Formation of Carbonyl Chloride in Carbon Tetrachloride Metabolism by Rat Liver in Vitro

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ABSTRACT

In order to identify intermediates of CCl₄ metabolism, whole, suitably fortified rat liver homogenates were incubated with ¹⁴CCl₄ in the presence and absence of "pools" of unlabeled suspected intermediates. In the presence of NADH or NADPH, incorporation of radioactivity was rapid and substantial in CO₂, lipid, protein, and the acid-soluble fraction. It was not influenced by the presence of large pools of unlabeled chloroform or formate, thus excluding these substances as obligatory intermediates. However, when incubated with L-cysteine, radioactivity incorporation in the acid-soluble fraction was almost doubled, and about one-third of the radioactivity of this fraction was identified as 2-oxothiazolidine 4-carboxylic acid. This substance is formed chemically by condensation of cysteine with carbonyl chloride and has been identified previously by others as a product of chloroform metabolism by liver microsomes in the presence of L-cysteine. Based on current knowledge of CCl₄ metabolism, the following aerobic pathway is envisioned: microsomal cleavage to Cl⁻ and -CCI₃ and oxidation of the latter to the unstable intermediate, CI₃COH, which loses HCI to yield COCl₂. COCl₂ is likely to be the major source of CO₂ from CCl₄ but is probably not the intermediate that binds to lipid and protein. The addition of glutathione had no effect on CCl₄ metabolism in rat liver homogenate, suggesting that glutathione S-transferases, which catalyze other dehalogenation reactions, do not play a role in CCl₄ metabolism.

INTRODUCTION

Carbon tetrachloride is a potent hepatotoxin and hepatocarcinogen in several species including humans (9) and is a prototype of a large number of toxic organohalides the massive industrial production and widespread use of which make them environmental hazards of great public concern. Despite a wealth of data on its toxic effects, there is as yet little definitive information on its metabolism or its mode of carcinogenic action (6, 23). Like many carcinogens, it is activated for hepatic action (6, 23). Like many carcinogens, it is activated for hepatic action (6, 23). Like many carcinogens, it is activated for hepatic action (6, 23).

The present study was undertaken to shed light on the metabolism of carbon tetrachloride, with the hope of furthering our understanding of its carcinogenicity. We have used the whole, unfractionated rat liver homogenate as a model experimental system to define the conditions for its conversion to CO₂, binding to lipid and protein, conversion to water-soluble intermediates and to identify possible metabolic intermediates.

MATERIALS AND METHODS

Preparation of Liver Homogenate. Male Sprague-Dawley rats, weighing 200 to 250 g, obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass., were decapitated; the livers, weighing approximately 12 g, were homogenized in 2 volumes of 0.25 M mannitol by 4 or 5 passes of a Teflon pestle in a coaxial, motor-driven homogenizer, while cooling in ice.

Carbon Tetrachloride. The ¹⁴CCl₄ was obtained from Amer sham/Searle Corp., Arlington Heights, Ill., and for most experiments was diluted with unlabeled reagent grade CCl₄ to give approximately 60,000 dpm/µmol of ¹⁴CCl₄. For the high-activity experiments used for identification of 2-oxothiazolidine 4-carboxylic acid, the ¹⁴CCl₄ was 10 times more active at 600,000 dpm/µmol. The ¹⁴CCl₄ was uniformly added as a solution in 0.2 ml dimethyl sulfoxide (DMSO) to facilitate accurate measurement and to enhance its solubility in the medium. At this concentration of DMSO, there were no deleterious effects on O₂ uptake or on ¹⁴CCl₄ metabolism. The ¹⁴CCl₄ solutions were assayed for purity of CCl₄ by gas chromatography.

Nicotinamide adenine nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo. (best available grade), and other reagents were top grades obtained from usual commercial sources. Glass-distilled water was used for all reagents.

The following basic procedure for incubation and subsequent separation of products was subject to minor modifications as dictated by individual experiments. A volume of 0.5 ml of the homogenate, equivalent to 167 mg of liver, was added to ice-chilled 18-ml Warburg vessels carrying a center well and a single glass-stoppered side arm. Components were added to the main compartment to make a final volume of 2.5 ml of a mixture containing 24 mM KCl, 40 mM potassium phosphate buffer (pH 8.2), 2 mM MgCl₂, 2 mM ATP, 1.6 mM NADPH, and 10 µmol ¹⁴CCl₄. A fluted filter paper strip (18 x 40 mm) was placed in the center well, 0.2 ml of 5 M NaOH was added to absorb CO₂, 3 0.2 ml of 2 M H₂SO₄ was pipetted into the side
arm, 0.2 ml of the $^{14} \text{CCl}_4$ in DMSO was added last, and the flasks were quickly placed on manometers and immersed in a bath at 37.5°. After temperature equilibration for 5 min, the taps were closed, and the flasks were shaken at approximately 100 oscillations/min. Readings of $O_2$ uptake were taken at regular intervals for 30 min; then acid was tipped in and the shaking was continued for 15 min to allow complete absorption of metabolic CO$_2$. Two ml of toluene were added through the side arm to collect unreacted $^{14} \text{CCl}_4$; the flasks were quickly removed, covered with foil, and chilled in ice. The filter papers were removed, the vessel contents were transferred to 15-ml centrifuge tubes and spun briefly, and the toluene layer was drawn off. Portions were taken for radioactivity assay in a toluene:POPOP:scintillation mixture; in some instances, 1.0 µl was injected into a Glawon gas chromatograph (Glowon Corp., Willow Grove, Pa.) with injection port at 37°, an electron capture detector at 70°, and voltage at 35 V. We found in this manner that approximately 60 to 70% of the added $^{14} \text{CCl}_4$ was recovered unchanged with a retention time of 1.3 min, and there was no detectable chloroform or hexachloroethane in the recovered $^{14} \text{CCl}_4$.

The alkali-soaked papers were dried overnight in a vacuum and extracted with 1.8 ml water, and 1 ml was counted in 10 ml of scintillation mixture with the following composition: p-xylene, 2250 ml; Triton X114, 750 ml; POPOP, 9 g; and POPOP, 0.6 g.

The aqueous layer was extracted with two 1-ml portions of ethyl ether to remove residual traces of $^{14} \text{CCl}_4$, and the aqueous layer (together with a thin layer of semisolid that collected at the interface) was separated from the tissue residue by centrifugation. A measured portion was counted in Formula 963 scintillator fluid.

The tissue residue was extracted successively 3 times with ethanol and ethyl ether, the extracts were evaporated to dryness in a stream of N$_2$ and taken up in chloroform:methanol (20:1), a portion was evaporated to dryness as above in a scintillation vial, and this lipid fraction was counted in a toluene scintillator.

The residue was dried thoroughly, and a weighed portion was dissolved in Protosol and counted in Bray's solution. This fraction was termed the protein fraction, since others reported that there was no detectable chloroform or hexachloroethane in the recovered $^{14} \text{CCl}_4$.

The aqueous layer was reextracted into ethyl acetate after acidification. The ethyl acetate-soluble material had 18,900 dpm or 32% of the initial radioactivity. A portion was streaked on a soft thin-layer 10- x 20-cm silica chromatography plate, developed with chloroform:ethanol:acetic acid (80:20:10), and counted. Ninety-eight % of the applied radioactivity appeared in bands with RF between 0.45 and 0.65, exactly where the authentic acid migrated, as visualized by staining with iodine (Chart 1).

**Identification of 2-Oxothiazolidine 4-Carboxylic Acid as a CCl$_4$ Metabolite.** Two experiments were conducted with 10 vessels, each containing 10 µmol $^{14} \text{CCl}_4$ and 12.5 µmol (5 mm) L-cysteine, incubated and treated as described in Table 1, except for a 10-fold higher radioactivity; i.e., 6 x 10$^6$ dpm/vessel. The acid-soluble fractions from 4 vessels were combined to give a total of 59,100 dpm. This solution was adjusted to pH 1.0, extracted with ethyl acetate, reextracted into the aqueous phase with 0.02 M K$_2$HPO$_4$ buffer (pH 8), acidified, and reextracted in ethyl acetate. The process was repeated twice, after which essentially all of the ethyl acetate-soluble radioactivity was drawn into the alkaline aqueous phase and was reextracted into ethyl acetate after acidification. The ethyl acetate-soluble material had 18,900 dpm or 32% of the initial radioactivity. A portion was streaked on a soft thin-layer 10- x 20-cm silica chromatography plate, developed with chloroform:ethanol:acetic acid (80:20:10), and counted. Ninety-eight % of the applied radioactivity appeared in bands with RF between 0.45 and 0.65, exactly where the authentic acid migrated, as visualized by staining with iodine (Chart 1).

**Crystalization to Constant Specific Activity.** Another ethyl acetate extract of the acid-soluble fraction, partially purified as described above, containing 10,900 dpm was evaporated to dryness, taken up in ethanol, 98 mg of synthetic acid were added as carrier, and the mixture was subjected to 4 successive recrystallizations from ethyl acetate:hexane. The recoveries of each of the 4 crystallizations were: 74.5, 58.7, 46.5, and 28.3 mg, and the corresponding specific radioactivities were 101, 113, 115, and 108 dpm/mg. The calculated radioactivity of 98 mg containing a total of 10,900 dpm = 10,900/98 = 111 dpm/mg.
Mass Spectrometry. Another portion of the acid-soluble fractions was purified as above by acid:base extraction and thin-layer chromatography followed by repeated acid-base extraction, and portions were used for mass spectrometry on a Hitachi-Perkin Elmer RMU-6H instrument with direct probe inlet. Source temperature was 200° and best probe temperature was 100 to 200°. Ionization voltage was 70 eV, acceleration voltage was 1.5 kV, and current was 70 μA. As shown in Chart 2, the synthetic material had major peaks at 147 (the molecular ion), 102, and 74. Despite some extraneous peaks, the presence of peaks at 147, 102, and 74, with nearly the same peak height ratios as given by the synthetic compound, provides further evidence for the metabolic formation of 2-oxothiazolidine 4-carboxylic acid from CCU. This same product gave a single radioactive peak and a single iodine vapor spot at R, 0.58 on thin-layer chromatography with a GF hard-layer silica plate, developed as described earlier.

RESULTS

Properties of the experimental system are depicted in Table 1. Incubation at 37.5° with 10 μmol of CCU and 1.6 mM NADPH resulted in rapid CCU metabolism, with substantial conversion to CO₂ and water-soluble products and binding to lipids and protein. By 30 min, approximately 7.5% of the added ¹⁴CCl₄ was accounted for in these products; 60 to 70% of the initial radioactivity added was recovered by extraction of the flask contents with toluene. That this was unreacted ¹⁴CCl₄ was established by gas chromatography, which yielded a single peak with a retention time of 1.3 min. O₂ uptake was measured as a criterion of tissue viability. It was strictly linear for 30 min and about half-maximal without added nucleotides. However, metabolism of ¹⁴CCl₄ to CO₂, and to acid-soluble material, and protein and lipid binding with no added nucleotide were less than 10% of that at the optimal concentration of 1.6 mM. No remarkable differences were observed between NADH and NADPH at 1.6 mM, but the latter was the better substrate at lower concentrations. There was no significant additive effect when both nucleotides, each at 1.6 mM, were present, nor did the addition of an NADPH-regenerating system consisting of glucose 6-phosphate and glucose-6-phosphate dehydrogenase enhance ¹⁴CCl₄ metabolism. Formate Not an Obligatory Intermediate. To assess the role of formate as an intermediate of CCl₄ metabolism in liver, the classical isotope trapping technique was used. Experiments were conducted in which ¹⁴CCl₄ was incubated with nonlabeled formate, on the assumption that any metabolic formate would be "trapped" by the pool of unlabeled formate and thereby lower the radioactivity in the metabolic products and enhance radioactivity in the acid-soluble fraction. The data of Table 2 are typical of many experiments which failed to show such effects. Whether 1 or 10 μmol ¹⁴CCl₄ were added, the addition of 1 or 10 μmol of formate did not lower the incorporation of radioactivity in any of the products and did not increase the radioactivity in the acid-soluble fraction. These results are considered to exclude formate as an obligatory intermediate.

Table 1: Effect of NADH and NADPH on ¹⁴CCl₄ metabolism in rat liver

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>O₂ uptake</th>
<th>CO₂</th>
<th>Acid-soluble</th>
<th>Lipid</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>44 ± 5</td>
<td>27 ± 5</td>
<td>37 ± 6</td>
<td>14 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>NADH</td>
<td>60 ± 2</td>
<td>373 ± 17</td>
<td>159 ± 10</td>
<td>59 ± 4</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>NADPH</td>
<td>60 ± 3</td>
<td>464 ± 33</td>
<td>210 ± 8</td>
<td>72 ± 2</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>NADH + NADPH</td>
<td>60 ± 2</td>
<td>472 ± 21</td>
<td>215 ± 11</td>
<td>65 ± 2</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>76 ± 3</td>
<td>572 ± 13</td>
<td>232 ± 8</td>
<td>87 ± 7</td>
<td>111 ± 2</td>
</tr>
<tr>
<td>NADPH + regenerating system</td>
<td>63 ± 2</td>
<td>460 ± 2</td>
<td>235 ± 18</td>
<td>58 ± 4</td>
<td>107 ± 6</td>
</tr>
</tbody>
</table>

Formation of Chloroform. Under the conditions used for ¹⁴CCl₄ metabolism, analysis of the toluene fractions by gas-liquid chromatography revealed little or no chloroform production. However, since chloroform is metabolized more rapidly than ¹⁴CCl₄ in rat liver (26), it could have been formed and utilized. "Trapping" with nonlabeled chloroform was therefore used to make a more definitive assessment of its formation.

Table 2: Effect of added formate on ¹⁴CCl₄ metabolism in rat liver

<table>
<thead>
<tr>
<th>Additions</th>
<th>CO₂</th>
<th>Acid-soluble</th>
<th>Lipid</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μmol ¹⁴CCl₄ Formate</td>
<td>None</td>
<td>125 ± 24</td>
<td>42 ± 7</td>
<td>29 ± 13</td>
</tr>
<tr>
<td>1 μmol ¹⁴CCl₄ Formate</td>
<td>137 ± 26</td>
<td>44 ± 7</td>
<td>47 ± 17</td>
<td>55 ± 11</td>
</tr>
<tr>
<td>10 μmol ¹⁴CCl₄ Formate</td>
<td>139 ± 27</td>
<td>46 ± 8</td>
<td>43 ± 17</td>
<td>47 ± 13</td>
</tr>
<tr>
<td>10 μmol ¹⁴CCl₄ Formate</td>
<td>348 ± 7</td>
<td>154 ± 5</td>
<td>57 ± 6</td>
<td>83 ± 6</td>
</tr>
</tbody>
</table>

In other experiments not shown, we found that ¹⁴CCl₄ metabolism was proportional to its concentration up to 4 μmol but was toxic at 10 μmol. Neither phenobarbital nor 3-methylcholanthrene pretreatment influenced ¹⁴CCl₄ metabolism. Addition of β-diethylaminomethyl-2,2-diphenylpentanoate (SKF-525A) had no effect up to 0.1 mM, but KCN at 1 mM inhibited all metabolic conversions by 40 to 50%.

Chart 2. Mass spectra of authentic 2-oxothiazolidine 4-carboxylic acid (top) and the ¹⁴CCl₄ metabolite (bottom). Mass spectra were obtained as described in the text. Top, results with 4.7 μg of the synthetic acid dissolved in 10 μl of ethyl acetate and evaporated in the mass spectrometer; bottom, mass spectrum obtained by eluting the radioactive material from a silica plate with radioactivity representing approximately 4 μg of acid. The peak at 149 is an impurity, appearing in blank runs.
Experiments were conducted with either 1 or 10 \( \mu \text{mol} \) of \(^{14}\text{CCl}_4\), to which were added 0, 1, or 10 \( \mu \text{mol} \) of nonlabeled \( \text{CHCl}_3 \). As shown in Table 3, there was no decrease of radioactivity incorporation into \( \text{CO}_2 \), lipid, protein, or acid-soluble fractions. We assume, therefore, that under aerobic conditions chloroform is formed in minimal amounts, if at all, from \(^{14}\text{CCl}_4\) by rat liver; or, if formed, it does not mix with an exogenous pool of chloroform.

**Formation of Carbonyl Chloride.** Recent studies demonstrated that carbonyl chloride (phosgene) is a metabolite of chloroform in rat liver microsomes. Incubation of \( \text{CHCl}_3 \) in the presence of L-cysteine led to the formation of 2-oxothiazolidine 4-carboxylic acid, a product formed chemically by condensation of L-cysteine with carbonyl chloride (15, 20). To test for the possible formation of carbonyl chloride in hepatic carbon tetrachloride metabolism, we added 5 \( \text{mm} \) L-cysteine to our basic system, together with 10 \( \mu \text{mol} \) of \(^{14}\text{CCl}_4\). As shown in Table 4, the presence of cysteine resulted in moderate decreases in \( \text{CO}_2 \) formation and protein binding and somewhat increased lipid binding, but the most striking effect was a pronounced increase of radioactivity in the acid-soluble fraction.

The presence in this fraction of the above-mentioned thio acid was established by the identity of migration of radioactivity with the synthetic material in thin-layer chromatography (Chart 1), by retention of a constant specific radioactivity on repeated recrystallization, and by the appearance of characteristic mass peaks when the mass spectrographs of the synthetic and metabolic material were compared (Chart 2). The mass spectrum exhibits impurities still present in the metabolic product; however, the presence of the molecular ion peak at 147 and the other major peaks at 102 (the oxothiazolidine ring) and 74 (the fragment, \( -\text{SCH}_2\text{CNH} \)) helps to confirm the identity of the metabolic product, and further support is provided from the similarity in the ratios of the peak heights, 36:100:60 versus 31:100:80.

**Lack of Effect of Glutathione.** In contrast with the marked action of L-cysteine on the incorporation of radioactivity in the acid-soluble fraction, glutathione had no significant effect on the disposition of \(^{14}\text{CCl}_4\) in these experiments as shown in Table 4, Experiment 2. We were led to test this substance for 2 reasons. Rubinsteind and Kanics (26) found that, although glutathione had little or no effect on \( \text{CCl}_4 \) metabolism, it markedly increased the oxidation of chloroform by rat liver homogenate; it is becoming increasingly recognized that one or more of the isozymes of glutathione S-alkyltransferase dehalogenate certain organohalides (10, 11). Our results indicate that neither glutathione nor the \( \text{S} \)-transferases play a role in those metabolic conversions measured by us.

**DISCUSSION**

The formation of 2-oxothiazolidine 4-carboxylic acid in our system demonstrates the metabolic formation of carbonyl chloride from \( \text{CCl}_4 \) as shown previously for \( \text{CHCl}_3 \) (15, 20) and emphasizes basic similarities in the metabolism of both chloroalkanes. An important question raised by this finding concerns a possible role of phosgene in hepatocarcinogenesis. It is conceivable that products of lipoperoxidation are responsible for \( \text{CCl}_4 \) hepatocarcinogenesis; indeed, malonaldehyde has been reported to be carcinogenic (27) as well as mutagenic (17). Phosgene, an intermediate of both \( \text{CHCl}_3 \) and \( \text{CCl}_4 \) metabolism, deserves special consideration as a carcinogen. A search of the literature has not yet revealed any data on COCl\(_2\) carcinogenicity or mutagenicity, although it is highly toxic to lungs exposed acutely or chronically to low levels (19). Its 2 highly reactive chlorines suggest that it could act on DNA or other macromolecules in ways similar to those of the bifunctional alkylating agents. The lack of binding of \( \text{CCl}_4 \) carbon to DNA argues against this possibility, however. Studies on the possible mutagenicity of phosgene are now under way (conducted by Nobuto Yamamoto of Fels Research Institute).

**Mechanism of Phosgene Formation.** The formation of phosgene as an intermediate may be placed within the framework of our current knowledge of \(^{14}\text{CCl}_4\) metabolism as outlined in Chart 3. A large body of experimental data indicates that the first step is a rapid reductive formation of the trichloromethyl (-\( \text{CCl}_3 \)) radical by complexing with one or more of the P-450 cytochromes (21, 23). Anaerobically, this radical may undergo several reactions, namely, addition of a proton and electron to yield chloroform (2, 5, 7, 29), binding to lipids (8, 24, 30, 32) and proteins (29, 30) (but not to nucleic acids) (24, 30, 31), dimerization to hexachloroethane (5, 29), and further reductive dechlorination to yield carbon monoxide, presumably via the carbene, \( \text{CCl}_2 \), as suggested by Wolf et al. (35). Chloroform (1, 30) and methylene dichloride (13, 14, 22, 25) also yield CO, presumably via the same carbene intermediate.

The repeatedly observed formation of chloroform from \( \text{CCl}_4 \), both in vivo and in vitro and in several species, might at first

ACKNOWLEDGMENTS

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