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HEPATIC CARBONYL SULFIDE METABOLISM

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SUMMARY: Carbonyl sulfide (COS) is rapidly metabolized by isolated rat hepatocytes, as determined by COS disappearance. However, upon termination of the reaction by acidification much of the metabolized COS reappears in the headspace of the reaction vessel. The COS disappearance when determined after acidification is equal to the formation of carbon dioxide and an inorganic sulfur containing compound(s). The metabolism of COS by hepatocytes is inhibited by acetazolamine but not by carbon disulfide or inhibitors of the cytochrome P-450 containing monooxygenase system. Upon subcellular fractionation, the majority of hepatic COS metabolizing activity is found in the cytosol. Additional experiments with a partially purified enzyme indicate that COS is a substrate for hepatic carbonic anhydrase.

INTRODUCTION

Carbonyl sulfide (COS) is a waste gas resulting from industrial procedures, such as coal gasification (1). It is also the predominant volatile product of carbon disulfide (CS_2) metabolism by isolated hepatic microsomes (2,3). The toxicity of CS_2, a commonly used industrial solvent, has been recognized and investigated for over a century (4). COS itself is metabolized by rat hepatic microsomal preparations to carbon dioxide (3). The hepatic microsomal metabolism of both CS_2 and COS results in the covalent binding of sulfur to microsomal macromolecules (2,3,5). Furthermore, DeMatteis and coworkers have recently shown that CS_2 is hepatotoxic in rats with the severity of the lesion being positively correlated to the hepatic concentration of cytochrome P-450 (6). The relationship between the covalent binding of sulfur to hepatic macromolecules and the hepatotoxicity of CS_2 is unclear, and presently under investigation in our laboratory. During the course of these studies we observed that CS_2 metabolism by isolated rat hepatocytes in contrast to hepatic microsomes (2,3) yielded no COS (7). This finding prompted us to examine COS metabolism in more detail. The results of these studies indicate a role for carbonic anhydrase in the metabolism of COS.

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MATERIALS AND METHODS

[^{14}C] labeled CS_2, sodium bicarbonate, and potassium thiocyanate were obtained from Amersham/Searle, Arlington Heights, Ill., as was ^{35}S-CS_2. [^{35}S] potassium thiocyanate was obtained from New England Nuclear. Unlabeled COS was purchased from Matheson, Morrow, Georgia. Acetazolamine and bovine erythrocyte carbonic anhydrase were purchased from Sigma Scientific Co., St. Louis, Mo. [^{14}C] and [^{35}S] labeled COS was prepared from [^{14}C] and [^{35}S] thiocyanate as described elsewhere (8).

Rat hepatocytes were prepared from adult male Sprague-Dawley rats (190-210 gm) according to the method of Zhalten and Stratman (9). After isolation, cells were suspended and incubated in Krebs-Heinsleit buffer fortified with 1.5% gelatin. For metabolic studies with COS, cells (2.5-5.0 mg/ml, as determined by the biuret reaction) were incubated in a final volume of 2.0 ml in sealed 25 ml Erlenmeyer flasks at 37° C. Reactions were initiated by the injection of COS (a gas at room temperature, 30 μl = 1 μmole) through the serum stopper. Reactions were terminated by the addition of 0.2 ml 40% trichloroacetic acid (TCA) or (when indicated) 0.4 ml 10% triton X-100. Subcellular fractions of the liver were prepared by standard methods (10). The subcellular fractions were resuspended and incubated with 1 μmole COS in 50 mM sodium phosphate buffer, pH 7.6. In all experiments companion flasks of boiled hepatocytes (or subcellular fractions) served as controls.

Headspace disappearance of COS was determined by gas chromatography (11). The formation of [^{14}C]CO_2 from [^{14}C] COS was determined by separating CO_2 from COS using gas chromatography (11) and collecting the CO_2 in ethanolamine (33% v/v in 2-methoxyethanol). Trapped [^{14}C]CO_2 was quantitated by liquid scintillation counting.

The formation of nonvolatile, [^{35}S]-labeled metabolites was determined as follows: after the termination and deproteinization of the reactions by the addition of TCA, the incubation solutions were neutralized (pH 7.0) by the addition of 4N K_2CO_3. Following centrifugation to remove the precipitate, a 0.6 ml fraction of the resulting supernatant was combined with methanol (1.0 ml) in a glass vial and evaporated to dryness in a vacuum oven. This effectively removes unreacted COS and other volatile sulfur compounds. The residue was dissolved in 0.6 ml water, followed by 2 ml methanol and 10 ml of scintillation cocktail. Radioactivity was determined by scintillation counting.

Thiosulfate ($SSO_3^=$) and hydrogen sulfide (HS^-) were determined by the ferricyanide method of Sorbo (12). As this method gives a positive response to both $SSO_3^=$ and HS^-, cadmium acetate addition was used to discriminate between the two anions. Thus, upon termination of an incubation an aliquot of the supernatant was first assayed for total "Sorbo positive material". A second aliquot was first treated with cadmium acetate to precipitate HS^-, and the assay repeated, the second time detecting only $SSO_3^=$. The HS^- concentration was determined as the difference between the initial reading and that obtained following the cadmium acetate treatment.

RESULTS

Initial experiments, in which the reactions were terminated by the addition of Triton X-100 and cooling to 0° (a procedure which results in cell disruption with no change in pH), demonstrated that isolated hepatocytes actively metabolized COS (30±5 nmoles/mg cell protein/min.) as measured by COS disappearance. This rapid disappearance of COS was not inhibited by the cytochrome P-450 monooxygenase

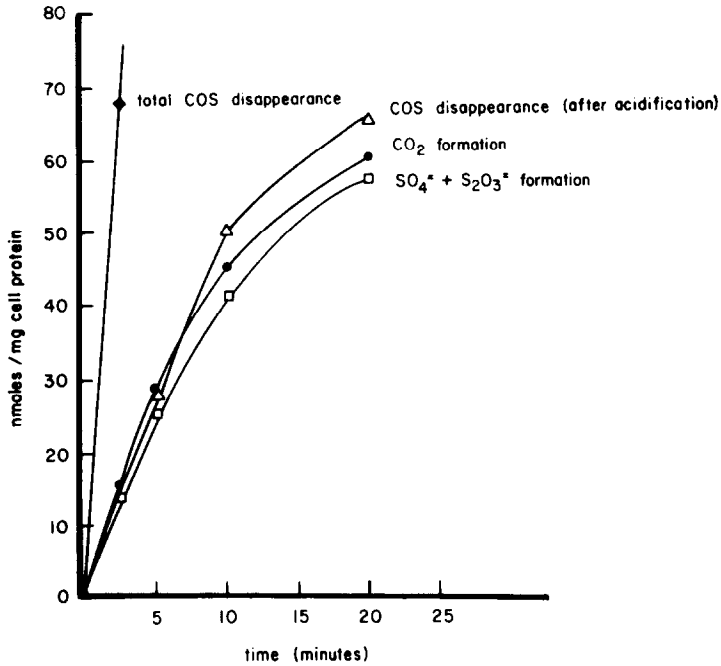

Figure 1. Carbonyl sulfide metabolism by isolated hepatocytes. Cells (2.5-5.0 mg/ml) were incubated with 1 μmole [^{14}C] or [^{35}S]COS in a final volume of 2.0 ml. COS disappearance was determined following termination of the reaction using Triton X-100 (total COS disappearance) and trichloroacetic acid [COS disappearance (after acidification)]. CO_2 and sulfate plus thiosulfate were determined as described in Materials and Methods. Each point represents the mean of three separate experiments.

inhibitors SKF 525-A, 4-methylpyrazole or metyrapone or the cytochrome P-450 monooxygenase substrate carbon disulfide. When the reactions were terminated by acidification with TCA the apparent rate of COS disappearance was less than that observed when Triton X was used (Fig. 1). Further, the rate of COS disappearance when assayed following acidification was nearly equal to the rate of formation of [^{14}C]CO_2 from [^{14}C]COS and nonvolatile sulfur from [^{35}S]COS. Using [^{14}C]COS, no label was found as nonvolatile metabolites, confirming that CO_2 is the sole carbon containing metabolite of COS and that the nonvolatile sulfur compounds contain only the sulfur atom of the parent compound.

The metabolism of [^{35}S]COS by hepatic subcellular organelles was also examined (Table 1). Addition of NADPH did increase COS metabolism by the microsomal fraction, confirming earlier published results (3). However, the majority of the metabolic activity towards COS clearly rested in the cytosol. The cytosolic

Table 1: Rat Hepatic Subcellular Distribution of Metabolic Activity Toward [^{35}S]Carbonyl Sulfide

Subcellular Fraction	Rate of formation of nonvolatile sulfur (nmole/mg protein/30 min)
Whole homogenate	30.0±2.0
Nuclei	6.0±1.0
Mitochondria	4.2±0.8
Lysosomes	10.0±3.2
Microsomes:	
-NADPH	2.5±1.5
+NADPH	14.5±1.5
Cytosol	118.0±12.0

Following the in situ perfusion of the liver through the portal vein, the liver was homogenized (25%) in 1.15% KCL, and subcellular fractions were prepared by standard techniques of differential centrifugation (10). Sedimented fractions were resuspended in 50 mM phosphate, pH 7.5. One μmole of [^{35}S]-COS (0.1 μCi/μmole) was incubated with 1-5 mg protein of each fraction in a final volume of 2.0 ml at 37°C for 30 min. Where indicated, microsomal incubations contained 0.15 mM NADPH. The results are from three separate experiments (mean ± S.D.). Assay procedures are described in the Methods and Material Sections.

localization of COS metabolism plus the structural similarity between COS and CO_2 led us to consider the possible involvement of carbonic anhydrase (I.U.B. 4.2.1.1.) in COS metabolism. Therefore, we examined the effect of acetazolamine, a carbonic anhydrase inhibitor (15) on [^{35}S]COS metabolism by rat liver cytosol and observed that 0.5 mM acetazolamine inhibited [^{35}S]COS metabolism by 50% (from 115±3 to 58±2 nmoles nonvolatile [^{35}S] sulfur/mg cytosolic protein/30 min.).

The metabolism of COS by bovine erythrocyte carbonic anhydrase was also examined (Table 2). The metabolism of [^{14}C]COS to [^{14}C]CO_2 by carbonic anhydrase (Experiment 1) was inhibited by acetazolamine and by heat inactivation of the enzyme. An examination of the sulfur containing products of the reaction of COS with carbonic anhydrase was also carried out (Experiment 2, Table 2). The initial experiments revealed the production of volatile and nonvolatile sulfur containing products. The nonvolatile product was thought to be thiosulfate on the basis of its

Table 2: COS Metabolism by Bovine Erythrocyte Carbonic Anhydrase

Incubation Conditions	Product Formation (nmoles/0.2 mg protein/30 min)		
	CO_2	Hydrogen Sulfide	Thiosulfate
Experiment #1			
Carbonic anhydrase	161±17	---	---
+ acetazolamine	60±5	---	---
Heat inactivated carbonic anhydrase	12±2	---	---
Experiment #2			
Carbonic anhydrase	---	120±14	22±4
+ acetazolamine	---	90±10	6±2
Heat inactivated carbonic anhydrase	---	20±5	10±2

Crude bovine erythrocyte (0.2 mg) carbonic anhydrase was incubated with 1 μmole of [^{14}C] COS (CO_2 formation) or unlabeled COS (sulfur product formation) in 2.0 ml Krebs-Hiensliet Carbonate Buffer at 37°C. The final concentration of acetazolamine was 0.5 mM. The carbonic anhydrase was inactivated by heating to 100°C for five minutes. Hydrogen sulfide and thiosulfate were determined as described in Materials and Methods. The values are the means of four experiments ± S.D.

positive reaction with ferricyanide (12). On the basis of its volatility, precipitation with cadmium acetate and oxidation by hemin to thiosulfate (12) a volatile product of the reaction was judged to be hydrogen sulfide. The production of hydrogen sulfide and thiosulfate was also inhibited by acetazolamine and by heat inactivation of the enzyme.

The effect of acetazolamine on COS metabolism by isolated hepatocytes was also examined (Table 3). Acetazolamine inhibits COS disappearance, regardless of the method used to terminate the reactions. Furthermore, acetazolamine also inhibits COS metabolism to CO_2 to the same extent as the COS disappearance when the reactions were terminated using TCA.

Table 3: The Effect of Acetazolamine on COS Metabolism by Isolated Hepatocytes

	COS Disappearance (nmoles/mg cell protein/10 min)		CO_2 Formation (nmoles/mg cell protein/10 min)
	Triton X-100	TCA	
Control	173 + 19	52 + 9	68 + 7
+ 1 mM acetazolamine	93 + 11	4.3 + 2	9.2 + 3

Isolated rat hepatocytes (2-5 mg cell protein/ml) were incubated with 2 ml Krebs-Hiensliet buffer at 37°C for 10 min in sealed 25 ml Erlenmeyer flasks. COS (1 μmole) was injected through the seal to initiate the reaction. Reactions were terminated by adding either Triton X-100, or TCA and headspace COS was determined by gas chromatography (11). CO_2 formation was determined after termination of the reaction using TCA. These values are the means ± S.D. of three separate experiments.

DISCUSSION

On the basis of these results, we propose that carbonic anhydrase is the enzyme chiefly responsible for rat hepatic COS metabolism by the following pathway:

$$S = C = O + H_2O \underset{\text{Carbonic Anhydrase}}{\rightleftharpoons} HS - \underset{\underset{O}{\|}}{C} - OH \xrightarrow{H_2O} HS^- + HCO_3^- + 2H^+$$

Carbonic anhydrase catalyzes the formation of monothiocarbonic acid (H_2CO_2S) which is converted back to COS upon acidification of the medium. It appears that the monothiocarbonic acid is also hydrolyzed to bicarbonate and hydrogen sulfide. It is unclear whether this latter reaction is enzymatically catalyzed, but it is known that monothiocarbonate breaks down to CO_2 and HS^- spontaneously (13). According to Sorbo (14), the liver rapidly oxidizes hydrogen sulfide to thiosulfate ($S_2O_3^=$) and sulfate ($SO_4^=$). The oxidation of $[^{35}S]COS$ to $[^{35}S]SO_4^=$ and $S_2O_2^=$ by hepatocytes has been reported previously (7). Thus, the oxidation of SH^- would pull the above reaction to the right.

There is a relatively large carbonic anhydrase activity[1] in the rat liver. This activity is more than adequate to account for the rates of COS metabolism presently reported. Furthermore, both the lungs and blood contain high levels of carbonic

[1] In our laboratory, rat liver cytosol was determined to have carbonic anhydrase activity between 4 and 5 Wilbur-Anderson units/mg protein as measured by the method of Wilbur and Anderson (17).

anhydrase (15), and both would be the organs of primary exposure when COS is inhaled in vivo. Hence, the action of carbonic anhydrase on COS with the subsequent release of hydrogen sulfide may be the mechanism of toxicity of COS. Indeed, the production of hydrogen sulfide from COS was earlier suggested to be the ultimate cause of COS toxicity (14). Further, unreported results by Neal and Alarie suggested COS and H_2S are acutely toxic by the same mechanism, that is, central respiratory arrest (16). The present results provide a molecular basis for this hypothesis.

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