Abstract. CCl₄ exerts its toxicity through its metabolites, including the free radicals CCl₃ and CCl₃00'. Oxygen strongly inhibits the hepatic cytochrome P-450-mediated formation of CCl₃ from CCl₄ and promotes the conversion of CCl₃ to CCl₃00'. Both these free radicals injure the hepatocyte by causing lipid peroxidation and binding covalently to cell structures. A reduced glutathione (GSH)-dependent mechanism can protect the liver microsomal membrane against CCl₄-induced damage under aerobic conditions but not under anaerobic conditions (Burk, R. F., K. Patel, and J. M. Lane, 1983, Biochem. J., 215:441-445). Experiments were carried out using rat liver microsomes to examine the effect of O₂ tensions found in the liver and of GSH on CCl₄-induced covalent binding and lipid peroxidation. An NADPH-supplemented microsomal system was used. CCl₄ or ¹⁴CCl₄ was added to the sealed flask that contained the system, and after 20 min CHCl₃ production, thiobarbituric acid-reactive substances (an index of lipid peroxidation), and covalent binding of ¹⁴C were measured. O₂ tensions of 0, 1, 3, 5, and 21% were studied. Increases in O₂ tension caused a fall in CHCl₃ production, which indicated that it decreased CCl₃ GSH had no significant effect on CHCl₃ production at any O₂ tension. Lipid peroxidation and covalent binding of ¹⁴C fell progressively as O₂ tension was increased from 1 to 21%. The addition of GSH decreased both lipid peroxidation and covalent binding, but did so better at the higher O₂ tensions than at the lower ones.

These results indicate that low O₂ tensions such as are found in the centrilobular areas of the liver favor conversion of CCl₄ to free radical products which cannot be detoxified by the GSH-dependent mechanism. They suggest that hyperbaric O₂ might decrease free radical formation in the liver in vivo and promote formation of CCl₃00' from CCl₃. This should result in diminished CCl₄-induced lipid peroxidation and liver damage. Rats given CCl₄ (2.5 ml/kg) were studied in metabolic chambers. Production of CHCl₃ and ethane, the latter an index of lipid peroxidation, were measured. Rats in two atmospheres of 100% O₂ produced much less CHCl₃ and ethane than rats in air. This strongly suggests that hyperbaric O₂ is decreasing free radical formation from CCl₄ and/or promoting the formation of CCl₃00' from CCl₃. These results provide the rationale for the use of hyperbaric O₂ in the treatment of CCl₄ ingestion.

Introduction

The hepatotoxicity of CCl₄ results from its metabolic conversion to free radical products (1). Evidence of CCl₃ formation from CCl₄ was provided in the 1960's by the identification of CHCl₃ (2) and C₂Cl₄ (3) in tissues of animals that were given CCl₄. More recently, spin-trapping experiments have verified directly

Scheme 1

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the production of CCl₃ from CCl₄ by the rat liver microsomal cytochrome P-450 system (4).

The major reactions of CCl₃ with liver microsomes (Scheme 1) appear to be covalent binding (5) and H⁺ abstraction from polyunsaturated fatty acids (PUFA).¹ The H⁺ abstraction results in CHCl₃ and PUFA⁺ formation. PUFA⁺ can enter into cross-linking reactions or lipid peroxidation, depending on the O₂ tension.

In 1978, Packer et al. (6) reported that O₂ reacts very rapidly with CCl₃ to yield CCl₃O₂⁺. Thus, the presence of O₂ when CCl₃ is formed results in the formation of a second radical species as shown in Scheme 1. Because this reaction is so rapid, O₂ can be expected to compete with microsomal constituents for CCl₃.

CCl₃O₂⁺ is many orders of magnitude more reactive with PUFA than is CCl₃ (7), and therefore is a potent initiator of lipid peroxidation. It or some of its breakdown products can bind covalently to microsomes as well (8). CCl₃O₂⁺ seems unlikely to serve as a source of CHCl₃ (9). Therefore, although both CCl₃ and CCl₃O₂⁺ can cause membrane injury through covalent binding and lipid peroxidation, CHCl₃ arises only from CCl₃ and may serve as an index of CCl₃ concentration.

O₂ inhibits the cytochrome P-450-mediated formation of CCl₃ from CCl₄. This inhibition is profound, but is not complete even under 21% O₂ (10, 11). Thus, O₂ diminishes CCl₃ concentration by (1) inhibiting its formation and (2) promoting its conversion to CCl₃O₂⁺. The highest concentration of CCl₃ in a microsomal system will occur then under anaerobic conditions, and its concentration should fall as O₂ tension increases. The effect of O₂ on CCl₃O₂⁺ concentration will be more complex. As O₂ tension rises, a greater fraction of the CCl₃ that is present will be converted to CCl₃O₂⁺, but a smaller amount of CCl₃ will be formed. Thus, the highest CCl₃O₂⁺ concentration should be achieved at an intermediate O₂ tension. At higher O₂ tensions, virtually all the CCl₄-derived free radicals will be CCl₃O₂⁺, but the total conversion of CCl₄ to radicals can be expected to be small, so CCl₃O₂⁺ concentration will decline.

In an earlier study with NADPH-supplemented rat liver microsomes (11), we found that substantial amounts of CCl₄ disappeared from the flask under anaerobic conditions. CHCl₃ appeared and covalent binding occurred. When the atmosphere in the flask was 21% O₂, no disappearance of CCl₄ could be detected by the method used. No CHCl₃ production was detected. A small amount of CCl₄ metabolism was occurring, however, because covalent binding and lipid peroxidation were detected.

It seems likely that nearly all the CCl₄-derived free radicals present under the anaerobic conditions (see Scheme 1) were CCl₃ and nearly all those present under 21% O₂ were CCl₃O₂⁺. This has potential importance because reduced glutathione (GSH) inhibited covalent binding and lipid peroxidation under 21% O₂, but had no effect on covalent binding or CHCl₃ formation under anaerobic conditions, which suggests that a GSH-dependent mechanism can protect the membrane against CCl₃O₂⁺ but not against CCl₃.

We undertook the present study to investigate the relevance of these observations to the hepatotoxicity of CCl₄. Experiments were designed to examine the effect of O₂ tensions present in the liver and the effect of GSH on CCl₄ metabolism and the injurious processes of lipid peroxidation and covalent binding. The effect of hyperbaric O₂ in vivo was also assessed as a potential treatment for CCl₄ ingestion.

**Methods**

**Animals.** Male Sprague-Dawley strain rats were purchased from Timco Laboratories, Houston, TX. They were given food and water ad libitum and were housed in rooms with alternating 12-h light and dark cycles. Rats used in the experiments depicted in Figs. 3 and 4 were fed standard laboratory rations and rats from which microsomes were prepared (Figs. 1 and 2) were fed a nutritionally adequate semisynthetic diet (12). Experiments were begun between 8 and 10 a.m.

Rats were killed by cervical dislocation and exsanguination. Hepatic microsomes were prepared as described before (13) and were used for experiment on the day of preparation.

**Microsomal incubations.** The microsomal incubations were carried out in a volume of 5 ml in 25-ml Erlenmeyer flasks that were sealed with rubber septums. They were shaken in a water bath at 37°C, and the buffer used (microsomal incubation buffer) was 50 mM Tris-HCl, pH 7.5, with 140 mM NaCl and 50 mM EDTA. When used, GSH was added to a concentration of 1 mM.

The incubations reported in Figs. 1 and 2 were carried out under O₂ or N₂ mixtures. O₂ concentration is indicated on the figures. The gas mixtures were made with a three-tube flowmeter (Matheson Gas Products, Newark, NJ) and solutions and buffers used were equilibrated by bubbling the buffer and solutions to be added with the gas mixture for 15 min. Then the flask that contained the incubation mixture without CCl₄ and NADPH was flushed with the gas mixture for 5 min at 37°C before the flask was sealed and CCl₄ (10 μl vol in ethanol) was injected. After a further 5 min equilibration, the incubation was started by injection of NADPH. Flask protein concentrations varied from 1.14 to 1.60 mg/ml. NADPH concentration was 400 μM. After 20 min of incubation, flask atmosphere was sampled for CHCl₃ analysis. Then the sealed flask was chilled on ice for several minutes before it was opened. A sample was taken immediately for assay of thiobarbituric acid-reactive substances when the flask was opened.

For the experiment in Fig. 1, 1 mM CCl₄ was used. For the one in Fig. 2, 72 μM ¹⁴C-labeled CCl₄ (2.8 Ci/mol, sp act) was used. To determine covalent binding to microsomes, 1 ml of 10% trichloroacetic acid was added to each flask after cooling and the microsomes were pelleted by centrifugation. The pellet was washed three times with 5 ml ethyl acetate to remove ¹⁴C-labeled material that was not covalently bound. Then the pellet was digested in a scintillation vial by 0.5 ml Protosol and ¹⁴C was determined after addition of 10 ml of Ready-Solv EP (Beckman Instruments, Palo Alto, CA).

**In vivo studies.** The in vivo studies were carried out using airtight plexiglas metabolism chambers (14). Each chamber contained soda lime to remove carbon dioxide and anhydrous CaSO₄ to remove water vapor. Hyperbaric conditions were maintained by pressurization of the O₂-filled chambers to two atmospheres using compressed 100% O₂. An air atmosphere was maintained by attachment of the chambers, sealed with air inside, to a respirometer that contained 100% O₂. As

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¹. Abbreviations used in this paper: PUFA, polyunsaturated fatty acids; GSH, reduced glutathione.
O₂ was consumed by the rat, it was replaced by O₂ from the respirometer, which maintained the original O₂ concentration. 3 h after the chamber measurements were begun, the chambers were flushed with room air for 3 min and then resealed for a subsequent 3-h experimental period under an air atmosphere. Chamber atmosphere was sampled through a rubber septum with a gas-tight syringe and needle and was injected directly into the gas chromatograph for analysis.

Rats given phorone (2,6-dimethyl-2,5-heptadien-4-one) received 250 mg/kg i.p. 2 h before CCl₄ was given. The phorone was dissolved in corn oil and 2 ml of the mixture per kilogram was injected. Controls received corn oil injections. Liver glutathione content was reduced to 4% of control 2 h after this phorone administration, as determined in a separate experiment (data not shown).

CCl₄ was mixed with an equal volume of mineral oil and 0.5 ml of the mixture was given by stomach tube per 100 g body weight immediately before the rat was put into the metabolism chamber. CHCl₃ in chamber atmosphere was used to determine its production from CCl₄. Because CHCl₃ can be metabolized by rats, it was necessary to determine the extent to which CHCl₃ would influence chamber CHCl₃ levels. Separate experiments were performed (results not shown).

Injection of CHCl₃ into a chamber that contained a rat resulted in rapid disappearance of the CHCl₃ from the chamber atmosphere. CHCl₃ did not disappear, however, when the rat in the chamber was treated with CCl₄ simultaneously. Therefore, CHCl₃ in chamber atmosphere represented production under these conditions. The absence of metabolism was probably due to CCl₄-induced destruction of cytochrome P-450.

**Assays.** Liver glutathione was determined by the recirculating assay of Tietze (13) as modified by Griffith (16). Thiobarbituric acid-reactive substances were measured as described previously (14). Ethane was determined in chamber atmosphere as described previously (14). CHCl₃ was measured using a Porasil C (80:100) column in a Hewlett-Packard 5880A gas chromatograph with a flame-ionization detector. Oven temperature was 80°C. Chamber atmosphere (1 ml) was injected directly into the instrument. CHCl₃ in flask atmosphere was measured by its direct injection into the gas chromatograph. Standard curves for the chamber studies were made by dilution of CHCl₃ in gas glass vessels. Standard curves for the microsomal experiments were made by injecting CHCl₃ into 25-ml Erlenmeyer flasks that contained 5 ml of buffer.

**Materials.** CCl₄ and phorone were purchased from Aldrich Chemical Co., Milwaukee, WI and CHCl₃ from American Scientific Products, Houston, TX; Protosol and ¹⁴C-CCl₄ were supplied by New England Nuclear, Boston, MA; NADPH was purchased from Boehringer-Mannheim Biochemicals, Indianapolis, IN; GSH, disodium EDTA, and Tris were from Sigma Chemical Co., St. Louis, MO.

**Statistics.** Statistical significance was determined with *t* test.

### Results

The O₂ tension in the rat liver varies from the equivalent of 7% of an atmosphere of O₂ to almost 0% (18). The regions around the central veins have the lowest O₂ tensions. For this study of CCl₄ metabolism by liver microsomes, we chose three different O₂ concentrations in the range found in the liver, as well as 0 and 21% O₂ (Figs. 1 and 2).

Figure 1A demonstrates the effect of O₂ tension on CHCl₃ formation by microsomes. As O₂ tension increased, CHCl₃ formation decreased. Under 21% O₂, no CHCl₃ production was detected. Because CHCl₃ is derived from CCl₄, these results indicate that increasing the O₂ tension decreases CCl₄ concentration. GSH had no detectable effect on CHCl₃ formation. These results support our earlier suggestion that GSH has little or no effect on CCl₄ metabolism (11).

Fig. 1B shows the effect of O₂ and GSH on CCl₄-induced lipid peroxidation. The greatest amount occurred under 1% O₂. Further increments in O₂ were associated with decrements in lipid peroxidation. No significant lipid peroxidation occurred under anaerobic conditions because the process is O₂-dependent. CHCl₃ production (Fig. 1A) and lipid peroxidation (Fig. 1B) were both diminished by increases in O₂ tension above 1%,

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**Figure 1.** Effect of O₂ concentration and GSH on CHCl₃ production from CCl₄ (A) and CCl₄-induced lipid peroxidation (B) in rat liver microsomes. Open bars indicate that no GSH was present; shaded bars indicate that 1 mM GSH was present. Incubations were carried out as described in Methods. Values are mean±1 SD (*n* = 4 animals).

**Figure 2.** Effect of O₂ concentration and GSH on covalent binding of ¹⁴C-derived from ¹³C-labeled CCl₄ Open bars indicate that no GSH was present; shaded bars indicate that 1 mM GSH was present. Incubations were carried out as described in Methods. Values are mean±1 SD (*n* = 4 animals).
but the diminution in CHCl₃ production was the greater. At 21% O₂, no CHCl₃ production could be detected, yet significant lipid peroxidation occurred.

GSH protected against lipid peroxidation but did so best at the higher O₂ tensions. Lipid peroxidation was inhibited 83–88% by GSH under 3–21% O₂, but under 1% O₂ the inhibition was only 50%. This result is consistent with the hypothesis that the GSH-dependent protection is effective against CCl₃0’0 but not against CCl₁.

Fig. 2 demonstrates that covalent binding decreases as O₂ tension increases. It, like lipid peroxidation, did not fall as profoundly as did CHCl₃ production, particularly at the higher O₂ concentrations. This is illustrated in Fig. 3, which shows that the respective decreases in CHCl₃ production and in covalent binding were the same when O₂ tension rose from 0 to 1%. Successive increases in O₂ tension led to progressively more profound drops in CHCl₃ production without comparable drops in covalent binding. As O₂ tension rose from 5 to 21%, CHCl₃ production ceased completely, but covalent binding fell only by 33%. GSH inhibited covalent binding under 3–21% O₂ but had no effect on it at 0 or 1% O₂ (Fig. 2). These results are consistent with the GSH-dependent protection, being effective against CCl₃0’0 but not against CCl₁.

Based on these results, raising hepatic O₂ tensions could be expected to lessen CCl₄ injury. The higher O₂ tension would inhibit the formation of CCl₁ by the cytochrome P-450 system and promote the conversion of CCl₁ to CCl₄, which can be detoxified by a GSH-dependent mechanism. If this supposition is correct, raising hepatic O₂ tension with hyperbaric O₂ might constitute effective treatment for CCl₄ poisoning.

Using metabolic chambers, we studied the effect of hyperbaric O₂ on CHCl₃ production from CCl₄ in rats, and also measured ethane production as an index of lipid peroxidation. The effect of GSH depletion was examined as well. The hyperbaric O₂ sharply depressed CHCl₃ production by the rats. GSH depletion had no statistically significant effect on CHCl₃ production, although it appeared to augment the hyperbaric O₂ effect at 2 and 3 h. The inhibition was relieved when hyperbaric O₂ was replaced by air after 3 h. All rats produced the same amount of CHCl₃.

![Figure 3. Comparison of the O₂ effect on CHCl₃ production and covalent binding. Values used here were calculated from the data presented in Figs. 1 A and 2 without GSH addition. These values are ratios and indicate the effect of the indicated increment in O₂ concentration on CHCl₃ production (solid bars) and on covalent binding (shaded bars).](image)

Figure 3.

![Figure 4. Effect of hyperbaric O₂ and GSH depletion on CCl₄ metabolism to CHCl₃ in the rat. The experiment was carried out as described in Methods. All rats received 2.5 ml CCl₄/kg at zero time and were put into the metabolism chambers. Treatments were as follows: •, air atmosphere; ○, hyperbaric O₂ atmosphere from 0–3 h; □, air atmosphere and depleted of GSH; △, hyperbaric O₂ atmosphere from 0–3 h and depleted of GSH. All rats were in a normobaric air atmosphere from 3 to 6 h. Values shown are averages of four rats. Brackets indicate 1 SD.](image)

Figure 4.

Figure 5 shows that hyperbaric O₂ sharply depressed ethane production after CCl₄ administration, which indicated that it prevented CCl₄-induced lipid peroxidation. GSH depletion had a striking effect on lipid peroxidation. It doubled ethane production by animals breathing air. The ethane production by animals in hyperbaric O₂ was so low, however, that no effect of GSH depletion could be seen. The hyperbaric O₂ inhibition of ethane production was not reversed when the animals were returned to an air atmosphere (Fig. 5). These in vivo experiments lend support to the hypothesis that O₂ and GSH are protective against the hepatotoxicity of CCl₄.

**Discussion**

Fig. 6 is a proposed scheme of the early free radical portion of CCl₄ metabolism. It serves as a basis for discussing the present results. The conversion of CCl₄ to CCl₁ is catalyzed by cytochrome P-450 (19). O₂ inhibits this reaction. Once CCl₁ has been formed, it reacts very rapidly with O₂ to give CCl₁0’0 a much more reactive radical than CCl₁ (6, 7). If little or no O₂ is present, CCl₁ can bind covalently to lipids and proteins or abstract an H⁺ usually from a PUFA. The PUFA‘
Figure 5. Effect of hyperbaric O₂ and GSH depletion on lipid peroxidation. These data are from the same experiment shown in Fig. 3 and the symbols used are the same.

Figure 6. Scheme to explain O₂ and GSH effects on CCl₄ metabolism by rat liver microsomes.

GSH can then react with other free radicals, which leads to polymerization, or, if any O₂ is present, PUFA - can undergo lipid peroxidation.

CCl₃00' is very reactive and is a good initiator of lipid peroxidation (7). It, or compounds derived from it, can bind covalently also (8). Our earlier work indicated that GSH can affect the metabolism of CCl₃00', preventing membrane damage by it, but that GSH could not protect against CCl₁ (11). Those conclusions are supported by the experiments shown in Figs. 1 and 2. GSH was more efficient in blocking CCl₄-induced lipid peroxidation and covalent binding at the higher O₂ tensions, where CCl₃00' formation would be favored, than at the lower ones, where CCl₁ concentration would be higher.

Mico and Pohl (8) have recently presented evidence that further reactions of CCl₃00' yield an electrophilic chlorine and phosgene, both of which are potentially toxic metabolites. The present studies were undertaken before that report and did not measure production of these metabolites. Consequently, determination of the role of GSH in their formation and detoxification will require further studies.

This scheme (Fig. 6) can potentially be used to understand the pattern of liver injury by CCl₄. In the well-oxygenated periportal areas, cytochrome P-450 produces a small amount of CCl₁, which is rapidly converted to CCl₃00'. CCl₁00' is very reactive and may destroy the activating cytochrome P-450 by direct attack or by damage to the adjacent membrane (11, 19, 20). GSH protects the rest of the cell against CCl₁00'.

In the poorly oxygenated centrilobular regions of the liver, CCl₁ is produced in large quantities by cytochrome P-450. Because O₂ tension is low, little CCl₁ is converted to CCl₃00'. CCl₁, being less reactive than CCl₃00', can diffuse farther before reacting and therefore should not destroy the cytochrome P-450 which activates CCl₄ to the extent that CCl₃00' does. Thus, there is much greater metabolism of CCl₄ under these conditions (11). CCl₁ can bind covalently and can cause lipid peroxidation (if some O₂ is present). GSH does not appear to inhibit these events. Because there is an O₂ gradient between the periportal and the centrilobular regions, some areas will have O₂ tensions in which both CCl₁ and CCl₃00' are present. Since the O₂ tension is relatively low, CCl₁ may diffuse away from the activating cytochrome P-450 before reacting with O₂, to produce the highly reactive CCl₃00'. This may protect the activating cytochrome P-450 and allow more CCl₄ metabolism. In those areas, perhaps analogous to the 1 and 3% O₂ in Figs. 1 and 2, GSH would be expected to exhibit significant protection. This may explain the protective effect of GSH against lipid peroxidation that was observed in air-breathing animals (Fig. 5).

The entire hepatic lobule would be well oxygenated under hyperbaric O₂. GSH protects the membrane remote from the activating cytochrome P-450 species from CCl₃00', but under well-oxygenated conditions it should have a small effect because of the small CCl₃00' production. This is a potential explanation for the failure of GSH depletion to cause increased ethane production in CCl₄-treated rats under hyperbaric O₂ (Fig. 4). Also contributing to the lowering of ethane production by hyperbaric O₂ could be reaction of O₂ with the ethyl radical, as suggested by Cohen (21).

This scheme (Fig. 6) can explain why hypoxic conditions are associated with increased lipid peroxidation and hepatotoxicity from CCl₄ (22, 23). Likewise, it provides the rationale for the use of hyperbaric O₂ in the treatment of CCl₄ poisoning. European workers have shown that prolonged treatment with hyperbaric O₂ protects rats against CCl₄-induced liver damage (24, 25), and there have been case reports of its efficacy in human CCl₄ poisoning (26). Hyperbaric O₂ should inhibit the metabolism of CCl₄ to free radicals and promote the formation of CCl₁00' in all parts of the liver lobule. That should result in decreased lipid peroxidation and covalent binding, but still allow destruction of the activating cytochrome P-450 with cessation of CCl₄ metabolism (11). Further studies are needed to determine the significance of CCl₄ metabolism to CHCl₃, which takes place under normoxic conditions even after a period of hyperbaric O₂ exposure (Fig. 4).

Hyperbaric O₂ would appear to be a logical treatment for CCl₄ poisoning. Available data (24-26) indicate it should be instituted as soon after ingestion as possible and that exposure should be maintained for as long as possible. This will allow
excretion of CCl₄ while inhibiting its conversion to injurious free radical intermediates. Conversely, hypoxia should be avoided.

These findings may have relevance to the hepatotoxicity of other agents. Halothane metabolism has recently been shown to yield a peroxy radical under aerobic conditions (7), and the GSH-dependent microsomal free radical scavenging mechanism might protect against it as well as against CCl₄0².

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References


