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OXIDATIVE METABOLISM OF CARBON DISULFIDE BY ISOLATED RAT HEPATOCYTES AND MICROSOMES*

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Abstract—The oxidative metabolism of carbon disulfide (CS_2) was investigated in isolated rat hepatocytes and liver microsomes. In microsomes, CS_2 metabolism was increased by phenobarbital pretreatment of the rats and decreased with pretreatment of the rats with cobaltous chloride. In both microsomes and hepatocytes, CS_2 metabolism was inhibited by SKF-525A. Carbon dioxide (CO_2) was the major volatile metabolite of CS_2 in hepatocytes, and carbonyl sulfide (COS) was the major volatile metabolite in microsomal incubations. Addition of cytosol to microsomal incubations shifted the predominant volatile metabolite from COS to CO_2 but did not change total volatile metabolite formation. Acetazolamide, a carbonic anhydrase inhibitor, significantly decreased COS metabolism but not CS_2 metabolism in isolated hepatocytes or microsomes fortified with dialyzed cytosol. When $[^{18}O]H_2O$ was included in incubations of microsomes and CS_2, a substantial portion of the resulting COS was $[^{18}O]$ enriched, indicating that the oxygen atom was derived from water. These data are consistent with the hypothesis that (1) CS_2 is oxidized predominantly by the cytochrome P-450 containing monooxygenase system, and (2) the product of this reaction is an unstable intermediate which reacts with water to form monothiocarbonate and reactive sulfur species. Monothiocarbonate is the hydrated form of COS. In intact hepatocytes, it is metabolized predominantly to CO_2 and hydrogen sulfide. Unmetabolized monothiocarbonate can be dehydrated to COS. The majority of the reactive sulfur species and hydrogen sulfide are oxidized to nonvolatile sulfur compounds, including sulfate, but by different mechanisms.

In rats, carbon disulfide (CS_2) is acutely hepatotoxic [1–3]. Hepatotoxicity, however, is observed only in animals treated with phenobarbital prior to CS_2 administration [2, 4]. There is evidence that such toxicity requires metabolic activation by the cytochrome P-450 containing monooxygenase system [3–5], but the relationship between metabolism and toxicity of CS_2 remains obscure. It is also known that the metabolism of CS_2 results in the irreversible inhibition of the cytochrome P-450 containing monooxygenase system [3, 5–9]. As yet, there is no apparent cause and effect relationship between CS_2-mediated inhibition of cytochrome P-450-dependent reactions and hepatocellular damage. The mechanism of CS_2-mediated inhibition of cytochrome P-450-catalyzed reactions has received considerable attention [3, 5–9], but other aspects of CS_2 metabolism have received less scrutiny. In this report, we describe studies of oxidative CS_2 metabolism in isolated rat hepatocytes and hepatic microsomes. The objective of these studies was to further define the mechanisms involved in CS_2 metabolism. In particular, the relationship between CS_2 and carbonyl sulfide (COS) metabolism was examined. COS is of interest because it is a metabolite of CS_2, but is not hepatotoxic. Rather, it causes death by acute respiratory failure [10], an effect which is due to its metabolism first to monothiocarbonate and then to hydrogen sulfide (H_2S) and carbon dioxide (CO_2). In this paper, we report data which suggest that the initial oxidation of CS_2 is catalyzed predominantly by the cytochrome P-450 containing monooxygenase system and that monothiocarbonate, not COS, is the more immediate product of this oxidation step.

METHODS

Animals. Male Sprague–Dawley rats (150–175 g) were purchased from Harlan Industries (Indianapolis, IN). They were housed in hanging wire cages and permitted access to laboratory chow and water *ad lib*. In experiments where rats were treated with phenobarbital (to increase hepatic cytochrome P-450), three doses of 80 mg/kg, i.p., were administered 72, 48 and 24 hr, respectively, prior to killing the animals. In experiments where rats were treated with cobaltous chloride (to decrease hepatic cytochrome P-450), four doses of 30 mg/kg, s.c., were administered 48, 36, 24 and 12 hr, respectively, prior to sacrifice.

Chemicals. Radiolabeled compounds were obtained from the Amersham Corp. (Arlington Heights, IL). The specific activity of CS_2 ($[^{14}C$ or $^{35}S]$) ranged from 0.1 to 0.6 mCi/μmole. The radiochemical purity was specified by the manufacturer to be 99% or greater. Radiolabeled COS was prepared according to the method of Chengelis and Neal [11]. $[^{18}O]H_2O$ was purchased from Schwarz-Mann (Spring Valley, NY). Unlabeled CS_2 was obtained from Matheson, Coleman & Bell (Norwood, OH).

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Carbonyl sulfide was purchased from Matheson Gas (Marrow, GA). $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and collagenase were obtained from the Sigma Chemical Co. (St. Louis, MO). SKF-525A was a gift of the Smith Kline & French Co. (Philadelphia, PA). Aquasol scintillation mixture and NCS tissue solubilizer were obtained from New England Nuclear (Boston, MA) and Amersham respectively. All other reagents were purchased from the Fisher Chemical Co. (Atlanta, GA) and were the best available grade.

Hepatocyte incubations. Hepatocytes were prepared by the method of Zahlten and Stratman [12] and incubated (2–3 mg cell protein/ml) with 0.05–0.2 mM CS_2 in a final volume of 2.0 ml under an atmosphere of 95% O_2–5% CO_2 at 37° in Teflon-sealed 25-ml Erlenmeyer flasks. Cell viability was routinely assessed by Trypan blue exclusion and was not affected by CS_2. In preliminary experiments, isolated cells maintained linear rates of glucose oxidation and urea synthesis for at least 4 hr. The incubation medium was Kreb's carbonate buffer fortified with 1.5% gelatin as described elsewhere [12]. Protein was determined by the Biuret reaction [13]. All incubations were conducted in triplicate.

Microsomal incubations. Hepatic microsomes were prepared by differential centrifugation as described elsewhere [14]. In the experiment summarized in Table 3, microsomes were washed twice by resuspension followed by centrifugation for 30 min at 100,000 *g* prior to incubation with CS_2. Reactions were conducted at 37° in Teflon sealed 25-ml Erlenmeyer flasks containing microsomal protein, 1 to 1.5 mg/ml, 0.6 mM CS_2, an NADPH-generating system (0.15 mM $NADP^+$, 2.5 mM glucose-6-phosphate, 6.5 mM $MgCl_2$, 1.0 I.U./ml glucose-6-phosphate dehydrogenase) and 100 mM potassium phosphate buffer, pH 7.4. In one set of experiments, the supernatant fraction (i.e. the cytosolic fraction) from the 100,000 *g* pellet was saved and dialyzed twice (50:1) against 100 mM potassium phosphate, pH 7.4. This dialyzed cytosol was included in microsomal incubations (experiments detailed in Table 3) to the final concentration of 2 mg cytosolic protein per ml incubation. The final incubation volume was 2.0 ml. All incubations were conducted in triplicate.

Assays of CS_2 metabolites. When $[^{14}C]CS_2$ was the substrate, reactions were terminated by the addition of 0.4 ml of 20% trichloroacetic acid (TCA). $[^{14}C]COS$ and $[^{14}C]CO_2$ present in the headspace were determined by gas radiochromatography as described by Thornsberry [15] and modified by Chengelis and Neal [16]. With $[^{35}S]CS_2$, reactions were terminated by the addition of 2 vol. of ice-cold ethanol. Protein was removed by centrifugation. A 2-ml portion of the resulting supernatant fraction was evaporated to dryness in a vacuum oven. This procedure removed all unmetabolized CS_2. The residue dissolved in 0.6 ml of distilled water. Scintillation mixture (15 ml) was added, and radioactivity was determined by liquid scintillation counting. The resulting radioactivity represented nonvolatile sulfur containing metabolites of CS_2. Because the molecular nature of these metabolites has not been fully established, the results (i.e. "nmoles formed" on Fig. 1, and Tables 1–3) described are as molar CS_2 equivalents.

The time course of CS_2 metabolite(s) formation was examined in both isolated hepatocytes and microsomes. The time points studied ranged from 2.5 to 60 min. In separate experiments the effects of SKF-525A (0.05 mM), an inhibitor of the cytochrome P-450 containing monooxygenase system, and acetazolamide (0.5 to 1.0 mM), an inhibitor of carbonic anhydrase, on CS_2 metabolism were investigated.

Gas chromatography/mass spectrometry. Gas chromatography–mass spectrometry was performed on the COS produced by the microsomal metabolism of CS_2. The final incubation volume was 2.0 ml and contained, in addition to the NADPH-generating system described above, 1.3 mg of microsomal protein, 20 mM sodium phosphate (pH 7.4), and 0.6 mM CS_2. Incubations were conducted for 30 min at 37° under an atmosphere of 95% O_2/5% CO_2 in Teflon-sealed 10-ml Erlenmeyer flasks. $[^{18}O]H_2O$

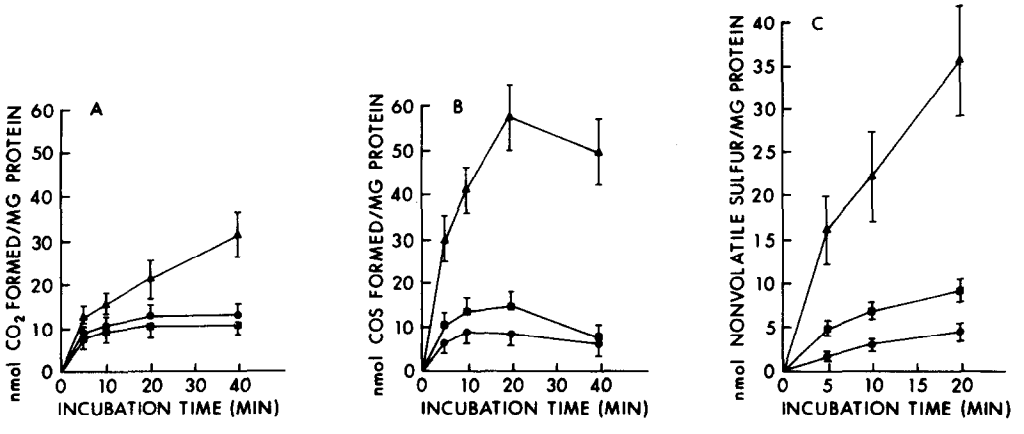

Fig. 1. Carbon disulfide metabolism by rat hepatic microsomes. Animals were untreated (■) or treated with either phenobarbital (▲) (80 mg/kg for 3 days) or cobaltous chloride (●) (30 mg/kg b.i.d. for 2 days), and hepatic microsomes were prepared as described under Methods. Microsomes (1 mg protein/ml) and CS_2 (0.6 mM) were incubated in the presence of an NADPH-generating system at 37°. The results are the means ± SD of three experiments: (A) $[^{14}C]CO_2$ formation from $[^{14}C]CS_2$; (B) $[^{14}C]COS$ formation from $[^{14}C]CS_2$; and (C) nonvolatile $[^{35}S]$sulfur formation from $[^{35}S]CS_2$.

Table 1. Effect of SKF-525A or acetazolamide on the metabolism of [^{14}C]- and [^{35}S]CS_2 by hepatocytes or microsomes isolated from phenobarbital-treated rats

Metabolite	Addition	Metabolite formation (nmoles/mg protein/10 min) Microsomes	Hepatocytes
[^{14}C]CO_2	None	13.1 ± 3.3	16.5 ± 3.8
	SKF-525A	8.9 ± 4.4	10.4 ± 2.8*
	Acetazolamide		12.7 ± 3.7
[^{14}C]COS	None	21.1 ± 6.5	1.76 ± 0.60
	SKF-525A	6.4 ± 3.5*	0.69 ± 0.30
	Acetazolamide		2.50 ± 0.90
Nonvolatile [^{35}S]sulfur	None	22.3 ± 7.2	16.6 ± 4.4
	SKF-525A	12.9 ± 4.2*	9.5 ± 2.8*
	Acetazolamide		13.9 ± 4.1

The incubation conditions are described under Methods. Concentrations were 0.2 mM CS_2 (hepatocytes) or 0.6 mM CS_2 (microsomes) and 0.05 mM SKF-525A or 0.5 mM acetazolamide. Values are the means ± SD of three (hepatocytes) or four (microsomes) separate experiments.

* Significantly different than the control incubation ("None") ($P < 0.05$).

Table 2. Effect of acetazolamide on nonvolatile sulfur formation from [^{35}S]CS_2 and [^{35}S]COS by isolated hepatocytes

Addition	Metabolite formation with [^{35}S]COS (nmoles/mg protein/10 min)	with [^{35}S]CS_2 (nmoles/mg protein/5 min)
None	45.3 ± 11.2	8.2 ± 2.2
1 mM Acetazolamide	10.0 ± 3.2*	7.8 ± 2.2

Hepatocytes isolated from untreated animals were incubated with CS_2 and COS as described under Methods. The final concentrations of [^{35}S]CS_2 and [^{35}S]COS were 0.2 and 0.5 mM respectively. Values are the means ± SD of three separate experiments.

* Significantly different from the incubation not containing acetazolamide ($P < 0.01$).

Table 3. Effect of hepatic cytosol and acetazolamide on the metabolism of [^{14}C]- or [^{35}S]CS_2 by hepatic microsomes isolated from phenobarbital-treated rats

Metabolite	Addition	Metabolite formation (nmoles/mg protein/10 min)
[^{14}C]CO_2	None	8.4 ± 1.1
	Cytosol (C)	31.3 ± 1.5*
	Acetazolamide (A)	4.5 ± 0.5
	A + C	27.3 ± 3.6*
[^{14}C]COS	None	21.6 ± 1.8
	Cytosol (C)	1.2 ± 0.5*
	Acetazolamide (A)	27.6 ± 3.2
	A + C	3.9 ± 0.8*
Total volatile ([^{14}C]CO_2 + COS	None	30.0 ± 2.9
	Cytosol (C)	32.5 ± 2.0
	Acetazolamide (A)	32.1 ± 3.7
	A + C	31.2 ± 4.4
Nonvolatile [^{35}S]sulfur	None	18.8 ± 0.7
	Cytosol (C)	32.2 ± 0.4*
	Acetazolamide (A)	17.9 ± 0.3
	A + C	28.4 ± 0.3*

The microsomal incubation conditions were as described under Methods. Microsomes were isolated from phenobarbital-induced animals and were included in the incubation at a final concentration of 1.5 mg protein/ml. The final concentration of CS_2 was 0.6 mM. Where indicated, dialyzed cytosol was added to a final concentration of 2 mg protein/ml. The final concentration of acetazolamide was 0.5 mM. Values are the means ± SD from four experiments.

* Significantly different than the control ("None") value for this metabolite ($P < 0.05$).

was included in the incubations at a final concentration of 23.3 atoms percent excess. Reactions were terminated by the addition of 0.2 ml of 1 N HCl. An aliquot (25 μl) of the headspace was removed using a gas tight syringe and injected into a Finnigan 3200 Gas Chromatograph/Mass Spectrometer. Gas chromatography was carried out as described by Thornsberry [15]. For electron ionization mass spectroscopy, an ionizing current of 70 eV was used and the column effluent was scanned at 150-msec intervals. Retention times and mass spectral data were analyzed to determine increases at mass peak 62 of COS. The main mass peak for COS is 60, the same as its formula weight.

Statistics. Data presented in Tables 1–3 were analyzed for statistical significance by one-way analysis of variance followed by Newman–Keul's test of multiple comparisons [17].

RESULTS

Pretreatment of rats with cobaltous chloride or phenobarbital caused decreases or increases, respectively, in the extent of CS_2 metabolism, as determined by the formation of [^{14}C]COS and nonvolatile [^{35}S]sulfur compounds, by hepatic microsomes (Fig. 1). The effects on [^{14}C]CO_2 formation were not as marked, but followed the same trend. Cobaltous chloride and phenobarbital treatments resulted in microsomal cytochrome P-450 concentrations of 0.47 and 2.1 nmoles/mg protein, respectively, compared to control concentrations of 1.2 nmoles/mg protein. In addition, the metabolism of CS_2 was biphasic in that there was an initial period of rapid metabolite formation followed by a period (after 5 min) of slower metabolism. CS_2 metabolism in hepatocytes was also biphasic (data not presented).

In microsomal incubations, the amounts of nonvolatile sulfur products were always less than the amounts of the volatile products. This was expected because [^{35}S]CS_2 was labeled in one position and COS was the predominant volatile product in microsomes. Hence, during the formation of [^{35}S]COS, approximately half of the resulting nonvolatile [^{35}S]sulfur compounds were radiolabeled. When [^{35}S]CS_2 was the substrate, [^{35}S]COS was the only detectable volatile metabolite. No hydrogen sulfide ([^{35}S]H_2S) was detected. No nonvolatile radioactive metabolites of [^{14}C]CS_2 were found following either hepatocyte or microsomal incubation.

The metabolism of CS_2 in hepatocytes and microsomes is further compared in Table 1. Although [^{14}C]CO_2 was the predominant volatile metabolite in hepatocytes, small amounts of [^{14}C]COS were detected from incubations of hepatocytes with [^{14}C]CS_2. The ratio of CO_2 to COS was approximately 20 to 1 in hepatocytes isolated from phenobarbital-treated rats. In microsomes from phenobarbital-treated rats, the ratio of CO_2 to COS was approximately 0.4–0.6 to 1.

SKF-525A significantly inhibited CS_2 metabolism in hepatocytes ([^{14}C]CO_2 and nonvolatile [^{35}S]sulfur metabolites) and microsomes ([^{14}C]COS and nonvolatile [^{35}S]sulfur metabolites) from phenobarbital-treated animals (Table 1). SKF-525A did not statistically decrease the amounts of [^{14}C]CO_2 produced by microsomes nor [^{14}C]COS produced by hepatocytes. These are not the predominant products in these respective systems. Lack of statistical significance may be due to the relatively small amounts and large standard deviations.

Acetazolamide did not significantly inhibit CS_2 metabolism regardless of the metabolite examined. The effects of acetazolamide on CS_2 and COS metabolism in hepatocytes were further compared (Table 2). The metabolism of [^{35}S]COS but not [^{35}S]CS_2 was inhibited significantly (by almost 78%) by 1 mM acetazolamide.

The addition of dialyzed cytosol (used as a source of carbonic anhydrase) to microsomal incubations containing CS_2 led to an increase in CO_2 formation at the expense of COS (Table 3), but with no change in total volatile metabolites. There was a concomitant increase in nonvolatile sulfur compounds. The addition of acetazolamide had no significant effect on the metabolism of CS_2 by microsomes fortified with cytosol. Acetazolamide caused a slight decrease in CO_2 formation in incubations containing microsomes without cytosol; however, this decrease was not statistically significant and not accompanied by a change in the amounts of nonvolatile sulfur formed. Cytosol alone had no ability to metabolize CS_2 (data not presented).

The source of the oxygen atom in the COS produced by metabolism of CS_2 was investigated by incubating microsomes and CS_2 in a buffer enriched (23 atoms percent excess) with [^{18}O]H_2O (Table 4). The M + 2 peak of COS was enriched to 26.9 atoms percent excess in the incubation containing [^{18}O]H_2O. Hence, the oxygen in COS formed by the microsomal oxidation of CS_2 was derived from water. No [^{18}O] enrichment was detected when COS was incubated with only the buffer enriched with [^{18}O]H_2O.

DISCUSSION

CS_2 is a substrate for the cytochrome P-450 containing monooxygenase system [3–7]; however, previous reports did not address the possible involvement of other enzymes in the initial oxidation of CS_2. Here we reported that the hepatic metabolism of CS_2 corresponded well with the amounts of cytochrome P-450 present. These data suggest that the initial step of oxidative CS_2 metabolism was catalyzed largely, if not exclusively, by the cytochrome P-450 containing monooxygenase system. This hypothesis is further supported by the observations that (1) SKF-525A inhibited CS_2 metabolism to the same extent in both hepatocytes and microsomes; (2) addition of cytosol to microsomal incubates of CS_2 shifted the spectrum of metabolites but not the total; (3) cytosol alone had no CS_2 metabolizing ability; and (4) the microsomal oxidation of CS_2 requires NADPH [6, 7].

COS is a putative metabolite of CS_2 both *in vitro* [5, 6] and *in vivo* [18], and is metabolized to CO_2 via a pathway catalyzed in part by cytosolic carbonic anhydrase [10, 16]. Therefore, we expected that CO_2 would be the predominant volatile metabolite of CS_2 with hepatocytes, while COS would predominate

Table 4. Mass spectrometry of COS produced by hepatic microsomes incubated with CS_2 in an $[^{18}O]H_2O$ enriched system

Conditions	Mass peaks (%) 60	62	Atoms % excess
(1) Complete incubation $-[^{18}O]H_2O$	100	4.9	
(2) Complete incubation $+[^{18}O]H_2O$	100	31.8	26.9
(3) COS + buffer	100	5.0	0.1
(4) COS + buffer $+[^{18}O]H_2O$	100	4.7	−0.2

Complete incubations consisted of 1.3 mg of twice washed microsomal protein, 0.6 μmole of CS_2 and an NADPH-generating system (described under Methods) in 2 ml (final volume) of 20 mM sodium phosphate buffer, pH 7.4, for 30 min at 37° in Teflon-sealed Erlenmeyer flasks. Reactions were terminated by the injection of 0.2 ml of 1 N HCl. Under conditions 3 and 4, 1 μmole of COS was incubated with the 20 mM sodium phosphate buffer for 30 min at 37°. Under conditions 2 and 4, incubations were enriched with $[^{18}O]H_2O$ to a final concentration of 23.3 atoms percent excess.

with microsomes [4, 5–7]. However, acetazolamide, at concentrations which inhibited COS metabolism, did not inhibit the metabolism of CS_2 to CO_2 in either isolated hepatocytes or microsomes fortified with cytosol. We, therefore, hypothesize that the product of microsomal oxidation of CS_2 is not COS *per se*, but its hydrated form, monothiocarbonate (Fig. 2, step 2). This would bypass the hydrolysis of COS by carbonic anhydrase and, hence, accommodate the lack of effect of acetazolamide. Monothiocarbonate and COS exist in solution in equilibrium. Acidification causes a quantitative shift toward COS, in the same manner that bicarbonate dehydrates to CO_2. Since the microsomal incubations were terminated by the addition of TCA, this suggested pathway is consistent with the observation that COS is a metabolite of CS_2. However, the amounts of COS detected in these experiments are greater than those which exist at physiological pH, and should now be considered to be indicative of the total amounts of monothiocarbonate and COS present at the end of the incubation period.

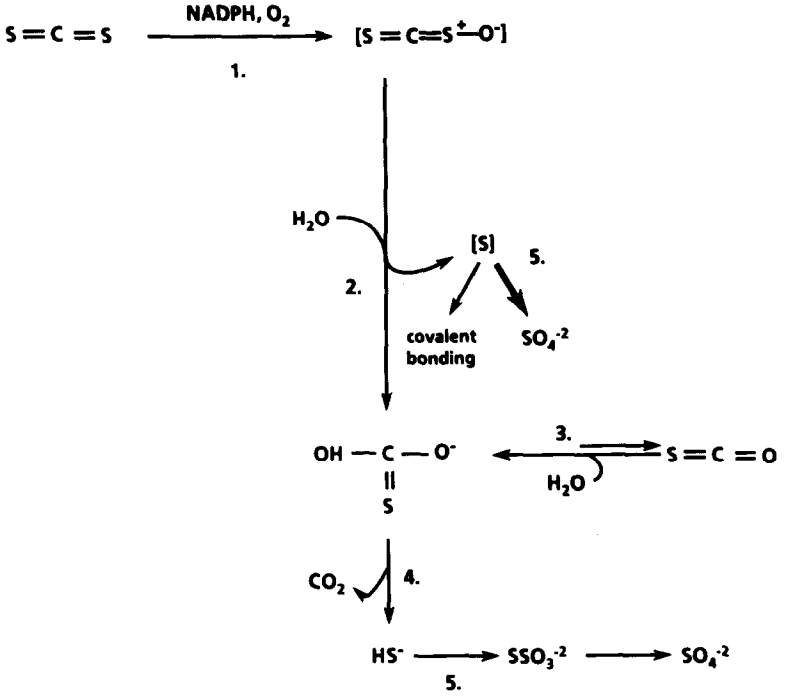

Fig. 2. Simplified scheme of oxidative carbon disulfide and carbonyl sulfide metabolism in rat liver. Step 1 is catalyzed by the cytochrome P-450 containing monooxygenase. The resulting intermediate breaks down in the presence of water (Step 2) producing monothiocarbonate, and reactive sulfur which either binds to microsomal macromolecules or is oxidized to sulfate. Step 3 is the equilibrium reaction between carbonyl sulfide and monothiocarbonate, catalyzed by carbonic anhydrase. Step 4 is the conversion of monothiocarbonate to carbon dioxide and hydrogen sulfide ion, which is oxidized (Step 5) to thiosulfate and sulfate.

This hypothesis is also consistent with the [^{18}O]COS mass spectral data. In fact, the degree of [^{18}O] enrichment suggests that both oxygens in monothiocarbonate are derived from [^{18}O]H_2O. This is because, in solution, monothiocarbonate equilibrates with water. COS was not enriched with [^{18}O]H_2O when incubated in the absence of hepatic enzymes. This indicates that a simple exchange of oxygen between H_2O and *de novo* COS (from CS_2) did not occur. To contain oxygen from [^{18}O]H_2O, COS most likely existed in solution as monothiocarbonate.

Like many other substrates of the cytochrome P-450 containing monooxygenase system, the immediate product of CS_2 oxidation (Fig. 2, step 1) is probably an unstable intermediate [5–7], which breaks down to the monothiocarbonate and a reactive sulfur species [6, 7]. The structure of the intermediate shown in Fig. 2 is that suggested by Neal and Halpert [19]. The exact nature of this intermediate, however, remains to be firmly established.

The biphasic nature of CS_2 metabolism reported here is consistent with the previously published reports [3–7] that CS_2 is a suicide substrate, due to the binding of a reactive sulfur species, for cytochrome P-450. However, the stoichiometry between [^{14}C]- and [^{35}S]CS_2 reported here suggests that macromolecular binding of sulfur probably plays a minor role in the overall disposition of CS_2. Most of the reactive sulfur species is metabolized to (more stable) nonvolatile sulfur compounds, but by mechanisms that are not yet understood.

The second sulfur atom (that removed during the conversion of COS to CO_2) is further metabolized by a different mechanism than the first sulfur atom. H_2S is a metabolite of COS [16] and, although none was detected, H_2S was probably a metabolite of CS_2 in isolated hepatocytes. As shown by Sörbo [20, 21] and later confirmed by Bartholomew *et al.* [22], H_2S is rapidly metabolized by rat liver first to thiosulfate and, subsequently, to sulfate. This rapid metabolism could account for the lack of observable H_2S formation from CS_2. In preliminary experiments, approximately 50% of the nonvolatile metabolites of [^{35}S]CS_2 were tentatively identified as sulfate (SO_4^{2-}) by precipitation with barium (data not presented). Sulfate is a product of CS_2 metabolism *in vivo* [23]. Hence, the present data and hypothesis are consistent with previous publications.

In summary, the data presented here support the hypothesis that (1) CS_2 is oxidized in rat liver predominantly by the cytochrome P-450 containing monooxygenase system, and (2) the product of this reaction is an unstable intermediate which reacts with water to form monothiocarbonate and a reactive sulfur species. Monothiocarbonate exists in solution in equilibrium with, and can be dehydrated to form, COS. In intact hepatocytes, however, monothiocarbonate is further metabolized to CO_2 and H_2S (and, thence, to thiosulfate and sulfate). The present evidence suggests that the two sulfur atoms of CS_2 are oxidized to sulfate by two distinct pathways.

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