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# Effect of Thiol-binding Reagents on the Metabolism of *Chromatium* D

R. E. HURLBERT
*Department of Bacteriology and Public Health, Washington State University, Pullman, Washington 99163*

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Thiosulfate, intracellular sulfur, and acetate utilization by *Chromatium* D were inhibited by iodoacetamide, *N*-ethylmaleimide (NEM), *p*-hydroxymercuribenzoate (CMB), and $HgCl_2$. Pyruvate, sulfide, and sulfite utilization were insensitive to iodoacetamide, and were less sensitive to NEM, CMB, and $HgCl_2$ than thiosulfate, intracellular sulfur, and acetate utilization. The effect of the thiol-binding reagents on cell viability was dependent upon the method of exposure; cells exposed to the thiol-binding reagents in the presence of thiosulfate were protected from the killing action of these agents, but not from their inhibitory effect. Although the inhibitory effects of the thiol-binding reagents could, in some cases, be attributed to their effect on viability, they were inhibitory under conditions where viability was unaffected. The most straightforward results were obtained with iodoacetamide, which revealed a sharp separation in sensitivities between the systems tested but had no effect on cell viability. The results are consistent with the hypothesis that thiols are involved in thiosulfate, intracellular sulfur, and acetate utilization.

Sulfenyl derivatives have been suggested as intermediates in the oxidation of inorganic sulfur compounds by both thiobacilli (5, 9, 13) and *Thiorhodaceae* (8). Trudinger reported that several thiol-binding reagents inhibit the oxidation of thiosulfate by *Thiobacillus neapolitanus* (9); however, no studies on the effect of thiol-binding reagents on the oxidation of reduced inorganic sulfur compounds by *Thiorhodaceae* have been reported. Trudinger (9) did not determine whether the observed inhibition of thiosulfate oxidation was the result of specific damage to the thiosulfate oxidizing system or simply the result of a general disruption of cellular metabolism as a result of the toxicity of the reagents employed.

*Chromatium* D is capable both of photolithotrophic growth on carbon dioxide and a variety of reduced inorganic sulfur compounds, including elemental sulfur, sulfide, sulfite, and thiosulfate, and of photo-organotrophic growth on a number of organic compounds (2, 8). Because of these characteristics, *Chromatium* D seemed a suitable organism for studying the effects of thiol-binding reagents on both sulfur and carbon metabolism in *Thiorhodaceae*, and for determining whether any specificity is involved.

In the present study, the effects of several thiol-binding reagents on thiosulfate, intracellular sulfur, sulfide, sulfite, pyruvate, and acetate utilization by *Chromatium* D were investigated.

## MATERIALS AND METHODS

*Organism and culture media.* The culture of *Chromatium* strain D, its stock maintenance, and its growth in liquid medium were as previously described (3). Thiosulfate-grown cells were employed in all cases.

*Preparation and incubation of suspensions.* Cultures were incubated until growth was complete (4 to 11 days); then the cells were sedimented at room temperature by centrifugation at 2,500 × *g* for 15 min. The cells were washed once in a phosphate-bicarbonate buffer solution [0.1 M potassium phosphate buffer (*p*H 7.15)–0.06 M sodium bicarbonate; final *p*H 7.7] previously flushed with an $N_2$ plus 5% $CO_2$ gas mixture, and were resuspended in the same solution to a concentration of 0.2 to 0.4 mg of protein per ml. The cell suspensions were distributed in glass vessels in a 30 C water bath, 25 cm from a bank of 100-w incandescent bulbs. All experiments were done under anaerobic conditions (2).

*Analytical methods.* Thiosulfate, pyruvate, radioactivity, and viable counts were determined as previously described (2). Sulfide and sulfite were determined by iodine titration under conditions that minimized autooxidation.

Whole cell protein was determined by a modification of the Biuret procedure of Hurlbert and Lascelles (3). The modification consisted of washing the cells

in 2.5% trichloroacetic acid prior to pigment extraction; this procedure increased the effectiveness of the pigment extraction and resulted in more consistent results. Bovine serum albumin was used as a protein standard.

*Chemicals.* *N*-ethylmaleimide (NEM), *p*-hydroxymercuribenzoate (CMB), and iodoacetamide were obtained from the Sigma Chemical Co., St. Louis, Mo., and were used without further purification. Fresh solutions of NEM, CMB, iodoacetamide, sulfide, and sulfite were prepared daily. CMB was dissolved in 0.1 M tris(hydroxymethyl)aminomethane, *p*H 8.0. Outer labeled $^{35}S$-thiosulfate was obtained from New England Nuclear Corp., Boston, Mass., and sodium acetate-*1*-$^{14}C$ was obtained from Calbiochem, Los Angeles, Calif.

## RESULTS

*Effect of thiol-binding reagents on thiosulfate utilization.* Of the thiol-binding reagents tested, $HgCl_2$ was the most effective inhibitor of thiosulfate utilization, followed, in order of their inhibitory action, by NEM, CMB, $CdCl_2$, and iodoacetamide. Lead chloride did not have a significant effect on thiosulfate utilization at the concentration employed (0.1 mM). Iodoacetamide, $CdCl_2$, NEM, and CMB required a lag before their full inhibitory effects were expressed (Fig. 1).

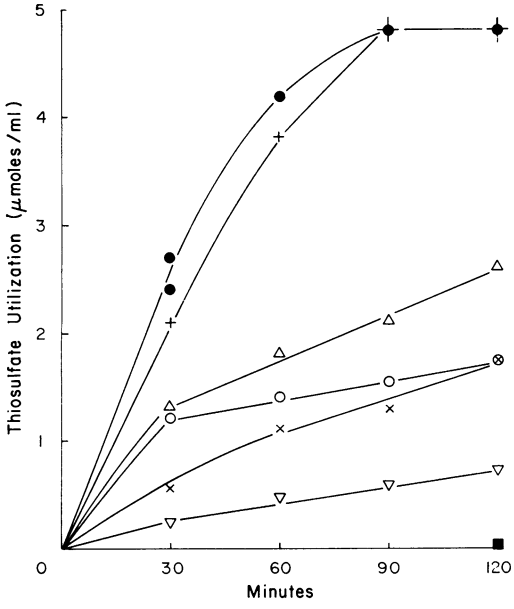


FIG. 1. *Effect of thiol-binding reagents on thiosulfate utilization. Thiosulfate (5 μmoles/ml) was added to a washed-cell suspension in the dark. The cell suspension was distributed to vessels in the light containing the appropriate thiol-binding reagent (0.1 μmole/ml), and thiosulfate utilization in the absence of thiol-binding reagent (●) and in the presence of NEM (▽), CMB (×), $HgCl_2$ (■), iodoacetamide (△), $CdCl_2$ (○), and $PbCl_2$ (+) was determined.*

In an attempt to reverse the inhibition of thiol-binding reagents, cells were exposed to 0.1 mM NEM, CMB, $HgCl_2$, and iodoacetamide for 10 min under standard conditions; then the cells were washed twice with 5 mM sulfide, L-cysteine, or reduced glutathione in the phosphate-bicarbonate buffer solution. After the first wash, the cells were suspended in the second wash solution and were exposed to the light for 10 min before further centrifugation. Finally, the cells were washed once in thiol-free phosphate-bicarbonate buffer solution and were resuspended to the original volume in the same solution. Thiosulfate was added to a final concentration of 10 mM, and the cells were incubated as before.

Not only did none of the thiol reagents completely reverse the inhibition of any of the thiol-binding reagents, but, in some cases, thiol exposure actually enhanced the inhibition of the thiol-binding reagents (Table 1). Cysteine stimulated thiosulfate utilization in the control and CMB-exposed cells, but had no effect on the other systems. Sulfide partially restored thiosulfate utilization in iodoacetamide-treated cells, but inhibited utilization in CMB- and $HgCl_2$-treated cells; $HgCl_2$-treated cells turned black upon exposure to sulfide and remained so throughout the subsequent treatments. Gluta-

TABLE 1. *Reversal of inhibition by thiols*[a]

| Inhibitor | Thiosulfate utilization following thiol wash (μmoles/ml)[b] | | | |
|---|---|---|---|---|
| | None | $Na_2S$ | Cysteine | Glutathione |
| None | 2.57 | 2.45 | 3.12 | 2.45 |
| CMB | 0.81 | 0.51 | 1.32 | 0 |
| NEM | 0.41 | 0.56 | 0.56 | 0.39 |
| Iodoacetamide | 1.17 | 1.52 | 1.16 | 0.31 |
| $HgCl_2$ | 0.41 | 0.11 | 0.32 | 0.42 |

[a] Cells were incubated in the presence of 0.1 mM solutions of the thiol-binding reagents for 10 min. Each suspension was then divided and washed twice with a phosphate-bicarbonate buffer solution containing 5 mM of the indicated thiols. After the first thiol wash, the cells were suspended in the wash solution in the light for 10 min. Following a final wash in phosphate-bicarbonate buffer solution, the cells were resuspended to their original volume and gassed with $N_2$ plus 5% $CO_2$; $S_2O_3^=$ (10 μmoles/ml) was then added.

[b] After 90 min of incubation.

thione inhibited thiosulfate utilization by CMB- and iodoacetamide-treated cells (Table 1).

*Effect of preincubation with thiosulfate on inhibitory action of thiol-binding reagents.* Trudinger observed that NEM, CMB, and iodoacetamide react chemically with thiosulfate, and that the addition of thiosulfate just prior to the addition of these thiol-binding reagents to suspensions of *T. neapolitanus* prevented the inhibition of thiosulfate oxidation by these reagents (9). Although it is clear that prior addition of thiosulfate to *Chromatium* D suspensions does not prevent the inhibition of thiosulfate utilization by the thiol-binding reagents (Fig. 1), it was felt that this treatment might significantly decrease their inhibitory action. To test this possibility, the effect of preincubation with thiol-binding reagents on subsequent thiosulfate utilization was compared to the effect of these reagents on cells actively metabolizing thiosulfate.

Preincubation with thiosulfate had little effect

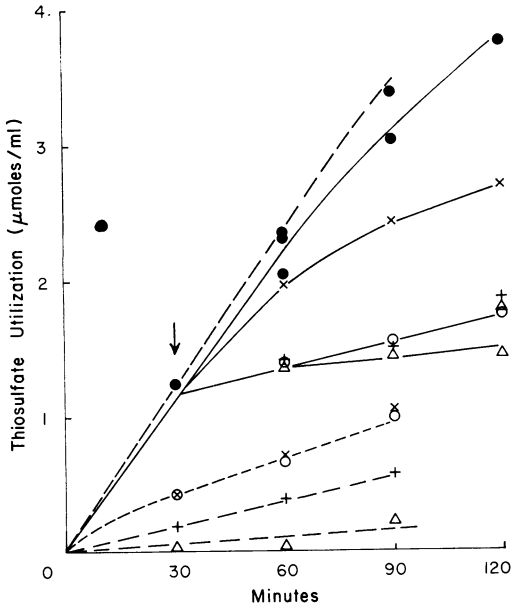


FIG. 2. *Effect of order of exposure of cells to thiol-binding reagents on inhibition of thiosulfate utilization. The washed-cell suspension was divided in half. One half was distributed to tubes containing the respective thiol-binding reagents (0.1 μmole/ml). After incubation for 10 min, thiosulfate (10 μmoles/ml) was added, and thiosulfate utilization (broken lines) was followed. Thiosulfate (10 μmoles/ml) was added to the other half of the cell suspension, which was then distributed to the incubation vessels. After 30 min of incubation (at the arrow), thiol-binding reagents were added (0.1 μmole/ml) and subsequent thiosulfate utilization was followed (solid lines); ●, controls; ○, CMB; +, NEM; ×, iodoacetamide; and △, $HgCl_2$.*

on the inhibition of CMB, NEM, and $HgCl_2$, but it did partially protect the cells from inhibition by iodoacetamide (Fig. 2).

*Effect of thiol-binding reagents on sulfide, sulfite, and intracellular sulfur metabolism.* Sulfide and sulfite utilization were inhibited by 0.1 mM CMB, NEM, and $HgCl_2$, but not by iodoacetamide at the same concentration. Although the sulfide- and sulfite-utilizing systems were inhibited by CMB and NEM, they were less sensitive than the thiosulfate-utilizing system (Table 2).

A threefold increase in the concentration of iodoacetamide did not result in any inhibition of sulfite or sulfide utilization, whereas thiosulfate utilization was inhibited by 85%.

Previous studies have shown that the outer sulfur atom of thiosulfate is converted to intracellular sulfur by *Chromatium* D and that the intracellular sulfur is subsequently oxidized to sulfate, which is secreted into the medium (2, 8). Furthermore, the conversion of intracellular sulfur to sulfate has been observed to be inhibited by molecular oxygen (2). To determine the effect of thiol-binding reagents on the oxidation of intracellular sulfur, cells were exposed to outer-labeled $^{35}S$-thiosulfate, allowed to accumulate $^{35}S$-intracellular sulfur, washed free of the $^{35}S$-thiosulfate, and incubated in the presence of 0.1 mM thiol-binding reagents in a thiosulfate-free medium. The conversion of intracellular sulfur to sulfate was almost completely inhibited by all the thiol-binding reagents employed (Fig. 3).

*Effect of thiol-binding reagents on pyruvate and acetate utilization. Chromatium* D will utilize thiosulfate and pyruvate or acetate simultaneously (Fig. 4). To further study the sensitivity of the thiosulfate-utilizing system to thiol-binding reagents, the effect of thiol-binding agents on the

TABLE 2. *Effect of thiol-binding reagents on sulfide, thiosulfate, and sulfite utilization*[a]

| Substrate | Substrate utilized (%)[b] | | | |
|---|---|---|---|---|
| | CMB | NEM | $HgCl_2$ | Iodoacetamide |
| $S_2O_3^{2-}$ | 36 | 16 | 0 | 54 |
| $S^{2-}$ | 58 | 29 | 0 | 100 |
| $SO_3^{2-}$ | 83 | 64 | 0 | 100 |

[a] Washed cells were incubated anaerobically under standard conditions for 20 min in the presence of the thiol-binding reagents (0.1 μmole/ml). Substrate was then added (5 μmoles/ml), and incubation was continued. After 65 min, substrate disappearance was determined.

[b] Percentage of unexposed cells.

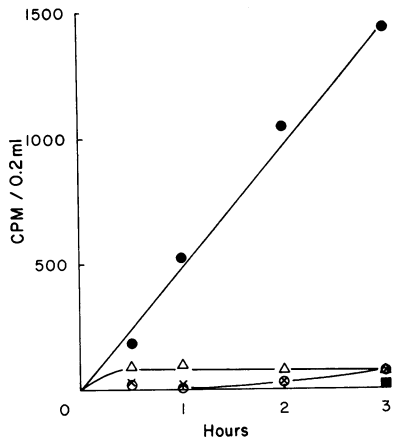


FIG. 3. *Effect of thiol-binding reagents on the metabolism of intracellular sulfur. Washed cells were incubated with outer-labeled $^{35}S$-thiosulfate (10 μmoles/ml, 660 counts/min per μmole) under standard conditions for 95 min; then they were harvested, washed twice with phosphate-bicarbonate buffer, and resuspended in the same solution to their original volumes. The cell suspension was distributed to tubes containing no thiol-binding reagent (●), CMB (○), NEM (×), iodoacetamide (△), or $HgCl_2$ (■), and was incubated under standard conditions with continuous flushing with $N_2$ plus 5% $CO_2$. (All thiol-binding reagents were at a final concentration of 0.1 mM.) At the indicated times, samples were collected; the cells were removed by centrifugation and the radioactivity of a portion of the supernatant fluid was determined.*

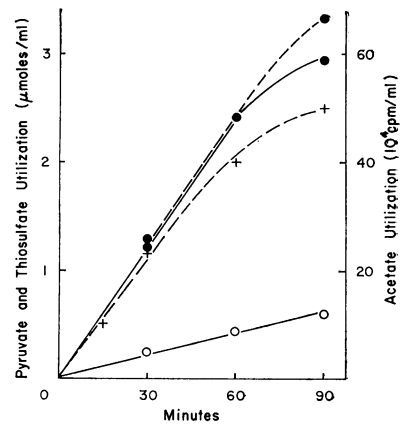


FIG. 4. *Simultaneous utilization of thiosulfate and organic compounds. Thiosulfate (5 μmoles/ml) and pyruvate (2 μmoles/ml) or acetate-1-$^{14}C$ (2 μmoles/ml, 1 μc) were added to washed cell suspensions, which were then incubated under standard conditions with continuous flushing with $N_2$ plus 5% $CO_2$. Thiosulfate (●) and pyruvate (○) utilization were determined as described in Materials and Methods. Acetate (×) utilization was followed by collecting 0.5-ml cell samples on Millipore filters and measuring the incorporation of label into the cells. In one experiment (solid lines), the organic substrate was pyruvate, and in the other (broken lines), it was acetate.*

simultaneous utilization of thiosulfate and pyruvate or acetate was determined.

Pyruvate utilization was inhibited by higher concentrations of CMB, NEM, and $HgCl_2$, but the inhibition was significantly less than that of the thiosulfate-utilizing system at the same concentrations. In addition, at lower concentrations of these thiol-binding reagents, pyruvate utilization was either unaffected or actually stimulated, whereas thiosulfate utilization was still severely inhibited (Fig. 5A, C, D). The apparent stimulatory effect of the thiol-binding compounds on pyruvate utilization is an artifact and is due to the fact that thiosulfate represses pyruvate utilization; i.e., when thiosulfate utilization is inhibited, this repression is released and pyruvate utilization is apparently stimulated. The most marked difference in sensitivity was obtained with iodoacetamide; there was no inhibition of pyruvate utilization at the highest concentration of iodoacetamide employed (0.3 mM), whereas at the same concentration thiosulfate utilization was 90% inhibited (Fig. 5B).

On the other hand, the sensitivity of acetate utilization to the thiol-binding reagents was qualitatively similar to that of the thiosulfate system, in that acetate utilization was inhibited by all the thiol-binding agents tested, including iodoacetamide (Table 3, Fig. 6).

*Effect of thiol-binding reagents on viability.* The manner of exposure to the thiol-binding reagents has a marked effect on cell viability. Exposure of the cells to $HgCl_2$, CMB, and NEM in the absence of thiosulfate resulted in a significant loss in viability during the experimental period, whereas the addition of thiosulfate prior to the addition of these thiol-binding reagents completely protected the cells from the lethal actions of CMB and $HgCl_2$, and partially from NEM. Iodoacetamide did not have a killing effect under either set of exposure conditions (Table 4).

## DISCUSSION

The inhibitory reagents used in this study are thought to affect biological systems by their reaction with critical thiol groups (14–16). On the basis of this assumption, the data presented are consistent with the hypothesis that thiol groups are required for the oxidation of thiosulfate, intracellular sulfur, and acetate by *Chromatium* D. However, since conclusive proof of this

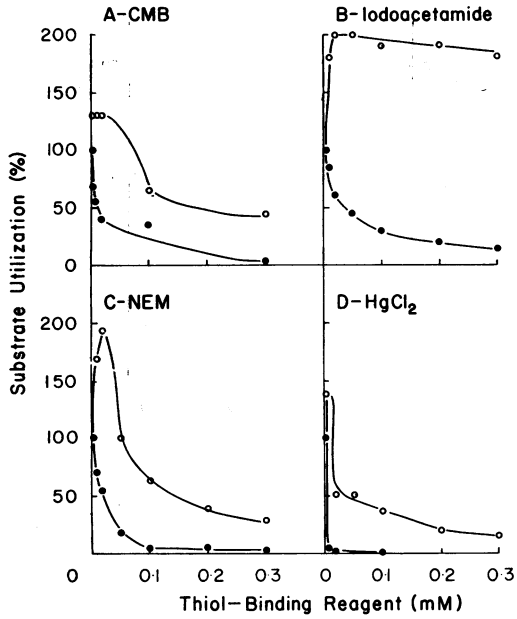


FIG. 5. *Effect of thiol-binding reagents on thiosulfate and pyruvate utilization by Chromatium D. Washed cells were distributed to tubes containing thiol-binding reagents and were incubated anaerobically for 20 min. Thiosulfate (5 μmoles/ml) and pyruvate (2 μmoles/ml) were added to each vessel, and incubation continued until approximately 50 to 70% of the thiosulfate was utilized in the control vessel. Thiosulfate (●) and pyruvate (○) utilization was determined in the cell-free supernatant fluid.*

hypothesis must await a study of the isolated systems, and since some of the inhibitors employed can react with nonthiol groups (1, 15), the involvement of thiols in these processes must be considered tentative.

Selective inhibition of thiol-binding reagents of whole cell systems has been previously reported (15, 16). The selectivity has been explained by concluding either that the sensitive systems contain critical thiol groups that react directly with the thiol-binding reagents or that the inhibition is the summation of the effects of the thiol-binding reagents and numerous other, but less directly related, groups. The insensitive systems are assumed either to lack the sites to react with the inhibitors or to have sites which are inaccessible to the thiol-binding reagents (e.g., because of conformational or permeability barriers; 15, 16).

The variety of results obtained when cells exposed to the various thiol-binding reagents were washed with thiols illustrate the complexity of the system under investigation (Table 1). However, reasonable explanations for some of

TABLE 3. *Effect of thiol-binding reagents on acetate utilization*[a]

| Inhibitor | Substrate utilization | |
|---|---|---|
| | Acetate[b] | Thiosulfate[c] |
| | | *μmoles/ml* |
| None | 53,540 | 2.71 |
| Iodoacetamide | 33,362 | 1.32 |
| CMB | 28,590 | 1.26 |
| NEM | 483 | 0.36 |
| $HgCl_2$ | 12 | 0 |

[a] Washed cells were incubated under standard conditions with the thiol-binding reagents (0.1 μmole/ml) for 20 min. Thiosulfate (5 μmoles/ml) and acetate-*1*-$^{14}C$ (2 μmoles/ml, 1 μc) were then added, and the incubation was continued with continuous gassing with $N_2$ plus 5% $CO_2$.

[b] Counts per min per ml in cell after 60 min of incubation.

[c] Incubated for 75 min.

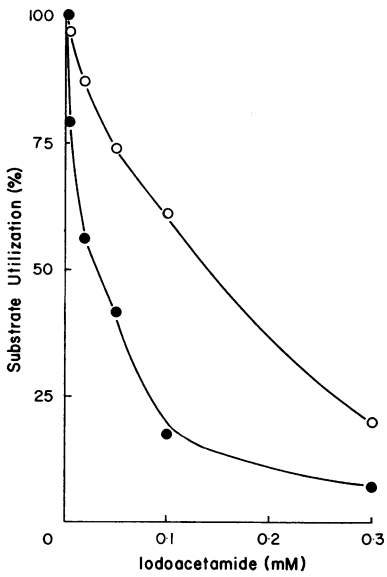


FIG. 6. *Effect of iodoacetamide concentration on the simultaneous utilization of thiosulfate and acetate. Washed cells were incubated with iodoacetamide under standard conditions for 20 min; then thiosulfate (5 μmoles/ml) and acetate-1-$^{14}C$ (2 μmoles/ml, 1 μc) were added. Thiosulfate (●) and acetate (○) utilization were determined as described in Fig. 4.*

these results are available. The enhancement of CMB and $Hg^{++}$ inhibition by sulfide could be due to the precipitation of HgS or the aromatic derivative at critical sites; the observation that $Hg^{++}$-treated cells turn black upon exposure to sulfide supports this possibility. Conversely, the slight reversal of iodoacetamide inhibition by

TABLE 4. *Effect of thiol-binding reagents on cell viability*

| Inhibitor | Concn | $10^6$ viable cells/0.5 ml | | | | | |
|---|---|---|---|---|---|---|---|
| | | Initial | | 20 min | | 90 min | |
| | | A[a] | B[b] | A | B | A | B |
| | mM | | | | | | |
| None | — | 191, 166[c] | 196, 214 | 214, 174 | 158, 202 | 285, 302 | 185, 185 |
| Iodoacetamide | 0.1 | 191, 166 | 196, 214 | 204, 181 | 164, 182 | 151, 160 | 177, 205 |
| | 0.3 | 191, 166 | 196, 214 | — | 151, 137 | — | 170, 185 |
| CMB | 0.1 | 191, 166 | 196, 214 | 172, 176 | 104, 116 | 177, 167 | 133, 137 |
| NEM | 0.1 | 191, 166 | 196, 214 | 172, 161 | 135, 164 | 9, 9 | 10, 8 |
| $HgCl_2$ | 0.1 | 191, 166 | 196, 214 | 187, 182 | 0.5, 0.5 | 183, 186 | 0.1, 0.1 |

[a] Thiosulfate (5 μmoles/ml) was added to cells; cells were then distributed to incubation vessels containing thiol-binding reagents.

[b] Cells were incubated for 20 min, under standard conditions, with the thiol-binding reagents before thiosulfate was added (5 μmoles/ml). The final determination was made 90 min after the addition of the thiosulfate.

[c] Duplicate determinations.

sulfide might be due to a partial removal of the inhibitor from the affected site; such reversibility of iodoacetamide inhibition has been reported (16). On the other hand, there does not seem to be a simple explanation for the different effects of cysteine and reduced glutathione.

The several metabolic systems of *Chromatium* D investigated in this study can be divided, on the basis of their response to iodoacetamide, into a sensitive and an insensitive group; the sensitive group includes the thiosulfate, intracellular sulfur, and acetate utilization systems, and the insensitive group includes the sulfite, sulfide, and pyruvate utilization systems. Although the iodoacetamide-insensitive group was inhibited by the other thiol-binding reagents, the degree of inhibition was always significantly less than with the sensitive group.

The unique behavior of iodoacetamide suggests that its mode of action is different in some way from that of $Hg^{++}$, PMB, or NEM. Although iodoacetamide is known to react rapidly with thiol groups at the *p*H employed in this study (16), it has also been shown to react with methionine and histidine under certain conditions (1), and it must be considered that the reactions of these nonthiols might be responsible for the unique action of iodoacetamide. Alternately, it may be that the permeability characteristics of iodoacetamide are such that only a limited number of sensitive groups are available to react with it. The more effective inhibition of $Hg^{++}$, NEM, and CMB could then be due to their more effective penetration and reaction with additional sensitive sites in the cell. This hypothesis is supported by the observation that at low concentrations these reagents fail to inhibit pyruvate utilization while still inhibiting thiosulfate utilization (Fig. 5). The repressive effects of thiosulfate on pyruvate utilization and its reversal by the thiol-binding reagents were not investigated but they may be the result of competition for some common requirement (e.g., adenosine triphosphate).

Although the location of the postulated iodoacetamide-sensitive groups can only be speculated about, there is some evidence to suggest that they may be located on or at the cell membrane. Several investigations have shown that the S–S bond of thiosulfate is cleaved at the cell membrane of *Chromatium* D (2, 8); thus, any sensitive groups required for this cleavage would be located there. Smith (8) has demonstrated that an enzyme (rhodanese) in *Chromatium* D extracts catalyzes the cleavage of the S–S bond of thiosulfate, and has reported that this enzyme is insensitive to cadmium ions and iodoacetate. This would suggest that rhodanese is not a target of the thiol-binding reagents; however, Smith was only able to demonstrate rhodanese activity with a nonphysiological acceptor ($CN^-$) for the sulfide product of the reaction. Thus, the physiological acceptor for the sulfide is presently unknown, but it may be a thiol (reduced lipoic acid), as was found for beef liver rhodanese (11). Such a requirement could render the reaction sensitive to thiol-binding reagents.

Since thiosulfate cleavage at the cell membrane of *Chromatium* D is usually accompanied by the deposition of the outer sulfur atom of thiosulfate into sulfur granules (2, 8), it is not unreasonable to speculate that the sulfur granules, as well as thiol groups required for their metabolism, are located at the cell membrane; this assumption would be consistent with the observed sensitivity

of the intracellular sulfur utilization system to iodoacetamide as well as to the other thiol-binding reagents.

Although little is known about the intermediary metabolism of reduced sulfur compounds by *Chromatium* D, and hence of the involvement of thiol groups in these processes, there is evidence to indicate that thiol groups are important in the metabolism of reduced sulfur compounds by other organisms (4, 6, 7, 11), and it would seem likely that similar reactions may occur in *Chromatium* D. For example, sulfhydryl compounds have been shown to be involved in the reductive cleavage of thiosulfate by yeast (4), *T. thioparus* (6), and beef liver rhodanese (11).

Trüper and Hathaway (10) have recently reported that the sulfur granules of *Chromatium* D are composed of orthorhombic sulfur. Thiol-requiring mechanisms for the utilization of elemental sulfur and polysulfides have been proposed by Vishniac and Santer (12) and Vishniac and Trudinger (13).

The difference in sensitivity of the two organic metabolic systems investigated in this study further illustrates the selectivity of inhibition that can be obtained with thiol-binding reagents. The apparent proportionality between the inhibition of thiosulfate and acetate utilization (Table 3) is probably due to the fact that both systems have critical groups sensitive to the inhibitors used. This explanation is supported by the observation that, though both systems are sensitive to iodoacetamide, the sensitivity is quantitatively different (Fig. 6).

The fact that simultaneous exposure of the cells to thiosulfate and the thiol-binding reagents protects them from loss in viability (Table 4), yet has little influence on the inhibitory effects of thiosulfate utilization (Fig. 1, 2), further illustrates the selective nature of the thiol-binding reagents and the particular sensitivity of the thiosulfate-utilizing system. It is also interesting to note that all viability determinations were carried out in a lithotrophic medium with thiosulfate as the reduced sulfur substrate; thus, the inhibition of thiosulfate utilization that occurred in the absence of a loss in viability in viability is fully reversible.

ACKNOWLEDGMENTS

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