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N_4-HYDROXYLATION OF SULFAMETHOXAZOLE BY CYTOCHROME P450 OF THE CYTOCHROME P4502C SUBFAMILY AND REDUCTION OF SULFAMETHOXAZOLE HYDROXYLAMINE IN HUMAN AND RAT HEPATIC MICROSOMES

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ABSTRACT:

The N_4-hydroxylation of sulfamethoxazole (SMX) to its hydroxylamine (SMX-HA) metabolite is the first step in the formation of reactive metabolites responsible for mediating hypersensitivity reactions associated with this compound. In rat hepatic microsomes, the NADPH-dependent oxidation of SMX to SMX-HA was increased 3-fold by pretreatment of rats with phenobarbital. Other cytochrome P450 (CYP) inducers were ineffective. The constitutive and induced SMX *N*-hydroxylation activities were inhibited by tolbutamide, and induction of SMX-HA activity paralleled the induction of progesterone 21-hydroxylase activity, a marker for CYP2C6. SMX *N*-hydroxylation in phenobarbital-treated rat hepatic microsomes was inhibited 70% by anti-CYP2C6 antisera. Thus, the N_4-hydroxylation of SMX by rat hepatic microsomes was mediated by members of the CYP2C subfamily, probably CYP2C6. In a panel of human microsomes, SMX-HA formation correlated with tolbutamide hydroxylase activity ($r = 0.75$; $p = 0.01$); CYP2C9 content ($r = 0.79$; $p < 0.01$) and was inhibited 70% by 500 μM tolbutamide and 90% by 100 μM sulfaphenzole. Recombinant CYP2C9 catalyzed the *N*-hydroxylation of SMX. SMX-HA formation in human hepatic microsomes was therefore mediated predominantly by CYP2C9. CYP-mediated reduction of SMX-HA to SMX was markedly induced in dexamethasone and phenobarbital-treated rat hepatic microsomes, and was attributed to CYP3A and CYP2B forms. In uninduced rat and human hepatic microsomes, SMX-HA reduction was mediated predominantly by an NADH-dependent microsomal hydroxylamine reductase under aerobic conditions. Under anaerobic conditions, troleandomycin at ≥1 μM inhibited the reduction of SMX-HA in human hepatic microsomes by 45%, whereas sulfaphenazole had no effect. Therefore, members of the human CYP3A subfamily may catalyze the reduction of SMX-HA under hypoxic conditions. Individuals with high levels of CYP2C9 and/or a decreased ability to reduce SMX-HA may be at increased risk for SMX adverse reactions.

The N_4-hydroxylation of SMX[1] and other sulfonamides to their hydroxylamine metabolites leads to the formation of reactive metabolites thought to mediate ADR to this class of drugs (1–5). Previous studies have suggested that genetically determined metabolic variation plays an important role in determining susceptibility to sulfonamide hypersensitivity reactions (1). The marked increase in the incidence of sulfonamide ADR in patients with AIDS (6–8) suggests that environmental modulation of metabolism may be an additional determinant of susceptibility. To understand the role of genetic and environmental variation of drug metabolism in predisposing individuals to sulfonamide ADR, we have been characterizing the formation and disposition of SMX-HA in humans (1, 2, 9–12).

[1] Abbreviations used are: SMX, sulfamethoxazole; ADR, adverse drug reaction; AIDs, acquired immunodeficiency syndrome; SMX-HA, sulfamethoxazole hydroxylamine; CYP, cytochrome P450; BNF, β-naphthoflavone; PB, phenobarbital; SAL, saline; DEX, dexamethasone; PYR, pyridine; EROD, ethoxyresorufin O-deethylation; PROD, pentoxyresorufin O-dealkylation; PNPH, *p*-nitrophenol; ENDM, erythromycin *N*-demethylation; PBS, phosphate-buffered saline; PCA, perchloric acid; DMSO, dimethylsulfoxide; CHLOR, chlorzoxazone; TAO, troleandomycin; SDS-PAGE, sodium doceyl sulfate-polyacrylamide gel electrophoresis; ANF, 7,8-benzoflavone; TOLB, tolbutamide; SPZ, sulfaphenazole; ERYTH, erythromycin.

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Previous studies have shown that SMX *N*-hydroxylation by human (2), mouse (13), and dog (14) microsomes is an NADPH-dependent process, but the specific CYP responsible have not been not identified. *N*-Hydroxylation of SMX was catalyzed predominantly by constitutive CYP in mouse hepatic microsomes, but the activity was induced up to 2-fold by BNF and PB pretreatment of mice (13). Flavin-containing monooxygenases were not involved in the metabolism of SMX (13). We have also observed microsomal reduction of SMX-HA to SMX (15). The objective of the current investigation was to identify the human and rat enzymes responsible for the formation of SMX-HA and its subsequent reduction to SMX.

Materials and Methods

Chemicals and Reagents. All routine chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), and HPLC solvents were obtained from Fisher Scientific (Malvern, PA). SMZ-HA was obtained from Dalton Chemical Co. (Toronto, Ontario, Canada) and was >99% pure by HPLC, with SMX (0.25%) being the major contaminant.

Instrumentation. A Beckman DU-640 spectrophotometer (Beckman Instruments, Palo Alto, CA), a Perkin-Elmer LS-50B luminescence spectrophotometer system with fluorescence data manager software (Perkin-Elmer, Exton, PA), and a Shimadzu LC-10A series HPLC system equipped with 2 LC-10AD pumps, an SL-10A autoinjector, an SCL-10A system controller, an SPD10-A UV spectrophotometric detector, and EZChrom HPLC software (Shimadzu Scientific Instruments, Inc., Columbia, MD) were used in analytical work.

Microsomes. Rat hepatic microsomes were prepared from freshly isolated livers of 200–225 g male CRL(CD)BR SD strain rats (Charles River Laboratories, Raleigh, NC) treated with 0.9% SAL, DEX (150 mg/kg/day in SAL

with 2% Tween 80), BNF (80 mg/kg/day in corn oil), PYR (100 mg/kg/day in SAL), or PB (80 mg/kg/day in SAL) administered intraperitoneally at 24-hr intervals for 72 hr. Three animals were included in each group. Animals were housed in climate-controlled rooms and treated in accordance with the provisions of the Merck Institutional Animal Care and Use Committee guidelines.

Animals were euthanized by carbon dioxide asphyxiation and livers removed. Livers were rinsed and homogenized in 2 volumes of ice-cold 0.05 M Tris:0.15 M KCl buffer (pH 7.4). The homogenate was centrifuged at 9,000*g* for 20 min. The supernatant was collected and centrifuged at 100,000*g* for 60 min. The supernatant was decanted, the pellet resuspended in the Tris/KCl buffer, and centrifuged again at 100,000*g* for 60 min. The final pellet was resuspended in a volume of Tris/KCl buffer equivalent to one-half the original liver weight. Microsomal protein concentration was determined using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as a standard.

Human microsomal samples suspended at 20 mg microsomal protein/ml in 0.25 M sucrose were obtained from Human Biologics, Inc. (Phoenix, AZ). Individual samples HBI 2, 3, 5, 6, 7, 9, 10, 11, 12, and 13 were used. In some experiments, pooled samples of microsomes were used. Pool I contained equal volumes of microsomes from individuals 2, 3, 5, 6, and 11, and pool II contained equal volumes of microsomes from individuals 6 and 7.

Microsomes prepared from human lymphoblastoid cells expressing native CYP1A1 (control) or recombinant CYP1A1, CYP1A2, CYP2C8, CYP2C9, CYP2E1, or CYP3A4 were obtained from Gentest Corp. (Woburn, MA).

Microsomal Enzyme Activities. The following CYP-dependent activities were measured, with minor modifications of methods previously published.

Alkoxyresorufin O-Dealkylase. EROD and PROD were measured according to Burke *et al.* (16), with substrate concentrations of 0.5 μM and an NADPH concentration of 250 μM. Microsomal protein concentration was adjusted to achieve a linear reaction rate for at least 1 min.

PNPH. PNPH was measured as described previously (17).

ENDM. ENDM was measured as described previously (18).

Progesterone 21-Hydroxylation. Progesterone 21-hydroxylase activity (19) was measured by incubating 0.125 mg microsomal protein with 1 mM NADPH and 100 μM progesterone (added in 5 μl methanol) in 500 μl of PBS (pH 7.4) at 37°C for 5 min. These conditions resulted in a linear production of deoxycorticosterone (21-hydroxyprogesterone). The reaction was terminated by the addition of an equal volume of acetonitrile. After centrifugation, 50 μl of the supernatant was analyzed by HPLC. The mobile phase was 27.5:27.5:45 (v/v) of methanol:acetonitrile:water, with a flow rate of 1.5 ml/min and UV detection at 240 nm. A 45 mm Ultrasphere 5 μM C_{18} guard column and a 150 mm Ultrasphere 5 μM C_8 column (Beckman Instruments, Palo Alto, CA) were used for separation. Retention times were 7.9 and 22.6 min for deoxycorticosterone and progesterone, respectively.

SMX-HA Formation. *N*-Hydroxylation of SMX to SMX-HA was determined essentially as described previously (2, 13), except that the reaction was terminated by the addition of 1/10 volume of 15% PCA. This resulted in better chromatography of SMX-HA, although two additional peaks resulting from an interaction of SMX, NADPH, and PCA were observed. These peaks did not interfere with the analysis of SMX-HA. Incubations contained 1 mg/ml of microsomal protein, 1 mM NADPH, 1 mM ascorbic acid, and 1 mM SMX in PBS. Reaction mixtures were incubated in a shaking water bath for 15 min for rat microsomes and 1 hr for human microsomes. Formation of SMX-HA was linear under these conditions. SMX-HA was quantitated by HPLC (2, 13) using a mobile phase of 20:80:1:0.05 acetonitrile:water:glacial acetic acid: triethylamine, with a flow rate of 1.5 ml/min and a 150 × 4.6 mm Ultracarb 5 μM ODS (30) C_{18} column with a 30 × 4.6 mm guard column (Phenomenex, Torrance, CA). Detection was at 254 nm. Retention times were 6.5 and 8.7 min for SMX-HA and SMX, respectively.

SMX-HA Reduction. Reduction of SMX-HA to SMX was measured under the same reaction conditions as noted, except that the SMX-HA concentration was 200 μM, and incubation times were 15 and 30 min for rat and human microsomes, respectively. In some incubations, NADH (1 mM) was used in place of NADPH, and the reaction was conducted at pH 6.3. The reaction was terminated by the addition of an equal volume of methanol to eliminate any possible error from an interaction between SMX, NADPH, and PCA, although this was not detectable at low concentrations of SMX. Additionally, nitroso-SMX formed from the oxidation of SMX-HA covalently binds to protein through acid labile bonds that liberate SMX in an acid environment.[2] SMX was quantitated by HPLC under the same conditions as SMX-HA.

Human Hepatic Microsomal Activities. The activities of known CYP substrates in the human hepatic microsomes used in this study were provided by Human Biologics, Inc. and were determined according to published protocols. Selected activities were confirmed by measurement of activity or by immunoblotting in our laboratory (data not shown).

Activities with Recombinant Human CYP Enzymes. Activity in recombinant human CYP enzymes was measured with 3 mg/ml microsomal protein in the presence of an NADPH regenerating system consisting of 0.65 mM NADP, 1 mM $MgCl_2$, 2.4 mM glucose-6-phosphate, and 2 units/ml of glucose-6-phosphate dehydrogenase. Incubations were conducted for 2 hr at 37°C.

Inhibition Studies. Chemical inhibitors at the concentrations indicated in the figure legends were added in DMSO, except for CHLOR that was added in 60 mM KOH (10 μL to 1 ml). Control incubations contained the appropriate vehicle. Except for TAO, inhibitors were preincubated with microsomes for 3 min before addition of NADPH to initiate the reaction. TAO was either added 3 min before initiation of the reaction with NADPH (competitive inhibition) or was preincubated with human microsomes for 30 min at 37°C in the presence of NADPH, but with no ascorbic acid before initiating the reaction (noncompetitive inhibition). The drug, ascorbic acid, and additional NADPH were added at the time of reaction initiation.

Antibody inhibition studies were performed with rabbit antirat CYP2B1 and rabbit antirat CYP3A1 IgG fractions (2 mg IgG/0.25 mg microsomal protein) (Human Biologics Inc.) or goat antirat CYP2C6 antisera (5, 10, or 20 μl in a 100 μl incubation containing 0.0625 mg microsomal protein; expressed as μl because IgG content of sera is unknown) (Dacchei Pure Chemicals Co., Ltd., Tokyo, Japan). Antibodies or antisera were preincubated with microsomes for 15 min at room temperature before measuring activity as described, except that a microsomal protein concentration of 0.25 mg/ml was used and the incubation volume was 100 μl. Incubation times were 60 min for the formation of SMX-HA and 30 min for its reduction. Control incubations contained an amount of preimmune IgG equivalent to the maximum amount added for anti-CYP2B1 and anti-CYP3A1 antibodies. No preimmune sera was available for the anti-CYP2C6 sera. Therefore, results are expressed compared with a standard incubation mixture. For anaerobic incubation conditions, nitrogen and carbon monoxide were deoxygenated by passing through a solution containing 0.05% 2-anthraquinone sodium sulfonate, 0.5% sodium dithionite, and 0.1 N NaOH before sparging the reaction mixture on ice for 5 min. Reactions were performed with a 250 μl incubation volume. The substrate was then added, and sparging continued for an additional 3 min before sealing the reaction tube and transferring to a 37°C water bath.

Immunoblotting. Hepatic microsomes (6.25, 10, 12.5, or 25 μg) were resolved on SDS-PAGE gels (10% and 12%; Bio-Rad Laboratories) and transferred by semidry electroblotting to nitrocellulose membranes. CYP2C9 (55 kDa band) and CYP2C8 (52 kDa band) were detected using an antirat CYP2C11 and anti-CYP2C6 antibodies (Dacchei Pure Chemicals Co., Ltd.), which recognize both human CYP. An additional band of <50 kDa molecular weight was also detected. This was not conclusively identified, but is presumed to be CYP2C19 because its content correlates with *S*-mephenytoin hydroxylation ($r = 0.76$; $p = 0.05$). CYP3A4 was detected using the antirat CYP3A1. The bound IgG was then visualized using the ECL detection system (Amersham Corp., Arlington Heights, IL). To compensate for the semiquantitative nature of SDS-PAGE electrophoresis and immunoblotting and the limited range of autoradiographic film, four separate gels were run and several exposures made to ensure response within the linear range. The mean of results of the four blots were used in determining correlation coefficients. The resultant autoradiographs were quantified by densitometry using the Bio-Rad Imaging Densitometer and Molecular Analyst 1.1.1 Software (Bio-Rad Laboratories).

Statistical Analysis. Statistical analysis was performed by analysis of variance, and the Bonferonni/Dunn *t* test was used to compare means. Pearson's correlation coefficient (r; significance determined by Fisher's r to z transformation) was used to determine correlations. All statistical analyses were performed using Statview (Abacus Concepts, Berkeley, CA) on a Macintosh IIci computer (Apple Computer, Inc., Cupertino, CA). Results are re-

[2] A. E. Cribb, unpublished observations.

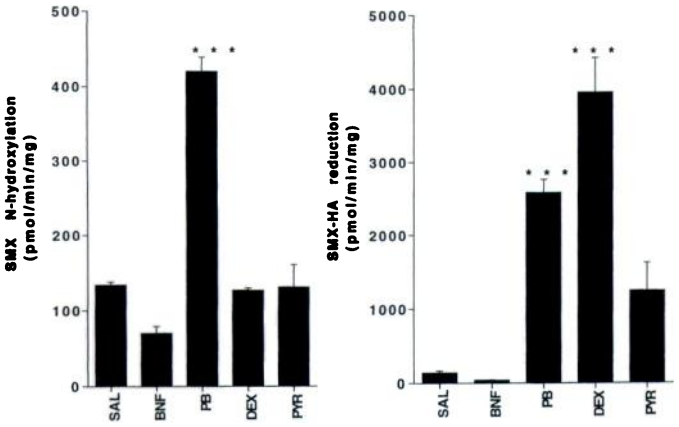

FIG. 1. *Effect of CYP inducers on the metabolism of SMX to SMX-HA (left) and the reduction of SMX-HA to SMX (right) by rat hepatic microsomes at pH 7.4, with NADPH as a cofactor.*

Incubation conditions were as described in *Materials and Methods* and represent the mean ± SE of results obtained from three individual animals in each group.

ported as mean ± SE. Statistical significance was taken to be at $p < 0.05$ and is reported as: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

N_4-Hydroxylation of SMZ. N_4-Hydroxylation of SMX was observed in human and rat hepatic microsomes. In the rat, PB pretreatment increased SMX-HA formation by hepatic microsomes 3-fold, but other inducers were ineffective (fig. 1). This induction correlated most closely with the induction of progesterone 21-hydroxylase activity, a marker for CYP2C6 induction (table 1). Formation of SMX-HA was not inhibited by ANF, CHLOR, or TAO, but TOLB inhibited its formation in a concentration-dependent fashion in microsomes from SAL-treated and PB-treated rats (fig. 2). Anti-CYP2C6 antisera inhibited the formation of SMX-HA by 70% at the maximum concentration used in hepatic microsomes from PB-treated rats (fig. 2). The concentration of anti-CYP2C6 serum (10 μl/0.0625 mg) inhibiting SMX-HA formation by 55% also inhibited progesterone 21-hydroxylation (CYP2C6-mediated) activity by 55%, but did not inhibit progesterone 6β-hydroxylation (CYP3A-mediated) and only inhibited 16α-hydroxylation (predominantly CYP2C11-mediated) by 15%.

The rate of *N*-hydroxylation in a panel of 10 human hepatic microsomes was 34 ± 4 pmol/mg/min (100 pmol/nmol CYP/min) at an SMX concentration of 1 mM, with a 4-fold variation (table 2). The apparent K_M for SMX *N*-hydroxylation determined in microsomal pool I was 1,200 μM and an Eadie-Hofstee transformation produced a linear plot, consistent with a single enzyme. The rate of SMX-HA formation was significantly correlated with TOLB methyl-hydroxylation (fig. 3; $r = 0.75$; $p = 0.01$), but did not correlate significantly with any other marker activities, including *S*-mephenytoin hydroxylation (table 3). SMX *N*-hydroxylation and TOLB methyl-hydroxylation were significantly correlated with CYP2C9 content, as determined by immunoblotting ($r = 0.79$, $p < 0.01$; $r = 0.91$, $p < 0.05$, respectively) (fig. 4). SMX *N*-hydroxylation was not correlated with CYP2C8 content ($r = 0.52$; $p = 0.12$).

The formation of SMX-HA was inhibited in a sample of pooled human microsomes 70% by 500 μM tolbutamide, 50% by 25 μM diclofenac, and 90% by 100 μM SPZ (figs. 5 and 6). ANF (10 μM) was a relatively poor inhibitor, reducing activity by 34% at a concentration usually associated with almost complete inhibition of CYP1A2 activity. As was observed in the rat, TAO was a weak inhibitor of SMX-HA formation, whether coincubated with SMX or preincubated with microsomes and NADPH for 30 min before assessing *N*-hydroxylation (fig. 6).

Using commercially available recombinant CYP expressed in a lymphoblastoid cell line, we were able to detect SMX-HA formation with CYP2C9 (405 pmol/3 mg microsomal protein/2 hr; ~113 pmol/nmol CYP/min) and CYP1A2 (110 pmol/3 mg pmol/3 mg microsomal protein/2 hr; ~8 pmol/nmol CYP/min). CYP1A1, 2C8, 2E1, and 3A4 did not produce detectable metabolites.

Microsomal Reduction of SMX-HA. Microsomal reduction of SMX-HA to SMX was observed in human and rat hepatic microsomes under aerobic conditions. Reduction of SMX-HA did not occur in rat or human hepatic microsomes in the absence of either NADPH or NADH (data not shown).

Pretreatment of rats with PB or DEX markedly induced the NADPH-dependent microsomal reduction of SMX-HA (fig. 1) by 19- and 29-fold, respectively, compared with SAL-treated rat hepatic microsomes. This induction correlated closely with the induction of ENDM activity in DEX-treated rats, but was much less than the induction of PROD in PB-treated rats (table 1). A nitrogen atmosphere increased SMX-HA reduction in hepatic microsomes from DEX-treated rats (fig. 7), whereas carbon monoxide inhibited activity by 90%. NADH was a preferred cofactor in SAL-treated rat hepatic microsomes, and showed greater activity at pH 6.3; however, NADH was unable to support the induced SMX-HA reductase activity in DEX-treated rat hepatic microsomes.

A series of inhibition studies were performed under aerobic conditions with hepatic microsomes from DEX- and PB-treated rats with NADPH as a cofactor. ERYTH and TAO were effective inhibitors of SMX-HA reduction in microsomes from DEX-treated rats, but were less effective in microsomes from PB-treated rats (fig. 8). TOLB did not inhibit SMX-HA reduction. Anti-CYP3A antibodies were highly inhibitory to both DEX and PB microsomes, whereas anti-CYP2B antibodies were only effective in PB-treated rat microsomes (37% inhibition).

NADPH-dependent reduction of SMX-HA was observed in human hepatic microsomes (table 2), and in a sample of pooled human microsomes the K_M for the reaction was 33 μM with a linear Eadie-Hofstee transformation. The mean rate of aerobic NADPH-dependent reduction of SMX-HA was 88 ± 22 pmol/mg/min, but did not correlate with any of the marker activities for specific CYP, with NADPH-cytochrome *c* reductase activity or cytochrome b_5 content (table 3). NADPH-dependent SMX-HA reduction could be markedly increased by a nitrogen atmosphere (up to 22-fold in some experiments), but carbon monoxide, although preventing the increase in an anaerobic atmosphere, did not inhibit the control activity (fig. 7). Reduction of SMX-HA was not increased in microsomes prepared from lymphoblastoid cells transfected with CYP1A1, CYP1A2, CYP2C9, CYP2E1, or CYP3A4 under aerobic conditions. TAO (1–100 μM) preincubated with microsomes in the presence of NADPH under aerobic conditions before measuring SMX-HA reduction under anaerobic conditions resulted in a maximal inhibition of 45% at concentrations ≥1 μM (fig. 6). Clotrimazole produced a similar degree of inhibition, whereas SPZ had no effect (fig. 6).

The results obtained with human hepatic microsomes suggested an additional enzyme was responsible for the reduction of SMX-HA under aerobic conditions. NADH was a superior cofactor to NADPH in human hepatic microsomes at pH 7.4, and NADH-dependent reduction was further enhanced at pH 6.3 (fig. 7) so that the mean SMX-HA reductase activity in human hepatic microsomes was 366 ±

TABLE 1

Effect of CYP inducers on SMX-HA formation and reduction by rat hepatic microsomes[a]

Inducer	CYP Form Induced	Marker Activity	Relative Marker Activity	Relative SMX-HA Formation	Relative SMX-HA Reduction[b]
BNF	CYP1A1/2	EROD	×615	×0.5	×0.3
PB	CYP2B1/2	PROD	×122	×3.1***	×18.7***
	CYP2C6/7	Progesterone 21-hydroxylase	×3		
DEX	CYP3A1	ENDM	×21	×1	×28.7***
	CYP2B1/2	PROD	×12		
PYR	CYP2E1	PNPH	×4	×1	×9

[a] All values are expressed relative to results obtained in uninduced male rats. Activity in uninduced male rats was 134 ± 4 pmol SMX-HA formed/min/mg and 138 ± 28 pmol SMX formed/min/mg. Marker activities in uninduced male rats were: EROD, 18.4 ± 1.4 pmol/min/mg; PROD, 6.2 ± 0.1 pmol/min/mg; progesterone 21-hydroxylase, 562 ± 74 pmol/min/mg; ENDM, 298 ± 225 pmol/min/mg; PNPH, 1.18 ± 0.02 nmol/mg/min.

[b] With NADPH as a cofactor at pH 7.4.

*** $p < 0.001$.

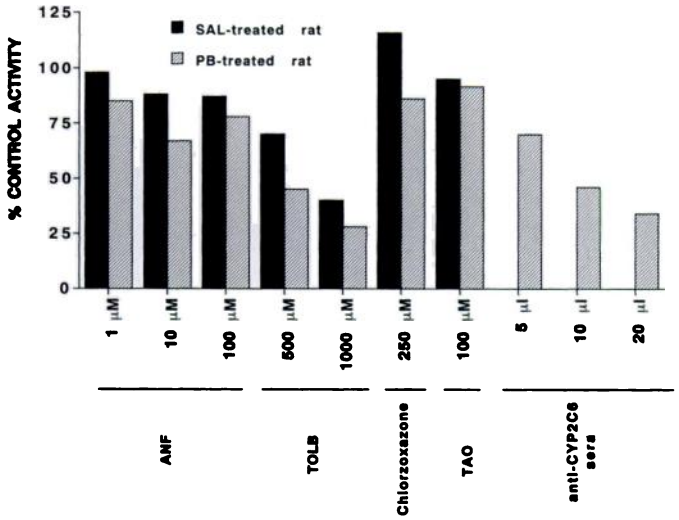

FIG. 2. *Effect of anti-CYP2C6 antisera and CYP substrates on the oxidation of SMX to SMX-HA in SAL and PB-treated rat microsomes.*

Results are presented as the percentage of control activity of incubations containing 1% DMSO, except for CHLOR in which the control contained 1% 60 mM KOH. Results are the mean of duplicate determinations. Inhibition of SMX-HA formation by anti-CYP2C6 is expressed as a percentage of control incubations containing nonimmune IgG fractions and represent duplicate determinations using 5, 10, or 20 µl of antisera/100 µl incubation containing 0.0625 mg protein. (For details, see *Materials and Methods*.)

82 pmol/min/mg (table 2). SMX-HA reduction with NADH cofactor at pH 6.3 was correlated with the NADPH-dependent reduction observed at pH 7.4 (table 3; fig. 9).

Discussion

The N_4-hydroxylation of SMX to form SMX-HA by CYP enzymes in human and rat hepatic microsomes and the reduction of SMX-HA to regenerate the parent SMX have been characterized in rat and human hepatic microsomes. The *N*-hydroxylation of SMX is a bioactivation pathway (1, 2), whereas the retroreduction observed must be considered a detoxification pathway. It was therefore important to understand the enzymes responsible for these competing pathways.

The 3-fold induction of SMX *N*-hydroxylation in rat hepatic microsomes observed after pretreatment with PB and lack of effect of other CYP inducers suggested that a constitutively expressed, PB-inducible form of CYP was responsible. The similar induction of progesterone 21-hydroxylase activity, a specific marker for the PB-inducible CYP2C6 in the rat (20), indicated that this form, in contrast with the CYP2B forms that were markedly induced, was responsible for activity in the male rat. The pattern of induction of SMX *N*-hydroxylation observed is the same as for TOLB methyl-hydroxylation (21, 22). Hence, the form(s) of rat CYP metabolizing these two drugs are similarly controlled, if not the same enzyme. The rat CYP responsible for TOLB methyl-hydroxylation is thought to be a CYP2C enzyme, but has not been conclusively identified (21, 22). ANF at a concentration of 10 µM produced 25% inhibition, much less than observed for substrates of the CYP1A enzymes but very similar to that observed for TOLB (21), and BNF did not induce SMX *N*-hydroxylation, so the CYP1A family does not contribute significantly. TAO was a weak inhibitor of SMX-HA formation and this, coupled with the lack of induction by DEX, rules out the involvement of CYP3A enzymes. TOLB was the only effective inhibitor of SMX oxidation in both SAL- and PB-pretreated rat microsomes. CYP2C11 is a constitutive enzyme that is generally not induced but rather depressed by PB (23) and therefore is unlikely to be a major form involved in the metabolism of SMX to SMX-HA, although it has been

TABLE 2

N-Hydroxylation of SMX to its hydroxylamine and the NADH/NADPH-dependent reduction of the hydroxylamine in human hepatic microsomes

The *N*-hydroxylation of SMX to form SMX-HA was conducted with 1 mg/ml microsomal protein, 1 mM NADPH, 1 mM ascorbic acid, and 1 mM SMX for 1 hr at 37°C. The reduction of SMX-HA to SMX was performed with 1 mg/ml microsomal protein, 1 mM ascorbic acid, 200 µM SMX-HA, and either 1 mM NADH at pH 6.3 or 1 mM NADPH at pH 7.4 for 30 min. Sample preparation and HPLC conditions were as described in *Materials and Methods*. Results are the mean of at least duplicate determinations.

Human Liver Sample	SMX *N*-Hydroxylation	NADPH-Dependent SMX-HA Reduction	NADH-Dependent SMX-HA Reduction
	pmol/min/mg	*pmol/min/mg*	*pmol/min/mg*
HBI 2	56	49	198
HBI 3	22	45	515
HBI 5	35	97	421
HBI 6	31	41	192
HBI 7	41	79	143
HBI 9	25	267	990
HBI 10	43	112	290
HBI 11	15	19	109
HBI 12	28	75	439
HBI 13	42	94	361
Mean ± SE	34 ± 4	88 ± 22	366 ± 82

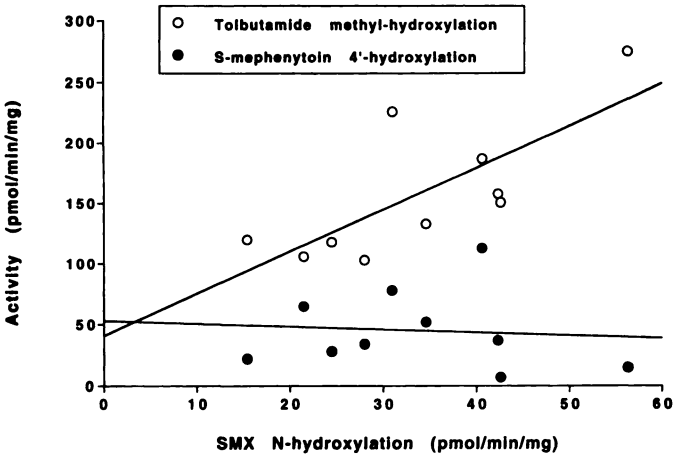

FIG. 3. *Correlation of SMX-HA formation with TOLB methyl-hydroxylation and S-mephenytoin hydroxylation in 10 human liver microsomes.*

SMX *N*-hydroxylation correlated with TOLB ($r = 0.75$; $p = 0.01$), but not mephenytoin ($r = 0.08$; $p = 0.81$) hydroxylation.

TABLE 3

Correlation of SMX-HA formation and reduction with marker activities in human hepatic microsomes[a]

Marker Activity	CYP Form	SMX-HA Formation		SMX-HA Reduction[b]	
		r	*P* Value	*r*	*P* Value
Caffeine-3-demethylation	CYP1A2	0.35	0.33	−0.38	0.29
Coumarin 7-OH	CYP2A6	0.01	0.99	−0.20	0.59
Benzphetamine *N*-DM	CYP2C8?	0.37	0.30	−0.20	0.59
TOLB hydroxylase	CYP2C9	0.75	0.01	−0.28	0.43
S-Mephenytoin hydroxylase	CYP2C19	0.08	0.81	−0.20	0.58
Dextromethorphan *O*-DM	CYP2D6	0.16	0.66	0.13	0.73
CHLOR 6-OH	CYP2E1	0.59	0.08	0.17	0.63
Testosterone 6β-OH	CYP3A	0.21	0.57	−0.41	0.23
NADH-dependent SMX-HA reduction	Non-CYP450	−0.31	0.38	0.85	0.002

OH, hydroxy; DM, demethylation.

[a] Marker activities provided by Human Biologics, Inc., as part of Hepatoscreen Test Kit, except for SMX-HA reduction.

[b] With NADPH as a cofactor at pH 7.4.

recently shown to be involved in the oxidation of the N_1-substituent ring of sulfamethazine (24). CYP2C7 is a closely related form of rat CYP that is also induced by PB (25) and could therefore be contributing to the oxidation of SMX, but it does not support progesterone 21-hydroxylase activity (20). Inhibition of SMX *N*-hydroxylation by anti-CYP2C6 antisera is also strongly supportive of a role for CYP2C6, although the weak cross-reactivity with CYP2C11 does not conclusively eliminate a contribution by CYP2C11. Together, these results support the identification of CYP2C forms, probably CYP2C6, as the major rat CYP responsible for the *N*-hydroxylation of SMX.

SMX N_4-hydroxylation was observed in human hepatic microsomes as previously reported (2). Correlation studies with marker activities in the 10 human liver samples revealed a significant correlation only with TOLB methyl-hydroxylation. TOLB methyl-hydroxylation can be catalyzed by CYP2C8, CYP2C9, and CYP2C19 recombinant enzymes; however, the activity is attributable primarily to the CYP2C9 enzyme in human hepatic microsomes (26–29). TOLB and diclofenac, substrates primarily for CYP2C9 (26–30), were both effective inhibitors of SMX oxidation. Inhibition by SPZ

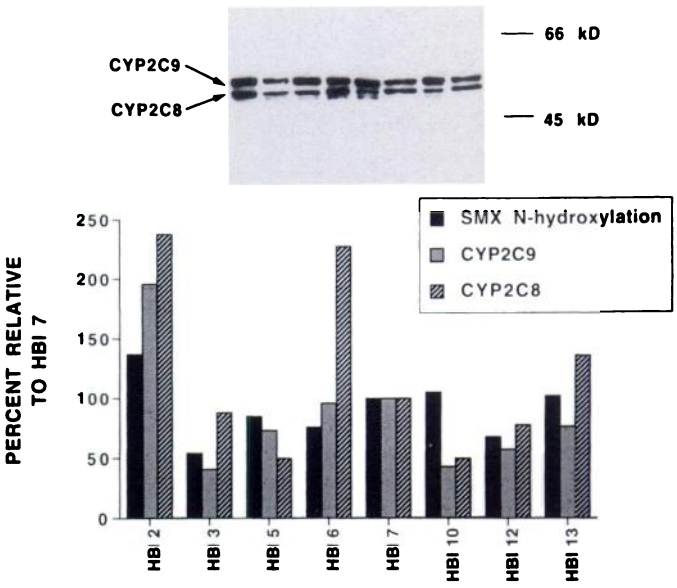

FIG. 4. *Correlation of CYP2C9 content in human liver microsomes with SMX N-hydroxylation.*

A representative immunoblot is shown in which 25 μg of microsomal protein was resolved on a 16-cm 10% polyacrylamide gel, transferred to nitrocellulose, and detected with a rat anti-CYP2C6 antibody. CYP2C9 (55 kDa), CYP2C8 (52 kDa), and an unidentified protein thought to be CYP2C19 (<50 kDa) (29) were detected. SMX *N*-hydroxylation, CYP2C9 content, and CYP2C8 content are shown expressed relative to the sample HBI 7. The order of samples on the immunoblot is the same as the order of samples in the graph. Quantitation of immunoblots was performed as described in *Materials and Methods*. SMX *N*-hydroxylation correlated with CYP2C9 content ($r = 0.79$; $p < 0.01$), but not with CYP2C8 content ($r = 0.52$; $p = 0.12$).

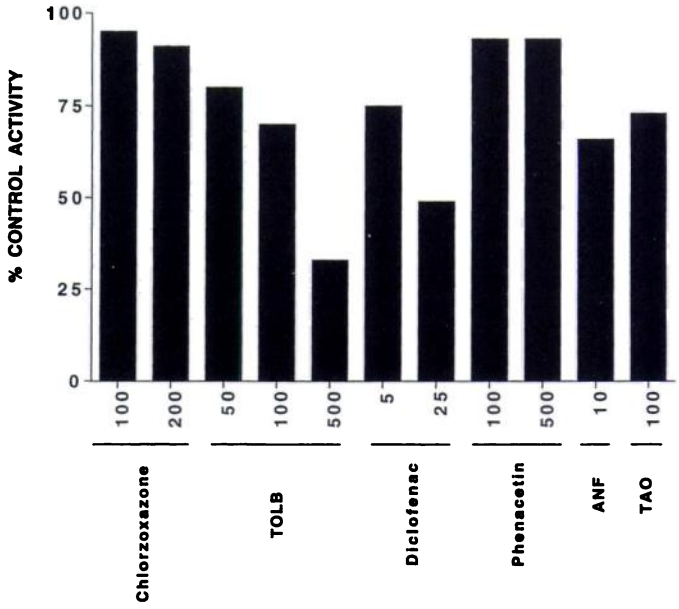

FIG. 5. *Effect of CYP inhibitors on the oxidation of SMX to SMX-HA in a sample of pooled human microsomes (pool 1).*

Results are the mean of duplicate determinations and are expressed relative to a control incubation containing 1% DMSO, except for CHLOR, in which case the control incubation contained 1% of a 60 mM KOH solution.

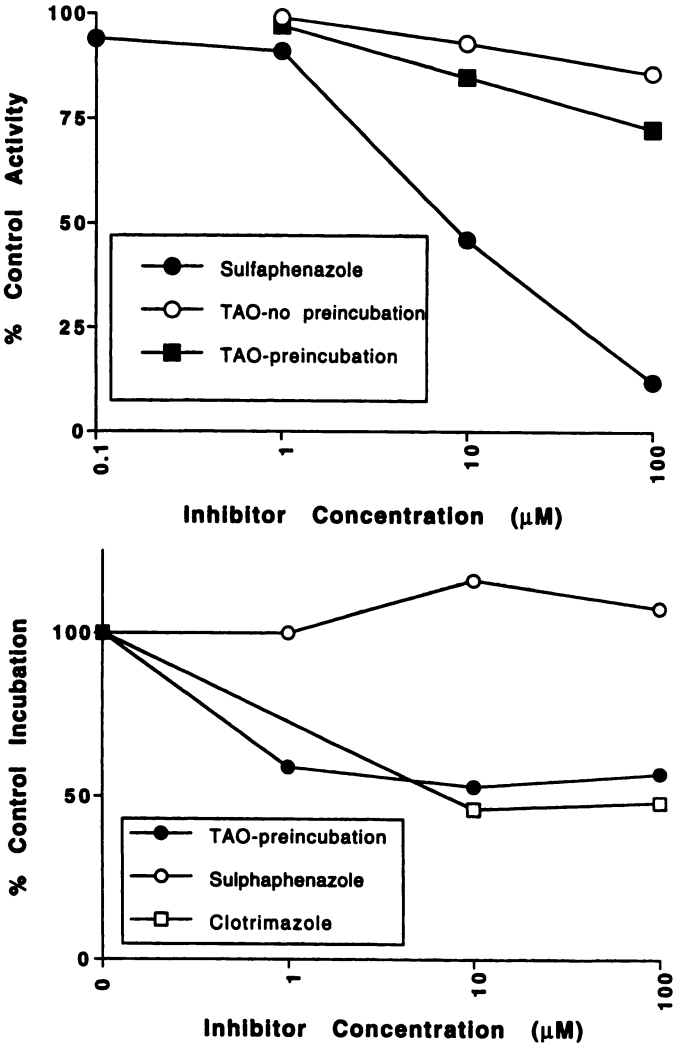

FIG. 6. *Effect of inhibitors on the formation and reduction of SMX-HA in human microsomes.*

(mditTop) Inhibition of SMX-HA formation by SPZ and TAO were assessed in a sample of pooled human microsomes (pool II). TAO was added either at the start of the incubation or was incubated with microsomes for 30 min before the addition of SMX. (*Bottom*) The effect of preincubation with TAO or the addition of SPZ or clotrimazole on the reduction of SMX-HA in a sample of pooled human microsomes (pool II) was assessed under a nitrogen atmosphere.

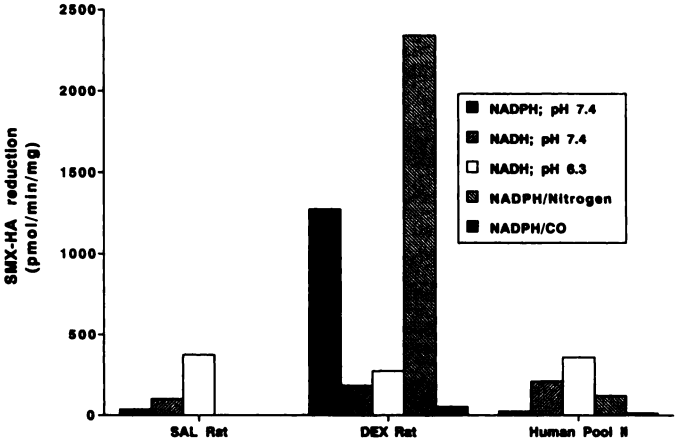

FIG. 7. *Microsomal reduction of SMX-HA in rat and human hepatic microsomes.*

The ability of NADH and NADPH to serve as cofactors for the microsomal reduction of SMX-HA was assessed in the SAL-treated rat, the DEX-treated rat, and human hepatic microsomes at pH 7.4 and pH 6.3. The effect of anaerobic atmosphere (nitrogen) or carbon monoxide on the reduction of SMX-HA was assessed as described in *Materials and Methods*. Results represent the mean of at least duplicate determinations.

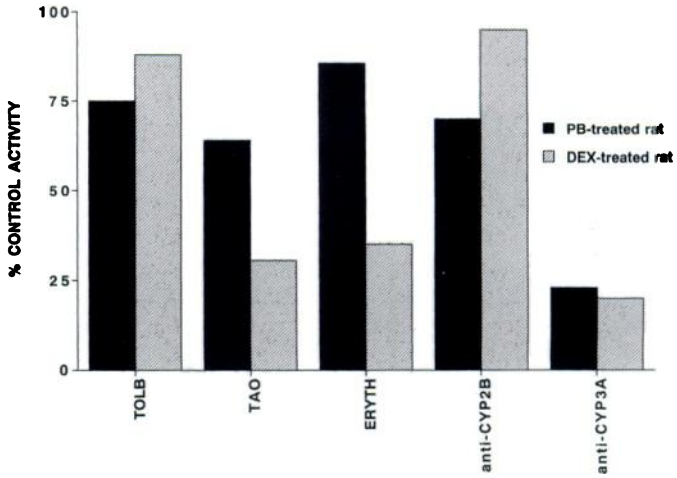

FIG. 8. *Effect of TOLB (1 mM), TAO (100 μM), ERYTH (400 μM), and anti-CYP2B and anti-CYP3A (8 mg IgG/mg microsomal protein) on SMX-HA reduction by PB- and DEX-treated rat hepatic microsomes.*

Results are the mean of duplicate determinations and are compared with control incubations containing vehicle only or with nonimmune IgG fractions. (For details, see *Materials and Methods*.)

was also consistent with a major role for the CYP2C9 enzymes (27, 28). The inhibition by SPZ observed was very similar to that observed for phenytoin and TOLB (27, 28) with human microsomes and recombinant CYP2C9. SPZ was metabolized (as observed by HPLC) during the 60-min incubation period used for SMX *N*-hydroxylation, so that the inhibition at a given concentration of SPZ cannot be directly compared with concentrations used in shorter incubations. SMX-HA formation by recombinant CYP2C9 was detected but was not observed with recombinant CYP2C8. The turnover/nmol CYP in the lymphoblastoid cells is limited by the low expression of NADPH-cytochrome *c* reductase (the cells used did not have cotransfection of reductase); therefore, the activity observed is consistent with CYP2C9 being the major SMX *N*-hydroxylation in human microsomes. We cannot rule out a role for CYP2C8 based on these data because of the low expression of CYP2C8 in the system used, but the inhibition by SPZ and a lack a correlation of SMX-HA formation with CYP2C8 content indicate that CYP2C8 does not make a major contribution. The ability of SPZ to inhibit CYP2C19 has not been assessed, and CYP2C19 can metabolize TOLB (29) so the ability of these compounds to inhibit SMX *N*-hydroxylation does not rule out a minor contribution by CYP2C19. However, the lack of correlation the *S*-mephenytoin hydroxylase and a positive correlation with CYP2C9 content and TOLB methyl-hydroxylation are strong evidence for a minor role of CYP2C19 in determining SMX *N*-hydroxylation.

ANF and TAO produced only limited inhibition at concentrations normally associated with marked inhibition of CYP1A2 (31) and CYP3A (32, 33) forms, respectively. In a study with three individual livers (data not shown), ANF at 1 μM produced <20% inhibition, whereas 100 μM produced 70% inhibition. The inhibition produced

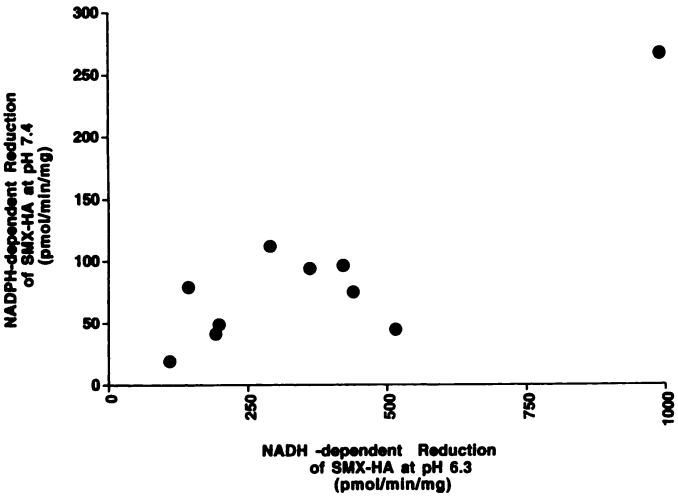

FIG. 9. *Correlation of NADH- and NADPH-dependent reduction of SMX-HA at pH 6.3 and pH 7.4, respectively, in human hepatic microsomes.*

Reduction of SMX-HA was assessed as described in *Materials and Methods* in a group of 10 human hepatic microsomes. Each *point* represents the mean of at least duplicate determinations ($r = 0.85$; $p = 0.002$).

by ANF was similar to the inhibition of TOLB hydroxylation observed in human microsomes (24) and is consistent with the involvement of CYP2C9 (33), but not with metabolism by CYP1A2. Further, phenacetin, a CYP1A2 substrate (31), had no effect on SMX-HA formation. The involvement of CYP3A forms can be ruled out because the inhibition by TAO was weak, and there was no correlation with testosterone 6β-hydroxylation, a marker of CYP3A activity. Western blotting with an antirat CYP3A antibody confirmed that testosterone 6β-hydroxylase activity correlated with the quantity of immunoreactive protein, whereas SMX-HA hydroxylation did not (data not shown). The formation of SMX-HA showed a weak, nonsignificant correlation with CHLOR hydroxylation (table 1), but CHLOR, a CYP2E1 substrate (34), had no inhibitory effect on the oxidation of SMX. Thus, CYP1A2, CYP2E1, and CYP3A do not contribute significantly to SMX-HA formation in human hepatic microsomes.

We therefore conclude, based on the aforementioned data, that CYP2C9 is the major CYP responsible for the *N*-hydroxylation of SMX in human hepatic microsomes. This is consistent with the observation of Back *et al.* (35) that SMX was an inhibitor of TOLB hydroxylation *in vitro*, albeit an order of magnitude less potent than sulfamethizole and several orders less potent than SPZ. SMX is also an inhibitor of TOLB metabolism *in vivo* (36).

The reduction of SMX to SMX-HA had a markedly different CYP specificity in the rat. PB and DEX pretreatment markedly enhanced the NADPH-dependent, carbon monoxide-sensitive reduction of SMX-HA, implicating CYP2B and CYP3A forms. TAO and ERYTH were effective inhibitors in DEX-treated rat hepatic microsomes, but were less effective in hepatic microsomes from PB-treated rats. The induction of SMX-HA reduction in DEX-treated rat microsomes was similar in magnitude to the induction of ERYTH *N*-demethylase activity, whereas the induction of SMX-HA reduction in PB-treated rat microsomes was less than induction of the CYP2B-mediated PROD activity, but greater than the induction of CYP3A-mediated activities. Partial inhibition by anti-CYP2B antibodies in the PB-treated rat microsomes suggests that, in addition to a major role for CYP3A, CYP2B enzymes make a significant contribution to SMX-HA reduction in PB-treated rat microsomes. Therefore, inducible CYP3A and CYP2B enzymes can catalyze the reduction of SMX-HA; however, constitutively expressed forms of rat CYP displayed little SMX-HA reductase activity. The ability of NADH to support SMX-HA reduction in uninduced rat hepatic microsomes with a pH optimum of 6.3, but not the CYP-dependent reduction observed in induced hepatic microsomes, supports a role for the previously described NADH-dependent hydroxylamine reductase (37, 38) in uninduced rat microsomes.

The reduction of SMX-HA back to SMX by human hepatic microsomes under aerobic conditions was metabolized predominantly by the NADH-dependent hydroxylamine reductase (37, 38) whether NADPH or NADH was the cofactor. The aerobic NADPH-dependent activity at pH 7.4 did not correlate with any of the CYP marker activities, total CYP, NADPH-cytochrome *c* reductase, or cytochrome b_5 content; however, it was significantly correlated with the NADH-dependent reduction at pH 6.3 (table 3). Anaerobic conditions induced with nitrogen enhanced the NADPH-dependent SMX-HA reductase activity, but a carbon monoxide atmosphere did not. Carbon monoxide, however, did not reduce activity below that observed under aerobic conditions. This suggests that human microsomal CYPs are capable of mediating the reduction of SMX-HA but do not make a major contribution in an aerobic environment. The anaerobic, NADPH-dependent reduction of SMX-HA was partially inhibited by TAO and clotrimazole, suggesting that CYP3A forms may be partially responsible (32, 39). The lack of inhibition by SPZ suggests that CYP2C enzymes do not contribute significantly to the reduction of SMX-HA. This is consistent with the results observed in rat microsomes, where CYP3A were the major forms mediating the reduction of SMX-HA. The ability of NADH to serve as a cofactor with greater activity at pH 6.3 is consistent with the previously described NADH-dependent hepatic microsomal hydroxylamine reductase (37, 38) that was also recently shown to reduce pentamidine hydroxylamines (40). We have been unable to detect NADH or NADPH-dependent reduction of SMX-HA in cytosolic fractions.[2]

The objective of these studies was to provide information regarding human and rat CYP microsomal metabolism of SMX. Data in rats were obtained to both validate methodology and provide a basis for attempts to establish an animal model of sulfonamide hypersensitivity reactions by manipulation of bioactivation and detoxification pathways. Whether metabolism by CYP2C9 at the N_4-position is common to most or all of the sulfonamide antimicrobials is unknown. It is interesting to note that the *N*-hydroxylation of dapsone, a sulfone, seems to be predominantly by CYP3A4 (41), whereas many carcinogenic arylamines are metabolized by CYP1A2 (26). In the rat, *N*-hydroxylation of sulfanilamide was induced 2-fold by 3-methylcholanthrene, but not by PB (42). As expected, it is apparent that the arylamine moiety *per se* does not determine the specificity of CYP metabolism, but rather it is determined also by the *para*-substituent of the arylamine. Nevertheless, the ability of several different sulfonamide antibacterials to inhibit TOLB metabolism or clearance suggest that metabolism by the CYP2C subfamily may be common to this class of drugs (35, 36, 43).

The major pathways of microsomal metabolism at the N_4-position of SMX are summarized in fig. 10. As indicated in this figure, further oxidation of SMX-HA to its more reactive nitroso-SMX may also be mediated by CYP,[2] as well as occurring spontaneously in the absence of reducing agents (8). The covalent binding of CYP-dependent SMX metabolites to microsomal protein *in vitro* is due to nitroso-SMX and not SMX-HA (8).[2] Although nitroso-SMX is more toxic than SMX-HA when applied directly to isolated cells, this may be primarily the result of its enhanced ability to penetrate the cell and does not necessarily implicate it as the ultimate toxin. Indeed, previous studies

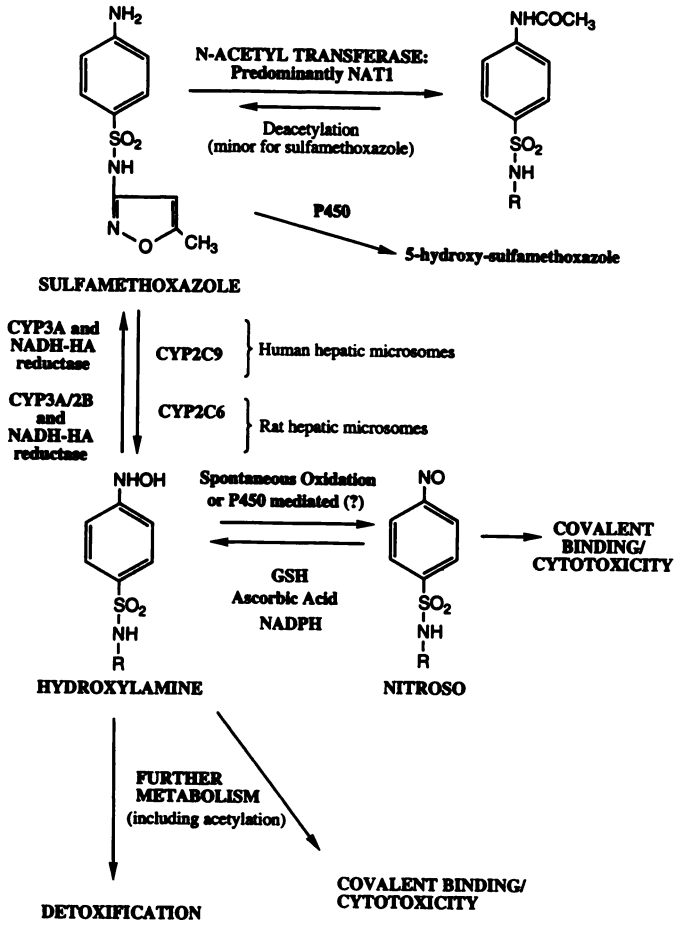

FIG. 10. *Proposed pathways of metabolism at the N_4 position of SMX in rat and human livers.*

This partial scheme of metabolism of SMX is based on results presented in this study and in refs. 2, 8, 9, and 15. NAT1, monomorphic *N*-acetyltransferase; GSH, glutathione.

suggest that, in cells with a normal complement of glutathione, nitroso-SMX does not mediate toxicity (8). We have recently identified the acetoxy-derivative, produced by *O*-acetylation of SMX-HA, as a further metabolite of SMX-HA (15). The ultimate toxic and covalent binding species of SMX *in vivo* have not been identified nor has the relative contribution of direct cytotoxicity and indirect immune-mediated cytotoxicity to the clinical expression of sulfonamide hypersensitivity reactions been resolved. Nevertheless, the formation and disposition of SMX-HA are clearly important factors in determining susceptibility to sulfonamide hypersensitivity reactions, because SMX-HA is a necessary intermediate.

Individuals deficient in TOLB and phenytoin hydroxylation (attributed to CYP2C9) have been described (45, 46). These individuals would presumably be at a reduced risk for sulfonamide ADR. There is between a 10- to 30-fold variation in TOLB hydroxylase activity (44, 47) in humans. It seems reasonable to suggest that individuals with high levels of CYP2C9 may be at an increased risk of sulfonamide hypersensitivity reactions, particularly if coupled with the slow acetylator phenotype and the previously postulated detoxification defect (1). On the other hand, because reduction of SMX-HA would be a protective pathway, individuals who have a decreased capacity for this reaction would be at an increased risk. It is not known whether a deficiency in the microsomal NADH-dependent hydroxylamine reductase represents the postulated detoxification defect. More work is required to understand the genetic and environmental factors predisposing to sulfonamide ADR, but a complete understanding of the bioactivation and detoxification of SMX as a model sulfonamide is a necessary part of investigations of this phenomenon.

References

1. N. H. Shear, S. P. Spielberg, D. M. Grant, B. K. Tang, and W. Kalow: Differences in metabolism of sulfonamides predisposing to idiosyncratic toxicity. *Ann. Intern. Med.* **105,** 179–184 (1986).
2. A. E. Cribb, and S. P. Spielberg: Sulfamethoxazole is metabolized to the hydroxylamine in humans. *Clin. Pharmacol. Ther.* **51,** 522–526 (1992).
3. M. Kiese: The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines and mechanisms of ferrihemoglobin formation. *Pharmacol. Rev.* **18,** 1091–161 (1966).
4. R. Eyanagi, H. Shigematsu, K. Yoshida, and K. Yoshimura: Metabolism and nephrotoxicity of phenacetin and sulfanilamide. *J. Pharmacobiodyn.* **8,** 95–105 (1985).
5. I. Yamamoto, and R. Eyanagi: Positive skin reaction induced by 4,4′-azoxybenzenedisulfonamide in relationship to the sulfanilamide allergy. *Int. Arch. Allergy Appl. Immun.* **54,** 538–541 (1977).
6. F. M. Gordin, G. L. Simon, C. B. Wofsy, and J. Mills: Adverse reactions to trimethoprim-sulfamethoxazole in patients with the acquired immunodeficiency syndrome. *Ann. Intern. Med.* **100,** 495–499 (1984).
7. B. L. Lee, D. Wong, N. L. Benowitz, and P. M. Sullam: Altered patterns of drug metabolism in patients with acquired immunodeficiency syndrome. *Clin. Pharmacol. Ther.* **53,** 529–535 (1993).
8. A. Carr, B. Tindall, R. Penny, and D. A. Cooper: *In vitro* cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. *Clin. Exp. Immunol.* **94,** 21–25 (1993).
9. A. E. Cribb, M. Miller, A. Tesoro, and S. P. Spielberg: Peroxidase-dependent oxidation of sulfonamides by monocytes and neutrophils from humans and dogs. *Mol. Pharmacol.* **38,** 744–751 (1990).
10. A. E. Cribb, M. Miller, J. S. Leeder, J. Hill, and S. P. Spielberg: Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione. *Drug Metab. Dispos.* **19,** 900–906 (1991).
11. A. E. Cribb, H. Nakamura, D. M. Grant, M. A. Miller, and S. P. Spielberg: Role of polymorphic and monomorphic human arylamine *N*-acetyltransferases in determining sulfamethoxazole metabolism. *Biochem. Pharmacol.* **45,** 1277–1282 (1993).
12. R. J. Riley, A. E. Cribb, and S. P. Spielberg: Glutathione transferase μ deficiency is not a marker for predisposition to sulphonamide toxicity. *Biochem. Pharmacol.* **42,** 696–698 (1991).
13. A. E. Cribb, and S. P. Spielberg: Hepatic microsomal metabolism of sulfamethoxazole to the hydroxylamine. *Drug Metab. Dispos.* **18,** 784–787 (1990).
14. A. E. Cribb, and S. P. Spielberg: An *in vitro* investigation of predisposition to sulfonamide idiosyncratic toxicity in dogs. *Vet. Res. Commun.* **14,** 241–252 (1990).
15. A. E. Cribb: An exploration of metabolic determinants of sulfamethoxazole idiosyncratic reactions. Ph.D. Thesis, University of Toronto, Toronto, Ontario, Canada, 1991.
16. M. D. Burke, S. Thompson, C. R. Elcombe, J. Halpert, T. Haparanta, and T. T. Mayer: Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem. Pharmacol.* **34,** 3337–3345 (1985).
17. D. R. Koop: Hydroxylation of *p*-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isozyme 3a. *Mol. Pharmacol.* **29,** 399–404 (1986).
18. M. A. Arlotto, A. J. Sonderfan, C. D. Klaassen, and A. Parkinson: Studies on the pregnenolen-16alpha-carbonitrile-inducible form of rat liver microsomal cytochrome P-450 and UDP-glucuronosyl transferase. *Biochem. Pharmacol.* **36,** 3859–3855 (1987).
19. J. Halpert, J.-Y. Law, and C. Balfour: Specific inactivation by 17β-substituted steroids of rabbit and rat liver cytochromes P-450 responsible for progesterone 21-hydroxylation. *Mol. Pharmacol.* **34,** 139–141 (1989).

20. D. C. Swinney, D. E. Ryan, P. E. Thomas, and W. Levin: Regioselective progesterone hydroxylation catalyzed by eleven rat hepatic cytochrome P-450 isozymes. *Biochemistry* **26,** 7073–7083 (1987).
21. M. E. Veronese, M. E. McManus, R. Laupattarakasem, J. O. Miners, and D. J. Birkett: Tolbutamide hydroxylation by human, rabbit and rat liver microsomes and by purified forms of cytochrome P-450. *Drug Metab. Dispos.* **18,** 356–361 (1990).
22. P. M. Bélanger, and S. St.-Hilaire: The characteristics of the microsomal hydroxylation of tolbutamide. *Can. J. Physiol. Pharmacol.* **69,** 400–405 (1991).
23. S. Bandiera: Expression and catalysis of sex-specific cytochrome CYP450 isozymes in rat liver. *Can. J. Physiol. Pharmacol.* **68,** 762–768 (1990).
24. R. F. Witkamp, S. M. Nijmeijer, H. Yun, J. Noordhoek, and A. S. J-. P. A. M. Van Miert: Sulfamethazine as a model compound to assess sex hormone-dependent cytochrome P-450 activity in rats. *Drug Metab. Dispos.* **21,** 441–446 (1993).
25. P. Soucek, and I. Gut: Cytochromes P-450 in rats: structures, functions, properties, and relevant human forms. *Xenobiotica* **22,** 83–103 (1992).
26. P. K. Srivastava, C. Yun, P. H. Beaune, C. Ged, and F. P. Guengerich: Separation of human liver microsomal tolbutamide hydroxylase and (*S*)-mephenytoin 4′-hydroxylase cytochrome P-450 enzymes. *Mol. Pharmacol.* **40,** 69–79 (1991).
27. M. E. Veronese, P. I. Mackenzie, C. J. Doecke, M. E. McManus, J. O. Miners, and D. J. Birkett: Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome CYP4502C9. *Biochem. Biophys. Res. Comm.* **175,** 1112–1118 (1991).
28. M. E. Veronese, C. J. Doecke, P. I. Mackenzie, M. E. McManus, J. O. Miners, D. L. P. Rees, R. Gasser, U. A. Meyer, and D. J. Birkett: Site-directed mutation studies of human liver cytochrome P-450 isoenzymes in the CYP2C subfamily. *Biochem. J.* **289,** 533–538 (1993).
29. J. A. Goldstein, M. B. Faletto, M. Romkes-Sparks, T. Sullivan, S. Kitareewan, J. L. Raucy, J. M. Lasker, and B. I. Ghanayem: Evidence that CYP2C19 is the major (*S*)-mephenytoin 4′-hydroxylase in humans. *Biochemistry* **33,** 1743–1752 (1994).
30. T. Leemann, C. Transon, and P. Dayer: Cytochrome $CYP450_{TB}$ (CYP2C): a major monooxygenase catalyzing diclofenac 4′-hydroxylation in human liver. *Life Sci.* **52,** 29–34 (1992).
31. M. A. Butler, M. Iwasaki, R. P. Guengerich, and F. F. Kadlubar: Human cytochrome $P\text{-}450_{PA}$ (P-4501A2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and *N*-oxidation of carcinogenic arylamines. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 7696–7700 (1989).
32. X. J. Zhou, X. R. Zhou-Pan, T. Gauthier, M. Placidi, P. Maurel and R. Rahman: Human liver microsomal cytochrome CYP450 3A isozymes mediated vindesine biotransformation. *Biochem. Pharmacol.* **45,** 853–861 (1993).
33. T. K. H. Chang, F. J. Gonzalez, and D. J. Waxman: Evaluation of triacetyloleandomycin, alpha-naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes CYP450. *Arch. Biochem. Biophys.* **311,** 437–442 (1994).
34. R. Peter, R. Böcker, P. H. Beaune, M. Iwasaki, F. P. Geungerich, and C. S. Yang: Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem. Res. Toxicol.* **3,** 566–573 (1990).
35. D. J. Back, J. F. Tjia, J. Karbwang, and J. Colbert: *In vitro* inhibition studies of tolbutamide hydroxylase activity of human liver microsomes by azoles, sulphonamides and quinolines. *Br. J. Clin. Pharmacol.* **26,** 23–29 (1988).
36. J. M. Hansen, and L. K. Christensen: Drug interactions with oral sulphonylurea hypoglycaemic drugs. *Drugs* **13,** 24–34 (1977).
37. F. F. Kadlubar, E. M. Mckee, and D. M. Ziegler: Reduced pyridine nucleotide-dependent *N*-hydroxy amine oxidase and reductase activities of hepatic microsomes. *Arch. Biochem. Biophys.* **156,** 46–57 (1973).
38. F. F. Kadlubar, and D. M. Ziegler: Properties of a NADH-dependent *N*-hydroxy amine reductase isolated from pig liver microsomes. *Arch. Biochem. Biophys.* **162,** 83–92 (1974).
39. J. J. Sheets, J. I. Mason, C. A. Wise, and R. W. Estabrook: Inhibition of rat liver microsomal cytochrome P-450 steroid hydroxylase reactions by imidazole antimycotic agents. *Biochem. Pharmacol.* **35,** 487–491 (1986).
40. B. Clement, and F. Jung: *N*-hydroxylation of the antiprotozoal drug pentamidine catalyzed by rabbit liver cytochrome P-450 2C3 or human liver microsomes, microsomal retroreduction, and further oxidative transformation of the formed amidoximes. *Drug Metab. Dispos.* **22,** 486–500 (1994).
41. C. M. Fleming, R. A. Branch, G. R. Wilkinson, and F. P. Guengerich: Human liver microsomal *N*-hydroxylation of dapsone by cytochrome P-4503A4. *Mol. Pharmacol.* **41,** 975–980 (1992).
42. R. Eyanagi, H. Shigematsu, K. Yoshida, and H. Yoshimura: Enhancement of sulfanilamide N^4-hydroxylase activity in kidney and liver microsomes of rats by pre-treatment with 3-methylcholanthrene type-polychlorinated biphenyl. *J. Pharm. Dyn.* **5,** 853–858 (1982).
43. B. Lumholtz, K. Siersbaek-Nielsen, L. Skovsted, J. Kampmann, and J. M. Hansen: Sulfamethizole-induced inhibition of diphenylhydantoin, tolbutamide, and warfarin metabolism. *Clin. Pharmacol. Ther.* **17,** 731–734 (1975).
44. M. E. Veronese, J. O. Miners, D. L. P. Rees, and D. J. Birkett: Tolbutamide hydroxylation in humans: lack of bimodality in 106 healthy subjects. *Pharmacogenetics* **3,** 86–93 (1993).
45. J. O. Miners, L. M. H. Wing, and D. J. Birkett: Normal metabolism of debrisoquine and theophylline in a slow tolbutamide metaboliser. *Aust. N.Z. J. Med.* **15,** 348–349 (1985).
46. M. R. Vasko, R. D. Bell, D. D. Daly, and C. E. Pippenger: Inheritance of phenytoin hypometabolism: a kinetic study of one family. *Clin. Pharmacol. Ther.* **27,** 96–103 (1980).
47. M. V. Relling, T. Aoyama, F. J. Gonzalez, and U. A. Meyer: Tolbutamide and mephenytoin hydroxylation by human cytochrome CYP450s in the *CYP2C* subfamily. *J. Pharmacol. Exp. Ther.* **252,** 442–447 (1990).