MOLECULAR PHARMACOLOGY, 26:112–116

MOL PHARM

Free Radicals *in Vivo*

Covalent Binding to Lipids

CHARLES V. SMITH, HELEN HUGHES, AND JERRY R. MITCHELL[1]

Institute for Lipid Research and Department of Medicine, Baylor College of Medicine, Houston, Texas 77030

Received November 28, 1983; Accepted March 1, 1984

SUMMARY

As one means of determining the extent to which free radical metabolites are involved in the interaction of hepatotoxic drugs with target tissues, we have measured the covalent binding to hepatic lipids of carbon tetrachloride, acetaminophen, 2-furamide, furosemide, dimethylnitrosamine, and bromobenzene. Transesterification of the Folch lipid fraction was required to distinguish radioactive label present but not covalently bound to alkyl residues through radical addition or combination reactions. Although all hepatotoxins were covalently bound to hepatic protein in the range of 1–2 nmoles/mg, thereby confirming tissue alkylation by reactive metabolites under the present experimental conditions, only carbon tetrachloride gave significant covalent binding to the alkyl residues of hepatic lipids (4.34 nmoles/mg). Thus, although these data further support the already well-documented role of a free radical in the reaction of carbon tetrachloride with target tissue molecules, none of the other hepatotoxins gave similar indications. Dimethylnitrosamine did give significant covalent binding to lipids, but the removal of the binding by transesterification indicates that the binding apparently resulted from electrophilic attack on nucleophilic centers present in phospholipids rather than from radical attack on electroneutral alkyl residues of the lipids.

INTRODUCTION

The interaction of reactive metabolites with cellular molecules is thought to be responsible for many, if not most, acute tissue lesions caused by xenobiotics (1). The nature of this interaction probably is determined largely by the chemical nature of the reactive metabolite (2). Presently, two major classes of reactive metabolites are perceived to be electrophilic intermediates or organic radicals that either alkylate nucleophilic sites or initiate lipid peroxidation directly by hydrogen atom abstraction or perhaps indirectly by generation of reactive oxygen species.

There is an ever-growing interest in free radicals as possible toxic intermediates produced during the metabolism of a wide variety of substances including carbon tetrachloride (3) and, more recently, acetaminophen (4). The evidence for the involvement of a free radical metabolite in the interaction of carbon tetrachloride with target tissues is comprehensive. In contrast, despite the electron paramagnetic resonance evidence for the microsomal formation *in vitro* of an acetaminophen-derived free radical (4), there is no evidence that such a radical reacts measurably with cellular constituents *in vivo*, and demonstration of such an interaction should be an essential part of any hypothesis regarding the nature of a reactive metabolite or mechanisms of toxicity.

There are three general types of chemical mechanism by which a free radical intermediate might alter cellular molecules. One is by electron transfer to molecular oxygen, generating superoxide and other reactive oxygen species. Oxidant stress produced *in vivo* by reactive oxygen species can be determined by measurement of GSSG in bile (5), blood, or tissue (6). We have demonstrated that hepatotoxic doses of acetaminophen do not produce oxidant stress *in vivo* (5, 6); if a reaction of this nature does occur, it is quantitatively too minor *in vivo* to demonstrate.

Free radicals may also alter cell macromolecules by hydrogen atom abstraction reactions, perhaps with the initiation of a radical chain autoxidation of polyunsaturated fatty acids. The products of this reaction can be characterized via the production of increased conjugated diene chromophores or by formation of lipid hydroxy acids. We have previously shown that hepatotoxic doses of acetaminophen do not produce measurable increases in either conjugated dienes (7) or in lipid hydroxy acids (8) *in vivo*.

The third general mechanism by which radical metabolites might alter tissue macromolecules is to become covalently bound, either by radical addition to carbon-

This work was supported by Grants GM 26611 and GM 13901 from the National Institute for General Medical Sciences.

[1] Burroughs Wellcome Scholar in Clinical Pharmacology.

0026-895X/84/040112-05$02.00/0
Copyright © 1984 by The American Society for Pharmacology and Experimental Therapeutics.
All rights of reproduction in any form reserved.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

carbon double bonds or by radical combination. We report here our investigations on the characterization of reactive metabolites of a selected series of hepatotoxins on the basis of the extent to which they become covalently bound *in vivo* to the alkyl residues of target tissue lipids.

MATERIALS AND METHODS

Adult male ICR mice and Sprague-Dawley rats were purchased from TIMCO (Houston, Tex.) and allowed food (Wayne lab blocks) and tap water ad libitum until being fasted 18 hr prior to receiving injections. The rats, employed only for the bromobenzene experiments, additionally had been treated with phenobarbital (80 mg/kg, i.p.) for each of the 4 consecutive days prior to the experiment. All drugs were administered between 8:00 and 9:00 a.m. and, following the indicated time interval, the animals were killed by decapitation and bled. The livers were removed, rinsed, and homogenized in 0.9% NaCl solution. A portion of this homogenate was added to 4 volumes of cold methanol for determination of covalent binding to protein by methods described previously (9). The balance of the homogenate was extracted with 20 volumes of 2:1 chloroform:methanol. Dilute saline (0.9%, 0.2 volume) was added for phase separation, and the lower (chloroform) phase was removed and evaporated under a stream of nitrogen to provide the crude lipid which we refer to as the Folch lipid (10). The transesterification of the Folch lipid was carried out by the addition of 2 ml of benzene, 5 ml of methanol, and 1 drop of concentrated sulfuric acid and warming to 60° for 3 hr. The mixtures were cooled, and the transesterified lipid was isolated by partitioning the reaction mixture between isooctane and water. The isooctane phase was separated, washed twice with additional portions of water, and transferred to a clean tube; solvent was evaporated under a stream of dry nitrogen.

The amount of Folch or transesterified lipid present was determined by gravimetric quantitation; radioactivity present was determined by liquid scintillation counting of a portion of the Folch lipid in benzene or the transesterified lipid in isooctane. Quench corrections were determined by internal standard addition.

Radiolabeled bromobenzene was purchased from ICN (Cleveland, Ohio); the other radiochemicals were obtained from New England Nuclear Corporation (Boston, Mass.). Labeled (^{14}C) bromobenzene, carbon tetrachloride, and dimethylnitrosamine were not purified further, but were greater than 98% radiochemically pure as determined by gas chromatographic analyses by the vendors. Acetaminophen, 2-furamide, and furosemide were labeled by tritium exchange and purified by thin-layer chromatography on silica gel by methods described previously (11–13). Acetaminophen was developed with ethyl acetate or ethyl acetate:methanol:acetic acid:water (12:6:0.2:1.8); 2-furamide was developed with chloroform:methanol (9:1) or benzene:ethyl acetate:formic acid (50:40:3); furosemide was developed with ethyl acetate:hexane:acetic acid (80:20:1) or with chloroform:methanol:acetic acid (89:6:5). Radiochemical purity was greater than 99% in all cases, as determined by co-chromatography of radiolabel with the appropriate authentic substance. Radioprofiles were determined with a Packard 7201 scanner and by scraping 0.5-cm bands of the developed plate into liquid scintillation vials, adding 0.5 ml of MeOH and 5 ml of ACS, and counting. Labeled compounds were diluted to specific activities of around 500 dpm/nmole and dissolved in the appropriate solvent for administration to animals. Carbon tetrachloride was dissolved in mineral oil, bromobenzene in corn oil, and the other substances in 0.9% NaCl solution. All other chemicals and solvents were reagent or high-pressure liquid chromatography grade and were purchased from Sigma Chemical Company (St. Louis, Mo.), Burdick & Jackson (Muskegon, Mich.), or Fisher Scientific Company (Pittsburgh, Pa.).

RESULTS

Measurable radioactivity was present in the Folch lipid extracted from the livers of the animals given the labeled hepatotoxins listed in Table 1. However, the data presented in Table 2 show that each of these parent compounds is readily extracted into chloroform with the lipid portion of a Folch procedure. Of the drugs examined, carbon tetrachloride, dimethylnitrosamine, and bromobenzene can be removed by repeated evaporation under a stream of nitrogen, although it should be noted that addition of excess unlabeled compound and several evaporation steps are necessary to remove even these substances effectively. For example, hepatic lipids were extracted by the Folch procedure from an untreated animal. To the chloroform solution of the hepatic lipids thus obtained were added 8.29×10^5 dpm of [^{14}C]carbon tetrachloride and 0.2 ml of unlabeled carbon tetrachloride. Three successive evaporations of solvent (3 ml of chloroform added between evaporations) yielded 28.8 mg of residue (lipid). This was taken up in 2 ml of benzene, and 0.5 ml was counted to show 800 dpm present in the sample (0.1% of the added ^{14}C). Chloroform (2 ml) and 0.5 ml of carbon tetrachloride were added, solvents were evaporated, and the residue was redissolved in benzene. Two hundred disintegrations per minute were present in the sample, indicating that two-thirds of the 600 dpm carried forward from the previous determination had been evaporated. This behavior was anticipated, and indeed the method described by Castro *et al.* (14) employs addition of unlabeled drug and a second evaporation. As expected, evaporation was completely ineffective in removing 2-furamide, furosemide, and acetaminophen from Folch lipid.

The transesterification of the Folch lipids, followed by partitioning between isooctane and water, removed virtually all of the radioactivity present in the hepatic lipids of all of the animals except those given carbon tetrachloride, as shown in Table 3. The values quoted in Table 3 for binding to transesterified lipid for the other drugs were calculated from radioactivity counted at close to background and therefore represent essentially no radioactivity present, within the limitations of the methods and specific activities employed. Control experiments showed that labeled 2-furamide, furosemide, and acet-

TABLE 1

Radioactivity in hepatic lipids of animals treated with selected hepatotoxins

Mice (M) or rats (R) were treated with the indicated drug as described, and after the appropriate time interval, the animals were killed and hepatic lipids were isolated by Folch extraction as described under Materials and Methods. Radioactivity present was determined by liquid scintillation counting, and lipid was quantitated gravimetrically. Data are expressed as means ± standard error of the mean (four animals per drug).

Drug	Dose	Time	Radioactivity in Folch lipid
	mg/kg	*hr*	*nmol/mg*
CCl_4 (M)	1500	1	3.49 ± 0.55
Dimethylnitrosamine (M)	40	3	2.36 ± 0.09
Furosemide (M)	400	3	0.44 ± 0.08
2-Furamide (M)	275	3	10.58 ± 3.35
Acetaminophen (M)	400	3	0.11 ± 0.05
Bromobenzene (R)	225	4	0.24 ± 0.03

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

aminophen were removed from transesterified lipid by isooctane-water partitioning.

The radioactivity present in the transesterified lipid of carbon tetrachloride-treated animals (Table 3) was higher per milligram of lipid than the corresponding value in the Folch lipids of these animals (Table 1). The total amount of radioactivity present was unchanged, however, and this apparent increase in specific activity of the lipid fractions correlates with a loss of around 30% of the mass of the Folch lipid in the transesterification procedure. Transesterified lipid fractions were recovered in greater than 90% efficiency from a repetition of the procedure, and the observed change in mass associated with the original transesterification of Folch lipid is probably the result of the loss of polar moieties such as phosphate, ethanolamine, glycerol, and similar species released from the alkyl residues during the reaction and extracted into water from isooctane.

Each of the chemicals employed gave covalent binding of metabolites to hepatic protein in the range of 1–2 nmole/mg of protein (Table 3). These data document the production of reactive metabolites of these substances in sufficient quantities to exceed cellular protective mechanisms operating *in vivo* and that the reaction of these metabolites with tissue macromolecules is readily measurable.

DISCUSSION

Previous studies of covalent binding of reactive metabolites to lipids have been restricted to the haloalkanes (14, 15). One major reason for this restriction has been the need for a method to remove radiolabel associated with unbound drug; in general, the haloalkanes and their metabolites are volatile enough to be removed by evaporation, whereas most other hepatotoxins are not. The major objective of the work described in this report, however, was to determine whether or not nonvolatile hepatotoxins such as acetaminophen interact with tissue molecules via free radical metabolites, as evidenced by the extent to which they become covalently bound to the nonpolar alkyl portions of tissue lipids *in vivo*. No evidence exists for the measurable interaction of putative radical metabolites of acetaminophen, furosemide, 2-furamide, bromobenzene, or dimethylnitrosamine with tissue macromolecules *in vivo* by electron transfer to molecular oxygen or hydrogen atom abstraction reactions as discussed above (1, 2, 5–8). Similarly, the present data provide no evidence for interaction of putative radical metabolites of the above compounds with tissue macromolecules *in vivo* by radical addition or combination reactions. The radioactivity present in the hepatic lipids isolated by Folch extraction from animals given hepatotoxic doses of the substances listed in Table 1 cannot be considered as evidence of radical reactions. As is shown in Table 2, 2-furamide, furosemide, and acetaminophen both partition into the chloroform phase of a Folch extraction and are insufficiently volatile to permit distinction of a covalently bound metabolite from the presence of the parent compound.

Although the radioactivity present in the hepatic Folch lipids of the animals given carbon tetrachloride, dimethylnitrosamine, or bromobenzene is not the result of the presence of the parent compounds, this radioactivity might represent any or all of three possible phenomena: the covalent binding of a free radical metabolite to the alkyl portion of lipid molecules; the covalent binding of an electrophilic metabolite to nucleophilic sites in the lipid, such as phosphodiester or ethanolamine functional groups in phospholipids (16); or unbound metabolites of the parent compounds that might partition with the

TABLE 2

Partition and evaporation of radiolabeled hepatotoxins during isolation of Folch lipid

For extraction, hepatic tissue from untreated animals was homogenized in 4 volumes of 0.9% NaCl solution, and the homogenate was added to 20 volumes of 2:1 chloroform:methanol. Tracer quantities (10^5–10^6 dpm) of the appropriate substance were added and the mixture was vortexed. Dilute saline (0.2 volume) was added and mixed and the phases were allowed to separate. A portion of the (lower) chloroform phase was counted, and the data presented are percentages of added radioactivity present in this phase (mean ± standard error of four determinations).

For evaporation, hepatic tissue from untreated animals was homogenized and extracted as described above. The chloroform phase was transferred to a clean tube, and 10^5–10^6 dpm of radiolabeled drug were added. Unlabeled drug was added, and the solvent was evaporated under a stream of nitrogen and redissolved in chloroform. This process was repeated four times. The final lipid product was redissolved in benzene and a portion was counted to determine the percentage of added radiolabel remaining (mean ± standard error of four determinations).

Drug	% Remaining with Folch lipid	
	Extraction	Evaporation
CCl_4	96.3 ± 4.8	<0.1
Dimethylnitrosamine	58.7 ± 5.4	<0.1
Furosemide	34.7 ± 0.7	98.9 ± 3.7
2-Furamide	61.7 ± 5.7	89.4 ± 4.2
Acetaminophen	38.6 ± 1.9	99.4 ± 2.3
Bromobenzene	94.1 ± 6.2	<0.1

TABLE 3

Covalent binding in vivo of selected hepatoxins to protein and transesterified lipid

Mice (M) or rats (R) were treated as described in Table 1. The Folch lipids thus obtained were transesterified and partitioned between isooctane and water, and the isooctane phase washed with dilute saline as described under Materials and Methods. Evaporation of the isooctane gave the transesterified lipid fraction, which was quantitated gravimetrically. Radioactivity was determined by liquid scintillation counting. Data are expressed as means ± standard error (four animals per drug). Covalent binding to hepatic protein was determined in these same animals as described under Materials and Methods (four animals per drug).

Drug	Dose	Time	Covalent binding	
			Transesterified lipid	Protein
	mg/kg	*hr*	*nmoles/mg*	*nmoles/mg*
CCl_4 (M)	1500	1	4.34 ± 0.64	1.30 ± 0.19
Dimethylnitrosamine (M)	40	3	0.06 ± 0.02	2.02 ± 0.19
Furosemide (M)	400	3	0.00 ± 0.01	0.95 ± 0.42
2-Furamide (M)	275	3	0.02 ± 0.10	1.47 ± 0.70
Acetaminophen (M)	400	3	0.01 ± 0.01	1.19 ± 0.22
Bromobenzene (R)	225	4	0.04 ± 0.01	1.31 ± 0.62

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

chloroform phase but, unlike the parent compounds, are not removed effectively by evaporation.

The transesterification procedure of the crude lipid extract chemically separates the polar phosphate, ethanolamine, and glycerol moieties from the nonpolar species such as the alkyl chains of fatty acids. Thus, substances that form electrophilic reactive intermediates that alkylate nucleophilic sites on phospholipids would show persistent binding to the Folch lipid but would be removed from the organic mixture by isooctane-water partitioning. Dimethylnitrosamine is known to alkylate nucleic acids *in vivo* to form phosphotriesters and other products (16). Although the formation of other nonvolatile metabolites is a possibility, the alkylation of phosphodiesters or other nucleophiles in tissue lipids provides a reasonable explanation for the data observed.

Bromobenzene could represent a similar example, but the ability of its reactive metabolites to alkylate phosphodiesters *in vivo* has not been established. The formation of bromophenols or bromocatechols might represent a more reasonable explanation of the observed data, but we did not investigate this question specifically. The key piece of information here is that bromobenzene did not produce significant quantities of metabolite bound to alkyl residues in the transesterified lipid (Table 3).

Similarly, essentially no radioactivity was present in the transesterified hepatic lipids of animals given 2-furamide, furosemide, or acetaminophen (Table 3). These data, combined with our previous reports of the absence of oxidant stress (5, 6), conjugated dienes (7), or lipid hydroxy acids (8) following hepatotoxic doses of acetaminophen *in vivo* would seem to preclude a quantitatively significant role for an acetaminophen free radical in the chemical interaction of acetaminophen-reactive metabolites with hepatic tissue molecules. The spin-trapped free radical observed by Rosen *et al.* (4) demonstrates that such a species can exist, but provides no information on the extent to which acetaminophen is metabolized through such a species, the nature or extent of the interaction of this radical with tissue molecules, or the applicability of the studies conducted *in vitro* to actual behavior *in vivo*. Rosen *et al.* (4) claim to have demonstrated an oxidant stress as evidenced by decreased ratios of GSH/GSSG following administration of acetaminophen; however, these data appear to have been obtained from samples of hepatic tissue subjected to isolation of a cytosolic fraction and storage for up to 4 days prior to analysis. The possibility of artifactual autoxidation of GSH (5, 6, 17) is of concern and makes interpretation of such data difficult. Since acetaminophen is a phenol, moreover, and phenols such as vitamin E are excellent antioxidants (18), the radical observed by Rosen *et al.* (4) may well be the result of antioxidant properties of acetaminophen (19, 20).

Carbon tetrachloride was the only hepatotoxin for which the radioactivity remained with the nonpolar fraction of transesterified lipid. This is consistent with the expected chemistry of the trichloromethyl radical, as has been demonstrated by the recent report of the mass spectral identification of an adduct of $\cdot CCl_3$ with cholesterol (21) or unsaturated fatty acids (22). Furthermore, the fact that essentially all of the radioactivity in the Folch lipids of the carbon tetrachloride-treated animals was recovered in the transesterified lipid fraction provides evidence that, for carbon tetrachloride, the interaction with nucleophilic sites in lipid by phosgene or other possible metabolites that react as electrophiles rather than as free radicals is quantitatively much less important than the radical addition or combination reactions of the trichloromethyl radical with tissue lipids. Previous studies (3, 14, 15, 21, 22) do not provide quantitative evidence *in vivo* for the relative importance of the two modes of interaction by the two types of metabolites (radical versus electrophile).

Within the limitations discussed above, the data presented here provide no evidence for the interaction of free radical metabolites with lipids for any of the hepatotoxins except carbon tetrachloride. The values quoted for covalent binding to transesterified lipid for the other substances correspond to measurements of radioactivity at close to background levels. Similar amounts of nonextractable radioactivity are seen in protein from nontarget tissues, e.g., skeletal muscle (9) of animals treated with hepatotoxins. Therefore, no further efforts were made to investigate the nature of these small amounts of radioactivity.

ACKNOWLEDGMENT

The skillful technical assistance of Marc Reilly is gratefully acknowledged.

REFERENCES

1. Mitchell, J. R., G. B. Corcoran, C. V. Smith, H. Hughes, and B. H. Lauterburg. Alkylation and peroxidation injury from chemically reactive metabolites, in *Biological Reactive Intermediates 2.* (R. Snyder, D. V. Parke, J. J. Kocsis, D. J. Jol, C. G. Gibson, and C. M. Witmer, eds.). Plenum Press, New York, 199–224 (1982).
2. Mitchell, J. R., H. Hughes, B. H. Lauterburg, and C. V. Smith. Chemical nature of reactive intermediates as determinant of toxicologic responses. *Drug Metab. Rev.* **13**:539–553 (1982).
3. Recknagel, R. O., A. A. Glende, Jr., R. L. Walker, and K. Lowry. Lipid peroxidation: biochemistry, measurement, and significance in liver cell injury, in *Toxicology of the Liver* (G. L. Plaa and W. R. Hewitt, eds.). Raven Press, New York, 213–241 (1982).
4. Rosen, G. M., W. V. Singletary, Jr., E. J. Rauckman, and P. G. Killenberg. Acetaminophen hepatotoxicity: an alternative mechanism. *Biochem. Pharmacol.* **32**:2053–2059 (1983).
5. Lauterburg, B. H., C. V. Smith, H. Hughes, and J. R. Mitchell. Biliary excretion of glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. *J. Clin. Invest.* **73**:124–133 (1984).
6. Adams, J. D., Jr., B. H. Lauterburg, and J. R. Mitchell. Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. *J. Pharmacol. Exp. Ther.* **227**:749–754 (1983).
7. Thorgeirsson, S. S., H. A. Sasame, J. R. Mitchell, D. J. Jollow, and W. Z. Potter. Biochemical changes after hepatotoxic injury from the doses of acetaminophen or furosemide. *Pharmacology* **11**:205–217 (1976).
8. Hughes, H., C. V. Smith, E. C. Horning, and J. R. Mitchell. HPLC and GC-MS determination of specific lipid peroxidation products *in vivo*. *Anal. Biochem.* **130**:431–436 (1983).
9. Jollow, D. J., J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette, and B. B. Brodie. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. *J. Pharmacol. Exp. Ther.* **187**:185–194 (1973).
10. Folch, J., M. Lees, and S. G. H. Sloane. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**:497–509 (1957).
11. Mitchell, J. R., D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette, and B. B. Brodie. Acetaminophen-induced hepatic necrosis I. Role of drug metabolism. *J. Pharmacol. Exp. Ther.* **187**:185–194 (1973).
12. Mitchell, J. R., W. L. Nelson, W. Z. Potter, H. A. Sasame, and D. J. Jollow. Metabolic activation of furosemide to a chemically reactive, hepatotoxic metabolite. *J. Pharmacol. Exp. Ther.* **199**:41–52 (1976).
13. McMurtry, R. J., and J. R. Mitchell. Renal and hepatic necrosis after

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012

metabolic activation of 2-substituted furans and thiophenes, including furosemide and cephaloridine. *Toxicol. Appl. Pharmacol.* **42**:289–300 (1977).
14. Castro, J. A., E. V. Cignoli, C. R. de Castro, and O. M. de Fenos. Prevention by cystamine of liver necrosis and early biochemical alterations induced by carbon tetrachloride. *Biochem. Pharmacol.* **21**:45–57 (1972).
15. Uehleke, H., and T. Werner. A comparative study on the irreversible binding of labeled halothane trichlorofluoromethane, chloroform, and carbon tetrachloride to hepatic protein and lipids *in vitro* and *in vivo*. *Arch. Toxicol.* **34**:289–308 (1975).
16. Margison, G. P., and P. G. O'Connor. Nucleic acid modification by N-nitroso compounds, in *Chemical Carcinogens and DNA* (R. L. Grover, ed.), Vol. 1. CRC Press, Boca Raton, Fla. 111–159 (1979).
17. Eberle, D., R. Clarke, and N. Kaplowitz. Rapid oxidation *in vitro* of endogenous and exogenous glutathione in bile of rats. *J. Biol. Chem.* **256**:2115–2117 (1981).
18. Pryor, W. A. *Free Radicals.* McGraw-Hill, New York, 326 (1966).
19. DuBois, R. N., K. E. Hill, and R. F. Burk. Antioxidant effect of acetaminophen in rat liver. *Biochem. Pharmacol.* **32**:665–670 (1983).
20. Reiter, R., and A. Wendel. Drug-induced lipid peroxidation in mice. IV. *In vitro* hydrocarbon evolution, reduction of oxygen and covalent binding of acetaminophen. *Biochem. Pharmacol.* **32**:665–670 (1983).
21. Ansari, G. A. S., M. T. Moslen, and E. S. Reynolds. Evidence for *in vivo* covalent binding of CCl_3 derived from CCl_4 to cholesterol of rat liver. *Biochem. Pharmacol.* **31**:3509–3510 (1982).
22. Trudell, J. R., B. Bosterling, A. J. Trevor. Reductive metabolism of carbon tetrachloride by human cytochromes P-450 reconstituted in phospholipid vesicles: mass spectral identification of trichloromethyl radical bound to dioleolyl phosphatidylcholine. *Proc. Natl. Acad. Sci. U. S. A.* **79**:2678–2682 (1982).

Send reprint requests to: Dr. Charles V. Smith, Baylor Building, Room 826E, Baylor College of Medicine, Houston, Tex. 77030.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 5, 2012