reaction rates of various concentrations of *N*-hydroxydebrisoquine with and without addition of *N*-methylhydroxylammonium chloride are shown in Fig. 4. Hydroxylammonium sulfate exerted the same effect as *N*-methylhydroxylammonium chloride.

Kinetics of the reduction of N-*hydroxydebrisoquine.* The reaction rate of the enzymatic formation of debrisoquine was practically linear up to an incubation time of 30 min. For quantitative measurements the samples were incubated for 10 min in order to obtain a sufficient amount of the metabolite.

The Lineweaver–Burk plot of the N reduction of *N*-hydroxydebrisoquine by rat hepatocytes revealed a linear course; the kinetic parameters obtained are shown in Fig. 5.

Qualitative and quantitative analyses of N-*hydroxydebrisoquine formed by microsomal N-hydroxylation of debrisoquine*

The qualitative and quantitative determination of *N*-hydroxydebrisoquine from *in vitro* incubation experiments is possible by selective extraction of the metabolite with diethyl ether at a pH value of 8.0. The combined and dried organic phase was evaporated to dryness, the residue taken up in eluent mixture, and subjected to HPLC analysis. By means of these biotransformation studies, *N*-hydroxydebrisoquine formed by the microsomal N-oxygenation of debrisoquine was detected. The incubations for the characterization of this transformation by microsomal enzymes were performed in the presence of *N*-methylhydroxylammonium chloride since these conditions resulted in an enhanced formation of the metabolite (see below). A typical chromatogram from the HPLC analysis is shown in Fig. 6.

By use of a diode array detector, the complete UV spectrum of the metabolite was recorded and found to be identical with that of the reference substance taken under the same conditions ($UVmax_1$ = 200 nm, $UVmax_2$ = 214 nm). The mass spectrum of the metabolite was measured by coupling the HPLC system to a mass spectrometer and use of the thermospray ionization procedure. In the same experiment, the daughter spectra of the protonated molecular ion and fragments were recorded by MS–MS. The spectra of the metabolite were compared with those of the reference substance recorded under identical conditions. Apart from differences in intensities, the main fragments were identical. As examples, daughter spectra of the molecular ions [MH^+ = 192 atomic mass unit (AMU)] of the reference substance and the metabolite are shown in Fig. 7.

Microsomal enzymes, cofactor requirements, and effect of hydroxylamines. Enzyme sources from rats and rabbits were used in the biotransformation studies. While the 9000 *g* supernatant and the microsomes showed enzyme activity, experiments with the 100,000 *g* supernatant did not give rise to measurable reactions. The microsomes from rabbit liver appreciably effected higher reaction rates than those from rat liver (Table 3). The microsomal N-hydroxylation required NADPH as cofactor. The addition of 2 mg superoxide dismutase or 2 mg catalase to the incubation mixtures did not influence the reactions (data not shown).

The concentration of *N*-hydroxydebrisoquine was proportional to the amount of protein employed up to a protein concentration of 0.5 mg/0.3 mL incubation mixture. The dependence of the reaction of the N-oxygenation on the concentration of *N*-methylhydroxylammonium chloride is shown in Fig. 8. The addition of as little as 0.5 mM gave rise to a

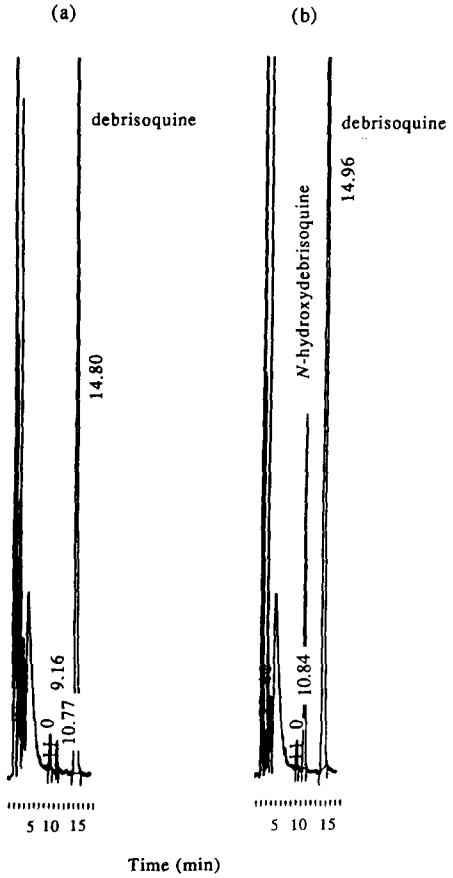

Fig. 6. Representative HPLC chromatogram of debrisoquine metabolism by 9000 *g* supernatant fractions of rat and rabbit liver homogenates. See Materials and Methods for details of reaction mixture content, incubation, sampling and analysis. Key: (a) omission of cofactor, (b) complete system.

7-fold increase in the amount of metabolite and this could not be increased further by larger amounts of the additive. In all experiments, the added amount of *N*-methylhydroxylamine was chosen as 6.0 mM.

Studies with polyclonal antibodies against P450 reductase. In the presence of polyclonal antibodies against P450 reductase from rabbits, the microsomal N-oxygenation of debrisoquine was diminished to approximately 42% of the uninhibited enzyme reactivity (Fig. 9).

Kinetics of the N-hydroxylation of debrisoquine. The reaction rate of the enzymatic formation of *N*-hydroxydebrisoquine. The reaction rate of the enzymatic formation of *N*-hydroxydebrisoquine was linear over a period of 1 hr. All experiments were performed with an incubation time of 45 min. The concentrations of debrisoquine, NADPH and *N*-methylhydroxylammonium chloride were chosen so that maximum reaction rates were achieved. This optimal composition of the incubation mixtures was determined as described for the N-reduction of *N*-hydroxydebrisoquine. The N-hydroxylation of debrisoquine by microsomes from rabbits followed Michaelis–Menten kinetics (Fig. 10).

Influence of pH and buffer. The effect of pH values between 5.5 and 7.8 on the microsomal N-oxygenation activity is shown in Table 2. The optimum pH value was determined as 6.7. The incubations were carried out in phosphate buffer (50 mM); incubations performed in Tris–HCl buffer (50 mM) in the pH range 7.0–9.2 proceeded at similar rates.

Incubations with reconstituted cytochrome P450 monooxygenase systems. Incubations were performed in the presence of enzyme preparations enriched in P450 with the addition of P450 reductase (see Materials and Methods). The reaction rates of incubations with and without the addition of *N*-methylhydroxylammonium chloride are listed in Table 4.

The reaction rates measured in reconstituted systems with purified cytochrome P450 2C3 are given in Table 5. No increase of the reaction rate upon addition of *N*-methylhydroxylammonium chloride was observed in these experiments. The addition of the P450 2C3 inducer 5-βPD (50 μM) [35] did not result in a higher reaction rate (0.012 ± 0.0013 nmol/min/nmol P450 2C3).

Incubations with flavin-containing monooxygenase. Incubations in the presence of flavin-containing monooxygenase obtained from porcine liver according to the procedure of Ziegler and Poulsen [26] did not provide any evidence for the participation of this enzyme system in the N-hydroxylation of debrisoquine. Up to 1 mg of the highly purified enzyme/incubation experiment (corresponding to approx. 70 mU) were employed in order to be able to detect even the slightest reaction. No metabolite could be detected even after addition of *n*-octyl-amine (3 mM), an activator of flavin-containing monooxygenase [36].

Qualitative analysis of N-*hydroxydebrisoquine formed by N-hydroxylation of debrisoquine using rat hepatocytes.* The formation of the metabolite *N*-hydroxydebrisoquine in incubation experiments with rat hepatocytes could not be detected even after enrichment attempts by selective extraction out with diethyl ether. Similarly, no *N*-hydroxydebrisoquine formation was detected after addition of 80 μM *N*-methylhydroxylammonium chloride.

Qualitative and quantitative analyses of the urea derivative of debrisoquine formed by microsomal transformation of N-*hydroxydebrisoquine*

Qualitative and quantitative analysis. The urea derivative of debrisoquine was unambiguously identified and quantified in *in vitro* incubation experiments with microsomal fractions by HPLC analysis and diode array detection. The procedure was similar to that used in the identification of debrisoquine (see above).

Subcellular distribution and cofactor requirements. The reaction rates for the formation of the urea derivative of debrisoquine by microsomal enzymes obtained from rabbit livers are shown in Table 6. Enzyme activity was only present in the microsomal fractions. A protein concentration of 0.5 mg microsomal protein/0.2 mL incubation mixture

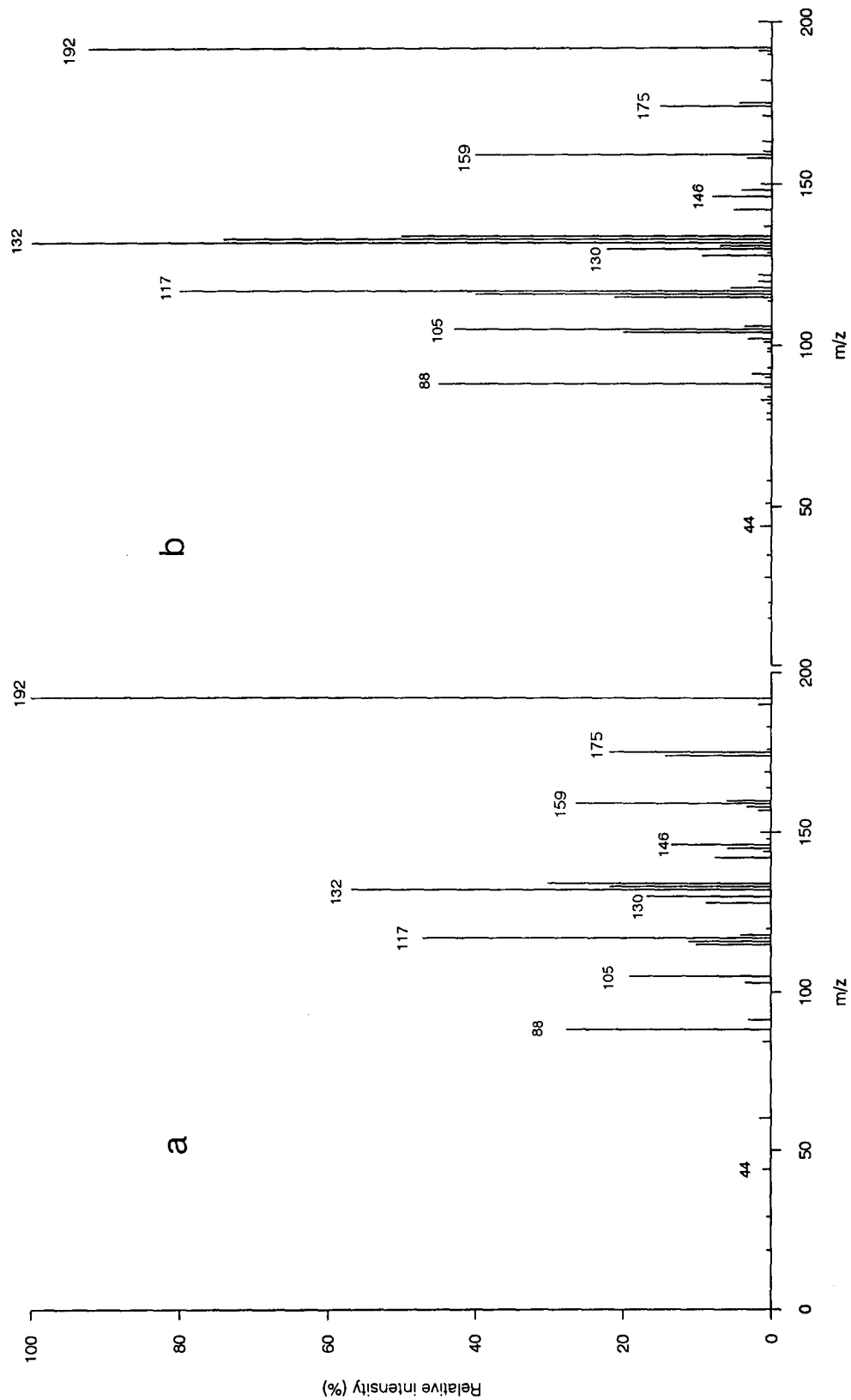

Fig. 7. Mass spectrum of the MH^+-cation (m/z = 192) of synthetic *N*-hydroxydebrisoquine (a) and of the metabolite *N*-hydroxydebrisoquine (b). See Materials and Methods for details of reaction mixture content, incubation, sampling and analysis.

Table 3. Rates of debrisoquine N-oxygenation by microsomal enzymes of rabbit and rat liver

Species	nmol *N*-hydroxydebrisoquine/min/mg
Rabbit	0.1269 ± 0.004
Rat	0.0390 ± 0.001

Rates ± SD were calculated from three determinations with microsomes isolated from liver homogenates of 10 rabbits or 15 rats; incubations were carried out as described in Materials and Methods.

proved to be optimal with regard to sample work-up and analysis.

From experiments with a cofactor concentration of 0.5 mM NADPH/incubation mixture, this could be discounted as a limiting factor. No turnover was measurable at time $t = 0$, or in the absence of protein. Generally the reaction rates with microsomal fractions were very low, deviated significantly depending on the rabbit liver used or were not measurable. Thus, only investigations using enzyme sources from one rabbit liver are listed in Table 6.

Effect of catalase and superoxide dismutase. The influence of catalase and superoxide dismutase in microsomal fractions from rabbit liver homogenates on the formation of the urea derivative of

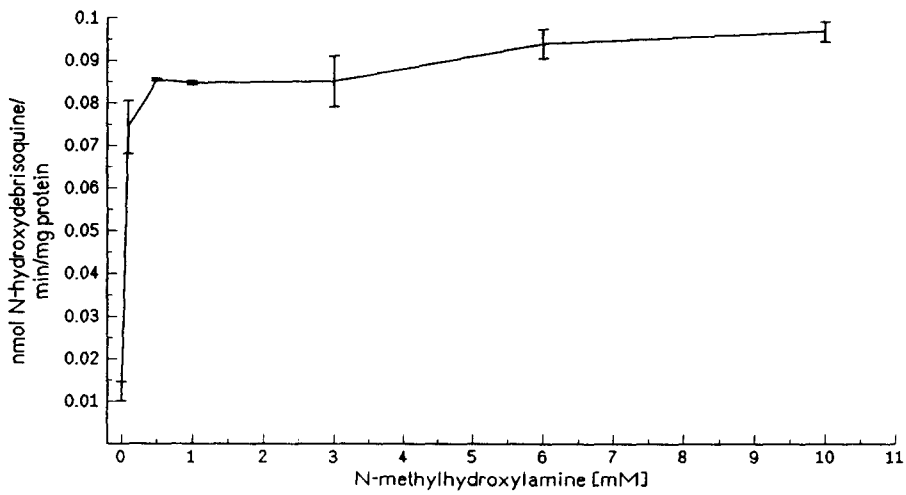

Fig. 8. Formation of *N*-hydroxydebrisoquine by microsomes of rabbit liver homogenates as a function of *N*-methylhydroxylamine concentration. Each point is the mean of two different experiments (two different incubations with an enzyme preparation from 10 rabbit livers, two determinations each) ± SD. See Materials and Methods for details of reaction mixture content, incubation, sampling and analysis.

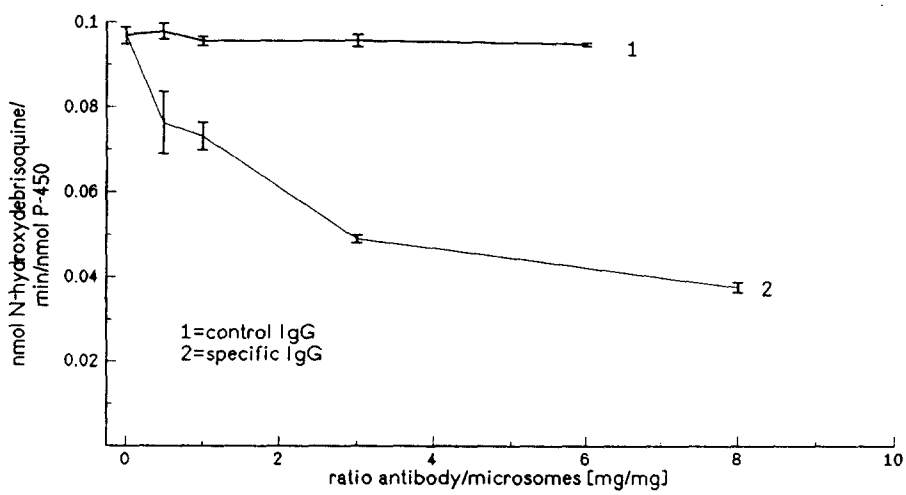

Fig. 9. Inhibition by a polyclonal antibody against rabbit NADPH cytochrome P450 reductase of the N-hydroxylation of debrisoquine catalysed by rabbit liver microsomes. Each point is the mean of two different experiments (two different incubations with an enzyme preparation from 10 rabbit livers, two determinations each) ± SD. Incubations were carried out as described in Materials and Methods.

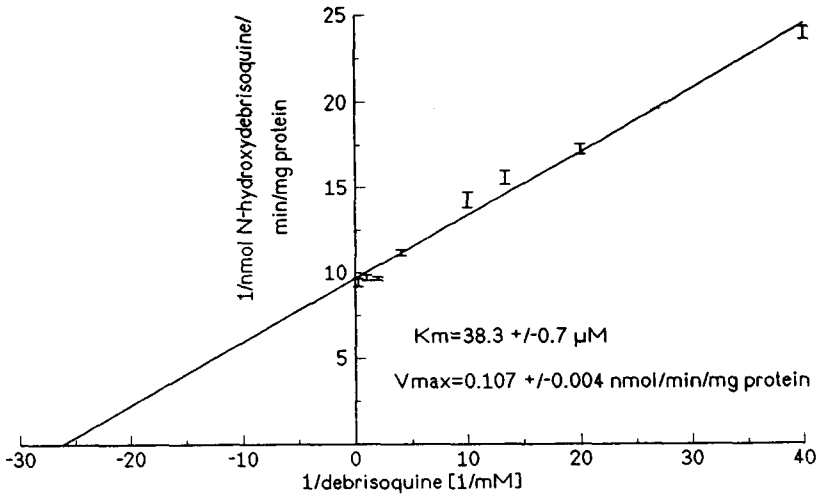

Fig. 10. Lineweaver–Burk plot of the N-hydroxylation of debrisoquine by microsomes measured by *N*-hydroxydebrisoquine formation in the incubation mixture. Each point is the mean of two different experiments (two different incubations with an enzyme preparation from 10 rabbit livers, two determinations each) ± SD. Incubations were carried out as described in Materials and Methods.

Table 4. Effect of *N*-methylhydroxylamine on the rate of N-hydroxylation of debrisoquine by reconstituted cytochrome P450 oxidase system

Incubation mixture	nmol *N*-hydroxydebrisoquine/ min/nmol P450
− CH_3NHOH	0.0052 ± 0.0007
+ CH_3NHOH	0.0300 ± 0.0030

Values are presented as the means of two different experiments ± SD; reaction mixture contained the components described in Materials and Methods, except for the addition of *N*-methylhydroxylamine (6 mM).

Table 5. Effect of *N*-methylhydroxylamine on the rate of N-hydroxylation of debrisoquine by P450 2C3 in reconstituted systems

Incubation mixture	nmol *N*-hydroxydebrisoquine/ min/nmol P450
+ CH_3NHOH	0.013 ± 0.002
− CH_3NHOH	0.014 ± 0.001

Values are presented as the means of two different experiments ± SD; reaction mixture contained the components described in Materials and Methods, except for the addition of *N*-methylhydroxylamine (6 mM).

debrisoquine from *N*-hydroxydebrisoquine is also shown in Table 6. The formation of the product was significantly suppressed by the addition of catalase or superoxide dismutase.

Incubations with reconstituted cytochrome P450 oxidase systems. Data on incubations with the reconstituted P450 oxidase system are listed in Table 7. A high rate of formation of the urea derivative of debrisoquine was observed (1.88 nmol/min/nmol P450). No significant reaction rates were observed in control incubations. The replacement of NADPH by H_2O_2 resulted in a significant increase in the rate of formation of the metabolite while the use of H_2O_2 in the absence of cytochrome P450, P450 reductase and NADPH resulted in very low reaction rates.

Similar to the experiments with microsomes, the rate of formation of the urea derivative of debrisoquine was markedly reduced by the addition of catalase or superoxide dismutase.

Incubations with flavin-containing monooxygenase. The urea derivative of debrisoquine was

Table 6. Rates of the formation of the urea derivative of debrisoquine by microsomal transformation of *N*-hydroxydebrisoquine and influence of NADPH, catalase and superoxide dismutase

Incubation mixture (microsomes)	pmol urea derivative of debrisoquine/ min/mg protein
Complete	71.3 ± 5.8
− NADPH	2.4 ± 0.13
+ Catalase (100 U)	29.2 ± 5.1
+ Superoxide dismutase (100 U)	11.6 ± 3.2

Values are presented as the means of three determinations with one enzyme preparation (one rabbit); reaction mixture contained the components described in Materials and Methods, except for the addition or omission of the components indicated in the table. All values statistically different from control (complete incubation mixture) with $P < 0.001$ (Student's *t*-test).

Table 7. Formation of the urea derivative of debrisoquine by transformation of *N*-hydroxydebrisoquine in incubations with reconstituted cytochrome P450 oxidase systems and influence of NADPH, H_2O_2, catalase and superoxide dismutase

Incubation mixture	nmol urea derivative of debrisoquine/min/nmol P450	N
Complete incubation	1.880 ± 0.12	7
− NADPH	0.100 ± 0.04	4
− NADPH cytochrome P450 reductase	0.100 ± 0.03	2
− Cytochrome P450	0.320 ± 0.07	3
− Cytochrome P450 and NADPH cytochrome P450 reductase	0.096 ± 0.04	3
+ Catalase (100 U)	0.520 ± 0.08	4
+ Catalase (200 U)	0.510 ± 0.07	2
+ Superoxide dismutase (100 U)	0.230 ± 0.05	3
+ H_2O_2 (1 mM), − NADPH	3.050 ± 0.15	2
+ H_2O_2 (1 mM), − cytochrome P450, P450 reductase, − NADPH	0.188 ± 0.10	2

Rates ± SD were calculated from N determinations with microsomes isolated from liver homogenates of 10 rabbits. Reaction mixture contained the components described in Materials and Methods, except for the addition or omission of the components indicated in the table. All values statistically different from control (complete incubations) with $P < 0.001$ (Student's *t*-test).

Table 8. Formation of the urea derivative of debrisoquine by transformation of *N*-hydroxydebrisoquine in incubations with flavin containing monooxygenase and influence of NADPH, octylamine, catalase and superoxide dismutase

Incubation mixture	nmol urea derivative of debrisoquine/min/mg protein
Complete incubation	0.37 ± 0.05
− NADPH	0.04 ± 0.01*
+ Octylamine (1 mM)	0.45 ± 0.03
+ Catalase (100 U)	0.26 ± 0.02
+ Superoxide dismutase (100 U)	0.05 ± 0.01*

Values are presented as the means of three different experiments ± SD with one enzyme preparation (one hog liver); reaction mixture contained the components described in Materials and Methods, except for the addition or omission of the components indicated in the table.

* Statistically different from control (complete incubation mixture) with $P < 0.001$ (Student's *t*-test).

also detected in incubation experiments with flavin-containing monooxygenase. Increasing amounts of the metabolite were obtained with increasing amounts of the enzyme. Above about 80 μg FMO/incubation mixture the reaction rate decreased and, therefore, all incubations were performed with the optimum protein concentration of 73 μg FMO/incubation mixture. The rates of product formation as well as the effects of *n*-octylamine, catalase, and superoxide dismutase are listed in Table 8.

Qualitative analysis of the urea derivative of debrisoquine formed by transformation of N-hydroxydebrisoquine using rat hepatocytes. The urea derivative of debrisoquine could not be detected in incubations with rat hepatocytes even under widely varied conditions.

DISCUSSIONS

Reduction of N-hydroxydebrisoquine to debrisoquine

Of the three investigated transformations, the reduction of *N*-hydroxydebrisoquine was the one that proceeded rapidly when catalysed by microsomal fractions (Table 1) and by rat hepatocytes (Fig. 4). Thus, the enzymatic reduction of an *N*-hydroguanidine has been demonstrated for the first time.

It was found that microsomes both from rat liver and from rabbit liver apparently contain two enzyme systems capable of catalysing the reduction (Fig. 3), albeit with markedly different K_m values. In the case of hepatocytes, on the other hand, the Michaelis–Menten kinetics did not show this biphasic course but rather only the enzyme with the markedly lower K_m value and, hence, higher affinity (Fig. 5). At present, this enzyme is being purified from microsomes for separate investigations. Nevertheless, the data presented here already allow the conclusion that this is the same microsomal enzyme system which reduces hydroxylamines to amines [33, 34], amidoximes to amidines [37], and *N*-hydroxyisothioureas to isothioureas [38]. Charac-

teristics comparable with those for the reduction of *N*-hydroxydebrisoquine were found for all three, above-mentioned reactions. The features in agreement include the existence of a microsomal reaction (Table 1), the pH optimum of approx. 6.3 (Table 2), the preference for NADPH rather than NADPH as cofactor (Table 1), and the lack of sensitivity towards atmospheric oxygen. Thus, organisms evidently possess an effective catalytic system for the reduction of N-hydroxylated derivatives of compounds possessing strongly basic functional groups. In the case of the reduction of amidoximes to amidines, this process can be classified as a detoxification process since, for example, benzamidoxime (in contrast to benzamidine) has a genotoxic potential [39]. The question of whether or not this is also valid for *N*-hydroxyguanidines is being examined at present. Although no investigations on the genotoxicity of *N*-hydroxyguanidines have been reported, *N*-hydroxyguanidines such as *N*-hydroxydebrisoquine do exhibit antihypertensive properties [11]. On the basis of the present results, it may be assumed that *N*-hydroxyguanidines are also reduced to guanidines *in vivo* so that at least a part of the activity may be attributed to the guanidines and not to the corresponding *N*-hydroxyguanidines; thus *N*-hydroxyguanidines could function as prodrugs.

The consideration that hydroxylamines [33, 34] and *N*-hydroxyguanidines could possibly be reduced by the same enzyme system prompted the addition of hydroxylamine and *N*-methylhydroxylamine to the incubation mixtures in the study of the reduction of N-hydroxydebrisoquine. These alternative substrates did indeed suppress the reduction of *N*-hydroxydebrisoquine both in experiments with microsomes and with hepatocytes (Fig. 4).

N-Hydroxylation of debrisoquine

The above results were also extremely important for the characterization of the reverse reaction, the N-hydroxylation of debrisoquine. When attempts were made to detect this transformation in the absence of hydroxylamines, *N*-hydroxydebrisoquine could be detected after incubation with microsomal fractions from rabbit liver (Fig. 8); however, the yield of the metabolite was appreciably increased upon addition of *N*-methylhydroxylamine which evidently suppressed the instantaneous back reaction (Fig. 8). Thus, all the microsomal biotransformation mixtures for the characterization of the N-oxygenation reaction were supplemented with a concentration of *N*-methylhydroxylamine (6 mM) necessary for the optimal suppression of the back reaction (Fig. 8). In this way, the kinetic parameters could also be determined (Fig. 10). The HPLC analytical method employed in this work (Fig. 6) in combination with mass spectrometry (Fig. 7) provided unambiguous evidence for the formation of *N*-hydroxydebrisoquine and, accordingly, the first ever demonstration of the N-hydroxylation of a strongly basic, non-physiological guanidine (pK_a value of debrisoquine = 12.5 [8]), while the previously employed TLC methods [6] were insufficiently sensitive for detection in the absence of hydroxylamine.

The N-oxygenation is a microsomal process (Table 3), it requires NADPH as cofactor and exhibits a pH optimum of 6.7 (Table 2). These properties were suggestive of a typical monooxygenase-dependent reaction for which both the flavin-containing monooxygenase [36] as well as, by analogy to the N-hydroxylation of amidines [3], the cytochrome P450 monooxygenase system could be taken into consideration. Experiments with purified FMO excluded the first possibility while the presence of polyclonal antibodies against cytochrome P450 reductase gave rise to an appreciable inhibition of the microsomal reaction (Fig. 9). The catalysis by cytochrome P450 was further substantiated by reactions using reconstituted systems (Tables 4 and 5).

Although the participation of cytochrome P450 in the N-hydroxylation of debrisoquine was unequivocally established, further experiments are necessary to identify which isoenzymes, in addition to 2C3 of the rabbit (Table 5), are also able to catalyse the transformation. The physiological significance of the N-hydroxylation of an exogenous substance by the cytochrome P450 isoenzyme 2C3—which participates in the catabolism of progesterone—has already been discussed in connection with the N-hydroxylation of benzamidine [20]. Since, in contrast to the reaction with benzamidine [20], no increase in the N-hydroxylation of debrisoquine in the presence of 5-βPD was observed, it may be assumed that the thus activated 6βL subform of this isoenzyme is not involved but rather the 6βH subform [35].

As with the N-hydroxylation of benzamidines [20], the microsomal fractions from rat liver showed an appreciably higher activity than those from rats (Table 3). This could be attributed to the fact that the cytochrome P450 isoenzymes catalysing this reaction are present in lower concentrations in the rat. This is supported by the observation that N-hydroxydebrisoquine could not be detected after incubations of debrisoquine with rat hepatocytes even in the presence of hydroxylamines. However, it is also feasible that the retroreaction in rat hepatocytes is not suppressed by hydroxylamines to the same extent as is the case for incubations with microsomal fractions.

It is clear that metabolic cycles consisting of N-oxygenation and N-reduction have to be taken into account in the metabolism of nitrogen-containing functional groups, as we have previously demonstrated for amidines and amidoximes [4] and as was also previously known for amines [40]. The present work has proved that this is also valid for the guanidine/*N*-hydroxyguanidine system. As a result of such metabolic cycles, individual processes can easily be overlooked not only in *in vivo* studies but also in work with microsomal fractions or hepatocytes. However, when purified enzymes are employed it can be shown that the enzymatic prerequisites both for the oxidation as well as for the reduction are present. Thus, only the reaction of debrisoquine with cytochrome P450 2C3 was not influenced by the addition of *N*-methylhydroxylamine (Table 5), whereas higher yields of *N*-hydroxydebrisoquine (Table 4) were obtained in the presence of this compound even with reconstituted

systems enriched in the cytochrome P450 system [20]. Therefore, the reductase system is apparently also still present as an impurity in the P450 preparation used [20].

The N-hydroxylation of debrisoquine by cytochrome P450 is surprising since, in most cases, an N-oxygenation by this enzyme system can only be observed when a competing N-dealkylation is not possible due to the absence of α-hydrogen atoms (hydrogen atoms on the carbon atom adjacent to the nitrogen atom) [40–42] or when a ring hydroxylation can be excluded as in the case of the completely ring-fluorinated *N,N*-diphenylguanidine [5]. This product formation by cytochrome P450 can be explained mechanistically even when an N-oxygenation does occur in exceptional cases [40–42]. Thus, the N-dealkylation of debrisoquine with its α-H atoms has been reported previously [8]. However, it appears that an N-oxygenation is also possible when the nitrogen and the α-H atoms are incorporated into a ring system such as that of tetrahydroisoquinoline. Further examples of this reaction are now under investigation.

An *N*-oxide of debrisoquine could also be formed by N-oxygenation of the ring nitrogen. However, no indications for such a metabolite were obtained. In the conversion of arginine to NO (EDRF) by NO synthases it is accepted that N^G-hydroxyarginine is formed in the first step [14, 15]. There is considerable evidence that this transformation proceeds analogously to a P450-catalysed reaction [43]. The N-hydroxylation of amidines to amidoximes [3, 20] by this enzyme and, in particular, the analogous transformation of the guanidine moiety of debrisoquine presented here support this hypothesis.

Transformation of N*-hydroxydebrisoquine to the urea derivative of debrisoquine*

NO synthases liberate NO from N^G-hydroxyarginine and the final product from the transformation of arginine is the corresponding urea citrulline [14, 15]. An analogous conversion of *N*-hydroxydebrisoquine to the corresponding urea was detected in several systems (Tables 6–8). Although the transformation with microsomal fractions was very low and poorly reproducible (Table 6), high rates of conversion were obtained with both the reconstituted cytochrome P450 oxidase system (Table 7) and with FMO (Table 8). However, the investigations also revealed that at least part of the formation of the urea derivative of debrisoquine was dependent on the presence of H_2O_2 and O_2^-. While the N-hydroxylation of debrisoquine was not influenced by the addition of catalase and superoxide dismutase (data not shown), the rates of formation of the urea decreased in the presence of these enzymes (Tables 6–8). Although catalase had no significant effect on FMO-catalysed reactions, superoxide dismutase was strongly inhibitory (Table 8). It is known that O_2^- is released in reactions of purified FMO with hydroxylamines and that this species oxidizes the hydroxylamines [44]. A similar behavior appears to be possible in the case of the *N*-hydroxyguanidines. Thus, although the formation of the superoxide ion is an enzymatic process, its reaction with the substrate is not enzymatic. This is in accord with the observation that the reaction with FMO is not significantly increased by the activator *n*-octylamine [36].

In reactions with cytochrome P450, in addition to the monooxygenase activity (Eqn 1), the oxidase (Eqn 2) and peroxidase (or peroxygenase) activities (Eqn 3) also have to be taken into account [45].

$$H^+ + RH + NADPH + O_2 \rightarrow R\text{-}OH + NADP^+ + H_2O \quad (1)$$

$$H^+ + NADPH + O_2 \rightarrow NADP^+ + H_2O_2 \text{ (via } O_2^-) \quad (2)$$

$$R_1OOH + RH \rightarrow ROH + R_1OH \text{ (or oxidation with } O_2^-). \quad (3)$$

The formation of H_2O_2 in the oxidase activity can occur by way of O_2^- [46]. The oxidase and peroxidase activities occur when the substrate is bound to the enzyme but then uncouples the catalytic cycle and finally undergoes an indirect oxidation (peroxidase activity) by the oxygen species formed in the NADPH reduction [47]. When H_2O_2, an organic hydroperoxide, or O_2^- is present, the peroxidase activity can operate directly and the presence of NADPH is not necessary [48].

Both oxidase and peroxidase activity are operative in the transformation of *N*-hydroxydebrisoquine. This is most clearly apparent in the reactions with the reconstituted cytochrome P450 oxidase system (Table 7). Experiments with microsomal fractions are more problematic since the microsomes may always contain certain amounts of catalase and superoxide dismutase [47]. The differing contents of these enzymes could also be responsible for the widely varying rates of reactions observed in the experiments with microsomes and also for the low conversion rates in reactions with the 9000 *g* supernatant.

From the data in Table 7, it is clear that H_2O_2 alone is able to catalyse the reaction. This purely chemical oxidation of *N*-hydroxyguanidines by widely differing oxidation agents was recently reported for other members of this compound class [49].

All the reaction rates given in Table 7 are not the result of a purely hydrolytic transformation of *N*-hydroxydebrisoquine but depend on an oxidative process. It is still not clear whether a hydroxylated compound (as shown in Eqn 3) or another oxidation product is initially formed, also the further reaction course has not been elucidated so far. We have already demonstrated (data not shown) that xanthine oxidase and peroxidases are also able to transform *N*-hydroxydebrisoquine into its urea derivative.

The reaction rates with H_2O_2 alone are much lower than those in completely reconstituted systems containing either NADPH or H_2O_2 (Table 7). The high activity in the complete system with H_2O_2 can be explained in terms of the peroxidase activity of cytochrome P450. However, high yields of the urea derivative were also obtained in the complete system containing NADPH (Table 7). These observations are in favor of an oxidase activity with subsequent peroxidase activity of cytochrome P450 which only occurs in high yields in the presence of all components

of the reconstituted system. This assumption is reflected by the influence of catalase and, especially, superoxide dismutase. Hence, this oxidation resembles the transformation of the hydroxylamine *N*-hydroxyphentermine [47].

It is of interest that peroxy derivatives have also been discussed as the oxidizing species in the conversion of N^G-hydroxyarginine to citrulline and NO by NO synthases [43].

Summary and perspectives

This work on the reactions of debrisoquine has demonstrate, in *in vitro* studies, a previously unknown route for the N-hydroxylation of a non-physiological guanidine by cytochrome P450 involving the usual monooxygenase activity of the enzyme. The resultant *N*-hydroxyguanidine decouples monooxygenases (cytochrome P450, FMO), while the H_2O_2 and O_2^- in particular thus formed transform the *N*-hydroxyguanidine further into the corresponding urea derivative. Further work is necessary to elucidate the exact mechanism of this last step, particular attention should be paid to the possible formation of NO. This is at present being investigated with other guanidines and *N*-hydroxyguanidine derivatives. The relevance of such a transformation *in vivo* is also of major interest. The availability of *N*-hydroxyguanidines *in vivo* will depend to a large extent on the degree of the reduction back to guanidines, a reaction that has also been demonstrated in the present work. The oxidative conversion of *N*-hydroxyguanidines *in vivo* by the oxidase and peroxidase activities of monooxygenases (cytochrome P450, FMO) will be restricted, since the presence of catalase and superoxide dismutase will rapidly destroy the reactive oxygen species. This assumption is supported by the observation that the urea derivative of debrisoquine could not be detected after incubations of *N*-hydroxydebrisoquine with rat hepatocytes. On the other hand, the nitro derivative of *p*-chlorphentermine has also been found *in vivo* [50]. Such oxidase and peroxidase activities of cytochrome P450 are of significance for the formation of this metabolite [47].

On the whole, several substances have to be taken into consideration as precursors of NO (ERDF). The physiological substrate is arginine which is apparently only accepted with very high substrate specificity by NO synthases [12, 13]. N^G-Hydroxyarginine is also transformed by NO synthases into NO [14, 15]. Interestingly, it has recently been reported that hemoproteins in the presence of H_2O_2 or organic hydroperoxides are also able to convert N^G-hydroxyarginine into citrulline and NO *in vitro* [51]. In further investigations *in vitro* with rat liver microsomes by the same group [52], the importance of cytochrome P450 in this transformation was accentuated. It is possible that in this case the oxidase and peroxidase activities of the enzyme could also play decisive roles. The authors [52] do not describe the influence of catalase and superoxide dismutase on the microsomal formation of citrulline. Thus, the possibility for the transformation of physiological N^G-hydroxyarginine corresponds to the oxidation of non-physiological *N*-hydroxydebrisoquine demonstrated by us in this work. However, the importance of the oxidation of N^G-hydroxyarginine *in vivo* by enzymes other than NO synthases remain to be studied [52]. Although N^G-hydroxyarginine is an intermediate in the transformation of arginine by NO synthases, the compound is not released from the enzyme but rather undergoes further transformation *in situ* [14].

Of the non-physiological compounds, in the so-called organic nitrates and some other compounds, information of NO has already been demonstrated (see Ref. 53 for a review).

As shown by the present work, non-physiological guanidines and aminoguanidines as well as their N-hydroxylated derivatives must also be taken into consideration. Numerous drugs contain such guanidine or aminoguanidine functional groups [1, 2]. Previous investigations in our laboratory have shown that amidine functions, which are also present as the active group in numerous medicinal agents, can undergo N-hydroxylations to amidoximes by cytochrome P450 both *in vitro* [3, 4, 20, 23, 41] and *in vivo* [4, 54]. The back reduction of the amidoximes is here also of major relevance for the concentration of this class of compounds *in vivo* [37, 54]. Further transformations of amidoximes to the corresponding amides as citrulline analogs *in vitro* by cytochrome P450 [55] and *in vivo* [54] have been recently reported. Our investigations (unpublished results) have shown that the transformation of benzamidoxime *in vitro* by cytochrome P450 is again attributable to the oxidase and peroxidase activities of this enzyme.

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