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METABOLIC ACTIVATION OF 1-NAPHTHOL BY RAT LIVER MICROSOMES TO 1,4-NAPHTHOQUINONE AND COVALENT BINDING SPECIES

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Abstract—1-Naphthol was metabolized by rat liver microsomes, in the presence of an NADPH-generating system, both to methanol-soluble metabolites including 1,4-naphthoquinone and an uncharacterized product(s) (X) and also to covalently bound products. NADH was much less effective as an electron donor than NADPH. Metyrapone, SKF 525-A and carbon monoxide all inhibited the metabolism of 1-naphthol to 1,4-naphthoquinone and to covalently bound products suggesting the involvement of cytochrome P-450 in at least one step in the metabolic activation of 1-naphthol to reactive products. Ethylene diamine, which reacts selectively with 1,2-naphthoquinone but not 1,4-naphthoquinone, did not affect the covalent binding whereas glutathione, which reacts with both naphthoquinones, caused an almost total inhibition of covalent binding. These and other results suggested that 1,4-naphthoquinone, or a metabolite derived from it, was responsible for most of the covalent binding observed and that little if any of the binding was due to 1,2-naphthoquinone.

Recently we have shown that 1-naphthol exhibits a selectively toxic action to short-term organ cultures of human colonic tumor tissue compared to normal colonic tissue from the same patients [1, 2]. In addition 1-naphthol has been shown to be toxic to colonic tumour cell lines [1, 2] and isolated rat hepatocytes *in vitro* [3]. As these results suggested that 1-naphthol may be of potential value as an antitumour agent, we wished to understand its possible mechanisms of toxicity to normal and tumor cells. Over the last 10–15 years it has become increasingly clear that many chemicals require metabolic activation in order to exert their toxicity [4, 5]. Much evidence has been accrued to suggest that in many but not all cases such reactive metabolites will bind covalently to critical cellular macromolecules and so in some as yet undefined way elicit a toxic reaction [6, 7]. Thus in order to understand better the mechanism of toxicity of 1-naphthol, we have studied its metabolism and covalent binding. For these studies, rat liver microsomes have been utilized because they are both well-characterized model systems and 1-naphthol is toxic to isolated hepatocytes [3].

In vivo and in intact cellular systems, such as isolated hepatocytes, 1-naphthol is extensively metabolized to both its glucuronic acid and sulphate ester conjugates [8–10]. Relatively little is known of the possible oxidative metabolism of 1-naphthol. One early study provided indirect evidence that it may be metabolized by microsomes to 1,2-dihydroxynaphthalene [11]. Hesse and Mezger [12] also showed that 1-naphthol was metabolized by rat liver microsomes in the presence of an NADPH-generating system to covalently bound products. The nature of the reactive products was not identified although the possible involvement of quinone or semiquinone metabolites was suggested [12]. Recently we have obtained indirect evidence for the formation of naphthoquinone metabolites following incubation of 1-naphthol with either rat liver microsomes or isolated rat hepatocytes [3, 13]. 1-Naphthol, 1,2- and 1,4-naphthoquinone stimulated microsomal superoxide anion formation in the presence of reduced pyridine nucleotides [13]. The toxicity of 1-naphthol and the naphthoquinones to hepatocytes was potentiated by dicoumarol, an inhibitor of DT-diaphorase (NAD(P)H; quinone oxidoreductase) [14], and the enhanced toxicity was preceded by an increased depletion of cellular GSH [3]. These findings are compatible with the metabolism of 1-naphthol to 1,2- and/or 1,4-naphthoquinone which then redox cycle producing active oxygen species.

The present study provides the first direct evidence for the formation, by an NADPH-dependent microsomal mixed function oxidase reaction, of naphthoquinone metabolites of 1-naphthol, which then either as the quinone or semiquinone bind covalently to microsomal protein. Evidence is presented which suggests that 1,2-naphthoquinone or 1,2-naphthosemiquinone were not the major binding species despite their high chemical reactivity.

MATERIALS AND METHODS

Materials. All chemicals were purchased from the Sigma Chemical Co. Ltd., Poole, Dorset, U.K. or the British Drug House Chemical Co. Ltd., Poole, Dorset, U.K. unless otherwise stated. All solvents were obtained from May and Baker Ltd., Dagenham, U.K., except for dimethyl sulphoxide (DMSO) which was purchased from the Sigma Chemical Co. HPLC grade methanol was obtained from Rathburn Chemicals Ltd., Peebleshire, Scotland. Copper/zinc superoxide dismutase (3300 units/mg), prepared from outdated human blood, was a gift from Dr. J. V. Bannister, University of Oxford. Catalase (beef

liver, 260,000 units/ml) and glucose-6-phosphate dehydrogenase (approx 500 units/ml) were purchased from Boehringer Mannheim. SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl) was kindly provided by Smith Kline and French, Welwyn Garden City, U.K. 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 p.l.c., Buckinghamshire and Aquasol for liquid scintillation counting from New England Nuclear Ltd., Edinburgh, Scotland. The purity of the labelled 1-naphthol was checked by high pressure liquid chromatography and found to be >99%.

Rat liver microsomes. Washed liver microsomes were prepared from the livers of 150–300 g male Sprague–Dawley rats, from Olac Ltd., Shaw's Farm, Blackthorn, Bicester, U.K., by the method of Ernster *et al.* [15], as previously described [13]. All animals were allowed water and laboratory chow *ad lib.* Microsomes were used fresh on the day of preparation.

Metabolism of [1-^{14}C]-1-*naphthol by rat liver microsomes.* Rat liver microsomes (1 mg/ml) were incubated in 0.12 M Tris–HCl shaking at 37°, pH 7.4, for 5–60 min with 10–500 μM [1-^{14}C]-1-naphthol (11.2–56.0 μCi/μmole) in the presence of an NADPH-generating system consisting of 1 mM $NADP^+$, 12.5 mM glucose-6-phosphate, 1.2 mM $MgCl_2$ and 1 unit/ml of glucose-6-phosphate dehydrogenase. At the end of the incubation period, the microsomal protein was precipitated by the addition of 2 volumes of methanol containing authentic standards of 1-naphthol, 1,2- and 1,4-naphthoquinone. The protein was removed by centrifugation at 2000 *g* for 2 min and stored at −20° for analysis of covalent binding. The supernatant fraction was analysed by HPLC on an Altex ODS Ultrasphere column (15 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 280 or 330 nm and 0.5 ml fractions collected directly into scintillation vials. Typical elution times (min) were as follows: 1,2-naphthoquinone 5,6, 1,4-naphthoquinone 8.2 and 1-naphthol 11.9. The radioactivity in the fractions was determined by liquid scintillation counting after the addition of 3.5 ml of Aquasol to each vial. The supernatant fractions were analysed for metabolites either immediately or after storage at −20° for 24–48 hr. All the metabolic products were stable for this time under these conditions. Selected samples were analysed under anaerobic HPLC conditions as previously described by Greenlee *et al.* [16]. The elution conditions were as described above except that the solvents were bubbled extensively with nitrogen and ascorbate (18 mg/ml) was added to the samples directly before injection onto the column. Under these conditions the quinones were converted to their corresponding hydroquinones and typical elution times (min) were as follows 1,4-dihydroxynaphthalene 6.0, 1,2-dihydroxynaphthalene 8.0 and 1-naphthol 11.9.

Determination of covalent binding. The microsomal protein pellets prepared from incubations containing [1-^{14}C]-1-naphthol, as described above, were analysed for covalent binding essentially as described by Kappus and Remmer [17]. The pellets were washed with successive 2 ml aliquots of the following solvents; 70% methanol, 100% methanol, acetone:chloroform 4:1 (v/v) until the level of radioactivity in the washes was equal to background. The pellets were then dissolved in 1 N NaOH and aliquots taken for protein assay by the method of Lowry *et al.* [18], and liquid scintillation counting following neutralization by 1 N HCl and the addition of 3.5 ml of Aquasol. Control reactions were carried out with heat inactivated microsomes or without an NADPH-generating system. Potential modifiers of the covalent binding of 1-naphthol metabolite(s) to microsomal protein were included in some incubations. The modifiers were added at concentrations which did not inhibit the NADPH-generating system.

N-*demethylase assay.* The mixed function oxidase activities of all microsomal preparations and the effects of various possible modifiers were measured using the *N*-demethylation of aminopyrine (1 mM). Microsomes (1 mg/ml) were incubated at 37° for 15 min in 0.12 M Tris–HCl pH 7.4 in the presence of aminopyrine and an NADPH-generating system. The production of formaldehyde was measured by the method of Nash [19].

RESULTS

Microsomal metabolism of [1-^{14}C]-1-*naphthol to methanol-soluble and covalently bound products*

[1-^{14}C]-1-Naphthol was metabolized by rat liver microsomes in the presence of an NADPH-generating system in a time and concentration dependent manner to both methanol-soluble and covalently bound products. HPLC analysis of the methanol-soluble radioactivity following microsomal metabolism of [1-^{14}C]-1-naphthol (20 μM) showed the presence of at least two major and one minor metabolic products associated with radioactivity (Fig. 1). One of the major products, eluting at 8.2 min, co-chromatographed with 1,4-naphthoquinone (Figs. 1a and b). Under anaerobic HPLC conditions, as described in Materials and Methods, in which naphthoquinones are converted to their corresponding hydroquinones this radioactive product comigrated with authentic 1,4-dihydroxynaphthalene (results not shown). The formation of 1,4-naphthoquinone as well as the other metabolic products was clearly dependent on the presence of both NADPH (Fig. 1c) and rat liver microsomes (results not shown) and similarly no metabolites were observed in the presence of heat inactivated microsomes. After 10 and 60 min incubation, 1,4-naphthoquinone accounted for 7.3 ± 2.1 and 8.8 ± 1.6% (mean ± S.E.) respectively of the methanol-soluble radioactivity. At these times, methanol-soluble radioactivity accounted for 82.7 ± 5.6 and 40.6 ± 6.5% (mean ± S.E.) respectively of the total radioactivity.

The other major metabolic product(s) associated with radioactivity eluted between 2.5 and 4 min (Figs. 1a and b). The amount of radioactivity associated with this product(s) increased significantly

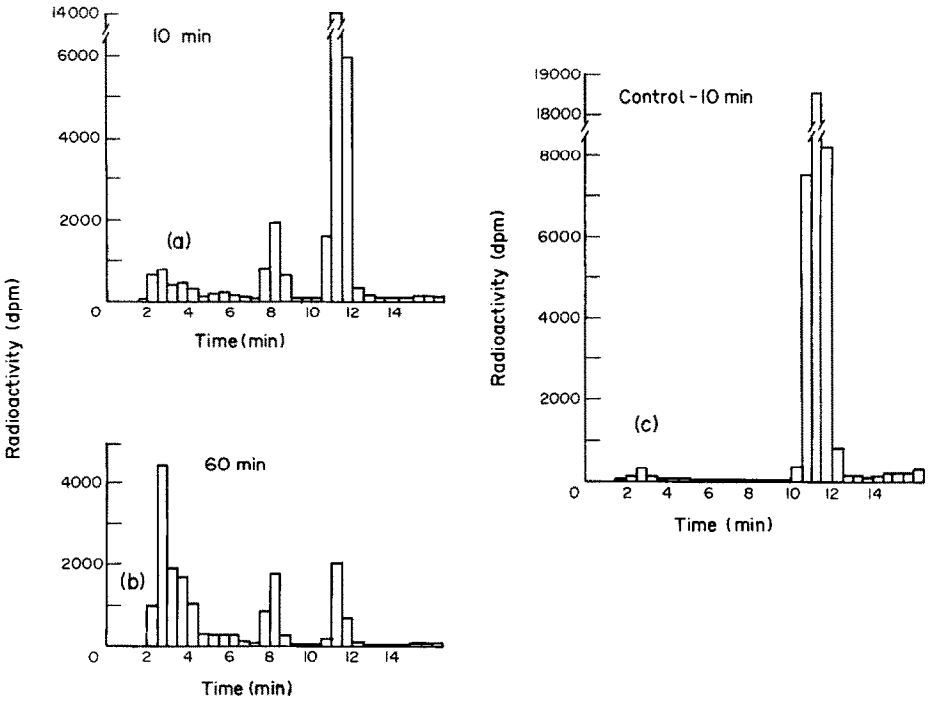

Fig. 1. HPLC separation of rat liver microsomal metabolites of [1-^{14}C]-1-naphthol. [1-^{14}C]-1-Naphthol (20 μM) was incubated with rat liver microsomes (1.0 mg protein/ml) in 0.12 M Tris–HCl pH 7.4 for 10 or 60 min in the presence (a and b) or absence (c) of an NADPH-generating system. The reaction was terminated by the addition of ice cold methanol containing cold 1-naphthol, 1,2- and 1,4-naphthoquinone as standards. The microsomal protein was removed by centrifugation and the supernatant solution analysed by HPLC as described in Materials and Methods. 1-Naphthol, 1,4-naphthoquinone, 1,2-naphthoquinone and the unidentified product(s) (X) eluted at 11.4, 8.2, 5.6 and 2.0–4.0 min after injection respectively. The results shown are from one experiment typical of four.

between 10 and 60 min (Figs. 1a and 1b) in contrast to 1,4-naphthoquinone. In the majority of experiments, in addition to the two major metabolic products a small amount of radioactivity eluted at approximately 5.6 min and cochromatographed with authentic 1,2-naphthoquinone (Figs. 1a and b). Thus after 10 min incubation, only 0.5 ± 0.26% of the methanol-soluble radioactivity eluted with 1,2-naphthoquinone. The very small amounts of radioactivity associated with this minor product prevented its accurate quantitation.

During the incubation, the marked decrease in the amount of methanol-soluble radioactivity noted above was accompanied by a concomitant time-dependent increase in covalently bound products associated with the microsomal proteins (Fig. 2). Covalently bound products accounted for only 8.1 ± 2.3% of [1-^{14}C]-1-naphthol (20 μM) after 5 min of incubation, whereas after 60 min 59.9 ± 4.8% was covalently bound (Fig. 2). The time-dependent increase in covalent binding was accompanied by a corresponding increase in the metabolic product(s) which eluted between 2.5 and 4 min as well as a decrease in 1-naphthol, such that only 11.6 ± 5.1% of unmetabolized substrate was left after 60 min (Fig. 2). However, no clear time-dependent increase in the amounts of either 1,4- or 1,2-naphthoquinone was observed (Fig. 2). Although results with 1,2-naphthoquinone are shown, they are only an approximation because of the difficulty in quantifying such small amounts.

The effects of varying 1-naphthol concentration on both metabolism and covalent binding were also studied. Increase in substrate concentration (10–500 μM) resulted in a concentration-dependent increase in covalent binding and in the product(s) eluting between 2.5–4 min as well as a smaller increase in 1,4-naphthoquinone (Fig. 3). A marked decrease in the overall % metabolism was observed with increasing substrate concentration; thus after 10 min 49.7 ± 8.4% of 1-naphthol was metabolized at 10 μM whereas only 2.7 ± 0.51% was metabolized at 500 μM (Fig. 3).

Effect of modifiers on covalent binding

In order to gain further insight into the metabolic activation of 1-naphthol as well as the nature of the binding species, the effects of a number of differing agents on both metabolism and covalent binding were studied. The metabolic activation of [1-^{14}C]-1-naphthol (20 and 500 μM) by rat liver microsomes to covalently bound products was clearly an NADPH-dependent reaction (Table 1). NADH could not substitute for NADPH and covalent binding was markedly inhibited when heat inactivated microsomes were used (Table 1). Covalent binding was also inhibited by SKF 525-A, metyrapone and carbon monoxide/oxygen (9:1, v/v) (Table 1). These results

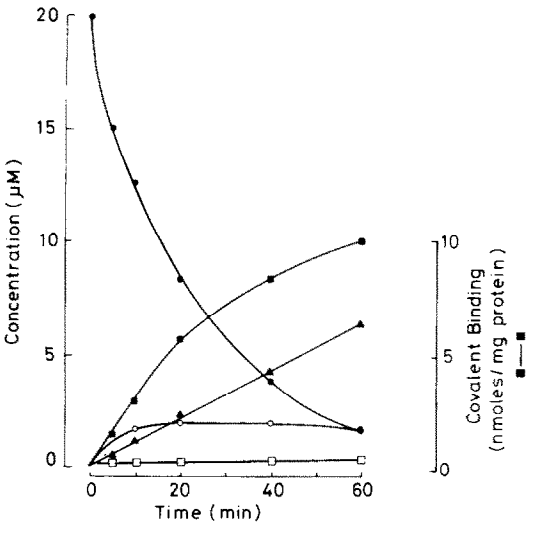

Fig. 2. Time-dependent metabolism and covalent binding of 1-naphthol (20 μM). [1-^{14}C]-1-Naphthol (20 μM) was incubated with rat liver microsomes (1.0 mg protein/ml) for 5–60 min in the presence of an NADPH-generating system. The disappearance of 1-naphthol (●——●) was accompanied by the formation of 1,2-naphthoquinone (□——□), 1,4-naphthoquinone (○——○), the early eluting unidentified product(s) (X) (▲——▲) as well as covalently bound species (■——■). The methanol-soluble metabolites were separated by HPLC as described in the legend to Fig. 1 and quantified after subtraction of appropriate controls. The covalently bound radioactivity (■——■) associated with the microsomal proteins is expressed in nmoles bound/mg protein and was determined after multiple solvent extractions as described in Materials and Methods. The results shown are from one experiment typical of four.

and the very similar inhibition by these agents of aminopyrine *N*-demethylase activity (Table 1), strongly support the involvement of cytochrome P-450 in the metabolic activation of 1-naphthol.

Further insight into the nature of the binding species and the possible role of active oxygen species in the metabolic activation of 1-naphthol (20 μM), was obtained by studying the effects of various agents on the level of covalent binding (Table 2).The most striking effect was the very marked inhibition of covalent binding of 1-naphthol by GSH (Table 2) and cysteine (preliminary results). A consistent but less dramatic inhibition of binding was observed in the presence of superoxide dismutase, dimethyl sulphoxide and ascorbic acid (Table 2) whilst catalase had no effect. Lysine and ethylene diamine, agents reported to react with *o*-quinones [20, 21], definitely did not inhibit covalent binding and possibly caused a slight increase (Table 2). When the effects of all these modifiers were studied on the covalent binding of a higher concentration of 1-naphthol (500 μM), similar trends were observed, although somewhat more variability was noted (results not shown). At the concentrations used, none of the modifiers caused any alteration in mixed function oxidase activity as assessed by *N*-demethylation of aminopyrine (results not shown). The effects of ascorbic acid were not determined because it interfered with the assay procedure.

Glutathione is known to react with quinones, semiquinones and other reactive electrophiles [22]. The non-enzymic reactivity of glutathione with both 1,4-naphthoquinone (Figs. 4a and c) and 1,2-naphthoquinone (Figs. 4d and f) was confirmed by the disappearance of the u.v. absorbing peaks (330 nm) from the elution profiles of these quinones, when they were mixed with GSH prior to the HPLC analy-

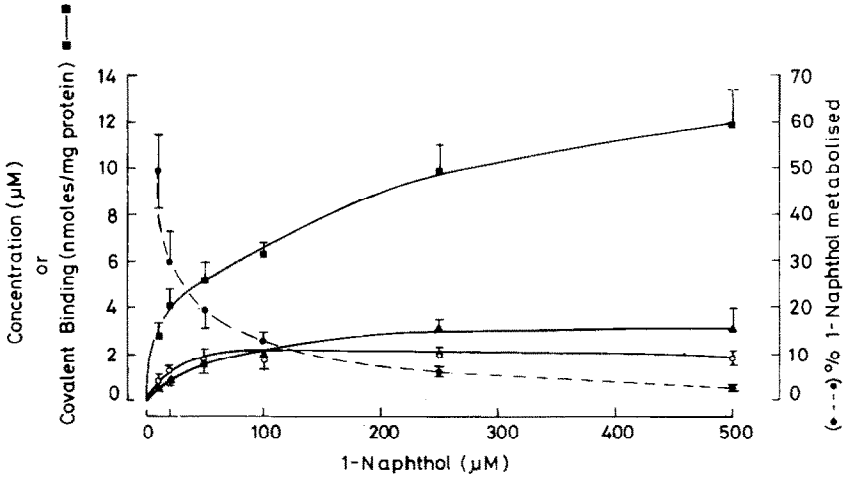

Fig. 3. Concentration dependence of metabolism and covalent binding of 1-naphthol. [1-^{14}C]-1-Naphthol (10–500 μM) was incubated for 10 min with rat liver microsomes (1.0 mg protein/ml) and an NADPH-generating system. 1,4-Naphthoquinone (○——○), and the early eluting product(s) (X) (▲——▲) were separated by HPLC from 1-naphthol (●——●) as described in the legend to Fig. 1 and Materials and Methods. The covalent binding (■——■) was determined as described in the legend to Fig. 2 and expressed as nmoles bound/mg protein. The results shown are the mean values from three experiments ± S.E.M.

Table 1. Effects on covalent binding of [1-^{14}C]-1-naphthol by factors affecting microsomal mixed function oxidase activity

Incubation mixture	Inhibition of control covalent binding* (%)		Inhibition of control aminopyrine *N*-demethylase activity (%)
	20 μM	500 μM	
−NADPH	97.7 ± 1.6 (7)†	76.0 ± 5.1 (7)	97.5 ± 6.6 (8)
+NADH (1 mM)−NADPH	87.7 ± 3.2 (3)	82.3 ± 5.5 (3)	83.6 ± 11.3 (4)
Heat inactivated microsomes	84.3 ± 17.7 (3)	84.0 ± 20.7 (6)	87.4 ± 16.4 (4)
$CO:O_2$ (9:1 atm.)	53.0 ± 6.5 (3)	11.3 ± 7.0 (3)	75.9 ± 21.8 (3)
Metyrapone (1 mM)	64.3 ± 7.5 (3)	14.3 ± 12.6 (3)	78.8 ± 3.9 (3)
SKF 525-A (0.25 mM)	72.3 ± 2.5 (3)	35.3 ± 5.1 (3)	98.8 ± 1.8 (4)

[1-^{14}C]-1-Naphthol (20 or 500 μM) was incubated for 10 min with freshly isolated rat liver microsomes (1.0 mg protein/ml) in the presence or absence of an NADPH-generating system or a possible modifier. The covalent binding was determined as described in Materials and Methods. Results are expressed as the mean ± S.D. of at least 3 determinations.

* Control covalent binding levels at 20 and 500 μM were 4.9 ± 2.2 and 12.2 ± 2.9 nmoles bound/mg protein respectively (Mean ± S.D., N = 7).

† The number in parentheses represents the number of experiments.

sis. Under similar conditions ethylene diamine, an *o*-quinone trapping agent [20], reacted selectively with 1,2-naphthoquinone (Figs. 4d and e) and not with 1,4-naphthoquinone (Figs 4a and b). By contrast L-lysine, an agent previously reported [21] to react with *o*-quinones did not react with either 1,2- or 1,4-naphthoquinone (results not shown).

Effect of modifiers on the metabolism of [1-^{14}C]-1-*naphthol*

In order to understand further the modification in the levels of covalent binding by a number of selected agents, their effects on the metabolism of [1-^{14}C]-1-naphthol were also studied (Table 3). SKF 525-A, metyrapone and carbon monoxide : oxygen (9 : 1 v/v) all caused an inhibition in the formation of 1,4-naphthoquinone and the unknown early eluting product(s) (X), which was accompanied by a decrease in the percentage of 1-naphthol metabolized (Table 3). Glutathione caused a significant decrease in the amounts of 1,4-naphthoquinone and 1,2-naphthoquinone (results not shown) together with an increased formation of the early eluting product(s) (X) (Table 3). When the metabolism of 1-naphthol was studied in the presence of glutathione for longer periods of time (60 as opposed to 10 min), 65–83% of the total radioactivity was associated with the early eluting product(s) (X) as opposed to 4–19% in its absence.

In contrast, ethylene diamine caused small but not significant increases in the amounts of 1,4-naphthoquinone, the early eluting product(s) (X) and the overall metabolism of 1-naphthol after incubation with rat liver microsomes and an NADPH-gen-

Table 2. Effects of various agents on the covalent binding of [1-^{14}C]-1-naphthol (20 μM)

Modifier	Concentration (mM)	Change in covalent binding of [1-^{14}C]-1-naphthol (%)
Glutathione	2	−88.7 ± 2.1
Lysine	10	+8.0 ± 3.0
Ethylene diamine	20	+17.0 ± 9.6
Ascorbic acid	1	−32.7 ± 3.1
Superoxide dismutase	40 μg/ml	−25.3 ± 3.2
Catalase	260 U/ml	+2.3 ± 11.6
DMSO	100	−19.0 ± 6.2

[1-^{14}C]-1-Naphthol (20 μM) was incubated for 10 min with rat liver microsomes in the presence of an NADPH-generating system and one of the modifiers in the table. The covalent binding was determined as described in Materials and Methods and the results expressed as % change in the level of covalent binding observed in incubations in the absence of any modifier (3.49 ± 1.19 nmoles/mg protein).

Results are expressed as mean ± S.D. (N = 3).

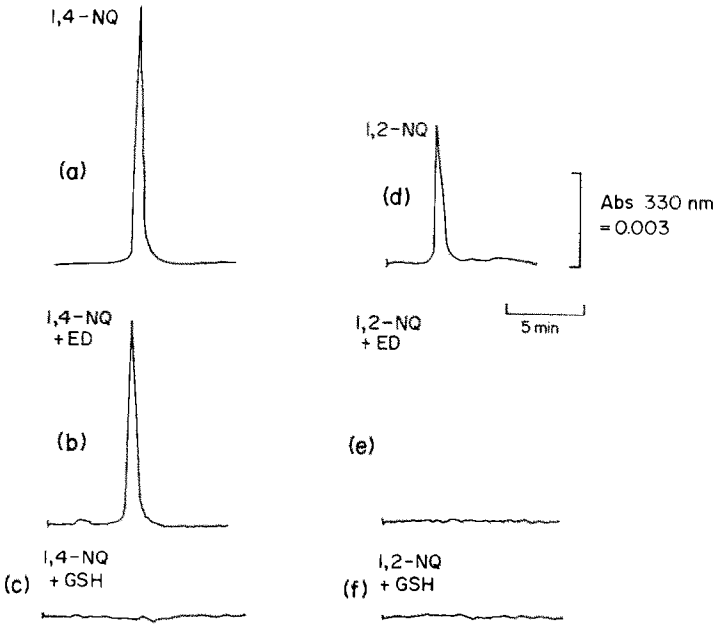

Fig. 4. The reactivity of 1,2- and 1,4-naphthoquinone with glutathione and ethylene diamine (ED). 1,4- or 1,2-naphthoquinone (0.2 mM) was incubated alone (Figs 4a and d) or in the presence of 20 mM ethylene diamine (Figs. 4b and e) or reduced glutathione (1 mM) (Figs. 4c and f), at 37° for 5 min, prior to HPLC analysis as described in Materials and Methods. The resultant elution profiles (330 nm) show that ethylene diamine reacted selectively with 1,2-naphthoquinone whereas glutathione reacted with both 1,2- and 1,4-naphthoquinone.

erating system (Table 3). This increased metabolism correlated with the small stimulation in the level of covalent binding observed in the presence of this compound (Table 2). The levels of 1,2-naphthoquinone formation in control incubations could not be accurately quantitated due to the low conversion, usually less than 1%, to this metabolite. However, it was noted that incubations containing either ethylene diamine or glutathione contained less radioactivity in the region where 1,2-naphthoquinone migrates (5.6 min). As both these compounds react with 1,2-naphthoquinone (Fig. 4), this lends further support to the suggestion that the small amount of radioactivity eluting at 5.6 min is associated with 1,2-naphthoquinone.

The most marked effect caused by superoxide dismutase was a decrease in the amount of the early eluting product(s) (X). This was accompanied by

Table 3. Effects of various agents on metabolism of [1-^{14}C]-1-naphthol (20 μM)

Modifier	Concentration (mM)	% Change in: Total 1-naphthol metabolism	% Change in: Unknown product(s) (X)	% Change in: 1,4-Naphthoquinone
SKF 525-A	0.25	−73.4 ± 6.2	−80.0 ± 7.4	−87.8 ± 1.2
Metyrapone	1.0	−59.3 ± 3.0	−37.4 ± 5.8	−58.8 ± 5.7
$CO:O_2$	9:1 (atmosphere)	−52.2 ± 5.2	−63.9 ± 6.3	−30.4 ± 8.6
Glutathione	2.0	−18.0 ± 2.6	+452.2 ± 101.7	−96.5 ± 2.0
Ethylene diamine	20.0	+16.1 ± 5.9	+9.6 ± 15.4	+19.6 ± 17.4
Superoxide dismutase	40 μg/ml	+11.6 ± 5.2	−54.4 ± 8.2	+25.7 ± 9.1

The percentage change in total 1-naphthol metabolism (i.e. methanol-soluble and covalently bound products), the early eluting product(s) (X) and 1,4-naphthoquinone were quantified, as described in Materials and Methods, following incubation of [1-^{14}C]-1-naphthol for 10 min with rat liver microsomes (1.0 mg protein/ml) in the presence of an NADPH-generating system and a possible modifier. In control incubations containing no modifiers, 8.0 ± 1.4 μM of 1-naphthol was metabolized forming 1.24 ± 0.07 μM 1,4-naphthoquinone and 1.53 ± 0.38 μM of product(s) (X). The results are expressed as the mean ± S.E.M. of at least three determinations

small increases in both 1,4-naphthoquinone, 1,2-naphthoquinone (results not shown) and in the amount of 1-naphthol metabolized (Table 3).

DISCUSSION

[1-^{14}C]-1-Naphthol was metabolized by rat liver microsomes in an NADPH-dependent reaction to: (i) methanol-soluble metabolites which cochromatographed with 1,4-naphthoquinone and an early eluting product(s) (X) (Figs. 1a and b); and (ii) reactive metabolites which bound irreversibly to microsomal proteins (Figs. 2 and 3 and Table 1). The metabolism of 1-naphthol, the formation of the two major metabolic products and the covalent binding were all markedly inhibited by the microsomal mixed function oxidase inhibitors, SKF 525-A, metyrapone and carbon monoxide : oxygen (9 : 1 v/v) (Tables 1 and 3). These observations together with the almost parallel effects observed on the inhibition of both covalent binding of 1-naphthol and aminopyrine *N*-demethylase activity (Table 1) support very strongly the involvement of the microsomal mixed function oxidase, cytochrome P-450, in most, if not all the metabolic activation of 1-naphthol to naphthoquinones and suggests that these metabolites are essential intermediates in the activation of 1-naphthol to covalently bound products. Naphthoquinones may arise following hydroxylation of 1-naphthol to either 1,2-dihydroxynaphthalene [11] or 1,4-dihydroxynaphthalene and their subsequent oxidation either enzymically or by autoxidation.

The suggestion that 1-naphthol is metabolized by microsomal cytochrome P-450 is in agreement with the observations that it both forms a type I binding spectrum with rat liver microsomes [23] and also inhibits certain microsomal mixed function oxidation reactions [24]. Our findings are also in broad agreement with Hesse and Mezger [12] who showed that following microsomal metabolism of [1-^{14}C]-1-naphthol, radioactive material became irreversibly bound to microsomal proteins and that this binding required active microsomes, oxygen and NADPH and was partially inhibited by carbon monoxide. However, in contrast to our study, they did not identify any methanol-soluble metabolites of 1-naphthol. In addition they did not observe an inhibition of covalent binding of 1-naphthol by SKF 525-A and therefore did not implicate cytochrome P-450 in the activation of 1-naphthol. This may have been due in part to the higher concentration of naphthol used in their study as it was noted that some effects of the mixed function oxidase inhibitors (metyrapone and carbon monoxide) were less pronounced at higher substrate concentrations (Table 1). Whilst the present study demonstrates a key role for cytochrome P-450 in the metabolic activation of 1-naphthol by isolated rat liver microsomes we cannot exclude the activation of 1-naphthol by other enzymes or systems under different conditions (M.T. Smith *et al.*, personal communication). Another possible reactive metabolite of 1-naphthol which might be formed is naphthoxy radical. As this species has not been detected in a biological system and its reactivity with any of the modifiers used in this study has not been documented, one cannot assess its contribution.

Hesse and Mezger [12] suggested, but did not prove, the involvement of quinones or semiquinones in the irreversible binding of 1-naphthol to microsomal proteins. Glutathione caused a profound inhibition of both the covalent binding of 1-naphthol (20 μM) to microsomal proteins (Table 2) and also the amounts of 1,2-naphthoquinone (results not shown) and 1,4-naphthoquinone (Table 3) formed together with a concomitant increase in the amount of early eluting product(s) (X) (Table 3). Glutathione was also shown to react with both 1,2- and 1,4-naphthoquinone (Fig. 4). These results are compatible with the suggestion that the binding species arise either directly from 1,2- and/or 1,4-naphthoquinone or after some subsequent reaction such as their reduction to the corresponding naphthosemiquinone. These experiments also gave some insight into the possible identity of the early eluting product(s) (X) (Figs. 1a and b). One distinct possibility, in particular in experiments with added glutathione (Table 3), was that X is a glutathione conjugate of 1,2- and/or 1,4-naphthoquinone.

Whilst many studies have tried to relate covalent binding and toxicity, only in a few instances have the binding species been unequivocally identified. This is often due to the high reactivity and thus the short half-life of the metabolite. Thus whilst the data presented so far are more compatible with 1,4-naphthoquinone or a further metabolite being the major binding species, the very low levels of 1,2-naphthoquinone detected may have been due to it or a metabolite binding very rapidly. Further information on the nature of the binding species was provided by the experiments with ethylene diamine, a known *o*-quinone trapping agent [20, 21] which reacted much more readily with 1,2-naphthoquinone than with 1,4-naphthoquinone (Figs. 4d and e). Thus the finding that the addition of ethylene diamine did not inhibit the covalent binding or overall metabolism of 1-naphthol (Tables 2 and 3) nor the formation of 1,4-naphthoquinone and the early eluting product(s) (X) (Table 3) suggested that little if any of the covalent binding was due to 1,2-naphthoquinone or a metabolite derived from it. This is in contrast to the importance of covalent binding and the high chemical reactivity of 1,2-naphthoquinone with proteins and other constituents of the lens, which have been implicated in the cataractogenic activity of naphthalene [25, 26].

The relatively small inhibition of covalent binding of 1-naphthol by ascorbic acid and superoxide dismutase (i.e. 33 and 25% respectively—Table 2) suggested that 1-naphthol was not activated primarily by superoxide anions as is believed to be the case for α-methyldopa and related catechols [27]. The lack of effect of catalase and the very small effect of dimethyl sulphoxide on the binding of 1-naphthol (Table 2) showed that there was little if any role for hydrogen peroxide or hydroxyl radicals in the binding reaction.

There are some marked similarities and interesting differences between the metabolic activation of 1-naphthol reported in this study and structurally related phenol. In an analogous manner to 1-naphthol, phenol was metabolically activated by rat liver microsomes from phenobarbitone pretreated rats in an NADPH-dependent reaction to hydroquinone,

catechol and covalently bound products and these reactions were inhibited by carbon monoxide, SKF 525-A and metyrapone [28]. The ratio of hydroquinone:catechol formed was 20:1 [28] and the amount of 1,4-naphthoquinone was much greater than the amounts of 1,2-naphthoquinone. The most striking difference between the two substrates appeared to be the ratio of benzoquinone or 1,4-naphthoquinone to covalent binding. Thus after 30 min, the amounts of benzoquinone formed and covalent binding from phenol (1 mM) were approximately 140 and 5 nmoles/mg protein respectively (Fig. 2, Ref. 28). In marked contrast to this with 1-naphthol, the covalent binding increased almost linearly for 60 min and far exceeded the amounts of 1,4-naphthoquinone, which quickly reached a plateau (Fig. 2). The most likely explanation for this was the more ready one electron reduction of 1,4-naphthoquinone than benzoquinone to a semiquinone and subsequent covalent binding. This is compatible with the one electron redox potentials of 1,4-naphthoquinone and benzoquinone, which are −100 mV and +100 mV respectively [29, 30], and thus, 1,4-naphthoquinone may be more readily reduced to 1,4-naphthosemiquinone by certain microsomal reductases such as NADPH-cytochrome P-450 reductase [31].

In conclusion, 1-naphthol is metabolized by rat liver microsomes in a cytochrome P-450 dependent reaction to 1,4-naphthoquinone, a product(s) (X), which may in part be glutathione conjugates of naphthoquinones, and to covalently bound products. The covalently bound species are derived primarily from 1,4-naphthoquinone most likely via 1,4-naphthosemiquinone and not from 1,2-naphthoquinone. These data are compatible with our previously proposed mechanism of toxicity of 1-naphthol to hepatocytes involving covalent binding and/or active oxygen species formation subsequent to naphthoquinone formation [3]. The relevance of these findings to the selective toxicity of 1-naphthol to human colonic tumour tissue is currently being investigated.

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