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MECHANISM OF METABOLIC ACTIVATION OF CHLOROFORM BY RAT LIVER MICROSOMES

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Abstract—In this investigation, we attempted to determine if reactive metabolites other than phosgene ($COCl_2$) are involved in the metabolic activation of chloroform ($CHCl_3$) in rat liver microsomes. This problem was approached by determining whether the formation of $COCl_2$ can account for the metabolism of $CHCl_3$ to covalently bound product, carbon dioxide (CO_2), and chloride ion (Cl^-). It was found that the levels of covalent binding of [^{14}C]$CHCl_3$ and [^{14}C]CO_2 formation decreased proportionately when [^{14}C]$COCl_2$ was trapped as 2-oxothiazolidine-4-carboxylic acid by the addition of cysteine to the incubation mixture. The amount of this product corresponded closely to the sum of the decreases in covalent binding and CO_2 formation. [^{36}Cl]Chloride was formed from [^{36}Cl]$CHCl_3$ under the same conditions that produced $COCl_2$ from $CHCl_3$. In addition, when ^{14}C-, ^{3}H-, or ^{36}Cl-labelled $CHCl_3$ was incubated with liver microsomes under a variety of conditions, only the ^{14}C-label was appreciably bound irreversibly to microsomal protein. These results support the view that $COCl_2$ is the major, if not the only, reactive metabolite formed from $CHCl_3$ in rat liver microsomes.

In 1847, chloroform ($CHCl_3$) was introduced clinically as a general anesthetic [1]. Its potential hepatic, renal, and cardio-toxic properties, however, led to a decline in its use in human medicine; in 1912, the Committee on Anesthesia of the American Medical Association condemned its use for major surgery.

Chloroform is still used widely as an industrial solvent and chemical intermediate. Until recently it was also employed as a preservative and flavor enhancer in pharmaceutical products such as cough medicines, mouth washes, and dentifrices. In 1976, these uses of $CHCl_3$ were banned by the Food and Drug Administration following the finding of the National Cancer Institute that $CHCl_3$ induced liver cancer in mice and renal tumours in male rats [2]. The potential carcinogenic activity of $CHCl_3$ [2, 3] has led to further concern since $CHCl_3$ has been found as a contaminant in drinking water purified by chlorination [4].

The results of several investigations suggest that a reactive metabolite of $CHCl_3$ is responsible for its hepatotoxicity and possibly its carcinogenicity and renal toxicity [5]. For example, when mice are treated with [^{14}C]$CHCl_3$, the extent of hepatic necrosis parallels the amount of ^{14}C-label bound irreversibly to liver protein [6]. In addition, both hepatic necrosis and binding are potentiated by pretreatment of animals with phenobarbital, a known inducer of liver microsomal metabolism, and are inhibited by pretreatment with the inhibitor piperonyl butoxide [6]. The finding that $CHCl_3$ administration significantly decreases the level of liver glutathione in rats pretreated with phenobarbital further suggests that a reactive metabolite is produced [7, 8]. Perhaps the clearest evidence that a metabolite of $CHCl_3$ is responsible for its hepatotoxicity is the observation that deuterium-labelled chloroform ($CDCl_3$) is two to three times less hepatotoxic than $CHCl_3$ [9, 10].

Recently, phosgene ($COCl_2$) was identified as a metabolite when $CHCl_3$ was incubated with rat liver microsomes [11–13] and when it was administered to living rats [14]. The formation of $COCl_2$, like the covalent binding of [^{14}C]$CHCl_3$ to liver microsomes, is oxygen dependent and appears to be catalyzed by a phenobarbital inducible form of cytochrome P-450 [6, 9, 10, 12, 15, 16]. Moreover, deuterium-labelled chloroform ($CDCl_3$) is metabolized to $COCl_2$ by liver microsomes [9] and in living rats [14] at approximately half the rate of $CHCl_3$. Thus, it seems likely that $COCl_2$ is responsible, at least in part, for the hepatotoxicity produced by $CHCl_3$.

In this investigation, we have attempted to determine if reactive metabolites other than $COCl_2$ are involved in the metabolic activation of $CHCl_3$ in rat liver microsomes. This problem has been approached by determining whether the formation of $COCl_2$ can account for the metabolism of $CHCl_3$ to covalently bound product, carbon dioxide (CO_2), and chloride ion (Cl^-).

MATERIALS AND METHODS

Materials

[^{3}H]Water (1.81 mCi/mmole), [^{14}C]chloroform (4.55 mCi/mmole) and [^{36}Cl]chloroform (0.290 mCi/mmole) were purchased from the New England Nuclear Corp., Boston, MA. [^{36}Cl]Sodium chloride solution (1.06 μCi/ml) and [^{14}C]sodium carbonate solution (60 mCi/mmole, 5.0 mCi/ml) were obtained from the Amersham/Searle Co., Arlington Heights,

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IL. NADP, NADH, and glucose-6-phosphate were purchased from Sigma Chemical Co., St. Louis, MO. Glucose-6-phosphate dehydrogenase was obtained from CalBiochem-Behring, La Jolla, CA. Silver nitrate was purchased from J. T. Baker, Phillipsburg, NJ. A scintillation mixture, consisting of 0.4% BBOT [2,5-bis-(5-*tert*-butyl-benzoxazoyl) thiophene], 0.8% naphthalene, and 40% 2-methoxyethanol in toluene was purchased from Yorktown Research, Hackensack, NJ.

Methods

Preparation of [^{3}H]$CHCl_3$. Chloroform (1 ml), 10 N sodium hydroxide (40 μl) and [^{3}H]H_2O (1 ml, 100 mCi, 1.81 mCi/mmole) were placed in a sealed 3-ml reaction vial and mixed at room temperature in the dark under an atmosphere of nitrogen. After 23 hr, the reaction mixture was acidified with concentrated hydrochloric acid (40 μl). The upper acidic aqueous layer was separated from the lower $CHCl_3$ layer, which was then washed with a solution of saturated sodium chloride (2 ml, ten times) to remove residual [^{3}H]H_2O. The washed $CHCl_3$ was dried over sodium sulphate and then distilled in a micro-distillation apparatus to yield [^{3}H]$CHCl_3$, 0.35 ml, 0.76 mCi/mmole.

Preparation of microsomes. Male Sprague–Dawley rats (180–200 g) were obtained from Hormone Assay Laboratories, Chicago IL. The animals were allowed free access to water and food (Purina Lab Rat Chow) and were pretreated with phenobarbital (80 mg/kg, in saline, i.p.) 72, 48, and 24 hr before the experiment. At least three rats were employed in each experiment. The animals were decapitated and their livers were homogenized in 3 vol. of 0.02 M Tris–1.15% KCl buffer (pH 7.4). The homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant fraction was recentrifuged for 60 min at 100,000 *g*. The resultant microsomal pellet was resuspended in 0.02 M Tris–KCl buffer and recentrifuged at 100,000 *g* for 60 min. The washed microsomal pellet was resuspended in 0.02 M Tris–KCl buffer to a concentration of microsomal protein of 1.1 mg/ml. Protein concentration was determined by the method of Lowry *et al.* [17].

Incubation mixtures. Microsomal incubation mixtures in a total volume of 1 ml contained 1 mg microsomal protein, 0.10 mM NADH, 0.20 mM NADP, 2.00 mM glucose-6-phosphate, 2.0 mM magnesium chloride, 1 unit glucose-6-phosphate dehydrogenase, 20 mM Tris buffer (pH 7.4), 153 mM KCl, 1.0 mM or 3.34 mM radiolabeled $CHCl_3$ (added in 10 μl of dimethylformamide), and in some cases other reagents as specified below. The incubations were conducted in a sealed vial (rubber septum) at 37° for 10 min under an atmosphere of air unless otherwise indicated. Prior to each experiment, the radiolabeled $CHCl_3$ derivatives were purified by preparative gas chromatography employing a Perkin–Elmer 900 gas chromatograph that was equipped with a glass column (6 ft × 4 mm i.d.), packed with Porapak Q, 100/120 mesh. The injector, column, and detector temperatures were 200, 140, and 240° respectively. Nitrogen was the carrier gas (approximately 20 ml/min) and, under these conditions, $CHCl_3$ had a retention time of 10 min.

Measurements of [^{14}C]CO_2, *covalent binding of* [^{14}C]$CHCl_3$ *to microsomal protein, and trapped* $COCl_2$ *as 2-oxothiazolidine-4-carboxylic acid.* [^{14}C]$CHCl_3$ (1.0 mM, 1 mCi/mmole) was incubated with microsomes and cofactors (as described above) in the presence or absence of cysteine (0.5 mM). Each reaction flask also contained a CO_2 trap consisting of a removable plastic well that contained sodium hydroxide (10 μl of an 8% solution absorbed onto filter paper, 1 cm square). After 10 min of incubation the reactions were stopped by the addition of 1 N hydrochloric acid (300 μl) through the rubber septum. The acidic reaction mixtures were allowed to stand at room temperature for 3 hr to insure complete absorption of [^{14}C]CO_2 into the sodium hydroxide traps. The rubber septa were then removed and the amounts of [^{14}C]CO_2, covalently bound product to protein, and trapped $COCl_2$ were measured as follows.

(*a*) [^{14}C]CO_2. The CO_2 traps were transferred to centrifuge tubes, and 1 ml of water was added. The resulting alkaline aqueous solutions were extracted with ethyl ether (2 ml, five times), and the trapped CO_2 was then determined by counting an aliquot (0.5 ml) of the washed alkaline solutions. The specificity of the assay for CO_2 was tested by counting another aliquot of washed alkaline solution after it was made acidic with 3 N hydrochloric acid. More than 90 per cent of the initial radioactivity was lost after acidification, confirming that most of the radioactivity in the traps was in the form of [^{14}C]sodium carbonate. The efficiency of recovery of [^{14}C]CO_2 from the incubations was determined to be at least 73 per cent by conducting incubations with [^{14}C]sodium carbonate (7.0×10^4dpm) in place of [^{14}C]$CHCl_3$.

(*b*) *2-Oxothiazolidine-4-carboxylic acid.* After removal of the CO_2 traps from the reaction vessels, methanol (4 ml) was added to precipitate microsomal protein. After centrifugation, the methanolic aqueous solutions were evaporated to dryness by removing the methanol under nitrogen and the water by lyophilization. The residue was dissolved in water (200 μl), followed by the addition of saturated sodium bicarbonate (10 μl). An aliquot (100 μl) of the resulting solution was then analyzed for [^{14}C]$COCl_2$ as [^{14}C]-2-oxothiazolidine-4-carboxylic acid as described previously [9].

(*c*) *Covalent binding of* ^{14}C*-label to microsomal protein.* The microsomal protein pellets recovered after centrifugation of the methanolic reaction mixture were washed with methanol–ether (3:1, 5 ml, five times). After aspiration of the final wash, which contained virtually no radioactivity, the protein was dissolved in 1 N sodium hydroxide (1 ml). The covalently bound radioactivity was determined by counting an aliquot (0.5 ml) of the alkaline solution [18]. Protein was determined by the method of Lowry *et al.* [17].

Control reactions were performed with heat-denatured microsomes. The amounts of [^{14}C]CO_2, [^{14}C]$CHCl_3$ covalent binding to protein, and [^{14}C]-2-oxothiazolidine-4-carboxylic acid formed in these reactions were subtracted from the results to obtain corrected values.

[^{36}Cl]*Chloride ion determination.* [^{36}Cl]Chloro-

form (3.45 mM, 0.290 mCi/mmole) was incubated for 10 min with microsomes and cofactors as described above. The reactions were stopped by the addition of 10% trichloroacetic acid (1 ml). Precipitated protein was removed by centrifugation and the supernatant fractions were transferred to new tubes. The microsomal pellets were resuspended with 5% trichloroacetic acid (1 ml) and centrifuged; the supernatant fractions were combined with the 10% trichloroacetic acid extracts. Silver nitrate solution (3 M, 1 ml) was added to the combined trichloroacetic acid extracts and a precipitate of silver chloride was formed immediatley. The precipitate was washed with methanol (5 ml, three times) to remove residual ^{36}Cl-labeled organic material. The third methanol wash contained virtually no radioactivity. The washed silver chloride was mixed with concentrated ammonium hydroxide (2 ml), and a 0.5-ml aliquot was then counted. Control reactions were performed with heat-denatured microsomes; the amount of Cl^- released from these reactions was subtracted from the Cl^- results to obtain corrected values. The efficiency of Cl^- recovery from the incubations was determined to be at least 95 per cent by conducting incubations with [^{36}Cl]sodium chloride (2.35×10^4 dpm per incubation) in place of [^{36}Cl]$CHCl_3$.

Covalent binding of [^{14}C]-, [^3H]- *and* [^{36}Cl]$CHCl_3$ *to microsomal protein determination.* [^{14}C]-, [^3H]- or [^{36}Cl]Chloroform (3.45 mM, diluted with nonradioactive $CHCl_3$ to 0.290 mCi/mmole) was incubated for 10 min with cofactors as described above. The reactions were stopped by the addition of 10% trichloroacetic acid (1 ml). The reaction mixtures were centrifuged and the resulting pellets (precipitated protein) were washed with 5% trichloroacetic acid (1 ml, one time) and methanol–ether (3 : 1, 5 ml, five times). After aspiration of the final wash, which contained virtually no radioactivity, the amount of covalently bound radiolabel was determined as described above.

RESULTS

Effect of trapping $COCl_2$ *on irreversible binding to microsomal protein and* CO_2 *formation from* [^{14}C]$CHCl_3$

[^{14}C]Chloroform was converted to nearly equal amounts of covalently bound product and [^{14}C]CO_2 when incubated with liver microsomes from phenobarbital-pretreated rats (Fig. 1). The levels of both

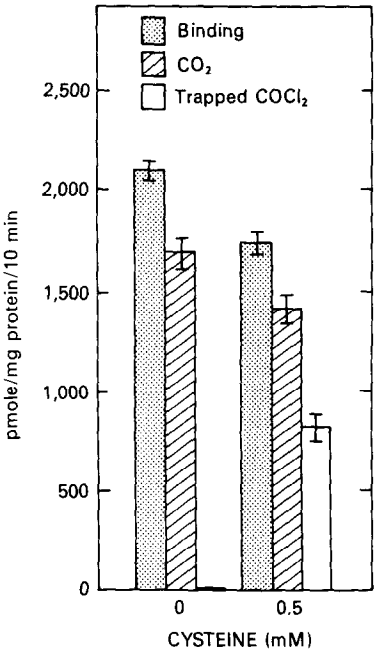

Fig. 1. Effect of trapping $COCl_2$ as 2-oxothiazolidine-4-carboxylic acid on the metabolism of [^{14}C]$CHCl_3$ to covalently bound product (microsomal protein) and CO_2 in liver microsomes from phenobarbital-pretreated rats.

covalent binding and CO_2 formation decreased proportionately when 0.5 mM cysteine was included in the incubation mixture. The total amount of these decreases corresponded quite closely to the amount of [^{14}C]$COCl_2$ trapped as [^{14}C]-2-oxothiazolidine-4-carboxylic acid.

Effect of incubation conditions on release of Cl^- *from* [^{36}C]$CHCl_3$

Larger amounts of ^{36}Cl-labelled Cl^- were released from [^{36}Cl]$CHCl_3$ when the incubations were conducted in air rather than in nitrogen (Table 1). The amount of ^{36}Cl-labeled Cl^- formed was signficantly decreased when the incubations were performed in the absence of NADPH or in the presence of $CO : O_2$ or SKF 525-A (Table 1).

Effect of incubation conditions on binding of ^{14}C-^3H- and ^{36}Cl-*labelled* $CHCl_3$ *to microsomal protein*

When ^{14}C-labelled $CHCl_3$ was incubated in an atmosphere of air with liver microsomes from phenobarbital-pretreated rats, maximum amounts of cov-

Table 1. Effects of various incubation conditions on formation of [^{36}Cl]Cl^- from [^{36}Cl]$CHCl_3$ in liver microsomes of phenobarbital-pretreated rats*

Incubation conditions	[^{36}Cl]Cl^- formed† [nmoles·(mg protein)$^{-1}$·10 min^{-1}]
Complete system in air	43.9 ± 2.8
+N_2	11.0 ± 0.2
+$CO : O_2$ (8:2)	3.3 ± 0.4
−NADPH	3.2 ± 0.6
+SKF 525-A (1 mM)	3.5 ± 0.3

* Chloride ion was measured as outlined in Materials and Methods with alterations of atmospheres, or deletions and additions, as noted.

† Each result is the mean ± S.E. of three incubations.

alent binding of the ^{14}C-label to microsomal protein were observed (Table 2). The amount of bound ^{14}C-label was significantly decreased when the reactions were conducted in an atmosphere of N_2 or $CO:O_2$ or in the absence of NADPH or the presence of SKF 525-A. In contrast to these results, negligible amounts of the radiolabels of [^3H]- or [^{36}Cl]$CHCl_3$ were bound covalently to microsomal protein under any of the incubation conditions.

DISCUSSION

It was proposed previously [9–14] that $CHCl_3$ is metabolically activated through an oxidative dechlorination mechanism by cytochrome P-450 in liver microsomes (Fig. 2). This mechanism explains (1) how $CHCl_3$ is metabolized to chloride ion and carbon dioxide in liver *in vitro* [19–23] and *in vivo* [22, 24–26], (2) how [^{14}C]$CHCl_3$ binds covalently to liver protein *in vitro* [6, 7, 15, 16] and *in vivo* [6, 16, 27], (3) how $CHCl_3$ can deplete liver glutathione [7, 8, 28], and (4) possibly how it is bioactivated into a hepatotoxic metabolite.

The following facts support the mechanism outlined in Fig. 2. First, the hydroxylation of aliphatic C–H bonds has been observed with other compounds [29]. If this reaction occured with $CHCl_3$, the product, trichloromethanol (Cl_3C–OH), would spontaneously dehydrochlorinate to $COCl_2$. This prediction is based upon the fact that trifluoromethanol (F_3C–OH), the only trihalomethanol derivative that has been synthesized, spontaneously dehydrofluorinates to carbonyl fluoride (COF_2) at temperatures above −20° [30, 31]. Second, phosgene has been identified as a metabolite of $CHCl_3$ in microsomes *in vitro* [11–13] and *in vivo* [14]. Third, phosgene reacts with various nucleophiles. For example, it reacts with water to produce carbon dioxide and chloride ion [32], with protein *in vitro* [33] and *in vivo* [34], and with glutathione*. Fourth, most importantly, several correlations exist between the formation of $COCl_2$ and other metabolic processes, as would be expected from the pathways outlined in Fig. 2. For instance, when $COCl_2$ is trapped with cysteine as 2-oxothiazolidine-4-carboxylic acid in liver microsomes, proportionate decreases occur in both covalent binding of the metabolite to protein and the formation of CO_2 [Fig. 1]. In addition, the metabolism of $CHCl_3$ to $COCl_2$ [12], Cl^- (Table 1), and covalently bound product in liver microsomes [6, 15, 16; Table 2] are all oxygen-dependent processes.

Cytochrome P-450 apparently mediates all of these reactions since the formation of $COCl_2$ [9] and Cl^- (Table 1) and the covalent binding of [^{14}C]$CHCl_3$ to microsomal protein [6, 15, 16; Table 2] *in vitro* are inhibited by SKF 525-A and an atmosphere of $CO:O_2$ but require NADPH and O_2 [35]. It also appears that a phenobarbital inducible form of cytochrome P-450 is involved in the formation of $COCl_2$ *in vitro* [10], in the depletion of liver glutathione *in vivo* [8], and in the hepatotoxicity produced by $CHCl_3$ [10]. Moreover, the rate-determining step for the formulation of $COCl_2$ *in vitro* [9] and *in vivo* [14], for the depletion of liver glutathione *in vivo* [8], and for the hepatotoxicity produced by $CHCl_3$ [9, 10] is cleavage of the C–H bond of $CHCl_3$.

There are at least three additional mechanisms that can be proposed for the metabolic activation of $CHCl_3$ by liver microsomes. The process outlined in Fig. 2 involves a direct insertion of activated oxygen across the C–H bond of $CHCl_3$. This proposal is supported by a number of observations with other compounds in which the hydroxylation of aliphatic C–H bonds occurs with retention of configuration [29]. The results of a recent paper, however, show that a significant amount of epimerization occurs during the hydroxylation of a C–H bond of norbornane *in vitro* [36]. This finding suggests that cytochrome P-450 catalyzes this reaction by an initial hydrogen abstraction to give a carbon radical intermediate. If this process occurred with $CHCl_3$, then the initial step in the hydroxylation would involve the formation of trichloromethyl radical ($Cl_3C\cdot$), which is believed to be the major reactive metabolite of CCl_4 [34, 37, 38]. This intermediate appears to bind irreversibly to protein and lipid [34, 37, 38], to abstract hydrogen atoms from lipids and other potential sources of hydrogen atoms such as thiols

* L. R. Pohl, unpublished results.

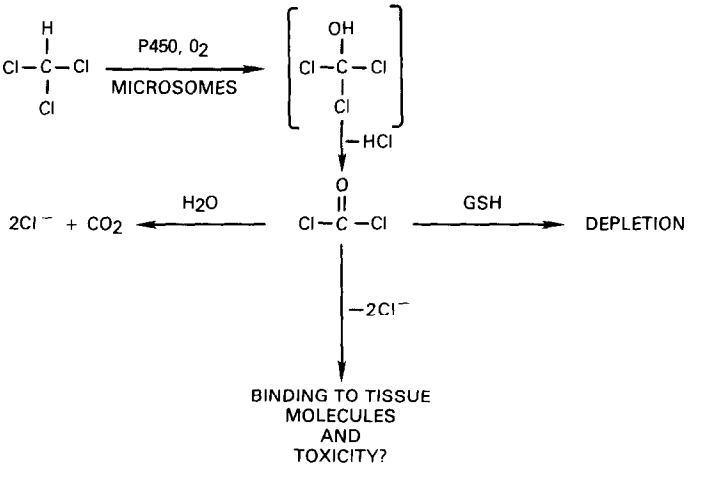

Fig. 2. Metabolic pathways that may explain the metabolism and hepatotoxicity of $CHCl_3$.

Table 2. Effects of various incubation conditions on covalent binding of ^{14}C-, ^{3}H-, or ^{36}Cl-labelled $CHCl_3$ to protein from liver microsomes of phenobarbital-pretreated rats*

Incubation conditions	Covalent binding† [nmoles·(mg protein)$^{-1}$·10 min^{-1}]		
	[^{14}C]$CHCl_3$	[^{3}H]$CHCl_3$	[^{36}C]$CHCl_3$
Complete system in air	5.5 ± 0.6	<0.1	<0.1
+N_2	1.9 ± 0.5	<0.1	<0.1
+CO:O_2 (8:2)	0.3 ± 0.1	<0.1	<0.1
−NADPH	<0.1	<0.1	<0.1
+SKF 525-A (1 mM)	<0.1	<0.1	<0.1

* Covalent binding of ^{14}C-, ^{3}H-, or ^{36}Cl-labeled $CHCl_3$ to microsomal protein was measured as outlined in Materials and Methods with alterations of atmospheres, or deletions and additions, as noted.

† Each result is the mean ± S.E. of three incubations.

to form $CHCl_3$ [22, 23, 37], or to react with oxygen to form phosgene [39].

Another alternative pathway for the bioactivation of $CHCl_3$ involves an abstraction of a hydrogen ion from $CHCl_3$ to produce trichloromethyl carbanion ($Cl_3C:^-$) as an initial intermediate. This intermediate could be hydroxylated to produce $COCl_2$, abstract a hydrogen ion to form $CHCl_3$, or eliminate chloride ion to form the reactive electrophile dichloromethyl carbene ($Cl_2C:$) which is known to add to double bonds or insert between C–H bonds [40]. The reductive dechlorination activation of $CHCl_3$ to dichloromethyl radical ($Cl_2HC\cdot$) is a third potential pathway for the metabolic activation of $CHCl_3$. This radical intermediate would be expected to undergo reactions similar to $CCl_3\cdot$ and therefore bind irreversibly to tissue molecules or abstract hydrogen atoms from lipids and other potential sources of hydrogen atoms to form dichloromethane (CH_2Cl_2).

The negligible amounts of binding of either the ^{36}Cl-label or the ^{3}H-label of $CHCl_3$ under various incubation conditions (Table 2), however, indicates that $CCl_3\cdot$, $Cl_2C:$, or $Cl_2HC\cdot$ is not a major reactive intermediate of $CHCl_3$. This conclusion is supported by the previous inability to detect CH_2Cl_2 as a metabolite of $CHCl_3$ *in vitro* [23] and *in vivo* [26, 27] and by the observation that the deuterium of $CDCl_3$ did not exchange to form $CHCl_3$ when $CDCl_3$ was administered to mice [41].

The results of the present investigation, therefore, indicate that $COCl_2$ is the major if not the only reactive metabolite of $CHCl_3$ in rat liver microsomes. This compound appears to be formed by an initial direct insertion of activated oxygen. Once produced, $COCl_2$ can lead to the formation of CO_2 and Cl^-. In addition, it may react covalently with various tissue molecules such as protein and glutathione [42].

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