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INTERACTION OF 4-METHYLBENZALDEHYDE WITH RABBIT PULMONARY CYTOCHROME P-450 IN THE INTACT ANIMAL, MICROSOMES, AND PURIFIED SYSTEMS

DESTRUCTIVE AND PROTECTIVE REACTIONS

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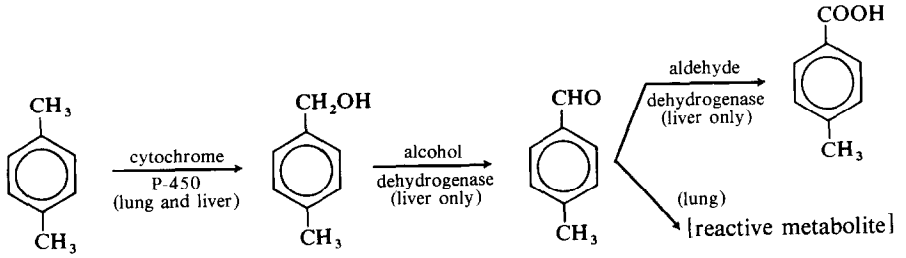
Abstract—About 50 per cent of rabbit pulmonary cytochrome P-450 is destroyed by treatment of the intact animal, microsomes, or systems reconstituted from purified pulmonary mono-oxygenase components with 4-methylbenzaldehyde. The loss of the cytochrome is accompanied by an equimolar loss of heme. The action of 4-methylbenzaldehyde requires the presence of NADPH and O_2 and appears to result from cytochrome P-450-catalyzed metabolism. Selective destruction of one of the known forms of rabbit pulmonary cytochrome P-450 does not account for the lack of complete destruction of pulmonary P-450 by 4-methylbenzaldehyde; loss of about 50 per cent of each form of the cytochrome occurs *in vivo* and in reconstituted systems. However, form II is affected to a greater extent than form I when microsomes are incubated with 4-methylbenzaldehyde. The portion of the cytochrome not degraded by 4-methylbenzaldehyde appears to be protected by some factor produced from 4-methylbenzaldehyde during the incubation. This factor also protects against complete destruction of the cytochrome by cumene hydroperoxide

Numerous exogenous compounds can be oxidatively metabolized by the pulmonary cytochrome P-450-dependent mono-oxygenase system (see review in Ref. 1). In general, cytochrome P-450-mediated metabolism is the intitial step in the elimination of many foreign compounds from the body. However, the metabolism of some chemicals by this system is thought to be a requisite step in the development of some pulmonary and respiratory tract cancers [2–4] and aromatic hydrocarbon-related bronchiolar necrosis [5]. It has been suggested that pulmonary cytochrome P-450 also acts as an oxygen sensor in the vasoconstrictor response to alveolar hypoxia [6]. Alterations of the pulmonary mono-oxygenase system (i.e. by induction or inhibition), therefore, could have a significant effect on a number of important processes in the lung.

An industrial solvent, 1,4-dimethylbenzene (*p*-xylene), has been reported by Patel *et al.* [7] to produce a pulmonary-specific effect on the mono-oxygenase system in rabbits and rats. Administration of *p*-xylene to these animals results in the destruction of approximately 50 per cent of the pulmonary cytochrome P-450 without altering the hepatic cytochrome content [7]. The maximum effect occurs within 4 hr and is not altered by increases in either dose or time [7]. *In vitro*, *p*-xylene is metabolized to 4-methylbenzoic acid by NADPH-fortified hepatic or pulmonary microsomes when alcohol and aldehyde dehydrogenases are present in the incubations. These enzymes are found in hepatic but not in pulmonary microsomal preparations [7]. The metabolism of *p*-xylene to 4-methylbenzoic acid does not alter the cytochrome P-450 concentration. However, approximately 50 per cent of the cytochrome P-450 is lost when (1) *p*-xylene and alcohol dehydrogenase or (2) 4-methylbenzaldehyde are/is incubated with pulmonary microsomes and NADPH. It appears, therefore, that the following reactions are involved in the metabolism of *p*-xylene and the destruction of pulmonary cytochrome P-450 (see below).

We have further investigated the interaction of 4-methylbenzaldehyde with pulmonary cytochrome P-450 in order to ascertain the following: (1) the



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requirements for the interaction in microsomes and in mono-oxygenase systems reconstituted from purified components; (2) the nature of the apparent loss of cytochrome P-450; (3) the selectivity of the effect with respect to the two known forms of rabbit pulmonary cytochrome P-450 [8, 9]; and (4) the reason(s) for the effect being limited to only 50 per cent of the cytochrome P-450. The findings presented describe the general mechanisms involved in the partial destruction of pulmonary cytochrome P-450 by 4-methylbenzaldehyde.

MATERIALS AND METHODS

Treatment of animals and preparation of microsomes. Adult, male rabbits (New Zealand White, 2 to 2.5 kg) were obtained from Dutchland Laboratory Animals, Inc. (Delware, PA). Rabbits given 4-methylbenzaldehyde (50 mg/kg body weight, given by injection into a marginal ear vein) were killed 1 hr after treatment. Pulmonary microsomes were prepared as described by Bend *et al.* [10]. For some experiments, the lungs were perfused with Krebs–Ringer solution (200 ml) via the pulmonary artery prior to the preparation of microsomes.

Preparation of pulmonary mono-oxygenase components. Pulmonary cytochrome P-450 (forms I and II) and NADPH-cytochrome P-450 reductase and hepatic phospholipids for the reconstitution of the mono-oxygenase system were isolated by the method of Wolf *et al.* [11]. (Differences between the substrate specificities, monomeric molecular weights, and spectral properties of rabbit pulmonary cytochrome P-450 I and II have been reported [11].) Microsomes from the lungs of twenty rabbits were used as the starting material for the preparation of the enzymes. Isolation and separation of forms I and II of the cytochrome from the microsomes of individual rabbit lungs was achieved as follows. The microsomes, containing about 70 mg protein and 15–30 nmoles cytochrome P-450, were solubilized with sodium cholate (1 mg/mg of protein) and applied to a column (1 × 5 cm) of DEAE-cellulose equilibrated with 10 mM phosphate (pH 7.7), 20% glycerol, 0.1 mM dithiothreitol (DTT), and 0.1 mM EDTA (10 mM buffer A). Form I of the cytochrome (molecular weight 51,000) was eluted with 10 mM buffer A containing 0.2% Emulgen 911 and 0.1% sodium cholate. Form II (molecular weight 53,000) was eluted with 75 mM buffer A also containing 0.2% Emulgen 911 and 0.1% sodium cholate.

Treatment of microsomes and reconstituted mono-oxygenase systems with 4-methylbenzaldehyde. The effect of 4-methylbenzaldehyde on pulmonary cytochrome P-450 was investigated *in vitro* by incubating microsomes (1 mg protein/ml), solubilized microsomes (1 mg protein/ml), or reconstituted systems (0.2 nmole cytochrome, 300 units reductase, 100 μg phospholipid, and 25 μg sodium cholate/ml) with 4-methylbenzaldehyde (1 mM) and NADPH (1 mM). Active reconstituted mono-oxygenase systems were formed by incubation of the phospholipid, sodium cholate, cytochrome, and reductase at 37° for 15 min prior to the addition of the buffer (0.1 M HEPES, pH 7.6), substrate and NADPH. The specific contents of the preparations used were 8–10 nmoles cytochrome/mg of protein for cytochrome I, 4–6 nmoles/mg for cytochrome II, and approximately 10,000 units/mg of protein for NADPH–cytochrome P-450 reductase. Cumene hydroperoxide (0.03 mM) was substituted for NADPH in some incubations. All incubations were carried out at 37°. Oxygen was removed from incubations according to the method of Matsubara *et al.* [12].

Analytical methods. Cytochrome P-450 concentrations in hemoglobin-free microsomes from perfused lungs and in purified systems were determined by the method of Omura and Sato [13]; in microsomes contaminated with hemoglobin, the method of Estabrook *et al.* [14] was used. Lipid peroxidation was measured using the thiobarbituric acid assay [15] according to

Table 1. Factors affecting the destruction of cytochrome P-450 by 4-methylbenzaldehyde in microsomes prepared from the lungs of 4-methylbenzaldehyde-treated and untreated rabbits

	Components added to the incubations							
				Microsomal source				Cytochrome P-450 content (% microsomal)
Expt. No.*	NADPH (1 mM)	O_2	4-Methylbenzaldehyde (1 mM)	Treated rabbits	Untreated rabbits	P-1§	S-1§	
1	–	+	–	–	+	–	–	100
2	+	+	–	–	+	–	–	92
3	–	+	+	–	+	–	–	102
4	+	+	+	–	+	–	–	48
5	+	–†	+	–	+	–	–	94
6	–	+	–	–	+	–	+	47
7	+	+	+	–	–	+	+	46
8	+	+	+	–	–	+	–	34
9	–	–†	–	+	–	–	–	50
10	+	+	+	+	–	–	–	5
11	+	+	+	+	–	–	+	45

* Results are reported for a single experiment. All experiments were repeated at least three times with similar results.

† Oxygen was removed using glucose oxidase, and the hydrogen peroxide formed was removed by catalase.

‡ Rabbits were treated with 4-methylbenzaldehyde (50 mg/kg) 1 h before they were killed.

§ P-1 and S-1 refer to the pellet and supernatant fractions obtained by centrifugation of a microsomal suspension following incubation with 4-methylbenzaldehyde, NADPH and O_2 for 15 min at 37°.

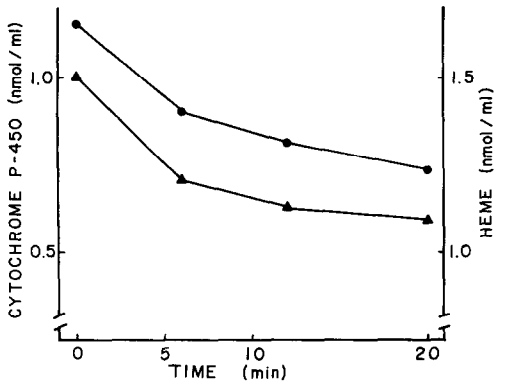

Fig. 1. Loss of cytochrome P-450 (▲—▲) and heme (●—●) in hemoglobin-free pulmonary microsomes incubated with 4-methylbenzaldehyde and NADPH. Microsomes prepared from perfused lungs were incubated at 37° for the indicated times. The incubation mixtures initially contained cytochrome P-450 (1 μM), heme (1.65 μM), 4-methylbenzaldehyde (1 mM) and NADPH (1 mM).

the method of Wills [16]. Protein was estimated by the method of Lowry *et al.* [17] and heme content was determined according to Falk [18].

Materials. All chemicals and reagents were purchased from commercial sources at the highest grade of purity available.

RESULTS

Treatment of rabbits with 4-methylbenzaldehyde resulted in the destruction of approximately 50 per cent of the pulmonary cytochrome P-450 within 1 hr (Table 1, Expt. 9). The extent of destruction was not increased by repetitive or prolonged treatment. Similar results were obtained *in vitro* by the addition of 4-methylbenzaldehyde to NADPH-fortified pulmonary microsomes (Table 1, Expts. 3 and 4). Maximum destruction occurred *in vitro* within 15–20 min; some incubations were run for as long as 90 min and no further destruction was noted. The destructive process was found to require O_2 as well as NADPH (Table 1, Expts. 3, 4 and 5). No cytochrome was destroyed when NADH was substituted for NADPH.

Formation of cytochrome P-420 and loss of heme were determined after incubation of microsomes, prepared from perfused lungs, with 4-methylbenzaldehyde and NADPH, in order to determine the nature of the destructive process. No hemoglobin could be spectrally detected by the addition of carbon monoxide to these microsomes. Figure 1 shows that the decrease in cytochrome P-450 concentration observed with time was paralleled by a loss of heme content. The cytochrome P-420 concentration (less than 5% of the total heme) did not change with time.

Two sets of conditions were found for which greater than 50 per cent destruction of the cytochrome could be obtained. First, microsomes were incubated with 4-methylbenzaldehyde, O_2 and NADPH for 15 min and then resedimented by centrifugation. The pellet (P-1) was then incubated as above and additional destruction of the cytochrome P-450 was noted (Table 1, Expt. 8). Second, incubation of microsomes (prepared from 4-methylbenzaldehyde-treated rabbits) with 4-methylbenzaldehyde, O_2 and NADPH resulted in the destruction of about 90 per cent of the remaining cytochrome (Table 1, Expt. 10). When the supernatant fraction (S-1), obtained in the same manner as P-1, was added to incubations containing P-1 or microsomes from treated animals, additional destruction of the cytochrome P-450 was inhibited completely (Table 1, Expts. 7 and 11). The supernatant fraction from incubations of microsomes with NADPH or 4-methylbenzaldehyde did not inhibit further destruction of the cytochrome. Addition of S-1 to microsomes from untreated rabbits did not block the initial destruction of the cytochrome. In fact, S-1 alone catalyzed the destruction of 50 per cent of the cytochrome (Table 1, Expt. 6).

The above results indicated that 50 per cent of the pulmonary cytochrome P-450 was readily destroyed by 4-methylbenzaldehyde and that destruction of the remaining cytochrome was prevented by some factor produced during the reaction.

The possibility that one form of the cytochrome was preferentially destroyed was investigated by isolating forms I and II from rabbits treated with 4-methylbenzaldehyde and from microsomes incubated with 4-methylbenzaldehyde, O_2 and NADPH. Separation of the forms of the cytochrome was carried out by chromatography of cholate-solubilized microsomes on DEAE-cellulose, as described in Materials and Methods. The ratio of form I to form II isolated from untreated microsomes or microsomes incubated with NADPH was consistently between 1.5 and 1.6 to 1 in these

Table 2. Cytochrome P-450 levels and the ratio of form I and II in microsomes incubated with 4-methylbenzaldehyde and in microsomes from rabbits treated with 4-methylbenzaldehyde

System treated	Treatment*		Cytochrome P-450		Ratio of I to II recovered from DEAE†
	4-Methylbenzaldehyde	NADPH (1 mM)	(nmoles/mg)	(% Control)	
Microsomes	–	–	0.44	100	1.56
Microsomes	–	+	0.42	95	1.56
Microsomes	+	+	0.18	41	3.65
Whole animal	+	–	0.20	46	1.75

* Treatment consisted of a 15-min incubation *in vitro* or administration of 4-methylbenzaldehyde *in vivo*, as described in Table 1.

† Results are shown for a single experiment. These experiments were repeated three times and similar results were obtained each time.

Table 3. Effect of 4-methylbenzaldehyde on the cytochrome P-450 levels in sodium cholate-solubilized microsomes

Components present in incubation *	Cytochrome P-450 (nmoles/mg protein)
Solubilized microsomes† (1 mg/ml)	0.31 ± 0.02
Solubilized microsomes, NADPH (1 mM)	0.30 ± 0.04
Solubilized microsomes + 4-methylbenzaldehyde (1 mM)	0.32 ± 0.02
Solubilized microsomes + NADPH (1 mM) + 4-methylbenzaldehyde (1 mM)	0.14 ± 0.03‡

* Incubations were for 30 min at 37°.

† Microsomes were solubilized by treatment with sodium-cholate (1 mg/mg of protein) for 30 min followed by dialysis for 48 hr at 4°.

‡ Significantly different from control, $P < 0.01$.

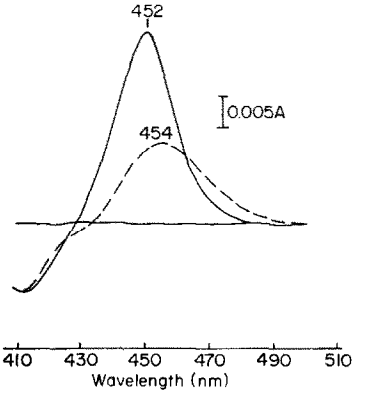

Fig. 2. Ferrous cytochrome P-450-carbon monoxide difference spectra of purified cytochrome P-450 I before (——) and after (– – –) incubation with 4-methylbenzaldehyde in a reconstituted system. Incubations were for 20 min at 37°. The reconstituted system is described in Materials and Methods.

experiments. (It should be noted that the ratio of I to II was 1:1 when a gradient of KCl [0 to 0.5 M] was used to elute cytochrome II from the DEAE [7].) The procedure using 75 mM buffer A was employed here for convenience and because of its excellent reproducibility. Results obtained from rabbits treated with 4-methylbenzaldehyde showed that equivalent amounts of both forms of the cytochrome had been destroyed (Table 2). However, incubation of pulmonary microsomes with 4-methylbenzaldehyde, O_2 and NADPH appeared to destroy a greater proportion of form II than form I (Table 2).

A difference between the effects of *in vivo* and *in vitro* treatment was also noted when the spectrum of the ferrous cytochrome P-450–carbon monoxide complex was examined. Treatment of microsomal preparations resulted in a 1 nm shift in the λ_{max} of the spectrum (from 451 to 452 nm), whereas no significant shift was caused by treatment *in vivo*. In addition, cytochrome I isolated from 4-methylbenzaldehyde-treated microsomes had a λ_{max} of 454 nm as compared to 452 nm when isolated from the pulmonary microsomes of either treated or untreated rabbits. The λ_{max} of cytochrome II (450 nm) was not altered in any of the experiments.

The possible importance of the intact microsomal membrane and the position of the cytochrome in the membrane to the destructive effect of 4-methylbenzaldehyde was investigated by using cholate-solubilized microsomes and reconstituted mono-oxygenase systems. When solubilized microsomes were incubated with 4-methylbenzaldehyde, O_2 and NADPH, 50 per cent of the cytochrome was destroyed (Table 3). This destruction was accompanied by a 1 nm shift in the λ_{max} of the spectrum of the ferrous cytochrome–carbon monoxide complex. In reconstituted systems, cytochromes I and II were destroyed to a similar extent (Table 4). The rates of destruction were similar for both forms. In agreement with results obtained using microsomes, the λ_{max} for cytochrome I shifted from 452 to 454 nm following partial destruction in the reconstituted system, and the λ_{max} of cytochrome II was not altered (Table 4, Fig. 2). No formation of cytochrome

Table 4. Effect of 4-methylbenzaldehyde on pulmonary cytochromes I and II in reconstituted mono-oxygenase systems *

Treatment†	Cytochrome I			Cytochrome II			Cytochromes I + II		
	nmoles	% Control	λ_{max} (nm)	nmoles	% Control	λ_{max} (nm)	nmoles	% Control	λ_{max} (nm)
None	0.40	100	452	0.40	100	450	0.40	100	451
NADPH	0.34	85	452	0.35	88	450	0.35	88	451
4-Methylbenzaldehyde	0.40	100	452	0.40	100	450	0.40	100	451
NADPH + 4-methylbenzaldehyde	0.17	43	454	0.16	40	450	0.18	45	452

* Results similar to those shown were obtained in repeated experiments.

† Treatments were carried out by the addition of the designated compounds to reconstituted systems containing cytochrome P-450 (0.2 nmole/ml), NADPH–cytochrome P-450 reductase (300 units/ml), phospholipids (100 μg/ml) and sodium cholate (25 μg/ml). The incubations were run for 15 min at 37° in a total volume of 2 ml.

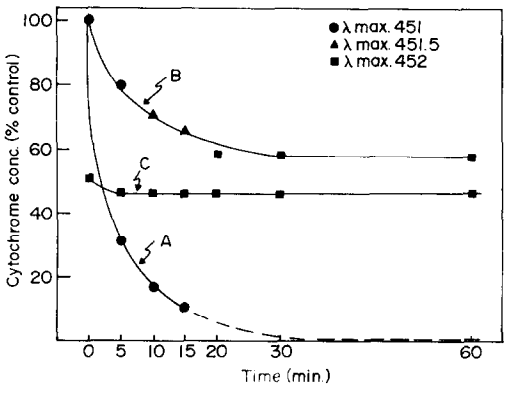

Fig. 3. Effect of 4-methylbenzaldehyde on the destruction of pulmonary cytochrome P-450 by cumene hydroperoxide: (A) microsomes incubated with cumene hydroperoxide (0.03 mM); (B) microsomes incubated with cumene hydroperoxide (0.03 mM) plus 4-methylbenzaldehyde (1.0 mM); and (C) microsomes preincubated with 4-methylbenzaldehyde (1.0 mM) plus NADPH for 20 min, followed by the addition of cumene hydroperoxide at time 0. The results shown were reproduced in a number of experiments.

P-420 was observed with the purified cytochromes.

Our results indicated that the partial destruction of cytochrome P-450 by 4-methylbenzaldehyde and the production of some protective factor were both mediated by cytochrome P-450. Therefore it was of interest to determine if cumene hydroperoxide, a compound known to mediate cytochrome P-450-catalyzed reactions and to destroy the cytochrome [19], could mediate the formation of the protective factor from 4-methylbenzaldehyde and thereby inhibit the cumene hydroperoxide-elicited destruction of the cytochrome. When cumene hydroperoxide was incubated with rabbit pulmonary microsomes, total destruction of the cytochrome P-450 took place within 20 min (Fig. 3). Addition of 4-methylbenzaldehyde to the incubations decreased the rate of destruction and resulted in a maximum cytochrome loss of 43 per cent (Fig. 3). In incubations containing cumene hydroperoxide and 4-methylbenzaldehyde, a shift in the λ_{max} of the cytochrome spectrum was observed; with cumene hydroperoxide alone, no spectral shift was observed. The addition of cumene hydroperoxide to microsomes that had been incubated with 4-methylbenzaldehyde and NADPH for 15 min had little effect on the remaining cytochrome P-450 (Fig. 3). Lipid peroxidation did not appear to play a role in the destruction of cytochrome P-450 in rabbit pulmonary microsomes by either cumene hydroperoxide or 4-methylbenzaldehyde. Analysis for this activity was negative in all incubations.

DISCUSSION

Rabbit pulmonary cytochrome P-450 can be partially destroyed *in vivo* and *in vitro* by 4-methylbenzaldehyde. The loss of cytochrome P-450 *in vitro* can be accounted for by an equal loss of heme. Although a previous report suggested that cytochrome P-420 is produced by this reaction [7], we were unable to detect the formation of P-420 in hemoglobin-free microsomes or in purified systems. Two possible explanations for this apparent discrepancy are: (1) cytochrome P-420 is formed, prior to the loss of heme, and detected in some systems due to differences in its stability; and (2) methemoglobin is formed by the reaction in hemoglobin-contaminated microsomes. This would result in an increase in absorbance near 420 nm (not due to the formation of cytochrome P-420) when the method of Estabrook *et al.* [14] is used for the determination of cytochrome P-450 [20]. The use of microsomes from perfused lungs eliminates this potential problem.

In vitro, the destructive reaction, which requires the presence of NADPH and O_2 or cumene hydroperoxide, occurs in microsomes, solubilized microsomes, and purified reconstituted systems. These results demonstrate that the effect of 4-methylbenzaldehyde on cytochrome P-450 is mediated by a P-450-dependent reaction.

Selective destruction of one of the forms of pulmonary cytochrome P-450 does not account for the loss observed either *in vivo* or *in vitro*. Although results obtained with microsomes showed a greater loss of cytochrome II than I (about 2:1), equal destruction of both forms occurred when rabbits or reconstituted systems were treated with 4-methylbenzaldehyde. The lack of complete destruction appears to be due to some factor produced during the initial reaction which protects against further destruction. The supernatant fraction from incubations of microsomes, 4-methylbenzaldehyde, NADPH, and O_2 completely inhibits the 4-methylbenzaldehyde-mediated destruction of the cytochrome in microsomes prepared from treated rabbits. Supernatant fractions from control incubations have no such effect. Furthermore, additional cytochrome can be destroyed after removal of the supernatant fraction and treatment of the pellet with 4-methylbenzaldehyde, NADPH and O_2. Why the supernatant fraction has no effect on the initial destruction of the cytochrome in microsomes prepared from untreated rabbits is not clear.

Although selective destruction of one form of pulmonary cytochrome P-450 cannot be demonstrated, the effect of 4-methylbenzaldehyde on each form is different. The λ_{max} of the ferrous cytochrome P-450-carbon monoxide difference spectrum of form I was altered by 4-methylbenzaldehyde in a reconstituted system. The spectral shift from 452 to 454 nm was also observed for cytochrome I recovered from microsomal incubations, and this probably accounted for the spectral shift seen in the spectrum from microsomes. No shift in the λ_{max} of cytochrome II occurred. It is possible that the cytochrome I preparation actually contains two forms of P-450, one of which is destroyed by 4-methylbenzaldehyde. This seems unlikely, however, because cytochrome I preparations from 4-methylbenzaldehyde-treated and untreated rabbits have identical spectra. The spectral shift may result from a metabolite–cytochrome complex which does not remain intact when microsomes are prepared from treated rabbits. The formation of a metabolite–cytochrome complex could account for the observation that the cytochrome remaining in microsomes after incubation with 4-methylbenzaldehyde is not readily destroyed, while the cytochrome in microsomes from treated rabbits is.

A number of compounds are known which destroy cytochrome P-450 either by removing the heme [21, 22] or by converting it to cytochrome P-420 [23–27].

It has been reported that the destructive effect of some compounds, like carbon tetrachloride [28], is due to the initiation of lipid peroxidation. This does not appear to be the case with 4-methylbenzaldehyde or cumene hydroperoxide in rabbit pulmonary microsomes.

The pulmonary-specific effect of 4-methylbenzaldehyde suggests that this compound could prove useful in attempts to elucidate the functions of cytochrome P-450 in the lung. Until the functions of this enzyme are clearly established, an evaluation of the potential consequences of exposure to *p*-xylene cannot be made. However, it seems unlikely that the destruction of 50 per cent of the pulmonary cytochrome P-450 could occur and not have any biological significance.

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