Reactions of Certain Trichloromethyl Sulfenyl Fungicides with Low Molecular Weight Thiols

In vivo Studies with Cells of *Saccharomyces pastorianus*

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The reactions of folpet with low molecular weight thiols from cells of *Saccharomyces pastorianus* were studied. Distribution of S^{35} in ethanol extracts of cells labeled with S^{35} Na_2SO_4 and treated with fungicide indicated that the radioactivity was associated with a series of new products which originated from GSH. No label could be detected in the disulfide in extracts from treated cells. When cells were allowed to react with S^{35} folpet, all or a portion of the released $SCCl_3$ moiety was contained in a series of derivatives having R_f values similar to many of the products found in treated cells labeled with Na_2SO_4. Fungistatic and fungitoxic effects were correlated with the destruction of GSH and incorporation of increasing amounts of S^{35} folpet into the low molecular weight fraction. Hypotheses are proposed to explain the absence of GSSG in treated cells and the mechanism and role of derivative formation in toxicity.

In a previous report (Siegel, 1970) it was demonstrated that *in vitro* oxidized glutathione (GSSG) was the primary product formed from the reaction of folpet with reduced glutathione (GSH). A small amount of the GSH was converted into a series of derivatives which contained all or a portion of the trichloromethylthio ($SCCl_3$) moiety released from the fungicide. It was suggested that the low yields of these new products were due to the reactivity characteristics of the $SCCl_3$ moiety.

In this paper the reactions of folpet with low molecular weight thiols, particularly GSH, from cells of *S. pastorianus* will be discussed from the standpoint of the products formed and the role of these reactions in toxicity.

MATERIALS AND METHODS

Cultural and Growth Conditions. The organism used in these studies was *Saccharomyces pastorianus* Hans. The growth medium was a glucose-glycine mineral salt solution, pH 6.0, containing: glucose, 20 g; glycine, 1.0 g; KH_2PO_4, 3 g; $MgCl_2$, 0.5 g, and distilled water to make 1 l. Vitamins and trace elements were added as previously described (Lukens and Sisler, 1958). Sulfur, equivalent to 4.96 mg per l., was added separately to the growth medium from a stock solution which contained $(NH_4)_2SO_4$, 1.5 g and $MgSO_4 \cdot 7H_2O$, 1 g per 100 ml.

In experiments in which the cells were to be labeled from a $Na_2S^{35}O_4$ source (20 to 60 mc per mM), 80 μCi was added to the growth medium just prior to inoculation. Cells were grown on a reciprocating shaker at 30° C and harvested during log-phase growth at approximately 1.2 mg dry wt per ml (30–36 hr). Cells were separated from the culture medium by centrifugation, washed twice with distilled water, and resuspended in a reaction medium (Siegel and Sisler, 1968a) to a final concentration of 1.75 mg dry wt per ml.

Reaction of $Na_2S^{35}O_4$ Labeled Cells with Folpet. Cells resuspended in the reaction medium were stirred at 30° C, and at 15 min intervals 6 μg per ml ($2 \times 10^{-5}M$) folpet in 95% ethanol was added until a total of 42 μg per ml ($1.4 \times 10^{-4}M$) of the fungicide was present. Immediately prior to each addition of fungicide, 30 ml of cell suspension (45 mg dry wt) was removed, centrifuged, and washed once with cold distilled water. The cells were extracted three times for 30 min each with 80% ethanol containing $1 \times 10^{-4}M$ *N*-ethylmaleimide (NEM). At 0 and 18 μg per ml ($6 \times 10^{-5}M$) concentrations of folpet, the cells were divided and one half the sample extracted with ethanol minus NEM. The ethanol extracts for each concentration of fungicide were combined and made to a final volume of 10 ml with 80% ethanol. The distribution and identification of S^{35} label in the ethanol soluble fraction were determined by use of paper radiochromatography as previously described (Siegel, 1970a).

The toxicity of folpet added to cells in the above mentioned sequence was determined. Immediately prior to the addition of each aliquot of fungicide, 2 ml of cell suspension was removed and allowed to incubate at 30° C for 1 hr. Aliquots of cells (0.3 ml) were added to test tubes containing 9.7 ml of glucose-mineral salt medium (Lukens and Sisler, 1958). Tubes were shaken at 30° C and rates of growth were determined by absorbance at 450 nm. The intervals between the absorbancy readings were corrected for the 15 min interval delay between each addition of fungicide. The effect of the fungicide on growth was measured by determining the time required for treated and untreated cells to attain log-phase growth (1.32 mg dry wt per ml).

Experiments were designed to measure the evolution of any sulfur gases from S^{35} labeled cells treated with either captan or folpet. Cell suspensions in reaction medium were prepared as previously described. Captan and folpet were added as 6 μg per ml aliquots at 5 min intervals until a total of 42 μg per ml were present. Prior to the addition of the fungicides and 60 min after the addition of the last aliquot, the cells and medium were analyzed for radioactivity (Siegel and Sisler, 1968a).

Reaction of Cells with S^{35} Labeled Folpet. Cultural conditions, preparation of cell suspensions, use of the reaction medium, concentrations of the fungicides, preparation of ethanol extracts, and paper radiochromatography were the same as described above, except the cells were reacted with S^{35} folpet (sp. act. 1–2 mCi per mM).

Determination of radioactivity in aqueous and nonaqueous solutions was by liquid scintillation spectrometry (Siegel, 1970).

S^{35} folpet was purchased from Mallinckrodt/Nuclear, Inc., St. Louis, Mo.

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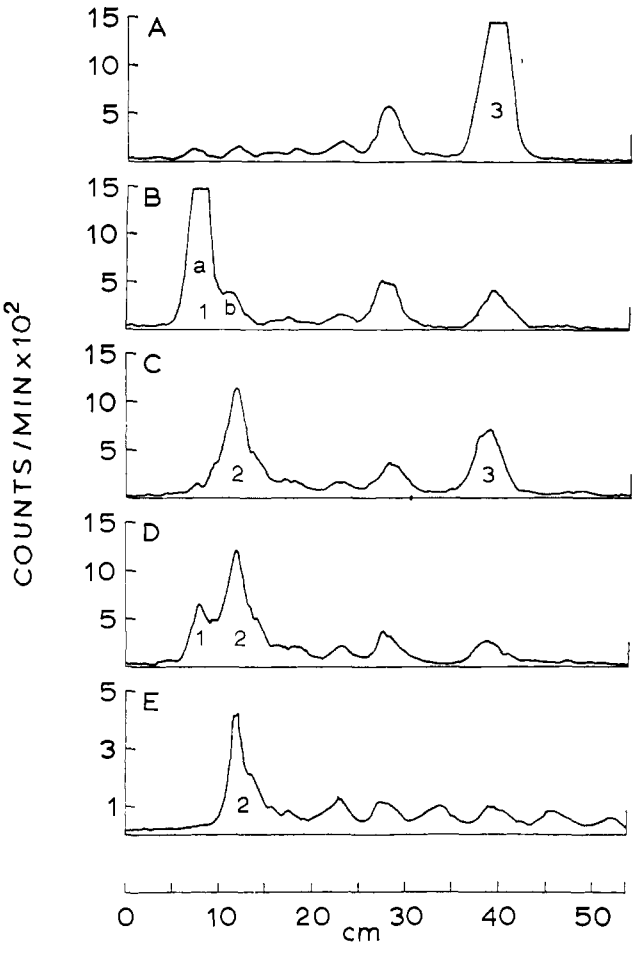

Figure 1. Radioactivity scans of paper chromatograms of ethanol extracts of yeast cells labeled with either S^{35} Na_2SO_4 or folpet. Chromatography was for 72 hr. Scans A and B, $S^{35}O_4$ labeled cells, not treated with fungicide and extracted with and without NEM, respectively. Scans C and D, $S^{35}O_4$ labeled cells treated with folpet and extracted with and without NEM, respectively. Scan E, unlabeled cells treated with S^{35} folpet and extracted without NEM

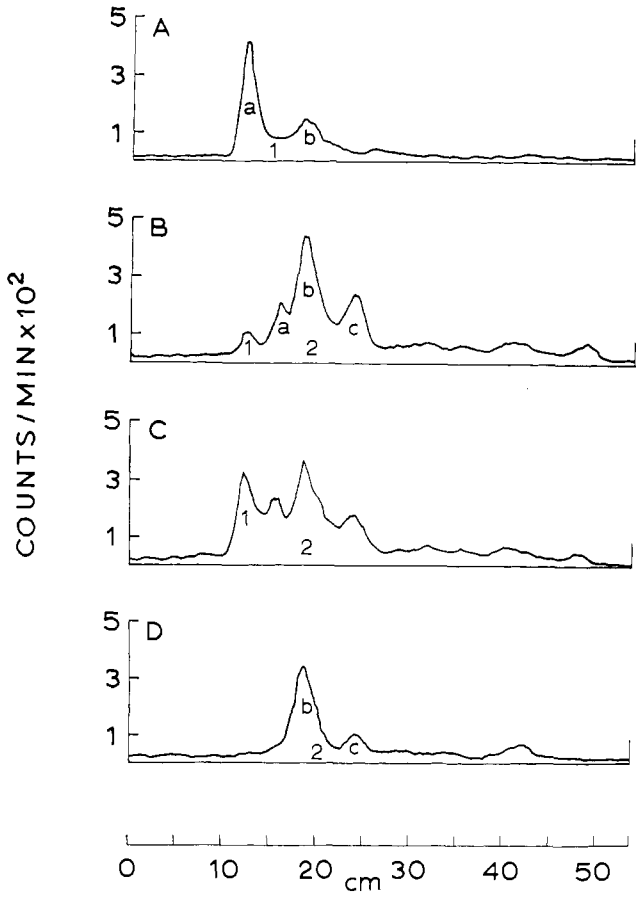

Figure 2. Radioactivity scans of paper chromatograms of ethanol extracts of yeast cells labeled with either S^{35} Na_2SO_4 or folpet. Chromatography was for 144 hr. Scan A, $S^{35}O_4$ labeled cells, not treated with fungicide and extracted without NEM. Scans B and C, $S^{35}O_4$ labeled cells treated with folpet and extracted with and without NEM, respectively. Scan D, unlabeled cells treated with S^{35} folpet and extracted without NEM

RESULTS

The growth medium used in these studies contained 4.96 mg per l. of sulfur. At this level of sulfur, 40 to 70% of the label was taken up by the cells with no appreciable reduction in growth. There were other possible sources of sulfur in the medium, notably sulfur present in the trace element solution and in the inoculum source.

Distribution of radioactivity, as determined by paper chromatography, in ethanol extracts from cells labeled with either S^{35} from SO_4 or folpet is illustrated in Figure 1. *N*-Ethylmaleimide was used to protect the reduced low molecular weight thiols during extraction of the cells (Ellis, 1966). Glutathione is the major thiol in yeast cells (Eddy, 1958) and was found to be the primary thiol in these extracts (Figure 1, scan A, peak 3). This compound was identified as the GSH-NEM complex on the paper chromatograms. When NEM was not used during the extraction procedure, air oxidation of all the reduced glutathione occurred (Figure 1, scan B, peak 1a). Under these conditions there was apparently a compound with an R_f similar to that of the GSH-NEM metabolite (Figure 1, scan A, peak 3). In addition, a small amount (15%) of an unknown product (Figure 1, scan B, peak 1b) was formed by air oxidation of GSH.

When $S^{35}O_4$ labeled cells were treated with folpet ($6 \times 10^{-5}M$) and extracted with ethanol and NEM, little GSSG could be recovered. Instead new products were formed (Figure 1, scan C, peak 2). Not all the GSH, however, was destroyed by the concentration of folpet used in this experiment. Scan D clearly indicates that when no NEM was used, extracts from treated cells contained some GSSG. The disulfide present was formed by air oxidation of the remaining unreacted GSH during ethanol extraction.

When unlabeled yeast cells were reacted with S^{35} folpet, the ethanol extract contained numerous labeled derivatives (Figure 1, scan E). The primary peak (2) corresponds to that in scan C.

Evidence which supports the above data is shown in Figure 2, in which the reactants were chromatographed for 144 hr. Peak 2, which represented new product or derivative formation in Figure 1, has now migrated a greater distance from the origin, furthering separation and resolution. Scan A illustrates the air oxidized extracts (minus NEM) from untreated $S^{35}O_4$ labeled cells. Peak 1a is GSSG; 1b the unknown previously mentioned. Scans B and C represent ethanol extract with and without NEM, respectively, from treated cells ($6 \times 10^{-5}M$ folpet). The area of peak 2 is seen to contain three distinct compounds, and GSSG is present in appreciable amounts (Figure 2, scan C, peak 1) only when

NEM was not used during ethanol extraction of the treated cells. The extract from S^{35} folpet treated cells contains areas of radioactivity (Figure 2, scan D, peaks 2b and c) which have the same R_f's as those in scans B and C.

The percent distribution of GSH in ethanol extracts from cells labeled with $S^{35}O_4$ and treated with 0 to 42 μg per ml (0 to 1.4 × $10^{-4}M$) folpet is illustrated in Figure 3. Approximately 40% of the total S^{35} in low molecular weight constituents was in the form of GSH. With increased concentrations of fungicide, the GSH present in the cell decreased until all was reacted with at 36 μg per ml of folpet. The reaction of folpet with low molecular weight ethanol soluble compounds can be further studied by reacting unlabeled cells with various concentrations of S^{35} folpet. Radioactivity and, hence, derivative formation increased in the ethanol soluble fraction up to 24 μg per ml (8 × $10^{-5}M$), where incorporation ceased. At this concentration of fungicide, approximately 75% of the GSH has been consumed. The relationship between destruction of GSH, incorporation of S^{35} label from folpet into the ethanol fraction, and toxicity is also illustrated by Figure 3. With each increase in concentration of folpet, treated cells required a longer time interval to attain the same level of growth as the untreated cells. From 6 to 18 μg per ml of fungicide, this interval was 13 to 20 hr, respectively, as compared to 12 hr for untreated cells. The interval increased dramatically at 24, 30, and 36 μg per ml, being 32, 49, and 78 hr, respectively. At 78 hr, only slight growth had occurred with the 42 μg per ml sample. Fungistatic effects therefore occurred up to 30 and 36 μg per ml, and fungitoxic effects occurred above 36 μg per ml. At 36 and 42 μg per ml of fungicide, less than 3% of the GSH remained in the treated cells.

Lukens (1969b) has suggested that "the loss of thiol sulfur from an organism treated with captan is evidence of irreversible thiol removal following treatment." This statement was based on the observation that S^{35} evolution, as CS_2, occurred from captan treated $S^{35}O_4$ labeled cells (Lukens, 1964). This observation could not be substantiated. In all experiments in which captan or folpet were reacted with $S^{35}O_4$ labeled cells, no label was lost from the treated samples. This supports the conclusions of Somers *et al.* (1967), who reported that cells of *Neurospora crassa* treated with captan evolved COS, which originated entirely from the sulfur in the fungicide.

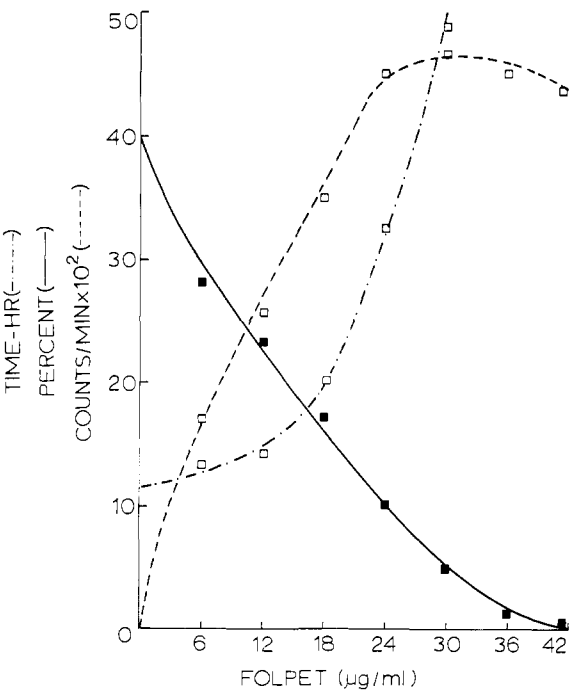

Figure 3. The relationship between toxicity and the percent distribution of S^{35} label in ethanol extracts from cells of *S. pastorianus* treated with various concentrations of folpet. (——) = % S^{35} in GSH from extracts of cells labeled with $Na_2S^{35}O_4$. (- - - -) = radioactivity in extracts of cells labeled with S^{35} folpet. (-·-·-) = time in hr required for cells to attain log-phase growth

DISCUSSION

When captan or folpet was reacted with GSH *in vitro*, the primary product was the disulfide (Siegel, 1970). This disulfide, however, was not detected in extracts from fungicide-treated cells. Instead, cellular GSH was apparently converted to a series of new products.

The cellular data conflict not only with test tube observations but also with a report that when *Neurospora crassa* conidia were incubated with S^{35} captan, the label was incorporated into two primary products, GSSG and a UV absorbing compound postulated to be a Thiazolidine ring derivative formed from GSH and $S{=}CCl_2$ (Richmond and Somers, 1968).

Why was GSSG apparently recovered from captan treated *Neurospora* conidia but not yeast cells, and how can the conversion of almost all the cellular GSH to products other than the disulfide be accounted for?

It is possible that product formation differed in the two species and that this accounts for the differences in formation and recovery of GSSG. To accept this hypothesis would discount many data which suggest that these fungicides have reaction mechanisms that are similar in a wide range of organisms (Lukens, 1969a,b; Siegel, 1971). A more likely explanation involves methodology, particularly chromatographic and radioscanning techniques, which appear to be crucial in separation and identification of labeled metabolites. The majority of the S^{35} labeled unknowns (Figure 1, scan C, D, and E, peak 2) have R_f values very close to that of authentic GSSG. Chromatography for short durations (24 to 36 hr) will not separate these compounds from GSSG. Chromatographic determinations for longer periods, coupled with co-chromatography of knowns, and conversion by air oxidation of GSH to GSSG in extracts from both treated and untreated cells suggest that, if the disulfide was present, it would have been recovered.

The lack of GSSG and the increased yields of new products in treated cells may be explained by the reactivity characteristics of the released $SCCl_3$ or $S{=}CCl_2$ moieties. *In vitro* low reactivity of the moieties with GSH was denoted by the presence of small amounts of derivatives and large quantities of gaseous products (Siegel, 1970). Reactivity of these moieties may be higher in cells due to the closed system involved and the presence of high concentrations of either thiols or their disulfides. Data support this hypothesis. Siegel and Sisler (1968a) reported that increasing the number of cells in the presence of S^{35} folpet appreciably decreased the amount of label lost as gaseous products and increased the concentration of label in the cells. Siegel (1970) demonstrated that increased yields of derivatives occurred when concentrations of the thiol were above those necessary to react all the fungicide, and when $S{=}CCl_2$ was reacted directly with GSH or GSSG. The amount of thiol or disulfide reacted was correlated to the concentrations of the $S{=}CCl_2$. Thus, GSSG may have been a reaction product but was destroyed before it could be re-

covered from treated cells. Alternatively GSSG may never be a product, but instead a direct transfer of the entire $SCCl_3$ moiety to cellular thiols occurs. Such a transfer mechanism has been demonstrated *in vitro* as $SCCl_3$-protein complexes which were shown to be unstable in the presence of other thiols (Siegel and Sisler, 1968b; Siegel, 1971). The formation and subsequent breakdown of a GS(H) $SCCl_3$ complex could yield a variety of products other than the disulfide, which may or may not contain any moiety from the fungicide.

One of the proposed mechanisms of action of captan and folpet is their irreversible reactions with proteins (Lukens and Sisler, 1958; Richmond *et al.*, 1967; Siegel and Sisler, 1968b; Siegel, 1971). Although the reactions of the fungicides with low molecular weight thiols have been considered a detoxification mechanism (Richmond and Somers, 1966) it is possible to correlate destruction of GSH with fungistatic and fungitoxic effects. The greater the loss of GSH, the slower the recovery of cellular growth. If little GSSG were present in treated cells, then regeneration of GSH by a reductase mechanism, as suggested by Richmond and Somers (1966), would not occur.

Our data may indicate that no single specific reactive site or type of reaction by the fungicide in the cell is responsible for toxicity. Fungicidal effects may be based on a complex multiplicity of reactions with both low and high molecular weight cellular constituents.

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