MOLECULAR PHARMACOLOGY, 16, 667-675

Involvement of Phenolic Metabolites in the Irreversible Protein-Binding of Aromatic Hydrocarbons: Reactive Metabolites of [^{14}C]Naphthalene and [^{14}C]1-Naphthol Formed by Rat Liver Microsomes

SIGRUN HESSE AND MARGOT MEZGER

Gesellschaft f. Strahlen- und Umweltforschung, Institut für Toxikologie und Biochemie, D-8042 Neuherberg/München, Germany

(Received August 29, 1978)
(Accepted March 23, 1979)

SUMMARY

HESSE, SIGRUN, AND M. MEZGER. Involvement of phenolic metabolites in the irreversible protein-binding of aromatic hydrocarbons: reactive metabolites of [^{14}C]-naphthalene and [^{14}C]1-naphthol formed by rat liver microsomes. *Mol. Pharmacol.* **16:** 667-675 (1979).

During microsomal metabolism of [^{14}C]naphthalene, radioactive material was irreversibly bound to microsomal protein. The binding seems to result predominantly from secondary oxidation of naphthol rather than from primary monooxygenation of the hydrocarbon. This is supported by the following observations: a) The metabolism of naphthalene was rapid, e.g., 100 nmoles were metabolized within 10 min, whereas protein-bound radioactivity increased for at least 80 min. b) Inhibition of the epoxide hydrase by trichloropropene oxide did not significantly affect the binding. c) Addition of UDP-glucuronic acid to the incubation mixture reduced the binding, presumably by conjugation of the phenolic metabolites. d) During microsomal metabolism of [^{14}C]1-naphthol, a major metabolite of [^{14}C]naphthalene, a considerable amount of protein-bound radioactivity was found, indicating the formation of reactive metabolites. e) The addition of unlabeled 1-naphthol decreased the binding of [^{14}C]naphthalene; this may be attributed to a dilution of the [^{14}C]labeled naphthol-pool derived from [^{14}C]naphthalene. Thus, the results are in agreement with our previous observations on the binding studies of 2,2'-dichlorobiphenyl and suggest that the major part of protein-bound metabolites of some aromatic hydrocarbons, formed *in vitro,* derive from secondary metabolism of the phenol(s).

INTRODUCTION

Recently it has been shown that a number of chemically and biologically inactive foreign compounds are converted by microsomal enzymes to reactive metabolites. Drug-induced carcinogenicity, mutagenicity and cytotoxicity have been attributed to the covalent binding of reactive intermediates to cellular macromolecules (for review see 1, 2), but in only a few cases have the binding metabolites been identified (3, 4). One group of intermediates that are thought to be responsible for covalent binding to cellular macromolecules are the arene oxides formed during metabolism of aromatic hydrocarbons (5). Our previous studies on the metabolism-dependent irreversible binding of the aromatic hydrocarbon [^{14}C]2,2'-dichlorobiphenyl suggested that the major part of the binding originates from intermediates other than the primary epoxide. These may be formed by secondary metabolism (6). It was of interest to know whether this is a more general phenomenon and applies to other aromatic hydrocarbons such as naphthalene. During

0026-895X/79/050667-09$02.00/0

the metabolism of naphthalene in microsomes two major metabolites are formed: naphthalene-1,2-dihydrodiol and 1-naphthol (7). Metabolism of the dihydrodiol is slow in the rat liver (8). It should be absent in microsomal incubations since no soluble dihydrodiol dehydrogenase is present (9). We assumed therefore that binding of naphthalene should preferentially be mediated via formation of 1-naphthol rather than via the dihydrodiol. The results indicate that [^{14}C]1-naphthol is metabolized in the presence of microsomes and a NADPH-generating system and that a high percentage of the [^{14}C]1-naphthol metabolites becomes bound to protein.

MATERIALS AND METHODS

Materials. [^{14}C]Naphthalene (specific activity 3.67 mCi/mmol) and [^{14}C]1-naphthol (specific activity 20.1 mCi/mmol) were obtained from Amersham Buchler, Braunschweig; TCPO and 7,8-benzoflavone from EGA-Chemie KG, Steinheim, Germany; SKF 525-A from Smith, Kline & French, Philadelphia, USA; superoxide dismutase (E.C. 1.15.1.1; 2900 units per mg protein) from Sigma Chemie GmbH, München, Germany; and hyamine hydroxide from W. Zinsser, Scintillators, Frankfurt, Germany. All other chemicals and biochemicals were purchased either from E. Merck, Darmstadt, or from Boehringer, Mannheim, Germany, at highest purity available.

Animal treatments. Male Wistar rats weighing 150–200 g were obtained from our animal breeding station. To induce the hepatic microsomal monooxygenase animals received 0.1% phenobarbital in their drinking water for 12 days.

Preparation of microsomes. Microsomes were prepared as described previously (10). Microsomal preparations were used immediately. In the following, microsomes obtained from untreated or phenobarbital-treated rats are referred to as control- or phenobarbital-microsomes, respectively. If not stated otherwise, experiments were done with phenobarbital-microsomes.

Determination of the microsomal metabolism of naphthalene and 1-naphthol. Naphthalene or 1-naphthol was incubated at 37° with microsomes (1 mg protein/ml) and the NADPH-generating system, consisting of 1 mM NADP, 8 mM sodium DL-isocitrate and 0.1 units isocitrate dehydrogenase (E.C. 1.1.1.42). For determination of naphthalene, a 1 ml aliquot of the incubation mixture was extracted with 4 ml n-hexane. To remove the phenolic metabolites 2 ml of the organic phase were shaken with 2 ml of 1 N-NaOH. The concentration of naphthalene in the hexane layer was determined by recording the UV-absorbance at 276 nm using a molar extinction coefficient of 6700. Naphthol was measured fluorimetrically following the procedure to determine phenolic products of benzo(a)pyrene (11): 1 ml of the incubation mixture was extracted with 4 ml n-hexane/n-propanol (3:1 v/v). A 2 ml aliquot of the organic phase was then shaken with 2 ml of 1 N-NaOH. The fluorescence of the aqueous-alkaline phase was determined with an Aminco-Bowman fluorimeter, using excitation and emission wavelengths of 328 nm and 468 nm, respectively. About 95% of added 1-naphthol could be recovered by this method. Metabolites of naphthalene were separated on a high pressure liquid chromatograph (Model Laboratory Data Control) fitted with a nucleosil 10 C_{18} column. The column was eluted with a reverse phase gradient system with methanol and water (40:60 initially, and 80:20 at the end). Fractions of 1 ml were collected and radioactivity was determined. Naphthalene and naphthol were identified by reference compounds. Dihydrodiol was identified by mass spectral analysis.

Determination of irreversibly bound [^{14}C]naphthalene and [^{14}C]1-naphthol. Irreversible binding of [^{14}C]labeled naphthalene and 1-naphthol was determined as described previously (6). [^{14}C]Naphthalene and [^{14}C]1-naphthol were added in 10 µl of methanol (specific activity 0.45 Ci/Mol). Inhibitors or other compounds used to modify binding of naphthalene and 1-naphthol were added.

Determination of lipid peroxidation. Lipid peroxidation was estimated by measuring the formation of malondialdehyde as described previously (10). For stimulation of lipid peroxidation, 0.1 mM $FeSO_4$, 2 mM

ADP and 0.2 mM ascorbic acid were added prior to the incubation.

RESULTS

Microsomal metabolism of naphthalene and 1-naphthol. Naphthalene was very rapidly metabolized in the presence of phenobarbital-microsomes and an NADPH-regenerating system. Within 10 min, 100 nmoles of naphthalene disappeared from the incubation mixture (Fig. 1). During this time, naphthol was formed reaching a concentration of about 12 μM. After 10 min the concentration of naphthol slowly decreased at a rate of about 0.3 nmol/min/mg of protein. When 200 nmoles of naphthalene were metabolized about 10% (phenobarbital-microsomes) to 50% (control-microsomes) of the substrate were left after a 15 min incubation period. Under these conditions the final concentration of free naphthol increased to 20–30 μM (phenobarbital-microsomes) and 3–5 μM (control-microsomes), respectively. High pressure liquid chromatography analysis of the metabolites formed from [^{14}C]naphthalene by phenobarbital-microsomes showed that the dihydrodiols comprised about half of the labeled metabolites that could be extracted with ethylacetate:acetone (4:1 v/v) from the incubation mixture. In the presence of 0.3 mM TCPO,[1] a potent inhibitor of the epoxide hydrase (12), the dihydrodiol peak was no longer detectable, but the naphthol peak increased about three-fold (data not shown). These results are in agreement with those described previously by Bock *et al.* (8) and Oesch and Daly (13).

When 1-naphthol (100 nmoles) was incubated with phenobarbital-microsomes, the initial metabolism was 3.5 nmol/min (Fig. 2). After 10 min the metabolism slowed down and came to a halt after 40 min, although about 30 nmoles of the substrate were left.

Irreversible binding of [^{14}C]naphthalene. During microsomal metabolism of [^{14}C]naphthalene, [^{14}C]radioactivity was irreversibly bound to microsomal protein. With control microsomes, 5 nmol [^{14}C]-naphthalene/15 min/mg of protein were bound. SKF 525-A inhibited the binding almost completely, but 7,8-benzoflavone had no such effect (Table 1). Phenobarbital pretreatment increased binding by 30%. This binding was completely inhibited in the presence of 100% nitrogen, while a carbon monoxide:oxygen ratio of 8:2 decreased the binding of radioactivity by 85%.

Figure 3 shows the time course of protein-binding of 0.1 mM naphthalene. Protein-bound radioactivity increased during the 120 min incubation period. The initial binding rate of 3.5 nmol [^{14}C]naphthalene/min/mg of protein decreased after 10 min. During the following 30 min only 40 nmol/mg protein were bound.

Irreversible binding of [^{14}C]1-naphthol. Binding also occurred when [^{14}C]1-naphthol was used as substrate for microsomal metabolism (Fig. 3). The binding of [^{14}C]1-naphthol was more than twice that of [^{14}C]-naphthalene (Table 1, Fig. 3). It was

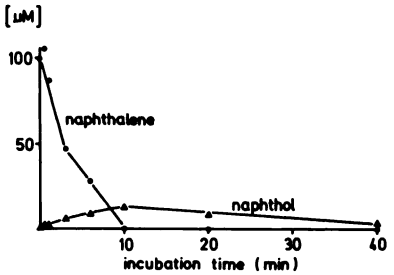

FIG. 1. *Disappearance of naphthalene from the incubation mixture and formation of naphthol*

Incubations were carried out with phenobarbital-microsomes (1 mg protein/ml) and 0.1 mM of naphthalene. For other conditions see MATERIALS AND METHODS.

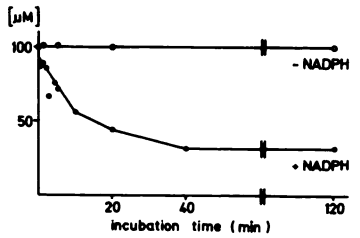

FIG. 2. *Disappearance of 1-naphthol during microsomal metabolism*

Incubations were carried out with phenobarbital-microsomes (1 mg protein/ml) and 0.1 mM 1-naphthol. Details of determination are given in MATERIALS AND METHODS.

[1] The abbreviations used are: TCPO, 1,1,1-trichloro-2-propene oxide; SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate.

TABLE 1

Effects of modification of monooxygenase activity on binding of [^{14}C]naphthalene and [^{14}C]1-naphthol

Experiments were carried out with 0.2 mM of substrate. For other conditions see MATERIALS AND METHODS. Values represent means ± S.D. and are corrected for binding of [^{14}C]radioactivity in the absence of NADP. Number of independent experiments are given in parentheses.

Conditions	bound radioactive material	
	[^{14}C]naphthalene	[^{14}C]1-naphthol
	(nmol/15 min/mg of protein)	
Control microsomes	5.0 ± 0.5 (6)	12.0 ± 0.7 (6)
+ SKF (1 mM)	0.2 ± 0.1 (6)	10.7 ± 0.5 (6)
+ 7,8-benzoflavone (0.05 mM)	5.6 ± 0.5 (3)	14.1 ± 1.1 (3)
Phenobarbital microsomes	6.6 ± 1.0 (6)	13.7 ± 1.9 (3)
− O_2 (100% N_2 atmosphere)	0.7 (2)	3.3 ± 1.3 (4)
+ N_2/O_2 (8:2 atmosphere)	8.7 (2)	14.2 ± 1.6 (4)
+ CO/O_2 (8:2 atmosphere)	1.1 (2)	8.8 ± 0.7 (4)

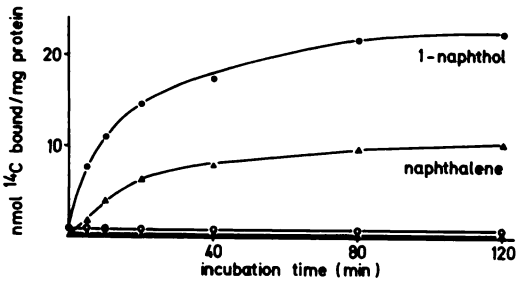

FIG. 3. *Irreversible binding of [^{14}C]naphthalene and [^{14}C]1-naphthol to microsomal protein in the presence (filled symbols) and in the absence (unfilled symbols) of a NADPH-regenerating system*

Incubations were carried out with phenobarbital-microsomes (1 mg protein/ml) and 0.1 mM of substrate.

not significantly stimulated by pretreatment of the rats with phenobarbital (Table 1). Addition of SKF 525-A or 7,8-benzoflavone did not significantly inhibit the binding, in either control- (Table 1) or in phenobarbital-microsomes (data not shown). Exposure to an atmosphere of 100% nitrogen decreased the binding by 75%, but in the presence of carbon monoxide the binding was inhibited by 40% (Table 1). The dependency of the degree of binding on the concentration of [^{14}C]1-naphthol is shown in a double reciprocal plot in Figure 4. The apparent K_m value of the reaction was about 20 μM, i.e., the range of concentrations of naphthol formed during the metabolism of naphthalene.

Influence of TCPO and UDP-glucuronic acid on the binding of [^{14}C]naphthalene and [^{14}C]1-naphthol. The possible involvement of phenols and dihydrodiols in the binding of naphthalene was investigated by examining the effects of TCPO and UDP-glucuronic acid.

Addition of TCPO to the incubation mixture did not significantly affect the binding of [^{14}C]naphthalene or of [^{14}C]1-naphthol during a 15 min incubation period (Table 2). Addition of UDP-glucuronic acid together with UDP-N-acetylglucosamine, an activator of the glucuronyltransferase system (8), decreased the rate of binding of [^{14}C]naphthalene by about 50% during the first few minutes to bring it to a halt after 7.5 min (Fig. 5A). UDP-glucuronic acid, at the concentration used, did not influence the total metabolism of naphthalene but it effectively inhibited the appearance of free naphthol (Fig. 5B). The binding of [^{14}C]1-naphthol measured after a 15 min incubation period was inhibited by 50% in the presence of UDP-glucuronic acid and UDP-N-acetylglucosamine (data not shown).

Effect of unlabeled 1-naphthol on the binding of [^{14}C]naphthalene. If the binding of [^{14}C]naphthalene occurs via the intermediary formation of naphthol, addition of

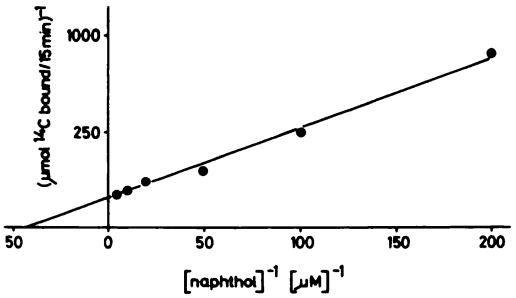

FIG. 4. *Double reciprocal plot of the amount of bound [^{14}C]radioactivity versus the concentration of [^{14}C]1-naphthol*

Incubations were carried out with phenobarbital microsomes (1 mg of protein/ml) for 15 min. Values are corrected for binding in the absence of NADP.

TABLE 2

Effects of various agents on the binding of [^{14}C]-naphthalene and [^{14}C]1-naphthol

Experiments were carried out with 0.2 mM of substrate. For other conditions see MATERIALS AND METHODS. Values represent means ± S.D. and are corrected for binding of [^{14}C]radioactivity in the absence of NADP. Number of independent experiments are given in parentheses.

Conditions	Bound radioactive material	
	[^{14}C]naphthalene	[^{14}C]1-naphthol
	(nmol/15 min/mg of protein)	
Complete	6.6 ± 1.0 (6)	13.7 ± 1.9 (3)
A		
+ TCPO (0.3 mM)	7.5 ± 1.6 (6)	12.3 ± 0.7 (4)
+ TCPO (0.4 mM)	7.0 ± 0.8 (4)	12.0 ± 0.9 (4)
B		
+ EDTA (0.1 mM)	7.7 ± 0.7 (4)	14.8 ± 1.7 (4)
+ Fe^{++}/ADP/ascorbic acid	0.3 ± 0.1 (4)	5.6 ± 1.0 (4)
C		
+ glutathione (1 mM)	2.5 ± 1.2 (4)	4.5 ± 1.4 (4)
+ cysteine (1 mM)	0.4 ± 0.1 (4)	0.2 ± 0.1 (4)
+ lysine (1 mM)	6.1 ± 0.4 (4)	10.4 ± 1.3 (4)
D		
+ superoxide dismutase (50 units)	9.0 ± 1.1 (4)	15.5 ± 0.4 (4)
+ catalase (600 units)	6.9 ± 0.6 (4)	12.6 ± 1.4 (4)
+ ascorbic acid (0.5 mM)	5.1 ± 0.5 (4)	9.2 ± 2.0 (4)

unlabeled 1-naphthol should lead to a decrease in the binding of radioactively labeled material. Figure 6 shows that addition of unlabeled 1-naphthol (10 μM) to the incubation mixture decreased the binding of [^{14}C]naphthalene. The rate of disappearance of naphthalene was not inhibited under the same conditions (Fig. 7).

Relationship of lipid peroxidation and binding of [^{14}C]naphthalene and [^{14}C]1-naphthol: Effect of EDTA and Fe^{++}, ADP and ascorbic acid. In agreement with the observations of others (14), lipid peroxidation in phenobarbital-microsomes was effectively inhibited by addition of 0.1 mM EDTA to the incubation mixture. On the other hand, addition of Fe^{++}, ADP and ascorbic acid stimulated a high rate of lipid peroxidation (data not shown). In contrast, EDTA had no inhibitory effect on the binding (Table 2). Furthermore, when lipid peroxidation was triggered by addition of Fe^{++}, ADP and ascorbic acid, the binding of [^{14}C]naphthalene decreased almost to zero, that of [^{14}C]naphthol to 40%.

Binding of [^{14}C]naphthalene and [^{14}C]-1-naphthol in the presence of glutathione

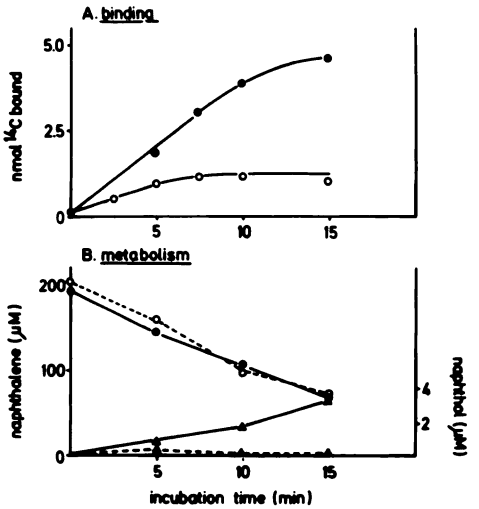

FIG. 5. *Effect of UDP-glucuronic acid on the binding of [^{14}C]naphthalene (A), on the total metabolism of naphthalene (B, circle) and on the formation of naphthol from naphthalene (B, triangles)*

Filled symbols: control incubations; unfilled symbols: incubations in the presence of 3 mM UDP-glucuronic acid and 3 mM UDP-N-acetylglucosamine. Incubations were carried out with control-microsomes (1 mg of protein/ml) and 0.2 mM of naphthalene.

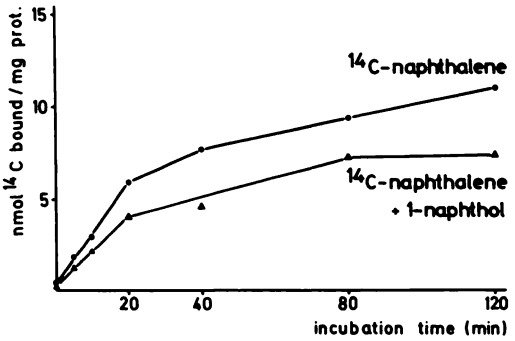

FIG. 6. *Irreversible binding of [^{14}C]naphthalene to microsomal protein in the presence of unlabeled 1-naphthol*

Incubations were carried out with phenobarbital-microsomes (1 mg protein/ml) and 0.1 mM of [^{14}C]-naphthalene. Other samples contained, in addition, 0.01 mM of unlabeled 1-naphthol.

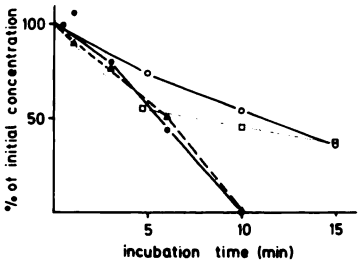

FIG. 7. Total metabolism of 0.1 mM naphthalene in the absence (—●—) and presence (- -▲- -) of 0.01 mM 1-naphthol, and of 0.2 mM naphthalene in the absence (—○—) and presence (····□····) of 1 mM glutathione.

and amino acids. Addition of glutathione (1 mM) decreased the binding of [^{14}C]naphthalene and of [^{14}C]1-naphthol by 60–70% (Table 2), but it did not inhibit the total metabolism of naphthalene (Fig. 7) or 1-naphthol (data not shown). Cysteine (1 mM) inhibited the binding even more effectively, whereas lysine (1 mM) had no effect.

Effects of superoxide dismutase and catalase on binding. Binding may involve the formation of semiquinones or quinones (15) which may be generated from catechols by mediation of superoxide anions (16). Therefore, the effect of superoxide dismutase was examined. As shown in Table 2, the binding of [^{14}C]naphthalene and of [^{14}C]1-naphthol was not inhibited in the presence of superoxide dismutase, but showed rather a slight increase. Catalase did not influence the degree of binding. Ascorbic acid slightly reduced the degree of binding.

DISCUSSION

Incubation of naphthalene with rat liver microsomes led to the formation of reactive metabolites that irreversibly bound to protein. This protein-binding was dependent on the oxidative metabolism of naphthalene because binding did not occur in the absence of NADPH or oxygen, after heat inactivation of the microsomes or in the presence of SKF 525-A. 7,8-Benzoflavone did not affect the binding. This indicates that a cytochrome P-450- rather than a cytochrome P-448-dependent monooxygenase was involved (17).

The findings of others (7) and our present observations on the formation of dihydrodiols indicate that epoxides are formed during the metabolism of naphthalene. If the primary epoxides were the binding species, the kinetics of binding should reflect the kinetics of primary metabolism of naphthalene. However, we found that the time course of binding did not parallel that of primary metabolism of naphthalene. This became evident when an amount of naphthalene was chosen that was metabolized within 10 min. Under these conditions the binding continued to increase for another 70 min. Therefore, the binding of reactive metabolites appears to occur at least partly during secondary metabolism. These results correspond to those previously obtained with ^{14}C-2,2′-dichlorobiphenyl (6).

Because epoxides do not appear to be the main binding species of [^{14}C]naphthalene, we have directed some efforts toward the question of which other reactive metabolites might be formed which bind to protein. Of major interest were semiquinones and quinones which are known to react covalently with nucleophilic sides of proteins (18). In principle, two pathways lead to diphenol-metabolites of aromatic hydrocarbons which can be further oxidized to semiquinones or quinones: a) dihydrodiols can be converted to catechols by a soluble dehydrogenase (9), and b) phenols may be further hydroxylated (19, 20). In microsomal incubations, lacking the soluble dehydrogenase, catechols are not likely to be formed via the dihydrodiol pathway. This is in agreement with the observation that TCPO, which inhibited dihydrodiol formation, did not decrease the degree of binding of naphthalene.

In contrast to the dihydrodiol, 1-naphthol, the second major oxidation product of naphthalene, is subject to microsomal metabolism to dihydroxy-naphthalene (19). Our results show that 1-naphthol was metabolized by microsomes in the presence of NADPH, and that during the metabolism of [^{14}C]1-naphthol radioactivity was irreversibly bound to microsomal protein. Thus, secondary metabolism of naphthalene leading to the formation of reactive metabolites may occur via naphthol. This is further supported by the following observations: 1) The binding of naphthalene as

well as of 1-naphthol was markedly reduced when UDP-glucuronic acid was added to the incubation mixture. Obviously this was due to trapping of naphthol by conjugation which reduced the amount of precursors for the formation of reactive metabolites. Furthermore the binding of naphthalene stopped to increase at that time, when naphthol was no longer detectable in the incubation mixture. 2) Addition of a small amount of unlabeled 1-naphthol decreased the binding of [^{14}C]naphthalene presumably by diluting the [^{14}C]labeled naphthol pool. 3) Less than 4% of the metabolites of [^{14}C]naphthalene and as much as 30% of the metabolites of [^{14}C]1-naphthol were bound to the protein. The former observation is in agreement with the findings of others (8). Since naphthol comprised only about 10% of the total naphthalene metabolites, the binding observed with naphthalene could largely be accounted for by the binding of naphthol. Thus, the data are compatible with the notion that most, if not all, of the binding species are formed via the metabolism of naphthol. This appears to contradict our finding that the binding of naphthalene did not significantly increase in the presence of TCPO, while TCPO increased the concentration of free naphthol about 3-fold. However, the apparent discrepancy might be resolved by taking the binding kinetics into consideration: The free concentrations of naphthol found in the absence and presence of TCPO were 1.5–4.5 times higher than the K_m value for the binding; within this substrate range the rate of binding increased by about 25% that is close to the margins of error of the binding measurements. At lower concentrations of free naphthol, changes such as those induced by TCPO should affect more strongly the binding. Indeed, in the presence of UDPG-glucuronic acid, which reduced the level of free naphthol, TCPO increased binding about three-fold (unpublished observations).

The question of whether or not a cytochrome P-450-dependent monooxygenase is involved in the formation of reactive metabolites from 1-naphthol can not be answered yet. Binding occurred only in the presence of active microsomes, oxygen, and NADPH, and was partly inhibited by carbon monooxide. But neither SKF 525-A nor 7,8-benzoflavone significantly decreased the amount of bound radioactive material. The involvement of lipid peroxidation in the activation of 1-naphthol to binding species could be ruled out since the addition of EDTA, at a concentration which inhibited lipid peroxidation, increased rather than decreased the binding, and furthermore, stimulation of the lipid peroxidation by addition of Fe^{++}, ADP and ascorbic acid markedly inhibited rather than increased the binding presumably by inactivating the metabolizing enzyme.

The nature of reactive metabolites formed from naphthol is not known. Hydroxylation of naphthol may conceivably lead to the formation of catechols or other diphenols that are easily oxidized to form reactive species such as semiquinones or quinones as has been shown for other phenolic compounds (15, 21). A number of observations indicate that the formation of semiquinones from catechols is a nonenzymatic reaction which, in some cases, is mediated by the action of superoxide anion formed during microsomal incubations (16). Our results show that addition of superoxide dismutase, which scavenges superoxide anion, led to a small increase and not to a decrease of the binding of naphthalene and 1-naphthol. This was in contrast to the binding of [^{14}C]2,2-dichlorobiphenyl (6). Also, ascorbic acid, another superoxide trapping agent, had only a weak inhibitory effect. These divergent results may be explained by differences in the chemical nature of 2,2′-dichlorobiphenyl and naphthalene. Differences in the redox potentials may cause the catechol of 2,2′-dichlorobiphenyl to be oxidized by superoxide anion and that of dihydroxynaphthalene by molecular oxygen. In this context it has been shown that 1,2-dihydroxynaphthalene is readily oxidized to 1,2-naphtho-quinone in air at neutral pH (22). It is also possible that the apparently different mechanisms of oxidation are due to the formation of different dihydroxy-metabolites: whereas a para-dihydroxy-metabolite may be formed from naphthalene, this appears unlikely for 2,2′-dichlorobiphenyl unless a chlorine

atom is eliminated. Unlike superoxide dismutase, which specifically scavenges superoxide anions, ascorbic acid reacts also with semiquinones and quinones (23). This could explain the inhibition of the binding of naphthalene and 1-naphthol by ascorbic acid which, however, is only minor compared to the strong inhibitory effect of ascorbic acid against the binding of 2,2′-dichlorobiphenyl. The binding of naphthalene and 1-naphthol is effectively inhibited by SH-group compounds such as glutathione and cysteine, which are known to react with electrophiles and radicals. No inhibition occurred in the presence of lysine which preferentially reacts with electrophiles like quinones (15, 24). Therefore, quinones are less likely to be involved in the binding than semiquinones.

In conclusion, our present results confirm and extend our previous observations on the binding of 2,2′-dichlorobiphenyl (6) that in microsomes the majority of bound metabolites of some aromatic hydrocarbons does not originate from the primary epoxides. The data indicate that other reactive species are formed via a secondary enzymatic activation of the phenolic intermediates.

ACKNOWLEDGMENTS

We wish to thank Dr. Friedrich Wiebel, Dr. Thomas Wolff and Prof. Helmut Greim for many fruitful discussions and critical readings of this manuscript. The excellent technical assistance of Mr. Josef Lichtmannegger is gratefully acknowledged. Mrs. Linda Lattmann and Dr. Dimitris Kotzias are thanked for registration of mass spectra.

REFERENCES

1. Gillette, J. R., J. R. Mitchell, and B. B. Brodie. Biochemical mechanisms of drug toxicity. *Ann. Rev. Pharmacol.* **14:** 271–288, 1974.
2. Gillette, J. R. Formation of reactive metabolites of foreign compounds and their covalent binding to cellular constituents, in *Handbook of Physiology* (Lee, D. M. K., H. L. Falk, S. D. Murphy, and S. R. Geiger, eds.). American Physiological Society, Bethesda, Maryland, 1977, 577–589.
3. Miller, J. A. and E. C. Miller. Chemical carcinogenesis: mechanisms and approaches to its control. *J. Nat. Cancer Inst.* **47:** V–XIV, 1971.
4. Weinstein, J. B., A. M. Jeffrey, K. W. Jennette, S. H. Blobstein, R. G. Harvey, C. Harris, H. Autrup, H. Kasai, and K. Nakamishi. Benzo(a)pyrene diol epoxides as intermediates in nucleic acid binding in vitro and in vivo. *Science* **193:** 592–595, 1976.
5. Daly, J. W., D. M. Jerina, and B. Witkop. Arene oxides and the NIH-shift: The metabolism, toxicity and carcinogenicity of aromatic compounds. *Experientia* **28:** 1129–1149, 1972.
6. Hesse, S., M. Mezger, and T. Wolff. Activation of ^{14}C chlorobiphenyls to protein-binding metabolites by rat liver microsomes. *Chem.-Biol. Interactions* **20:** 355–365, 1978.
7. Jerina, D. M., J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg, and S. Udenfriend. 1,2-naphthalene oxide as an intermediate in the microsomal hydroxylation of naphthalene. *Biochemistry* **9:** 147–155, 1970.
8. Bock, K. W., G. Van Ackeren, F. Lorch, and F. W. Birke. Metabolism of naphthalene to naphthalene dihydrodiol glucuronide in isolated hepatocytes and in liver microsomes. *Biochem. Pharmacol.* **25:** 2347–2356, 1976.
9. Ayengar, P. K., O. Hayaishi, M. Nakajima, and J. Tomida. Enzymic aromatization of 3,5-cyclohexydiene-1,2-diol. *Biochem. Biophys. Acta* **33:** 111–119, 1959.
10. Hesse, S. and T. Wolff. In vitro interactions of di-, tetra- and hexa-chlorobiphenyl with rabbit liver monooxygenase. *Biochem. Pharmacol.* **26:** 2043–2047, 1977.
11. Wiebel, F. J., J. K. Selkirk, H. L. Waters, and S. Brown. Activation of xenobiotics by monooxygenases: cultures of mammalian cells as analytical tool. *Arch. Toxicol.* **39:** 133–148, 1977.
12. Oesch, F., D. M. Jerina, J. W. Daly, and J. R. Rice. Induction, activation and inhibition of epoxide hydrase: an anomalous prevention of chlorobenzene-induced hepatotoxicity by an inhibitor of epoxide hydrase. *Chem.-Biol. Interact.* **6:** 189–202, 1973.
13. Oesch, F. and J. Daly. Conversion of naphthalene to *trans*-naphthalene dihydrodiol: evidence for the presence of a coupled aryl monooxygenase-epoxide hydrase system in hepatic microsomes. *Biochem. Biophys. Res. Commun.* **46:** 1713–1720, 1972.
14. Hochstein, P., K. Nordenbrand, and L. Ernster. Evidence for the involvement of iron in the ADP-activated peroxidation of lipids in microsomes and mitochondria. *Biochem. Biophys. Res. Commun.* **14:** 323–328, 1964.
15. Bolt, H. M. and H. Kappus. Irreversible binding of ethynyl-estradiol metabolites to protein and nucleic acids as catalyzed by rat liver microsomes and mushroom tyrosinase. *J. Steroid Biochem.* **5:** 179–184, 1973.
16. Nelson, S. D., J. R. Mitchell, E. Dybing, and H. A. Sasame. Cytochrome P-450-mediated oxidation

of 2-hydroxyestrogens to reactive intermediates. *Biochem. Biophys. Res. Commun.* **70:** 1157–1165, 1976.

17. Wiebel, F. J., J. C. Leutz, L. Diamond, and H. V. Gelboin. Aryl hydrocarbon (benzo(a)pyrene) hydroxylase in microsomes from rat tissue: differential inhibition and stimulation by benzoflavones and organic solvents. *Arch. Biochem. Biophys.* **144:** 78–86, 1971.
18. Marks, F. and E. Hecker. Metabolism and mechanism of action of oestrogens. XII. Structure and mechanism of formation of water-soluble and protein-bound metabolites of oestrone in rat-liver microsomes in vitro and in vivo. *Biochim. Biophys. Acta* **187:** 250–265, 1969.
19. Daly, J., J. K. Inscoe, and J. Axelrod. The formation of O-methylated catechols by microsomal hydroxylation of phenols and subsequent enzymatic catechol O-methylation. Substrate specificity. *J. Med. Chem.* **8:** 153–157, 1965.
20. Billings, R. E. and R. E. McMahon. Microsomal biphenyl hydroxylation: The formation of 3-hydroxybiphenyl and biphenyl catechol. *Mol. Pharmacol.* **14:** 145–154, 1978.
21. Sasame, H. A., M. M. Ames, and S. D. Nelson. Cytochrome P-450 and NADPH cytochrome C reductase in rat brain: formation of catechols and reactive catechol metabolites. *Biochem. Biophys. Res. Commun.* **78:** 919–926, 1977.
22. van Heyningen, R. and A. Pirie. The metabolism of naphthalene and its toxic effect on the eye. *Biochem. J.* **102:** 842–852, 1967.
23. Deamer, D. W., R. E. Heikkila, R. V. Panganamala, G. Cohen, and D. G. Cornwell. The alloxan-dialuric acid cycle and the generation of hydrogen peroxide. *Physiol. Chem. Physics* **3:** 426–430, 1971.
24. Jellinck, P. H. and L. Irwin. Interaction of oestrogen quinones with ethylene diamine. *Biochem. Biophys. Acta* **78:** 778–780, 1963.