JOURNAL OF BACTERIOLOGY, Feb., 1967, p. 550-559
Vol. 93, No. 2

Printed in U.S.A.

Metabolism of Thiosulfate and Tetrathionate by Heterotrophic Bacteria from Soil

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Received for publication 16 November 1966

ABSTRACT

Two heterotrophic bacteria that oxidized thiosulfate to tetrathionate were isolated from soil. The enzyme system in one of the isolates (C-3) was constitutive, but in the other isolate (A-50) it was induced by thiosulfate or tetrathionate. The apparent K_m for oxygen for thiosulfate oxidation by A-50 was about 223 μM, but, for lactate oxidation by A-50 or thiosulfate oxidation by C-3, the apparent K_m for oxygen was below 2 mM. The oxidation of thiosulfate by A-50 was first order with respect to oxygen from 230 μM. The rate of oxidation was greatest at *p*H 6.3 to 6.8 and at about 10 mM thiosulfate, and it was strongly inhibited by several metal-binding reagents. Extracts of induced A-50 reduced ferricyanide, endogenous cytochrome *c*, and mammalian cytochrome *c* in the presence of thiosulfate. A-50, once induced to oxidize thiosulfate, also reduced tetrathionate to thiosulfate in the presence of an electron donor such as lactate. The optimal *p*H for this reaction was at 8.5 to 9.5, and the reaction was first order with respect to tetrathionate. There was no correlation between the formation of the thiosulfate-oxidizing enzyme of A-50 and the incorporation of thiosulfate-sulfur into cell sulfur. Thiosulfate did not affect the growth rate or yield of A-50.

It has been recognized for many years that inorganic sulfur compounds are oxidized by heterotrophic bacteria (4–6). Indeed, some workers (29) consider that heterotrophs, as distinct from sulfur autotrophs such as thiobacilli, may play the dominant role in the oxidation of sulfur compounds in soils. The main heterotrophic organisms which have been identified with this process are *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Achromobacter stutzeri*, and the so-called *Thiobacillus trautweinii*, all of which oxidize thiosulfate quantitatively to tetrathionate (19–23). Similar organisms have been reported by Sijderius (Thesis, Univ. Amsterdam, Amsterdam, Netherlands) and by Baalsrud and Baalsrud (quoted by van Neil, 28).

Little is known of the mechanism of thiosulfate oxidation by heterotrophic bacteria. It is the purpose of this paper to describe some properties of an inducible thiosulfate-oxidizing system in a soil heterotroph. The same organism, once induced to oxidize thiosulfate, also reduced tetrathionate. Some experiments with a second organism possessing a constitutive enzyme are also described. No physiological role can at present be advanced for these enzymes.

MATERIALS AND METHODS

Organisms and growth conditions. The organisms, designated A-50 and C-3, were isolated from percolation units containing garden soil and elemental sulfur. Organism A-50 was kindly supplied by R. S. Swaby of the C.S.I.R.O., Division of Soils, Adelaide, Australia. Both organisms were motile, gram-negative, nonsporulating, pleomorphic rods. Organism A-50 utilized lactate, malate, succinate, glutamate, and aspartate as sole sources of carbon and energy, but not glucose, lactose, maltose, or sucrose. Growth in simple mineral medium containing one of these substrates was stimulated by small amounts of yeast extract. Organism C-3 utilized the same carbon substrates as A-50, but, in addition, it grew with carbohydrates as the sole carbon source. Under some conditions, a fluorescent pigment accumulated in the growth medium.

The basic media used were peptone-yeast extract medium, composed of 2% Difco peptone plus 0.5% Difco yeast extract, and lactate medium containing the following: K_2HPO_4, 7 g; KH_2PO_4, 2 g; trisodium citrate · $2H_2O$, 0.5 g; $MgSO_4$ · $7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g; Difco yeast extract, 0.5 g; sodium DL-lactate, 5 to 20 g in 1 liter. Modifications to these media are noted in the text. There was no major difference in the thiosulfate-oxidizing activity of bacteria grown in these two media. The bacteria were grown either in 1,800-ml penicillin flasks containing 200 ml

of medium or in 250-ml conical flasks containing 50 ml of medium. They were incubated at 28 C on a reciprocating shaker. Unless otherwise stated, the growth period was 16 hr.

The bacteria were harvested by centrifugation and were washed twice with 0.1 M potassium phosphate (*p*H 7). Where necessary to reduce endogenous respiration, the bacteria were shaken at 30 C for 1 to 2 hr between the first and second washes.

Preparation of cell-free extracts. Suspensions of bacteria in 0.1 M potassium phosphate were passed twice through a chilled high-pressure cell, described by Milner, Lawrence, and French (12), at 18,000 to 20,000 psi. The extract was centrifuged for 30 min at 10,000 rev/min in the SS-34 rotor of a Servall refrigerated centrifuge, and the supernatant liquid was retained (12,000 × *g* supernatant liquid). For some experiments, this supernatant liquid was then dialyzed overnight against 5 liters of 0.1 M potassium phosphate (*p*H 7). The supernatant liquid centrifuged at 12,000 × *g* was centrifuged for 1 hr at 40,000 rev/min in the 40 rotor of a Spinco model L ultracentrifuge. The supernatant liquid (144,000 × *g* supernatant liquid) was retained, and the precipitate (144,000 × *g* particles) was washed once with buffer and resuspended in 0.1 M phosphate (*p*H 7). All fractions were stored at −20 C.

Manometry. The conventional Warburg manometric technique was used. Filter paper and 0.2 ml of 2 N NaOH were placed in the center wells. The reactions were run at 30 C in 1.8 ml of 0.1 M potassium phosphate (*p*H 7); the flasks were shaken at 120 oscillations per min (amplitude of 4 cm).

Polarographic measurement of oxygen uptake. A Clarke electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) with a 0.001-inch Teflon membrane was used in conjunction with a temperature-controlled (30 C) reaction vessel similar to that described by Peel (14). The voltage generated across an appropriate resistor was recorded with a Varian G10 recorder adjusted to give a full scale deflection from 5 mv. The overall 95% response time of the system was about 12 sec. Unless otherwise stated, the reaction mixtures contained 5 ml of 0.1 M potassium phosphate and 100 μmoles of $Na_2S_2O_3$. The enzyme, substrate, and other additions (10 to 100 μliters) were injected with a microsyringe (Hamilton Co., Whittier, Calif.) through a fine hole in the top of the reaction vessel.

Since thiosulfate oxidation by A-50 was nonlinear with time (Fig. 2), the rate was defined arbitrarily as the reciprocal of the time taken to reduce the dissolved oxygen concentration from 184 to 46 μM. The specific activity of bacteria (units per gram of dry weight) is the rate of oxidation given by 1 g of bacteria suspended in 1 ml of reaction medium.

Reduction of ferricyanide. Unless otherwise stated, Thunberg tubes, flushed with O_2-free N_2, contained 5 μmoles of $K_3Fe(CN)_6$ and 40 μmoles of $Na_2S_2O_3$ in 3 ml of 0.13 M potassium phthalate (*p*H 5.2). The decrease in OD at 420 mμ was measured at 30 C.

Anaerobic metabolism of tetrathionate. Reactions were carried out at 30 C under O_2-free N_2 in Thunberg tubes and were stopped by the addition of one-half volume of 20% (v/v) acetic acid. After centrifugation, the thiosulfate produced was determined by titration of the supernatant liquid with standard iodine.

Spectra. These were measured with a Cary model 14R spectrophotometer by use of cuvettes with a 1-cm light path. Reduced minus oxidized difference spectra were obtained by adding extracts, which had been vigorously gassed with 100% oxygen for about 5 min, to the reference cuvette and to a cuvette charged with the appropriate substrate; the spectra were discarded if significant cytochrome reduction occurred in the reference cuvette. Such reduction was determined by recording the oxidized (oxygen) minus oxidized (ferricyanide) difference spectrum.

Radiochemical analysis. Radioactive sulfur was determined with a Nuclear-Chicago C210 gas-flow counter by the method described elsewhere (24) and also by scintillation-counting techniques by use of a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The scintillation fluid contained: naphthalene, 80 g; 2,5-diphenyloxazole, 5.0 g; 1,4-bis-2 (4-methyl-5-phenyloxazolyl) benzene, 0.5 g; dioxane, 385 ml; toluene, 385 ml; 100% ethyl alcohol, 230 ml. The radioactive sample (10 to 100 μliters) was mixed with 20 ml of this fluid for analysis. Radioactive areas on paper chromatograms were located by scanning the chromatograms with a locally constructed 4_π geiger scanner coupled to an Ecko Type N522B ratemeter and an Evershed and Vignoles 1-ma recorder. For quantitative analysis, the radioactive areas were cut into strips of approximately 1 × ⅛ inches (2.54 × 0.85 cm), and each strip was added directly to 20 ml of scintillation fluid and counted. Standards were absorbed on filter paper and counted in a similar manner. Duplicates agreed to within ±8%.

Chromatographic methods. Mixtures of sulfur compounds were separated by chromatography on 1-inch strips of Whatman no. 1 paper developed with pyridine–*n*-butanol–acetic acid–water (20:30:6:24) (27) or on Dowex 1 × 2 (26). Ammoniacal $AgNO_3$ was used to detect thiosulfate and polythionates on paper chromatograms.

Bacterial incorporation of S^{35}-thiosulfate. Bacteria, which had been exposed to S^{35}-thiosulfate, were washed four times with 0.1 M potassium phosphate (*p*H 7), containing 0.01 M unlabeled $Na_2S_2O_3$ [about 1 ml/mg (dry weight) of bacteria], and once with buffer alone. The bacteria were then suspended in 5% (w/v) trichloroacetic acid and centrifuged. The pellet was either dissolved in 0.1 N NaOH for scintillation counting or digested with $HCl—HNO_3—Br_2$ (25) for gas-flow counting.

Dry weights. Bacteria were dried to constant weight at 105 C. For growth experiments, dry weights were determined turbidimetrically with an EEL absorptiometer (Evans Electroselenium Ltd., Harlow, Essex, England) by use of a 1-cm cuvette, filter 605; 1 EEL unit corresponded to 3.7 μg (dry weight) of bacteria per ml.

Protein. The method of Lowry et al. (11) was used with bovine serum albumin as a standard.

Reagents. Potassium tetrathionate ($K_2S_4O_6$) was prepared by oxidation of thiosulfate with iodine (24, 25). Sodium thiosulfate ($Na_2S_2O_3$), labeled with S^{35} in either the bivalent or hexavalent sulfur atom,

was obtained from the Radiochemical Centre, Amersham, Bucks, England. Mammalian cytochrome *c* was a product of Calbiochem, Los Angeles, Calif.

RESULTS AND DISCUSSION

Oxidation of thiosulfate measured manometrically. Washed cells and extracts of A-50 grown in the presence of thiosulfate rapidly oxidized thiosulfate (Fig. 1). The rate of oxygen uptake in the presence of thiosulfate reverted to the endogenous rate when the theoretical oxygen for conversion of thiosulfate to tetrathionate had been consumed. Cells and extracts of A-50 grown in the absence of thiosulfate oxidized thiosulfate at less than 1% of the rate found with thiosulfate-grown bacteria. By contrast, thiosulfate was oxidized rapidly by washed cells of C-3 grown in the absence of thiosulfate; extracts of C-3, however, were completely inactive.

A single radioactive compound was obtained after complete oxidation of uniformly labeled S^{35}-thiosulfate by C-3 or by thiosulfate-grown A-50. This compound was indistinguishable from tetrathionate by chromatography on paper or Dowex 1 × 2 and reacted with sulfite to form thiosulfate and trithionate (2). Thus, both bacteria oxidized thiosulfate according to equation 1.

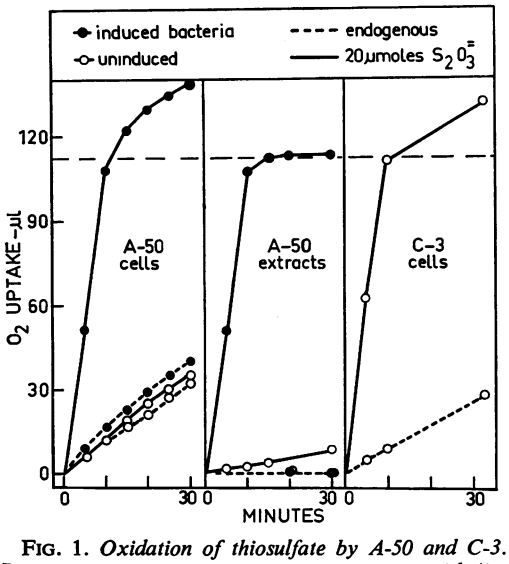

FIG. 1. *Oxidation of thiosulfate by A-50 and C-3. Bacteria were grown in peptone-yeast extract with (induced) or without (uninduced) 0.5% $Na_2S_2O_3 \cdot 5H_2O$. Concentrations per 1.8 ml were: induced A-50 cells, 14 mg (dry weight); uninduced A-50 cells, 16 mg (dry weight); induced and uninduced A-50 extracts (crude 12,000 × g supernatant liquid), 11 mg of protein; C-3 cells, 3.1 mg of dry weight. The horizontal broken line shows the theoretical oxygen consumption for the oxidation of thiosulfate to tetrathionate.*

$$2S_2O_3^{-2} \rightarrow S_4O_6^{-2} + 2e \quad (1)$$

Oxidation of thiosulfate measured with the oxygen electrode. The rate of thiosulfate oxidation by crude extracts of A-50 fell rapidly as the dissolved oxygen concentration decreased (Fig. 2). Similar results were given by washed cells and by 144,000 × *g* supernatant liquids and particles. The addition of 0.02% catalase or 0.02 M $K_2S_4O_6$ to the reaction mixtures did not affect the oxidation of thiosulfate by extracts of A-50, indicating that the fall in the rate of oxidation was not due to inhibition by the end product or by H_2O_2. Addition of oxygen restored the thiosulfate-oxidizing activity (Fig. 3), and the oxidation of thiosulfate appeared to be first order with respect to oxygen (Fig. 4). On the other hand, the oxidation of lactate by extracts of A-50 and of thiosulfate by washed cells of C-3 proceeded at linear rates until the oxygen concentration fell below about 2 µM (Fig. 2).

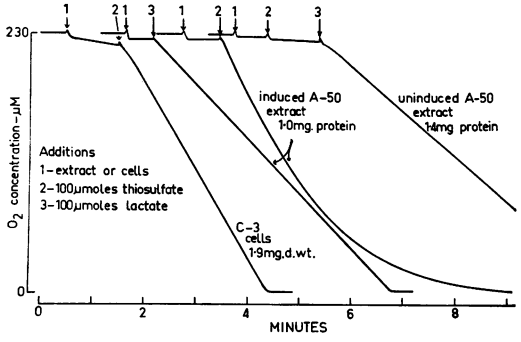

FIG. 2. *Polarographic determination of oxygen utilization in the presence of thiosulfate and lactate. C-3 cells were grown in peptone-yeast extract medium. Extracts were 12,000 × g supernatant liquids of A-50 grown in 2% lactate medium with (induced) or without (uninduced) 0.5% $Na_2S_2O_3 \cdot 5H_2O$.*

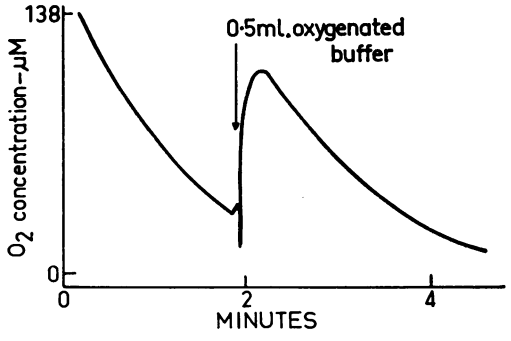

FIG. 3. *Restoration of thiosulfate oxidation by oxygen. Dialyzed 12,000 × g supernatant liquid of induced A-50 (see Fig. 2), 0.4 mg of protein per ml.*

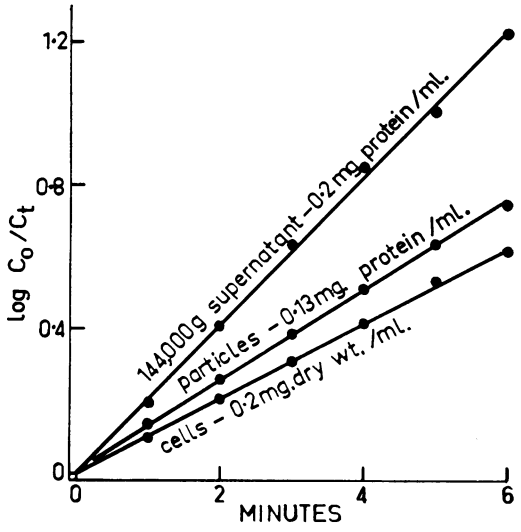

FIG. 4. *First-order plots of oxygen utilization in the presence of thiosulfate. The data were calculated from polarographic traces employing cells and extracts of thiosulfate-grown A-50. C_0 and C_t are the oxygen concentrations at zero-time and time t, respectively.*

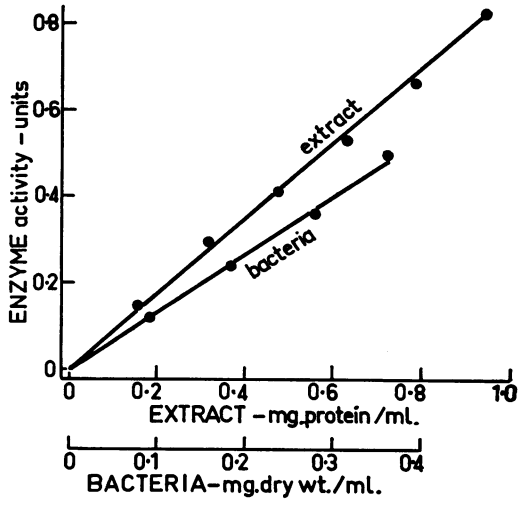

FIG. 5. *Effect of enzyme concentration on thiosulfate oxidation. Reaction mixtures contained either intact bacteria or a dialyzed 12,000 × g supernatant liquid. Bacteria were grown in peptone-yeast extract medium with 0.5% $Na_2S_2O_3 \cdot 5H_2O$. Oxygen uptake was measured polarographically. Enzyme unit = 1/t (184 to 46 mM O_2).*

Effect of enzyme concentration. For convenience, the reciprocal of the time taken to reduce the concentration of dissolved oxygen from 184 to 46 μM (80 to 20% of air saturation) was taken as a measure of thiosulfate-oxidizing activity by A-50. By use of these units, a linear relationship was found between activity and enzyme concentration (Fig. 5). With intact cells, reliable results were obtained only if the endogenous oxidation rate was less than about 15 μliters per hr per mg (dry weight).

The thiosulfate-oxidizing enzyme in extracts of thiosulfate-grown A-50 appeared to be saturated at oxygen concentrations above 407 μM (Table 1). Based on the rate of oxidation at high oxygen concentrations, the standard enzyme unit ($1/t$ 184 to 46 μM O_2) was equivalent to a maximal oxidation rate of 6.7 (± 0.3) μliters of O_2 per ml per min. This relationship was used to calculate V in equation 2 (3).

$$\frac{2.303}{t} \times \log \frac{C_0}{C_t} = \frac{V}{K_m} \qquad (2)$$

where V is the maximal velocity of oxidation, K_m is the oxygen concentration for half-maximal velocity, and C_0 and C_t are the oxygen concentrations at zero-time and time t, respectively; V/K_m was determined from the slopes of first-order plots of the type described in Fig. 4.

An average apparent K_m of 223 ± 12 μM for oxygen was obtained with intact cells, 12,000 × g supernatant liquids, 144,000 × g supernatant liquids, and 144,000 × g particles (two determinations on each).

TABLE 1. *Oxidation of thiosulfate by extracts of thiosulfate-grown A-50 at elevated oxygen concentrations*[a]

Initial O_2 concn	Initial rate of oxidation (μliters of O_2 per ml per min)	
	0.31 mg of protein	0.62 mg of protein
μM		
230 (air)	1.15	2.29
470	1.53	
500		3.06
670	1.56	

[a] The standard polarographic method was used with a dialyzed 12,000 × g supernatant liquid (742 units per mg of protein) except that the reaction vessel was charged with oxygenated buffers.

Inducible formation of the thiosulfate-oxidizing enzyme during growth of A-50. The thiosulfate-oxidizing enzyme was synthesized by A-50 during the exponential phase of growth (Fig. 6); the enzyme was fully induced within 4 hr. The maximal rate of thiosulfate utilization, however, occurred after growth had stopped, presumably because of insufficient oxygen in the medium

during the growth period. Measurements with oxygen electrode showed that, in experiments similar to that described in Fig. 6, the dissolved oxygen in the medium fell to less than 10 μM during the latter part of the exponential growth period and rose rapidly when growth ceased. Experiments on the simultaneous oxidation of lactate and thiosulfate by washed bacteria indicated that lactate did not inhibit thiosulfate oxidation.

Induced bacteria generally had an activity in the order of 500 to 800 units per g (dry weight), but occasionally bacteria with up to 1,700 units per g were obtained. No explanation has yet been found for this variation. Tetrathionate, but not trithionate, also induced the formation of the thiosulfate-oxidizing enzyme.

Other electron acceptors. Extracts of thiosulfate-induced A-50 reduced ferricyanide in the presence of thiosulfate (Fig. 7), and the reaction was linear until about 25% of the ferricyanide had been reduced. Extracts of induced cells had less than 1% of the activity of induced cell extracts on a protein basis. At *p*H 7, the rates of electron transfer from thiosulfate to ferricyanide and to oxygen (at saturating oxygen tensions) were comparable.

Endogenous cytochromes of the *c*-type were reduced when thiosulfate was added to extracts of induced but not of uninduced bacteria (Fig. 8).

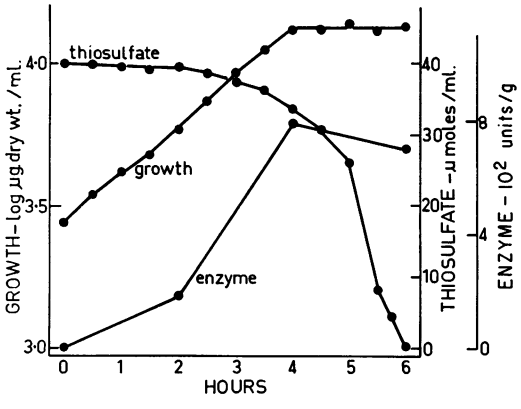

FIG. 6. *Inducible formation of the thiosulfate-oxidizing enzyme; 50 ml of an overnight culture of A-50 grown in 2% lactate medium was inoculated into 1% lactate medium containing 1% $Na_2S_2O_3 \cdot 5H_2O$ in a 2-liter flask. The medium was stirred at 1,300 rev/min with a 2-inch magnetic stirrer bar and incubated at 30 C; pH was maintained at 7 ± 0.1 by the controlled addition of 1 N HCl with a pH-stat. Growth was measured turbidimetrically, and thiosulfate, by iodine titration. At 2-hr intervals, 50 ml of the culture was centrifuged, the cells were washed with buffer, and the enzyme activity was measured polarographically.*

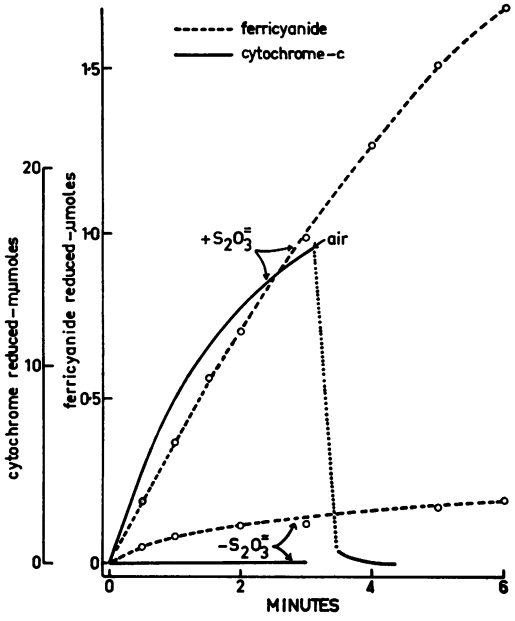

FIG. 7. *Reduction of ferricyanide and cytochrome c by extracts of thiosulfate-grown A-50 (dialyzed 12,000 × g supernatant liquids). Ferricyanide reduction as described in Materials and Methods, 0.11 mg of protein. Cytochrome c reduction, 1.6 mg of protein in 1 ml of 0.1 M phosphate (pH 7), 10 µmoles of $Na_2S_2O_3$, 20 mµmoles of mammalian cytochrome c; the cuvette was gassed with N_2 and cytochrome reduction was followed at 550 mµ at 25 C with a Cary spectrophotometer.*

A cytochrome *b*-type spectrum was obtained when dithionite-reduced extracts of A-50 were compared with thiosulfate-reduced extracts (Fig. 8). These results agree with the oxidation-reduction potential of the thiosulfate-tetrathionate couple [E_0' = 100 mv (30)] which is below that of most *c*-type cytochromes but above that of *b*-type cytochromes (13).

Further work will be necessary to determine whether or not cytochrome reduction is an integral part of the electron transport chain from thiosulfate to oxygen. Although a number of extraction and fractionation procedures have been tried, including the use of detergents and ion-exchange resins, the thiosulfate-oxidizing system has not been separated from bacterial cytochrome *c* or from a number of "particulate" components such as cytochrome *b* and ferrochelatase (Trudinger and Johnson, *unpublished data*).

Extracts of induced A-50 also reduced mammalian cytochrome *c* in the presence of thiosulfate, and the cytochrome was rapidly reoxidized on aeration (Fig. 7). Cytochrome *c* (0.01 to 0.1 mM), however, had no effect on the rate of thiosulfate

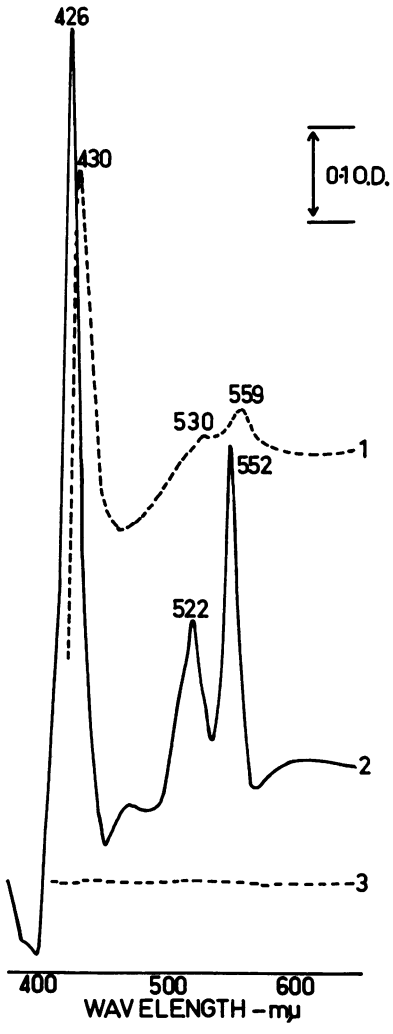

FIG. 8. *Reduction of endogenous cytochromes of A-50 by thiosulfate. Crude 12,000 × g supernatant liquids of A-50 grown as those in Fig. 2. Curves: (1) induced extract (7.5 mg protein per ml), reduced ($Na_2S_2O_4$) minus reduced ($Na_2S_2O_3$); (2) induced extract (16 mg protein per ml), reduced ($Na_2S_2O_3$) minus oxidized; (3) uninduced extract (10 mg protein per ml), reduced ($Na_2S_2O_3$) minus oxidized. Numbers are the absorption maxima in millimicrons.*

oxidation by the extracts with oxygen as electron acceptor.

Distribution of the thiosulfate-oxidizing enzyme in extracts of A-50. About 70 to 75% of the enzyme activity of extracts of A-50 was in the particle fraction sedimenting between 12,000 and 144,000 × *g* (Table 2). Similar results were obtained whether the activity was measured polarographically or by ferricyanide reduction.

Effect of pH and substrate concentration on thiosulfate oxidation by A-50. The rate of thiosulfate oxidation by extracts of thiosulfate-grown A-50, measured polarographically, was greatest at *p*H 6.3 to 6.8 (Fig. 9) and at thiosulfate concentrations above 10 μM. The reaction rate was, however, not greatly influenced by *p*H values between 5.5 and 8.0. The optimal *p*H value for the thiosulfate-ferricyanide reaction was about 5.2 (Fig. 9).

Stability of the thiosulfate-oxidizing enzyme of A-50. Crude extracts of thiosulfate-grown A-50 lost about 50% of their thiosulfate-oxidizing activity after 2 months at −20 C. The extracts were completely inactivated within 10 min at 60 C.

Effects of some inhibitors on thiosulfate oxidation. Thiosulfate oxidation by A-50 extracts was strongly inhibited by the metal-binding reagents azide, cyanide, and cupferron (Table 3). Diethyldithiocarbamate inhibited slightly, but 8-hydroxyquinoline, ethylenediaminetetraacetate, α-α'dipyridyl, bathocuproin sulfonate, and bathophenanthroline sulfonate (0.1 to 1 mM) were inactive. Extracts, after treatment with 0.1 mM KCN and subsequent removal of the KCN by dialysis, did not oxidize thiosulfate, but activity was not restored by incubation of the extracts with 10^{-7} to 10^{-3} M $FeSO_4$, $FeCl_3$, $CoCl_2$, $NiCl_2$, $MnCl_2$, $CuSO_4$ or $(NH_4)_6Mo_7O_{24}$.

Sulfite inhibited thiosulfate oxidation, apparently in a competitive manner (Table 3); cells or extracts of thiosulfate-grown A-50 did not oxidize sulfite.

Antimycin A (0.01 mM), 2*N*-nonyl-hydroxyquinoline-*N*-oxide (2 μg/ml), mepacrine (0.2 mM), glutathione (0.5 mM), 95% CO, and the thiol-binding reagents, *N*-ethyl maleimide, *p*-chloromercuribenzoate, and iodoacetamide (all 1 mM) did not inhibit. The slight inhibition by amytal (Table 3) was variable and probably not significant.

Anaerobic metabolism of tetrathionate. Thiosulfate- or tetrathionate-grown A-50 metabolized tetrathionate anaerobically with the formation of an iodine-reacting compound which was not separated from thiosulfate by chromatography on paper or Dowex 1 × 2, and iodine oxidized the compound to tetrathionate. Thiosulfate accounted for all the tetrathionate metabolized (Table 4, experiment 2), indicating that tetrathionate was reduced according to equation 3.

$$S_4O_6^{-2} + 2e \rightarrow 2S_2O_3^{-2} \qquad (3)$$

The reduction of tetrathionate required an electron donor such as lactate (Table 4, experiment 1), and bacteria grown in the absence of thiosulfate had only slight activity. The optimal

TABLE 2. *Distribution of thiosulfate oxidation in extracts of A-50*[a]

Expt no.	Fraction	Oxygen uptake	Ferricyanide reduction (μmoles of $Fe(CN)_6^{-3}$ per min per ml)
		units/ml	
1	Intact bacteria	41	—
	Supernatant liquid (12,000 × *g*)	23.3	—
	Supernatant liquid (144,000 × *g*)	4.2	—
	Particles (144,000 × *g*)	17.4	—
2	Intact bacteria	24.0	—
	Supernatant liquid (12,000 × *g*)	19.6	45.0
	Supernatant liquid (144,000 × *g*)	3.8	11.2
	Particles (144,000 × *g*)	13.1	33.3

[a] Bacteria were grown in either peptone-yeast extract (experiment 1) or 2% lactate media (experiment 2) containing 0.5% $Na_2S_2O_3 \cdot 5H_2O$. Enzyme activity was measured polarographically or by ferricyanide reduction.

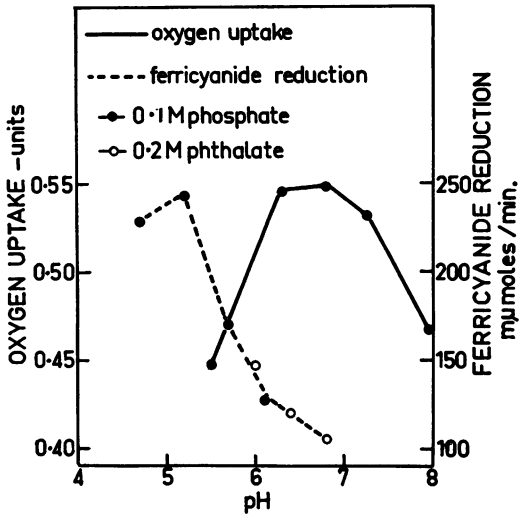

FIG. 9. *Effect of pH on oxygen utilization and ferricyanide reduction in the presence of thiosulfate. Dialyzed 12,000 × g supernatant liquid of induced A-50 grown as that of Fig. 1. Ferricyanide reduction, 0.1 mg of protein per ml; oxygen uptake (polarographic), 0.31 mg of protein per ml.*

*p*H value for tetrathionate reduction in the presence of lactate was about 8.5 to 9.5, and the reaction was first order with respect to tetrathionate (Fig. 10). Although extracts of A-50 grown on lactate and thiosulfate rapidly oxidized both these substrates (e.g., Fig. 2), the rate of reduction of tetrathionate by crude extracts was less than 5% of that of intact bacteria, and the rate was not increased by 1 mM nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, flavin mononucleotide, flavin adenine dinucleotide, or $MgCl_2$.

The reduction of tetrathionate by thiosulfate-grown A-50 suggests that the thiosulfate-oxidizing enzyme may catalyze a reversible reaction. The possibility cannot be excluded, however, that a separate tetrathionate-reducing enzyme is induced. Enzymes, which apparently catalyze the irreversible reduction of tetrathionate, are induced by tetrathionate in a number of facultative anaerobic bacteria (9, 10, 17, 18). These tetrathionate reductases, however, differ in a number of respects from that of A-50; in particular, their synthesis is suppressed by oxygen (15, 16) and is not induced by thiosulfate (16). Moreover, the maximal rate of tetrathionate reduction by the facultative anaerobes is obtained with less than 2 μM tetrathionate (16, 17), whereas the enzyme

TABLE 3. *Effects of some inhibitors on thiosulfate oxidation by extracts of A-50*[a]

Inhibitor	Concn	Per cent inhibition
	mM	
NaN_3	1.0	83–96
	0.1	57–73
KCN	0.1	100
	0.01	58–100
	0.001	18–23
Cupferron	0.02	94
	0.02	75
	0.002	48
	0.0002	20
Diethyldithiocarbamate	0.2	17
Amytal	20	0–39
Na_2SO_3 (with 10 mM $S_2O_3^{-2}$)	5	81
Na_2SO_3 (with 20 mM $S_2O_3^{-2}$)	5	60
Na_2SO_3 (with 50 mM $S_2O_3^{-2}$)	5	26

[a] Standard manometric or polarographic methods were used. Similar results were obtained for 12,000 × *g* supernatant fluids, 144,000 × *g*, supernatant fluids, and 144,000 × *g* particles.

TABLE 4. *Anaerobic metabolism of tetrathionate*[a]

Expt no.	Bacteria	Amt (μmoles) of $S_2O_3^{-2}$ formed			Amt of $S_4O_6^{-2}$ remaining after 30 min
		10 min	20 min	30 min	
					μmoles
1	Uninduced (7 mg)	0.85	1.20	—	—
	Induced (9 mg)	41.4	57.0	—	—
	Induced (9 mg) (no lactate)	1.35	1.35	—	—
2	Induced (9 mg)	—	—	49.5	25.5
3	Induced (13 mg)	—	—	81.5	9.0

[a] A-50 was incubated under O_2-free N_2 at 30 C with 50 μmoles of $K_2S_4O_6$ and 50 μmoles of sodium lactate in 5 ml of 0.2 M phosphate (*p*H 8). The bacteria were grown as described in Fig. 2. In experiment 1, thiosulfate was determined iodometrically; in experiments 2 and 3, uniformly labeled S^{35}—$K_2S_4O_6$ was used and the mixtures were analyzed by chromatography on paper (experiment 2) or on Dowex 1 × 2 (experiment 3).

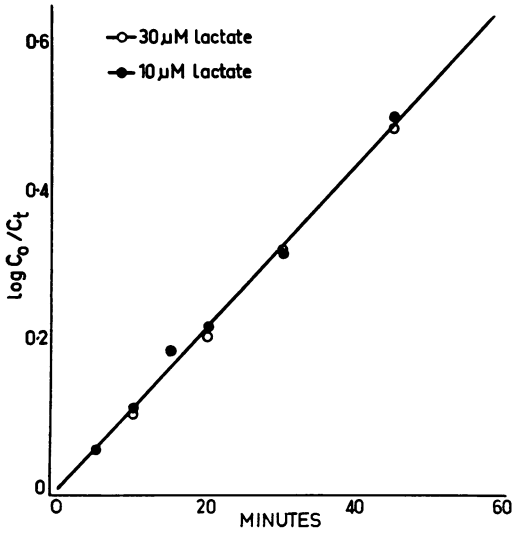

FIG. 10. *First-order plot of tetrathionate reduction by A-50 grown on 2% lactate and 0.5% $Na_2S_2O_3 \cdot 5H_2O$; 0.5 mg (dry weight) of organisms per ml, other conditions as for Table 4. C_0 and C_t are the concentrations of tetrathionate at zero-time and time t, respectively, calculated from the amount of thiosulfate produced.*

system in A-50 appears to have a low affinity for the substrate (Fig. 10). Attempts to demonstrate a sequential relationship in the induction of the thiosulfate oxidation and tetrathionate reduction by A-50, which might be expected if two separate enzymes were involved, were unsuccessful. In facultative anaerobic bacteria containing tetrathionate reductase, tetrathionate appears to be an electron acceptor for anaerobic growth (7, 8). Organism A-50, however, did not grow anaerobically in the presence or absence of 0.5 to 2% $K_2S_4O_6$ in the media used in this work.

Incorporation of S^{35}-thiosulfate by A-50. Thiosulfate sulfur was incorporated by A-50 during growth (Table 5); the bivalent sulfur was preferentially utilized [cf. *Escherichia coli* (1)]. Thiosulfate completely suppressed the incorporation of S^{35}-sulfate during the growth of A-50. Cysteine inhibited incorporation of S^{35}-thiosulfate by cultures of A-50 and also inhibited the formation of the thiosulfate-oxidizing enzyme to about the same extent (Table 5). This suggested that the oxidation of thiosulfate to tetrathionate might be an artifactual side reaction of an enzyme concerned with activation of thiosulfate for cell synthesis. Subsequent experiments, however, did not confirm this idea. No differences were found in the rates of S^{35}-labeled thiosulfate incorporation by induced and uninduced A-50 during the initial stages of growth (Fig. 11). No correlation was found between the amounts of thiosulfate incorporated and the thiosulfate-oxidizing activity of cells grown in different concentrations of thiosulfate (Table 6); at the lowest concentration of thiosulfate used, no significant induction of the enzyme occurred although there was extensive incorporation of the bivalent sulfur of thiosulfate.

The synthesis of the thiosulfate-oxidizing enzyme was not suppressed by 2% peptone or 0.5% yeast extract although incorporation of S^{35} from labeled thiosulfate was inhibited by 60 to 70% in both instances.

Effect of thiosulfate on the growth of A-50. Thiosulfate (0.1 to 2.0% $Na_2S_2O_3 \cdot 5H_2O$) had no effect on the growth rate or yield of A-50 in either peptone-yeast extract or lactate media, provided that the *p*H of the medium was con-

TABLE 5. *Incorporation of S^{35}-thiosulfate by A-50*[a]

Expt no.	Thiosulfate	Addition	Yield [mg (dry wt) per flask] of bacteria	S^{35} incorporation (μg of S^{35} per mg of bacteria)	Thiosulfate-oxidizing enzyme
					units/g
1	S^{35}—SO_3^{-2}	None	78	2.8	
	S—$S^{35}O_3^{-2}$	None	67	0.02	
2	S^{35}—SO_3^{-2}	None	60	3.2	625
	S^{35}—SO_3^{-2}	L-Cysteine (0.2 mg ml)	58	1.4	229

[a] Bacteria were grown for 16 hr in 50 ml of 2% lactate medium (minus sulfate) containing 25 mg of S^{35}-labeled $Na_2S_2O_3 \cdot 5H_2O$ (S^{35}—SO_3^{-2}, 2.5 × 10^6 counts/min; S—$S^{35}O_3^{-2}$, 4.5 × 10^6 counts/min; 3.2 μg of S^{35} per flask).

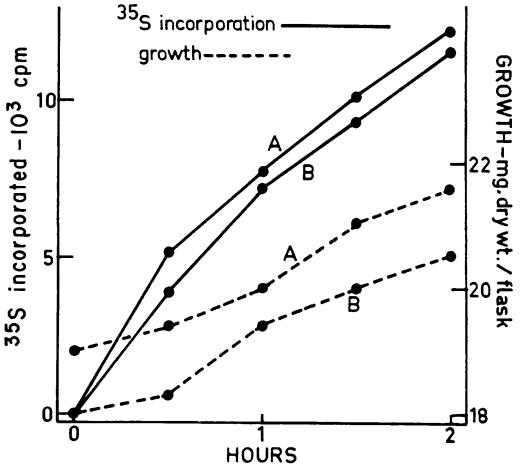

FIG. 11. *Incorporation of labeled thiosulfate by A-50 during the early stages of growth. Washed bacteria were incubated at 30 C in 11 ml of 2% lactate medium (minus yeast extract and sulfate) containing 5.5 mg of $Na_2(S^{35}—SO_3) \cdot 5H_2O$ (2.2 × 10^6 counts/min). (A) Inoculum, A-50 grown in 2% lactate medium. (B) Inoculum, A-50 grown in 2% lactate medium plus 0.5% $Na_2S_2O_3 \cdot 5H_2O$.*

TABLE 6. *Effect of thiosulfate concentration on thiosulfate incorporation and oxidation by A-50*[a]

Thiosulfate added	Yield in mg (dry wt) of bacteria	S^{35} incorporated (counts/min × 10^{-6})	Amt of S^{35} per mg of bacteria	Amt (units per g) of thiosulfate-oxidizing enzyme
mg			*μg*	
0.5	39	1.5	1.01	<20
5.5	41	0.24	1.66	375
25.5	41	0.05	1.60	592

[a] A-50 incubated 16 hr in 50 ml of 2% lactate medium (minus sulfate and yeast extract) containing $Na_2(S^{35}—SO_3) \cdot 5H_2O$ (2.5 × 10^6 counts/min).

trolled by adding acid to balance the alkalinity produced by the oxidation of thiosulfate (equation 4).

$$2Na_2S_2O_3 + \tfrac{1}{2}O_2 + H_2O \rightarrow Na_2S_4O_6 + 2NaOH \quad (4)$$

Thus, the thiosulfate-oxidizing enzyme does not appear to be involved in a detoxication process or in the energy metabolism of the bacterium.

ACKNOWLEDGMENT

The skilled technical assistance of L. Calis is gratefully acknowledged.

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