Biochemical Pharmacology, Vol. 38, No. 6, pp. 961–972, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

IN VIVO AND *IN VITRO* STUDIES OF THE HEPATOTOXIC EFFECTS OF 4-CHLOROPHENOL IN MICE*

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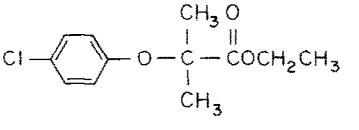
(*Received* 17 *May* 1988; *accepted* 10 *August* 1988)

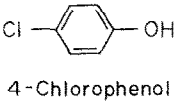
Abstract—4-Chlorophenol (4-CP) was studied for its toxicological effect on liver by using both *in vivo* and *in vitro* approaches. Male mice were administered 4-CP, 1.5 mmol/kg body weight, i.p., and were killed at 10, 20, 30 and 50 min after drug injection. Either i.p. or oral 4-CP administration significantly lowered total liver thiol levels by 20–30% after 30 min and 3 hr respectively. This time-dependent effect of 4-CP after i.p. treatment was enhanced when mice were pretreated with hepatic microsomal enzyme inducers (phenobarbital, 40 mg/kg body weight, b.i.d., 7 days; and β-naphthoflavone, 80 mg/kg body weight once daily, 4 days). Further, the microsomal cytochrome P-450 inhibitor, SKF 525-A, 75 mg/kg body weight injected i.p. to mice 30 min prior to 4-CP administration, blocked the reduction of liver thiol content produced by 4-CP. The results suggest that a chemically reactive intermediate of 4-CP may be formed in liver which is responsible for the observed decrease in liver thiol content. Other investigations were done to characterize the *in vitro* irreversible binding of [^{14}C]4-CP. [^{14}C]4-CP was bound irreversibly to mouse liver microsomal proteins in a concentration-dependent manner. Binding was NADPH dependent and gave a maximal binding of 12.0 nmol/mg protein/20 min and an apparent binding constant of 0.222 mM. [^{14}C]-Binding of 4-CP was increased by 155 and 127% in liver microsomes of phenobarbital- and β-naphthoflavone-treated mice respectively. By contrast, microsomal enzyme inhibitors [metyrapone, α-naphthoflavone, SKF 525-A, and CO : O_2 (4 : 1, v/v)] and selected nucleophilic compounds (glutathione, L-cysteine or L-lysine) significantly reduced [^{14}C]4-CP binding to mouse liver microsomes. An epoxide hydrolase inhibitor, cyclohexene oxide, did not alter the extent of irreversible binding, whereas scavengers of superoxide anions or agents that are reported to reduce accumulation of active semiquinone and quinone species (L-ascorbic acid, superoxide dismutase or epinephrine) decreased the binding of [^{14}C]4-CP to mouse liver microsomal proteins by 56, 31 and 92% respectively. The data suggest that semiquinone and quinone species of 4-CP may be the chemically reactive intermediates leading to the *in vivo* reduction of liver thiol levels. Since 4-CP is a minor contaminant and possible metabolite of clofibrate and chemically related hypolipidemic agents, 4-CP and its metabolites may be partly responsible for some of the hepatotoxic effects seen after long-term administration of this therapeutic class of drugs.

4-Chlorophenol (4-CP) is a locally acting antiseptic used in dental care, and it has been identified as a trace contaminant in clofibrate preparations [1–3] (Fig. 1). It is also a minor metabolite of some hypolipidemic clofibrate analogs [4, 5]. Several international pharmacopeias have specified the purity of clofibrate in pharmaceutical products and only a very low percentage of 4-CP is allowed to be present [6, 7]. However, the toxicological effects of 4-CP have not been adequately investigated. It is possible that chronic administration of clofibrate to patients may lead to a chronic exposure to 4-CP leading to adverse drug reactions such as hepatomegaly, hepatic microsomal enzyme induction and tumorigenicity [8, 9].

Epoxides are reactive intermediates of xenobiotics which are known to bind irreversibly with tissue macromolecules and cause drug toxicity [10–22]. Compounds that are structurally similar to 4-CP, such as trichlorophenols, bromobenzenes, bromophenols, and chlorobenzenes, have been demonstrated to be metabolically activated by hepatic mixed-function oxidase system [12–15]. The chemi-



Clofibrate



4-Chlorophenol

Fig. 1. Chemical structures of clofibrate and 4-chlorophenol (4-CP).

* This work was presented in part at the 67th Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, IL; *Fed Proc* **42**: 891 (Abstr. No. 3507), 1983.

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cally reactive intermediates formed are covalently bound to tissues, and the extent of binding is directly related to their toxicity [10–12, 23].

This study is designed to determine whether 4-CP is oxidized by the hepatic mixed-function oxidase system to form reactive epoxide intermediate or chemically reactive metabolites of 4-chlorocatechol which bind irreversibly to liver nucleophiles *in vivo* and *in vitro*. The epoxide of 4-CP may be a possible reactive electrophilic intermediate or be further converted to a 4-chlorocatechol which can be oxidized to semiquinone or quinone species as reactive metabolites. These two chemical intermediates have been proposed to be responsible for benzene, phenol and acetaminophen toxicity [24–26] and have been reported to be the major reactive metabolites formed in the metabolic biotransformation of chlorobiphenyls [27].

MATERIALS AND METHODS

Drugs and Chemicals

Compounds used in this study and their sources were as follows: phenobarbital sodium (Merck & Co. Inc., Rahway, NJ); β-naphthoflavone and 5-5′-dithiobis-(α-nitrobenzoic acid) (DTNB; Aldrich Chemical Co., Inc., Milwaukee, WI); glutathione (Eastman Kodak Co. Rochester, NY); SKF 525-A (Smith Kline & French Laboratories, Philadelphia, PA); metaphosphoric acid (Mallinkrodt Inc., Paris, KY); and colbaltous chloride, diethylmaleate, metyrapone, α-naphthoflavone, copper sulfate, sodium tartrate, bovine serum albumin, ethylenediamine tetra acetate, TRIZMA, NADP, NADH, NADPH, glucose-6-phosphate disodium salt and glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, MO).

4-Chlorophenol (purity >98%) used in this study was purchased from the Fisher Scientific Co. (Fair Lawn, NJ). It was purified by distillation under vacuum with an air condenser. The initial condensate, 5 ml, was discarded, and the fraction (boiling point = 217°) was collected and used in the experiments. The melting point of the colorless crystals was 43.2° (literature 43°).

[U-^{14}C]4-Chlorophenol of specific activity 20.4 mCi/mmol was obtained from New England Nuclear (Boston, MA). This labeled compound was diluted in methanol and purified by ascending thin-layer chromatography (TLC 0.25 mm thickness, silica gel GF, Analab Inc., North Haven, CT) using a solvent system of toluene:methanol:glacial acetic acid (76:20:4, by vol.). In initial work, 4-CP (R_f = 0.75) and an impurity (R_f = 0.80) were observed in TLC chromatograms. ^{14}C corresponding to 4-CP was recovered and rechromatographed with the same solvent system. Using a radiochromatographic scanner, [^{14}C]4-CP gave a single symmetrical peak with an R_f corresponding to 4-CP. Segments (0.5 cm) of adsorbent gel were also removed from the origin to the solvent front of the thin-layer plates and analyzed for ^{14}C by liquid scintillation spectrometry. The radiochemical purity of [^{14}C]4-CP used in these experiments exceeded 96%.

Animals and drug treatment

Adult male ICR mice, weighing 25–35 g, used in this study were purchased from Harlan Industries (Cumberland, IN). Animals were housed in a facility accredited by the American Association for the Advancement of Laboratory Animal Care and maintained on a 12-hr alternating light and dark cycle. Tap water and Purina rat chow were provided *ad lib.* 4-CP was dissolved in pure corn oil and administered by oral intubation or i.p. injection to mice at doses ranging from 1 to 3 mmol/kg body weight. Control animals were administered an equal volume of pure corn oil. Animals were killed by cervical dislocation or decapitation between 7:00 and 9:00 a.m.

Pretreatment regimens. Phenobarbital sodium was injected i.p. into mice in a dosage of 40 mg/kg body weight, b.i.d., for 7 consecutive days. β-Naphthoflavone was dissolved in warm corn oil and injected i.p. into mice once daily in a dosage of 80 mg/kg body weight for 4 days. Control mice received an identical volume of corn oil by i.p. injection. All animals were killed 16–18 hr after the last dose.

SKF 525-A was prepared in physiological saline solution and injected i.p. into mice at a 75 mg/kg body weight dose 30 min prior to 4-CP administration. Cobaltous chloride was prepared as an aqueous solution and injected s.c., at a dosage of 40 mg/kg to mice once daily for 2 days. Diethylmaleate was injected i.p. into mice at a volume of 0.8 ml/kg body weight 20 min prior to 4-CP administration.

Preparation of liver subcellular fractions

Control and drug-treated animals were killed by decapitation, and the abdominal cavity was opened surgically. Livers were isolated, placed in ice-cold 0.02 M Tris buffer containing 1.15% KCl (pH 7.4), and then blotted, weighed and homogenized in 3 vol. of 0.02 M Tris buffer, pH 7.4, with a Teflon–glass tissue homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min in a Sorvall refrigerated centrifuge (model RC-5B, Du Pont Instruments, Newtown, CT), and the 9000 *g* supernatant fraction was centrifuged at 105,000 *g* for 60 min in a Beckman Ultracentrifuge (model L5-75, Beckman Instruments, Palo Alto, CA) using a 50 Ti rotor head. The 105,000 *g* supernatant fractions were removed by decantation, and microsomal pellets were carefully resuspended by manual homogenization in a small volume of 0.02 M Tris buffer, pH 7.4, and recentrifuged at 105,000 *g* for 45 min. Recovered microsomal pellets were resuspended in 0.02 M Tris buffer, and the final suspensions were analyzed for protein content by the method of Lowry *et al.* [28] using bovine serum albumin (Fraction V) as a protein standard.

Assay of liver thiol levels

Determination of total thiol content in liver was done according to the method described by Owens and Belcher [29]. Livers were removed and placed in ice-cold 3% (w/v) metaphosphoric acid in doubly distilled water. Liver specimens were blotted, weighed and homogenized in 10 ml of ice-cold 3%

metaphosphoric acid. These homogenates were filtered through a 10–15 μm porosity sinter glass funnel by suction. The filtrates obtained were kept in an ice-bath under a nitrogen atmosphere, and total thiol content in these filtrates was assayed as follows. Reaction mixtures contained 1.5 ml of 0.5 M potassium phosphate buffer, pH 7.4, 30 μl of DTNB solution (39.6 mg DTNB, 15 mg $NaHCO_3$ mixed with 10 ml of 0.1 M potassium phosphate buffer, pH 7.4), and 0.5 ml of the recovered supernatant solutions. The samples were mixed thoroughly, and the intensity of the color developed from the reaction was measured at 412 nm. A glutathione standard curve was constructed for the estimation of total thiol content in these tissue samples. Final values were expressed as milligrams of total thiol content per gram of tissue wet weight (mg thiol/g tissue).

Determination of the irreversible binding of 4-chlorophenol to hepatic microsomal proteins

The irreversible binding of 4-CP to microsomal proteins, *in vitro*, was determined by a modification of the method of Jollow *et al.* [30]. Microsomal protein (10 mg in 1 ml) was preincubated for 10 min with 2 ml of 0.02 M Tris buffer, pH 7.4, or 2 ml of NADPH-regenerating system (containing NADP, 3.28 mg; glucose-6-phosphate, 32 mg; glucose-6-phosphate dehydrogenase, 4 I.U.; 0.05 ml of 1 M $MgCl_2$ and 0.02 M Tris buffer, pH 7.4, to a total of 2 ml). If a modifier of 4-CP binding was used, it was added to the incubation mixture in a volume of 0.1 ml and allowed to incubate at 37° with shaking (90 rpm) in air for 10 min. To study the binding of 4-CP to microsomal protein, [^{14}C]4-CP (0.05 to 0.3 μCi per incubation flask) was added, and flasks were incubated at 37° with shaking for 20 min. Reactions were terminated by transferring the mixtures into tubes containing 3.5 ml of hot (90°) trichloroacetic acid (TCA; 10%, w/v) with mixing. All tubes were centrifuged at 10,000 *g* for 20 min and protein precipitates were resuspended in a 3.5 ml volume of hot TCA solution (10%, w/v), washed by vortex mixing for 3 min, and recentrifuged. The protein precipitate was then washed successively in the following solvents: twice each in 3 ml ethanol:water (1:1, v/v), 3 ml of ether and 3 ml of chloroform:methanol (2:1, v/v). The final protein precipitate was digested by incubation at 80° with 1 ml of 0.1 N NaOH in a water bath. Samples were cooled at room temperature and divided into two parts for the measurements of protein [28] and the amount of radioactivity bound to the microsomal protein using liquid scintillation spectrometry and an internal standard for quench correction. Results were expressed as nanomoles of 4-CP bound per milligram of protein per 20 minutes.

In the preliminary experiments, the time course and substrate dependency of the enzymatic ^{14}C-binding of 4-CP in liver microsomes was determined. [^{14}C]4-CP was also found to bind irreversibly and equally to proteins of heat-denatured (100°, 5 min) or nontreated microsomes (data not presented). The extent of nonenzymatic ^{14}C-binding in nontreated microsomes was proportionate to the amount of radioactivity added and not modified by addition of unlabeled 4-CP. A series of flasks in which the NADPH-regenerating system was omitted were also included for the determination of the extent of this nonenzymatic ^{14}C-binding. Within each experiment, the amount of nonenzymatically bound ^{14}C was subtracted from the total binding of ^{14}C in the presence of NADPH and/or hepatic modifiers to give the net ^{14}C-binding (or NADPH-dependent binding) of 4-CP to microsomal proteins. In those experiments where the incubation atmosphere was modified, gas mixtures (80% CO:20% O_2) were bubbled into the incubation mixture for 3 min in a closed incubation system (using a serum bottle with rubber-stopper and needles for gas input and exit). The atmosphere above the incubation mixture was also displaced with that gas mixture. The system was preincubated at 37° for 10 min prior to the addition of [^{14}C]4-CP, which was injected into the incubation mixture through the serum bottle-stopper.

Statistical analysis

Significant differences between values obtained from control and drug-treated animals were tested at the level of 95% confidence ($P < 0.05$) using a two-tail *t*-test.

RESULTS

Effect of 4-chlorophenol on mice liver thiol levels after oral and intraperitoneal administration

To evaluate the dose-dependent effect of 4-CP, mice were administered 1, 2 and 3 mmol/kg body weight of 4-CP by oral intubation. Three hours after drug treatment, liver sections were taken and assayed for thiol content. As indicated in Fig. 2, 1 and 3 mmol/kg body weight doses of 4-CP did not alter liver thiol level, whereas the 2 mmol/kg body weight dose significantly lowered thiol content to about 78% of normal level. Lower doses of 4-CP (0.5 and 1 mmol/mg body weight) did not produce any effect on total liver thiol content and higher doses (4, 6 and 8 mmol/kg body weight) produced seizures and

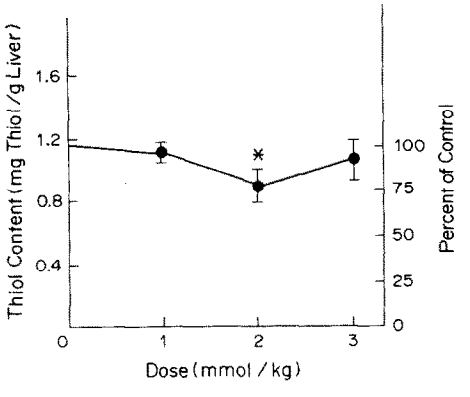

Fig. 2. Dose-dependent effect of 4-chlorophenol on mouse liver thiol levels. 4-Chlorophenol was administered orally to treated animals. Mice were killed 3 hr after drug treatment. Each value is the mean mg thiol content per g liver ± SE (left ordinate), and the values are also expressed as percent of control (right ordinate). Each point was obtained from a group of four to six animals. Key: (*) significantly different from the corresponding control value ($P < 0.05$).

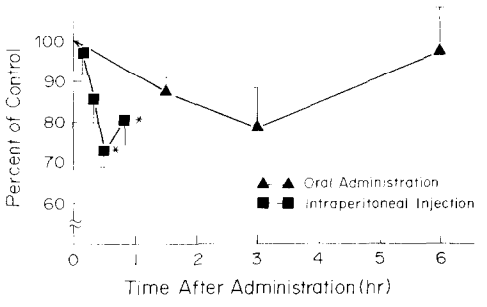

Fig. 3. Effects of different routes of drug administration on the time–course effect of 4-chlorophenol (4-CP) on mouse liver thiol levels. Each value was obtained from groups of four to six mice and is presented as percent of the corresponding control value ± SE. Oral and intraperitoneal dosing of 4-CP were 2 and 1.5 mmol/kg, body wt respectively. Key: (*) significantly different from the corresponding control value ($P < 0.05$).

death of the animals within 3 hr. This observation suggested that a 2 mmol/kg body weight dose of 4-CP would be suitable for subsequent experiments. In other experiments, mice were fasted overnight and 4-CP was given either orally in the dosage of 2 mmol/kg body weight or by i.p. injection with a lower dose of 4-CP (1.5 mmol/kg body weight). Animals were killed at different time periods after drug administration, and the pattern of changes in liver thiol content is shown in Fig. 3. By comparison to oral administration, i.p. injection of 4-CP resulted in a more rapid lowering of liver thiol content. In addition, a similar extent of reduction in thiol content (20–27%) was obtained even though the dose of 4-CP used for injection was less than that used with oral dosing (2.0 vs 1.5 mmol/kg body weight respectively). The decrease in liver thiol occurred with a more rapid onset by i.p. injection, and the rebound effect was seen after 30 min of drug treatment. In contrast, the maximum reduction and rebound of liver thiol levels occurred at later time periods and appeared to show greater variability in those animals given 4-CP orally. Based upon these results, the i.p. route of 4-CP administration was used in subsequent experiments.

Effects of various drug pretreatments on 4-CP lowering of liver thiol content

In preliminary experiments, we found that pretreatment of mice with hepatic enzyme modifiers, phenobarbital, β-naphthoflavone, and cobaltous chloride) produced an elevation in liver thiol levels. Therefore, mice in each modifier-pretreated group were used as their own control. In these studies, the time-dependent changes in liver thiol levels by 4-CP treatment were monitored and expressed as an absolute change in liver thiol content (Fig. 4). The results show that 4-CP induced a significant ($P < 0.05$) decrease in liver thiol levels in nondrug-pretreatment mice only after 30 min. By contrast, the onset of the reduction of liver thiol by 4-CP appeared more rapidly and was significant at all time periods in phenobarbital-pretreated mice. In addition, the magnitude of the change in liver thiol content by 4-CP was greater in phenobarbital-treated *versus* control animals at all time periods. With β-

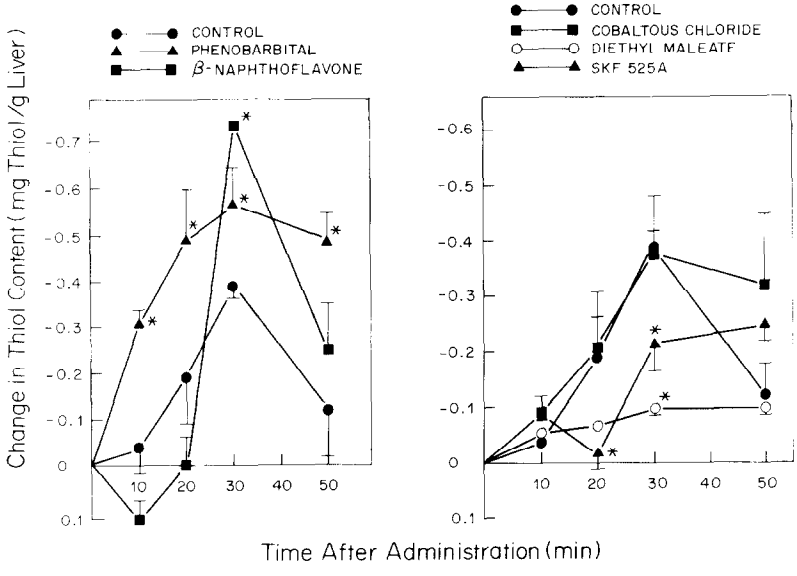

Fig. 4. Comparisons of the effect of pretreatment with hepatic microsomal enzyme inducers (phenobarbital, β-naphthoflavone) or inhibitors (SKF 525A, cobaltous chloride) and thiol modifier (diethylmaleate) on time-dependent effect of 4-chlorophenol on mouse liver thiol levels. Pretreatment regimens are given in Materials and Methods. The absolute changes in liver thiol content (mg/g liver) are presented versus time after 4-chlorophenol treatment (1.5 mmol/kg, body wt, i.p.). Negative values indicate a net decrease in thiol content as compared to the corresponding control group. Each value is the mean ± SE from groups of four to eight animals. Key: (*) significantly different from the corresponding control value ($P < 0.05$).

naphthoflavone pretreatment, a reduction in liver thiol content was found at 30 min after 4-CP administration, and the magnitude of this decrease in thiol level was approximately 2-fold greater than the corresponding control value in non-drug-pretreated animals.

The thiol lowering effect of 4-CP was diminished significantly and the time–course response was prolonged after SKF 525-A treatment (Fig. 4). Pretreatment with SKF 525-A significantly reduced the changes in thiol levels and 10 and 20 min after 4-CP administration. In contrast to SKF 525-A, no significant difference in the time profile of the reduction of thiol level (mg/g liver) was noted between cobaltous chloride treated and non-treated mice after 4-CP administration. Diethylmaleate treatment markedly lowered liver thiol levels to 19% of the normal value, and the absolute change in thiol content (mg/g liver) after 4-CP treatment was very small. However, when these values were converted to percentage of control, the percentage of change in liver thiol content was greater than the corresponding values obtained from nondrug-treated mice.

Irreversible binding of [^{14}C]-4-*chlorophenol to mouse liver microsomes*

The NADPH dependency of the binding of [^{14}C]4-CP to liver microsomes was studied (Fig. 5). In initial experiments, ^{14}C was found to bind irreversibly to liver microsomal proteins in the presence and absence of NADPH. To characterize the irreversible binding of ^{14}C to microsomal proteins, the time course for the covalent binding to [^{14}C]4-CP to mouse liver microsomes was monitored in the presence and absence of NADPH (Fig. 5A). We found that ^{14}C-binding in the absence of NADPH was rapid and increased by approximately 60% at 10 and 20 min. However, there was a linear increase in the total ^{14}C-binding in the presence of NADPH throughout a 30-min period. When the amount of NADPH-independent binding was subtracted from the total ^{14}C-binding, the net irreversible binding, which represents the NADPH-dependent binding process, was found to be linear throughout a 20-min period. Thus, in later experiments, a 20-min incubation period was used for the studies of the characteristics of [^{14}C]4-CP binding to mouse liver microsomal proteins.

To determine whether the NADPH-dependent and -independent processes of ^{14}C-binding to microsomal proteins were due to 4-CP, various concentrations of unlabeled 4-CP were added to incubation mixtures containing a constant amount of ^{14}C. As shown in Fig. 5B, the NADPH-independent binding of ^{14}C was unaffected by varying 4-CP concentrations and this represented 3.3 to 3.5% of the total radioactivity added to the reaction mixture. In contrast, the NADPH-dependent binding of ^{14}C to microsomal proteins was reduced proportionate to the amount of unlabeled 4-CP in the incubation mixture. At the lowest concentration of 4-CP used (80 μM), the NADPH-dependent binding of ^{14}C exceeded the NADPH-independent binding by 4-fold and accounted for 16% of the total ^{14}C added. These results indicated that the irreversible binding of ^{14}C in the absence of NADPH is a nonspecific process which is independent of 4-CP, and it may be suggested that this is due to the presence of a radiochemical impurity.

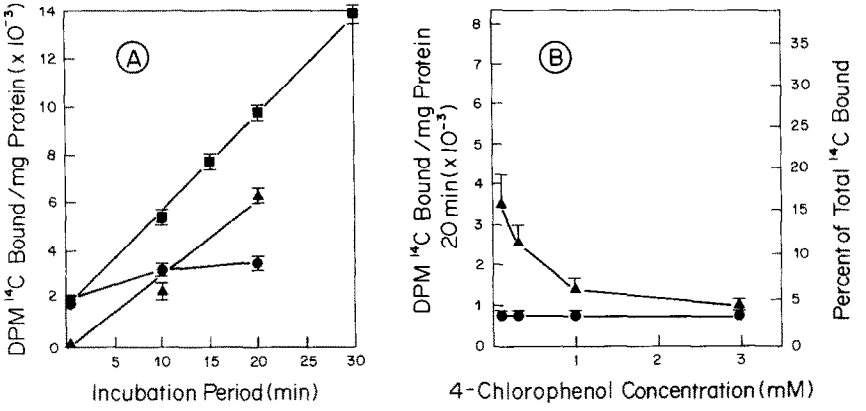

Fig. 5. Panel A: Time course of the nonenzymatic and enzymatic irreversible binding of [^{14}C]4-chlorophenol ([^{14}C]4-CP) to mouse liver microsomal proteins. Data are expressed as dpm ^{14}C bound/mg protein in the presence of 0.2 mM 4-CP. Each incubation mixture contained 0.3 μCi [^{14}C]4-CP and was incubated in the presence or absence of NADPH for 0, 10, 20, 30 and 40 min. Key: (■) total ^{14}C bound in presence of NADPH; (●) ^{14}C bound in absence of NADPH; and (▲) net ^{14}C bound due to presence of NADPH. Panel B: Effect of various concentrations of unlabeled 4-CP on the NADPH-dependent and NADPH-independent irreversible binding of [^{14}C]4-CP to mouse liver microsomes. Key: (●) ^{14}C bound in absence of NADPH and (▲) net ^{14}C bound due to presence of NADPH. Data are presented as ^{14}C bound per mg protein per 20 min (left ordinate) or as a percent of total ^{14}C bound (right ordinate) in the presence of various concentrations of 4-CP (0.1 to 3 mM; 0.1 μCi/incubation). Data are the means ± SD of four incubations. Enzymatic binding of ^{14}C to protein was determined in liver microsomes supplemented with an NADPH-generating system as described in Materials and Methods.

Table 1. Effect of reduced pyridine nucleotides on covalent binding of [^{14}C]4-chlorophenol ([^{14}C]4-CP) to mouse liver microsomal proteins

Incubation conditions	Covalent binding of [^{14}C]4-CP* (nmol/mg protein/20 min)
NADPH, 1 mM	12.6 ± 0.46
NADH, 1 mM	1.69 ± 0.18†
NADPH, 1 mM, + NADH, 1 mM	9.2 ± 1.22
NADPH, 0.5 mM	11.9 ± 0.30
NADH, 0.5 mM	1.76 ± 0.40†
NADPH, 0.5 mM, + NADH, 0.5 mM	11.3 ± 0.31

* Data are the means ± SE of triplicate incubations. Microsomal incubations were done with the pyridine nucleotide cofactor addition(s) as described in Materials and Methods. The concentration of 4-CP used was 0.2 mM.

† Significantly different ($P < 0.05$) from value obtained for 0.5 or 1 mM NADPH.

Since the enzymatically mediated binding of 4-CP to microsomal protein was higher in the presence of NADPH, we also determined whether NADPH or NADH was the pyridine cofactor required for this enzymatic binding process. Microsomal protein was incubated with 0.2 mM 4-CP (0.1 μCi per incubation) in the presence of either NADPH and/or NADH in various concentrations (Table 1). The amount of 4-CP bound to microsomes in the presence of NADPH was 7-fold greater than the amount bound when NADH was added. In addition, these results also indicate that the concentration of NADPH used in our experiments was present in a saturating amount. As shown in Table 1, when one-half of the amount of NADPH-regenerating system was used, the amount of ^{14}C bound to microsomal proteins was not changed. The combination of NADH and NADPH did not significantly increase or decrease the ^{14}C-binding, suggesting that NADH is not a competitor of this enzymatic process mediated by NADPH in mouse liver microsomes. In the remaining experiments, NADPH was included as the pyridine cofactor for the support of [^{14}C]4-CP binding to mouse liver microsomes.

Characteristics of the NADPH-mediated process of 4-chlorophenol binding to mouse liver microsomes

To determine the substrate dependency of 4-CP binding, mouse liver microsomes were incubated with various concentrations of 4-CP, and the kinetic results of a representative experiment are given in Fig. 6. The NADPH-dependent irreversible binding of [^{14}C]4-CP was a saturable process in concentrations of 0.08 to 3.0 mM with an irreversible binding constant (K_b) of 0.222 mM and a maximum binding (B_{max}) of 12.0 nmol per mg protein per 20 min.

Effect of hepatic metabolic inhibitors on 4-chlorophenol binding to liver microsomes

To assess whether 4-CP was converted to a chemically reactive metabolite by the mixed-function monooxygenase system, liver microsomes were incubated with metabolic inhibitors for 10 min prior to the addition of [^{14}C]4-CP, and the extent of ^{14}C was determined after 20 min. The results of the effect of selected metabolic inhibitors of liver microsomal cytochrome P-450 on 4-CP binding are given in Table 2. As shown, incubation of liver microsomes with 1 mM metyrapone, α-naphthoflavone or SKF 525-A significantly reduced the NADPH-dependent binding of [^{14}C]4-CP to 35, 0 and 31% of the corresponding control values respectively.

More direct evidence for the involvement of the hemoprotein, cytochrome P-450, in this binding process was obtained by partial replacement of the incubation atmosphere with carbon monoxide. When the binding of 4-CP was performed under an atmosphere of carbon monoxide:oxygen (4:1, v/v), the enzymatic binding of 4-CP was reduced to 13% of the control value (Table 2). This evidence suggested that 4-CP undergoes oxidative metabolism by cytochrome P-450 to a chemically reactive intermediate which is bound to microsomal proteins.

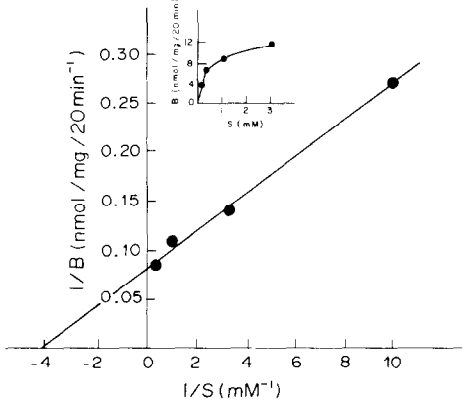

Fig. 6. Kinetic characteristics of the *in vitro* covalent binding of [U-^{14}C]4-chlorophenol to liver microsomal proteins. Data are expressed as ^{14}C bound (B) in nmol per mg protein per 20 min. Results are expressed as a double-reciprocal plot, and kinetic parameters were determined using linear regression analysis. Inset shows a Michaelis plot of the data. Each point is the mean of three incubations. Enzymatic binding of ^{14}C (0.1 μCi) to proteins was determined in liver microsomes supplemented with an NADPH-generating system as described in Materials and Methods. The concentration of 4-CP varied from 0.1 to 3.0 mM.

Table 2. Effect of selected metabolic inhibitors of the mixed-function monooxygenase system on the covalent binding of [^{14}C]4-chlorophenol ([^{14}C]4-CP) to mouse liver microsomal proteins

Treatment	NADPH-mediated [^{14}C]4-CP binding*	
	nmol/mg protein/20 min	% Control
Experiment 1		
Control	1.86 ± 0.21	100 ± 11
Metyrapone, 1 mM	0.64 ± 0.11†	34 ± 5
α-Naphthoflavone, 1 mM	0 ± 0.15†	0 ± 8
Experiment 2		
Control	5.25 ± 0.47	100 ± 9
SKF 525A, 1 mM	1.61 ± 0.47†	31 ± 9
$CO:O_2$, 4:1 (v/v)	0.71 ± 0.25†	14 ± 5

* Data are the means ± SE of triplicate incubations. Microsomal incubations were done with the pyridine nucleotide cofactor addition(s) as described in Materials and Methods. The concentration of 4-CP used was 0.2 mM.

† Significantly different ($P < 0.05$) from value obtained for 0.5 or 1 mM NADPH.

Effects of various nucleophiles and enzymes on 4-chlorophenol binding to mouse liver microsomes

In this series of experiments, the use of amino acids, glutathione, L-ascorbic acid, cyclohexene oxide, superoxide dismutase and epinephrine as agents which could modify the binding of reactive species of 4-CP to tissue macromolecules was investigated (Table 3). As shown, 1 mM glutathione, L-cysteine or L-lysine significantly reduced the binding of 4-CP to 53, 79 and 59% of the control, respectively, whereas glycine had no effect on the extent of 4-CP binding in liver microsomes. The differential ability of these amino acids to modify the extent of the NADPH-dependent ^{14}C-binding process is in agreement with the involvement of cytochrome P-450 in the formation of a reactive electrophilic intermediate of 4-CP.

The effects of L-ascorbic acid, cyclohexene oxide, superoxide dismutase and epinephrine on the binding of [^{14}C]4-CP to liver microsomes were also studied (Table 3). Only L-ascorbic acid (1 mM), superoxide dismutase (50 I.U.) and epinephrine (0.5 mM) significantly reduced the binding of 4-CP to 44, 69 and 8% of control respectively. In other experiments, preincubation with cyclohexene oxide (0.5 and 1 mM) did not modify significantly the ^{14}C-binding of 4-CP to liver microsomal proteins using low (0.2 mM) or high (1.0 mM) concentrations of 4-CP (data not presented).

DISCUSSION

The toxicological effects of 4-CP, a trace contaminant of clofibrate preparations, have not been investigated to date. In this study, the formation of a reactive intermediate in the metabolic biotransformation pathway of 4-CP is being proposed since compounds that are structurally similar to 4-CP have been shown to produce toxicity presumably through the formation of epoxides, semiquinones or quinones [24, 27, 31, 31] and covalent binding to tissue macromolecules. Phenol and chlorobiphenyls are similar cytotoxic compounds which are proposed to cause their toxic effects through semiquinone and quinone formation [25, 27], whereas bromobenzene and trichlorobenzene are shown to mediate their hepatoxicity via an epoxide intermediate [15, 31, 33, 34]. While our studies were in progress, Lau *et al.* [35] have proposed that the liver microsomal oxidation of 4-bromophenol to 4-bromocatechol leads to formation of a chemically reactive quinone species which is covalently bound to tissue proteins, *in vitro*.

Glutathione is available in liver in a high concentration, and any electrophilic metabolites formed in liver will undergo interaction with liver thiol forming adducts resulting in a lowering of liver thiol content [36]. To determine whether 4-CP is converted to a chemically reactive metabolite, *in vivo* experiments were done by administering a high dose of 4-CP to mice and monitoring time–course changes in liver thiol content. We found that acute administration of high doses of 4-CP (2 mmol/kg body weight orally or 1.5 mmol/kg body weight i.p.) significantly lowered liver thiol levels. A reduction in liver thiol levels is suggestive evidence that either 4-CP itself or the conversion to a reactive electrophilic species was involved in this thiol depleting action.

Our studies with 4-CP differ from results of bromobenzene and 4-bromophenol on liver thiol reduction, *in vivo* [11, 33, 34]. Intraperitoneal administration of bromobenzene significantly lowers liver thiol level in rats; however, the onset of action and the duration of thiol lowering effect of bromobenzene are much slower and longer [11] than those produced by 4-CP in our experiment. In addition, these investigators did not observe any alteration in liver thiol content after 4-bromophenol administration. 4-Bromophenol was reported to be a metabolite of bromobenzene, and it has been demonstrated to be further converted to bromocatechol [33–35]. Therefore, it is possible that a reactive intermediate of bromophenol is formed, and this metabolite may reduce liver thiol levels. Monks *et al.* [33, 34] reported that intraperitoneal administration of 4-bromophenol to rats does not lower liver thiol content when assayed hourly. As shown in this study (Fig. 3), changes in

Table 3. Effects of selected amino acids, L-ascorbic acid, cyclohexene oxide and superoxide dismutase on the covalent binding of [^{14}C]4-chlorophenol ([^{14}C]4-CP) to mouse liver microsomal proteins

Treatment	NADPH-mediated [^{14}C]4-CP binding*	
	nmol/mg protein/20 min	% Control
Experiment 1		
Control	2.75 ± 0.07	100 ± 3
Glutathione		
0.5 mM	2.02 ± 0.33†	73 ± 12
1.0 mM	1.45 ± 0.15†	53 ± 5
Cysteine		
0.5 mM	2.52 ± 0.38	92 ± 14
1.0 mM	2.16 ± 0.21†	79 ± 8
Glycine		
1 mM	2.62 ± 0.13	98 ± 5
L-Ascorbic acid		
0.5 mM	1.68 ± 0.8†	61 ± 3
1.0 mM	1.21 ± 0.25†	44 ± 9
Experiment 2		
Control	5.25 ± 0.47	100 ± 9
Lysine		
1.0 mM	3.1 ± 0.29†	59 ± 6
Cyclohexene oxide		
1.0 mM	4.11 ± 0.47†	78 ± 9
Superoxide dismutase		
50.0 I.U.	3.6 ± 0.12†	69 ± 2
Experiment 3		
Control	3.63 ± 0.20	100 ± 5
Epinephrine		
0.5 mM	0.30 ± 0.11†	8 ± 3

* Data are the means ± SE of three to four incubations. Microsomal incubations were done as described in Materials and Methods. The concentration of 4-CP used was 0.2 mM.

† Significantly different from the corresponding control value ($P < 0.05$).

liver thiol levels induced by 4-CP were very rapid. A significant reduction of liver thiol levels was observed 30 min after 4-CP injection, and a rebound effect was seen within 1 hr. After 30 min of drug administration, animals started to recover from 4-CP toxicity (convulsions and drowsiness) and seemed to react normally again by 50 min. Thus, the failure to detect changes in liver thiol after acute 4-bromophenol treatment [33, 34] may be due to a faster onset and a shorter duration of thiol lowering action of bromophenol as compared to bromobenzene. In this regard, Lau *et al.* [37] have reported that 2-bromophenol, rather than 4-bromophenol, may play a role in the *in vivo* toxicity by bromobenzene.

The observed reduction of liver thiol level after 4-CP treatment provides presumptive evidence that 4-CP, like bromobenzene [33, 34] and 2-bromophenol [37], may also be converted to an electrophilic metabolite. Our results further show that the *in vivo* thiol lowering effect of 4-CP in liver is due to the formation of a chemically reactive electrophilic intermediate by the microsomal cytochrome P-450-dependent drug-metabolizing system. Pretreatment of mice with phenobarbital, a cytochrome P-450 inducer, increased the onset, magnitude, and duration of the time–course effect of 4-CP on liver thiol reduction (Fig. 4). This result clearly suggested that a more electrophilic intermediate(s) of 4-CP was formed in the liver of phenobarbital-pretreated mice. Moreover, pretreatment of mice with β-naphthoflavone, another hepatic microsomal enzyme inducer, also significantly lowered liver thiol level over control levels after 4-CP administration. By contrast, animals pretreated with SKF 525-A, a metabolic inhibitor of the microsomal cytochrome P-450 system, showed a smaller reduction of liver thiol content after 4-CP administration (Fig. 4). These observations provided more evidence that metabolic activation of 4-CP by the liver microsomal cytochrome P-450 enzymes may be involved in the thiol lowering effect of 4-CP.

Cobaltous chloride has been shown to be an inhibitor of *de novo* synthesis of cytochrome P-450 [38] and thereby acts to decrease the activity of the hepatic microsomal mixed-function oxidase enzyme system. Treatment of rodents with cobaltous chloride has

also been shown to induce the activity of the hepatic microsomal cytochrome P-450 drug-metabolizing enzyme system [39]. In contrast to the other metabolic modifiers, cobaltous chloride pretreatment did not alter significantly the liver thiol lowering effect of 4-CP (Fig. 4). This nonsignificant effect of cobaltous chloride may also be due to the relatively high concentration of thiol present in liver induced by cobaltous chloride treatment (Fig. 4) which could possibly compensate for an increase in the amount of reactive intermediate produced. This effect, as well as the diverse action on cytochrome P-450 systems [38, 39], may mask the thiol lowering effect of 4-CP in cobaltous chloride pretreated mice. Nevertheless, the *in vivo* thiol lowering effect of 4-CP and the modification of this effect by pretreatment of animals with phenobarbital, β-naphthoflavone and SKF 525-A suggested that 4-CP undergoes metabolic conversion to an electrophilic species by the liver microsomal MFO system.

To further characterize the role of the microsomal drug-metabolizing enzyme system on the formation of a chemically reactive metabolite of 4-CP, we examined the irreversible binding of [^{14}C]4-CP to mouse liver microsomal proteins in the presence of NADPH and a variety of hepatic microsomal modifiers. The goal of this study was to establish whether 4-CP was metabolized by cytochrome P-450 to a reactive intermediate which is covalently bound to microsomal proteins. Enzymatic-mediated binding of [^{14}C]4-CP was specific for NADPH as the pyridine cofactor, and the NADPH-dependent enzymatic binding was found to be a saturable process with a K_b of 0.222 mM and a maximum binding of 12 nmol/mg protein/20 min. These binding characteristics for 4-CP are similar to the binding constant reported for the covalent binding of bromobenzene to rat liver microsomal proteins [35, 40]. Several compounds including bromobenzene and chlorobenzene have been reported to be capable of covalently binding to tissue macromolecules [12], and the degree of binding has been found to be directly proportional to the magnitude of hepatotoxicity or nephrotoxicity [12, 23].

A series of *in vitro* experiments were undertaken to further confirm the role of cytochrome P-450 in the formation of a reactive metabolite of 4-CP and irreversible binding to microsomal proteins. SKF 525-A, metyrapone and α-naphthoflavone, which are inhibitors of microsomal cytochrome P-450, were found to inhibit significantly the binding of [^{14}C]4-CP to microsomal proteins (Table 2). The most direct evidence for involvement of cytochrome P-450 was the finding that partial replacement of the incubation atmosphere with carbon monoxide strongly inhibited the irreversible binding of 4-CP to liver microsomes. Carbon monoxide is a potent inhibitor of hepatic monooxygenase activity and is a ligand for interaction with the reduced iron chelate of cytochrome P-450 [41]. These results strongly suggest that the proposed metabolic biotransformation of 4-CP by cytochrome P-450 is a reasonable one.

The observed capability of 4-CP to irreversibly bind with microsomal protein further confirms the suggestion of the role of metabolic activation on the biotransformation pathway of 4-CP. Accordingly, if a chemically reactive species is formed, then this electrophile should undergo interaction with nucleophilic tissue components (glutathione) forming premercapturic acid metabolites which will be further converted to corresponding cysteinyl adducts [36]. To verify indirectly whether thiol containing adducts are formed from an electrophilic intermediate of 4-CP in microsomes, various nucleophilic compounds were added to the incubation systems to determine whether the extent of covalent binding of [^{14}C]4-CP to microsomal proteins could be reduced (Table 3). Glutathione, L-lysine and L-cysteine inhibited the binding of 4-CP significantly, whereas glycine, which is an amino acid that does not contain an accessible reactive nucleophilic group, did not alter the irreversible binding of 4-CP. These results have provided more support, suggesting that 4-CP is converted to a chemically reactive and electrophilic metabolite.

To investigate whether the major chemically reactive metabolite of 4-CP in liver microsomes was an epoxide, the extent of ^{14}C-binding of 4-CP was examined in the presence of cyclohexene oxide (Table 3). If an epoxide is the major reactive metabolite in the biotransformation of 4-CP, inhibition of epoxide hydrolase by the addition of cyclohexene oxide [42] to the incubation system should result in an accumulation of this reactive intermediate and lead to an increase in the binding of ^{14}C to microsomal proteins. In this study, cyclohexene oxide did not potentiate the binding of 4-CP metabolite to mouse liver microsomal proteins either at low or high 4-CP concentrations. Moreover, cyclohexene oxide at the highest concentration used (1 mM) tended to inhibit the binding of ^{14}C to liver microsomal proteins. This evidence suggested that an epoxide was not the major reactive metabolite(s) of 4-CP which bound to mouse liver microsomal proteins.

Hesse *et al.* [27] and Lau *et al.* [37] have suggested that chemically reactive quinone species of catechol-derived metabolites of chlorobiphenols and 4-bromophenol are bound to tissue protein *in vitro*. In this regard, catechol compounds are presumed to bind to protein by forming highly reactive semiquinones and quinones [27, 37]; this reaction may be mediated in part by the presence of superoxide anions [43]. To assess this possibility, we investigated whether a semiquinone and quinone species of 4-chlorocatechol was formed in the metabolic pathway of 4-CP. As shown in Fig. 7, we propose that 4-CP may be oxidized by hepatic mixed-function oxidase system to a 4-chlorocatechol by a nonenzymatic rearrangement via an epoxide intermediate, and that 4-chlorocatechol might be further oxidized and converted to the corresponding semiquinone and quinone metabolites. Support for this proposed scheme is derived from the work of Lau *et al.* [35] showing that 4-bromocatechol is a microsomal cytochrome P-450-derived metabolite of 4-bromophenol, *in vitro*. If the semiquinone and/or quinone species of 4-chlorocatechol were major metabolites of 4-CP implicated in the irreversible binding process, the addition of superoxide dismutase and L-ascorbic acid should reduce the extent of the binding of [^{14}C]4-CP. As indicated in Table 3, the addition of superoxide

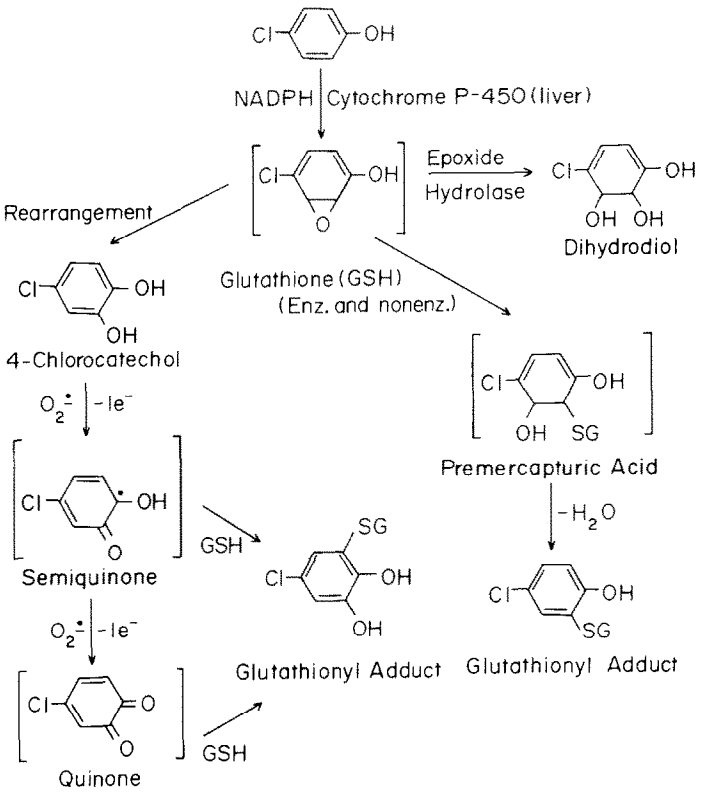

Fig. 7. Proposed metabolic pathways of 4-chlorophenol in mouse liver microsomes. Structures in brackets represent unstable intermediate metabolites.

dismutase markedly inhibited the degree of ^{14}C-binding of 4-CP to microsomal proteins. L-Ascorbic acid, a reagent that scavenges superoxide anion [44] and reduces accumulation of semiquinone or quinone species [45], inhibited the irreversible ^{14}C-binding of 4-CP to microsomal proteins. These findings are similar to those reported for the binding of dichlorobiphenyl to microsomal proteins [27] and 4-bromophenol [37]. The results of Hesse *et al.* [27] suggested that the majority of dichlorobiphenyl bound metabolite was not derived from an epoxide intermediate, but from other reactive species, possibly involving a semiquinone and quinone. Therefore, the proposed metabolic pathway for 4-CP (Fig. 7) may be similar to that reported for dichlorobiphenyl [27] and 4-bromophenol [35], but differs from that of naphthol [46].

Nelson *et al.* [43] reported that the irreversible binding of [6,7-^{3}H]-2-hydroxyestrogen requires microsomes, oxygen and NADPH. The NADPH could be replaced by xanthine and xanthine oxidase as a superoxide anion generating system, whereas superoxide dismutase inhibited the extent of both the NADPH and superoxide anion-dependent irreversible binding of 2-hydroxyestrogens. Moreover, addition of L-ascorbic acid, epinephrine or superoxide dismutase significantly reduced the degree of 2-hydroxyestradiol binding to microsomal proteins. It was proposed that 2-hydroxyestradiol possesses lower redox potentials than the superoxide anion radical and therefore undergoes oxidation to form the corresponding chemically reactive semiquinone and/or quinone species [43]. Based upon the similarity of the inhibitory effects of superoxide dismutase, epinephrine and L-ascorbic acid on the binding of [^{14}C]4-CP to mouse microsomal proteins, we suggest that the semiquinone or quinone species of 4-chlorocatechol is involved as intermediate and chemically reactive metabolites which are bound irreversibly to liver microsomal proteins (Fig. 7). Similarly, 4-bromocatechol formation and its subsequent oxidation to a quinone species by NADPH-supplemented liver microsomes have been proposed to account for increased covalent binding of 4-bromophenol to microsomal proteins [37]. Although our studies have not involved the elucidation of various 4-CP metabolites, the same types of chemically reactive metabolites, as reported for 4-bromophenol [35, 37], are likely formed as intermediates in the covalent binding process. In this regard, glutathionyl adducts formed either through an electrophilic epoxide intermediate and/or chlorocatechol metabolites are also likely conjugates of this proposed pathway of 4-CP metabolism (Fig. 7). Furthermore, studies are needed to confirm the relationships between tissue thiol depletion, production of chemically reactive metabolites and mercapturic acid conjugates, covalent binding to tissue proteins, *in vitro*, and *in vivo* hepatotoxicity for 4-CP in this species.

Acknowledgement—This work was supported by the National Institutes of Health (USPHS HL-12740).

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