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Thiosulfate Oxidation and Electron Transport in *Thiobacillus novellus*

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ABSTRACT

ALEEM, M. I. H. (Research Institute for Advanced Studies, Baltimore, Md.). Thiosulfate oxidation and electron transport in *Thiobacillus novellus*. J. Bacteriol. **90**:95–101. 1965.—A cell-free soluble enzyme system capable of oxidizing thiosulfate was obtained from *Thiobacillus novellus* adapted to grow autotrophically. The enzyme systems of autotrophically grown cells brought about the transfer of electrons from thiosulfate to molecular oxygen via cytochromes of the *c* and *a* types; the reactions were catalyzed jointly by thiosulfate oxidase and thiosulfate cytochrome *c* reductase. The levels of both of these enzymes were markedly reduced in the heterotrophically grown organism. Cell-free extracts from the autotrophically grown *T. novellus* catalyzed formate oxidation and enzymatically reduced cytochrome *c* with formate. Both formate oxidation and cytochrome *c* reduction activities were abolished under heterotrophic conditions. The thiosulfate-activating enzyme $S_2O_3^{-2}$-cytochrome *c* reductase, as well as thiosulfate oxidase, was localized chiefly in the soluble cell-free fractions, and the former enzyme was purified more than 200-fold by ammonium sulfate fractionation and calcium phosphate gel adsorption procedures. Optimal activity of the purified enzyme occurred at *p*H 8.0 in the presence of 1.67×10^{-1} M $S_2O_3^{-2}$ and 2.5×10^{-4} M cytochrome *c*. The thiosulfate oxidase operated optimally at *p*H 7.5 and thiosulfate concentrations of 1.33×10^{-3} to 3.33×10^{-2} M in the presence of added cytochrome *c* at a concentration of 5×10^{-4} M. Both enzymes were markedly sensitive to cyanide and to a lesser extent to some metal-binding agents. Although a 10^{-3} M concentration of *p*-hydroxymercuribenzoate had no effect on $S_2O_3^{-2}$-cytochrome *c* reductase, it caused a 50% inhibition of $S_2O_3^{-2}$ oxidase, which was completely reversed in the presence of 10^{-3} M reduced glutathione. Carbon monoxide also inhibited $S_2O_3^{-2}$ oxidase; the inhibition was completely reversed by light.

Among the sulfur bacteria, *Thiobacillus novellus* is the only facultative autotroph (Starkey, 1935*a*, *b*). Thus, Vishniac and Santer (1957) and Santer, Boyer, and Santer (1959) studied the growth characteristics of this organism under both autotrophic and heterotrophic conditions. There is as yet no information regarding the mechanism of action of the enzyme systems involved in inorganic sulfur metabolism by *T. novellus*. The present report is concerned with the electron-transport mechanisms involved in thiosulfate oxidation.

MATERIALS AND METHODS

A culture of *T. novellus*, kindly supplied by R. L. Starkey, was adapted to grow autotrophically, and mass cultures of the autotrophic strain were grown, as described by Vishniac and Santer (1957), in an inorganic medium containing thiosulfate as the oxidizable substrate. After 4 to 5 days of growth at 30 C, the cells were collected in a Sharples centrifuge, washed twice with cold distilled water, and stored at −15 C. The activity of the enzyme systems of the stored cells was stable for several months.

For the preparation of cell-free extracts, 5 g (wet weight) of cells were suspended in 25 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.2) containing 10^{-4} M ethylenediaminetetraacetic acid (EDTA-Na_2) and 10^{-4} M reduced glutathione. The cell suspension was treated for 20 min in a 10-kc Raytheon sonic disintegrator. The cell debris were removed by centrifugation at 20,000 × *g* for 60 min, and the supernatant fraction was used as the crude cell-free extract. The soluble 144,000 × *g* supernatant fraction was obtained by centrifugation of the 20,000 × *g* supernatant fraction at 144,000 × *g* for 60 min. The resultant pellet was termed P-144,000. Thiosulfate oxidation was measured by use of Warburg manometry or polarographically, as well as by the colorimetric procedure described by Sörbo (1957). The steady-state oxidized spectra of the cell-free extracts and their cytochrome *c* reductase activity were measured spectrophotometrically,

as described previously (Aleem and Nason, 1959; Aleem and Lees, 1963).

RESULTS

Thiosulfate and formate oxidation by cell-free extracts. Cell-free fractions 20,000 × *g* and 144,000 × *g* obtained from the autotrophic strain were found to catalyze the oxidation of thiosulfate and formate. The rates of oxidation were stimulated in the presence of added cytochrome *c* (Fig. 1). Particulate fraction P-144,000 was devoid of $S_2O_3^{-2}$ or formate oxidase activities. When the autotrophic strain was grown in the heterotrophic glutamate medium described

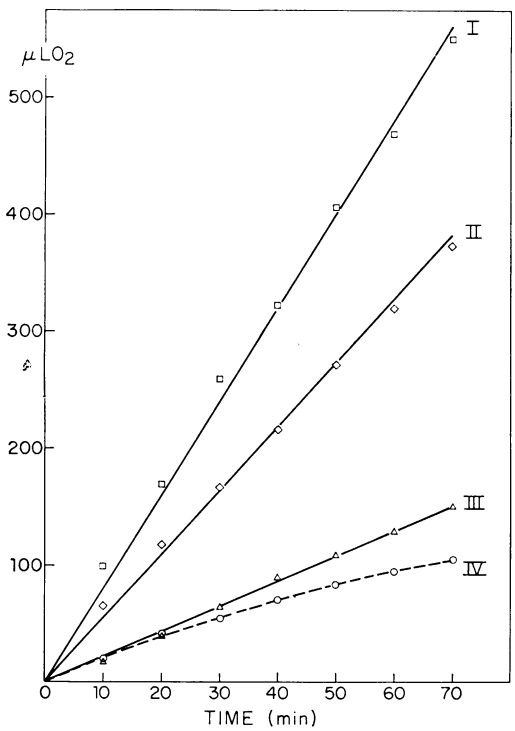

FIG. 1. *Effect of added mammalian cytochrome c upon thiosulfate and formate oxidations by Thiobacillus novellus cell-free extracts. The oxidation of thiosulfate or formate was measured by use of Warburg manometry. Reaction mixture, in a total volume of 3.0 ml, contained 100 μmoles of Tris (pH 7.5), 0.2 ml of enzyme equivalent to 4 mg of protein, and, where shown, 0.1 ml of 4% cytochrome c (horse heart type III, Sigma Chemical Co.) and 20 μmoles of thiosulfate or 20 μmoles of formate. The center well of the Warburg flask contained 0.2 ml of 20% KOH. Curves I and II represent the rate of thiosulfate oxidation in the presence and absence of added cytochrome c, and III and IV represent the rate of formate oxidation with and without added cytochrome c, respectively.*

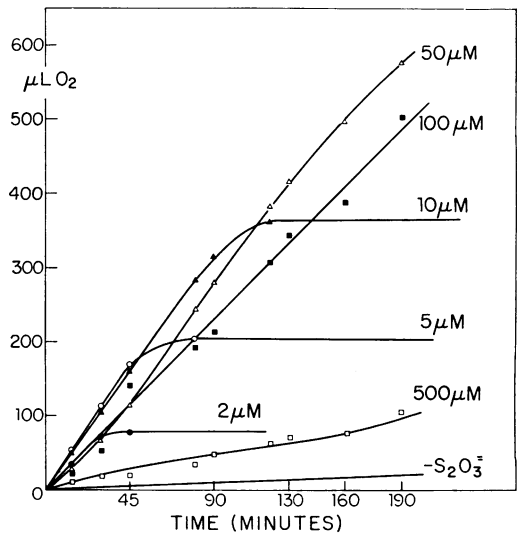

FIG. 2. *Utilization of O_2 by Thiobacillus novellus extracts in the presence of various concentrations of thiosulfate. Experimental conditions are the same as described in Fig. 1. Each Warburg flask contained cytochrome c with varying concentrations of thiosulfate, as noted.*

by Santer et al. (1959), the cell-free extracts lost their ability to oxidize thiosulfate and formate.

Oxygen uptake by the thiosulfate-oxidizing enzyme system was linear with time in the presence of 2 to 33 mM thiosulfate. However, 1.67×10^{-1} M $S_2O_3^{-2}$ caused about 77% inhibition of the thiosulfate oxidase (Fig. 2). The O_2 uptake in the absence of oxidizable substrate was almost negligible. The optimal *p*H for $S_2O_3^{-2}$ oxidation was 7.5, and a sharp drop in O_2 uptake was noted with increasing *p*H values (Fig. 3).

Effect of inhibitors upon thiosulfate oxidation. Intact cells or cell-free extracts from *T. novellus* were markedly sensitive to low concentrations of cyanide; 10^{-4} M cyanide caused 80 and 65% inhibition of $S_2O_3^{-2}$ oxidation by whole cells and cell-free extracts, respectively (Table 1). A 10^{-3} M concentration of *p*-hydroxymercuribenzoate inhibited 80 and 50% of the $S_2O_3^{-2}$ oxidase in intact cells and cell-free extracts, respectively. The inhibition in the latter case was completely reversed by 10^{-3} M reduced glutathione. Azide at a concentration of 2×10^{-3} M caused only 45% inhibition of the $S_2O_3^{-2}$ oxidase. Less effective were *o*-phenanthroline, diethyldithiocarbamate, arsenite, arsenate, and ethyl-xanthate. The $S_2O_3^{-2}$ oxidase system was also inhibited by Atebrin and amaytal; rotenone, however, was ineffective. A 40 and 25% inhibition of $S_2O_3^{-2}$ oxidation was caused by nonylhydroxyquinoline-

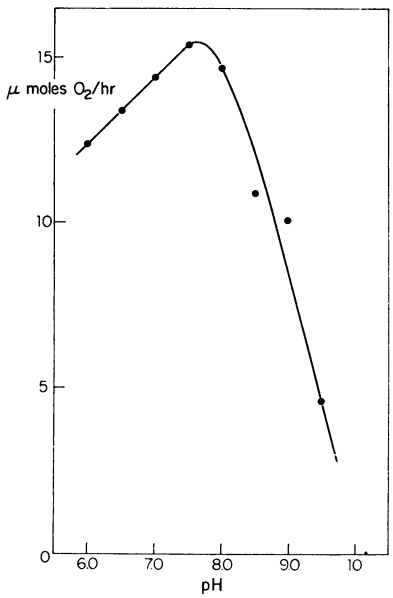

FIG. 3. *Influence of pH on the oxidation of thiosulfate. Experimental conditions are the same as described in Fig. 1. Each Warburg flask contained 10 μmoles of thiosulfate and 200 μmoles each of phosphate buffer (pH 6.0 to 7.0), Tris buffer (pH 7.5 to 9.0), and carbonate-bicarbonate buffer (pH 9.5 to 10).*

N-oxide and antimycin A, respectively, each at a concentration of 7 μg/ml. An atmosphere of 10% CO, 20% O_2, and 70% N_2 exhibited a 50% inhibition of thiosulfate oxidation; this inhibition was completely reversed in the presence of light.

Cytochrome systems. Washed cell suspensions of autotrophically grown *T. novellus* were pink and yielded reddish cell-free extracts, which appeared to be rich in cytochromes. Studies were therefore undertaken to elucidate the role of such cytochrome systems in thiosulfate oxidation. Examination of the cell-free extracts treated with 10^{-2} M $S_2O_3^{-2}$ in the Cary spectrophotometer caused the immediate appearance of absorption peaks in the region of 550, 520, and 420 mμ, which represent, respectively, α, β, and γ peaks of the cytochrome components of the *c* type, and in the region of 600 to 610 mμ, indicating the presence of cytochromes of the *a* and a_3 type. These absorption peaks increased in magnitude upon prolonged incubation (Fig. 4a). The cytochrome systems were also reduced by the addition of 10^{-2} M formate to the cell-free extracts (Fig. 4b). A similar phenomenon was observed when intact cells of the organism were incubated in the presence of added thiosulfate. In addition, it was observed that, when the autotrophic strain was grown on synthetic organic medium containing glutamate as the energy source, the amount of cytochromes in the cells or cell-free extracts was reduced to the extent of about 95% or more. As already mentioned, such cell-free extracts were unable to catalyze the oxidations of either $S_2O_3^{-2}$ or formate in the presence or absence of added cytochrome *c*.

Thiosulfate-activating enzyme ($S_2O_3^{-2}$-cytochrome c reductase). The reduction of endogenous or exogenous cytochrome *c* by thiosulfate in the presence of cell-free extracts of *T. novellus* is enzymatic and is apparently brought about by $S_2O_3^{-2}$-cytochrome *c* reductase. The enzyme activity resided in the soluble cell-free preparations and was stable for several months upon storage at 0 to −15 C. However, no absorption bands were observed when boiled cells or boiled cell-free extracts were treated with thiosulfate in the presence or absence of mammalian cytochrome *c*.

TABLE 1. *Effect of inhibitors on thiosulfate oxidase**

Fraction	Inhibitor	Inhibition
		%
Whole cells	Cyanide, 10^{-4} M	80
	p-Hydroxymercuribenzoate (PCMB), 10^{-3} M	81
	2,4-Dinitrophenol, 10^{-5} M	0
	2,4-Dinitrophenol, 5×10^{-5} M	36
Cell-free extracts	Cyanide, 10^{-4} M	65
	Carbon monoxide, 10% CO + 20% O_2 + 70% N_2	50
	Carbon monoxide + light, 10% CO + 20% O_2 + 70% N_2	0
	8-Hydroxyquinoline, 1.5×10^{-3} M	80
	Azide, 2×10^{-3} M	45
	PCMB, 10^{-3} M	50
	PCMB + reduced glutathione, 10^{-3} M	0
	Atebrin, 7×10^{-4} M	77
	Amytal, 2×10^{-3} M	53
	Nonylhydroxyquinoline-*N*-oxide, 7 μg/ml	40
	Antimycin A, 7 μg/ml	25

* The oxidase activity was measured polarographically with an oxygen electrode. Reaction mixture in a total volume of 1.5 ml contained: 50 μmoles of tris(hydroxymethyl)-aminomethane (Tris) buffer (*p*H 7.5), 0.1 ml of cell-free extract containing 2 mg of enzyme protein, 0.05 ml of 4% mammalian cytochrome *c*, and inhibitor concentration as shown. The reaction was started by the addition of 5 μmoles of thiosulfate.

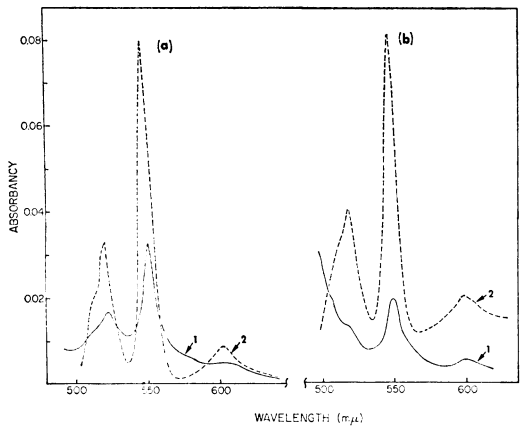

FIG. 4a. *Steady state oxidized difference spectra of Thiobacillus novellus cell-free fraction S-144,000 upon addition of thiosulfate. Traces 1 and 2 represent extent of reduction of endogenous cytochrome systems after 3 and 20 min of incubation of the cell-free extracts with thiosulfate. The Beckman cuvettes, in a total volume of 1.0 ml, contained 50 μmoles of Tris (pH 7.5), 0.1 ml of cell-free extract containing 4 mg of protein, and the test cuvette contained, in addition, 10 μmoles of thiosulfate. The difference spectra were obtained in a Cary model 14 spectrophotometer with sensitive slide wire.*

FIG. 4b. *Steady state oxidized difference spectra of Thiobacillus novellus cell-free fraction S-144,000 upon addition of formate. Conditions were the same as for Fig. 4a, except 10 mM formate was used instead of thiosulfate.*

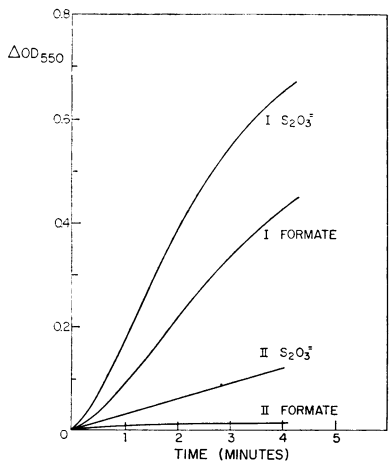

FIG. 5. *Levels of thiosulfate and formate-cytochrome c reductase activities in cell-free extracts from autotrophic and heterotrophic thiobacillus novellus. Experimental conditions were the same as for Table 2. Curves I and II represent activity of enzymes in autotrophic and heterotrophic cell-free extracts, respectively. In each case, 500 μg of enzyme protein was used.*

Compared with the cell-free extracts from *T. novellus* (autotrophic), the levels of thiosulfate and formate-cytochrome *c* reductases dropped 80 and 99%, respectively, in the cell-free extracts obtained from heterotrophically grown organism (Fig. 5). It was of interest, therefore, to purify the $S_2O_3^{-2}$-activating enzyme and study its characteristics. Of the various methods tried for the purification of $S_2O_3^{-2}$-cytochrome *c* reductase, ammonium sulfate precipitations, followed by calcium phosphate gel adsorption, yielded most satisfactory results (Table 2). The supernatant fraction (from 50 to 70% ammonium sulfate saturation), when treated with calcium phosphate gel, resulted in a greater than 200-fold increase in its specific activity. Some of the characteristics of the partially purified enzyme are given below.

Substrate optima and enzyme proportionality. Thiosulfate-cytochrome *c* reductase exhibited two substrate optima. In the presence of 10^{-4} M cyanide, the optimal activity of the enzyme occurred at 3.5 to 7.0 mM thiosulfate. However, the enzyme was inhibited by about 30 to 35% at this cyanide concentration. In the absence of cyanide, little or no activity was observed at such low concentrations of thiosulfate because of the rapid reoxidation of the reduced cytochrome *c* by cytochrome oxidase. A linear increase in the enzyme activity was observed in the presence of 67 to 167 mM $S_2O_3^{-2}$, with maximal activity occurring at the latter concentration which was found to inhibit $S_2O_3^{-2}$ oxidase (Fig. 2). Higher concentrations of $S_2O_3^{-2}$ proved to be somewhat inhibitory (Fig. 6). It was observed, however, that the rate of cytochrome *c* reduction was linear with time and proportional to the enzyme

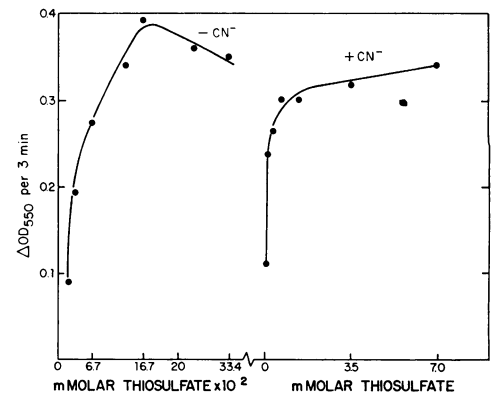

FIG. 6. *Substrate optima of thiosulfate-cytochrome c reductase in the presence and absence of added cyanide. Experimental conditions were the same as for Table 2, except varying concentrations of thiosulfate were used in the absence or presence of 10^{-4} M cyanide.*

TABLE 2. *Purification of thiosulfate-cytochrome c reductase from Thiobacillus novellus**

Fraction	Total units†	Total protein	Specific activity (units/mg of protein)	Fold purification
		mg		
I. Supernatant 8,000 × *g*	1,640	340.0	4.8	—
II. Precipitate from 50 to 70% ammonium sulfate saturation	1,300	41.25	31.7	7
III. Supernatant fluid from II	270	38.16	71.0	15
IV. Calcium phosphate gel eluate of III	426	0.80	533.0	110
V. Supernatant fluid from IV	12,450	11.13	1,102.0	228
VI. Calcium phosphate gel eluate of V	199	0.25	717.0	148
VII. Supernatant fluid from VI	6,261	6.02	1,041.0	215

* Reaction mixture in a total volume of 1.5 ml contained: 100 μmoles of Tris (pH 8.0), 2.5×10^{-4} M cytochrome *c* (horse heart type II, Sigma Chemical Co., St. Louis, Mo.), and 0.02 to 0.05 ml of enzyme preparation. The reaction was started by the addition of 250 μmoles of $Na_2S_2O_3$ to the test cuvette, and the reduction of cytochrome *c* was measured over a period of 3 min at 550 mμ in a Cary model 14 spectrophotometer.

† One unit of enzyme activity is represented by a net change in the absorbance at 550 mμ of 0.01 per 3 min.

TABLE 3. *Effect of inhibitors on thiosulfate-cytochrome c reductase**

Inhibitor	Concn	Inhibition
	M	%
Cyanide	5×10^{-4}	42
	1×10^{-3}	50
O-phenanthroline	3.4×10^{-3}	81
Diethyldithiocarbamate	1×10^{-3}	0
	7×10^{-3}	63
Potassium ethylxanthate	5×10^{-3}	25
8-Hydroxyquinoline	3.4×10^{-3}	15
p-Hydroxymercuribenzoate	5×10^{-3}	0
p-Chloromercurisulfonate	5×10^{-3}	0
Atebrin	5×10^{-3}	17
Amytal	2×10^{-3}	14
Antimycin A	50 μg/ml	3
	100 μg/ml	47
2-*n*-Heptyl-4-hydroxyquinoline-*N*-oxide	100 μg/ml	18

* Experimental conditions and reaction mixture were the same as described in Table 2.

concentrations either in the presence of 3.5 mM $S_2O_3^{-2}$ and 10^{-4} M CN^- or 167 mM $S_2O_3^{-2}$ without cyanide.

Cytochrome c and pH optima. Figure 7 reveals that the optimal activity of thiosulfate-cytochrome *c* reductase occurred at a 2.5×10^{-4} M concentration of added mammalian cytochrome *c*. The *p*H effect was rather sharp. A linear increase in enzyme activity occurred from *p*H 6.0 to 8.0, followed by a sharp and linear decrease with increasing *p*H values (Fig. 8).

Effect of inhibitors. Thiosulfate-cytochrome *c* reductase was sensitive to cyanide and *o*-phenanthroaline, but relatively less sensitive to other metal-binding agents, i.e., ethylxanthate, diethyldithiocarbamate, and 8-hydroxyquinoline (Table 3). The enzyme was not inhibited by 5×10^{-3} M *p*-hydroxymercuribenzoate or 5×10^{-3} M *p*-chloromercurisulfonate, and the respiratory-chain inhibitors were without effect. Concentrations (50 μg/ml) of antimycin A or 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide did not inhibit $S_2O_3^{-2}$-cytochrome *c* reductase activity. Thus far, the purified enzyme has shown no metal requirements.

DISCUSSION

Studies were undertaken to investigate the biochemical mechanisms involved in thiosulfate oxidation by *T. novellus* (autotrophic). In the presence of added mammalian cytochrome *c*, the cell-free extracts catalyze active $S_2O_3^{-2}$ oxidations using approximately 2 moles of O_2 per mole of $S_2O_3^{-2}$ oxidized. Similar O_2-uptake values were obtained by Santer et al. (1959), who used intact cells of the organism catalyzing SO_3^{-2} oxidation according to the equation: $Na_2S_2O_3 + 2O_2 + H_2O \rightarrow Na_2SO_4 + H_2SO_4$. In contrast to the chemoautotroph *Nitrobacter agilis* (Aleem and Nason, 1959, 1960), the particulate cell-free fractions of *T. novellus* are devoid of the inorganic substrate oxidase activity, which is chiefly localized in the soluble cell-free fractions. This soluble $S_2O_3^{-2}$ oxidase is markedly sensitive to low concentrations of cyanide and carbon monoxide. In addition, the $S_2O_3^{-2}$ oxidation is inhibited by Atebrin and amytal, which are potent inhibitors of the flavoprotein systems. The $S_2O_3^{-2}$ oxidase system also shows some sensitivity toward the

respiratory chain poisons, nonylhydroxyquinoline-*N*-oxide and antimycin A, which block oxidation of cytochrome *b*. However, in view of the relatively high levels of these agents required to partially inhibit $S_2O_3^{-2}$ oxidation, it is doubtful whether any flavin or cytochrome *b* components are involved in the process. The enzyme differs from the *p*-hydroxymercuribenzoate-insensitive thiosulfate-oxidizing enzyme from *T. thioparus* (Vishniac and Trudinger, 1962) in being sensitive to the sulfhydryl-group inhibitor. The activity of the mercuribenzoate-inhibited enzyme is completely restored by the addition of an equimolar concentration of reduced glutathione, indicating the involvement of sulfhydryl groups in thiosulfate oxidation.

The cell-free extracts from *T. novellus* grown on $S_2O_3^{-2}$ also oxidized formate; both oxidations are cyanide-sensitive and appear to be catalyzed by similar, if not the same, oxidase systems. In this respect, the organism differs from *T. thiocyanoxidans* which, when grown on $S_2O_3^{-2}$, would not oxidize formate unless grown on the thiocyanate (Happold, Jones, and Pratt, 1958). The bacterium, however, coupled thiosulfate oxidation to cytochrome *c* reduction and may be similar to *T. neapolitanus* (*Thiobacillus X*) in this respect (Trudinger, 1958, 1961).

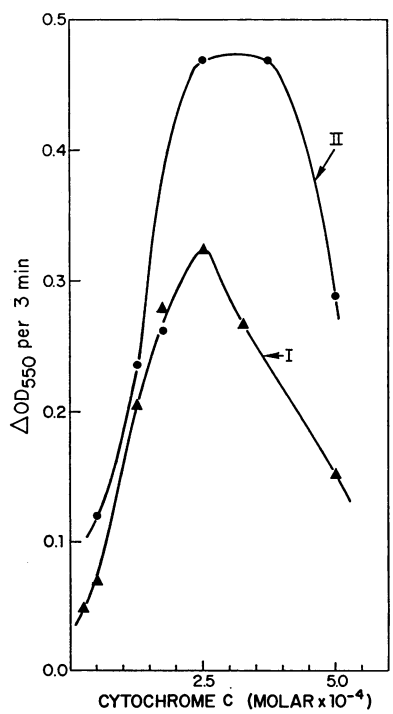

FIG. 7. *Cytochrome c optima of thiosulfate-cytochrome c reductase. Experimental conditions were the same as for Fig. 6. Curve I represents an enzyme activity in the presence of 3.5 mM thiosulfate and 10^{-4} M cyanide. Curve II represents activity in the presence of 167 mM thiosulfate without added cyanide.*

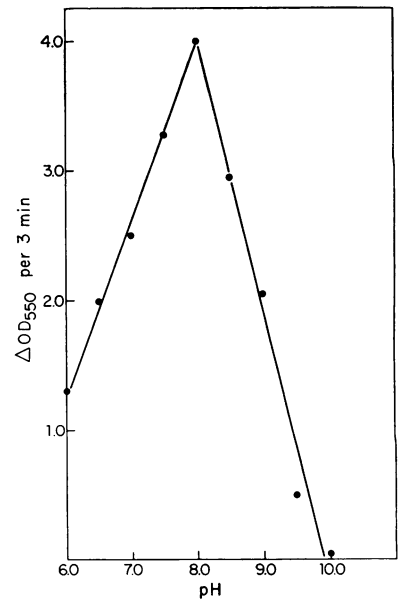

FIG. 8. *Effect of pH upon thiosulfate-cytochrome c reductase activity. Experimental conditions were the same as for Table 2. Same buffers were used as described in Fig. 3.*

The cell-free extracts of *T. novellus* (autotrophic) are richer in cytochrome systems by about 90- to 95-fold compared with the cell-free extracts obtained from heterotrophically grown organism; the addition of $S_2O_3^{-2}$ to the autotrophic cell-free extracts causes the enzymatic reduction of their cytochrome systems; and addition of mammalian cytochrome *c* stimulates $S_2O_3^{-2}$ oxidation. Thus, it is obvious that $S_2O_3^{-2}$ oxidation involves the enzymatic transfer of electrons from thiosulfate to molecular oxygen through cytochromes of the *c* and *a* type. In view of these observations, the electron-transport mechanisms concerned in $S_2O_3^{-2}$ oxidation in *T. novellus* may be presented as follows.

$$S_2O_3^{-2} \xrightarrow[\text{Reductase}]{S_2O_3^{-2}-\text{Cytochrome } c} \text{Cytochrome } c \rightarrow \begin{matrix}\text{Cytochrome oxidase} \\ \text{Cytochrome } (a + a_3)\end{matrix} \dashrightarrow O_2$$

$$\downarrow$$

$$CN^-, CO$$

ACKNOWLEDGMENTS

I am indebted to Bessel Kok and George Cheniae for helpful criticism.

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