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Catabolite Repression in the Facultative Chemoautotroph *Thiobacillus novellus*

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Several fermentable carbon sources were found to give rise to catabolite repression of all enzymes implicated in thiosulfate oxidation in the facultative chemoautotroph, *Thiobacillus novellus*. Glucose was found to elicit a strong repression. Glycerol, lactate, lactose, ribose, and pyruvate caused marked repression. In all cases, the repression could be relieved only by returning the cells to a medium devoid of such fermentable substrates. On the other hand, carbon compounds (amino acids and organic acids) that are metabolizable only aerobically caused transient or no repression of the thiosulfate oxidative system. All of the enzymes believed to participate in thiosulfate oxidation, except tetrathionase, were found to be simultaneously induced and repressed. This would suggest that tetrathionate may not be a necessary intermediate in the thiosulfate-oxidation pathway of *T. novellus*.

Members of the genus *Thiobacillus* can grow autotrophically, obtaining their energy from the oxidation of reduced inorganic sulfur compounds and simultaneously fix CO_2 by the reactions of the Calvin-Benson pathway. Fixation of CO_2 and the subsequent biosynthetic processes require adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide ($NADH_2$), which are generated during the oxidation of inorganic sulfur compounds (17). ATP is believed to be formed at several stages in the oxidation of a substrate such as thiosulfate and both substrate-linked and electron transport phosphorylations may occur. Charles and Suzuki (4) have shown that, in *Thiobacillus novellus*, oxidative phosphorylation is coupled to sulfite oxidation with a low P/O ratio. *T. novellus* is a facultative autotroph that can utilize reduced organic carbon compounds for growth in the absence of thiosulfate. This is not the case in another facultative autotroph, *T. intermedius*, studied by London and Rittenberg (7), which can grow only in a thiosulfate-mineral salts medium with a carbon source.

Prior to this, no effort had been made to study the physiological behavior of *T. novellus* when it is allowed to grow in a thiosulfate medium with organic substrates. Because the oxidation of thiosulfate results in the production of ATP, $NADH_2$, and such inorganic sulfur compounds as sulfite, sulfur, and sulfate, it seemed to us a convenient system to use in the study of catabolite repression. These thiosulfate-oxidizing enzymes are not only quite removed from organic intermediary metabolism, but do not share any common intermediates except energy compounds (e.g., ATP) and reducing power ($NADH_2$).

The phenomenon of catabolite repression is well known and has been reviewed in detail by Magasanik (10). Recent studies by Loomis and Magasanik (8) of the β-galactosidase in a mutant *Escherichia coli* that lacks hexose phosphate isomerase activity seem to indicate that either the trioses or pentoses may act as co-repressors of β-galactosidase synthesis. Moses and Prevost (13) on the other hand, suggested, as did Mandelstam (12), that the co-repressor of this enzyme may be a substance whose concentration is somewhat dependent on ATP or may even be ATP itself. The present paper reports indirectly on the probable participation of ATP or $NADH_2$ as an effector of catabolite repression of the enzymes of inorganic sulfur oxidation.

MATERIALS AND METHODS

Bacteria and growth conditions. T. novellus (ATCC 8093), a gift of R. L. Starkey, was converted to autotrophy as described by Charles and Suzuki (2). Cells were maintained in autotrophic condition by growth in 250-ml shake cultures. The medium used was that of Vishniac and Santer (18) with the following modification: 4.2 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of NH_4Cl, and 7.5 g of $Na_2S_2O_3 \cdot 5H_2O$ in 1 liter of distilled water containing

[1] Predoctorate fellow.

5 ml of trace element mixture at *p*H 8.0 (thiosulfate-mineral salts medium). One ml of a 0.2% phenol red suspension in water was added to each liter of culture medium to give an observable color, and the *p*H was adjusted to 9 by using 10 N NaOH. Autotrophic cells in shake flasks were grown for a period of 5 days with periodic *p*H adjustment to 9, using sterile 10% $NaHCO_3$. Such cells were used as inocula for large-scale 10-liter cultures in 12-liter carboys. The cells were grown under forced aeration at 30 C. Unless otherwise stated heterotrophic cells were produced by the addition of an appropriate organic substrate to the autotrophic culture after 2 days of growth. Both types of cells were harvested at the end of their exponential growth phase, which was 7 days for autotrophs and 60 hr for heterotrophs. Bacteria were harvested by centrifugation, washed twice in 0.01 M tris(hydroxymethyl)aminomethane (Tris) chloride buffer, *p*H 8, resuspended in 0.01 M phosphate buffer, *p*H 8, and starved by shaking for 1 to 2 hr at room temperature before use in manometric assays or alternatively stored in pellet form at −20 C.

Preparation of cell-free extracts. Starved cells were resuspended in 0.05 M Tris chloride buffer, *p*H 8, and were sonically treated as a 20% suspension of wet cells under an atmosphere of N_2 for 30 min in a water-cooled (5 C) Raytheon sonic disintegrator (10 kc/sec). Cell debris was removed by centrifugation at 12,100 × *g* for 20 min at 2 C.

Enzyme assays. Oxidation of reduced inorganic sulfur compounds was measured manometrically at 30 C in a Warburg respirometer and spectrophotometrically at 24 C.

Thiosulfate oxidation was estimated manometrically. The reaction mixture contained, in μmoles, Tris chloride (*p*H 8.0), 100; potassium phosphate (*p*H 8.0), 10; $Na_2S_2O_3$, 10; cytochrome *c*, 0.15; and enzyme and water to a total volume of 3 ml. The reaction was started by adding the thiosulfate solution from the side arm of the reaction vessel. Disappearance of thiosulfate was determined in parallel experiments by the thiocyanate method of Sörbo (15).

Sulfite oxidation was measured manometrically and spectrophotometrically by following the reduction of $Fe(CN)_6^{3-}$ or cytochrome *c*. The reaction mixture for the manometric assays contained, in μmoles, Tris chloride, *p*H 8, 50; Na_2SO_3 in 5 mM ethylenediaminetetraacetate (disodium), 10; cytochrome *c*, 0.15; enzyme; and water to a final volume of 3 ml. The reaction was started by the addition of Na_2SO_3. For the spectrophotometric assay of the enzyme, the reaction mixture contained, in μmoles: Tris chloride (*p*H 8.0), 5; $K_3Fe(CN)_6$, 1.5; or cytochrome *c*, 0.30; Na_2SO_3 in 5 mM EDTA, 10; enzyme; and water to a final volume of 3 ml. The reduction of $Fe(CN)_6^{3-}$ and cytochrome *c* was followed at 420 and 550 mμ, respectively, in a 1-cm cell with a Unicam SP700 spectrophotometer (Unicam, Baton Rouge, La.).

Sulfur oxidation was determined manometrically as described by Charles and Suzuki (3). The elemental sulfur suspension used was prepared as described by Suzuki and Silver (16).

Thiosulfate and polythionates (tetrathionate) were estimated according to the procedure of Sörbo (15) after the removal of proteins by acidification and centrifugation of the precipitate. When necessary, sulfate was determined by the colorimetric method of Letonoff and Reinhold (6), and any contaminating thiosulfate was estimated by the thiocyanate method. Protein was determined spectrophotometrically by the method of Warburg and Christian (19) and colorimetrically according to the procedure of Lowry et al. (9).

Disappearance of glucose from the medium was determined, after removal of the cells, by the Glucostat assay method (Worthington Biochemical Corp., Freehold, N.J.). Glutamate uptake was determined enzymatically by the use of beef liver glutamate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) as described by Bernt and Bergmeyer (1).

Growth. This was estimated by measuring the optical density at 530 mμ using a Klett-Summerson photoelectric colorimeter.

Chemicals. These were of the highest purity available commercially. Horse heart cytochrome *c* (Type II) was obtained from Sigma Chemical Co. Precipitated sulfur was purchased from Baker Chemical Co., N. Phillipsburg, N.J.

RESULTS

Effect of amino acids and organic acids on growth. Figure 1A shows that the growth rate and thiosulfate oxidation are roughly coincident for cells grown as shake cultures in the presence of glutamate. Very similar patterns were obtained when one of the following organic compounds, citrate, succinate, L-arginine, L-alanine, L-histidine, and L-asparagine, replaced glutamate in a thiosulfate-mineral salts medium. Thiosulfate was rapidly oxidized to sulfate and a small quantity of elemental sulfur. The quantity of sulfur formed was somewhat dependent on the rate of aeration and the carbon source used. There was significantly more sulfur precipitation when amino acids provided the carbon source than when organic acids were used for growth. Autotrophic growth was only 20 to 25% of that shown by heterotrophic cultures. The rates of thiosulfate oxidation, however, were approximately equal under both sets of growth conditions on a per-cell basis. The mean doubling time of heterotrophic cultures varied between 5 and 8 hr and was about 20 hr for autotrophs under the conditions used.

Transient repression. The results described in Fig. 1A were modified when the cells were grown under vigorous aeration (Fig. 1B). Organic compounds were rapidly utilized with a resultant transient repression of the thiosulfate-oxidation system. Breakdown of thiosulfate did not occur until sometime during the second or third generation. Once initiated, the oxidation was rapid and proceeded at the same rate as unrepressed cultures

(Fig. 1A). When cells in the transient repression phase were assayed by manometry for the activity of thiosulfate-oxidizing enzymes, practically no activity was found (Table 1).

Effect of glucose. A completely different response was demonstrated by cells that were grown in a thiosulfate-mineral salts medium with glucose, lactate, or glycerol serving as the carbon source. Thiosulfate utilization was completely inhibited when any one of these organic substrates served as the carbon source at a relatively high concentration of 0.05 M (Fig. 2A). The level of growth at which repression occurred was found to be dependent on the concentration of glucose used (Fig. 2B). About 80% of the thiosulfate was used up at 0.005 M, 15% at 0.025 M, and none at 0.05 M glucose concentration. In all cases, about 90% of the glucose was utilized. Similar results were obtained when glycerol or lactate replaced glucose as the carbon source. Such re-

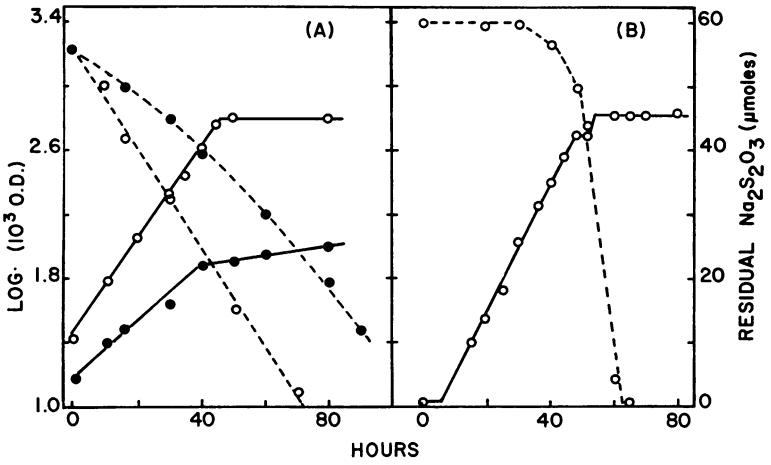

FIG. 1. *Effect of aeration and glutamate on growth and thiosulfate utilization. Solid lines represent growth and broken lines represent thiosulfate breakdown. (A) Culture shaken in glutamate-thiosulfate medium* (○) *or thiosulfate* (●). *(B) Culture grown with forced aeration in a glutamate-thiosulfate medium* (○).

TABLE 1. *Effect of aeration on oxidation of thiosulfate by whole cells of Thiobacillus novellus grown in autotrophic and heterotrophic conditions*

Incubation condition	Addition to 0.5% thiosulfate-mineral salts medium	Age of culture	Optical density (530 mμ)	O_2 uptake per hr per 20 mg (wet wt)[a]	Type of repression
		hr		*μmoles*	
Shake[b] or forced aeration	None	12	0.05	2.1	None
		24	0.09	6.7	
		36	0.12	8.3	
Shake[c]	Glutamate, 0.5%	12	0.15	1.5	None
		24	0.30	5.7	
		36	0.55	7.4	
Forced aeration	Glutamate, 0.5%	12	0.14	0.3	Transient
		24	0.33	0.2	
		35	0.52	1.2	
Shake	Glucose, 0.5%	12	0.10	0.5	Complete
		24	0.42	0.3	
		36	0.98	0.2	
Forced aeration	Glucose, 0.5%	12	0.13	0.4	Complete
		24	0.50	0.2	
		36	1.05	0.0	

[a] Sample of cells washed, starved, and assayed by manometry.

[b] Cultures (100 ml) in 250-ml Erlenmeyer flasks shaken on New Brunswick shakers at an excursion rate of 180 oscillations/min. Incubation at 30 C.

[c] Aerated by bubbling sterile air through 4-liter cultures.

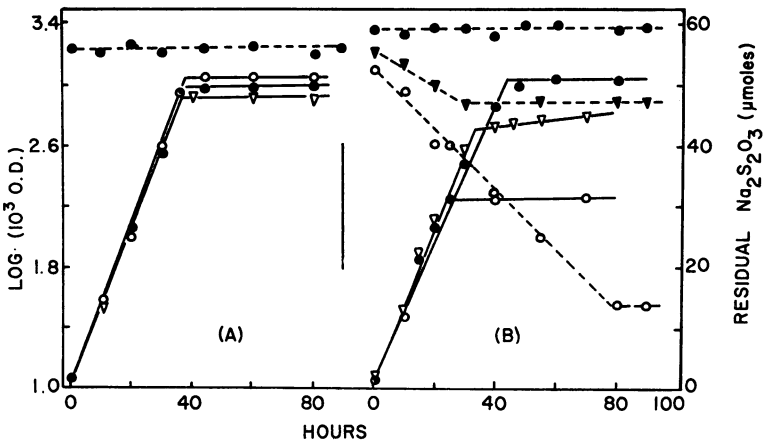

FIG. 2. *Growth of Thiobacillus novellus with fermentable substrates. (A) Growth with glucose, 0.05 M (○); glycerol, 0.05 M (●); and lactate, 0.05 M (▽). Broken line indicates thiosulfate level in all three cultures. (B) Growth with varying concentrations of glucose. Growth curves for glucose at 0.005 M (○); 0.025 M (▽); and 0.05 M (●). Broken lines represent thiosulfate oxidation for each appropriately indicated medium.*

pressed cells remained in this state even when transferred to a fresh thiosulfate medium as long as the carbon source present was a fermentable compound that, presumably, supplied the repressor continuously. If the cells were transferred to a medium with a carbon compound that could only be aerobically metabolized (e.g., glutamate, citrate, or succinate), the repression was released within 30 hr and thiosulfate oxidation became rapid and coincident with the growth of the cells.

London and Rittenberg (7) have reported that *T. intermedius*, another facultative chemoautotroph, required both organic carbon substrates and reduced inorganic sulfur compounds for maximal growth rates. The organism could not grow in the absence of thiosulfate, which presumably served as the energy source. *T. novellus* is different in this respect, because organic compounds could provide both carbon and energy for growth in the presence or absence of thiosulfate (Table 2). A second point of difference between the two autotrophs is that the organic compound is fully metabolized in the case of *T. novellus*, whereas less than 10% of the organic material is utilized by *T. intermedius*.

Induction and repression of thiosulfate oxidation. The results shown in Fig. 3 indicate that the thiosulfate-oxidizing system may be inducible. To test this theory, cells were grown in a thiosulfate-free medium for several generations with glutamate serving as the energy and carbon source. A sample of these cells was then assayed, by using a Warburg manometer, for their latent thiosulfate-oxidizing ability. Virtually no enzyme activity was present. Thiosulfate was then added to the culture during the exponential growth phase, and the disappearance of thiosulfate was followed colorimetrically. Cells sampled during the early phase of thiosulfate oxidation showed low oxidizing ability, but this increased rapidly and remained constant throughout the rest of the growth period (Fig. 4). The level of enzyme in such induced cells was roughly equivalent to that present in autotrophic and preadapted cells.

The route of thiosulfate breakdown in the thiobacilli is still controversial and, indeed, there may be a variety of reaction routes (14, and references therein). It seemed to us that the phenomenon of induction and repression of the thiosulfate-oxidizing enzymes could afford a useful tool in determining what enzymes are involved in the catabolism of thiosulfate. We therefore turned our

TABLE 2. *Utilization of organic substrate by Thiobacillus novellus*

Substrate[b]	Per cent substrate[a] used in 50 hr	
	Thiosulfate	Glutamate or glucose
Thiosulfate	35	
Glucose	—	88
Glucose + thiosulfate	0	80
Glutamate	—	91
Glutamate + thiosulfate	80	95

[a] Disappearance of glutamate and glucose was followed by enzymatic assays as described in Materials and Methods.

[b] Thiosulfate was at concentration of 0.5%, glucose at concentration of 1.0%, and glutamate at concentration of 0.5%.

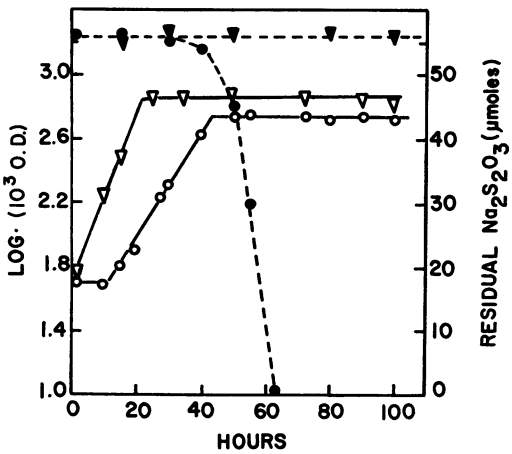

FIG. 3. *Growth and thiosulfate oxidation by glucose-grown cells* (▽) *and glutamate-grown cells* (○). *Thiosulfate oxidation for glucose-grown culture* (▼) *and glutamate-grown culture* (●).

attention to assays of the individual inorganic sulfur-oxidizing enzymes that are present in cells grown under repressed (glucose as carbon source), derepressed (glucose-grown cells transferred to glutamate medium), and nonrepressed (autotrophic cells) conditions. In all instances, thiosulfate was present at a concentration of 0.5%. Four major enzyme systems were studied. These were "tetrathionase," rhodanese, sulfur oxidase, and sulfite oxidase (sulfite:cytochrome *c* oxidoreductase).

Tetrathionate-forming system ("tetrathionase"). The presence of this enzyme was evaluated from the formation of polythionates (tetrathionate) in cell-free extracts of cells grown under repressed, derepressed, and nonrepressed conditions as defined above. Tetrathionate production was determined by the colorimetric method of Sörbo (15). Enzyme assay was conducted as described in Materials and Methods. "Tetrathionase" was detectable only in repressed cultures (Fig. 5).

Thiosulfate-cleaving system (rhodanese). The existence of a thiosulfate-cleaving enzyme was demonstrated in cell-free extracts of derepressed and nonrepressed cultures. Thiosulfate was oxidized rapidly and linearly to completion (Fig. 6) with no accumulation of polythionates. This rapid rate of thiosulfate oxidation corresponded to the pattern shown by cells during their exponential growth phase. In a parallel experiment, rhodanese activity was demonstrated in the same extracts by coupling thiosulfate-splitting to cyanide which acted as an electron acceptor according to the assay procedure of Charles and Suzuki (3). These results indicate that the following reactions are taking place in repressed and nonrepressed or derepressed cells.

$$SSO_3^{2-} + CN^- \xrightarrow{\text{rhodanese}} SCN^- + SO_3^{2-}\text{(nonrepressed)}$$

$$SSO_2^{2-} + SSO_3^{2-} \xrightarrow{\text{"tetrathionase"}} S_4O_6^{2-} + 2e\text{(repressed)}$$

Sulfur oxidase. An enzyme that catalyzes the oxidation of elemental sulfur with reduced glutathione as a necessary cofactor has been found in *T. novellus* by Charles and Suzuki (3). By using assay techniques similar to those used by these workers, the enzyme was detected only in nonrepressed and derepressed cells (Table 3). The presence of a strong sulfite-oxidase prevented the detection of sulfite which is the product of sulfur oxidation according to the reaction:

$$S + O_2 + H_2O \xrightarrow{\text{sulfur oxidase}} SO_3^{2-} + 2H^+$$

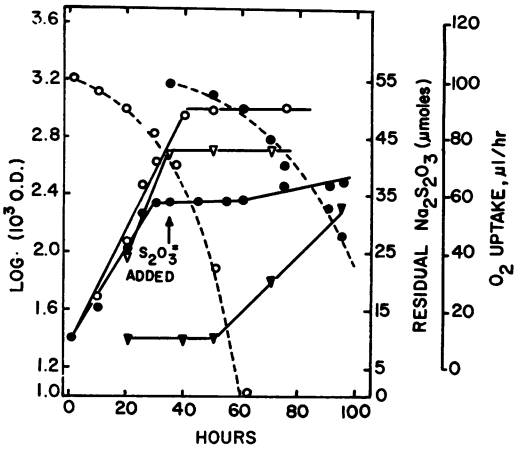

FIG. 4. *Induction of thiosulfate-oxidizing system in Thiobacillus novellus. Cells were initially exposed to glutamate, 0.05 M, and 0.5% thiosulfate. Growth curve* (○, *solid line*) *and thiosulfate utilization* (○, *dashed line*). *At intervals of 20, 36, 50, and 70 hr, samples were drawn from fermentors, the cells were washed by centrifugation, and 10 mg (wet weight) assayed by manometry for thiosulfate-oxidizing enzyme system* (▽). *The results are expressed as μliters of O_2 uptake/hr. In a parallel experiment, cells were grown in 0.01 M glutamate medium without thiosulfate until glutamate was exhausted (30 hr). At 36 hr, 0.5% thiosulfate was added and growth* (●, *solid line*) *and thiosulfate oxidation* (●, *dashed line*) *followed. Samples were drawn at intervals of 20, 36, 50, and 90 hr, and the thiosulfate-oxidizing enzyme system was assayed as described above* (▼).

Sulfite-oxidase. By using manometric and spectrophotometric techniques as described by Charles and Suzuki (4), the presence of an enzyme that catalyzes the conversion of sulfite to sulfate

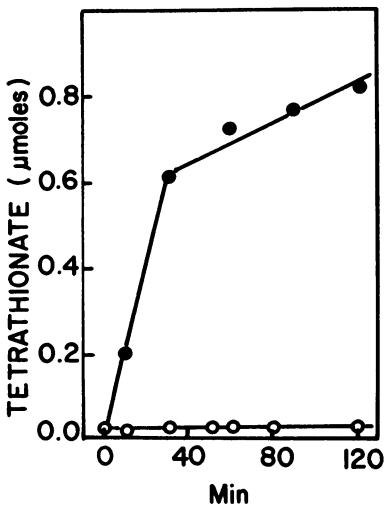

FIG. 5. *Assay for "tetrathionase" in cell-free extracts of bacteria grown in a thiosulfate medium with one of the following as carbon source:* L-*glutamate, citrate, succinate,* L-*arginine,* L-*histidine,* L-*alanine, and no organic carbon* (○)*; glucose, glycerol, lactate, ribose, and pyruvate* (●)*. Reaction mixture contained in μmoles: Tris chloride, pH 8, 200; phosphate buffer, pH 8, 10;* $Na_2S_2O_3$*, 10; reduced glutathione, 10; and protein extract, 20 mg.*

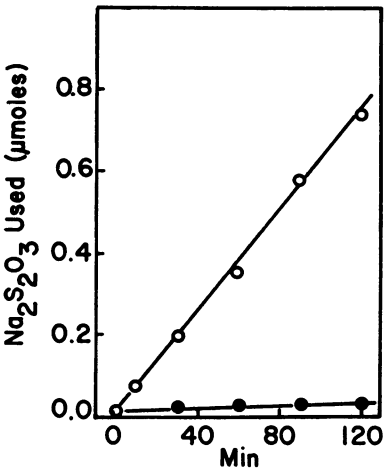

FIG. 6. *Assay for thiosulfate-oxidizing enzymes in cell-free extracts of bacteria grown in a thiosulfate medium with the carbon sources specified in Fig. 5. The thiosulfate-oxidizing enzyme system was assayed for in a reaction mixture of the same composition outlined in Fig. 5.*

TABLE 3. *Sulfur oxidase activity of Thiobacillus novellus grown in thiosulfate-mineral salts medium with organic supplements*[a]

Addition to medium	Sulfur oxidase	
	O_2 utilized/hr	Autotrophic
	μmoles	%
None	4.0	100
Glutamate, 0.5%	2.7	69
Citrate, 0.5%	3.0	75
Glucose, 0.5%	0.35	9
Lactate, 0.5%	0.15	4

[a] Reaction mixture contained, in μmoles: Tris chloride (*p*H 8), 100; potassium phosphate (*p*H 8), 50; sulfur, 32 mg; reduced glutathione, 5; cell-free extract, 10 mg of protein; and water to 3 ml. Assay by manometry.

as follows:

$$2SO_3^{2-} + 4 \text{ cytochrome } c\ Fe^{3+} + 2H_2O \xrightarrow{\text{sulfite oxidase}} 2SO_4^{2-} + 4 \text{ cytochrome } c\ Fe^{2+} + 4H^+$$

was detectable only in derepressed and nonrepressed cells. The oxidation of sulfite was dependent on either cytochrome *c* or $Fe(CN)_6^{3-}$. A strong cytochrome oxidase activity interfered with the reduction of cytochrome *c*, and cyanide could only partially inhibit this enzyme. Consequently, ferricyanide was used in all partially purified enzyme assays. The results are shown in Table 4.

DISCUSSION

Whole cells and cell-free extracts of *T. novellus* appear capable of oxidizing thiosulfate to sulfate without the intermediary formation of polythionates (3). The results reported in this paper support this. All of the enzymes involved in the breakdown of thiosulfate, except "tetrathionase," can be simultaneously induced and repressed. Tetrathionase could only be detected in heterotrophic cells that have had their thiosulfate-cleaving system repressed. We conclude from this that the two processes are not involved in the same metabolic pathway.

The degradation of glucose cannot provide the products of the thiosulfate-oxidizing enzyme system, notably sulfur, sulfite, and sulfate. The same is true for all the organic substrates used in this study. Nonetheless, some of them (notably, glucose, glycerol, and lactate) exert a strong repression on the thiosulfate-oxidizing enzyme system in *T. novellus*. Others, like glutamate,

TABLE 4. *Sulfite:cytochrome c oxidoreductase activity of Thiobacillus novellus grown in thiosulfate-mineral salts medium with organic supplements*

Addition to medium	Sulfite:cytochrome *c* oxidoreductase	
	Specific activity[a]	Per cent autotrophic
None	62.3	100
Glucose, 0.5%	0.0	0
Lactate, 0.5%	0.0	0
Glycerol, 0.5%	0.0	0
Citrate, 0.5%	60.0	93
Glutamate, 0.5%	63.5	102
Succinate, 0.5%	55.0	88

[a] Specific activity = 0.001 optical density change per min per mg of protein. Reaction mixture contained, in μmoles: Tris chloride, *p*H 8, 10; $K_3Fe(CN)_6^{3-}$, 1.5; Na_2SO_3 in 5 mM ethylenediaminetetraacetic acid, 5; 1.2 mg of protein; and water to a final volume of 3 ml. The reduction of ferricyanide was followed spectrophotometrically at 420 mμ.

citrate, and succinate do not cause any significant repression of these same enzymes. Mandelstam (12) reported that catabolite repression of the β-galactosidase enzyme in *E. coli* could be relieved by low concentrations of 2,4-dinitrophenyl (DNP), an uncoupler of oxidative phosphorylation, provided the metabolites are those that can be metabolized aerobically only. Fermentable substrates like glucose or galactose cause a repression that even DNP cannot release. The present report is strongly analogous to the work of Mandelstam, who had suggested that ATP or a similar compound may be responsible for the repression.

Cohn and Horibata (5) have shown that catabolite repression of β-galactosidase can be overcome by anaerobiosis, at which time the ATP level in the cell is expected to drop. Further support for the ATP concept comes from the studies of Moses and Prevost (13) with a mutant *E. coli* that shows a transient repression of β-galactosidase synthesis. The repression could be relieved by adenine and phosphate starvation. Under certain conditions, *T. novellus*, when grown in glutamate, succinate, or citrate does demonstrate a similar transient repression of the thiosulfate-oxidation system. This repression is relatively short-lived and is overcome within two or three generations of growth.

A rapid breakdown of glucose would result in the accumulation of high intracellular levels of ATP and other high-energy phosphate compounds in *T. novellus*. The rate of utilization of glucose is completely coincident with the growth rate, and the generation time of glucose heterotrophs decreases about fourfold over autotrophs. When glutamate is supplied, glutamate breakdown is less rapid with a correspondingly longer generation time of 8 hr compared to 5 hr for glucose. Clearly, the level of ATP and other high energy compounds would fluctuate under these conditions. In fact, during the transient repression phase when glutamate utilization is rapid, there is a temporary cessation of thiosulfate oxidation. A logical conclusion is that some common product of glucose and glutamate catabolism is the cause of the catabolite repression of thiosulfate oxidation. By definition (11), this effector may be a product of thiosulfate breakdown. The only candidates that fit this role are ATP and $NADH_2$, both of which are the sole common by-products of inorganic sulfur and organic metabolism. A probable tenet is that the cell is adjusting its energy imbalance by reducing the production of excess energy through some form of inhibition. In the case of *T. novellus*, thiosulfate oxidation and the concomitant ATP production would fall into this category.

ACKNOWLEDGMENTS

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