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Enantioselective *S*-Oxygenation of *para*-Methoxyphenyl-1,3-dithiolane by Various Tissue Preparations: Effect of Estradiol

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SUMMARY

Liver, kidney, and lung microsomes prepared from nonpretreated female Sprague-Dawley rats catalyze the NADPH- and oxygen-dependent *S*-oxygenation of *para*-methoxyphenyl-1,3-dithiolane. Studies on the biochemical mechanism of dithiolane *S*-oxygenation in liver, kidney, and lung microsomes suggest that this reaction is catalyzed in a diastereoselective and enantioselective fashion by the flavin-containing monooxygenase and, to a lesser extent, the cytochromes P-450. This conclusion is based on results examining the effects of selective cytochrome P-450 inhibitors and positive effectors, microsome heat-inactivation treatment, and alternate substrates for the flavin-containing monooxygenase. Liver and kidney microsomes prepared from ovariectomized female rats tended to have decreased *S*-oxygenase activity, compared with nonpretreated female rats, whereas ovariectomized rats pretreated with estradiol had markedly lower *S*-oxygenase activity. In contrast, lung microsomal *S*-oxygenase activity, which is low in pulmonary microsomes from nonpretreated female rats, increases 2–4-fold after ovariectomization and estradiol pretreatment. In female Sprague-Dawley rats, estradiol pretreatment is mainly responsible for the large decrease (or increase) in *S*-oxygenase activity observed in the tissues examined, although it is unlikely that estradiol alone controls flavin-containing monooxygenase *S*-oxygenase activity.

The flavin-containing monooxygenase and cytochromes P-450 catalyze the NADPH-dependent oxygenation of nitrogen- and sulfur-containing drugs, xenobiotics, and pesticides (1, 2). Flavin-containing monooxygenase-catalyzed *N*- and *S*-oxygenation can be distinguished from cytochrome P-450-catalyzed *N*- and *S*-oxidations by the use of various microsome treatments. For example, in contrast to cytochrome P-450, the flavin-containing monooxygenase from liver and kidney of rat is unusually sensitive to thermal inactivation (3–5). In addition, alternate competitive substrates and pH can be employed to specifically investigate *N*- or *S*-oxygenations due to the flavin-containing monooxygenase (2, 6). Another technique to distinguish *S*-oxygenases is to determine the stereoselectivity of the enzyme action (5, 6).

2-Aryl-1,3-dithiolanes offer a novel stereochemical probe to investigate monooxygenase-catalyzed *S*-oxygenation (7). Initial *S*-oxygenation of 2-aryl-1,3-dithiolanes produces two *S*-oxide diastereomers. Of two diastereomers that could form, attack of oxygen may occur on either the pro-*S* or pro-*R* sulfur atom in order to form *cis*- or *trans*-*S*-oxide diastereomers (8–11) (Fig. 1). Enzymatic *S*-oxygenation of 2-aryl-1,3-dithiolanes may occur with diasteretoptic selectivity and may also result in enantioenrichment of an *S*-oxide product by selection between the two enantiotopic sulfur atom lone pairs (8–10) (Fig. 2). An examination of the enantioselectivity and diastereoselectivity of enzymatic *S*-oxygenation may reveal which monooxygenase system is responsible for sulfoxidation. In addition, enantioselective *S*-oxygenation studies may also provide evidence for monooxygenase activity for cells or tissues not previously examined for monooxygenase activity because it has been determined, at least for the few cases thus far studied, that flavin-containing monooxygenase and cytochrome P-450 produce *S*-oxide enantiomers of opposite absolute stereochemistry (12).

The flavin-containing monooxygenase and cytochrome P-450 enzymes have been detected in most mammalian secretory tissue thus far examined (1, 2, 13). In many species the enzymes are present in very high concentrations in the liver, although kidney, lung, and other tissues also have considerable amounts of the enzyme activity. In contrast to cytochrome P-450, the flavin-containing monooxygenase cannot be induced by pretreating animals with xenobiotics (14), although changes in dimethylaniline *N*-oxidase activity induced by sex hormones have been observed in the liver and kidney of microsomes from mice (14, 15). Reports in the literature suggest that sex- or hormone-related differences in lung dimethylaniline *N*-oxidase activity of mice could not be observed (15).

In this report we describe studies on the stereoselective *S*-oxygenation of *p*-methoxyphenyl-1,3-dithiolane as a specific substrate to determine the diastereoselective and enantioselec-

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ABBREVIATIONS: MeOH, methanol; HPLC, high pressure liquid chromatography.

tive action of the microsomal flavin-containing and cytochrome P-450 monooxygenase *S*-oxygenase systems in female Sprague-Dawley rats. The effect of the steroid sex hormone 17β-estradiol on *S*-oxygenase activity was studied with microsome preparations from liver, kidney, and lung of female Sprague-Dawley rats.

Materials and Methods

Chemicals. *para*-Methoxybenzaldehyde, *para*-toluenesulfonic acid, 1,2-ethanedithiobis(trimethylsilane), sodium borohydride, and *n*-octylamine were obtained from Aldrich Chemical Co. Sodium *meta*-periodate and thiourea were purchased from Fisher Scientific. Aminobenzotriazole was a generous gift of Prof. Paul Ortiz de Montellano of this department. All of the compounds of the NADPH-generating system were obtained from Sigma Chemical Co. All other reagents and buffers were obtained commercially from the highest quality source.

***p*-Methoxyphenyl-1,3-dithiolane (1).** To a stirred solution of *p*-methoxybenzaldehyde (1 g, 8.1 mmol) in 5 ml of CH_2Cl_2 was added *para*-toluenesulfonic acid (20 mg) and 1,2-ethanedithiobis(trimethylsilane) (2.42 ml, 9.4 mmol). The resulting mixture was stirred at room temperature under nitrogen for 60 hr. The reaction mixture was evaporated and methanol (3 ml) and $NaBH_4$ (178 mg, 4.7 mmol) were added to reduce any trace of unreacted aldehyde. The mixture was extracted with CH_2Cl_2, dried, and chromatographed to give the dithiolane (1.3 g, 74%). ^{1}H NMR ($CDCl_3$): δ 7.50 (d, J = 8.25 Hz, 2 H, H_2 and H_5), 6.84 (d, J = 8.25 Hz, 2 H, H_3 and H_4), 3.8 (s, 3 H, CH_3O), 5.63 (s, 1 H, H_6), 3.50 (2 dd, J_{gem} = 15.1 Hz, J = 11.7 Hz, 2 H, H_7 and H_8), 3.34 (2 dd, J_{gem} = 15.1 Hz, J = 11.7 Hz, 2 H, H_9 and H_{10}); IR (neat): 3017, 2930, 1257, 1205 cm^{-1}; UV (MeOH) λ_{max} (ϵ): 234 (4697).

***p*-Methoxyphenyl-1,3-dithiolane *S*-oxide (2).** To a stirred solution of *p*-methoxyphenyl-1,3-dithiolane (1) (100 mg, 0.47 mmol) in CH_3OH (8 ml) were added 1.17 ml of aqueous sodium *meta*-periodate (0.47 mmol). The reaction mixture was stirred for 3 hr or until analysis by thin layer chromatography showed that no starting material remained. The crude reaction mixture was extracted with CH_2Cl_2, dried, and chromatographed on Whatman $PLCK_{18}F$ reverse phase preparative thin layer chromatography (eluent, 6:10:84, MeOH/ethyl acetate/hexanes) to give two diastereomers, a major *trans*-*S*-oxide diastereomer **2a** (R_F = 0.3, 44.5 mg, 45%), ^{1}H NMR ($CDCl_3$): δ 7.39 (d, J = 8.5 Hz, 2 H, aromatic C3- and C4-H), 5.36 (s, 1 H, C6-H), 3.80 (ddd, $J_{8,7}$ = 11.0 Hz, $J_{8,10}$ = 11.0 Hz, $J_{8,9}$ = 5.0 Hz, 1 H, H_8), 3.79 (s, 3 H, OCH_3), 3.58 (ddd, $J_{7,8}$ = 11.0 Hz, $J_{7,10}$ = 7.5 Hz, $J_{7,9}$ = 2.0 Hz, 1 H, H_7), 3.32 (ddd, $J_{9,10}$ = 14.5 Hz, $J_{9,7}$ = 2.0 Hz, $J_{9,8}$ = 5.0 Hz, 1 H, H_9), 2.89 (ddd, $J_{10,9}$ = 14.5 Hz, $J_{10,8}$ = 11.0 Hz, $J_{10,7}$ = 7.5 Hz, 1 H, H_{10}); mass spectrum (electron impact): m/z (relative intensity) 228 (M^+, 68), 165 (M^+-CH_4SO), 152 ($M^+-C_2H_8SO$, 100); UV (MeOH): λ_{max} (ϵ) 236 (9200), and the minor *cis*-diastereomer **2b** (R_F = 0.2, 34 mg, 30%), ^{1}H NMR ($CDCl_3$): δ 7.49 (d, J = 8.5 Hz, 2 H, aromatic C2- and C5-H), 6.91 (d, J = 8.5 Hz, 2 H, aromatic C3- and C4-H), 5.28 (s, 1 H, C6-H), 3.93 (ddd, $J_{8,7}$ = 10.5 Hz, $J_{8,10}$ = 10.5 Hz, $J_{8,9}$ = 5.5 Hz, 1 H, H_8), 3.80 (s, 3 H OCH_3), 3.51 (ddd, $J_{9,10}$ = 13.5 Hz, $J_{9,8}$ = 5.5 Hz, $J_{9,7}$ = 3.0 Hz, 1 H, H_9), 3.42 (ddd, $J_{7,8}$ = 10.5 Hz, $J_{7,10}$ = 7.0 Hz, $J_{7,9}$ = 3.0 Hz, 1 H, H_7), 3.12 (ddd, $J_{10,9}$ = 13.5 Hz, $J_{10,8}$ = 10.5 Hz, $J_{10,7}$ = 7.0 Hz,1,H_{10}); IR (CH_2Cl_2) both isomers: 3020, 2940, 2837, 1609, 1509, 1463, 1303, 1250, 1040 cm^{-1}; UV (MeOH) λ_{max} (ϵ): 236 (9200).

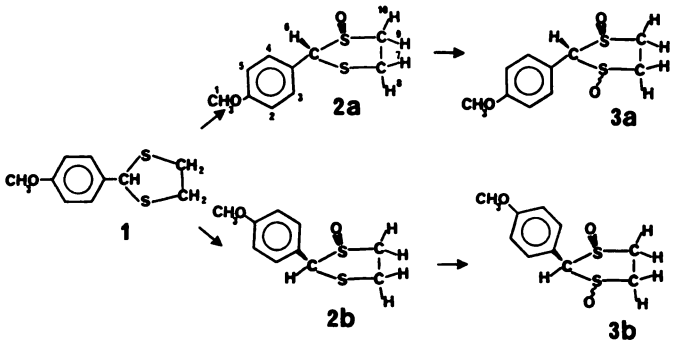

Fig. 1. Metabolic pathway for *S*-oxygenation of *para*-methoxyphenyl-1,3-dithiolane (1) to the (*trans*)-*S*-oxide diastereomer (2a) and the (*cis*)-*S*-oxide diastereomer (2b) in rat liver lung and kidney microsomes. Compound 3 is the *S*,*S*′-dioxide.

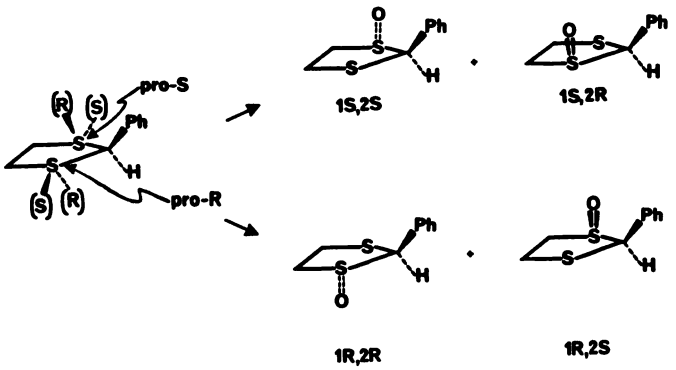

Fig. 2. Enantioselective *S*-oxygenation of *para*-methoxyphenyl-1,3-dithiolane.

Animals. Female white Sprague-Dawley rats, ages 2–3 months, obtained from Bantin and Kingman (Modesto, CA), were housed under standard conditions and allowed full access to food (Purina Lab Chow) and water. Rats were housed with a daily 14-hr light/10-hr dark cycle.

Estradiol administration. Estradiol was administered to ovariectomized rats from subdermally implanted sustained-release silastic tubes (16). Previously, the implants have demonstrated consistent estradiol release for up to 2 months (17). In addition, at the termination of the experiment, silastic tubes were examined and found to contain a significant amount of intact estradiol. The silastic tubes release estradiol at approximately 10–20 μg/day/animal (17). The tubes were implanted immediately after ovariectomy and left in place for approximately 6 weeks before the animals were killed. The average weight of the estradiol-pretreated rats was very similar to that of the control animals (258 and 252 g, respectively).

Tissue preparation. Animals were killed by decapitation, and livers, lungs, and kidneys were rapidly excised and chilled in cold 0.2 M sucrose. Microsome fractions were isolated by the method described earlier (13). To minimize inactivation of the flavin-containing monooxygenase, all steps were carried out as quickly as possible at 4°.

Metabolic incubation and product analyses. The incubation medium contained 50 mM potassium phosphate, pH 8.1, 0.5 mM $NADP^+$, 2.0 mM glucose-6-phosphate, 1 IU of glucose-6-phosphate dehydrogenase, and 0.8–2.0 mg of microsomal protein, in a total volume of 0.25 ml. After a brief temperature equilibration at 33°, the reaction was initiated by the addition of *p*-methoxyphenyl-1,3-dithiolane and the incubation was continued with shaking in the presence of air. At various time intervals, the reaction was stopped and analyzed for products by a procedure similar to the one described previously (6, 18, 19).

The assays were quenched by the addition of 4 volumes of cold dichloromethane. After thorough mixing, insoluble material was separated by a brief centrifugation. After filtration through a 4-μm nylon filter and evaporation, the extract was taken up in MeOH for separation and quantitation by HPLC (IBM model 9000 with a UV detector at 240 nm, fitted with a precolumn and 5-mm Altex Ultrasphere ODS reverse phase analytical column). The mobile phase consisted of acetonitrile/water (50:50, v/v). This system efficiently separates *p*-methoxyphenyl-1,3-dithiolane (1) and (*trans*)- and (*cis*)-*p*-methoxyphenyl-1,3-dithiolane *S*-oxide (2a and 2b), which have retention volumes of 11.8, 6.7, and 5.9 ml, respectively. The recovery of metabolites as judged by HPLC was greater than 90% and 98% of this recovered material was the sulfide or *S*-oxide. Quantitation was accomplished by compar-

ing the integrated area of the *S*-oxide or substrate HPLC peak, after taking into consideration the extinction coefficient values of each species at 240 nm.

Determination of *S*-oxide stereochemistry. The absolute stereochemistry of **2a** and **2b** was determined after correlating the *S*-oxide metabolites obtained with authentic synthetic *S*-oxides. The synthesis of optically active **2a** and **2b** followed the general procedure of Pitchen *et al.* (20). The optical purity of the product sulfoxides **2a** and **2b** was determined by NMR analysis (General Electric 500 MHz NMR, $CDCl_3$), using a chiral shift reagent tris[3-heptafluoropropyl(hydroxymethylene)-(−)-camphorato]europium(III). The synthetic (*trans*)-*S*-oxide **2a** was obtained with a maximum optical yield of 76% enantiomeric enrichment. The absorption spectra of sulfoxides **2a** and **2b** are characterized (JASCO J-500A spectropolarimeter) by an increase in absorption between 285 and 190 nm. A prominent positive CD band at 245 nm with a molar ellipticity [Θ] value of 16,278 was observed (21). This absorption band contains the information required for configuration correlation. A previous study of 1,3-dithiolane *S*-oxides established that a positive CD can be correlated with an (*R*)-sulfoxide configuration (22). Another previous study has also established the relationship between positive CD spectral absorption and the *R* absolute configurations of *S*-oxides (9), and the assignment of absolute configuration has been unequivocally determined by other methods. With synthetic materials of **2a** and **2b** to serve as standards, an efficient HPLC method to separate each (*cis*)- and (*trans*)-*S*-oxide enantiomer was developed. HPLC was performed with an IBM model 9000 fitted with a Daicel Chiralcel OD (25 cm × 0.46 cm i.d.) analytical column (Daicel Chemical Industries, New York). The mobile phase consisted of isopropanol/hexane (18:82, v/v). This system efficiently separates (retention volume) *p*-methoxyphenyl-1,3-dithiolane (**1**) (4–5 ml) from (1*R*,2*R*)-**2a** (12.5 ml), (1*S*,2*S*)-**2a** (18.0 ml), (1*R*,2*S*)-**2b** (22.9 ml), and (1*S*,2*R*)-**2b** (28.6 ml) (where the first assignment designates the *S*-oxide center) (Fig. 2). Quantitation was accomplished by comparing the integrated area of the *S*-oxide HPLC peak, after taking into consideration the extinction coefficient values of each species at 240 nm. Employing this method, dichloromethane extracts of metabolic reactions were evaporated to dryness (as described above), reconstituted in isopropanol/hexane (18:82, v/v), and analyzed by Chiralcel OD HPLC.

Other analytical methods. Heat-inactivated microsomes were prepared following the method previously described (1, 3). Microsomes were degassed with argon and placed in 60° water for 60 sec in the absence of NADPH. For an incubation, an appropriate aliquot of heat-inactivated microsomes was combined with 5 IU of catalase and the NADPH-generating system and the reaction was initiated as described above. The concentration of protein used in this study was determined by the method of Bradford (23).

Results

***S*-Oxygenase activity in rat liver, kidney, and lung microsomes.** The results shown in Tables 1–5 indicate that *para*-methoxyphenyl-1,3-dithiolane (**1**) is converted to its *S*-oxide (**2a** and **2b**) by rat kidney, liver, and lung microsomes supplemented with molecular oxygen and an NADPH-generating system. Preliminary studies demonstrated that *S*-oxide formation is linearly dependent on time (0–10 min) and protein concentration (0–2.5 mg of protein). In hepatic microsomes, *p*-methoxyphenyl-1,3-dithiolane *S*-oxide formation is stimulated by *n*-octylamine, which is a known activator of the hepatic flavin-containing monooxygenase (1, 3) and an inhibitor of cytochromes P-450 (24) (Table 1). In renal microsomes, the stimulatory effect of *n*-octylamine is less marked, due to the possibility that flavin-containing and cytochromes P-450 monooxygenases may contribute to *p*-methoxyphenyl-1-3-dithiolane *S*-oxygenation. *S*-Oxygenase activity is almost completely absent in microsomes that were heat treated under conditions that inactivate the flavin-containing monooxygenase but leave cytochrome P-450 active (1, 5) (Tables 1–4). In renal and hepatic microsomes, thiourea, a good alternate substrate for flavin-containing monooxygenase activity (1, 3), markedly decreased the rate of *S*-oxygenation and aminobenzotriazole, a potent mechanism-based inactivator of cytochromes P-450

TABLE 1

Effect of metabolism inhibitors on *S*-oxygenation of *para*-methoxyphenyl-1,3-dithiolane in rat hepatic microsomes

Incubations were performed as described in Materials and Methods for 10 min at 33°. Frozen microsomes were obtained from nonpretreated animals or ovariectomized animals treated with estradiol (E_2 + OVX microsomes).

Description	*S*-oxide formed (*cis*:*trans*)			
	Nonpretreated microsomes		E_2 + OVX microsomes	
	nmol/min/mg of protein			
Complete[a]	4.5 ± 0.4	(40:60)	1.1 ± 0.2	(32:68)
−NADPH GS[a]	ND[b]		ND	
+Heat inactivation	0.1 ± 0.1	(100:0)	0.1 ± 0.1	(100:0)
−*n*-Octylamine (4.0 mM)	1.3 ± 0.2	(55:45)	0.4 ± 0.1	(49:51)
+Thiourea (0.5 mM)	0.6 ± 0.1	(100:0)	0.5 ± 0.1	(100:0)
+Aminobenzotriazole (0.5 mM)	4.4 ± 0.3	(45:55)	1.2 ± 0.2	(33:67)

[a] The complete system contained 50 mM phosphate buffer, pH 8.1, the NADPH-generating system (GS), 300 μM substate, 4 mM *n*-octylamine, and 0.5 mg of microsomal protein. Data represent the average of three determinations ± standard deviation.
[b] ND, not detectable.

TABLE 2

Effect of metabolism inhibitors on *para*-methoxyphenyl-1,3-dithiolane *S*-oxygenation in rat kidney microsomes

Incubations were performed as described in Table 1.

Description	*S*-oxide formed (*cis*:*trans*)			
	Nonpretreated microsomes		E_2 + OVX microsomes	
	nmol/min/mg of protein			
Complete[a]	4.4 ± 0.6	(96:4)	0.5 ± 0.1	(0:100)
−NADPH GS	0.1 ± 0.1	ND[b]	0.1 ± 0.1	ND
+Heat inactivation	0.1 ± 0.2	(98:2)	0.1 ± 0.1	(2:98)
−*n*-Octylamine (4.0 mM)	3.8 ± 0.4	(68:32)	0.3 ± 0.2	(2:98)
+Thiourea (0.5 mM)	2.0 ± 0.3	(94:6)	0.3 ± 0.1	(0:100)
+Aminobenzotriazole (0.5 mM)	4.8 ± 0.4	(100:0)	0.5 ± 0.1	(0:100)

[a] The complete system is as described in Table 1.
[b] ND, not detectable.

TABLE 3

Effect of estradiol pretreatment on hepatic microsomal *para*-methoxyphenyl-1,3-dithiolane *S*-oxygenase activity

Microsome condition (replicates)	*S*-Oxygenase activity		
	Complete[a]	−NADPH GS[b]	+Heat inactivation[c]
	nmol of S-oxide/min/mg of protein		
Nonpretreated (9)	5.0 ± 0.6	0.01[d]	0.01[d]
Sham-treated (6)	5.7 ± 0.7	0.03	0.03
Ovariectomized (6)	4.7 ± 0.5	0.02	0.03
Ovariectomized + estradiol (6)	2.2 ± 0.3	0.02	0.03

[a] The complete system contained 50 mM phosphate buffer (pH 8.1), the NADPH-generating system, 300 μM substrate, 4 mM *n*-octylamine, and 0.8–1.5 mg of microsomal protein. Data represent the average 6–9 determination ± standard deviation.
[b] Incubations performed in the absence of an NADPH-generating system (GS).
[c] Microsomes were pretreated with heat inactivation, as described in Materials and Methods.
[d] The range of values was from 0.0 to 0.05 nmol/min/mg of protein.

TABLE 4

Effect of estradiol pretreatment on kidney microsomal *para*-methoxyphenyl-1,3-dithiolane *S*-oxygenase activity

Microsome condition (replicates)	*S*-Oxygenase activity		
	Complete[a]	−NADPH GS[b]	+Heat inactivation[c]
	nmol of S-oxide/min/mg of protein		
Nonpretreated (9)	1.3 ± 0.2	0.02[d]	0.01[d]
Sham-treated (6)	1.4 ± 0.2	0.01	0.02
+ Ovariectomized (6)	1.1 ± 0.1	0.01	0.02
+ Ovariectomized and estradiol (6)	0.3 ± 0.1	0.01	0.01

[a] The complete system is as described in Table 3.
[b,c] Incubations are described in Table 3.
[d] The range of values was from 0.0 to 0.04 nmol/min/mg of protein.

TABLE 5

Effect of estradiol pretreatment on lung microsomal *para*-methoxyphenyl-1,3-dithiolane *S*-oxygenase activity

Microsome condition (replicates)	*S*-Oxygenase activity		
	Complete[a]	−NADPH GS[b]	+Heat inactivation[c]
	nmol of S-oxide/min/mg of protein		
Nonpretreated (4)	2.1 ± 0.4	0.02[d]	1.2 ± 0.4[d]
Sham-treated (4)	1.9 ± 0.5	0.03	1.3 ± 0.4
+ Ovariectomized (4)	2.4 ± 0.5	0.03	1.6 ± 0.7
+ Ovariectomized and estradiol (4)	7.1 ± 0.5	0.02	5.1 ± 0.5

[a] The complete system contained 50 mM phosphate buffer (pH 8.1), the NADPH-generating system (GS), 300 μM substrate, and 0.6–2 mg of microsomal protein. Data represent the average of eight determinations ± standard deviation.
[b,c] Incubations were performed as described in Table 3.
[d] The range of values was from 0.0 to 0.06 nmol/min/mg of protein.

(25), did not inhibit *S*-oxygenation. In control experiments, preincubation of aminobenzotriazole with hepatic and renal microsomes from treated and nontreated animals led to a 40–50% inhibition of testosterone hydroxylase activity. These results suggest that the flavin-containing monooxygenase is responsible for *S*-oxygenation of *p*-methoxyphenyl-1,3-dithiolane in rat liver and kidney microsomes, although cytochromes P-450 or other enzymes may contribute to *S*-oxygenation. The involvement of other *S*-oxygenases may play a significant role in rat kidney, because other enzymes have been shown to oxidize sulfides in renal microsomes. As shown in Table 5, **1** is efficiently *S*-oxygenated by rat lung microsomes. That *S*-oxide formation is completely dependent on NADPH and is not decreased by heat inactivation is consistent with a role of the flavin-containing monooxygenase in *S*-oxygenation (26, 27), although the involvement of cytochromes P-450 cannot be ruled out. In separate studies, testosterone hydroxylase activity was used to measure isozyme levels and rates of cytochromes P-450 hydroxylation. Some differences in cytochrome P-450 isozyme levels were apparent but the overall level of testosterone hydroxylase activity did not vary greatly from various microsome preparations from a single tissue.[1]

para-Methoxyphenyl-1,3-dithiolane employed in this study has several features useful for investigation of *S*-oxygenase activity, because (a) *para*-methoxyphenyl-1,3-dithiolane (**1**) is an excellent substrate for both purified cytochrome P-450 and purified flavin-containing monooxygenases, (b) *S*-oxygenation of **1** stereoselectively produces two diastereomers stable to pyramidal inversion, and (c) during the short incubation times used in these studies, the *S*-oxide did not undergo further *S*-oxygenation to *S*,*S*′-dioxide (**3**) or other decomposition products. The diastereoselectivity of *S*-oxygenation of **1** was, therefore, examined in rat liver, kidney, and lung microsomes of pretreated and nonpretreated animals.

Rat liver microsomes prepared from nonpretreated animals *S*-oxygenated **1** with only modest stereoselectivity (Table 1). Rat liver microsomes prepared from ovariectomized animals pretreated with estradiol also gave only modest diastereoselective *S*-oxygenation of **1**. If diastereoselective *S*-oxygenation was observed to decrease, then this would tend to support the notion that the involvement of cytochromes P-450 or other enzymes in *S*-oxygenation of **1** increases as a consequence of ovariectomization and estradiol pretreatment. That this is not the case suggests that *S*-oxygenation is due at least in large part to the flavin-containing monooxygenase. Under conditions where flavin-containing monooxygenase activity was greatly reduced (i.e., microsome heat-inactivation or thiourea pretreatment), the amount of product observed was significantly reduced.

Surprisingly, in rat kidney microsomes, the major *S*-oxide from the metabolism of **1** is the (*cis*)-S-oxide diastereomer and the *S*-oxygenase stereoselectivity is quite great. Ovariectomized animals pretreated with estradiol also stereoselectively produced the *S*-oxide diastereomer from kidney microsomes. In agreement with the results obtained with rat liver microsomes, under conditions where flavin-containing monooxygenase activity is decreased, the minor *S*-oxide diastereomer is increased. These results suggest that in kidney microsomes cytochrome P-450 or other enzymes may contribute to *S*-oxygenation of **1** and produce the minor diastereomer. The use of *n*-octylamine, however, which stimulates the flavin-containing monooxygenase (1, 2) and inhibits cytochromes P-450 (24), can be employed to monitor flavin-containing monooxygenase enantioselectivity in various kidney and liver tissue preparations utilizing *para*-methoxyphenyl-1,3-dithiolane *S*-oxygenation. The results are presented below.

In contrast to rat hepatic and renal microsomes, heat inactivation of rat lung microsomes did not completely decrease the rate of *p*-methoxyphenyl-1,3-dithiolane *S*-oxide formation (Table 5). As shown in Tables 6 and 7, *n*-octylamine markedly decreased the rate of *S*-oxide formation in pulmonary microsomes. These results are consistent with a role of the flavin-containing monooxygenase in dithiolane *S*-oxide formation, because others have shown that lung flavin-containing monooxygenase activity is relatively insensitive to heat inactivation and that *n*-octylamine is a substrate for the lung flavin-containing monooxygenase (2, 26, 27). However, a role of pulmonary cytochrome P-450 in the *S*-oxygenation of *p*-methoxyphenyl-1,3-dithiolane cannot be ruled out.

Effect of ovarectomy and estradiol pretreatment on *S*-oxygenase activity. In agreement with previous reports (28), after ovariectomization, flavin-containing monooxygenase activity tended to be decreased in female rats. As shown in Tables 3 and 4, after ovariectomization, treatment of these animals with estradiol decreased kidney and hepatic microsomal *S*-oxygenase activity. The loss of *S*-oxygenase activity for ovariectomized rats pretreated with estradiol was significantly greater than for ovariectomized or sham-treated rats (Tables 3 and 4).

In contrast to the results obtained in hepatic and renal microsomes, *S*-oxygenase activity in various rat lung micro-

[1] J. R. Cashman and M. J. Presas, unpublished observations.

TABLE 6

S-Oxygenation of *para*-methoxyphenyl-1,3-dithiolane by rat microsome preparations

Incubations were performed as described in Materials and Methods. Fresh microsomes were obtained from nonpretreated female rats, female rats pretreated with estradiol, or female rats ovariectomized and pretreated with estradiol. Each incubation contained 50 mM phosphate buffer (pH 8.1), the NADPH-generating system, 0.5–1.9 mg of protein, and 300 μM substrate. Data represent the average of three or four determinations and the range of values was less than 5%.

Microsome preparation	S-Oxide formed		Diastereomeric excess[a]	Enantiomeric excess[b]	
	(*cis*)-S-Oxide	(*trans*)-S-Oxide		(*cis*)-S-Oxide (1*R*,2*S* 1*S*,2*R*)	(*trans*)-S-Oxide (1*S*,2*R* 1*R*,2*R*)
	nmol/min/mg of protein		%	%	
Nonpretreated					
Lung	0.1	2.9	93	40	49.4
Liver	1.6	2.4	20	95.8	17.9
Kidney	3.4	1.0	55	100.0	100
Estradiol alone					
Lung	0.1	0.5	67	86.3	45.3
Liver	0.4	0.6	26	78.4	12.8
Kidney	0.1	0.1	55	67.5	32.6
Estradiol + ovariectomization					
Lung	ND[c]	6.7	100	ND	70.6
Liver	0.4	0.7	25	92.2	17.0
Kidney	0.2	0.2	12	79.5	100

[a] Diastereometic excess is defined as % of major diastereomer − % minor diastereomer.
[b] Enantiomeric excess is defined for each (*cis*)- and (*trans*)-S-oxide diastereomer as % major enantiomer − % minor enantiomer.
[c] ND, not detectable.

TABLE 7

S-Oxygenation of *para*-methoxyphenyl-1,3-dithiolane by rat microsome preparations in the presence of *n*-octylamine

Incubations were performed as described in Table 6 except that 5 mM *n*-octylamine was present. Data represent the average of three or four determinations and the range of values was less than 6%.

Microsome preparation	S-Oxide formed		Diastereomeric excess[a]	Enantiomeric excess[b]	
	(*cis*)-S-Oxide	(*trans*)-S-Oxide		(*cis*)-S-Oxide (1*R*,2*S* 1*S*,2*R*)	(*trans*)-S-Oxide (1*S*,2*R* 1*R*,2*R*)
	nmol/min/mg of protein		%	%	
Nonpretreated					
Lung	ND[c]	1.4	100	ND	91.8
Liver	2.1	3.3	22	100	100
Kidney	3.9	0.8	66	95	100
Estradiol alone					
Lung	ND	2.3	100	ND	99.6
Liver	1.6	1.6	0	100	84.6
Kidney	0.5	3.1	72	ND	97.8
Estradiol + ovariectomization					
Lung	0.1	0.7	75	91	100
Liver	0.7	0.9	6	100	96.4
Kidney	ND	0.4	100	ND	98.0

[a] Diastereometic excess as defined in Table 6.
[b] Enantiomeric excess as defined in Table 6.
[c] ND, not detectable.

some preparations from pretreated animals followed a different pattern. As shown in Table 5, ovariectomization tends to cause a slight increase in *p*-methoxyphenyl-1,3-dithiolane *S*-oxygenase activity, compared with *S*-oxygenase activity detected in nonpretreated or sham-treated animals. When *S*-oxygenase activity was determined in lung microsomes from animals that had been ovariectomized and pretreated with estradiol, a large increase in dithiolane *S*-oxygenase activity was observed (Table 5). It has been shown by others (26, 27) that the pulmonary and hepatic forms of the flavin-containing monooxygenase are distinct. The results presented here are in agreement with other reports that suggest that the lung enzyme is induced almost 2-fold during the final stages of pregnancy (27) or by the administration of steroids (29).

Changes in total organ dithiolane *S*-oxygenase activity did not exactly follow changes in specific activity, because the organ size was also altered by ovariectomy and treatment of animals

TABLE 8

Changes in total organ *para*-methoxyphenyl-1,3-dithiolane S-oxygenase activity following animal pretreatment

Animals were nonpretreated, ovariectomized, or ovariectomized and pretreated with estradiol, as described in Materials and Methods. Incubations contained 50 mM phosphate buffer (pH 8.1), the NADPH-generating system, 300 μM substrate, 4 mM *n*-octylamine, and 0.7–1.7 mg of microsomal protein. Data represent the average of six to nine determinations ± standard deviation.

Treatment (replicates)	S-Oxygenase activity		
	Liver	Kidney	Lung[a]
	nmol of S-oxide/min/total organ		
Control (9)	9,061 ± 1,018	365 ± 36	145.6 ± 22
Sham-treated (6)	10,322 ± 1,403	415 ± 59	148.7 ± 31
Ovariectomized (6)	12,324 ± 1,645	688 ± 68	175.5 ± 28
+Ovariectomized and estradiol (6)	4,009 ± 436	128 ± 22	339.1 ± 37

[a] Data represent the average of four replicates ± standard deviations.

with estradiol pretreatment (Table 8). For example, ovariectomization and pretreatment of animals with estradiol decreased hepatic *S*-oxygenase specific activity 76% (Table 1), compared with nonpretreated animals, but it also increased liver weight 19% (data not shown) and total organ *S*-oxygenase activity decreased 55% (Table 8), compared with nonpretreated animals.

As in the liver, dithiolane *S*-oxygenase activity of the kidney was decreased by ovariectomization. In agreement with data from previous studies (15), ovariectomization and estradiol pretreatment of animals significantly decreased renal *S*-oxygenase activity, compared with nonpretreated or sham-treated rats (Table 8). For example, ovariectomization and estradiol pretreatment of female rats decreased renal *S*-oxygenase specific activity 89% (Table 2), compared with nonpretreated animals, but also increased kidney weight 13% and total organ *S*-oxygenase activity decreased 65% (Table 8), compared with nonpretreated animals. As a result, the decrease in total kidney *S*-oxygenase activity did not exactly parallel changes in specific *S*-oxygenase activity, because the kidney size was altered by the various treatments.

The change in total organ dithiolane *S*-oxygenase activity in lung microsomes as a function of various pretreatments did not follow the same pattern as that observed for liver and kidney microsomes. *S*-Oxygenase activity, low in lung microsomes from nonpretreated and sham-treated animals, increased greater than 2-fold for female rat lung microsomes isolated from ovariectomized and estradiol-pretreated animals (Tables 5, 6, and 8). In contrast to kidney and liver tissue obtained from ovariectomized and estradiol-pretreated animals, lung weights determined after pretreatment were unchanged compared with nonpretreated or sham-treated animals (data not shown).

Effect of ovariectomization and estradiol pretreatment on *S*-oxygenase stereoselectivity. *para*-Methoxyphenyl-1,3-dithiolane (**1**) was chosen to examine the stereoselectivity of *S*-oxygenation because we have previously observed that **1** is *S*-oxygenated in a variety of tissue preparations in a highly (i.e., 96–100%) diastereoselective process.[2] Thus, *S*-oxygenation of this substrate can be utilized to monitor enzyme stereoselectivity and results can suggest whether different forms of the same enzyme are emerging or disappearing as a consequence of animal pretreatment. The ability of monooxygenase enzymes to differentiate between enantiotopic lone pairs of prochiral sulfur atoms during *S*-oxygenation also provides, in principle, a further confirmation of enzyme-catalyzed *S*-oxygenation stereoselectivity (Fig. 2). For example, we have observed that, in hog liver microsomes, **1** is efficiently converted to the (*trans*)-S-oxide diastereomer with very high enantioselectivity.[2] These results have been confirmed with purified flavin-containing monooxygenase from hog and rat liver. In addition, rat and mouse liver cytochrome $P\text{-}450_{PB\text{-}B}$ catalyzes *trans* *S*-oxygenation of **1** but with enantioselectivity opposite to that observed for hepatic flavin-containing monooxygenase.[3]

As shown in Tables 1 and 6, the diastereoselectivity of nonpretreated hepatic *S*-oxygenation is extremely low. It is interesting to note that the slight degree of diastereoselectivity (i.e., 20% *trans* diastereomeric enrichment) observed in liver microsomes is opposite and much less than that observed in kidney microsomes. As shown in Tables 1 and 2, heat inactivation almost completely abolished *S*-oxygenation in both liver and kidney microsome preparations for all animal pretreatments. Further, from the small amount of *S*-oxide metabolite produced using the heat-inactivated microsomes, no change in the ratio of (*cis*)- to (*trans*)-*S*-oxide diastereomer formation was observed for kidney microsomes compared with non-heat-inactivated kidney microsomes. In liver microsomes pretreated with heat inactivation, the major *S*-oxide product is the *cis*-isomer, presumably as a consequence of cytochrome P-450 metabolism, which produces the (*cis*)-*S*-oxide diastereomer. That *S*-oxygenation is not due to concurrent nonenzymatic *S*-oxidation is apparent because (a) the ratio of diastereomers remains constant over the entire time course of the reaction, (b) for the case of the chemical oxidation, large concentrations of H_2O_2 (i.e., 100–200 mM) are required to effect the same degree of *S*-oxidation observed for the enzymatic case, and (c) incubations of **1** in the presence of catalase did not appreciably alter the stereoselectivity of *S*-oxygenation (data not shown).

As shown in Tables 6 and 7, we investigated the enantioselectivity of *p*-methoxyphenyl-1,3-dithiolane *S*-oxygenation (Fig. 2) for microsomes obtained from nonpretreated, estradiol-pretreated, and ovariectomized plus estradiol-pretreated animals in the presence and absence of *n*-octylamine. We sought to provide evidence as to whether the flavin-containing monooxygenase could produce the opposite *S*-oxide enantiomer to that produced by cytochrome P-450, as has been previously reported by others for other sulfide substrates (12).

In addition to a major *S*-oxide metabolite formed in each tissue homogenate, a minor *S*-oxide was also generally formed. In incubations performed in the absence of *n*-octylamine, it is clear that lung microsomes *S*-oxygenate *p*-methoxyphenyl-1,3-dithiolane with the greatest diastereoselectivity and the major product is the (*trans*)-*S*-oxide regardless of the animal pretreatment (Table 6). The microsomes that produce dithiolane *S*-oxide with lowest diastereoselectivity are the hepatic preparations, whereas kidney microsomes demonstrate modest to good diastereoselectivity. In all tissues examined, a striking preference for addition of molecular oxygen to the pro-*R* sulfur atom was observed. Thus, for nonpretreated animals, lung microsomes *S*-oxidize the pro-*R* sulfur of *p*-methoxyphenyl-1,3-dithiolane to an extent of 75.4% (i.e., 73.1% 1*R*,2*R*; 2.3% 1*R*,2*S*), with 24.6% attack of oxygen at the pro-*S* sulfur atom (i.e., 24% 1*S*,2*S*; 0.6% 1*S*,2*R*). Nonpretreated liver microsomes preferentially *S*-oxidize pro-*R* sulfur to an extent of 74% (i.e., 35% 1*R*,2*R*; 39% 1*R*,2*S*), with 26% attack at the pro-*S* sulfur atom (i.e., 24.5% 1*S*,2*S*; 1.5% 1*S*,2*R*). Nonpretreated kidney microsomes preferentially *S*-oxidize pro-*R* sulfur, by 100% (i.e., 22.7% 1*R*,2*R*; 77.3% 1*R*,2*S*). In general, ovariectomization and pretreatment of animals with estradiol actually increases the enantiomeric enrichment of the product *S*-oxides observed for the tissues examined (Table 6). The above outlined study was repeated and microsomal dithiolane *S*-oxygenase activity was determined in the presence of *n*-octylamine (Table 7). Because it is known that *n*-octylamine is a potent inhibitor of cytochromes P-450 (24), the enantioselectivity observed should be due in large part to the flavin-containing monooxygenase. Inspection of Table 7 demonstrates that, in general, an increase in diastereoselectivity and enantioselectivity of dithiolane *S*-oxygenation is observed. That *n*-octylamine increases the en-

[2] J. R. Cashman and L. D. Olsen, unpublished observations.
[3] L. M. Bornheim and J. R. Cashman, unpublished results.

antioselectivity and diastereoselectivity of pro-*R* *S*-oxide formation in hepatic and renal microsomes is in agreement with the suggestion that flavin-containing monooxygenase is in large part responsible for *S*-oxygenation of **1**. In other independent studies with purified enzymes, we confirm these results, which demonstrate that purified rat liver flavin-containing monooxygenase produces mainly the (1*R*,2*R*)-(*trans*)-*S*-oxide of **1** in 95% enantioselectivity. Further, highly purified rat liver cytochrome $P\text{-}450_{PB\text{-}B}$ produces the (1*S*,2*S*)-(*trans*)-*S*-oxide of *p*-methoxyphenyl-1,3-dithiolane in 95.5% enantioselectivity. [2,3]

Discussion

For microsomes prepared from female rat liver and kidney, *para*-methoxyphenyl-1,3-dithiolane *S*-oxygenase activity is markedly decreased by ovariectomy and estradiol pretreatment (Tables 3, 4, 6, and 7). Ovariectomization depresses *S*-oxygenase activity in both liver and kidney to a minor degree, but ovariectomization and estradiol pretreatment is mainly responsible for the large decrease in flavin-containing monooxygenase *S*-oxygenase activity observed. Administration of estradiol alone significantly decreases *S*-oxygenase activity in kidney, liver, and lung homogenates (Table 6). As pointed out by other workers, however, it is unlikely that estradiol alone determines the activity of the flavin-containing monooxygenase in female mice and this may also be the case for female rats (14, 15). Thus, in nonpretreated or sham-treated animals, hepatic *S*-oxygenase activity (5.0–5.75 nmol/min/mg of protein, respectively) was markedly decreased, compared with ovariectomized rats pretreated with estradiol (2.24 nmol/min/mg of protein). In contrast to what has been reported for mouse kidney, our results suggest that *S*-oxygenase activity, compared with nonpretreated or sham-treated rats (1.32–1.38 nmol/min/mg of protein, respectively), is significantly decreased in ovariectomized rats pretreated with estradiol (0.33 nmol/min/mg of protein). Estradiol-dependent differences in *S*-oxygenase specific activity are more pronounced in microsomal fractions than total organ *S*-oxygenase activity of liver or kidney (Table 8). This may simply be due to the fact that ovariectomization and estradiol pretreatment of female rats increases the weight of the tissue or it may be due to other more complicated mechanisms. Previous reports have demonstrated that the activity of the pulmonary flavin-containing monooxygenase is under the control of sex steroids (29) and/or other factors (14, 15). Results shown in Tables 5–8 demonstrate that ovariectomized female rats pretreated with estradiol have significantly greater pulmonary *S*-oxygenase activity than nonpretreated animals. This observation may have practical consequences for the isolation and purification of pulmonary flavin-containing monooxygenase activity, because previous studies have relied on pregnant animals (26, 27). In addition, the results of our studies of the pulmonary enzyme suggest a close link between induction of lung flavin-containing monooxygenase activity and estradiol administration or induction of some pituitary factor. Because estradiol administration markedly increases pituitary cell mass (16, 30) and elevates pituitary and serum levels of pituitary hormones such as prolactin (16) and growth hormone (31), we can not rule out the involvement of some pituitary factor in the control of flavin-containing *S*-oxygenase activity.

In addition to a modulation of flavin-containing monooxygenase activity, loss of *S*-oxygenase activity in kidney and liver microsomes and induction of pulmonary activity could arise from a variety of other possible mechanisms. For example, it has been shown that loss of *in vitro* rat cytochrome P-450 activity observed from microsome preparations prepared from growth hormone-treated animals is due to a decrease in cytochrome P-450 reductase activity (32). Growth hormone may also regulate cytochrome P-450 expression (33). Thus, loss of cytochrome P-450 activity could also account for our results. Hormones can also regulate the expression of different forms of cytochrome P-450 (34–36). Other possible mechanisms could involve selective loss of one form of cytochrome P-450 *S*-oxygenase enzyme activity or loss of some important cofactor. We find no evidence for a major loss of cytochrome P-450 activity in any of the tissue preparations, as assessed by testosterone hydroxylase activity. Changes in isozyme composition of cytochromes P-450 isozymes are apparent, however. Another possibility is that *S*-oxygenase enzyme activity is labile and enzyme activity is sensitive to isolation conditions and/or animal pretreatments. In an attempt to investigate these possibilities more carefully, we examined the diastereoselectivity and enantioselectivity of *S*-oxygenase activity recovered in microsomal fractions from female rats under various pretreatment conditions. As shown in Table 5, very modest stereoselectivity was observed for rat hepatic *S*-oxygenase activity. From these and other data, we conclude that stereoselective *S*-oxygenase activity in rat liver microsomes is very low and animal pretreatment does not alter *S*-oxygenation diastereoselectivity (Fig. 1). In order to investigate this more carefully, the enantioselectivity of hepatic *S*-oxygenation was investigated (Fig. 2). As shown in Table 6, the modest degree of enantioselectivity suggests that the flavin-containing monooxygenase is mainly responsible for *S*-oxygenation of **1**, but a significant contribution from cytochrome P-450 (i.e., primarily oxidizing the dithiolane at the pro-*S* sulfur atom) is also occurring. It is currently unclear why kidney microsome diastereoselectivity is distinct from hepatic or pulmonary *S*-oxygenase activity. It is possible that other *S*-oxygenases or other forms of the flavin-containing monooxygenase may be present in the rat kidney. That cytochrome P-450 is not making the major contribution to renal *S*-oxygenase activity is apparent from studies with metabolism inhibitors (Table 2) as well as the pronounced stereoselectivity of *S*-oxygenation of **1**, because preference for pro-*R* enantioselectivity has been seen for all flavin-containing monooxygenase preparations thus far examined. Pulmonary flavin-containing monooxygenase appears to be mainly responsible for *S*-oxygenation of **1**, because the high degree of enantioselectivity observed (i.e., primarily oxidizing the dithiolane at the pro-*R* sulfur atom) is opposite to the enantioselectivity observed for purified pulmonary cytochromes P-450 (data not shown). Estradiol pretreatment appears to coordinately decrease rat *S*-oxygenase activity in liver and kidney and increase *S*-oxygenase activity in lung microsomes.

The data reported here suggest that, at least for sulfide-containing chemicals, *S*-oxygenation catalyzed by microsomes isolated from rat liver or kidney from ovarectomized female animals pretreated with estradiol is markedly decreased. In agreement with this observation, previous studies have shown that rat liver flavin-containing monooxygenase appears to be subject to negative control by estradiol (35). The apparent inconsistency of our results with those of others (36) may be a consequence of the fact that we have employed *S*-oxygenase rather than *N*-oxidase activity to investigate the flavin-con-

taining monooxygenase. This may explain the results, because it has been demonstrated that nitrogen- and sulfur-containing substrates demonstrate unusual substrate specificities for the flavin-containing monooxygenase, depending upon the source of the enzyme (26, 27, 36). The issue of thermal lability of the flavin-containing monooxygenase activity may also be a factor in the modulation of hepatic and kidney *S*-oxygenase activity, but this has been carefully addressed in our studies because we take great effort to minimize this possibility. Finally, female rats pretreated with estradiol and ovariectomization gave lung microsomal *S*-oxygenase activity that demonstrated a 2.3-fold increase in activity, in agreement with previous reports (27, 36). A further possible complication to the results reported here is the apparent contradictory nature of properties of the flavin-containing monooxygenase from various tissues and species. Thus, a report from other workers (37) demonstrated little difference between rat liver and lung flavin-containing monooxygenase activity, whereas another report (38) using immunochemical probes showed that, in addition to a pulmonary form, a hepatic form of the flavin-containing monooxygenase was present in rat lung. The apparent lack of correlation between the relative amounts of protein, at least in rat lung, as determined by immunochemical methods, and the tissue enzyme activity may be a consequence of differential regulation and expression of pulmonary forms of the flavin-containing monooxygenase. The results of this study are consistent with the induction of the heat-stable form of the pulmonary flavin-containing monooxygenase by treatment of ovariectomized animals with estradiol. Another possibility is that the heat-stable form of the pulmonary flavin-containing monooxygenase is determined by pituitary factors and the heat-labile form of the pulmonary flavin-containing monooxygenase is under ontogenic control.

The results from this study suggest that (a) estradiol or some pituitary factor may play a role in determining the relative contribution of microsomal monooxygenase *S*-oxygenase activity in xenobiotic metabolism, (b) *S*-oxygenase activity of *para*-methoxyphenyl-1,3-dithiolane is highly diastereoselective in rat lung microsomes and less so in kidney and liver microsomes, and (c) the large preference for flavin-containing monooxygenase-catalyzed addition of molecular oxygen to the pro-*R* sulfur atom of *p*-methoxyphenyl-1,3-dithiolane is in distinct contrast to the preference for addition of oxygen by cytochrome P-450 to the pro-*S* sulfur atom and this may provide a useful stereochemical probe to investigate the presence of each monooxygenase in various tissue preparations.

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