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 CI- and -CCI3 and oxidation of the latter to the unstable intermediate, Cl2COH, which loses HCl 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 hepatotoxicity by the mixed-function oxidase system (6, 23); however, unlike many other carcinogens, it binds minimally to DNA (5, 24, 30, 35) and thus far has not been found to be mutagenic in microbial test systems (4, 16). Among reported metabolic reactions in liver are conversion to CO2 (18, 26), reduction to chloroform (2, 5, 7, 29) and hexachloroethane (5, 29), and binding to lipid and protein (7, 8, 24, 29, 30, 33). In the presence of oxygen, it enhances lipid peroxidation and malondialdehyde formation and destroys the endoplasmic reticulum, together with its associated enzymes (23). The trichloromethyl free radical, -CCI3, has been proposed as the key causal agent for these toxic effects (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 co-axial, motor-driven homogenizer, while cooling in ice.

Carbon Tetrachloride. The 14CCl4 was obtained from American-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 mM NaOH was added to absorb CO2, 0.2 ml of 2 mM H2SO4 was pipetted into the side...

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3 We avoided the use of organic bases such as Hyamine to absorb CO2 because they absorbed 14CCl4 vapor as well and thereby complicated the experiments.
arm, 0.2 ml of the $^{14}$CCI$_4$ 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 $^{14}$CCI$_4$; 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 $\mu$l was injected into a Glawol gas chromatograph (Glowall 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 $^{14}$CCI$_4$ was recovered unchanged with a retention time of 1.3 min, and there was no detectable chloroform or hexachloroethane in the recovered $^{14}$CCI$_4$.

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; PPO, 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 $^{14}$CCI$_4$, and the aequous 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 N2 and taken up in chloroform:methanol (2:1), a portion was streaked on a soft thin-layer 10 x 20-cm silica chromatography plate, developed with chloroform:ethanolic 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).

**Chart 1. Chromatography of acid-soluble $^{14}$CCI$_4$ metabolite, purified by repeated acid:base extraction as described in the text.**

\begin{itemize}
  \item 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.
  \item Ninety-eight % of the applied radioactivity appeared in bands with $R_f$ between 0.45 and 0.65, exactly where the authentic acid migrated, as visualized by staining with iodine (Chart 1).
\end{itemize}

**Synthesis of 2-Oxothiazolidine 4-Carboxylic Acid.** The synthesis was conducted essentially as described by Kaneko et al. (12), except for isolation of the product by repeated extraction with ethyl acetate from the acidified solution rather than by concentration of the aqueous reaction mixture. The product had a melting point of 173-174°* (reported by Kaneko et al., 171-172.5°). Analysis: C, 30.37; H, 3.03; S, 28.3 mg; and calculated for C$_2$H$_2$NSO$_3$: C, 32.65; H, 3.40; S, 21.76; neutralization equivalent, 154; calculated, 147.

**Identification of 2-Oxothiazolidine 4-Carboxylic Acid as a $^{14}$CCI$_4$ Metabolite.** Two experiments were conducted with 10 vessels, each containing 10 $\mu$mol $^{14}$CCI$_4$ and 12.5 $\mu$mol (5 mm) L-cysteine, incubated and treated as described in Table 1, except for a 10-fold higher radioactivity; i.e., $6 \times 10^6$ 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 $R_f$ 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.

\*Although $O_2$ uptake was not an important parameter of CCl$_4$ metabolism, it was convenient to measure and served as an index of tissue viability. It was determined in all experiments but was omitted from those tables in which no significant changes occurred. In all experiments, $O_2$ uptake was linear throughout the entire 30-min incubation.
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 1.8 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 Rs 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.5

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 unlabeled 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.

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 (28), it could have been formed and utilized. "Trapping" with unlabeled chloroform was therefore used to make a more definitive assessment of its formation.

Table 1

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>O2 uptake</th>
<th>CO2</th>
<th>Acid-soluble</th>
<th>Lipid</th>
<th>Protein</th>
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<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>
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<tr>
<td>NADH</td>
<td>60 ± 2</td>
<td>373 ± 17</td>
<td>150 ± 10</td>
<td>59 ± 4</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>NADPH + NADPH</td>
<td>60 ± 3</td>
<td>464 ± 33</td>
<td>210 ± 8</td>
<td>72 ± 2</td>
<td>92 ± 4</td>
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<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH + regenerating system</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 + formate</td>
<td>63 ± 2</td>
<td>460 ± 2</td>
<td>235 ± 18</td>
<td>58 ± 4</td>
<td>107 ± 6</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Additions</th>
<th>CO2</th>
<th>Acid-soluble</th>
<th>Lipid</th>
<th>Protein</th>
</tr>
</thead>
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<tr>
<td>1 μmol 14CCl4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate No</td>
<td>125 ± 24</td>
<td>42 ± 7</td>
<td>29 ± 13</td>
<td>43 ± 11</td>
</tr>
<tr>
<td>1 μmol 14CCl4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate No</td>
<td>137 ± 26</td>
<td>44 ± 7</td>
<td>47 ± 17</td>
<td>55 ± 11</td>
</tr>
<tr>
<td>10 μmol 14CCl4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate No</td>
<td>139 ± 27</td>
<td>46 ± 8</td>
<td>43 ± 17</td>
<td>47 ± 13</td>
</tr>
<tr>
<td>10 μmol 14CCl4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate No</td>
<td>348 ± 7</td>
<td>154 ± 5</td>
<td>57 ± 6</td>
<td>83 ± 6</td>
</tr>
<tr>
<td>10 μmol 14CCl4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate No</td>
<td>376 ± 23</td>
<td>145 ± 8</td>
<td>59 ± 21</td>
<td>70 ± 8</td>
</tr>
</tbody>
</table>

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

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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 CHCl\(_3\). As shown in Table 3, there was no decrease of radioactivity incorporation into 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 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 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, \(-\text{SCH}_2\text{CHNH}\)) 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. Rubinstein and Kanics (26) found that, although glutathione had little or no effect on 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 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 CCl\(_4\) as shown previously for 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 hepatic carcinogenesis. It is conceivable that products of liperoxidation are responsible for CCl\(_4\) hepatocarcinogenesis; indeed, malonaldehyde has been reported to be carcinogenic (27) as well as mutagenic (17). Phosgene, an intermediate of both CHCl\(_3\) and 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 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, CCl\(_2\), as suggested by Wolff 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 CCl\(_4\), both in vivo and in vitro and in several species, might at first
glance appear contradictory with our results, which indicate that CHCl₃ does not dilute the incorporation of ¹⁴C carbon into metabolites. However, CC₄ yields CHCl₃ most readily in vitro under anaerobic conditions, and its formation is inhibited by oxygen (7, 29). These findings would suggest minimal formation under normal physiological conditions. It is conceivable that chloroform may have been formed and undergone rapid further reaction, since in a preparation similar to ours Rubinstien and Kanics (26) found it to be more rapidly converted to CO₂ than CC₄. However, if CHCl₃ is formed as a transient, obligatory intermediate of CC₄ metabolism, it evidently is metabolized further without mixing with a large pool of free chloroform. Our results would also indicate that CHCl₃ does not compete successfully for a rate-limiting step in CC₄ metabolism, such as the initial binding of the halocarbon to cytochrome P-450 (23, 28). Wolf et al. (35) found that binding of CHCl₃ to reduced cytochrome P-450 was extremely slow compared to that of CC₄.

**Aerobic Metabolism of CC₄.** Aerobically, the CC₄ free radical could be oxygenated by the microsomal mixed-function oxidase system to yield trichloromethanol, Cl₂COH. This substance was suggested by Mansuy et al. (15) and Pohl et al. (20) as the precursor of phosgene formed metabolically from CHCl₃. Pohl et al. (20) clearly demonstrated that the phosgene must have been formed by an oxygenase reaction. CO₂ would be formed by the hydrolytic dechlorination of CC₄COH, and it seems highly likely that this is the pathway by which the microsomal oxygenase system converts CC₄ to CO₂. With the evidence that phosgene is the precursor of CO₂ from CC₄, it is necessary to consider that it might also be one of the reactive species that bind to lipids and protein. Reynolds (24) showed that ¹⁴CO₂ given to intact rats labeled liver protein (and lipids to a smaller extent), but the pattern was quite different from that of ¹⁴CC₄. Moreover, C₃H₅Cl₂ radioactivity was also stably incorporated into liver lipid and protein, pointing to the Cl₃C- radical rather than phosgene as the reactive form. Cessi et al. (3) also reported that ¹³C-phosgene given to rats labeled the liver proteins. The significance of phosgene as the form in which CC₄ binds to lipid and protein obviously needs further investigation.

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