Reactions of Certain Trichloromethyl Sulfenyl Fungicides with Low Molecular Weight Thiols

In vitro Studies with Glutathione

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The reactions of captan, folpet, or thiophosgene with reduced glutathione were studied using S^{35} labeled reactants. Oxidized glutathione was the primary product formed from the reaction of the thiol and intact fungicide. A small amount of the GSH was converted into a series of derivatives which contained all or a portion of the trichloromethylthio moiety released from the fungicide. The formation of derivatives appeared to be a function of the concentration of the thiol and reactivity of the $SCCl_3$ moiety. When thiophosgene was allowed to react directly with either GSH or GSSG, the amount of the thiol and the disulfide reacted and the number and amount of new products formed were dependent on the $S{=}CCl_2$ concentration. These results suggested that the reactions of the fungicides and their decomposition products may be more complex than previously shown.

Captan and related trichloromethylthio derivatives are widely used organic fungicides. The three commercial fungicides in this group are captan (*N*-trichloromethylthiotetrahydrophthalimide), folpet (*N*-trichloromethylthiophthalimide), and difolatan (*N*-trichloroethylthiotetrahydrophthalimide). The fungitoxic action of captan and folpet has been intensively studied. Lukens (1969a,b) and Siegel (1971a) have reviewed this subject and the postulated mechanism of action of these fungicides can be summarized as follows. In the reaction of $RNSCCl_3$ compounds with cell thiols, 1 mol of fungicide splits at the RN-S bond, forming the free imide, and 2 mol of the thiol are oxidized to disulfide. The liberated trichloromethylthio ($SCCl_3$) moiety can be either transferred directly to various cellular sites or can undergo dechlorination to yield thiophosgene ($S{=}CCl_2$). Thiophosgene is highly reactive and can either undergo rapid hydrolysis with water, yielding carbonyl sulfide (COS), or react with thiol and other cellular groups to form various addition products. The oxidation of thiols and the subsequent reactivity of the $SCCl_3$ moiety have been postulated to be the basis for toxicity of these fungicides. It is apparent that these trichloromethyl sulfenyl fungicides can enter into a variety of indiscriminate reactions, many of which could be responsible for toxic action. This has been demonstrated recently by the reaction of captan and folpet with both low and high molecular weight cellular constituents (Richmond and Somers, 1966, 1968; Richmond *et al.*, 1967; Siegel and Sisler, 1968a,b; Siegel 1971a).

MATERIALS AND METHODS

Reaction of Folpet and Captan with Reduced Glutathione (GSH). The products formed from the reaction of GSH and folpet were determined by labeling one of the reactants with S^{35} and subjecting the products to paper radiochromatography. The buffer used in all experiments contained $0.008M$ K_2HPO_4 and $0.002M$ citric acid, pH 7.6. Experiments were run at ambient temperatures (22–25° C).

Sulfur^{35} labeled GSH, $6 \times 10^{-5}M$ (sp. act. 5–10 mCi per mM), was reacted with $2 \times 10^{-5}M$ folpet in 17 × 100 mm test tubes containing 5 ml of buffer. Nitrogen was bubbled through the buffer for 30 min before the addition of the reactants. The reactants were stirred magnetically while a stream of nitrogen was blown into the tube above the surface of the solution. The reaction was terminated at the end of 30 min by the addition of 2 mequiv of *N*-ethylmaleimide (NEM) for each mequiv of the thiol originally present (Ellis, 1966). A control sample containing no fungicide was reacted in a similar manner. Air oxidation of $GS^{35}H$ to labeled GSSG was accomplished by bubbling air through the reaction mixture for 2 hr, followed by addition of NEM to the sample.

The conditions were the same when S^{35} folpet (sp. act. 1–2 mCi per mM) was reacted with unlabeled GSH, except the reaction mixture contained 20 ml of buffer and either 3 to 1 or 6 to 1 molar ratios of thiol to fungicide. No NEM was used to terminate the experiment. Determination of gaseous products from the reaction was by a previously described method (Siegel and Sisler, 1968a). At the end of 60 min, the reaction mixture was taken to dryness under vacuum at 45° C and resuspended in 1.5 ml of 80% ethanol.

The distribution and identification of S^{35} labeled products from the above reactions were determined by paper radiochromatography and cochromatography with known compounds. Aliquots were spotted on Whatman No. 1 filter paper and chromatographed in the descending direction for 12 to 144 hr with the upper phase of 1-butanol:acetic acid:water (4:1:5) solvent. Chromatograms were air dried and scanned for areas of radioactivity with a Nuclear Chicago Actigraph III scanner and digital integrator. After scanning, the chromatograms were developed for ninhydrin positive compounds with 0.1% ninhydrin spray reagent (Brinkmann Industries, Inc., Westbury, N.Y.).

Product formation was also determined in nonlabeling experiments in which captan ($5 \times 10^{-5}M$) was reacted with GSH at ratios of 3 to 1 to 12 to 1 of thiol to fungicide. These experiments were carried out under nitrogen and in buffer as previously described. At the end of 30 min the ultraviolet absorption spectra of the samples were determined from 340 to 220 nm in a Beckman DB spectrophotometer.

The rate of formation of the UV absorbing product(s) was determined at 269 nm in a model 2000 Gilford spectrophotometer. The rate of breakdown of folpet in the presence of the above mentioned concentrations of GSH was determined colorimetrically at 447 nm, as previously described (Siegel and Sisler, 1968a).

Reactions of S^{35} Labeled GSH and GSSG with Thiophosgene. $GS^{35}H$ ($6 \times 10^{-5}M$) was reacted with concentrations of thiophosgene from 1×10^{-4} to $1 \times 10^{-3}M$. The use of the buffer, nitrogen, NEM, and paper radiochromatography was the same as previously described.

$GS^{35}H$ ($6 \times 10^{-5}M$) in buffer was converted to labeled

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Table I. Chromatographic Distribution of the Reaction Products of Folpet and Reduced S^{35} Glutathione

Compounds	R_f	Control 0 Time % [b]	Control N_2 %	Control Air %	Treated [a] N_2 %
GSSG	0.12	9.7	9.5	78.2	65.2
GSH	0.42	82.0	81.0		4.6
I-1	0.16				9.2
I-2	0.20			12.9	8.1
I-3	0.28				6.2

[a] 3:1 molar ratio of GSH to fungicide. [b] % distribution of radioactivity.

Table II. Distribution of S^{35} from Folpet after Reaction with Reduced Glutathione

Fraction	Ratio [a] 6:1 % S^{35} [b]	Ratio [a] 3:1 % S^{35} [b]
Reaction medium at 60 min	48	35
Concentrated reaction medium	27	18
Total radioactivity after chromatography	12.5	8.8
Chromatographic distribution of radioactivity		
Compounds / R_f		
GSSG 0.07	0	
II-1 0.10	4.5	
II-2 0.17	3.7	
II-3 0.24	1.8	
II-4 0.40	1.3	
II-5 0.85	1.2	

[a] $3.6 \times 10^{-4}M$ or $1.8 \times 10^{-4}M$ GSH reacted with $6 \times 10^{-5}M$ S^{35} folpet. [b] % of total radioactivity added to the reaction medium.

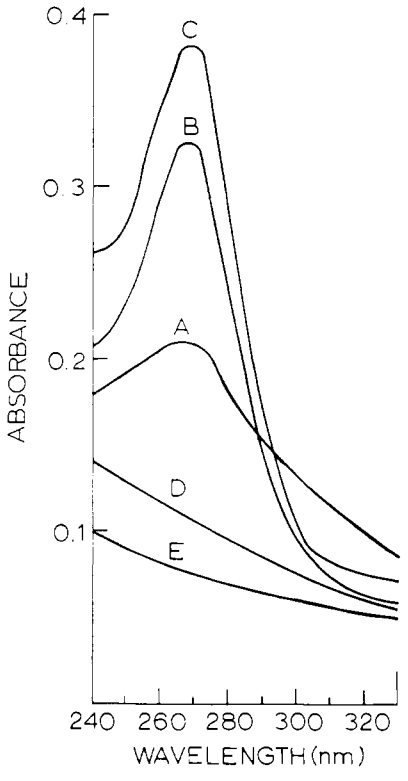

Figure 1. Ultraviolet absorption spectra of the reaction product(s) formed between various molar concentrations of GSH and captan. A, 3 to 1 ratio; B, 6 to 1 ratio; C, 12 to 1 ratio; D, GSSG blank ($3.0 \times 10^{-4}M$); E, GSH blank ($3.0 \times 10^{-4}M$)

GSSG by bubbling air through the reaction mixture. The S^{35} GSSG ($3.0 \times 10^{-5}M$) was reacted with various concentrations of thiophosgene ($2.5 \times 10^{-4}M$–$2.5 \times 10^{-3}M$) buffer. No nitrogen or NEM was used in these experiments.

Radioactivity of aqueous and nonaqueous samples was determined as previously described (Siegel, 1968a), except a model 3310 tri-carb liquid scintillation spectrometer was used for counting purposes.

RESULTS

Reactions of Folpet and Captan with Reduced Glutathione. Cysteine and peptides containing cysteine are extremely reactive and unstable. Ellis (1966) has discussed in detail the use of *N*-ethylmaleimide to stabilize S^{35} labeled thiols in both free and protein bound forms prior to chromatography. After chromatography, the $GS^{35}H$ referred to in this study was a complex of reduced glutathione and *N*-ethylmaleimide.

When folpet was reacted with $GS^{35}H$, the primary reaction product was oxidized glutathione (Table I). At 0 time, 82% of the label was in $GS^{35}H$. Air oxidation and folpet treatment resulted in the transfer of 78 and 65%, respectively, of the total label into GSSG. No oxidation of GSH or formation of new products occurred in the control sample under nitrogen. Two new products (I-1 and I-3), containing approximately 15% of the label, were formed by treatment of GSH with folpet. Compound I-2 was present in the air oxidized sample. Analysis of the samples at the end of the reaction period showed that no loss of S^{35}, as gaseous products, had occurred.

A similar pattern was also noted when S^{35} fungicide was reacted with unlabeled GSH (Table II). The maximum amount of label from folpet associated with the five unknown products was 12%. No label was associated with GSSG. Compounds II-1 and II-2 are the major new products formed in this reaction and appear to be similar to I-1 and I-3 (Table I).

It has been reported (Owens and Blaak, 1960; Richmond and Somers, 1966) that at molar ratios of approximately 3 to 1 of GSH to captan, all the fungicide was consumed. The data in Table I would indicate that at this ratio folpet reacted with all but 4.6% of the GSH. However, as the ratios were increased from 3 to 1 to 6 to 1, the total % label from S^{35} folpet remaining in the reaction mixture and hence in the unknowns was increased (Table II). At the 3 to 1 ratio more gas was evolved (65% *vs.* 52% for the 6 to 1 ratio) with a corresponding decrease of label in the reaction mixture and various reaction products. Ninety percent of the gaseous products was trapped in Viles' reagent and tentatively identified as COS. The identification of COS from the reaction of GSH and captan has been confirmed by Somers *et al.* (1967) on the basis of infrared and gas chromatographic analysis.

Ultraviolet absorption spectra, from 320 to 240 nm, indicated that one or more of the reaction product(s) produced have an absorption peak at 268–269 nm (Figure 1). Increasing the ratio of GSH to captan resulted in significant increases in the absorption peaks. The absorption at ratios of 6 to 1 and 12 to 1 was 35 to 40% greater, respectively, than the absorption of those product(s) formed at the 3 to 1 ratio.

The rate of formation of the UV absorption product(s) and the rate of decomposition of the fungicide was rapid (Figure 2). A large percentage (80%) of these reactions occurred within the first 4 min of the experiment.

Reaction of Thiophosgene with S^{35} GSH and GSSG. Thiophosgene and S^{35} labeled GSH were reacted in order to determine the number and distribution of the reaction products (Figure 3). Scan A represents the unreacted control under

Table III. Chromatographic Distribution of the Reaction Products of S^{35} Labeled GSSG[a] and Thiophosgene

Compounds	R_f	S=CCl_2 Concentration: Zero % [b]	$2.5 \times 10^{-4}M$ %	$5 \times 10^{-4}M$ %	$1 \times 10^{-3}M$ %	$2.5 \times 10^{-3}M$ %
III-1 (GSSG)	0.17	75	57	48	39	27
III-2	0.25	15	14	13	12	13
III-3	0.29–0.44		10	13	16	21
III-4	0.58		6	14	21	28

[a] $3 \times 10^{-5}M$ S^{35} GSSG. [b] % distribution of radioactivity.

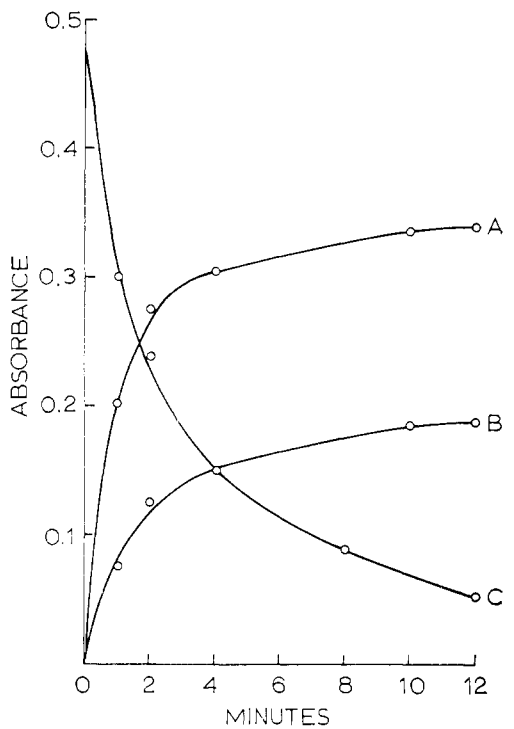

Figure 2. Rate of formation of ultraviolet absorbing product(s) and rate of decomposition of captan from the reaction of the fungicide with GSH. A and B, Rate of formation of UV absorbing product(s). Molar concentrations were 3 to 1 and 12 to 1 of thiol to fungicide, respectively. C, Rate of decomposition of captan. Molar concentration was 3 to 1 of GSH to fungicide

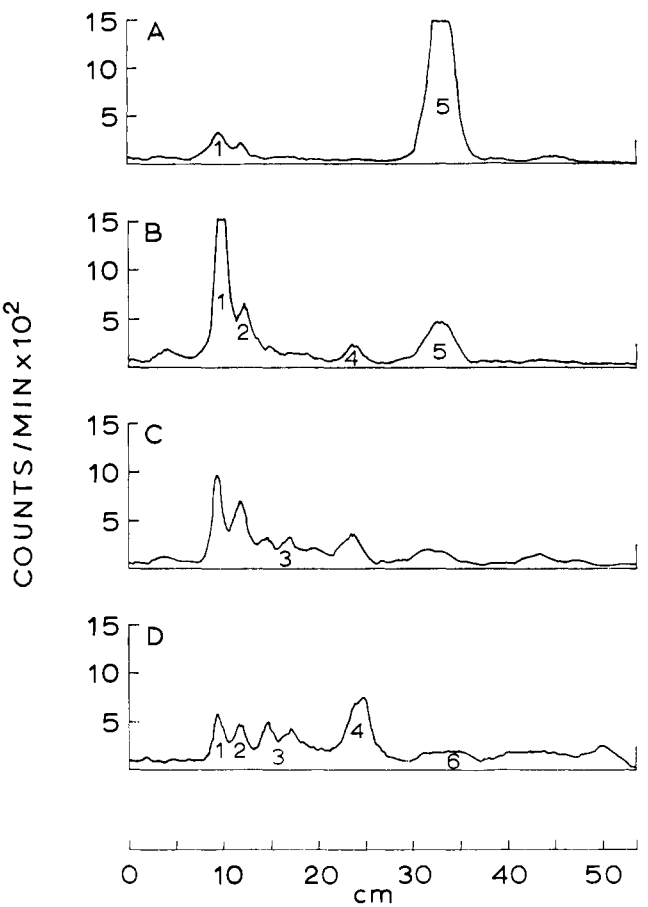

Figure 3. Radioactivity scans of paper chromatograms of the reaction products formed between $6 \times 10^{-5}M$ $GS^{35}H$ and various concentrations of thiophosgene. Scan A, control: B–D: $1 \times 10^{-4}M$, $3.3 \times 10^{-4}M$, and $1 \times 10^{-3}M$ S—CCl_2, respectively

nitrogen. Peaks 1 and 5 are GSSG and GSH, respectively. With increased concentrations of S=CCl_2 (scans B–D), destruction of GSH and the formation of new products occur. Two interesting phenomena are illustrated in scans B–D. At $1 \times 10^{-4}M$ S=CCl_2, the reaction resulted primarily in the formation of GSSG by oxidation of GSH with only small amounts of the label in new products. However, as the concentrations of S=CCl_2 were increased to $3.3 \times 10^{-4}M$ and $1 \times 10^{-3}M$, new metabolites were formed (Peaks 3, 4, and 6) apparently from GSSG since all the GSH had been reacted. The areas on the scans designated as numbers 3 and 6 represent two or three individual products.

That thiophosgene is capable of reacting with GSSG is further illustrated by the data in Table III. The amount of the label present in GSSG (III-1) in the reaction mixture at 0 time was 75% of the total added. Addition of $2.5 \times 10^{-4}M$ to $2.5 \times 10^{-3}M$ S=CCl_2 resulted in a decrease of the S^{35} in GSSG from 75% to 27%. Correspondingly, the % label in compounds III-3 and III-4 was increased. The area designated as III-3 contained a wide zone of radioactivity on the chromatogram (R_f 0.29–0.44) and may represent more than one compound. Compounds III-3 and III-4 appear to be similar to those in Figure 3 in the corresponding numbered peaks. However, the UV absorbing compound present when GSH was reacted with S=CCl_2 could not be recovered from the GSSG reaction.

DISCUSSION

Thiophosgene reacts with the amino and thiol groups of cysteine (Lukens and Sisler, 1958) or the cysteine contained in the tripeptide glutathione (Liu and Fishbein, 1967; Richmond and Somers, 1968) to form a UV absorbing thiazolidine ring compound.

Although a UV absorbing product was found in this study, the reaction of the fungicide and thiophosgene with GSH appeared to be more complex than previously indicated.

The primary product formed by the reaction of GSH and folpet was GSSG. Approximately 10 to 15% of the GSH was converted into at least two to five new unidentified prod-

ucts. These compounds contained all or a portion of the $SCCl_3$ moiety from the fungicide as they were formed in the presence of S^{35} folpet.

The formation of derivatives appears to be a function of the concentration of the thiol and reactivity of the released $SCCl_3$ moiety. Thiophosgene, the primary reactive decomposition product, tends to form faster than it is reacted with (Owens and Blaak, 1960; Siegel and Sisler, 1968a) and is highly unstable in aqueous solutions (Richmond and Somers, 1968). My data suggest that the UV absorbing compound(s) formed very rapidly as the intact fungicide was consumed. The release of large quantities of gaseous products (primarily COS) and the small amounts of derivatives produced, however, would indicate the unstable nature of the released $S{=}CCl_2$ and its apparent low reactivity with GSH. The product yields of this reaction *in vitro* can be increased by addition of excess concentrations of GSH in the presence of the intact fungicide. Both the labeling and UV absorption data support this conclusion.

Derivative formation was also demonstrated by directly reacting thiophosgene with GSH. Various concentrations of $S{=}CCl_2$ caused the thiol to be either partially or completely reacted, forming GSSG and a series of new products. The distribution of all the reaction products was dependent on the concentration of $S{=}CCl_2$ and the amount of the GSH reacted. It was demonstrated that some of the new products formed in the reaction of $S{=}CCl_2$ with GSH could also be found when thiophosgene was reacted with GSSG.

It is apparent that the released $SCCl_3$ moiety or its decomposition product $S{=}CCl_2$ is capable of reacting with both the reduced and oxidized forms of glutathione to produce a series of new products or derivatives. The new derivatives formed *in vitro* between intact fungicide and GSH are not the primary products of this reaction. However, the formation of new products or derivatives in fungicide treated cells of *Saccharomyces pastorianus* will be shown in a subsequent paper (Siegel, 1970), to predominate over the formation of GSSG.

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