In Vivo CHCl₃ Bioactivation, Toxicokinetics, Toxicity, and Induced Compensatory Cell Proliferation in B6C3F1 Male Mice

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Chloroform carcinogenicity has often been associated with acute tissue damage and consequent compensatory cell proliferation. However, available data do not fully support this hypothesis, and other biological factors may play a role in the tumor induction by chloroform. The purpose of this study was to characterize the in vivo CHCl₃ metabolism and the time course of toxic effects and of cell proliferation in the liver and kidney of B6C3F1 male mice dosed ip or by gavage with 150 mg CHCl₃/kg body wt. Microsomal phospholipid adducts attributed to ¹⁴CHCl₃ metabolism by both oxidative and reductive pathways were detected in both liver and kidney. The levels and composition of the adducts were similar in the liver and kidney of treated animals. In the liver, although no necrosis was histologically detectable, a transient cell proliferation was found starting at 24 and peaking at 48 hr post-treatment. Kidney toxicity was evident by biochemical and cytochemical methods at 5 hr after dosing and progressed to severe necrosis at 48 and 96 hr. An intense kidney cell regeneration began 48 hr after CHCl₃ treatment, became maximal at 96 hr, and was sustained for at least the following 3 days. These observations raise questions about the purely epigenetic action of chloroform in tumor induction since bioassays have found tumors in liver but not kidneys of CHCl₃-treated B6C3F1 mice.

Chloroform is an established rodent carcinogen (USDHHS, 1994). At dose levels close to the maximum tolerated dose, chloroform was found to be carcinogenic for the liver of male and female B6C3F1 mice and for the kidneys of male Osborne Mendel (OM) rats (NCI, 1976; Jorgenson et al., 1985). At lower doses, chloroform exposure induced kidney tumors in male IC1 mice, but was not carcinogenic for Sprague–Dawley (S.D.) rats, beagle dogs, or C57Bl, CBA, and CF1 mice (Roe et al., 1979; Heywood, et al., 1979; Palmer et al., 1979). Hepatic tumors were found in B6C3F1 mice treated by gavage with chloroform dissolved in corn oil but not when administered chloroform in drinking water (NCI, 1976; Jorgenson et al., 1985).

High doses of chloroform also rapidly induce acute liver damage in female and male mice and kidney necrosis in male mice (Eschenbrenner and Miller, 1945; Moore et al., 1982; Larson et al., 1993, 1994). Although DNA alkylation has been recently found in liver and kidneys after [¹⁴C]-chloroform administration to Wistar rats and BALB/c mice (Colacci et al., 1991), the genotoxic properties of chloroform are not yet clearly established (Rosenthal, 1987). A few authors have suggested that a causal association exists between tumors and chloroform-induced acute damage followed by compensatory cell proliferation (Eschenbrenner and Miller, 1945; Reitz et al., 1982, 1990; Larson et al., 1993). However, B6C3F1 male mice treated with chloroform develop acute kidney damage followed by intense compensatory renal cell proliferation but not kidney tumors (Larson et al., 1994). Moreover, acute kidney damage has not been assessed in CHCl₃-treated OM rats (NCI, 1976; Jorgenson et al., 1985; Reuber, 1979). Observations of CHCl₃-induced kidney damage and compensatory cell proliferation in rats were reported in a strain (F-344; Larson et al., 1993, 1995) not used in the CHCl₃ carcinogenicity bioassays.

Chloroform can be activated by liver and kidney microsomes through oxidative and reductive pathways (Mansuy et al., 1977; Pohl et al., 1977; Testai and Vittozzi, 1986; Testai et al., 1992; Smith et al., 1983; Ade et al., 1994). Metabolic formation of reactive electrophilic species is a plausible basis for both acute cellular toxicity and genotoxicity of chloroform (Ilett et al., 1973; Pohl et al., 1980; Rosenthal, 1987). Therefore a study of chloroform metabolism could yield key information relevant to the processes of initiation and promotion of chloroform-induced tumors. The well known oxidative pathway of chloroform activation produces phosgene in vivo as a major determinant for the acute liver and kidney toxicity (Pohl et al., 1980; Branchflower et al., 1984). However, the species-specific hepatocarcinogenic effect of this halomethane is not fully explained.

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by the biotransformation of CHCl₃ to phosgene nor to its terminal metabolite CO₂ (Brown et al., 1974; Reitz et al., 1978, 1990; Testai et al., 1992). Activation by the reductive pathway produces a dichloromethyl radical in several experimental systems (Tomasi et al., 1985; Testai et al., 1995). In vitro bioactivation to this radical by hepatic and renal microsomes from selected rodent strains produces adducts to phospholipids (PL), which are distinctly different from those of phosgene (Testai and Vittorazzi, 1986; Testai et al., 1990, 1992; De Biasi et al., 1992; Ade et al., 1994). No information on the occurrence and toxicological relevance of the reductive pathway in vivo is available.

We have therefore undertaken a study to determine if different pathways of chloroform biotransformation associated with toxicity and cell proliferation could account for the different susceptibility of the liver and the kidneys to CHCl₃-induced carcinogenicity in B6C3F1 male mice.

**MATERIALS AND METHODS**

**Chemicals.** [¹⁴C]Chloroform (about 3.0 mCi/mmol, radiocutaneous purity 99%) was obtained from Merck (Darmstadt, Germany). Tanax was supplied by Hoechst A.G. (Frankfurt am Main, Germany). Liquid scintillation cocktails, Aqualuma, Lipoluma, Lumasolve, Lumagel, and the CO₂ trap; were purchased from Lumac System A.G. (Basel, Switzerland). All substrates used in cytotoxic studies and 5'-bromo-deoxyuridine (BrdU) were purchased from Sigma Chemical (St. Louis, MO). Mouse monoclonal anti-BrdU antibodies were obtained from Becton & Dickinson (San Jose, CA). Commercial kits used to measure serum alanine transaminase (ALT), creatinine, triglycerides, and blood urea nitrogen (BUN) were from Boehringer GmBH (Mannheim, Germany). Reduced glutathione (GSH) and defatted bovine serum albumin were purchased from Serva, Feinbiochemicals (Heidelberg, Germany). All other reagents used were of the highest purity commercially available.

**Animals.** Male B6C3F1 mice (20–25 g body wt) from Charles River (Calco, Italy) were used. Mice were housed in solid-bottom shoebox-type polycarbonate cages with stainless steel wire-bar lids, using a wooden dust-free litter (Ditta Mucedoli, Italy) as bedding material. Mice were maintained on a 12-hr light cycle and provided with contaminant-free food (Ditta Mucedoli, Italy) and water on a 12-hr light cycle and provided with contaminant-free food (Ditta Mucedoli, Italy) and water ad libitum. Animals were treated at 9:00 AM sacri¢ced in an ether-saturated chamber 5, 8, 12, 24, 48, and 96 hr or 1 week after CHCl₃ treatment. Liver and kidneys were rapidly removed, ®xed in 70% ethanol, and processed for paraf®n embedding. Serial sections were immunohistochemically stained using a standard protocol to reveal BrdU radioactivity present in liver and kidneys was measured after tissue homogenization in 5 vol (w/v) of cold toluen for 5 min using an Ultraturrax homogenizer. The homogenate was centrifuged at 3000 rpm for 10 min, and 10 ml Lipoluma was added to 1 ml supernatant. Total body radioactivity was measured after digesting the carcasses of four animals with 1.5 m KOH in 20% ethanol (v/v) for 5 days. Samples of the digest (10 ml) were centrifuged (3000 rpm, 10 min); two aliquots of the supernatant (1 ml each) were decolorized with a few drops of NaClO, neutralized, and counted in 10 ml Aquamount, to measure radioactivity due to [¹⁴C]chloroform-derived alkali-soluble metabolites. Toluene-extractable radioactivity present in liver and kidneys was measured after tissue homogenization in 5 vol (w/v) of cold toluen for 5 min using an Ultraturrax homogenizer. The homogenate was centrifuged at 3000 rpm for 10 min, and 10 ml Lipoluma was added to 1 ml supernatant. Total body radioactivity was measured after digesting the carcasses of four animals with 1.5 m KOH in 20% ethanol (v/v) for 5 days. Samples of the digest (10 ml) were centrifuged (3000 rpm, 10 min); two aliquots of the supernatant (1 ml each) were decolorized with a few drops of H₂O₂ and added to 10 ml Lumagel:1.5 N HCl (9:1, v/v). Samples were kept in the dark overnight and then counted.

**Covalent binding of [¹⁴C]chloroform metabolites.** Mice were sacri¢ced 5 hr after CHCl₃ dosing with an ip injection of Tanax (5 ml/kg body wt). Microsomal preparations were obtained from liver and kidneys as previously described (Testai and Vittorazzi, 1986; Ade et al., 1994). Microsomal lipids were extracted from aliquots of 250 µl of microsomal suspension (equivalent to 1 g wet tissue), according to the method of Foch et al. (1957). Covalent binding of [¹⁴C]chloroform metabolites to microsomal PL moieties was measured by the method of De Biasi et al. (1992).

**Serum enzymes and tissue GSH.** Treated and control animals were sacri¢ced by means of cervical dislocation at 5 or 24 hr after CHCl₃ treatment. Serum ALT, creatinine, triglycerides, and BUN were measured with commercial kits and serum sorbitol-dehydrogenase activity (SDH) according to Rose and Henderson (1975). Liver and kidneys were rapidly removed and their cytosolic GSH contents were measured as described by Ellman (1959).

**Histology and BrdU immunohistochemistry.** CHCl₃-treated and control animals were injected twice with BrdU (100 mg/kg body wt) in 0.1 m phosphate buffer saline (pH 7.2) 2 and 1 hr before sacri¢ce. They were sacri¢ced in an ether-saturated chamber 5, 8, 12, 24, 48, and 96 hr or 1 week after CHCl₃ treatment. Liver and kidneys were rapidly removed, ﬁxed in 70% ethanol, and processed for parafﬁn embedding. Serial sections were cut at 4 µm nominal thickness. One set of sections was stained with hematoxylin and eosin (H&E) for morphological analysis. A second set was immunohistochemically stained using a standard protocol to reveal BrdU incorporation in ethanol-ﬁxed tissues (Schuate et al., 1987). Sections were incubated with an anti-BrdU mouse monoclonal antibody, followed by a biotin/alkaline-labeled streptavidin method (Biopac Division, Milano, Italy) with 3,3′-diamino-benzidine as the chromogen. Stained sections were counterstained with hematoxylin. Cells with incorporated BrdU were easily identiﬁed by their brown stained nucleus and were counted in the entire liver and kidney sections. The area of each section was measured with a calibrated image cytometer (see following) and BrdU labeling was expressed as the number of labeled cells per square millimeter of tissue. The average areas evaluated per animal were 30 and 27 mm² for liver and kidney sections, respectively. In animals with high proliferative activity, labeled nuclei were counted in two circular areas of 1.5 mm diameter randomly positioned on the section. BrdU labeling was then expressed as the number of positive nuclei per unit area.
TABLE 1
Recovery of $^{14}$C Radioactivity in Male B6C3F1 Mice after $^{[14]}$C Chloroform Administration

<table>
<thead>
<tr>
<th>Sample</th>
<th>0–24 hr</th>
<th>0–48 hr</th>
<th>0–72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expired $^{14}$CHCl$_3$</td>
<td>30.8 ± 5.0</td>
<td>31.2 ± 5.0</td>
<td>31.0 ± 5.4</td>
</tr>
<tr>
<td>Expired $^{14}$CO$_2$</td>
<td>48.2 ± 6.1</td>
<td>55.5 ± 6.7</td>
<td>56.7 ± 6.4</td>
</tr>
<tr>
<td>Urine</td>
<td>2.3 ± 1.0</td>
<td>2.8 ± 1.0</td>
<td>3.0 ± 1.1</td>
</tr>
<tr>
<td>Feces</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>Carcass</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Total recovery</td>
<td>92.1 ± 9.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. n.d., not determined. Groups of four male mice were dosed ip with $^{14}$CHCl$_3$ (150 mg/kg, equivalent to 1.26 mmol/kg body wt). Results represent means ± SD of data obtained from at least three different groups of treated mice.

Cytotoxicity. Small samples of liver and kidneys were attached to a piece of cork and quickly frozen in liquid nitrogen. Serial sections (8 μm nominal thickness) were cut with a motorized cryostat and collected on a glass coverslip precoated with 10% poly-L-lysine. The sections were air-dried and stored at −80°C in air-tight plastic boxes. Unfixed liver and kidney sections were stained for glucose-6-phosphatase (G6Pase; EC 3.1.3.9) and lactate dehydrogenase (LDH; EC 1.1.1.27) as previously described (Chieco et al., 1988). Intracellular content of calcium was examined with alizarin red S (McGee, 1985). Unfixed kidney sections were also stained for succinate dehydrogenase (SuDH; EC 1.3.99.1), glycerophosphate dehydrogenase (GPODH; EC 1.1.99.5), reduced nicotinamide adenine dinucleotide (NADH)-dehydrogenase (NADH-DH; EC 1.6.99.3), reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dehydrogenase (NADPH-DH; EC 1.6.2.4) as previously described (Chieco et al., 1988), for naphthyl acetate esterase (NACES; EC 3.1.1.1, EC 3.1.1.2, EC 3.1.1.6) with a hexazoniazid-pararosaniline method (Pearse, 1991) and for dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) following Lojda (1979).

Cytometry. Measurements on histological sections were performed with a computerized image analysis system (Cytometrica C&V, Bologna, Italy) using a CCD video camera (XC77ce, Sony, Kangara-Ken, Japan) connected to a Bravado frame grabber board (Truevision Indianapolis, IN). The system was calibrated for morphometric and absorbance measurements as previously described (Chieco et al., 1994). GPODH, LDH, and NADPH-DH were measured at a wavelength of 575 nm in three fields randomly selected in the cortex using a 10X objective. For NACES the absorbance of five fields in the cortical region was measured with a 10X objective at a wavelength of 520 nm. Threshold levels were set at 0.4 absorbance units (A.U.) for GPODH and LDH, at 0.1 A.U. for NADPH-DH, and at 0.2 A.U. for NACES. Section thickness was corrected by measuring a predetermined area on three different serial sections and reporting the original measure to the average of these. Measurements were expressed as integrated absorbance per square micrometer of tissue.

Statistical analysis. The ANOVA test was used to determine significant differences in cell proliferation and cytometry results between control and treated animals. $p < 0.05$ was considered significant. Data for biochemical markers of CHCl$_3$-induced toxicity from control and treated mice were compared using Student’s $t$ test.

RESULTS
Toxicokinetics and in Vivo Covalent Binding of $^{[14]}$C Chloroform
$^{[14]}$C Chloroform expiration rate in B6C3F1 male mice treated ip with $^{14}$CHCl$_3$ (1.26 mmol/kg body wt) was constant up to 1 hr after dosing, being equal to 232 ± 18 (90% confidence interval) μmol/kg body wt/hr. By 2 hr the mice had exhaled about 85% of total $^{14}$CHCl$_3$ expired during the first 24 hr post-treatment. $^{14}$CO$_2$ expiration was slow during the first 30 min, and then it increased reaching a rate of 102.5 ± 7.0 (90% confidence interval) μmol/kg body wt/hr which remained constant for about 3 hr. By 5 hr, the mice had exhaled 73% of the total $^{14}$CO$_2$ expired during the first 72 hr post-treatment. The cumulative recovery of $^{14}$CO$_2$ increased significantly until 24 hr post-treatment, attaining 90% of the total expired $^{14}$CO$_2$. Most $^{14}$C chloroform-derived radioactivity excreted in the urine was detected within 24 hr. The percentage of $^{14}$C radioactivity expired as $^{14}$CHCl$_3$ and $^{14}$CO$_2$ or excreted in the urine by 24, 48, and 72 hr is reported in Table 1, together with the estimated total recovery of $^{14}$C label.

Figure 1 shows the levels of $^{14}$CHCl$_3$ (toluene-extractable $^{14}$C label) in the liver, kidneys, and blood after a single ip injection of $^{14}$CHCl$_3$. Alkali-soluble radioactivity due to $^{14}$CHCl$_3$-derived metabolites ($^{14}$CO$_2$) in blood is also reported in Fig. 1. The halomethane was rapidly distributed to these tissues, as indicated by the peak levels detected at 10 min after $^{14}$CHCl$_3$ injection in liver, kidneys, and blood. The kinetics of $^{14}$CHCl$_3$ elimination were also similar in the liver, kidneys, and blood, with significant levels of toluene-soluble radiolabeled material no longer detectable after 3 hr. Blood level of $^{14}$CO$_2$ increased during the first hour after dosing, remained almost stable from 1 to 4 hr, and then slowly decreased.

Similar amounts of covalent adducts with PL were recovered in hepatic and renal microsome fractions at 5 hr after $^{14}$C chloroform injection. Specifically, the amounts of adducts to PL polar heads were 4.20 ± 1.30 and 3.95 ± 1.57 from at least three groups of four or five animals.
TABLE 2

<table>
<thead>
<tr>
<th>CHCl₃ dose (mg/kg body wt)</th>
<th>5 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic GSH (µmol/g)</td>
<td>150</td>
<td>3.1 ± 0.4 (6)</td>
</tr>
<tr>
<td>ALT (U/liter)</td>
<td>150</td>
<td>3.2 ± 1.2 (6)</td>
</tr>
<tr>
<td>(µmol/g)</td>
<td>150</td>
<td>144 ± 54 (8)</td>
</tr>
<tr>
<td>(mg/100 ml)</td>
<td></td>
<td>266 ± 130 (7)</td>
</tr>
<tr>
<td>SDH (U/liter)</td>
<td>150</td>
<td>126 ± 18 (6)</td>
</tr>
<tr>
<td>(µmol/g)</td>
<td>150</td>
<td>2069 ± 484 (4)*</td>
</tr>
<tr>
<td>Triglycerides (mg/100 ml)</td>
<td>150</td>
<td>80 ± 18 (6)</td>
</tr>
<tr>
<td>Renal GSH (µmol/g)</td>
<td>150</td>
<td>72 ± 9 (6)</td>
</tr>
<tr>
<td>(mg/100 ml)</td>
<td></td>
<td>3.0 ± 0.5 (5)</td>
</tr>
<tr>
<td>Creatinine (mg/100 ml)</td>
<td>150</td>
<td>0.9 ± 0.05 (6)</td>
</tr>
<tr>
<td>(µmol/g)</td>
<td>150</td>
<td>1.4 ± 0.3 (5)*</td>
</tr>
<tr>
<td>BUN (mg/100 ml)</td>
<td></td>
<td>1.1 ± 0.1 (5)*</td>
</tr>
</tbody>
</table>

Note. Results are expressed as means ± SD of data obtained from groups of five animals. Numbers in parentheses represent the number of independent determinations. Asterisks indicate results significantly different from their respective controls at *p < 0.002 (*) and **p < 0.02 (**)..

nmol PL-bound ¹⁴C/g tissue (means ± SD) and the amounts of adducts to the PL fatty acyl chains were 1.64 ± 0.52 and 1.37 ± 0.67 nmol PL-bound ¹⁴C/g tissue (means ± SD) in the liver and in the kidneys, respectively. If reactive metabolites (phosgene or dichloromethyl radicals) are necessary intermediates for the metabolism of chloroform to CO₂ and covalent adducts to cell components, then our data indicate that a very small percentage of reactive intermediates binds to hepatic and renal microsomal PL (about 0.03% of administered dose), while most intermediates are transformed to CO₂ (about 57% of the dose) (Table 1). Based on other reports of covalent binding to microsomal protein and to other subcellular fractions (Uehleke and Werner, 1975; Hill et al., 1975), we estimate that the total amount of radioactivity covalently bound to the liver and the kidneys represents less than 0.12% of the administered dose of ¹⁴CHCl₃.

Serum Chemistry and Tissue GSH

Serum SDH, creatinine and BUN were increased at 5 hr after ip administration of 150 mg/kg body wt CHCl₃ (Table 2). At 24 hr all serum parameters, except triglycerides, significantly differed from control values. A significant GSH depletion (50%) was found in the kidney at 5 and 24 hr post-treatment, but not in the liver.

Morphological and Immunohistochemical Analysis

On histological examination, chloroform was found to cause extensive cortical renal damage. At 5 hr after CHCl₃ treatment, scattered proximal tubules lined by necrotic epithelium were observed in one of three treated mice. At later time points all treated animals had similar pathologic changes spread all over the cortex (Figs. 2A and 2B). Necrotic tubules accounted for 15, 40, 25, and 10% of total tubules at 12, 24, 48, and 96 hr, respectively. At 48 hr, well-recognizable basophilic regenerating cells were scattered among necrotic areas. The extent of regeneration was higher at 96 hr and 1 week after exposure. Regenerating tubules were enriched in BrdU-positive nuclei. The time course of compensatory cell regeneration in the kidney is shown in Fig. 3B.

No morphological alterations were found in the liver of treated mice at any time point (5, 8, 12, 24, 48, 96, or 168 hr). An increased hepatocyte proliferation was observed starting at 24 hr after treatment and peaking at 48 hr (Fig. 3A).

Cytochemical Analysis

Cytochemical analysis was carried out at 5, 8, and 12 hr after CHCl₃ administration to detect damage preceding necrotic changes in the kidneys and the presence of subtle changes in the liver preceding increased proliferation. The enzymes selected are located in the cytoplasm (LDH) or in different parts of cell membranes, and changes in their activities are indicative of damage in the surrounding membrane or of cell metabolism alterations. Succinate dehydrogenase and GPDH are located in the internal and external leaflets of the inner mitochondrial membrane, respectively; G6Pase and NADPH-DH are present in the smooth endoplasmic reticulum; NACES is present mostly in mitochondrial and endoplasmic reticulum membranes; and DPP IV is located in the hepatocyte plasma membrane lining bile canaliculi and in the brush border membrane of renal tubular cells.
Kidneys of CHCl₃-treated animals showed increased cellular calcium in scattered tubules starting at 5 hr (Figs. 2C–2D). Microscopic areas with decreased LDH activity were observed in the cortex at 8 and 12 hr. A transient increase in NADPH-DH was observed in medullary rays at 5 hr after CHCl₃ administration, but not at 8 and 12 hr. NACES (Figs. 2E–2F) and GP0DH were definitely increased in several cortical tubules already at 5 hr. The time course of enzymatic
changes is summarized in Table 3. Activities of SuDH, NADH-DH, G6Pase, and DPP IV were unchanged.

Liver of treated animals showed activity levels of LDH and G6Pase similar to controls and no hepatocytes showed increased staining for calcium.

**DISCUSSION**

The observed kinetics of $^{14}$CHCl$_3$ and $^{14}$CO$_2$ expiration by the B6C3F1 mice after ip injection of 1.26 mmol $^{14}$CHCl$_3$/kg body wt compare well with the results of a previous study (Mink et al., 1986). At this dose level, most $^{14}$CO$_2$ was already expired, $^{14}$CHCl$_3$ was no longer detectable in the expired air, and very low levels of the halomethane were found in the metabolically competent tissues, by 5 hr. Therefore, the measurement of the adducts at 5 hr after the treatment provides representative information on chloroform bioactivation, while minimizing the possible occurrence of confounding secondary effects (Stevens and Anders, 1981).

We monitored $^{14}$CHCl$_3$ conversion to reactive metabolites in the liver and kidneys of male B6C3F1 mice using an assay which exploits the regioselectivity of binding to microsomal PL. Specifically, the reactive species formed by the reductive and the oxidative pathways of chloroform metabolism (namely, dichloromethyl radical and phosgene) were reported to produce adducts with PL fatty acyl chains and PL polar heads, respectively (De Biasi et al., 1992). This assay was also reported to selectively detect the formation of electrophiles and radicals produced from halomethanes in vivo (De Curtis et al., 1994). Our data indicate that the proportion of $^{14}$CHCl$_3$-derived radioactivity associated with the PL fatty acyl chains (29.0 ± 6.0% of total bound radioactivity in the liver and 21.6 ± 2.5% in the kidneys, means ± SE) was higher than that measured in vitro (10.5 ± 1.0 and 12.9 ± 1.7%) in air-equilibrated incubations of $^{14}$CHCl$_3$ with hepatic and renal microsomes, respectively (De Biasi et al., 1992; Ade et al., 1994), and in vivo in the liver of S.D. rats (10.4 ± 3.6%; Gemma et al., 1994). Therefore, a significant formation of CHCl$_2$ radical adducts did occur in both organs, although formation of adducts predominantly occurred from the oxidative pathway of chloroform metabolism. Notice, however, that the total amount of reactive metabolites formed from chloroform, as calculated by the expired $^{14}$CO$_2$, is extremely high, although we detected only a tiny fraction as covalent adducts. It is likely that an effective chemical or enzymatic scavenging occurs for reactive metabolites of chloroform (Testai et al., 1990; Ade et al., 1994). Therefore, the levels of covalent adducts cannot be considered a quantitative measure of chloroform activation in vivo. Neverthe-

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>Control mice</th>
<th>Treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 hr</td>
<td>8 hr</td>
</tr>
<tr>
<td>LDH</td>
<td>253.1 ± 32.2</td>
<td>238.1 ± 38.1</td>
</tr>
<tr>
<td>NADPH-DH</td>
<td>78.6 ± 15.9</td>
<td>123.0 ± 32.3*</td>
</tr>
<tr>
<td>NACES</td>
<td>242.6 ± 34.3</td>
<td>350.3 ± 58.0*</td>
</tr>
<tr>
<td>GPODH</td>
<td>231.1 ± 53.6</td>
<td>339.6 ± 69.5*</td>
</tr>
</tbody>
</table>

Note. Treated mice (4 or 5 animals per group) were dosed by gavage with CHCl$_3$ in corn oil (150 mg/kg body wt). Results are expressed as the integrated absorbance per square micrometer (A·10$^{-3}$/μm$^2$) and represent means ± SD of data obtained from three different sections of each kidney. Asterisks indicate results significantly different from their respective controls with $p < 0.05$. 
less, our results indicate similar amounts of both types of PL adducts are formed in the liver and kidneys of B6C3F1 male mice.

In spite of the metabolic similarity of the liver and the kidneys, overt toxic effects were detected only in the kidneys at 150 mg/kg body wt, which is close to the bioassay dose (NCI, 1976). Early tissue alterations include GSH depletion, as is typical for CHCl₃ acute nephrotoxicity (Smith et al., 1983; Docks and Krishna, 1976). At 5 hr calcium accumulation also was evident in a few tubules. Spotty LDH release from tubule cells was evident at 8 hr, and necrotic cells were morphologically detectable at 12 hr. These initial alterations progressed to massive renal necrosis, which was highest at 24 hr, accompanied by a reduced creatinine clearance. Since the activities of the membrane enzymes examined with cytochemical methods were not decreased at early times after chloroform treatment, the tubule cell necrosis does not seem to be preceded by specific damage to the mitochondrial, microsomal, or plasma membranes. No definitive explanation can be given for the early and remarkable increase of NACES in tubule cells of CHCl₃-treated mice. Since NACES is a marker of nonspecific esterases, its increase might be related to the processing of phosgene adducts through the cleavage of their ester and amide bonds. We also observed increased activity of mitochondrial GPODH in the kidneys of chloroform-treated mice. This enzyme is involved in PL synthesis and its activity could have been stimulated by the high Ca²⁺ cellular influx (Fisher et al., 1973; Wernette et al., 1981), which occurred early after chloroform exposure. Renal damage was followed by a high rate of cellular proliferation which started at 48 hr post-treatment and was sustained for longer than 5 days.

No histological nor cytochemical signs of cellular injury were detected in the livers of chloroform-treated mice at early time points (5, 8, or 12 hr). No evidence of GSH depletion, which is an early and sensitive alteration associated with acute chloroform hepatotoxicity, was found (Docks and Krishna, 1976; Brown et al., 1974; Ekstrom and Högberg, 1980). No hepatic injury was histologically detectable at later time points (24, 48, 96, and 168 hr). The normal serum levels of triglycerides throughout the experiment are consistent with a lack of liver dysfunction. The increases of serum SDH and ALT were the only alterations observed suggestive of hepatic damage. Such marked changes (Table 2) are not consistent with a histologically undetectable or limited damage of the liver. Nevertheless it is possible that these enzymes leak from liver cells due to membrane permeability changes. On the other hand, serum ALT is not liver specific and may reflect also renal injury (Zimmerman, 1978; Plaa and Hewitt, 1982), while SDH increase may be caused by CHCl₃-induced lysis of red cells (Belfiore and Zimmerman, 1970), which contain high SDH activity in mice (Agar, 1979). The weight of the evidence, therefore, favors the conclusion that no appreciable liver necrosis occurred in B6C3F1 mice treated with 150 mg CHCl₃/kg body wt. Such results are in agreement with previous reports that no hepatic necrosis occurred in male mice treated similarly (Eschenbrenner and Miller, 1945; Moore et al., 1982; Bull et al., 1986; Larson et al., 1994).

A transient cell proliferation was observed in the liver starting at 24 and peaking at 48 hr. Because of the lack of histologically detectable liver necrosis, this proliferation may represent a response of the liver to a mild cellular insult caused by CHCl₃, to products from the damaged kidney, or to circulating mediators produced to stimulate kidney cell regeneration.

Although extrapolations from single dose to multiple dose experiments are of limited value, some cautious comments might be appropriate. Tumor development in B6C3F1 mice has been related to chloroform-induced cytotoxicity followed by increased cell replication (Larson et al., 1993). However, weak hepatocyte proliferation was observed in the livers of male B6C3F1 mice after single or repeated chloroform treatments at the lowest carcinogenic dose (Larson et al., 1994; present results). On the other hand, renal tubule cells of male B6C3F1 mice underwent massive necrosis followed by intense proliferation (Larson et al., 1994; present results), but no tumors were reported in the kidney (NCI, 1976). Therefore, the role of cell proliferation in chloroform-induced kidney tumors is unclear.

Biological models (Cohen and Ellwein, 1990a,b) point out the importance of tissue necrosis-mediated cell proliferation in carcinogenesis. These models indicate that DNA mutations and tumors may also occur as a consequence of increased cell replication (epigenetic mechanism). Our observations raise questions about the role of cell proliferation as the only determinant of chloroform-induced tumor development, since bioassays have found tumors in liver but not kidneys of chloroform-treated B6C3F1 mice (NCI, 1976). Based on the present results, together with findings of DNA adducts of chloroform metabolites in the liver and kidneys in vivo (Pereira et al., 1982; Colacci et al., 1991), we suggest that a weak genotoxic action plus cell proliferation may result in tumor formation (as is the case of the liver), while massive necrosis, eliminating cells with damaged DNA, has an antagonistic effect resulting in no tumor formation (as is the case of the kidneys). The organ-specific carcinogenesis of chloroform in B6C3F1 mice seems therefore regulated by a balance between its cytotoxic and weak genotoxic effects. Such a balance is likely dependent mainly on the presence of differently effective cellular repair and protection systems (e.g., GSH) in hepatocytes and tubular cells, since reactive metabolite formation appeared similar in the two organs.

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