Chiral Sulfoxidations Catalyzed by Rat Liver Cytochromes P-450†

David J. Waxman,*,‡ David R. Light,§ and Christopher Walsh*

ABSTRACT: The chirality of sulfoxidation catalyzed by two cytochrome P-450 isozymes purified from phenobarbital-induced rat liver was studied by using 4-tolyl ethyl sulfide as a substrate. Both P-450 isozymes, termed PB-1 and PB-4, when reconstituted with purified rat liver NADPH–cytochrome P-450 reductase and cytochrome b_5, generated 4-tolyl ethyl sulfoxide which was predominantly in the *S*-(−) configuration. In the case of isozyme PB-1, the sulfoxide was 79 ± 1% *S* and was formed with a turnover of 41 min^{-1}; with isozyme PB-4, sulfoxide, 84 ± 1% *S*, was formed at 31 min^{-1}. In addition, PB-1 catalyzed oxygen transfer to the *p*-methyl group of the sulfide substrate to yield the (ethylthio)benzyl alcohol with a turnover of 6.8 min^{-1}, corresponding to a sulfur:carbon oxygenation partition ratio of 6:1. Isozyme PB-4 was ~80-fold less efficient at catalyzing this carbon hydroxylation, giving a sulfur:carbon ratio of ~375:1. In the absence of cytochrome b_5, turnover numbers were reduced to ~15% and 67% of the above values for PB-1 and PB-4, respectively, with no change in sulfoxide chirality. This fact, and the lack of improvement in chirality upon inclusion of scavengers for reactive oxygen species, suggests that the ~79–84% chirality observed for the sulfoxide product reflects an intrinsic lack of complete stereospecificity in these cytochrome P-450 catalyzed reactions. The enantiomeric composition of 4-tolyl ethyl sulfoxide generated in rat liver microsomal incubations was shown to reflect the relative contribution of cytochrome P-450 isozymes, which generate the *S*-(−) enantiomer preferentially, and of the flavin adenine dinucleotide (FAD) containing monooxygenase (EC 1.14.13.8), which as we have shown catalyzes (*R*)-(+)-sulfoxide formation [Light, D. R., Waxman, D. J., & Walsh, C. (1982) *Biochemistry* (preceding paper in this issue)]. Thus, the chirality of microsome-catalyzed sulfoxidation is shown to be modulated by factors which alter the relative participation of these two liver monooxygenases, such as phenobarbital induction, inclusion of inhibitors or activators (metyrapone and *n*-octylamine), and variation in sulfide substrate concentration.

The oxidative processing of xenobiotic compounds in the liver results primarily from the action of two types of enzymes, the flavin adenine dinucleotide (FAD)[1] containing microsomal monooxygenase and the cytochrome P-450 oxygenase isozymes [reviewed by Ziegler (1980) and Wislocki et al. (1980)]. The iron–oxygen reagent is more reactive chemically than the flavin–oxygen reagent; cytochromes P-450 can oxygenate carbon, nitrogen, and sulfur atoms while the FAD-containing monoxygenase can attack nitrogen and sulfur but not carbon sites. In the preceding paper (Light et al., 1982), we have analyzed the stereochemical outcome of oxygen transfer to divalent sulfur in several aryl alkyl sulfides, as catalyzed by the FAD-containing enzyme. These studies were greatly facilitated by analysis of incubation mixtures on a chiral stationary phase HPLC Pirkle-type column (Pirkle et al., 1981), a method which allows for the rapid, sensitive, and quantitative analysis of enzymically generated sulfoxide enantiomers. In this paper, we report the outcome of processing of *p*-tolyl ethyl sulfide (*p*Tol-S-Et) by several cytochrome P-450 isozymes purified from phenobarbital (PB)-induced rat liver. We include a comparison with the flavoprotein results to reconstruct the contributions of each enzyme type in microsomal sulfur oxygenation metabolism. We find that *p*Tol-S-Et shows very high turnover numbers with two of the isozymes isolated from PB-induced rat liver with stereoselectivity in favor of (*S*)-(−)-sulfoxide formation. This is in contrast to the generation of the (*R*)-(+)-sulfoxide enantiomer by the liver microsomal FAD-containing monooxygenase. In addition, one of the PB P-450 isozymes studied is also shown to catalyze oxygen transfer to the *p*-methyl group of the substrate in competition with S-oxygenation, allowing comparison of carbon vs. sulfur oxygenation with a pure P-450 isozyme. Results presented also demonstrate that the chirality of sulfoxidation and the partitioning between carbon and sulfur oxidation catalyzed by microsomal preparations can be modulated by inclusion of specific monooxygenase inhibitors or activators and by changes in sulfide concentration.

Materials and Methods

Materials

Microsomes. Male rats of Sprague-Dawley origin (35 days old, ~120 g each) were obtained from Charles River Breeding Laboratories and either were uninduced (control) or were given injections of NaPB (80 mg/kg body weight) in saline for 4 consecutive days. Animals were sacrificed on the fifth day and liver microsomes prepared by standard methods (Van der Hoeven & Coon, 1974) and then stored in liquid nitrogen after a final wash with 0.1 M KP_i (pH 7.4), 20% glycerol (v/v), and 0.1 mM EDTA. Specific contents of the microsomal heme proteins, determined by the method of Omura & Sato (1964), were found to be as follows: for control (uninduced) microsomes, P-450 = 0.92 nmol/mg and b_5 = 0.75 nmol/mg; for PB-treated microsomes, P-450 = 2.39 nmol/mg and b_5 = 0.76 nmol/mg. Thus, the P-450 was induced 2.6-fold, and the

† From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received November 30, 1981.* This research was supported by National Institutes of Health Grants GM21643 and 20011, National Cancer Institute Grant TE32-CA09112, and a grant from the Monsanto Co.

‡ Postdoctoral Fellow of the Damon Runyon–Walter Winchell Cancer Fund (DRG-439F).

§ Present address: Genentech, South San Francisco, CA 94081.

[1] Abbreviations: PB, phenobarbital; P-450, cytochrome P-450; b_5, cytochrome b_5; FAD-containing monooxygenase, EC 1.14.13.8, *N*,*N*-dimethylaniline monooxygenase; *p*Tol-S-Et, *p*Tol-SO-Et, and *p*Tol-SO_2-Et, *p*-tolyl ethyl sulfide, sulfoxide, and sulfone, respectively; *p*BzlOH-S-Et, *p*-(hydroxymethyl)phenyl ethyl sulfide; HPLC, high-pressure liquid chromatography; EDTA, disodium ethylenediaminetetraacetate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide.

0006-2960/82/0421-2499$01.25/0

cytochrome b_5 was uninduced.

Purified P-450s. P-450 PB-1 and P-450 PB-4 were purified from liver microsomes isolated from 40-day-old male Sprague-Dawley rats induced with 0.1% NaPB (drinking water) for 6 days essentially by using the method of West et al. (1979) with minor modifications to be described (D. J. Waxman and C. Walsh, unpublished experiments). The nomenclature P-450 PB-1, PB-2, PB-3, etc. is used to identify distinct isozymes isolated from PB-induced animals, with the isozymes numbered in order of their elution from diethylaminoethylcellulose (Whatman DE52). The P-450 PB-1 utilized in this study (specific content = 11.5 nmol/mg) appears to be equivalent to a major P-450 component migrating on DE52 in fraction A of West et al. (1979). P-450 PB-4 (specific content = 15.8 nmol/mg) appears equivalent to the major PB-induced P-450 isozyme characterized previously [i.e., fraction C of West et al. (1979), fraction B of Guengerich (1977), and P-450$_b$ of Ryan et al. (1979)] by a combination of structural (NH_2-terminal sequence), enzymatic, and immunochemical criteria (D. J. Waxman and C. Walsh, unpublished results).

Other Enzymes. Highly purified rat liver cytochrome P-450 reductase was that purified previously (Loosemore et al., 1980), and purified hog liver FAD-containing monooxygenase was that studied in the preceding paper (Light et al., 1982). Rat liver cytochrome b_5 was purified to protein homogeneity from the fraction tightly adsorbed to the first DE52 column of West et al. (1979) by a modification of the method of Strittmatter et al. (1978) (D. J. Waxman and C. Walsh, unpublished experiments). Rabbit liver b_5, kindly provided by Dr. Y. Takagaki of this department, was found to be indistinguishable from the rat enzyme in stimulating P-450-catalyzed reactions.

Other Materials. All substrates and product standards as well as HPLC columns and solvents were those described in the preceding paper (Light et al., 1982). PB, catalase, superoxide dismutase, and NADPH were purchased from Sigma. Deoxycholic acid (Calbiochem) was recrystallized from ethanol before conversion to its sodium salt. 4-Thiocresol was purchased from Aldrich.

Methods

Microsomal Incubations. Unless indicated otherwise, the standard microsomal assay contained 0.15 nmol of P-450/mL of 0.1 M KP_i (pH 7.4), 20% glycerol (v/v), and 20 μM EDTA ("assay buffer") containing 0.5 mM *p*Tol-S-Et and 0.5% MeOH (v/v). Stoppered samples were warmed to 37 °C (3 min) and the reactions initiated by the addition of NADPH to 1 mM. Samples were quenched, usually after 10 min, by a single extraction with an equal volume of hexane. In certain cases, samples were extracted once into 2 volumes of ethyl acetate which was then evaporated under a stream of N_2 (effecting a near-quantitative volatilization of *p*Tol-S-Et), dissolved in water (0.2 volume), and back-extracted once with hexane (0.2 volume). Direct extraction into hexane was preferred to the ethyl acetate method (despite the lower yield obtained) since nonenzymatic sulfoxidation during sample workup was minimized.

Reconstitution and Standard Assay of Purified P-450s. Purified P-450 isozymes (0.03 nmol) were mixed in assay buffer with cytochrome b_5 (0.034 nmol) and cytochrome P-540 reductase (0.0375 nmol), each containing minimal nonionic detergent, to give a final volume of 112.5 μL. Freshly sonicated dilauroylphosphatidylcholine (12.5 μL of 0.6 mg of lipid/mL of water) was then added, and the samples were incubated for 3–10 min at room temperature, after which *p*Tol-S-Et was added in 0.85 mL of assay buffer containing 25 μg of sodium deoxycholate to give 0.5 mM sulfide and 0.5% MeOH (v/v). Stoppered samples were warmed to 37 °C and reactions initiated by the addition of NADPH to 0.3 mM. Samples were hexane extracted as described for the microsomal incubations.

HPLC Analysis for Chiral Sulfoxides. Hexane extracts were kept stoppered and on ice until ready for analysis by direct injection (0.6–1.5 mL) onto a Waters HPLC system equilibrated and run in 95% hexane/5% 2-propanol (v/v). Analysis was performed by using one of the following systems: HPLC system A, consisting of a chiral Pirkle type IA column containing a 3,5-dinitrobenzoyl-D-phenylglycine chiral stationary phase (Pirkle et al., 1981; Regis Chemical Co.) run at 4 mL of solvent/min (e.g., as in Figure 1); HPLC system B, a Pirkle type IA column connected in series to the effluent of a μPorasil column (Waters) run at 4 mL/min (e.g., as in Figure 2); HPLC system C, a μPorasil column run at 8 mL of 96.5% hexane–3.5% 2-propanol (v/v) per min when rapid analysis was desired without resolution of sulfoxide enantiomers. Elution times for standard *p*Tol-S-Et, *p*BzlOH-S-Et, *p*Tol-SO_2-Et, (*S*)-*p*Tol-SO-Et, and (*R*)-*p*Tol-SO-Et were as follows: system A, 1.1, 3.7, 6.65, 6.2, and 6.6 min, respectively; system B, 1.4, 5.0, 7.8, 9.8, and 10.2 min, respectively; system C, 0.58, 1.3, 1.1, 3.3, and 3.3 min, respectively.

Compounds were detected by the absorbance at 220 or at 254 nm with quantitation based on the yields of extraction and recovery of standard compounds in the relevant concentration ranges. For the standard extraction from assay buffer into an equal volume of hexane (independent of the presence of 1 mg of microsomal protein/mL), the recovery by HPLC was found to be 15% for *p*Tol-SO-Et, 40% for *p*Tol-SO_2-Et, and 47% for *p*BzlOH-S-Et. Yields of duplicate samples were usually within 5–10% of each other.

Chiralities are expressed as the percent of the total sulfoxide product which is the *S* enantiomer [$\% S = [S/(S + R)] \times 100$]. Quantitation of the percent (*S*)-*p*Tol-SO-Et in a given enzymatic reaction mixture was found to be highly reproducible, with values generally within 1–2% from one experiment to another. Although most of the chirality determinations were made by integration of the areas under the two partially fused peaks (Hewlett-Packard Model 3390A integrator), percent *S* values were unchanged when determined by relative peak height or by integration after four to five recyclings, which usually effected a near-base-line resolution of the two enantiomers (e.g., see the inset of Figure 2B).

7-Ethoxycoumarin O-Deethylase Assays. Purified P-450s were reconstituted with b_5 and P-450 reductase as described above at 0.03 nmol of P-450/mL of assay buffer containing 0.5 mM 7-ethoxycoumarin (Aldrich) (added from a freshly prepared 50 mM solution in MeOH) and 25 μg/mL sodium deoxycholate. Reactions (0.4-mL samples) were initiated by addition of NADPH to 0.3 mM and incubated 3–10 min at 37 °C. Samples were quenched with 2 N HCl (50 μL) and extracted with $CHCl_3$ (0.9 mL). The $CHCl_3$ layer (0.6 mL) was transferred to a clean tube and then back-extracted with 30 mM $Na_2B_4O_7$ (pH 9.2) (2 mL). The fluorescent product (7-hydroxycoumarin) was determined with a Perkin-Elmer fluorescence spectrophotometer (Model MPF-4) by excitation at 370 nm (slit width = 10 nm) and emission at 455 nm (slit width = 12 nm) (Prough et al., 1978) in comparison with standard 7-hydroxycoumarin (Aldrich).

Results

Chirality of Microsomal Sulfoxidations. Liver microsomal FAD-containing monooxygenase catalyzes a chiral sulfoxidation of the model sulfide *p*Tol-S-Et to yield the *R*-(+)

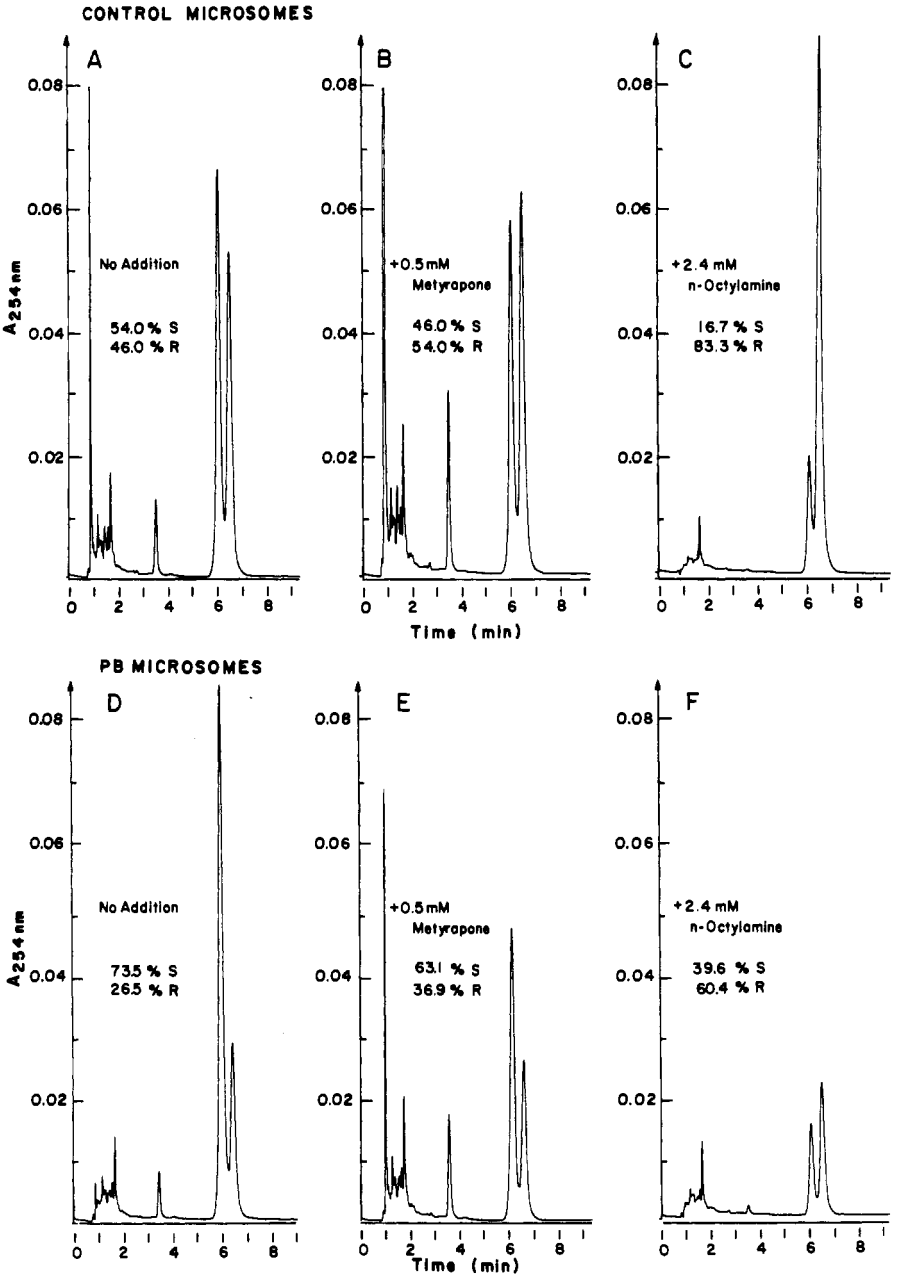
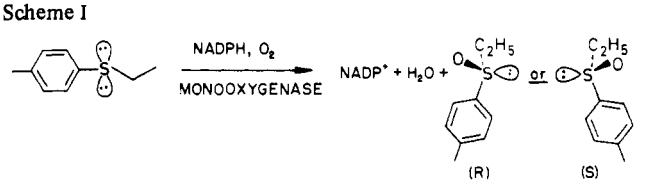


FIGURE 1: Processing of *p*Tol-S-Et by liver microsomes from control (A–C) and from PB-induced (D–F) rats. Microsomes (1.0 nmol of P-450/mL of assay buffer) were incubated with *p*Tol-S-Et under standard assay conditions for 20 min at 37 °C in the presence or absence of P-450 inhibitors, as indicated. Products were extracted with ethyl acetate (which gives a more efficient extraction of *p*Tol-SO-Et relative to *p*BzlOH-S-Et than does hexane) and analyzed on a chiral Pirkle column (HPLC system A). Quantitation of the relative formation of *p*BzlOH-S-Et (eluting at t = 3.7 min), (*S*)-*p*Tol-SO-Et (t = 6.2 min), and (*R*)-*p*Tol-SO-Et (t = 6.7 min) is shown in Table I. Similar samples analyzed by A_{220} with HPLC system B (as in Figure 2A) indicated that essentially no *p*Tol-SO_2-Et was formed in these relatively short microsomal incubations.

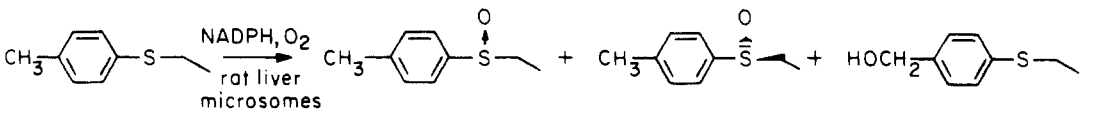
Scheme I



enantiomer of *p*Tol-SO-Et as the predominant product (Light et al., 1982). This reaction is also catalyzed by rat liver microsomes (which contain the FAD monooxygenase) excepting that both (*R*)-(+)- and (*S*)-(−)-*p*Tol-SO-Et are formed (Scheme I). In microsomes isolated from control (uninduced) animals, the sulfoxide formed is approximately 54% S, and in microsomes from PB-induced animals, it is 74% *S* (Figure 1A,D, Table I; Light et al., 1982). That this increase in chirality (and in overall sulfoxidation activity[2]) is likely due to the induction of cytochrome P-450 isozymes which catalyze formation of the *S* enantiomer preferentially was suggested by the experiments shown in Figure 1. Microsomes were incubated with *p*Tol-S-Et in the presence of either metyrapone (0.5 mM) or *n*-octylamine (2.4 mM) and the products analyzed on a chiral Pirkle column (Pirkle et al., 1981; Light et al., 1982). In the presence of metyrapone, reported to be a specific inhibitor of PB-induced P-450 isozymes (Luu-The et al., 1980), (*S*)-*p*Tol-SO-Et formation was inhibited to a greater extent than was (*R*)-*p*Tol-SO-Et formation such that the percent *S* was reduced from 54% to 46% in control microsomes and from 73% to 63% in PB-treated microsomes (Figure 1B,E

[2] Control microsomes catalyzed formation of 8.9 nmol of sulfoxide (mg of protein)$^{-1}$ min^{-1} vs. 17.9 nmol (mg of PB-induced microsomes)$^{-1}$ min^{-1} under standard assay conditions.

Scheme II



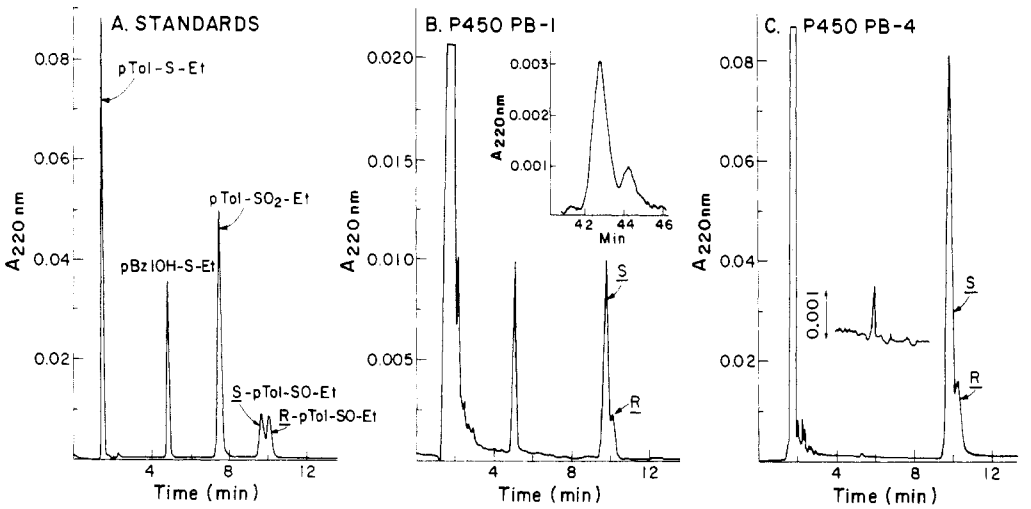


FIGURE 2: Processing of *p*Tol-S-Et by purified P-450 PB-1 and P-450 PB-4. Purified P-450 isozymes were reconstituted and incubated with *p*Tol-S-Et under standard assay conditions for 10 min (P-450 PB-1, panel B) or 60 min (P-450 PB-4, panel C) at 37 °C. Products were hexane extracted and analyzed in HPLC system B, permitting resolution of the four products shown in panel A (~4–6 nmol of each) with minimal loss of resolution of the chiral sulfoxides (e.g., compared to the resolution obtained in HPLC system A, Figure 1). Quantitation of the turnover number and percent (*S*)-sulfoxide formed by each P-450 is shown in Table II. Inset of panel B: Three recycles of the sulfoxide peaks improved the separation of *S* and *R* enantiomers (t = 42.6 and 44.7 min, respectively) without changing the percent *S* values significantly (82.0% *S* at the first elution and 79.5% *S* at the fourth). Inset of panel C: 10-fold scale expansion facilitating quantitation of the small amount of *p*BzlOH-S-Et (t = 5.2 min) formed during the prolonged incubation with P-450 PB-4.

and Table I). Metyrapone (0.5 mM) had no effect on the activity of the purified FAD-containing monooxygenase. When microsomes were incubated in the presence of *n*-octylamine, a hydrophobic ligand which inhibits reactions catalyzed by many P-450 isozymes and also stimulates FAD-containing monooxygenase activity (Ziegler et al., 1973), inhibition of (*S*)-*p*Tol-SO-Et formation was accompanied by an enhancement of (*R*)-*p*Tol-SO-Et production (Figure 1C,F and Table I). This is most readily apparent with control microsomes, where the FAD-containing monooxygenase to P-450 ratio is expected to be ~2.6 times that of the PB-treated microsomes [i.e., the extent of P-450 induction by PB, the FAD-containing monooxygenase being uninduced (Burke & Mayer, 1974)]. This higher FAD-containing monooxygenase to P-450 ratio in control microsomes is also reflected in the greater resistance of the total *p*Tol-SO-Et formation activity of control microsomes to the P-450 inhibitors employed (Table I).

In addition to sulfoxidation, both control and PB microsomes catalyzed hydroxylation at the *p*-methyl carbon of *p*Tol-S-Et to yield *p*BzlOH-S-Et (Scheme II) (Light et al., 1982). In contrast to FAD-containing monooxygenase, which does not oxygenate at carbon (Ziegler, 1980), many P-450-catalyzed carbon hydroxylations have been described [reviewed by Wislocki et al. (1980)], suggesting that *p*BzlOH-S-Et formation might be P-450 mediated. This is supported by the inhibition of *p*BzlOH-S-Et formation by *n*-octylamine (Figure 1C,F and Table I) and by reactions catalyzed by the purified P-450 isozymes (see below). The metyrapone stimulation of *p*BzlOH-S-Et formation (Figure 1B,E and Table I) is discussed below.

Reactions Catalyzed by Purified P-450s. We have recently purified several P-450 isozymes from PB-induced rats, including P-450 PB-1 (D. J. Waxman and C. Walsh) and P-450 PB-4, equivalent to the major PB-induced P-450 isozyme described by several other groups (see Materials). In order to ascertain whether the chiral sulfoxidation activity catalyzed by microsomes is retained by individual P-450s, we reconstituted purified isozymes in a dilauroylphosphatidylcholine system with purified rat liver cytochrome b_5 and cytochrome P-450 reductase. Samples were incubated with *p*Tol-S-Et and the enzymatic products analyzed by enantioselective chromatography using HPLC system B (Figure 2). P-450 PB-1 and P-450 PB-4 were each shown to catalyze chiral sulfoxidations with the *S* enantiomer as the major product (Figure 2B,C and Table II). In addition, P-450 PB-1 catalyzed carbon hydroxylation (*p*BzlOH-S-Et formation) at approximately one-sixth the rate of sulfur oxidation (*p*Tol-SO-Et formation)

Table I: Effects of P-450 Inhibitors on Microsomal Monooxygenations[a]

	% (*S*)-*p*Tol-SO-Et	product formation[b] *p*Tol-SO-Et	*p*BzlOH-S-Et
control microsomes			
standard assay	54.0	1.00	1.00
+metyrapone (0.5 mM)	46.0	1.00	2.44
+*n*-octylamine (2.4 mM)	16.7	0.90	0.02
PB microsomes			
standard assay	73.5	1.00	1.00
+metyrapone (0.5 mM)	63.1	0.63	2.31
+*n*-octylamine (2.4 mM)	39.6	0.31	0.18

[a] Microsomes were assayed for oxygenation of *p*Tol-S-Et in the presence of metyrapone or *n*-octylamine as shown in Figure 1 with quantitation based on product integration. [b] Relative activities are shown, with the standard assay samples defined as 1.00. Absolute activities for the microsomal incubations under standard assay conditions are expressed as turnover numbers and are included in Table II.

Table II: Chirality and Turnover Numbers for Oxygenation of *p*Tol-S-Et[a]

	chirality [% (*S*)-*p*Tol-SO-Et]	turnover no. (min^{-1} P-450^{-1}) *p*Tol-SO-Et	*p*BzlOH-S-Et	S:C
P-450 PB-1	78.5	41	6.8	6.0
P-450 PB-4	84.0	31	~0.09	~375
PB microsomes	75.3	7.5	0.63	11.9
control microsomes	54.4	9.7	1.2	8.1

[a] Shown are typical results obtained with purified P-450 and microsomes assayed under standard conditions. Values of percent (*S*)-sulfoxide were highly reproducible (±1-2%). The purified P-450s occasionally exhibited ~30% lower activity, with the chirality and the ratio of rates of sulfur to carbon oxygenation (S:C, last column) essentially unchanged.

Table III: Steady-State Kinetic Parameters for the Oxygenation of *p*Tol-S-Et[a]

	K_m[b] (μM) *p*Tol-SO-Et formation	*p*BzlOH-S-Et formation	V_{max} (min^{-1} P-450^{-1}) ± 10% *p*Tol-SO-Et	*p*BzlOH-S-Et
PB microsomes	35 ± 8	105 ± 15	8.0	0.75
control microsomes	40 ± 10	105 ± 15	10.5	1.4
P-450 PB-1	200 ± 30	350 ± 50	57	11.6
P-450 PB-4	30 ± 5	ND[c]	35	ND
FAD-containing monooxygenase	13 ± 2		48	0

[a] Determinations were made under standard assay conditions with product analysis by using HPLC systems B and C. Values listed reflect the range of several experimental determinations. V_{max} values are expressed as maximal turnover numbers. Values for the FAD-containing monooxygenase are taken from Light et al. (1982). [b] K_m values shown are for *p*Tol-S-Et as substrate leading to formation of the two products indicated. [c] ND designates not determined.

under standard assay conditions (Table II). P-450 PB-4 was a much less efficient catalyst for this carbon hydroxylation, the ratio of sulfur to carbon oxidation being approximately 60-fold higher than that with P-450 PB-1 (S:C = 6 for PB-1 vs. ~375 for PB-4).

Unlike P-450 PB-4, which is metyrapone sensitive (>90% loss of 7-ethoxycoumarin *O*-deethylase activity at 0.5 mM metyrapone) and which gives rise to a distinct difference spectrum [equivalent to the "P_{446}" described by Hildebrandt et al. (1969)] when metyrapone is bound to the reduced enzyme, P-450 PB-1 is insensitive to this heme iron ligand (D. J. Waxman and C. Walsh, unpublished results). It is thus likely that the metyrapone stimulation of *p*BzlOH-S-Et formation, seen both in control and in PB microsomes (Figure 1B,E) but not with purified P-450 PB-1 (not shown), reflects an increased flux through P-450 PB-1 (or through PB-1-like isozymes) in microsomal populations as a consequence of inhibition of the metyrapone-sensitive P-450s. This increase in flux can be understood in light of the observation that P-450-catalyzed microsomal activities are often cytochrome P-450 reductase limited (Miwa et al., 1978), a fact which might also explain the higher turnover numbers determined for the purified P-450s as compared to those for microsomes (Table II). K_m and V_{max} values determined with microsomes and with purified isozymes for conversion of *p*Tol-S-Et to *p*Tol-SO-Et and to *p*BzlOH-S-Et (Table III) indicate that the higher turnovers seen with the purified P-450s reflect higher V_{max} values, rather than lower K_m values.

Table IV: Effects of Cytochrome b_5 on Substrate Oxygenation[a]

	P-450 PB-1	P-450 PB-4
(A) cytochrome b_5, stimulation of product formation[b]		
(1) *p*Tol-SO-Et	6.6	1.5
(2) *p*BzlOH-S-Et	8.5	not determined
(3) 7-hydroxycoumarin	6.0	1.8
(B) chirality [% (*S*)-*p*Tol-SO-Et]		
$+b_5$	78.5	84.0
$-b_5$	77.2	83.5

[a] Purified P-450s were incubated with *p*Tol-S-Et (entries A1, A2, and B) or 7-ethoxycoumarin (entry A3) under standard assay conditions after reconstitution with lipid and P-450 reductase in the presence or absence of cytochrome b_5. Product analysis and quantitation were as described under Methods. [b] Values expressed as ratio of initial velocities in the presence of cytochrome b_5 to those in the absence of b_5.

Racemic *p*Tol-SO-Et appeared to be a relatively poor substrate for the purified P-450 isozymes, which catalyzed its conversion to the sulfone at <5% the rate of *p*Tol-SO-Et formation under standard assay conditions. As expected, only small amounts of *p*Tol-SO_2-Et were formed upon prolonged incubation of microsomes with *p*Tol-S-Et (data not shown). 4-Thiocresol, a product expected from methylene hydroxylation of *p*Tol-S-Et followed by S-dealkylation, was not detected in any of the incubation mixtures. Other possible products, e.g., those derived from aromatic ring hydroxylation or from ω-hydroxylation of the ethyl group, were not determined in this study.

Influence of Cytochrome b_5 on Processing of pTol-S-Et. Several recent studies suggest that cytochrome b_5 may mediate electron transfer between cytochrome P-450 reductase and P-450 in microsomes or in a reconstituted system containing purified components [e.g., see Hildebrandt & Estabrook (1971), Imai (1981), and Bonfils et al. (1981)]. In this study, P-450-catalyzed reactions were studied in a reconstituted system containing a small molar excess of both cytochrome b_5 and cytochrome P-450 reductase over P-450 (see Methods). Under these conditions, a 6-fold enhancement of the rate of sulfoxidation catalyzed by P-450 PB-1 and a 1.5-fold stimulation of the activity of P-450 PB-4 were seen relative to the same reactions performed in the absence of cytochrome b_5 (Table IV). Despite these significant b_5-induced rate accelerations, the chirality of the sulfoxide product was unchanged, suggesting that the relative affinities of the P-450s for the presumably distinct modes of sulfide binding leading to (*S*)- vs. (*R*)-*p*Tol-SO-Et are unaffected by cytochrome b_5. Similar b_5-induced rate enhancements were seen when 7-ethoxycoumarin *O*-deethylase activity was determined (Table IV), in which case the increase in substrate turnover reflects an increase in V_{max} with no change in K_m (unpublished results).

Influence of Scavengers for Reactive Oxygen Species. The observation that the sulfoxidation reactions catalyzed by purified P-450 isozymes were less than 100% chiral (i.e., the sulfoxide produced was only 78–85% *S*) might suggest the presence of a nonenzymatic, achiral component which contributes to *p*Tol-SO-Et formation. Several experiments indicated that this was unlikely. In control incubations of cytochrome P-450 reductase and cytochrome b_5 in the absence of P-450, sulfoxide levels were reduced to less than 2% of the enzymatic levels. Deletion of NADPH from the standard reaction mixtures gave a similar result. These observations reduce the possibility of significant sulfoxidation formation during sample workup, consisting of a single extraction into

Table V: Influence of Scavengers for Activated Oxygen on Sulfur Oxidations[a]

	P-450 PB-1		P-450 PB-4		PB microsomes		control microsomes	
	velocity	chirality	velocity	chirality	velocity	chirality	velocity	chirality
standard assay	1.00	79.5	1.00	84.5	1.00	74.8	1.00	59.4
+catalase (5 μg/mL)	0.83	82.0	0.79	85.9	0.85	74.6	0.83	60.4
+superoxide dismutase (5 μg/mL)	0.69	81.8	0.79	86.0	0.88	75.9	0.86	58.5
+mannitol (0.1 M)	0.78	81.6	0.94	86.2	not determined		not determined	

[a] Purified P-450s and microsomes were incubated with *p*Tol-S-Et under standard assay conditions in the absence ("standard assay") or presence of the scavengers indicated. Velocities are expressed as rates of *p*Tol-SO-Et formation relative to the standard assay incubations and chiralities as percent (*S*)-*p*Tol-SO-Et. Analysis and quantitation were performed by using HPLC system B.

hexane followed by direct injection onto an HPLC column (a procedure designed to keep adventitious sulfoxidation to a minimum). It could be possible, however, that enzyme-generated reactive oxygen species might contribute to achiral sulfux oxidation occurring in solution. Such reactive species are known to account for the P-450-mediated conversion of ethanol to acetaldehyde, a process which is inhibited by activated oxygen scavengers such as superoxide dismutase and mannitol (Ingelman-Sundberg & Johansson, 1981). Inclusion of either catalase, superoxide dismutase, or mannitol (traps for H_2O_2, $O_2^{-}\cdot$, and OH·, respectively) in either purified P-450 or microsomal incubations did not influence the chirality of the sulfoxide product (Table V), indicating that such reactive species could not account for much achiral sulfoxide formation in solution. Inhibition of overall sulfoxidation (10–30%, Table V) was somewhat greater than the inhibition of *p*BzlOH-S-Et formation in the same experimental samples (0–10%, data not shown). Moderate inhibition (10–20%, data not shown) of P-450-catalyzed O-deethylation of 7-ethoxycoumarin by these reactive oxygen scavengers was also observed.

Modulation of Microsomal Activities by Changing Sulfide Concentrations. Conversion of *p*Tol-S-Et to *p*Tol-SO-Et in PB microsomes is mediated by at least five distinct enzymes: the FAD-containing monooxygenase (Light et al., 1982), P-450 PB-1, P-450 PB-4, P-450 PB-5,[3] and a fourth, partially purified isozyme, P-450 PB-2/PB-3 (not shown). Each of the rat liver PB-450s tested thus far catalyzes formation of *p*Tol-SO-Et which is from ~75% *S* to 85% *S*, depending on the isozyme employed. Thus, the steady-state kinetic parameters determined in PB microsomes (and similarly in control microsomes) reflect a composite picture of these and possibly other activities. As such, it might be possible to modulate the chirality of microsomal sulfoxidations not only by addition of inhibitors and activators (Figure 1) but also by changing the substrate concentration. Figure 3 shows that, in the case of control microsomes, decreasing the sulfide concentration from 500 to 10 μM decreases the percent (*S*)-sulfoxide produced from 54 to 28%. Thus, at low substrate concentrations, the chirality of the sulfoxide product (72% *R*) is much closer to that formed by purified FAD-containing monooxygenase (95% *R*; Light et al., 1982). This finding is readily understood in terms of higher K_m values observed for P-450 as compared to those for the flavoprotein-mediated sulfoxidations (Table III). That such a decrease in percent (*S*)-sulfoxide was not observed with PB microsomes reflects the probability that, even at 10 μM *p*Tol-S-Et, the bulk of sulfoxidation is still P-450 mediated as a consequence of the 2.6-fold induction of total P-450 relative to flavoprotein. The small but reproducible decrease in chirality of *p*Tol-SO-Et formed by purified P-450 PB-1 at lower substrate concentrations (Figure 3) might reflect small

[3] A PB-induced isozyme which is very similar to PB-4 both structurally and catalytically, yet can be distinguished readily by its particular chromatographic and enzymatic properties (D. J. Waxman and C. Walsh, unpublished experiments).

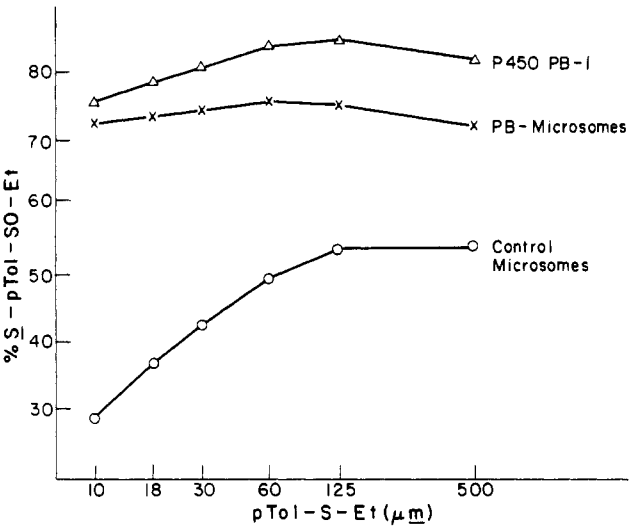


FIGURE 3: Influence of *p*Tol-S-Et concentration on chirality of sulfoxidation. Microsomes and reconstituted P-450 PB-1 were incubated under standard assay conditions with various concentrations of *p*Tol-S-Et for 10 min at 37 °C following which samples were hexane extracted and analyzed for percent (*S*)-*p*Tol-SO-Et by using HPLC system B.

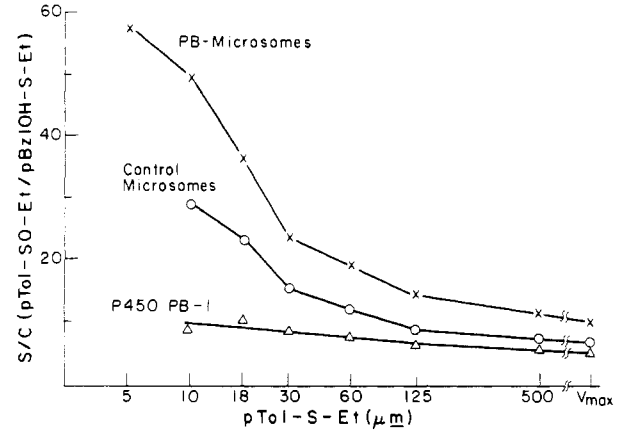


FIGURE 4: Influence of *p*Tol-S-Et concentration on the ratio of rates of oxygenation at sulfur to oxygenation at carbon (S:C). Samples were incubated and analyzed as described in Figure 3.

differences in the substrate binding affinities leading to the two enantiomeric sulfoxides.

Significant *p*BzlOH-S-Et formation was seen only with purified P-450 PB-1, suggesting that this isozyme is responsible for the majority of the benzylic hydroxylation detected in microsomes. Upon decreasing the substrate concentration, we observed a significant increase in the ratio of rates of *p*Tol-SO-Et formation to *p*BzlOH-S-Et production (S:C ratio) (Figure 4). Thus, in PB microsomes, S:C = 10.7 at V_{max} vs. 57 at 5 μM *p*Tol-S-Et, and in control microsomes, S:C = 7.5 at V_{max} vs. 29 at 10 μM *p*Tol-S-Et. These findings probably reflect the lower K_m values determined for *p*Tol-SO-Et for-

mation relative to *p*BzlOH-S-Et production (Table III). Similarly, the smaller increase (~1.8-fold) in S:C seen with purified P-450 PB-1 is consistent with the kinetic parameters determined for *p*Tol-S-Et with respect to the individual oxidation products. These findings underscore the importance of substrate concentration as a parameter which influences the regio- and enantioselectivity of monooxygenase reactions catalyzed in vivo.

Discussion

Cytochrome P-450 catalyzed sulfoxidations have now been studied both with rat liver microsomes and with purified P-450s reconstituted with cytochrome P-450 reductase and cytochrome b_5 by using *p*Tol-S-Et as a model substrate. HPLC analysis using a chiral Pirkle column [Pirkle et al., 1981; also see Light et al. (1982)] permitted simple, rapid, and highly sensitive resolution and quantitation of the two sulfoxide enantiomers formed. Both in the microsomal system and with the reconstituted purified enzymes, the heme proteins were shown to catalyze chiral sulfoxidations with the *S*-(−) enantiomer of *p*Tol-SO-Et as the predominant product (75–85% of the total sulfoxide formed). These findings can be contrasted with those described in Light et al. (1982), where it is shown that the other major sulfoxidizing enzyme of mammalian liver microsomes, the FAD-containing monooxygenase, catalyzes conversion of *p*Tol-S-Et (and of other sulfides) to the (*R*)-(−)-sulfoxide. Differences in the K_m values and inhibitor sensitivities of the flavoprotein vs. heme protein monooxygenases allowed us to modulate the net chirality of microsome-mediated sulfoxidations (Figures 1 and 3), indicating that substrate concentration as well as the presence of inhibitors and activators probably plays an important role in determining the relative contribution of each of these two enzyme systems (and thus the net chirality of sulfoxidation) in vivo.

Inclusion of scavengers for activated oxygen species such as catalase, superoxide dismutase, or mannitol, did not alter (i.e., improve) the chiral purity in the P-450-mediated sulfoxidations (Table V). Thus, the possibility that enzyme-generated reactive oxygen species such as those implicated in the P-450-supported oxidation of ethanol to acetaldehyde (Ingelman-Sundberg & Johansson, 1981) might mediate achiral sulfoxidation and thus lower the net chirality observed significantly is unlikely. This is also supported by the lack of an increase in net chirality upon inclusion of cytochrome b_5 in the reconstituted system, despite the 1.5–6-fold increase in the rates of enzyme-mediated sulfur oxidation (Table IV). Cytochrome b_5 stimulation of P-450-catalyzed reactions is now well documented and, in most cases, is accompanied by decreased formation of H_2O_2 and other reactive oxygen species, resulting in an improved coupling of NADPH consumption to substrate oxygenation [e.g., see Imai (1981) and Imai & Sato (1977)]. Thus, the <100% (*S*)-sulfoxide formation does not reflect the presence of a significant achiral (i.e., nonenzymatic) component but rather a moderate stereoselectivity between enzymatic oxidation products, with *S*:*R* = ~3–6, depending on the P-450 isozyme employed. It should be emphasized, however, that even in the case of P-450 isozyme PB-4, where *S*:*R* ~ 6, the mode of sulfide binding leading to (*S*)-*p*Tol-SO-Et would be favored over that leading to the *R* enantiomer by only ca. 0.9 kcal/mol. Lack of absolute stereo- and/or regiospecificity is typical of liver P-450 isozymes as noted previously in polycyclic hydrocarbon and in warfarin metabolism (Brunström & Ingelman-Sundberg, 1980; Fasco et al., 1978). The possibility that the P-450 isozymes studied yield 100% chiral sulfoxides when situated in the more native lipid environment of the microsomal membrane cannot, however, be excluded at this point.

In addition to sulfur oxidation, both control and PB-induced microsomes catalyzed hydroxylation of *p*Tol-S-Et at the *p*-methyl group to yield the benzyl alcohol *p*BzlOH-S-Et as a major product. Differences in K_m values determined for *p*Tol-S-Et leading to formation of *p*Tol-SO-Et vs. *p*BzlOH-S-Et in microsomes enabled us to modulate the distribution between products derived from carbon vs. sulfur oxidation by changing the substrate concentration (Figure 4). That purified P-450 PB-1, but not P-450 PB-4, catalyzed this carbon hydroxylation efficiently indicates that these two P-450s are distinct isozymes. In addition, these isozymes are readily distinguished by their molecular weights, chromatographic mobilities, metyrapone sensitivities, and peptide maps (D. J. Waxman and C. Walsh, unpublished results). That P-450 PB-4 catalyzes *p*BzlOH-S-Et formation at ~1.3%, the rate of P-450 PB-1 (Table II), suggests an upper limit for possible contamination of isozyme PB-4 by PB-1. Similarly, immunochemical analysis indicates that the contamination of PB-1 by PB-4 is <3% (D. J. Waxman and C. Walsh, unpublished results).

In contrast to the participation of several microsomal monooxygenases in *p*Tol-SO-Et formation (S-oxygenation), we have detected significant benzylic hydroxylation with only one of our purified P-450 PB isozymes, P-450 PB-1. This suggests that P-450 PB-1 might be the predominant isozyme mediating *p*BzlOH-S-Et formation in PB-induced and possibly also in control microsomes. If so, one might explain the lower K_m values determined for *p*BzlOH-S-Et formation with the microsomes (K_m ~ 100 μM) as compared to when P-450 PB-1 is reconstituted in the nonmembranous dilauroylphosphatidylcholine system (K_m ~ 350 μM) as reflecting a more favorable binding of substrate when the heme protein is incorporated into the microsomal lipid bilayer. The kinetics obtained with purified rabbit liver cytochromes P-450 are markedly dependent on the nature of the lipid system used in reconstitution of monooxygenase activity (Ingelman-Sundberg & Johansson, 1980).

In contrast to *n*-octylamine, which inhibits both sulfoxidation and benzylic hydroxylation, metyrapone stimulates benzylic hydroxylation while inhibiting sulfoxidation catalyzed by either control or PB-induced microsomes (Figure 1 and Table I). As metyrapone does not stimulate benzylic hydroxylation catalyzed by purified P-450 PB-1, this microsomal stimulation probably reflects an overall increase in electron flow from cytochrome P-450 reductase to P-450 PB-1 as a consequence of the inhibition of electron transfer to the metyrapone-sensitive P-450 isozymes. Such an increase in monooxygenase flux through P-450 PB-1 is consistent with the observation that P-450-catalyzed microsomal reactions are often P-450 reductase limited (Miwa et al., 1978). A similar explanation might also account for the 2–3-fold stimulation of acetanilide hydroxylation effected by metyrapone treatment of rat liver microsomes (Leibman, 1969).

The chirality of P-450-mediated sulfoxidation reactions has been the subject of previous investigations. In a study utilizing 2-substituted thianes as substrates, it was concluded that the cytochromes P-450 employed (both purified and microsomal) did not oxidize sulfur stereospecifically, as indicated by the formation of equimolar mixtures of *cis*- and *trans*-sulfoxides (Takahashi et al., 1978). However, the *enantiomeric* composition of each of the sulfoxide diastereomers was not reported. Previous studies of microsome-catalyzed sulfoxidations using aryl alkyl sulfides have been interpreted in terms of either

a P-450-mediated nonstereospecific (Kexel & Schmidt, 1972) or a stereospecific (Takata et al., 1980) reaction. However, in neither case was the participation of the FAD-containing monooxygenase considered. If this is borne in mind, the "achiral sulfoxidation" of 4-thioanisidine seen with uninduced rat liver microsomes (Kexel & Schmidt, 1972) is, in fact, in good agreement with our finding of a *net* 54% (*S*)-*p*Tol-SO-Et formation catalyzed by a similar fraction (Figure 1A). In addition, the strong substituent dependence of product chirality seen by Takata et al. (1980) [e.g., 73% (*S*)-sulfoxide, derived from microsomal incubations with *p*Tol-S-*t*-butyl as substrate, 40% (*S*)-sulfoxide from *p*TolCH$_2$-S-*t*-butyl, and 23% (*S*)-sulfoxide from Bzl-S-*t*-butyl] is unlikely to reflect the influence of steric bulk on the enantioselectivity of a given P-450, as suggested by those authors, but rather changes in the relative participation of P-450 and the FAD-containing monooxygenase. It should be possible to distinguish between these two possibilities by examining the chiral outcome either of microsomal incubations with some of the aryl alkyl sulfides used in that study in the presence of inhibitors or activators (e.g., as in Figure 1) or, alternatively, from incubations of the sulfides with individual, purified P-450 isozymes.

Our observation that purified P-450 PB-1 can oxygenate *p*Tol-S-Et either at sulfur or at carbon indicates that substrate can bind with either end adjacent to the heme iron oxygenating agent in the enzyme active site. Whether P-450-mediated carbon hydroxylation proceeds by a distinct mechanism from sulfoxidation is not clear. Mechanistic studies with rabbit liver P-450 LM$_2$ suggest that carbon hydroxylations may involve radical mechanisms (Groves et al., 1980). However, stereochemical studies demonstrating retention of configuration at carbon (Caspi et al., 1981; D. Arigoni, unpublished experiments) would indicate a tight coupling of oxygen delivery to hydrogen abstraction, i.e., before a radical could rotate. Similarly, in the case of P-450-mediated sulfoxidation, it has been proposed that one-electron transfer processes are involved (at the rate-determining step) (Watanabe et al., 1981). Sulfoxidation would thus proceed via a sulfur radical cation Fe^{IV}–oxo pair which then effects oxygen transfer. In this view, the oxygen transfers to CH_3 carbon or to divalent sulfur are very similar.

These heme protein mediated radical processes are probably distinct from the oxygen transfer process carried out by either of the flavoprotein monooxygenases described in the preceding paper (Light et al., 1982) where a nucleophilic attack of substrate sulfur on a 4a-flavin hydroperoxide is the likely oxygen transfer step. A third monooxygenase type, the copper-dependent dopamine β-hydroxylase, also oxygenates sulfur-containing substrate analogues with exclusive (*S*)-sulfoxide stereochemistry (May & Phillips, 1980). The change in stereochemical outcome for heme protein vs. flavoprotein vs. copper protein is, however, unlikely to bear any relationship to the enzymatic oxygen transfer mechanism employed. In addition to sulfoxide formation, it is also known that each liver monooxygenase enzyme type (flavoprotein and heme protein) will oxygenate thiono sulfur atoms [e.g., –C(=S)–, –P(=S)–] in such substrates as thioacetamide, carbon disulfide, and parathion, where the sulfur atoms are much less nucleophilic (Poulsen et al., 1974; Kamataki et al., 1976). In those instances, the initial (*S*)-oxides are often much less stable that those studies here, and the mechanism of oxygen transfer is not yet clear.

Finally, although microsome-catalyzed NADPH oxidation in the presence of sulfoxides proceeding at ~20–30% the rate with sulfides has been reported (suggesting a relatively efficient conversion of sulfoxide to sulfone) (Fukushima et al., 1978), we see only very slow secondary oxygenation of the sulfoxides to the sulfones with either the flavoenzyme or the purified heme protein monooxygenases, a fact that has permitted the stereochemical determinations described in these two papers.

At this juncture, we suggest that the availability of Pirkle-type HPLC systems with chiral stationary phases provides a convenient and highly sensitive technique for probing related questions of mechanism, stereochemistry, and relative microsomal participation [see, e.g., Prough & Ziegler (1977)] of these and other oxygenases (including related isozymes) in oxygenations of a wide variety of substrates including drugs, pesticides, and industrial toxins. The results reported here suggest that the *net* stereochemical outcome in sulfur atom processing in microsomes and in vivo reflects not only the enantioselectivity of soluble sulfoxide reductases (Kexel & Schmidt, 1972; Anders et al., 1980) but also the competitive contributions of flavoprotein and heme protein monooxygenases in producing sulfoxides of opposite chirality.

References

Anders, M. W., Ratnayake, J. H., Hanna, P. E., & Fuchs, J. A. (1980) *Biochem. Biophys. Res. Commun. 97*, 846–851.

Bonfils, C., Balny, C., & Maurel, P. (1981) *J. Biol. Chem. 256*, 9457–9465.

Brunström, A., & Ingelman-Sundberg, M. (1980) *Biochem. Biophys. Res. Commun. 95*, 431–439.

Burke, M. D., & Mayer, R. T. (1974) *Drug Metab. Dispos. 2*, 583–588.

Caspi, E., Shapiro, S., & Piper, J. U. (1981) *Tetrahedron 20*, 3535–3543.

Fasco, M. J., Vatsis, K. P., Kaminsky, L. S., & Coon, M. J. (1978) *J. Biol. Chem. 253*, 7813–7820.

Fusushima, D., Kim, Y. H., Iyanagi, T., & Oae, S. (1978) *J. Biochem. (Tokyo) 83*, 1019–1027.

Groves, J. T., Krishnan, S., Avaria, G. E., & Nemo, T. E. (1980) in *Biomimetic Chemistry* (Dolphin, D., et al., Eds.) pp 227–289, American Chemical Society, Washington, DC.

Guengerich, F. P. (1977) *J. Biol. Chem. 252*, 3970–3979.

Hildebrandt, A. G., & Estabrook, R. W. (1971) *Arch. Biochem. Biophys. 143*, 66–79.

Hildebrandt, A. G., Leibman, K. C., & Estabrook, R. W. (1969) *Biochem. Biophys. Res. Commun. 37*, 477–485.

Imai, Y. (1981) *J. Biochem. (Tokyo) 89*, 351–362.

Imai, Y., & Sato, R. (1977) *Biochem. Biophys. Res. Commun. 75*, 420–426.

Ingelman-Sundberg, M., & Johansson, I. (1980) *Biochemistry 19*, 4004–4011.

Ingelman-Sundberg, M., & Johansson, I. (1981) *J. Biol. Chem. 256*, 6321–6326.

Kamataki, T., Belcher, D. H., & Neal, R. A. (1976) *Mol. Pharmacol. 12*, 921–932.

Kexel, H., & Schmidt, H. L. (1972) *Biochem. Pharmacol. 21*, 1009–1017.

Leibman, K. (1969) *Mol. Pharmacol. 5*, 1–9.

Light, D. R., Waxman, D. J., & Walsh, C. (1982) *Biochemistry* (preceding paper in this issue).

Loosemore, M., Light, D. R., & Walsh, C. (1980) *J. Biol. Chem. 255*, 9017–9020.

Luu-The, V., Cumps, J., & Dumont, P. (1980) *Biochem. Biophys. Res. Commun. 93*, 776–781.

May, S. W., & Phillips, R. S. (1980) *J. Am. Chem. Soc. 102*, 5981–5983.

Miwa, G. T., West, S. B., & Lu, A. Y. H. (1978) *J. Biol. Chem. 253*, 1921–1929.

Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.

Pirkle, W. H., Finn, J. M., Schreiner, J. L., & Hamper, B. C. (1981) *J. Am. Chem. Soc. 103*, 3964–3966.
Poulsen, L. L., Hyslop, R. M., & Ziegler, D. M. (1974) *Biochem. Pharmacol. 23*, 3431–3440.
Prough, R. A., & Ziegler, D. M. (1977) *Arch. Biochem. Biophys. 180*, 363–373.
Prough, R. A., Burke, M. D., & Mayer, R. T. (1978) *Methods Enzymol. 52*, 372–377.
Ryan, D. E., Thomas, P. E., Korzeniowski, D., & Levin, W. (1979) *J. Biol. Chem. 254*, 1365–1374.
Strittmatter, P., Fleming, P., Connors, M., & Corcoran, D. (1978) *Methods Enzymol. 52*, 97–101.
Takahashi, T., Kim, Y. H., Fukushima, D., Fujimori, K., & Oae, S. (1978) *Heterocycles 10*, 229–235.
Takata, T., Yamazaki, M., Fujimori, K., Kim, Y. H., Oae, S., & Iyanagi, T. (1980) *Chem. Lett.*, 1441–1444.
Van der Hoeven, J. A., & Coon, M. J. (1974) *J. Biol. Chem. 249*, 6302–6310.
Watanabe, Y., Numata, T., Iyanagi, T., & Oae, S. (1981) *Bull. Chem. Soc. Jpn. 54*, 1163–1170.
West, S. B., Huang, M.-T., Miwa, G. T., & Lu, A. Y. H. (1979) *Arch. Biochem. Biophys. 193*, 42–50.
Wislocki, P. G., Miwa, G. T., & Lu, A. Y. H. (1980) in *Enzymatic Basis of Detoxification* (Jakoby, W. B., Ed.) Vol. 1, pp 135–182, Academic Press, New York.
Ziegler, D. M. (1980) in *Enzymatic Basis of Detoxification* (Jakoby, W. B., Ed.) Vol. 1, pp 201–227, Academic Press, New York.
Ziegler, D. M., McKee, E. M., & Poulsen, L. L. (1973) *Drug Metab. Dispos. 1*, 314–321.

Oligothymidylate Analogues Having Stereoregular, Alternating Methylphosphonate/Phosphodiester Backbones as Primers for DNA Polymerase†

Paul S. Miller,* Nancy D. Annan,‡ Kevin B. McParland, and Stephen M. Pulford§

ABSTRACT: Oligothymidylate analogues having stereoregular, alternating methylphosphonate/phosphodiester backbones, d-T*p*(TpT*p*)$_4$T isomers I and II and d-T*p*(TpT*p*)$_3$T(pT)$_{1-5}$ isomers I and II, were prepared by methods analogous to the phosphotriester synthetic technique. The designations isomer I and isomer II refer to the configuration of the methylphosphonate linkage, which is the same throughout each isomer. Analogues with the type I methylphosphonate configuration form very stable duplexes with poly(dA) while those with the type II configuration form either 2T:1A triplexes or 1T:1A duplexes with poly(dA) of considerably lower stabilities. The oligothymidylate analogues were tested for their ability to initiate polymerizations catalyzed by *Escherichia coli* DNA polymerase I or calf thymus DNA polymerase α on a poly(dA) template. Neither d-T*p*(TpT*p*)$_4$T nor d-T*p*(TpT*p*)$_3$TpT served as initiators of polymerization while d-T*p*(TpT*p*)$_3$T(pT)$_{2-5}$ showed increasing priming ability as the length of the 3′-oligothymidylate tail increased. Analogues with type I methylphosphonate configuration were more effective initiators than the type II analogues at 37 °C. The apparent activation energies of polymerizations initiated by d-T*p*(TpT*p*)$_3$T-(pT)$_{4\text{ and }5}$ isomer I were greater than those for reactions initiated by isomer II or d-(Tp)$_{11}$T. The results suggest that DNA polymerase interacts with the charged phosphodiester groups of the primer molecule and may help stabilize primer/template interaction. At least two contiguous phosphodiester groups are required at the 3′ end of the analogue primers in order for polymerization to occur. Interactions between the polymerase and primer also appear to occur with phosphodiester groups located at sites remote from the 3′-OH polymerization site and may be influenced by the configuration of the methylphosphonate group.

Oligonucleotide analogues having nonionic alkyl phosphotriester or methylphosphonate backbones have served as models for studying the influence of backbone structure on the conformation and interactions of nucleic acids (Miller et al., 1971; Pless & Ts'o, 1977; Miller et al., 1979; Kan et al., 1980). As a result of their ability to form complexes with complementary nucleic acid sequences, these noncharged analogues have been used to probe and regulate cellular nucleic acid function both in the test tube and in living cells (Miller et al., 1974, 1977, 1981; Barrett et al., 1974; Jayaraman et al., 1981). In a recent publication we described the synthesis and physical properties of novel oligothymidylate analogues having methylphosphonate groups of fixed configuration arranged in an alternating manner with negatively charged phosphodiester groups throughout the backbone of the oligonucleotide (Miller et al., 1980a). The structure of the analogues may be written as d-T*p*(TpT*p*)$_4$T[1] where *p* denotes 3′–5′ methylphosphonate linkages with either *R* or *S* configuration throughout and p denotes 3′–5′ phosphodiester linkages.

These oligothymidylate analogues are able to form complexes with both poly(dA) and poly(rA). The stoichiometries and stabilities of the complexes are dependent upon the configuration of the methylphosphonate group. Although the

† From the Division of Biophysics, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205. *Received October 2, 1981.* This work was supported by a grant from the National Institutes of Health (1-RO3-GM 25795).

‡ Present address: Department of Chemistry, Goucher College, Baltimore, MD 21204.
§ Present address: Genex Laboratories, Rockville, MD 20852.

[1] Abbreviations: d-N*p*N, an oligonucleotide having a 3′–5′ internucleoside methylphosphonate linkage; d-N*p*N, an oligonucleotide having a 3′–5′ *p*-chlorophenyl phosphotriester linkage; MST, (mesitylenesulfonyl)tetrazole; TSNI, (*p*-toluenesulfonyl)-4-nitroimidazole; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; CD, circular dichroism. The symbols used to represent protected nucleosides and oligonucleotides follow the IUPAC–IUB Commission on Biochemical Nomenclature (1970) recommendations.

0006-2960/82/0421-2507$01.25/0