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FORMATION OF GLUTATHIONE ADDUCTS OF CARBON TETRACHLORIDE METABOLITES IN A RAT LIVER MICROSOMAL INCUBATION SYSTEM*

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Abstract—Metabolism of CCl_4 by rat liver microsomes causes damage to the membrane. GSH diminishes that damage. One mechanism of GSH protection has been characterized. It involves formation of diglutathione carbonate from two molecules of GSH and one molecule of phosgene, an oxygenated metabolite of CCl_4. The present studies were done to seek other GSH adducts of CCl_4 metabolites and to examine the effect of oxygen tension on their formation. Incubations were carried out in sealed flasks under oxygen concentrations ranging from 0.14 to 21% at 37°. The CCl_4 concentration was 72 μM. $^{14}CCl_4$ and ^{35}S-GSH were used to label metabolites. High pressure liquid chromatographic analysis of the aqueous phase demonstrated two GSH adducts of CCl_4 metabolites. One adduct was oxygen-dependent and was identified as diglutathione carbonate by its co-elution with a diglutathione carbonate standard. Its formation showed no evidence of saturation when GSH concentrations as high as 10 mM were used, indicating that the overall process was nonenzymatic. Formation of the other adduct was greatest under the lowest oxygen concentration studied and none occurred at oxygen tensions of 5% or greater. Based on experiments with radiolabeled CCl_4 and GSH, this metabolite appeared to be a product of one molecule each of CCl_4 and GSH. Formation of this adduct had enzymatic characteristics. It was saturable with respect to GSH with an apparent K_m of 70 μM, and other thiol compounds that were tested could not substitute for GSH. The adduct was unstable during isolation attempts and was not characterized further. Formation of these two GSH adducts could account for some of the protection by GSH against CCl_4 injury.

Metabolism of carbon tetrachloride (CCl_4) by hepatic cytochrome P-450 yields a variety of free radicals and electrophiles. CCl_4 is reductively activated to CCl_3· and to dichlorocarbene [1, 2] with CCl_3OO·, electrophilic chlorine, and phosgene arising from secondary reactions involving oxygen [3–6]. Covalent binding of these reactive metabolites to cell constituents and the initiation of lipid peroxidation by some of them are considered to be the basic mechanisms of CCl_4 injury [7].

Reduced glutathione (GSH) protects microsomal membranes against such injury. Inclusion of GSH in a microsomal incubation system diminishes covalent binding of CCl_4 metabolites and lipid peroxidation [8]. This suggests that GSH is able to quench some of the CCl_4 metabolites. One glutathione adduct of a CCl_4 metabolite has been identified by Pohl's group [6]. It is diglutathione carbonate and it arises from the reaction of two GSH molecules with phosgene, an oxygenated metabolite of CCl_4. We carried out this study to determine if other GSH-dependent CCl_4-derived metabolites are formed.

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MATERIALS AND METHODS

Male Sprague–Dawley rats were purchased from Harlan Sprague–Dawley, Houston, TX. They were fed Teklad pelleted rat diet and given water *ad lib*. When they reached a body weight of 250–300 g, they were used for preparation of hepatic microsomes as described previously [8]. The isolated microsomes were resuspended and centrifuged to decrease GSH contamination and were used on the day of preparation.

A pH 7.4, 50 mM Tris–HCl buffer containing 150 mM KCl and 50 μM Na_2EDTA was used as the microsomal suspension and incubation medium. The incubations were carried out in sealed 25-ml Erlenmeyer flasks at 37° in a shaking water bath. The volume incubated was 2.5 or 5 ml, and the microsomal protein concentration was 1.1 to 2.4 mg/ml. The oxygen tension of the flask atmosphere (except for those incubated under air) was adjusted by flushing the flask with nitrogen for 15 min and then injecting the oxygen needed to give the desired final concentration using a gas-tight syringe. Reagents were purged with nitrogen. The commercial nitrogen which was purchased was found to contain 0.47% oxygen with a method measuring auto-oxidation of benzylviologen [9]. This was lowered to 0.14% by an oxygen-scrubbing system [10].

$^{14}CCl_4$ (4 Ci/mol) was added as 10 μl of an etha-

nolic solution to give a CCl_4 concentration of 72 μM in a 5-ml incubate for the experiment presented in Table 1 or as 5 μl to give the same concentration in a 2.5-ml incubate for the other experiments. CCl_4 is distributed in the head space, water, and microsomal membranes. Berger *et al.* [11] recently determined, using $^{14}CCl_4$ in a hepatocyte suspension, that approximately 50% of the ^{14}C in the incubation flask was in the cells. Thus, we suspect that the CCl_4 concentration in the microsomal membranes was greater than 72 μM because of this partitioning. This low concentration of CCl_4 was chosen for several reasons: (1) CCl_4 concentrations of 10 mM or more may lead to changes in membrane properties due to solvent effects [11]; (2) hepatic concentrations of CCl_4 after *in vivo* administration are typically in this range [12]; and (3) very high rates of radical generation associated with high concentrations of CCl_4 would be expected to deplete oxygen locally, which would complicate interpretation of results.

After a 5-min preincubation, the reaction was started by the injection of NADPH to give a concentration of 300 μM. In all experiments except those presented in Table 1, an NADPH-regenerating system was used [13]. At 15 min of incubation, the reaction was stopped by the addition of solid trichloroacetic acid to give a 10% concentration. The flask was left open in a hood overnight to allow $^{14}CCl_4$ and volatile metabolites to escape. After centrifugation of the flask contents, the supernatant fraction was decanted and used for study of trichloroacetic acid-soluble metabolites, referred to henceforth as aqueous metabolites. The pellet was used for determination of covalent binding of $^{14}CCl_4$ metabolites in some experiments. It was resuspended in 10% trichloroacetic acid and centrifuged five times. The pellet was digested as described before [8] and ^{14}C was determined by liquid scintillation counting.

The aqueous metabolites were analyzed using a Beckman model 332 high pressure liquid chromatography (HPLC) system equipped with a reverse phase ODS column (250 mm × 5 mm). A gradient of 0.4% acetic acid and methanol was used as shown in Fig. 1A. The flow rate was 1 ml/min, and 0.5-ml fractions were collected for liquid scintillation counting.

Diglutathione carbonate was synthesized by reacting equimolar amounts of GSH and diphosgene (trichloromethyl-chloroformate) in a pH 8, 1 M bicarbonate buffer for 1 hr under nitrogen. The compound was isolated using HPLC as described above. It rechromatographed on HPLC as a single peak.

$^{14}CCl_4$ and ^{35}S-GSH were supplied by New England Nuclear, Boston, MA, and had specific radioactivities of 4 Ci/mol and 291.5 Ci/mmol respectively. The Sigma Chemical Co. of St. Louis, MO, supplied GSH, GSSG, cysteine, cystine, coenzyme A, cysteamine, penicillamine, propylthiouracil, pantethine, and lipoamide. NADPH and dithioerythritol were from Boehringer Mannheim Biochemicals, Indianapolis, IN. Diphosgene was purchased from the Alfa Division of the Ventron Corp., Danvers, MA.

RESULTS

CCl_4 metabolism by liver microsomes was studied under nitrogen and air with and without GSH addition. Table 1 shows that covalent binding and aqueous metabolite formation were lower under air than under nitrogen, consistent with the known inhibitory effect of oxygen on CCl_4 metabolism. Under air, GSH depressed covalent binding and increased aqueous metabolite formation in a reciprocal fashion. Phosgene is formed under these conditions, and this increase in aqueous metabolite formation could possibly be caused by production of diglutathione carbonate. Under nitrogen, reciprocal changes did not occur. GSH had no effect on covalent binding. However, it caused a large increase in aqueous metabolite formation. This increase cannot be explained by the formation of diglutathione carbonate because phosgene production is dependent on oxygen. Therefore, this finding indicated the existence of a GSH-dependent CCl_4 metabolite other than diglutathione carbonate.

HPLC was used to study the CCl_4 metabolites in the aqueous phase. Eight $^{14}CCl_4$-derived metabolites were resolved. Two of these metabolites were GSH dependent.

The effect of oxygen on the GSH-independent and the GSH-dependent metabolites is shown in Fig. 1. In the absence of GSH (panel A), six labeled metabolites were formed with retention times of 5.5, 18.5, 31.5, 39, 47, and 49.5 min. They were given the designations I–VI in the order of their elution. Metabolites I, III, IV and V were minor metabolites

Table 1. GSH effect on distribution of CCl_4 metabolites in microsomal incubations under nitrogen and air*

Atmosphere	GSH (mM)	Carbon derived from CCl_4† Bound covalently (nmol)	In aqueous phase (nmol)
Nitrogen	0	53 ± 7.3	3.1 ± 1.3‡
	1	50 ± 4.5	17 ± 1.3‡
Air	0	2.4 ± 0.67§	0.73 ± 0.05‖
	1	0.42 ± 0.16§	2.2 ± 0.75‖

* Incubations were carried out in a volume of 5 ml. Protein concentration of the microsomes in the flasks ranged from 1.4 to 2.2 mg/ml with a mean of 1.7 ± 0.36.

† Values are means ± SD, N = 4.

‡‖ Pairs with the same superscript are significantly different by Student's *t*-test, $P < 0.05$.

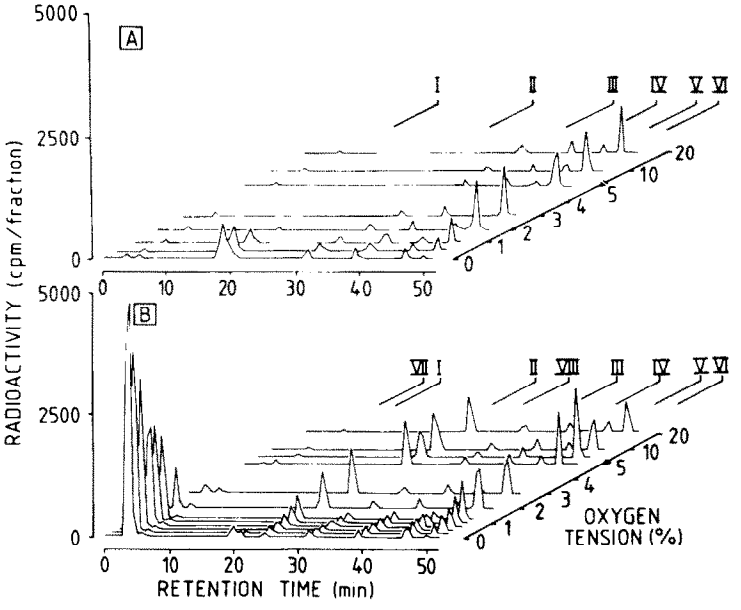

Fig. 1. HPLC separation of ^{14}C-containing aqueous metabolites produced by microsomal metabolism of $^{14}CCl_4$ at different oxygen tensions. Panel A presents chromatograms from incubations carried out with no GSH added. Panel B presents chromatograms from incubations carried out with 1 mM GSH. Incubation and chromatographic conditions are described in Materials and Methods. Control incubations without NADPH were carried out and contained less than 200 cpm. The metabolites accounted for 75–85% of the ^{14}C in the aqueous phase.

(<0.01 nmol/mg · 15 min), and their formation was independent of the oxygen tension. The remaining two metabolites showed a characteristic oxygen dependence. Metabolite II was formed maximally in the presence of the lowest oxygen tension and was no longer detectable when the oxygen tension was raised above 2%. Metabolite VI was oxygen dependent and reached maximal concentrations at approximately 3% oxygen.

In the presence of 1 mM GSH, striking changes in the metabolite pattern took place (panel B). Two new metabolites appeared. Metabolite VII, which eluted at 3.7 min, was formed maximally at the lowest oxygen tension and declined with increasing oxygen tensions. Half-maximal levels were reached at 1.5% oxygen, and at 5% oxygen none could be detected. A second GSH-dependent metabolite (metabolite VIII, retention time 24.7 min) was oxygen dependent. Its concentration increased as oxygen tension rose to 3% but did not increase further at higher oxygen tensions.

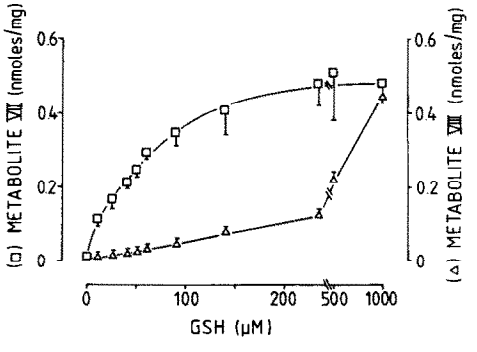

Fig. 2. Formation of aqueous metabolites VII and VIII as a function of GSH concentration. The incubations were carried out under 1% oxygen as described in Materials and Methods.

Both GSH-dependent metabolites were formed to a significant extent under 1% oxygen. Figure 2 shows the GSH concentration dependence of their formation at this oxygen tension. Formation of metabolite VII was saturable with an apparent K_m for GSH of 70 μM. Formation of metabolite VIII increased linearly as GSH concentration was increased. Additional experiments (not shown) with GSH concentrations up to 10 mM confirmed the linear relationship of GSH concentration with metabolite VIII formation, suggesting strongly that it is a non-saturable process.

The specificity of metabolite formation for GSH was studied using various thiol compounds as possible acceptors of reactive CCl_4 intermediates. Under nitrogen, metabolite VII was the only GSH-dependent metabolite formed. Under 5% oxygen, metabolite VIII was the only one formed. Therefore, the effects of thiol and disulfide compounds were studied under these conditions. In Table 2, the effects of several thiols and disulfides on aqueous metabolite formation are shown. Under nitrogen, aqueous metabolite formation was stimulated 226% by GSH, 24% by cysteine, and 47% by penicillamine. GSSG, cystine, dithioerythritol, cysteamine, propylthiouracil, lipoamide, pantetheine, and coenzyme A did not affect aqueous metabolite formation. Under 5%

Table 2. Effects of thiol and disulfide compounds on formation of aqueous metabolites of CCl_4 under nitrogen and under 5% oxygen

Compound	Metabolites formed* (nmol/mg·min) Nitrogen	5% Oxygen
None	0.34 ± 0.03	0.22 ± 0.01
GSH	1.11 ± 0.02†	0.39 ± 0.04†
GSSG	0.36 ± 0.08	0.16 ± 0.03
Dithioerythritol	0.35 ± 0.04	0.23 ± 0.01
Cysteine	0.42 ± 0.08†	0.37 ± 0.01†
Cystine	0.35 ± 0.05	0.20 ± 0.02
Penicillamine	0.50 ± 0.11†	0.24 ± 0.02
Propylthiouracil	0.32 ± 0.10	0.23 ± 0.03
Cysteamine	0.33 ± 0.05	0.25 ± 0.04
Pantethine	0.25 ± 0.10	0.20 ± 0.03
Coenzyme A	0.30 ± 0.03	0.20 ± 0.02
Lipoamide	0.36 ± 0.10	0.20 ± 0.01

* Values are means ± SD, N = 4.

† Significantly different from control (no thiol added) by Student's *t*-test, $P < 0.05$.

oxygen, GSH increased aqueous metabolite formation by 77% over control and cysteine increased it 68%. Therefore, stimulation of aqueous metabolite formation by GSH does not reflect a general thiol effect. Among the thiols that were effective, there was a relative specificity for GSH at very low oxygen tensions, but at 5% oxygen cysteine had almost the same effect as GSH.

The nature of the GSH-dependent metabolites as GSH adducts was demonstrated in experiments carried out with labeled GSH and CCl_4. Some incubations were performed with ^{14}C-labeled CCl_4 and unlabeled GSH and others with ^{35}S-labeled GSH and unlabeled CCl_4. Figure 3 shows that there were two ^{35}S-labeled metabolites with retention times identical to metabolites VII and VIII. The two main ^{35}S peaks on the chromatogram with retention times of 5.5 and 16.5 min represent GSH and GSSG respectively. Metabolite VIII co-chromatographed with synthetic diglutathione carbonate, the reaction product of two molecules of GSH with one molecule of phosgene [6]. Based on the specific radioactivities of the ^{35}S-GSH and $^{14}CCl_4$ and a ratio of 2 GSH sulfurs to 1 CCl_4 carbon in metabolite VIII, it was calculated that metabolite VII contained 1.03 GSH sulfurs per CCl_4 carbon. Thus, it appears that metabolite VII is generated from equimolar quantities of CCl_4 and GSH.

DISCUSSION

GSH is involved in a variety of detoxification processes. Formation of the two GSH adducts of CCl_4 metabolites observed in this work is likely to be responsible for some of the GSH protection against CCl_4-induced membrane injury. Formation of

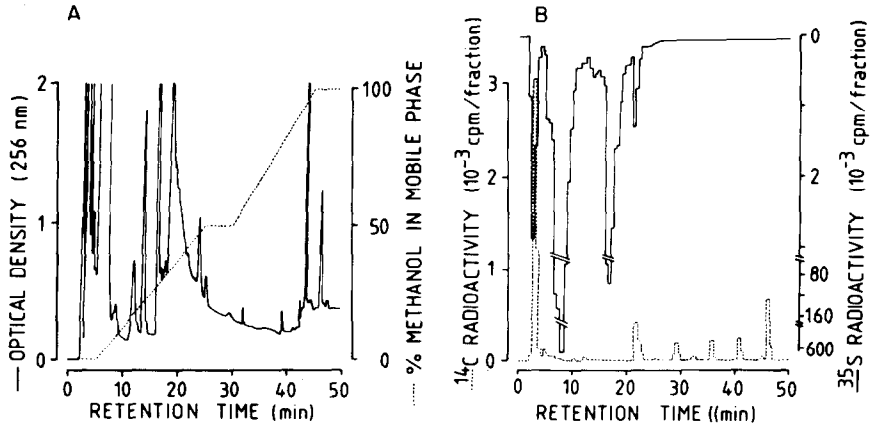

Fig. 3. HPLC analysis of GSH-dependent aqueous metabolites of CCl_4. The incubations were carried out as described in Materials and Methods under 1% oxygen and at a microsomal protein concentration of 1.6 mg/ml. Panel A presents the u.v. absorbance profile (solid line) and the gradient shape (broken line). Panel B shows the results when the incubation was carried out with $^{14}CCl_4$ (2.1×10^6 cpm) and non-radioactive GSH (broken line) or ^{35}S-GSH (9×10^6 cpm) and non-radioactive CCl_4 (solid line). Calculation of the stoichiometry of C derived from CCl_4 to S derived from GSH in metabolite VII (see text) was based on these experiments.

diglutathione carbonate during CCl_4 metabolism was first described by Pohl *et al.* [6]. The overall formation of this compound is apparently nonenzymatic as judged from the linear relationship between its formation with GSH concentration (Fig. 2). However, it is unclear why GSH was effective in increasing aqueous metabolites under 5% oxygen and other thiol compounds, except cysteine, were not. The effectiveness of cysteine can be explained by its ability to undergo thiozolidine formation when it reacts with phosgene [14, 15]. The efficiency of GSH, therefore, may be a reflection of a peculiar feature of diglutathione carbonate formation such as enzymatic support of a partial reaction.

Metabolite VII, which was formed only under low oxygen tensions, has not been characterized completely. It appears to be a GSH adduct of a CCl_4 metabolite with equimolar amounts of both compounds. Instability of the compound frustrated our attempts to isolate sufficient quantities for identification. The strong inhibition of metabolite VII formation by oxygen suggests that it is derived from one of the primary products of CCl_4 reduction, $CCl_3\cdot$ or dichlorocarbene. $CCl_3\cdot$ is known to bind covalently to microsomes [16] so the lack of effect of GSH on covalent binding under nitrogen when metabolite VII formation was stimulated (Table 1) argues against the involvement of CCl_3. Dichlorocarbene is the source of carbon monoxide [2], and we have observed that GSH diminishes carbon monoxide formation under these conditions.* This is consistent with an interaction between dichlorocarbene and GSH. Thus, metabolite VII may be a GSH adduct of dichlorocarbene or of a compound arising from it.

The formation of metabolite VII appears to be enzymatic. Figure 2 shows that it was saturable with an apparent K_m for GSH of 70 μM. Table 2 shows that GSH was the most effective thiol tested for trapping CCl_4 metabolites in the aqueous phase under low oxygen tensions. This is consistent with an enzymatic process specific for GSH.

Formation of these adducts likely accounts for some of the protection by GSH against CCl_4 injury. However, GSH protection against lipid peroxidation is likely to have some other basis because it occurs at oxygen tensions above those supporting metabolite VII formation and phosgene is not known to initiate lipid peroxidation. Therefore, other GSH-dependent protective mechanisms remain to be identified.

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* R. Reiter and R. F. Burk, unpublished observation.

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