Cumene hydroperoxide-dependent oxidation of *NNN'*-tetramethyl-*p*-phenylenediamine and 7-ethoxycoumarin by cytochrome *P*-450

Comparison between the haemoproteins from liver and olfactory tissue

Celia J. REED* and Francesco DE MATTEIS

MRC Toxicology Unit, MRC Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

The interaction of cytochromes *P*-450 of the liver and olfactory epithelium of male hamsters with cumene hydroperoxide (CHP) has been characterized with regard to the ability of CHP to (1) support 7-ethoxycoumarin-*O*-de-ethylase (ECOD) activity, (2) support the oxidation of *NNN'*-tetramethyl-*p*-phenylenediamine (peroxidase activity) and (3) cause inactivation of cytochrome *P*-450. In the liver, CHP was found to support both ECOD and peroxidase activities while causing only minimal inactivation of cytochrome *P*-450. In contrast, in the olfactory epithelium CHP was virtually unable to support ECOD activity, peroxidase activity was 4-fold greater than in the liver, and extensive inactivation of cytochrome *P*-450 occurred. The reasons for these differences have been investigated with particular reference to the mode of cytochrome *P*-450-catalysed decomposition of CHP, that is, via homolytic or heterolytic cleavage of the hydroperoxide dioxygen bond. In both tissues, cumenol (2-phenylpropan-2-ol) was the major product of CHP decomposition detected. The radical scavenger nitrosobenzene inhibited cumenol formation by 84% in the olfactory epithelium, but by only 38% in the liver. This may indicate that dioxygen-bond scission occurs predominantly homolytically in the nasal tissue, whereas there is a balance between homolysis and heterolysis in the liver. It is suggested that the inability of CHP to support ECOD activity in the olfactory epithelium and the extensive inactivation of cytochrome *P*-450 that it causes both stem from decomposition of the hydroperoxide occurring homolytically rather than heterolytically in this tissue.

INTRODUCTION

In addition to its role as a mono-oxygenase, catalysing oxygen and NADPH-dependent substrate hydroxylations, cytochrome *P*-450 is also able to function as a peroxidase or a peroxygenase in reactions supported by hydroperoxides and other artificial oxygen donors. As a peroxidase, cytochrome *P*-450 couples the decomposition of peroxides with one-electron oxidation of *NNN'*-tetramethyl-*p*-phenylenediamine (TMPD) (Hrycay & O'Brien, 1971), NADPH (Hrycay & O'Brien, 1973), diaminobenzidine (O'Brien, 1978), alcohols (Rahimtula & O'Brien, 1977), phenols (O'Brien, 1978) and other peroxidase substrates. The substrate specificity for the hydroperoxide is relatively broad and includes linoleic acid hydroperoxide (Hrycay & O'Brien, 1971), cumene hydroperoxide (CHP) (Hrycay & O'Brien, 1971), various steroid hydroperoxides (Hrycay & O'Brien, 1972) and H₂O₂ (Hrycay & O'Brien, 1971). As a peroxygenase, cytochrome *P*-450 catalyses reactions in which one of the peroxide oxygens is incorporated into the substrate, and which include *O*-dealkylation (Rahimtula & O'Brien, 1975), amine oxidation (Kadlubar *et al.*, 1973), aromatic hydroxylation (Rahimtula & O'Brien, 1974) and *N*-demethylation (Jeffery *et al.*, 1977).

In vitro, the prosthetic haem of cytochrome *P*-450 is rapidly degraded by organic hydroperoxides to,

primarily, hematinic acid and methylvinylmaleimide (Schaefer *et al.*, 1985). The precise mechanism of this destruction is unclear, but may involve oxidation of essential thiol groups by either the hydroperoxide (Hrycay & O'Brien, 1971) or peroxy radicals formed during decomposition of the hydroperoxide (O'Brien, 1978).

In a previous paper (Reed *et al.*, 1988) we described the extremely rapid, CHP-dependent oxidation of TMPD by cytochrome *P*-450 of the hamster olfactory epithelium. In the present study we have compared hamster olfactory and hepatic cytochromes *P*-450 with regard to (a) their ability to catalyse a CHP-dependent peroxygenase reaction (the *O*-de-ethylation of 7-ethoxycoumarin) and (b) their sensitivity to CHP-dependent inactivation. We have also explored various explanations for the unusually high peroxidase activity of the olfactory cytochrome *P*-450.

EXPERIMENTAL

Materials

7-Ethoxycoumarin, NADPH, TMPD, catalase, horse heart cytochrome *c* and acetophenone were obtained from Sigma Chemical Co.; glucose 6-phosphate, glucose-6-phosphate dehydrogenase and glucose oxidase were

Abbreviations used: TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine; CHP, cumene hydroperoxide; ECOD, 7-ethoxycoumarin-*O*-de-ethylase.

* Present address and address for correspondence and reprint requests: Department of Pharmacy, University of Manchester, Oxford Road, Manchester, U.K.

purchased from Boehringer-Mannheim; 7-hydroxycoumarin, 2-phenylpropan-2-ol (cumenol) and nitrosobenzene from Aldrich Chemical Co.; cumene hydroperoxide from Fluka AG, Buchs, Switzerland; methanol and tetrahydrofuran (both h.p.l.c. grade) from BDH Chemicals and from Rathburn Chemicals respectively; other chemicals used were of the highest quality available commercially.

Methods

Male Syrian hamsters were between 6 and 8 weeks old and were allowed food and water *ad libitum*. Animals were killed either by decapitation or by a dose of Expiral (sodium pentobarbitone, 200 mg/ml) given by the intraperitoneal route (0.3 ml/100 g) and followed by decapitation while the animals were under deep anaesthesia. Olfactory epithelium and liver were obtained and microsomes prepared as described previously (Reed *et al.*, 1986).

7-Ethoxycoumarin *O*-de-ethylase (ECOD), NADPH:cytochrome *c* reductase and cytochrome *P*-450-dependent peroxidase activities were measured as described previously (Reed *et al.*, 1986, 1988). In order to estimate rates of CHP-supported ECOD activity, product formation had to be measured after 30 s incubation periods, since the reaction was non-linear at longer incubation times (presumably due to inactivation of the cytochrome *P*-450). To minimize the errors inherent in such short incubations, the following precautions were taken: (i) samples were equilibrated to 37 °C before the addition of prewarmed NADPH-generating system or 7-ethoxycoumarin; (ii) after addition of reagents, samples were vortex-mixed briefly to ensure complete and rapid mixing and were immediately replaced at 37 °C; (iii) after addition of 1 M-HCl to terminate the reaction, samples were vortex-mixed and placed on ice.

When incubations were carried out under hypoxic conditions, oxygen was removed from the system by a combination of bubbling all solutions with nitrogen and including an oxygen-scavenging system (final concentra-

tions: D-glucose, 60 mM; glucose oxidase, 12.5 units/ml; and catalase, 600 units/ml). All flasks and tubes were fitted with rubber stoppers, and addition or removal of reagents was with a syringe through the rubber stopper.

H.p.l.c. was carried out on a Waters Associates μ Bondapak C_{18} column according to the method of Griffin & Ting (1978), except that both acetophenone and cumenol were detected at 210 nm. CHP was redistilled under vacuum for those experiments involving h.p.l.c. analysis.

Protein concentrations were estimated by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The probability, *P*, of the significance of the difference between two sets of results was calculated by using Student's *t* test. Values of *P* < 0.05 were considered to indicate that two sets of results were statistically significantly different.

RESULTS

Cumene hydroperoxide-supported ECOD activity

In the liver, CHP was able to support ECOD activity at a rate that was 62 % of that seen with NADPH (Table 1, sample 2). In contrast, CHP was virtually unable to support ECOD activity in the olfactory epithelium (Table 1, sample 7). It will be noted that the rates of NADPH-supported ECOD activity shown in Table 1 are considerably higher for both tissues than those reported previously (Reed *et al.*, 1986) and those shown in Table 2 (below). The reason for these discrepancies is unknown, but the results of Table 1 were from four separate experiments conducted over a period of 4 weeks, which gave consistent results; the percentage activities, if not the absolute values, were reproducible at a later date. Several possible explanations for this lack of olfactory activity were explored: (a) interference in the fluorescence characteristics of the product, 7-hydroxycoumarin, (b) metabolism of 7-ethoxycoumarin by an alternative pathway, and (c) extensive inactivation of the olfactory

Table 1. CHP-supported ECOD activity in hepatic and olfactory microsomes from male hamsters

Microsomes were incubated aerobically with substrate (S; 0.1 mM) in the presence of NADPH and/or CHP (75 μ M) for 30 s. NADPH was supplied by a NADPH-generating system (De Matteis & Sparks, 1973). Where preincubations were carried out, the microsomes were first incubated for 30 s with CHP, CHP and NADPH, or CHP and substrate, and then the other reagents were added for a further 30 s incubation. Results are means $\pm$ S.D. of at least three determinations, each on tissue pooled from two animals.

Tissue	Sample	Incubation conditions	ECOD activity	
			(nmol/min per mg of protein)	(%)
Liver	1	S + NADPH, 30 s	21.13 $\pm$ 9.00	100
	2	S + CHP, 30 s	13.15 $\pm$ 4.21	62
	3	S + NADPH + CHP, 30 s	13.16 $\pm$ 4.70	62
	4	CHP, 30 s, then S + NADPH, 30 s	10.32 $\pm$ 1.95	49
	5	CHP + NADPH, 30 s, then S, 30 s	9.43 $\pm$ 2.43	45
Olfactory epithelium	6	S + NADPH, 30 s	125.74 $\pm$ 21.61	100
	7	S + CHP, 30 s	1.76 $\pm$ 0.82	1
	8	S + NADPH + CHP, 30 s	37.92 $\pm$ 11.06	30
	9	CHP, 30 s, then S + NADPH, 30 s	27.88 $\pm$ 9.21	22
	10	CHP + NADPH, 30 s, then S, 30 s	27.26 $\pm$ 6.34	22
	11	CHP + S, 30 s, then NADPH, 30 s	35.98 $\pm$ 8.99	29

Fig. 1. CHP-supported ECOD activity in liver and olfactory epithelium

Suspensions of hepatic (○, ●) and olfactory (□, ■) microsomes were diluted to give samples of comparable cytochrome *P*-450 concentration. The reaction was initiated by the addition of 75 μ M-CHP and incubations were for 30 s. Assay of product formation was as described under 'Methods' section. Open and closed symbols represent two separate experiments, each on tissue pooled from five hamsters. Note that results in liver were approx. 60% of the rates seen with NADPH, whereas in the olfactory epithelium the results given were only 1–5% of the corresponding rates seen with NADPH.

cytochrome *P*-450 due to the higher hydroperoxide-to-haem ratio in the olfactory samples as compared with the hepatic.

(a) The fluorescence spectra of NADPH- and CHP-generated 7-hydroxycoumarin were found to be identical, and were also identical with that of an authentic sample of 7-hydroxycoumarin. Furthermore, incubation of 7-hydroxycoumarin with 75 μ M-CHP at 37 °C for 5 min in either the presence or absence of hepatic or olfactory microsomes had no effect upon the intensity of the fluorescence of the 7-hydroxycoumarin (results not shown).

(b) When hepatic microsomes were incubated with 5 μ M-7-ethoxycoumarin at 37 °C in a spectrofluorimeter cell, a decrease in fluorescence (excitation 326 nm, emission 390 nm) was observed after the addition of 75 μ M-CHP, and this was assumed to be due to substrate utilization. No such decrease could be detected in samples containing olfactory microsomes (results not shown).

(c) CHP-supported ECOD activity was also measured in the liver and olfactory epithelium at comparable cytochrome *P*-450 concentrations. Product formation was linearly related to cytochrome *P*-450 concentration in both tissues, but was consistently lower in the olfactory epithelium as compared with the liver (Fig. 1).

Cumene hydroperoxide-dependent inactivation of cytochrome *P*-450

The inability of CHP to support ECOD activity in the olfactory epithelium does not appear to be due to a lack of interaction between the peroxide and the haemoprotein, as evidenced by the considerable inactivation of olfactory cytochrome *P*-450 caused by CHP, and also by the ability of olfactory microsomes to metabolize cumene hydroperoxide (see below). When olfactory microsomes were preincubated with CHP before estimation of NADPH-supported ECOD activity, there was a marked (78%) loss of activity, as compared with samples which

received no preincubation [Table 1, sample 9 ($n = 4$) compared with sample 6 ($n = 4$), $P < 0.01$]. Furthermore, incubation of olfactory microsomes with substrate, NADPH and CHP resulted in considerably lower ECOD activity than incubation with substrate and NADPH alone [Table 1, sample 8 ($n = 4$) compared with sample 6 ($n = 4$), $P < 0.01$]. Since CHP was found to have no effect upon (i) levels of NADPH or (ii) NADPH:cytochrome *P*-450 reductase (as judged by the ability to reduce cytochrome *c*) (results not shown), these observations suggest that CHP-dependent destruction of cytochrome *P*-450 was occurring. The presence of either 7-ethoxycoumarin or NADPH during the preincubation with CHP afforded no significant protection against CHP-dependent loss of ECOD activity (Table 1, samples 10 and 11 compared with sample 9). Some CHP-dependent loss of ECOD activity was also observed in the liver [Table 1, sample 4 ($n = 3$) compared with sample 1 ($n = 4$)], but this loss was not statistically significant ($P > 0.05$). These differences between the two tissues were apparent even when incubations were carried out at comparable protein concentrations, and thus do not appear to be dependent on the different CHP/protein ratios usually encountered when incubating microsomes from the two tissues. We conclude that olfactory cytochrome *P*-450 is more readily destroyed by CHP than hepatic cytochrome *P*-450. However, the inability of CHP to support ECOD activity with microsomes from nasal tissue cannot be due entirely to rapid destruction of cytochrome *P*-450, as residual activity could be readily demonstrated in the presence of NADPH (Table 1, sample 9). It is more likely that CHP interacts in a different way with the cytochromes from the two tissues and that the mode of interaction with the olfactory cytochrome is such that little ECOD activity occurs.

To investigate the role of lipid peroxidation in this inactivation of cytochrome *P*-450, the ability of CHP to support ECOD activity and the effect of preincubation

Table 2. CHP-supported ECOD activity in hepatic and olfactory microsomes from male hamsters: effect of anaerobic incubation

Anaerobic preparation and assay of production formation was as described under 'Methods'. All preincubations or incubations were for 30 s. NADPH was provided by an NADPH-generating system, and CHP was used at a final concentration of 75 μ M. Results are means of two separate determinations (individual values in parentheses), each on tissue pooled from three animals.

Sample	Incubation conditions	ECOD activity			
		Liver		Olfactory epithelium	
		(nmol/min per mg of protein)	(%)*	(nmol/min per mg of protein)	(%)*
1	Aerobic incubation with NADPH	3.78 (3.90; 3.65)		33.63 (33.06; 34.19)	
2	Anaerobic preparation; then aerobic incubation with NADPH	2.53 (2.70; 2.36)	67	21.33 (27.06; 15.60)	63
3	Anaerobic incubation with NADPH	0.75 (0.71; 0.78)	30	3.35 (4.69; 2.01)	16
4	Aerobic incubation with CHP	2.16 (2.32; 1.99)	57	1.47 (1.19; 1.74)	4
5	Anaerobic incubation with CHP	1.42 (1.65; 1.19)	56	0.99 (1.19; 0.79)	5
6	Aerobic preincubation with CHP, then aerobic incubation with NADPH	1.11 (1.20; 1.01)	29	7.49 (9.84; 5.14)	22
7	Anaerobic preincubation with CHP, then aerobic incubation with NADPH	0.77 (0.74; 0.80)	30	2.43 (2.81; 2.05)	11

* Results for aerobic incubations (samples 2, 4 and 6) are compared with sample 1, whereas those for anaerobic incubations (samples 3, 5 and 7) are compared with sample 2.

with CHP on NADPH-supported ECOD activity were determined under anaerobic conditions. To achieve anaerobiosis, all samples and solutions were extensively bubbled with nitrogen in the presence of an oxygen-scavenging system (see under 'Methods' for details). This procedure caused loss ($\sim 35\%$) of ECOD activity in both liver and olfactory epithelium (compare, in Table 2, sample 2 with sample 1) but complete anaerobiosis was apparently not achieved as NADPH was still able to support ECOD activity to some extent (Table 2, sample 3). Such hypoxic conditions afforded no protection against CHP-dependent inactivation of cytochrome *P*-450 in either the liver or the olfactory tissue, nor was there any effect upon CHP-supported ECOD activity in either tissue (Table 2).

Peroxidase activity of cytochrome *P*-450

We have previously reported (Reed *et al.*, 1988) that, utilizing CHP as the peroxide substrate, the olfactory cytochrome *P*-450 is extremely active in the oxidation of TMPD, a reaction of a peroxidase nature. In liver microsomes, peroxidase activity was found to be 17.3 nmol/min per mg of protein (11.4 nmol/min per nmol of cytochrome *P*-450), whereas, in olfactory microsomes, peroxidase activity was 76.7 nmol/min per mg of protein (123.6 nmol/min per nmol of cytochrome *P*-450). We have now attempted to explore the reasons for this tissue-specific difference in peroxidase activity.

The peroxidase activity of both hepatic and olfactory microsomes appeared to obey typical Michaelis-Menten kinetics. In both tissues, Lineweaver-Burk plots of activity at increasing CHP concentration were mono-

phasic, and the calculated K_m values for the two tissues were not significantly different, being $11.4 \pm 5.5 \mu$ M in the liver and $5.7 \pm 2.5 \mu$ M in the olfactory epithelium (means $\pm$ S.D. for three observations in both cases).

Decomposition of cumene hydroperoxide

The iron-catalysed decomposition of CHP can proceed via either heterolytic or homolytic scission of the oxygen-oxygen bond [Blake & Coon (1981*a,b*) and references cited therein]. Heterolytic cleavage results in cumenol formation, homolytic scission forms a cumyloxy-radical intermediate and, ultimately, either cumenol or acetophenone plus a methyl radical (Scheme 1). The products of CHP arising from the interaction of this hydroperoxide with hepatic and olfactory microsomes were analysed by h.p.l.c.

In both tissues CHP was metabolized almost exclusively to cumenol. Acetophenone production could be detected, but at a rate that was $< 1\%$ that of cumenol formation, and which was below the level of sensitivity of the assay under the experimental conditions routinely used. Cumenol production was inhibited by KCN (10 mM) in both the liver (77%) and the olfactory epithelium (67%) and was thus considered to be cytochrome *P*-450-dependent.

If results were expressed per mg of microsomal protein, the hepatic rate of cumenol formation was double that observed in the olfactory epithelium (Table 3). Thus the specific activity [nmol of cumenol formed/min per nmol of cytochrome *P*-450, calculated by using concentrations of cytochrome *P*-450 reported previously (Reed *et al.*, 1986)] was virtually identical in the two tissues, being

Scheme 1. Homolytic and heterolytic pathways of haem-catalysed decomposition of CHP

17.7 in the liver and 18.6 in the olfactory epithelium. Inclusion of TMPD in the incubations caused a 2-fold increase in the hepatic rate of cumenol formation (Table 3). In contrast, TMPD increased olfactory cumenol production by a factor of 17. In both tissues rates of cumenol formation in the presence of TMPD were

considerably lower than rates of TMPD oxidation (peroxidase activity), and the ratio of the two activities was found to be very similar in the liver and olfactory epithelium [(B)/(A) in Table 3]. However, it should be noted that when the TMPD-dependent cumenol formation was taken (by subtracting the appropriate rate in the absence of TMPD), then ratios of peroxidase activity to cumenol formation became 3.0 and 1.6 for liver and olfactory epithelium respectively.

The above experiments cannot differentiate between cumenol formed by heterolytic cleavage of the oxygen-oxygen bond and that arising from homolytic scission. Terabe & Konaka (1972) demonstrated that the cumyloxy radical can be efficiently trapped by nitrosobenzene in organic solvents, and Griffin (1980) reported similar results in a study of aqueous systems containing haemoproteins. Thus it was considered that nitrosobenzene should inhibit production of cumenol via the cumyloxy-radical intermediate (homolytic pathway), but not via the heterolytic pathway. Table 4 shows the effect of nitrosobenzene on the decomposition of CHP to cumenol effected by hepatic and olfactory microsomes. This reaction was inhibited in both tissues, but to a much greater extent in the olfactory epithelium (85 %) than in the liver (38 %).

DISCUSSION

The results described above highlight a major difference between the cytochromes *P*-450 of the liver and the olfactory epithelium, namely their mode of interaction with the organic hydroperoxide CHP. In the liver CHP is able to support ECOD activity; in the olfactory epithelium it is not. In the liver CHP causes some inactivation of cytochrome *P*-450; in the olfactory epithelium this destruction is considerably more substantial. Hepatic cytochrome *P*-450 can utilize CHP for the oxidation of TMPD, but the olfactory haemoprotein is far more efficient at this reaction. We would like to suggest that these three facets of the interaction with CHP are closely interrelated and may stem from differences in the cytochrome *P*-450-catalysed peroxide dioxygen-bond cleavage.

The virtual inability of CHP to support ECOD activity in the olfactory epithelium appears to be an intrinsic feature of the catalytic mechanism of olfactory cytochrome *P*-450. The inactivation of ECOD activity after

Table 3. Cumenol formation and peroxidase activity in hepatic and olfactory microsomes from male hamsters

Cumenol formation was measured as described under 'Methods'. Incubations were for 1 min at 37 °C and contained 75 μM -CHP and, where appropriate, 0.2 mM-TMPD. Peroxidase activity was measured at 37 °C with 75 μM -CHP and 0.2 mM-TMPD, to allow direct comparison with cumenol formation. Values given are initial rates, which remained linear for at least 1 min. Results for cumenol formation in the absence of TMPD are means $\pm$ S.D. of three separate determinations, each on tissue pooled from four animals. Other results are means $\pm$ S.D. of four determinations, each on tissue from a single animal.

Tissue	(A) Cumenol formation (nmol/min per mg of protein)		(B) Peroxidase activity (nmol/min per mg of protein)	Ratio (B)/(A)
	- TMPD	+ TMPD		
Liver	20.32 $\pm$ 4.33	45.39 $\pm$ 11.15	75.77 $\pm$ 18.32	1.67 $\pm$ 0.07
Olfactory epithelium	10.78 $\pm$ 2.27	180.60 $\pm$ 34.28	268.82 $\pm$ 65.19	1.49 $\pm$ 0.18

Table 4. Effect of nitrosobenzene on cumenol formation by hepatic or olfactory microsomes incubated with CHP

Cumenol formation was measured as described under 'Methods'. Incubations contained 75 μ M-CHP, 0.2 mM-TMPD and, where appropriate, 1 mM-nitrosobenzene and were for 1 min at 37 °C. Results are means $\pm$ S.D. of four separate determinations, each on tissue from a single animal.

Tissue	Cumenol formation (nmol/min per mg of protein)		Inhibition by nitrosobenzene (%)
	– Nitrosobenzene	+ Nitrosobenzene	
Liver	33.88 $\pm$ 6.16	20.98 $\pm$ 4.46	38 $\pm$ 4
Olfactory epithelium	142.07 $\pm$ 24.81	23.41 $\pm$ 11.51	84 $\pm$ 6

incubation with CHP (Table 1) and the linear relationship between CHP-supported ECOD activity and olfactory cytochrome *P*-450 concentration (Fig. 1) are both evidence for an interaction between the hydroperoxide and the haemoprotein. However, the nature of this interaction appears to be such that mono-oxygenation does not readily occur. One possible explanation is that the active site of the olfactory cytochrome *P*-450 is such that CHP and 7-ethoxycoumarin cannot be accommodated concurrently. Blake & Coon (1981a) found that CHP-dependent hydroxylation of toluene by rabbit cytochrome *P*-450 LM₂ is sensitive to substitution on both the peroxide and the toluene, and concluded that the organic functionality of the peroxide is still present in the oxidizing agent responsible for hydroxylation. Kinetic analyses with rabbit liver cytochromes *P*-450 have demonstrated an 'ordered Bi Bi' kinetic sequence in which the substrate binds before the peroxide (Koop & Hollenberg, 1980). If such kinetics also occur with olfactory cytochromes *P*-450, then the CHP-dependent inactivation of cytochrome *P*-450 in the presence of substrate (Table 1, sample 11) suggests that both substrate and peroxide can be accommodated in the active site.

Blake & Coon (1980, 1981b) described the interaction of rabbit cytochrome *P*-450 LM₂ (*P*-450) with organic hydroperoxides (XOOH) to form two spectral intermediates, C and D:

In their model, C is on the route to substrate hydroxylation, whereas D is an inconsequential side product. Should a similar situation exist for all cytochromes *P*-450, it is conceivable that, in the olfactory epithelium, the formation of D is energetically favourable in comparison to the de-ethylation of 7-ethoxycoumarin.

It should be noted that the olfactory epithelium is not the only extrahepatic tissue in which peroxides are unable to support mono-oxygenation reactions. Adrenal, kidney, placenta and lung are all unable to utilize hydroperoxides for cytochrome *P*-450-dependent substrate hydroxylation (O'Brien, 1978).

In addition to their ability to support drug metabolism, organic hydroperoxides also cause inactivation of both microsomal and purified cytochromes *P*-450 (Hrycay & O'Brien, 1971, 1972; Nordblom *et al.*, 1976; Jeffery *et al.*, 1977), and degradation of the haem prosthetic group. This inactivation of cytochrome *P*-450 may involve the

oxidation of essential thiol groups by either the hydroperoxide (Hrycay & O'Brien, 1971) or peroxy radicals formed during decomposition of the hydroperoxide (O'Brien, 1978). Generalized lipid peroxidation may be partly responsible for the loss of cytochrome *P*-450 activity, and protection is afforded by antioxidants, hydrogen donors (such as NADPH and TMPD) and some drug or steroid substrates (O'Brien, 1978). However, the extreme sensitivity of the olfactory cytochrome *P*-450 to destruction by CHP does not appear to be due to lipid peroxidation, as no protection was afforded by carrying out the incubation under hypoxic conditions (Table 2). The question remains as to why the olfactory cytochrome *P*-450 is so sensitive to CHP. One possibility is that this form of cytochrome *P*-450 may have a greater affinity for CHP than the cytochromes of the liver. However, no significant difference in K_m values for CHP was found between liver and olfactory microsomes, at least as measured by oxidation of TMPD. Alternatively the two groups of haemoproteins may interact with CHP to form different intermediates, with that formed by the olfactory cytochrome being the more reactive and thus more destructive.

If the cytochromes *P*-450 of the liver and olfactory epithelium interact with CHP to form different intermediates, then what is the nature of these intermediates? The iron-catalysed decomposition of organic hydroperoxides has been extensively studied (Kharasch *et al.*, 1950; Blake & Coon, 1981a; Lee & Bruce, 1985; Thompson & Wand, 1985; Wand & Thompson, 1986) and is generally accepted to proceed via either heterolytic or homolytic cleavage of the dioxygen bond (Scheme 1). The iron-oxo intermediate represented on the right hand side of Scheme 1 ($Fe^V=O$) is generally accepted as the iron-co-ordinated species arising from heterolytic dioxygen-bond cleavage (see the references in White & Coon, 1980). Homolytic scission may instead generate the equivalent of a hydroxyl radical, which remains co-ordinated to the protoporphyrin iron as $Fe^{IV}-OH$ [Scheme 1; and see White & Coon (1980) and references cited therein]. There is considerable evidence in the literature for hydroperoxide-supported mono-oxygenation reactions occurring by a mechanism involving heterolytic dioxygen-bond cleavage (Rahimtula *et al.*, 1974, 1978; Nordblom *et al.*, 1976; Gustafsson *et al.*, 1976; Akhrem *et al.*, 1977; McCarthy & White, 1983). On the other hand, homolysis appears to be the best candidate for destruction of cytochrome *P*-450. The $Fe^V=O$ intermediate formed during heterolysis is

generally considered to be similar to, or identical with, that formed during cytochrome *P*-450-catalysed reactions involving NADPH and O₂, and, since little significant enzyme inactivation occurs under normal turnover conditions, this intermediate is not inherently destructive. In contrast, homolysis results in the formation of radical species which are potentially extremely reactive.

With both hepatic and olfactory cytochromes *P*-450 cumenol was by far the main product of CHP decomposition found. In the olfactory epithelium, cumenol formation could be inhibited to greater than 80% by nitrosobenzene (Table 4). In contrast, hepatic cumenol production was inhibited by only 40% by nitrosobenzene (Table 4). This would be compatible with a predominantly homolytic pathway in the olfactory epithelium, with a more equal balance between the two pathways in the liver. We cannot exclude the possibility that the olfactory cytochrome *P*-450 itself might be more susceptible to inhibition or inactivation by nitrosobenzene than the liver cytochrome, as experiments designed to investigate this were hampered by interference with the ECOD assay by nitrosobenzene. All the results hitherto discussed could therefore be explained by a model in which heterolytic cleavage leads to 7-ethoxycoumarin *O*-de-ethylation, whereas homolytic cleavage causes destruction of cytochrome *P*-450. Thus, in the liver, where there is a balance between the two routes (in favour of heterolysis), we see both de-ethylation and, to a lesser extent, destruction. In the olfactory epithelium, homolysis predominates, resulting in very little ECOD activity and extensive inactivation of cytochrome *P*-450.

The question remains as to why the hepatic and olfactory cytochromes *P*-450 catalyse the decomposition of CHP via alternative pathways. Alkyl peroxides readily undergo homolytic oxygen-oxygen-bond scission and, unless the hydroperoxide contains a good leaving group (e.g. a peracid), such one-electron reduction predominates (Marnett *et al.*, 1986). However, there is a shift from homolytic to heterolytic dioxygen-bond cleavage of alkyl hydroperoxides by metallo-tetraphenylporphyrin complexes when the trans-axial ligand is made imidazole (Mansuy *et al.*, 1984), and the strong electron-donor property of the thiolate ligand may also facilitate heterolytic cleavage. Poulos & Kraut (1980) have identified charged groups at the active site of cytochrome *c* peroxidase which facilitate heterolytic dioxygen-bond cleavage, and a similar situation may exist with some cytochromes *P*-450. It is conceivable that the active sites of olfactory cytochromes *P*-450 lack the structural features (i.e. appropriately positioned amino acid residues) necessary to facilitate heterolytic dioxygen-bond cleavage; alternatively the thiolate ligand may be more readily displaced in the presence of an organic peroxide.

The extremely rapid peroxidatic oxidation of TMPD occurring in olfactory microsomes remains unexplained. However, it is possible that the cumyloxy radical may oxidize TMPD very effectively; Griffin *et al.* (1980) have proposed a similar homolytic mechanism for the *P*-450-catalysed CHP-dependent oxidation of aminopyrine to its characteristic aminopyrine radical species.

In conclusion, we have shown that hepatic and olfactory cytochromes *P*-450 interact with CHP with very different consequences, and have suggested that the basic reason for these differences lies in the mode of CHP dioxygen-bond cleavage. The validity of this hypothesis

and its implications regarding the activation of molecular oxygen by olfactory cytochrome *P*-450 remains to be elucidated.

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