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CYTOCHROME P-450 DEPENDENT METABOLIC ACTIVATION OF 1-NAPHTHOL TO NAPHTHOQUINONES AND COVALENT BINDING SPECIES

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Abstract—1-Naphthol was metabolised by a fully reconstituted cytochrome P-450 system in the presence of NADPH to methanol-soluble and covalently bound products. The formation of 1,4-naphthoquinone, the major methanol-soluble product at early time points, showed an almost total dependence on cytochrome P-450, NADPH-cytochrome P-450 reductase and NADPH, and to a lesser extent on dilauroylphosphatidylcholine. The metabolism was rapid and detectable levels of 1,4-naphthoquinone were formed within 30 sec. 1,4-Naphthoquinone formation was dependent on the concentration of both cytochrome P-450 (0.05–0.04 μM) and 1-naphthol (5–50 μM). Whereas 1,4-naphthoquinone was the major product observed at early time points, additional products were observed after prolonged incubation. In the absence of NADPH and NADPH-cytochrome P-450 reductase, 1-naphthol was metabolised, in a cumene hydroperoxide- and cytochrome P-450-dependent reaction, to 1,2- and 1,4-naphthoquinone and covalently bound products. Glutathione and ethylenediamine inhibited both the NADPH- and cumene hydroperoxide-dependent formation of covalently bound products. These data show that cytochrome P-450 catalyses the activation of 1-naphthol to naphthoquinone metabolites and covalently bound species, the latter most likely being derived from naphthoquinones.

Our recent studies have shown that 1-naphthol is toxic to freshly isolated rat hepatocytes [1]. As this toxicity was preceeded by glutathione depletion and potentiated by dicoumarol, a potent inhibitor of DT-diaphorase [2], we hypothesised that the toxicity of 1-naphthol was mediated via naphthoquinone formation [1, 3, 4]. Naphthoquinones may be toxic either by redox cycling, which generates reactive semiquinone intermediates and active oxygen species thus leading to a depletion of cellular glutathione (GSH), or by covalent binding to cellular macromolecules [5, 6].

In common with many chemicals the toxicity of 1-naphthol may be dependent on prior metabolic activation [7, 8]. Whilst the detoxification of 1-naphthol to 1-naphthyl-β-D-glucuronide and 1-naphthyl sulphate has been studied both *in vivo* and *in vitro* [9–11] far less is known of its possible Phase I metabolic activation to toxic products. A study by Hesse *et al.* [12] showed that [1-^{14}C]-1-naphthol is metabolised by rat liver microsomes in an NADPH-dependent reaction to covalently bound products and they suggested that naphthoquinones and/or naphthosemiquinones may be formed. In agreement with this suggestion, we found that 1-naphthol stimulated NADPH-dependent microsomal oxygen consumption and superoxide anion formation [3, 4]. Later we demonstrated by HPLC that in the presence of an NADPH-generating system, [1-^{14}C]-1-naphthol was metabolised by rat liver microsomes to methanol-soluble products, including, 1,4-naphthoquinone, and covalently bound species most likely derived from 1,4-naphthoquinone [13]. This 1,4-naphthoquinone most probably arose by autooxidation of 1,4-dihydroxynaphthalene. The formation of these products was inhibited by SKF 525-A, metyrapone and CO:O_2 (9:1) atmosphere strongly implicating a role for cytochrome P-450 in the metabolic activation of 1-naphthol [13]. Replacement of NADPH by cumene hydroperoxide led to the formation of 1,2- and 1,4-naphthoquinone as well as covalently bound products (Cohen, d'Arcy Doherty and O'Brien, unpublished observations), indicating that in common with many chemicals 1-naphthol may also be activated in peroxide-dependent microsomal reactions [14, 15].

In order to substantiate the role of cytochrome P-450 in the NADPH- and peroxide-dependent metabolism of 1-naphthol by rat liver microsomes we decided to study its metabolism using an isolated cytochrome P-450 preparation in a reconstituted system. This study provides definitive proof that 1-naphthol is metabolised by cytochrome P-450 to methanol-soluble naphthoquinone metabolites and covalent binding species. These results do not exclude the possibility that enzymes such as peroxidases (unpublished observations and M. T. Smith *et al.*, personal communication), may also be capable of activating 1-naphthol. These reactions may be important in mediating 1-naphthol toxicity to isolated rat hepatocytes.

MATERIALS AND METHODS

Materials

All chemicals were purchased from the Sigma

Chemical Co. (Poole, Dorset, U.K.) or the British Drug House Chemical Co. (Poole, Dorset, U.K.) unless otherwise stated. All solvents were obtained from May and Baker (Dagenham, U.K.) except for HPLC grade methanol which was obtained from Rathburn Chemicals (Peebleshire, Scotland). 1,2- and 1,4-Naphthoquinone were obtained from Fluka (Switzerland).

[1-^{14}C]-1-Naphthol (56.0 μCi/μmole) was obtained from Amersham International (Buckinghamshire, U.K.) and Aquasol for liquid scintillation counting from New England Nuclear (Edinburgh, Scotland).

Methods

Purification of cytochrome P-450 *and* NADPH-*cytochrome* P-450 *reductase.* Cytochrome P-450 was purified from the hepatic microsomes of male Wistar albino rats (150–200 g, University of Surrey Breeders) which had been pretreated with sodium phenobarbitone (0.1% w/v in their drinking water for 6 days) as previously described [16]. The cytochrome P-450 isoenzyme isolated represents the form eluting from hydroxyapatite and DEAE-Sephacel columns at 90 and 35 mM ionic strength respectively as described by Guengerich [17] and exhibited a monomeric mol. wt of 53,330 daltons as determined by calibrated sodium dodecyl sulphate polyacrylamide gel electrophoresis. The isoenzyme was purified to electrophoretic homogeneity, exhibited a specific content of 16–18 nmoles haemoprotein/mg protein and was catalytically competent as judged by the rapid turnover of the model substrate benzphetamine (200 nmoles formaldehyde formed/min/nmole cytochrome P-450). In addition, the cytochrome P-450 preparation was uncontaminated by NADPH-cytochrome P-450 reductase.

Intact, detergent-solubilised NADPH-cytochrome P-450 reductase was purified to electrophoretic homogeneity from the hepatic microsomes of phenobarbitone-induced rats by a modification [16] of the procedure described by Yasukochi and Masters [18]. The reductase exhibited a monomeric mol. wt of 78,000 daltons, a specific content of 11.8 nmoles/mg protein and a specific activity of 19 Units/mg protein at 25° (1 Unit of activity is defined as 1 μmole cytochrome reduced per min). In addition, the purified reductase preparation was free from contamination by cytochrome P-450.

Metabolism of [1-^{14}C]-1-*Naphthol.* [1-^{14}C]-1-Naphthol (5–100 μM, 56 μCi/μmole) was incubated at 37° in a shaking waterbath with sonicated L-α-dilauroylphosphatidylcholine (30 μg/ml), cytochrome P-450 (0.2 μM), NADPH-cytochrome P-450 reductase (0.6 U/ml), sodium deoxycholate (50 μg/ml), $MgCl_2$ (15 mM) and NADPH (5 mM) in 30 mM potassium phosphate, 24 mM Tris–HCl buffer, pH 7.5. The reaction was stopped at various times by the addition of 2 vol. of ice-cold methanol containing unlabelled 1-naphthol, 1,2- and 1,4-naphthoquinone as standards. Incubations containg cumene hydroperoxide (50 μM) instead of NADPH did not contain cytochrome P-450 reductase. Reactions were started by the addition of NADPH or cumene hydroperoxide. The methanol-soluble metabolites were analysed by HPLC following protein precipitation by centrifugation at 2000 *g* for 2 min. Analysis was carried out on an Altex ODS Ultrasphere column (25 cm) fitted with a pre-column packed with Ultrapack ODS. Samples (50 μl) were injected onto the column and eluted at a flow rate of 1 ml/min in a linear 15-min gradient from 40 to 90% methanol in water. The column eluate was continuously monitored at 254 or 330 nm and 0.5 ml fractions collected directly into scintillation vials. Typical elution times were 1,2-naphthoquinone 10.6, 1,4-naphthoquinone 14.5 and 1-naphthol 16.8 min. The radioactivity in the fractions was determined by liquid scintillation counting after addition of 3.5 ml of Aquasol to each vial. Selected samples were analysed under anaerobic HPLC conditions as previously described by Greenlee *et al.* [19]. The elution conditions were as described above except that the water contained 1% acetic acid, the solvents were bubbled with nitrogen, and ascorbate (18 mg/ml) was added to samples directly before injection onto the column. Under these conditions the quinones are converted to the corresponding hydroquinones and typical elution times were as follows: 1,4-dihydroxynaphthalene 10.0, 1,2-dihydroxynaphthalene 14.1 and 1-naphthol 16.7 min.

Determination of covalent binding. Incubations were prepared as described above except that bovine serum albumin (BSA) was included at a final concentration of 10 mg/ml. After methanol precipitation of the protein at the end of the incubation period, the amount of covalently bound material was assessed as previously described [13].

RESULTS

Metabolism of [1-^{14}C]-1-*naphthol to methanol-soluble products by cytochrome* P-450 *in the presence of* NADPH

Incubation of [1-^{14}C]-1-naphthol in the presence of a fully reconstituted cytochrome P-450 system and NADPH led to the formation of methanol-soluble products. HPLC analysis of these products showed that, at early time points, the predominant metabolite cochromatographed with 1,4-naphthoquinone whilst no significant amount of radioactivity coeluted with 1,2-naphthoquinone [Fig. 1(a)]. Further support for the identity of the major methanol-soluble metabolite as 1,4-naphthoquinone was obtained when, after reduction with ascorbate, it cochromatographed with authentic 1,4-dihydroxynaphthalene (results not shown). The formation of 1,4-naphthoquinone was highly dependent on cytochrome P-450 [Fig. 1(b)], NADPH-cytochrome P-450 reductase and NADPH and to a lesser extent on the presence of dilauroylphosphatidylcholine (Table 1). The metabolism was rapid with detectable levels of 1,4-naphthoquinone being formed within 30 seconds [Fig. 2(a)]. The rate of 1,4-naphthoquinone formation increased with the concentration of cytochrome P-450 [Fig. 2(b)] and increasing substrate concentration up to 50 μM [Fig. 2(c)]. The apparent K_m of 1-naphthol for this reaction was 17 μM which is in good agreement with that previously obtained with rat liver microsomal preparations [12, 13].

When the fully reconstituted cytochrome P-450 system was incubated in the presence of glutathione

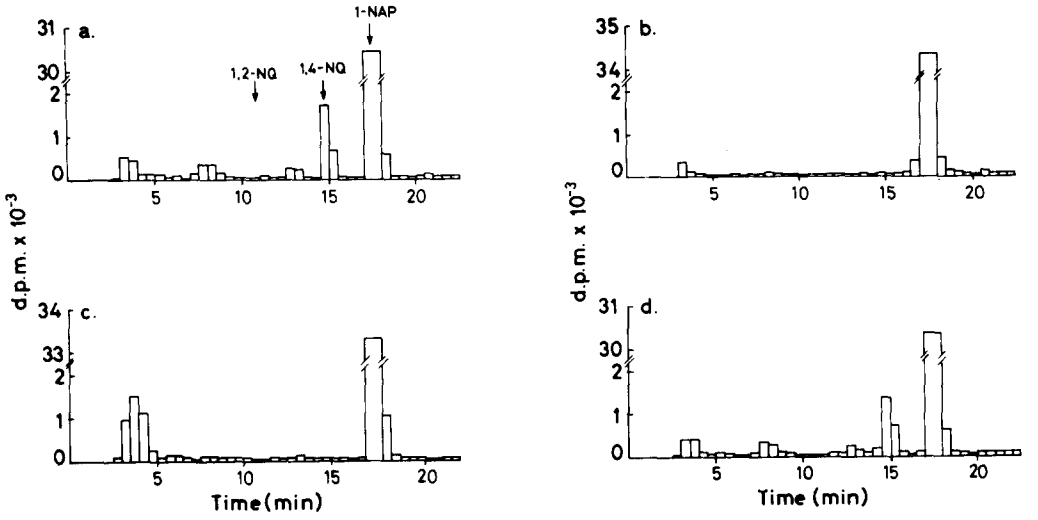

Fig. 1. HPLC separation of the metabolites of [1-^{14}C]-1-naphthol formed in the reconstituted cytochrome P-450 system in the presence of NADPH. [1-^{14}C]-1-Naphthol (20 μM) was incubated for 2 min at 37° with a reconstituted monooxygenase system containing NADPH-cytochrome P-450 reductase (0.6 U/ml), dilauroylphosphatidylcholine (30 μg/ml) and NADPH (5 mM) in the presence (a), (c), (d) or absence (b) of cytochrome P-450 (0.2 μM). Glutathione (1 mM) [Fig. 1(c)] or ethylenediamine (20 mM) [Fig. 1(d)] were included in some incubations. The reactions were terminated by the addition of cold methanol containing 1-naphthol, 1,2- and 1,4-naphthoquinones as cold standards. Following protein precipitation, the methanol-soluble metabolites were analysed by HPLC as described in Materials and Methods. The radioactivity profiles shown are from one experiment, typical of five.

(1 mM) [Fig. 1(c)], which reacts with both 1,2- and 1,4-naphthoquinone [13], the radioactivity which cochromatographed with 1,4-naphthoquinone disappeared with the concomitant formation of a new metabolite(s) which was associated with similar amounts of radioactivity and eluted between 3 and 4.5 min [Fig. 1(c)]. This is where GSH conjugates of 1,2- and 1,4-naphthoquinone elute in this system (results not shown). In contrast to the results with glutathione, when ethylenediamine, which reacts specifically with 1,2-naphthoquinone [13], was added to the fully reconstituted cytochrome P-450 system, little or no alteration was observed in the metabolite profile [compare Figs 1(a) and (d)].

While 1,4-naphthoquinone was the major metabolite observed at early time points [Fig. 1(a)], after 10 min of incubation three additional cytochrome P-450 dependent products were present in approximately similar amounts as 1,4-naphthoquinone. Their elution times were approximately 3.5, 8.0 and 13.0 min and their rates of formation were also increased with increasing cytochrome P-450 and substrate concentrations. However, after 2 min incubation, with the highest concentration of cytochrome P-450 (0.4 μM) or 1-naphthol (100 μM), the amount of each of these products ranged from 12 to 61% of the 1,4-naphthoquinone concentration confirming that 1,4-naphthoquinone is the major methanol-sol-

Table 1. Requirements for the metabolism of [1-^{14}C]-1-naphthol to 1,4-naphthoquinone by a reconstituted cytochrome P-450-dependent monooxygenase system

Incubation mixture	1,4-Naphthoquinone formation (% control)
Complete	100
−Cytochrome P-450	12.0 ± 3.7
−NADPH-Cytochrome P-450 reductase	10.7 ± 3.8
−NADPH	9.7 ± 5.1
−Dilauroylphosphatidylcholine	62.4 ± 23.6

The control incubation mixture contained a fully reconstituted monooxygenase system as described in the legend to Fig. 1 and was analysed by HPLC for metabolites following 2 min incubation at 37°.

1,4-Naphthoquinone formation is expressed as a percentage of the control (±S.D.) for 3–4 separate experiments. The rate of 1,4-naphthoquinone formation in control incubations was 2.5 ± 0.7 nmoles/nmole cytochrome P-450/min.

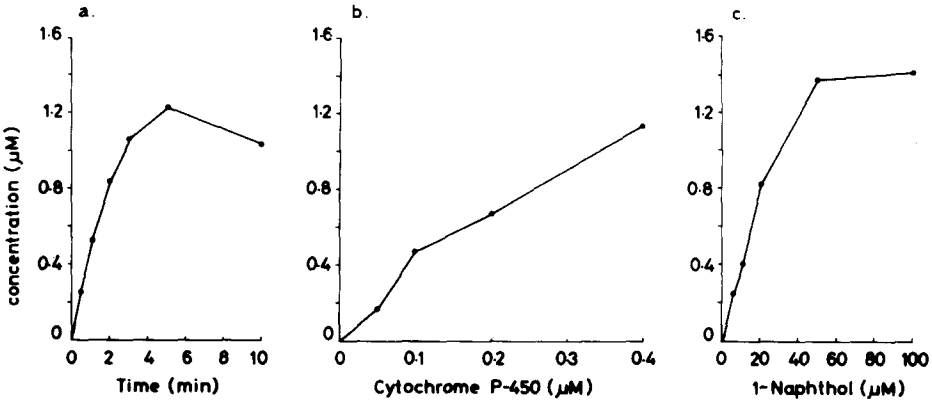

Fig. 2. Effect of time of incubation, cytochrome P-450 and 1-naphthol concentrations on the NADPH-dependent metabolism of 1-naphthol to 1,4-naphthoquinone. [1-^{14}C]-1-Naphthol (20 μM) was incubated with a fully reconstituted monooxygenase system in the presence of NADPH (5 mM), as described in the legend to Fig. 1, varying (a) time of incubation, (b) the concentration of cytochrome P-450 or (c) the concentration of [1-^{14}C]-1-naphthol. The amounts of 1,4-naphthoquinone formed are from one of two similar experiments and have been corrected for the low background observed in the absence of cytochrome P-450.

uble metabolic product formed in this system at early time points.

Cumene hydroperoxide-dependent metabolism of [1-^{14}C]-1-*naphthol to methanol-soluble products by cytochrome* P-450

[1-^{14}C]-1-Naphthol (20 μM) was metabolised to methanol-soluble products by cytochrome P-450 in a cumene hydroperoxide-dependent reaction. In contrast to the NADPH-dependent metabolism of 1-naphthol described above, products which co-chromatographed with both 1,2- and 1,4-naphthoquinone were detected [Fig. 3(a)]. The formation of these products was dependent on cytochrome P-450 [Fig. 3(b)] and cumene hydroperoxide but independent of NADPH-cytochrome P-450 reductase (results not shown). 1,4-Naphthoquinone was consistently the major methanol-soluble metabolite detected, being approximately 1.4–1.9-fold greater in amount than 1,2-naphthoquinone. Under these conditions 0.50 ± 0.10 μM 1,4-naphthoquinone was formed representing a 2.5% conversion to this methanol-soluble product. Inclusion in the reaction mixture of glutathione (1 mM) [Fig. 3(c)], which reacts with 1,2- and 1,4-naphthoquinone [13], resulted in the disappearance of the radioactivity associated with both of these quinones and the appearance of a peak between 3.5 and 4.5 min containing an equivalent amount of radiolabel [compare Figs 3(a) and 3(c)]. In contrast, inclusion of ethylenediamine (20 mM), which reacts specifically with 1,2-naphthoquinone [13], resulted in the disappearance of radioactivity coeluting with 1,2-naphthoquinone whilst no decrease was observed in the level of radioactivity associated with 1,4-naphthoquinone [compare Figs. 3(a) and 3(d)]. Experiments with cold standards indicated that the major product from the reaction of ethylenediamine with 1,2-naphthoquinone eluted close to 1-naphthol, making its detection impossible in incubations of [1-^{14}C]-1-naphthol, cumene hydroperoxide and cytochrome P-450 in the presence of ethylenediamine.

During the course of these experiments, a variation in the percentage recovery of radioactivity injected onto the HPLC column was noted. Recovery of radioactivity from incubations lacking cytochrome P-450 ranged from 90 to 100% whereas in the presence of cytochrome P-450 the recovery was 77–92%. This may be indicative of the formation of further reactive metabolites of 1-naphthol in the presence of cytochrome P-450 which do not elute from the column. Such products may be polymerisation products of 1-naphthol (P. J. O'Brien, personal communication). Some evidence for the formation of more nonpolar metabolites of 1-naphthol was obtained by comparing the levels of radioactivity eluting later than 1-naphthol in the presence or absence of cytochrome P-450 [Figs 3(a) and (b)].

Metabolism of [1-^{14}C]-1-*naphthol to covalent binding species by the reconstituted cytochrome* P-450 *system*

[1-^{14}C]-1-Naphthol (20 μM) was metabolised by the fully reconstituted cytochrome P-450 system in both NADPH- and cumene hydroperoxide-dependent reactions to covalently bound species (Table 2). In both systems there was an absolute dependence on cytochrome P-450 for the formation of the binding species. A time-dependent increase in covalent binding was observed, rising from 5.2 ± 3.9% of the total radioactivity at 2 min to 24.8 ± 7.3% after 30 min. The corresponding figures for the cumene hydroperoxide dependent reaction were 3.0 ± 1.0% and 9.5 ± 0.5% respectively. No methanol soluble metabolites could be detected in these incubations containing BSA (2 and 10 min) however, the extent of metabolism of 1-naphthol to covalently bound products was comparable to the levels of methanol-soluble products detected in the absence of BSA.

Inclusion of glutathione (1 mM) in the reaction mixtures markedly inhibited the covalent binding in

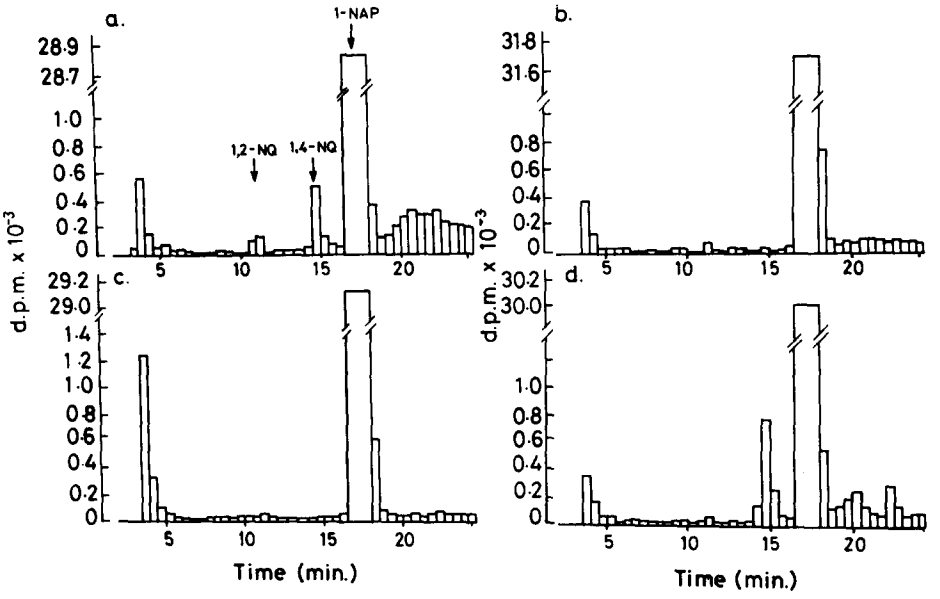

Fig. 3. HPLC separation of the metabolites of [1-^{14}C]-1-naphthol formed in a reconstituted cytochrome P-450 system in the presence of cumene hydroperoxide. [1-^{14}C]-1-Naphthol (20 μM) was incubated at 37° for 2 min with (a)(c)(d) or without (b) cytochrome P-450 (0.2 μM) in the presence of dilauroylphosphatidylcholine (30 μg/ml) and cumene hydroperoxide (50 μM) prior to HPLC analysis as described in Materials and Methods and Fig. 1. Some incubations contained either glutathione (1 mM) [Fig. 3(c)] or ethylenediamine (20 mM) [Fig. 3(d)]. The data are from one experiment typical of three.

both systems (Table 2). Ethylenediamine (20 mM) caused a lesser inhibition than glutathione and appeared to inhibit the cumene hydroperoxide dependent reaction to a greater extent than the binding observed in the presence of NADPH (Table 2). The data presented in Table 2 are from the results obtained after an incubation of 10 min but similar results were obtained at 2 and 30 min, although at 2 min the data were more difficult to quantify due to the lower levels of covalent binding observed at this time point. As the inclusion of glutathione or ethylenediamine in reactions carried out in the absence of BSA had been shown to have no significant effect on the extent of metabolism of 1-naphthol to methanol-soluble products (Figs 1 and 3), the extensive inhibition of binding caused by these agents cannot be explained by a gross inhibition of metabolism.

DISCUSSION

We have recently reported that 1-naphthol is metabolised by rat liver microsomes in an NADPH-dependent reaction to 1,4-naphthoquinone and covalently bound species [13]. As this metabolism was inhibited by classical cytochrome P-450 inhibitors such as metyrapone, SKF 525-A and $CO:O_2$ (9:1) atmosphere, we concluded that cytochrome P-450

Table 2. Requirements for the metabolism of [1-^{14}C]-1-naphthol to covalently bound species by the reconstituted cytochrome P-450 system

Incubation mixture	% of control covalent binding	
	NADPH	Cumene hydroperoxide
Complete	100	100
−Cytochrome P-450	8.9 ± 9.9	5.3 ± 9.4
−NADPH-Cytochrome P-450 reductase	8.4 ± 3.5	—
−Cumene hydroperoxide	—	9.3 ± 8.6
+ Glutathione (1 mM)	20.2 ± 4.2	4.7 ± 5.9
+ Ethylenediamine (20 mM)	59.8 ± 5.3	37.2 ± 13.3

Incubations containing [1-^{14}C]-1-naphthol (20 μM) and either NADPH (5 mM) or cumene hydroperoxide (50 μM) were set up as described in Materials and Methods. Bovine serum albumin was included in the incubations at a final concentration of 10 mg/ml. Following incubation at 37° for 10 min, the protein was precipitated by 2 vol. of methanol prior to analysis for covalently bound metabolites. The results are the means of 5–6 determinations ± S.D. Control levels of binding in the presence of NADPH and cumene hydroperoxide were 0.25 ± 0.08 and 0.13 ± 0.03 nmoles/mg protein, representing 12.7 and 6.5% of the total radioactivity in the incubations respectively.

played a major role in the metabolic activation of 1-naphthol by rat liver microsomes. Hesse and Metzger [12] have previously studied the NADPH-dependent metabolism of 1-naphthol to covalently bound species in rat liver microsomes and concluded that cytochrome P-450 may not be involved as binding was not significantly decreased by SKF 525-A or 7,8-benzoflavone. The apparent discrepancy between these results may be explained by the higher concentration of 1-naphthol used in their studies (cf. Table 1, ref. [12]). The current study was undertaken to provide definitive proof that 1-naphthol is metabolised by cytochrome P-450.

1-Naphthol was metabolised by a fully reconstituted cytochrome P-450 system, in the presence of NADPH, to methanol-soluble and covalently bound species. Within 30 sec a detectable level of 1,4-naphthoquinone, the major early product, was formed [Fig. 2(a)] in a reaction which depended on cytochrome P-450, NADPH-cytochrome P-450 reductase, NADPH and to a lesser extent dilauroylphosphatidylcholine (Table 1). One of the most likely metabolic intermediates in the formation of 1,4-naphthoquinone is 1,4-dihydroxynaphthalene which, under our standard incubation conditions, would autooxidise and thus comigrate with 1,4-naphthoquinone. Although 1,4-naphthoquinone was the major metabolic product observed at early time points, following 10 min incubation, three further metabolic products were present in the methanol-soluble fraction in comparable amounts. The identity of these products is at present unknown but they may represent further metabolic products of 1,4-dihydroxynaphthalene or 1,4-naphthoquinone. For example, 2-hydroxy-1,4-naphthoquinone comigrates with the product eluting at 3.5 min (unpublished observations). The high concentrations of these products, formed in the fully reconstituted cytochrome P-450 system, may be due to the optimal conditions for further metabolism of primary metabolites as has been observed with other substrates such as benzo(a)pyrene [20].

If NADPH was replaced by cumene hydroperoxide, 1-naphthol was metabolised in a cytochrome P-450 dependent, NADPH-cytochrome P-450 reductase independent, reaction to 1,2- and 1,4-naphthoquinone [Figs 3(a) and (b)]. In order to further investigate the generation of reactive species in the NADPH- and cumene hydroperoxide-dependent metabolism of [1-^{14}C]-1-naphthol by cytochrome P-450, the covalent binding of reactive intermediates formed in these reactions was investigated. It was not possible to measure directly the level of covalent binding to the proteins in the reconstituted cytochrome P-450 system due to the low concentration of protein present in these incubation mixtures. Therefore the ability of cytochrome P-450 to generate reactive metabolites of 1-naphthol, which would bind covalently to cellular macromolecules, was assessed in incubations in which exogenous protein (BSA) had been added. Cytochrome P-450 catalysed the time dependent metabolism of [1-^{14}C]-1-naphthol to covalent binding species in NADPH- or cumene hydroperoxide-dependent reactions (Table 2).

In order to study the nature of the covalent binding species the effects of glutathione and ethylenediamine on the metabolism and covalent binding reactions were investigated. Glutathione, which is well known to react with electrophiles [21], reacts with both 1,2- and 1,4-naphthoquinone [13], whereas ethylenediamine, which has been used as an orthoquinone trapping agent [22], reacts specifically with 1,2-naphthoquinone [13]. Neither of these agents significantly inhibited the extent of either NADPH- or cumene hydroperoxide-dependent metabolism of 1-naphthol to methanol-soluble products (Figs 1 and 3). Inclusion of glutathione in these incubation mixtures caused the disappearance of the radioactivity which coeluted with both 1,4-naphthoquinone [Figs 1(c) and 3(c)] and also with 1,2-naphthoquinone [Fig. 3(c)] in incubations with cumene hydroperoxide. In incubations with glutathione, an early eluting product(s) was observed, which was associated with an equivalent amount of radioactivity to that which comigrated with 1,2- and 1,4-naphthoquinone in the absence of glutathione. This product(s) most likely represents glutathione conjugates of these naphthoquinones, with which it coelutes (unpublished observations). Ethylenediamine had no significant effect on the radioactivity profiles obtained following HPLC analysis of incubation mixtures containing NADPH [Fig. 1(d)], whereas in reaction mixtures containing cumene hydroperoxide it caused the disappearance of the radioactivity which coeluted with 1,2-naphthoquinone [Fig. 3(d)]. This was most likely due to reaction of metabolically formed 1,2-naphthoquinone with ethylenediamine but we cannot exclude the possibility that ethylenediamine had a specific inhibitory effect on the formation of 1,2-naphthoquinone although it did not inhibit the formation of 1,4-naphthoquinone (Figs 1 and 3).

The greater inhibition of covalent binding of [1-^{14}C]-1-naphthol by glutathione compared to ethylenediamine (Table 2) suggested that some if not most of the binding was due to 1,4-naphthoquinone or a metabolite derived from it. However, ethylenediamine also caused a significant inhibition of binding, indicating that 1,2-naphthoquinone or a structurally related compound may also be involved in the binding. This was not surprising in the reactions containing cumene hydroperoxide, where 1,2-naphthoquinone was observed as a methanol-soluble product [Fig. 3(a)]. However, a lesser though significant inhibition of NADPH dependent-binding was observed in the presence of ethylenediamine (Table 2) even though no methanol-soluble 1,2-naphthoquinone had been detected [Fig. 1(a)]. This is in contrast to our previously reported observation on the NADPH-dependent metabolism of 1-naphthol to binding species by rat liver microsomes, where glutathione but not ethylenediamine lowered the level of covalent binding observed [13]. The reason for this discrepancy is unclear but may in part be due to different metabolic capabilities of the isolated cytochrome P-450 system from phenobarbitone-induced rats compared to the control microsomes used in the previous study. It is possible that the species which is inhibited from binding in the fully reconstituted cytochrome P-450 system in the presence of ethylenediamine is not 1,2-naphthoquinone *per se* but another metabolic product having ortho

quinone groups which would enable it to interact with ethylenediamine. Such a product need not necessarily arise from 1,2-naphthoquinone but could be formed by the further metabolism of 1,4-naphthoquinone or 1,4-dihydroxynaphthalene to a compound such as 4-hydroxy-1,2-naphthoquinone. The further metabolism of primary products which is more likely in the reconstituted cytochrome P-450 system than in a microsomal incubation may at least, in part, explain the differences in the effect of ethylenediamine in these systems.

In summary, this study shows conclusively that 1-naphthol is metabolised by cytochrome P-450 in both NADPH- and cumene hydroperoxide-dependent reactions to naphthoquinones and covalently bound species.

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