# EVIDENCE FOR THE FORMATION OF A PROTEIN BOUND HYDRODISULFIDE RESULTING FROM THE MICROSOMAL MIXED FUNCTION OXIDASE CATALYZED DESULFURATION OF CARBON DISULFIDE

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## SUMMARY

The nature of the sulfur released during the microsomal metabolism of carbon disulfide ($CS_2$) has been examined. Following incubation with [$^{35}$S]$CS_2$, microsomes were reisolated by Sephadex chromatography and incubated with cyanide ($CN^-$). Approximately 45% of the [$^{35}$S] bound to the microsomes was released in a form which appears to be identical to thiocyanate ($SCN^-$). Incubation of microsomes with unlabled $CS_2$ followed by incubation with [$^{14}$C] $CN^-$ also resulted in recovery of what appeared to be [$^{14}$C]$SCN^-$. On the basis of these results, it is proposed that a portion of the sulfur released in the microsomal metabolism of $CS_2$ reacts with the sulfhydryl groups of cysteine residues in the microsomal proteins to form a hydrodisulfide.

The metabolism of carbon disulfide ($CS_2$) to carbonyl sulfide (COS) by the liver mixed function oxidase (MFO) enzyme system results in the release of sulfur which binds covalently to the microsomal membrane (1-3). It has been proposed that sulfur is released as singlet atomic sulfur which, because of its electrophilicity, should readily react with membrane nucleophiles (1). Because of its nucelophilicity, the sulfhydryl group of cysteine would be the most probable site of attack by singlet sulfur. This would result in the formation of a hydrodisulfide (R-S-S-H). It has been reported that a hydrodisulfide is present in the active sites of the enzymes xanthine oxidase (4) and aldehyde oxidase (5). Reaction of these enzymes with $CN^-$ leads to the release of thiocyanate ($SCN^-$). The formation of $SCN^-$ is thought to result from the attack of $CN^-$ on the terminal sulfur of the hydrodisulfide (R-S-S-H) releasing $SCN^-$ and

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forming the corresponding mercaptide ($RS^-$ or RSH). The reaction of synthetic benzyl hydrodisulfide with $CN^-$ also leads to the formation of $SCN^-$ and α-toluenethiol (6).

The present study was undertaken to determine whether there was any evidence indicating that the sulfur released as a result of the hepatic MFO catalyzed metabolism of $CS_2$ *in vitro* was covalently binding to cysteine side chains in microsomal proteins to form a hydrodisulfide.

## METHODS AND MATERIALS

Massey and Edmondson (4) have shown that the $SCN^-$ formed on reaction of $CN^-$ with metabolically active xanthine oxidase could be effectively separated from unreacted $CN^-$ using columns of Sephadex G-25. This procedure was used in these studies for the separation of $SCN^-$ from other low molecular weight compounds used in the incubations.

Liver microsomes were isolated from phenobarbital pretreated rats as previously described (1). Incubations were carried out in stoppered 50 ml Erlenmeyer flasks for 30 min at 37°C in 0.05 M Hepes buffer, pH 7.8, containing $1 \times 10^{-3}$M EDTA, 40 mg of microsomal protein, an NADPH generating system (5 units glucose-6-phosphate dehydrogenase, 10 mg NADP and 100 mg glucose-6-phosphate) and $5.9 \times 10^{-3}$M [$^{35}$S] $CS_2$ [0.325 μCi/μmole] in a total volume of 2.8 ml. Control incubations lacked the NADPH generating system. Following the incubations, the flasks were placed on ice and evacuated through the stopper using a high volume vacuum pump. The vacuum was released by $N_2$. This procedure was repeated four additional times. The purpose of this procedure was to remove as much unreacted $CS_2$ and its product, COS, as possible. Following this the control and experimental incubations were each chromatographed over a Sephadex G-25 column (1.5 cm x 30 cm bed volume) equilibrated with 0.05M Hepes, pH 7.8, containing $1 \times 10^{-3}$M EDTA to separate the remainder of the non-covalently bound radioactivity from the microsomes. The flow rate was adjusted to 6 ml per hour and one ml fractions collected. An aliquot (25 μl) of each fraction was diluted to 2 ml with distilled $H_2O$ and analyzed for protein at 280 nm. An equal aliquot was assayed for radioactivity by scintillation counting following addition of 10 ml of Aquasol (New England Nuclear). Only small amounts of radioactivity corresponding to low molecular weight compounds (fractions 32-38) were eluted from the columns. The fractions from the columns corresponding to microsomes (fraction 15-21) were pooled and concentrated to 2 ml using an Amicon model 52 ultrafiltration cell and a XM-300 membrane. The control and experimental microsomes were next incubated separately with 100 mM $CN^-$ for 1 hr at 0-4°C in a stoppered flask. The incubation mixtures were then reapplied to the Sephadex G-25 columns, eluted as described above and assayed for radioactivity and protein.

Experiments with [$^{14}$C] $CN^-$ and unlabeled $CS_2$ were carried out as described above with the exception that the concentration of $CN^-$ was reduced to 1 mM.

The $SCN^-$ formed in the various experiments was quantitated by radioactivity or using the colorimetric procedure of Sörbo (7). Protein was determined by the biuret method (8). Radiolabeled $CS_2$ was a product of Amersham Searle and [$^{14}$C]KCN was obtained from New England Nuclear. NADP and glucose-6-phosphate were products of the Boehringer-Mannheim Corporation and glucose-6-phosphate dehydrogenase was obtained from the Sigma Chemical Company.

## RESULTS AND DISCUSSION

Microsomes were incubated in the presence of [$^{35}$S] $CS_2$ either with or without (control) an NADPH generating system and isolated from the incubation mixture by Sephadex G-25 chromatography. The column fractions containing the microsomes from the experimental and control incubations were pooled separately and a sample taken for the determination of the amount of [$^{35}$S] bound to microsomes. The pooled fractions were next concentrated, incubated with 100 mM $CN^-$ and rechromatographed on Sephadex G-25.

The amount of radioactivity bound to the microsomes incubated with [$^{35}$S] $CS_2$ in the presence and absence of NADPH and the effect of incubation with $CN^-$ on the amount of radioactivity bound to these microsomes is shown in Table I. As previously reported (1), incubation of microsomes with [$^{35}$S]$CS_2$ in the presence of NADPH results in an approximate 3-6 fold increase in the amount of [$^{35}$S] bound to the microsomes as compared to those incubated with [$^{35}$S]$CS_2$ in the absence of NADPH. These studies had also shown that the majority (>80%) of the [$^{35}$S] bound in the presence of NADPH was not bonded to the carbon atom

TABLE I

Effect of the Presence and Absence of NADPH and Cyanide on the Amount of [$^{35}$S] from [$^{35}$S]$CS_2$ Bound to Liver Microsomes*

| Incubation Conditions | [$^{35}$S] Bound nmole/mg Protein |
|---|---|
| $CS_2$ minus NADPH (before $CN^-$) | 3.0 |
| $CS_2$ plus NADPH (before $CN^-$) | 9.2 |
| $CS_2$ minus NADPH (after $CN^-$) | 3.0 |
| $CS_2$ plus NADPH (after $CN^-$) | 5.3 |

* The reaction conditions and procedure for isolation of microsomes from the incubation mixture are given in the METHODS AND MATERIALS.

of $CS_2$ whereas, all of the radioactivity bound to microsomes in the absence of NADPH is thought to be largely dithiocarbamates and trithiocarbonates formed in the reaction of $CS_2$ with amines and sulfhydryl groups respectively (1). As shown in Table I the incubation of microsomes with $CN^-$ leads to the release of approximately 40% of the bound sulfur in the case of microsomes labeled with [$^{35}S$] in the presence of NADPH but had no effect on the [$^{35}S$] bound to microsomes incubated in the absence of NADPH.

Figure I shows the elution profile of absorbance at 280 nm and radioactivity resulting from Sephadex G-25 chromatography of the $CN^-$ treated experimental ([$^{35}S$] $CS_2$ plus NADPH) and control ([$^{35}S$] $CS_2$ minus NADPH) microsomes described in Table I. As can be seen $CN^-$ treatment results in the release of two low molecular weight labeled fractions from the experimental microsomes and one labeled fraction from the control microsomes. Seventy-five percent of the [$^{35}S$] released from the experimental microsomes elutes in fractions 46-56.

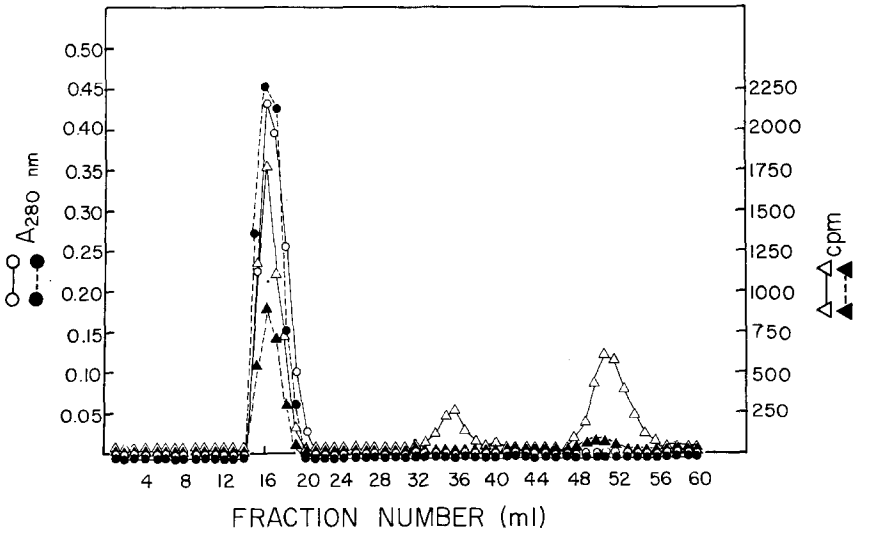


Figure 1. Elution profile of absorbance at 280 nm (circles) and radioactivity (triangles) from columns of Sephadex G-25 to which were applied either $CN^-$ treated experimental ([$^{35}S$] $CS_2$ plus NADPH), open circles and open triangles, or control microsomes ([$^{35}S$] $CS_2$ minus NADPH), closed circles and closed triangles. See the text for additional details.

This elution volume is the same as that reported by Massey and Edmondson (4) for $SCN^-$ using very similar chromatographic conditions. The total amount of radioactivity eluted in fractions 32-38 and 46-56 of Figure 1 is almost identical to the decrease in radioactivity seen on treatment of the experimental microsomes with $CN^-$ (Table 1).

In an attempt to establish the identity of the radioactive material in fractions 46-56, Figure 1, these fractions were pooled and reduced in volume to 1 ml by evaporation under nitrogen. An equal volume of the eluting buffer was reduced in volume to serve as a control. The total radioactivity of this fraction, calculated from the original specific activity of $[^{35}S]CS_2$, represented 119 nmoles of sulfur. When this fraction was assayed by the colorimetric procedure of Sorbo (7), the fraction was found to contain 125 nmoles of $SCN^-$. The same calculations on fractions 46-56 from chromatography of control microsomes incubated with $CN^-$ indicated less than 13 nmoles of $SCN^-$ by the colorimetric test (7) (lower limit of accuracy) and 9 nmoles of $[^{35}S]$.

Fractions 46-56 from the chromatography of an additional incubation of $CN^-$ with experimental microsomes were reduced in volume (2 ml), 500 nmoles of unlabeled $SCN^-$ added and the sample re-chromatographed over Sephadex G-25. As shown in Figure 2, the authentic $SCN^-$ co-chromatographed with the $[^{35}S]$ labeled compound.

These data indicated the $[^{35}S]$ labeled compound in fractions 46-56 from the Sephadex columns was $SCN^-$. As an additional proof microsomes were incubated with unlabeled $CS_2$ in the presence and absence of NADPH followed by incubation with $[^{14}C]CN^-$. The results of these experiments are shown in Figure 3. The $[^{14}C]CN^-$ likewise releases from microsomes incubated with unlabeled $CS_2$ in the presence of NADPH a labeled compound which elutes in fractions 46-56. The total radioactivity in this peak from the experimental microsomes ($CS_2$ plus NADPH) represents 84 nmoles of $[^{14}C]$ while the colorimetric assay gives 79 nmoles of $SCN^-$. Again, a small amount of radioactivity with an elution volume identical to $SCN^-$ was released on incubation of the control microsomes with $[^{14}C]CN^-$.

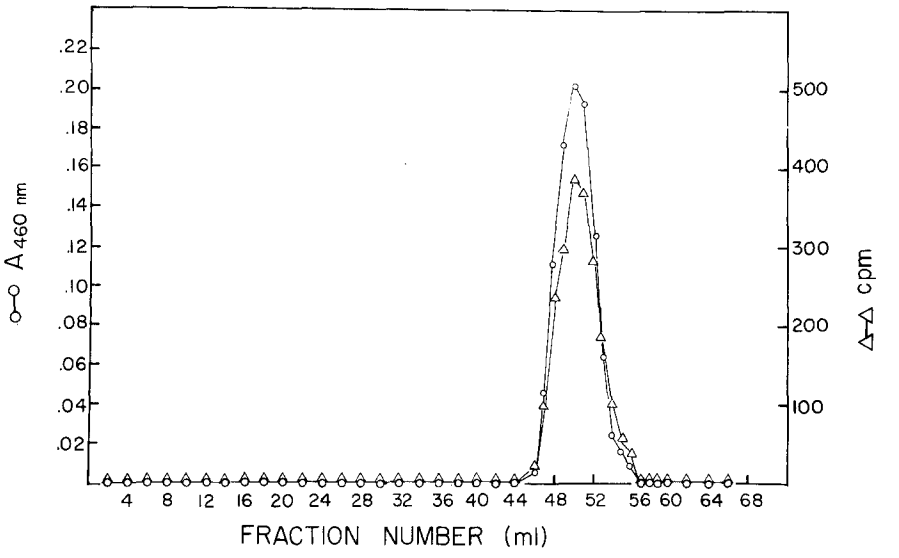


Figure 2. Elution profile of radioactivity (fractions 46-56, Figure 1) and unlabeled thiocyanate from a column of Sephadex G-25. The thiocyanate was determined colorimetrically (7) (open circles). The radioactivity is indicated by open triangles.

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It should be noted that less radioactive material corresponding to $SCN^-$ was recovered in the incubation using $[^{14}C]CN^-$ than from that using $[^{35}S]CS_2$. This is most likely due to the fact that the $CN^-$ concentration was reduced from 100 mM to 1 mM in the experiment using $[^{14}C]CN^-$. This was because of difficulties in eluting all the $[^{14}C]CN^-$ from the Sephadex column before the appearance of the peak corresponding to $SCN^-$ if a concentration of $CN^-$ greater than 1 mM was used.

The identity of the radiolabeled compound or compounds in fractions 28-38 of Figure 1 is presently unknown. The elution volume is the same as that of other low molecular weight compounds used in the incubations, namely $[^{35}S]CS_2$, NADP(H) and the unreacted $[^{14}C]CN^-$.

Since a compound which appeared to be identical to $SCN^-$ can be released by $CN^-$ from microsomes incubated with $CS_2$ in the presence of NADPH, it appears that a significant portion (approximately 50%) of the sulfur which is released

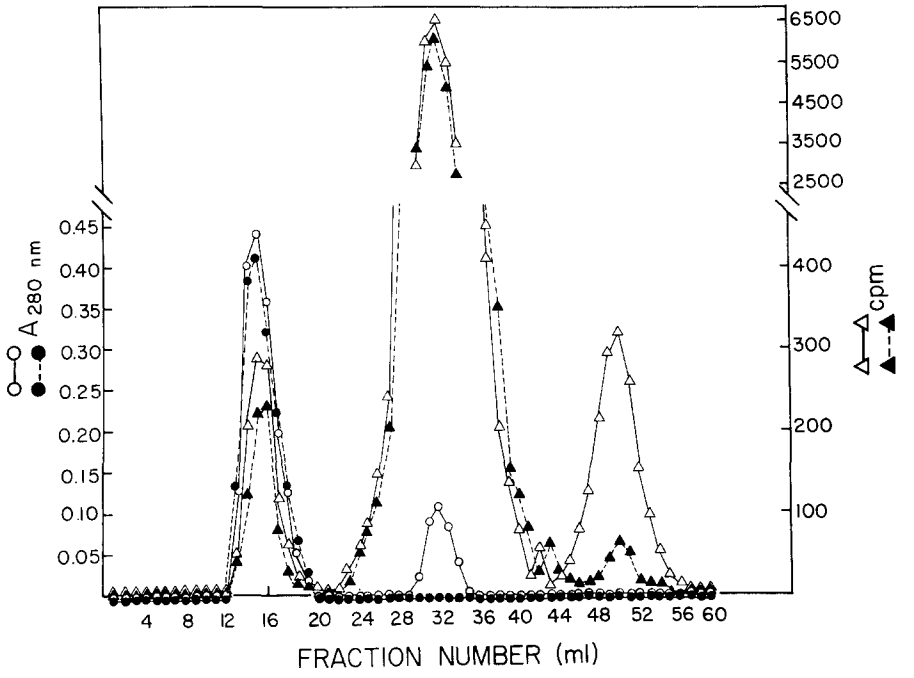


Figure 3. Elution profile of absorbance at 280 nm (circles) and radioactivity (triangles) from columns of Sephadex G-25 to which were applied either [$^{14}$C]CN$^-$ treated experimental ($CS_2$ plus NADPH),open circles and open triangles, or control microsomes ($CS_2$ minus NADPH), closed circles and closed triangles. See the text for additional details.

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in the MFO metabolism of $CS_2$ is bound to the microsomal proteins in the form of a hydrodisulfide.

These data lend support to the previously proposed mechanism (1) for the MFO catalyzed metabolism of $CS_2$ to COS in which it was postulated that the sulfur is released as the singlet form of atomic sulfur. Such a species of sulfur would be highly electrophilic and readily attack protein RSH groups to form the corresponding hydrodisulfide.

Analogous to carbenes and nitrenes, the singlet sulfur atom is capable of carbon hydrogen insertion reactions (9,10). This type reaction may be responsible for a portion of the sulfur which is bound in the presence of NADPH, but which is not released on treatment of the microsomes with CN$^-$.

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