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Oxalate, Formate, Formamide, and Methanol Metabolism in *Thiobacillus novellus*

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Thiobacillus novellus was able to grow with oxalate, formate, formamide, and methanol as sole sources of carbon and energy. Extensive growth on methanol required yeast extract or vitamins. Glyoxylate carboligase was detected in extracts of oxalate-grown cells. Ribulose bisphosphate carboxylase was found in extracts of cells grown on formate, formamide, and thiosulfate. These data indicate that oxalate is utilized heterotrophically in the glycerate pathway, and formate and formamide are utilized autotrophically in the ribulose bisphosphate pathway. Nicotinamide adenine dinucleotide-linked formate dehydrogenase was present in extracts of oxalate-, formate-, formamide-, and methanol-grown cells but was absent in thiosulfate- and acetate-grown cells.

Since the discovery of the first facultatively autotrophic strain of *Thiobacillus novellus* (45), few such thiobacilli have been described (19, 26, 47). Extensive studies on sulfur metabolism, energetics of the electron transport system, and regulation of ribulose bisphosphate carboxylase (RuBPCase) have been restricted, however, to *T. novellus* (1-3, 11, 12, 28, 29). The purification and regulation of RuBPCase in *T. intermedius* (36) and *Thiobacillus* A2 (13) have recently been described. *T. novellus* can easily be transferred from an autotrophic to a heterotrophic medium, but, according to conflicting reports (42, 45, 47), its ability to utilize organic compounds is restricted. Taylor and Hoare (47) reevaluated the heterotrophic potential of *T. novellus* ATCC 8093 and reported that both *T. novellus* and *Thiobacillus* A2 grow on formate and methanol and that *Thiobacillus* A2 does not utilize oxalate. In an earlier study, Shethna (Indian J. Biochem. 4:34, 1967) had indicated that *T. novellus* could utilize oxalate heterotrophically and formate autotrophically, but the details of the metabolic patterns with these two substrates were not known. From the work of these authors and others (11, 42, 44, 45), it appears that information on oxalate and C_1 metabolism in *T. novellus* is limited. Among facultative chemolithotrophs, only *Micrococcus denitrificans* (15) has been screened for the utilization of oxalate and C_1 compounds like methane, methanol, formate, methylamine, etc., which are generally utilized by facultative methylotrophs (4). Hence, we decided to examine the potential for oxalate and C_1 metabolism in *T. novellus*.

Currently there is a vigorous quest to elucidate the factors that regulate the biochemistry of autotrophy in this organism. Some of the important findings are that every cell of *T. novellus* has autotrophic and heterotrophic potential (42) and that only thiosulfate can induce RuBPCase (28). This enzyme has been purified from thiosulfate-grown cells, and its properties and modes of regulation have been studied (29).

Shethna (Indian J. Biochem. 4:34, 1967) had reported the resemblance of *T. novellus* to *Pseudomonas oxalaticus* in its metabolic pattern of growth on oxalate and formate. *P. oxalaticus* assimilates oxalate carbon heterotrophically but shifts over to an autotrophic mode of carbon assimilation with formate as the sole carbon source (6). Since formate is formed as an intermediate even during growth on oxalate, it is of interest to study the biochemical basis of heterotrophic-autotrophic interconversions in *P. oxalaticus* (M. Knight et al., Proc. Soc. Gen. Microbiol. 3:p7, 1975) and *T. novellus*. *P. oxalaticus* is, to date, the only organism known to utilize oxalate and formate by different pathways (6, 21; M. Knight et al., Proc. Soc. Gen. Microbiol. 3:p7, 1975). Therefore, in the study presented here we investigated the metabolic pathways operating in *T. novellus* grown on oxalate, formate, formamide, and methanol, as well as on mixed substrates.

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MATERIALS AND METHODS

Maintenance and growth of *T. novellus*. *T. novel-*

lus (NCIB 9113) was maintained on nutrient agar slants and was subcultured at 4-week intervals.

For all growth studies, the mineral base medium of Chandra and Shethna (8) was used but with 6.26 g of K_2HPO_4 and 1.90 g of KH_2PO_4 per liter. The carbon sources added to the medium were sterilized separately. In tests for vitamin requirements, the media were prepared in glass-distilled water and autoclaved after the addition of vitamins.

To obtain thiosulfate-grown *T. novellus*, the inorganic medium of Santer et al. (42) was used.

All liquid cultures (100 or 200 ml) were contained in 500-ml Erlenmeyer flasks and were always incubated at 30°C on a rotary shaker (250 rpm). Growth was followed by measuring the increase in turbidity in a Bausch & Lomb Spectronic 20 colorimeter at 650 nm and by using tubes with a 1-cm light path. Doubling time was calculated as the time required for a doubling of absorbance values in the logarithmic phase of growth. The cells were always harvested and washed at 0 to 4°C in a Sorvall RC2-B centrifuge.

Determination of cell yields on oxalate and methanol. The yields were determined by the method of Vary and Johnson (50). After maximum growth had occurred, as determined by turbidity measurements, the cells were harvested, washed two to three times with water, and suspended in 2 to 3 ml of water. The suspensions were dried at 100°C in preweighed beakers. Yields were calculated as grams (dry weight) of cells formed per gram of substrate added to the medium.

Cultivation of cells for manometric and enzymatic studies. The inocula were prepared by transferring cells from nutrient agar slants into the appropriate media. The following growth conditions were used.

Cells were grown in 16.3 mM potassium oxalate medium with 150 μg of biotin per 100 ml and an initial pH of 7.0 to 7.2 for nearly 60 h, until an absorbance of 0.30 to 0.35 was reached. During growth, when the pH rose to 8.0 to 8.2, it was corrected to 7.0 to 7.2 with 1.33 M oxalic acid. (pH corrections were necessary to maintain growth and to prevent enzyme inhibition during growth on oxalate, formate, formamide, acetate, and thiosulfate medium. The initial pH of all growth media was 7.0 to 7.2.) A 20-ml amount of this culture was inoculated per 200 ml of four different media, viz., 16.3 mM potassium oxalate, 25 mM potassium oxalate plus 25 mM sodium formate (O+F), 25 mM potassium oxalate plus 25 mM sodium thiosulfate (O+T), and 25 mM sodium formate plus 25 mM sodium thiosulfate (F+T). All of these media contained 150 μg of biotin per 100 ml. The oxalate-grown cells were harvested after 33 h of incubation at an absorbance at 650 nm (A_{650}) of 0.28, and the pH was corrected with oxalic acid during growth. O+T-, O+F-, and F+T-grown cells were harvested when the absorbances were 0.20, 0.14, and 0.33 after 19, 22, and 37 h of growth, respectively. The pH was corrected with 2 N HCl during growth. All of these cells were in the logarithmic phase.

Formate (30 mM)-grown cells were used as an inoculum (30 ml) for 200 ml of a similar medium, and an additional 30 mmol of formate was added after 36 h. After 3 to 4 days of incubation, the logarithmically growing cultures were harvested at an A_{650} of 0.22. The pH was corrected with 2 N HCl during growth.

Cells grown in formamide (44 mM) plus 0.02% yeast extract (YE) (25 ml) were inoculated into 200 ml of similar medium. After 21 h of incubation, the logarithmically growing cultures were harvested at an A_{650} of 0.20. The pH was corrected with 2 N HCl during growth.

Cells grown in methanol (62 mM) plus 0.02% YE (10 ml), at an absorbance of 0.70, were used as inoculum for 200 ml of a similar medium. After 3 to 4 days of incubation, the logarithmically growing cultures were harvested at an A_{650} of 0.50 to 0.70.

Cultures in thiosulfate media (42) were harvested after 4 to 5 days of incubation at an A_{650} of 0.11. The pH was maintained in the neutral range by addition of 2 N NaOH during growth.

Cells grown in 48 mM acetate (15 ml) were used as the inoculum for 200 ml of a similar medium. After 3 to 4 days of incubation, the logarithmically growing cultures were harvested. The pH was corrected with 2 N HCl during growth.

The harvested cells were washed three times with 0.025 M sodium phosphate buffer (pH 7.0) and stored at −20°C for 2 to 3 days until used for enzyme studies, but were used immediately after harvesting for manometric studies.

Preparation of cell extracts. Extracts in 0.025 M potassium phosphate buffer (pH 7.0) were obtained either by grinding with glass powder or by disrupting in a Raytheon model DF 101 ultrasonic oscillator for 10 min at 0°C. The supernatant fraction obtained after centrifugation at 27,000 × *g* for 15 min (Sorvall RC2-B) was used for enzyme assays within 24 h, during which time it was stored at −20°C.

Protein determination. The Folin-Ciocalteau reagent was used for protein determination, as described by Lowry et al. (27).

Chemicals. All of the cofactors and purified fine chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Enzyme assays. All spectrophotometric assays were carried out in a Pye-Unicam SP-500 series 2 spectrophotometer.

Nicotinamide adenine dinucleotide (NAD)-linked formate dehydrogenase (EC 1.2.1.2) and formaldehyde dehydrogenase were assayed by the method of Chandra and Shethna (9), using 50 μmol of formate or 66 μmol of formaldehyde. One unit of activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of NAD per min.

NAD-linked formate dehydrogenase was also assayed in extracts of thiosulfate-grown cells under anaerobic conditions in a manometer. Double sidearm Warburg flasks contained (in a total volume of 2.6 ml) 2 μmol of NAD, 4 μmol of ferricyanide, extract, and water in the main compartment. The flasks were flushed with O_2-free N_2 for 15 min. The reaction was started by tipping in 50 μmol of formate from the first side arm and was terminated by the addition of 0.5 ml of 2 N HCl from the second side arm. One unit of activity was defined as the amount

of enzyme that catalyzed the evolution of 1 μmol of CO_2 per min.

Dichlorophenolindophenol (DCPIP)-linked formate, formaldehyde, and methanol dehydrogenase were assayed by a modification of the method of Anthony and Zatman (5). One milliliter of the reaction mixture contained 0.1 mmol of tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.8), 0.1 μmol of phenazine methosulfate (PMS), 0.1 μmol of DCPIP, and 15 μmol of NH_4Cl. The reaction was started by the addition of 50 μmol of formate, 66 μmol of HCHO, or 25 μmol of methanol. One unit of activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of DCPIP per min. The E_{mM} of DCPIP was taken as 16.2 at 600 nm.

Glyoxylate carboligase and oxalyl-coenzyme A (CoA) decarboxylase (EC 4.1.1.8) were assayed by the method of Blackmore and Quayle (7). One unit of activity was defined as the amount of enzyme that catalyzed the evolution of 1 μmol of CO_2 per min.

Oxalyl-CoA reductase (EC 1.2.1.17) was assayed by the method of Blackmore and Quayle (7). One unit of activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of NADP per min.

Hydroxypyruvate reductase (EC 1.1.1.29) and L-serine glyoxylate aminotransferase were assayed by the method of Blackmore and Quayle (7). One unit of activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADH per min.

Catalase was assayed by the method of Fujii and Tonomura (17). One unit of activity was defined as the amount of enzyme required for a decrease in A_{240} of 1.0/min.

Methanol oxidase was assayed manometrically essentially by the method of Sahm (40). Warburg flasks contained (in a total volume of 2.3 ml) 25 μmol of potassium phosphate buffer (pH 7.0), 7 to 15 mg of protein, and water in the main compartment; 0.2 ml of 20% KOH in the center well; and 100 μmol of methanol in the side arm. One unit of activity was defined as the amount of enzyme that catalyzed the uptake of 1 μl of O_2 per h after endogenous O_2 uptake values were subtracted. Control flasks contained all of the components listed above except the enzyme extract.

Formamidase was assayed by the method of Halpern and Grossowicz (20). The assay mixture contained (in 1 ml) 2 mg of protein, 20 μmol of formamide, 7.5 μmol of potassium phosphate buffer (pH 7.0), and water. After incubation at 37°C for 2.5 h, the ammonia formed was estimated by diffusion, according to the Mortenson (31) method. Ammonia was determined colorimetrically by the nesslerization method.

RuBPCase (EC 4.1.1.39) was assayed by the method of Blackmore and Quayle (6), but using 500 to 700 μg of protein per 1 ml of assay mixture and incubating at 37°C for 5 min before terminating the reaction with 0.06 ml of 1 N HCl and heating at 100°C for 5 min. One unit of activity was defined as amount of enzyme that catalyzed the formation of 1 nmol of phosphoglyceric acid per min.

RESULTS

Growth studies. *T. novellus* utilized oxalate, ethanol, formamide, formate, and methanol (Table 1). Biotin accelerated growth on oxalate, but it did not affect the maximum growth. Hence, in all studies with oxalate and mixed substrates, biotin was added to the medium. Studies of growth patterns on 16.3 and 25 mM oxalate indicated an average doubling time of 11 h. Oxalate at 270 mM inhibited growth. The cell yield on 25 mM oxalate, when maximum growth had occurred (absorbance of 0.27, Fig. 1) was 0.024 g (dry weight) of cells per g of oxalate.

YE accelerated growth on formate and formamide (Table 1), but biotin did not affect growth on formate. Growth on formate occurred when the initial concentration was 44 mM or less; concentrations of 74 mM and above inhibited growth. Studies of the growth pattern on 44 mM formate medium to which 44 mmol of formate was added after 40 and 64 h of growth indicated an average doubling time of 32 h.

TABLE 1. *Utilization of C_1 and C_2 compounds by T. novellus*[a]

Substrate	Concn (mM)	A_{650}[b]	
		Without YE	With 0.02% YE
Nil		0.04	0.12
Oxalate[c]	16.3	0.19	NT
Formate	44	0.22	0.30[d]
Methylamine	65	0.04	0.08
Ethanol	43	0.26	NT
Ethylamine	44	0.05	0.12
Methanol	62	0.11	0.90
Formamide	44	0.16	0.30[d]
Trimethylamine	34	0.05	0.09
Formaldehyde	67	0.07	0.06
H_2-CO_2-air[e]		NG	NT

[a] Subcultures were prepared on nutrient agar slants. Cells from these slants were washed aseptically three times in 0.025 M phosphate buffer (pH 7.0) and suspended in the same buffer. A 1- to 2-ml amount of this suspension was then used as the inoculum for 100 ml of liquid medium in a 500-ml Erlenmeyer flask, which was then incubated on a rotary shaker. See text for composition of medium. The purity of the cultures when growth had occurred was checked by streaking on nutrient agar plates.

[b] Maximum turbidity obtained in 2 to 13 days. NT, Not tested; NG, no growth.

[c] Biotin (150 μg/100 ml of 16.3 mM oxalate) was added. In absence of biotin, the maximum turbidity was obtained 2 to 3 days later than in its presence.

[d] In presence of YE, the maximum turbidity was attained 2 to 3 days earlier than in its absence.

[e] Tested as described by Chandra and Shethna (8).

The growth patterns on mixed substrates with oxalate-grown cells as the inoculum are shown in Fig. 1. The growth pattern on F+T medium was essentially similar to that on O+F medium. The final extent of growth on O+F or O+T medium was nearly twofold higher than on 25 mM oxalate alone. There was no indication of a diauxic growth phenomenon on mixed substrates. The average doubling times on O+T, O+F, and F+T media were 10.5, 13, and 13 h, respectively.

T. novellus grew poorly on methanol; extensive growth on methanol required the addition of YE (Table 1). The growth rate on methanol was sensitive to different concentration of YE (Table 2). Calcium pantothenate or a mixture of vitamins also supported extensive growth (Table 2). When cells grown on 62 mM methanol plus 0.02% YE (10 ml of a 6- to 7-day-old culture with an absorbance of 0.70) were inoculated into a similar medium, the highest turbidity obtained was similar to that of cells transferred from nutrient agar to methanol (Fig. 2). Also the adaptation to growth on methanol did not overcome the requirement of YE for extensive growth. The cell yield on 62 mM methanol plus 0.02% YE when the culture had reached a maximum absorbance of 0.85 to 0.90 (Fig. 2) was between 0.23 to 0.26 g (dry weight) of cells per g of methanol after subtracting the dry weight of cells obtained in the same period from 0.02% YE medium without methanol.

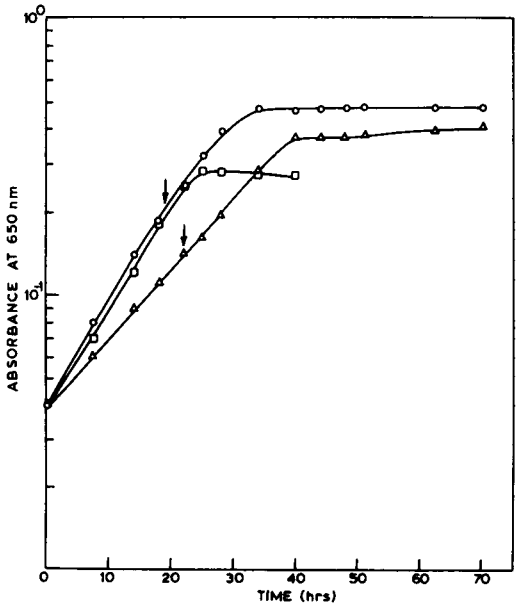

FIG. 1. *Growth pattern of T. novellus on mixed substrates. Symbols: □, 25 mM oxalate; ○, 25 mM oxalate plus 25 mM thiosulfate; △, 25 mM oxalate plus 25 mM formate. Arrows indicate absorbance at which O+T- and O+F-grown cells were harvested for enzymatic studies.*

TABLE 2. *Effect of YE and vitamins on T. novellus growth on methanol*[a]

Addition[b]	A_{650}[c]	Doubling time[d] (h)
Nil	0.11	
YE (0.002%)	0.40	110
YE (0.012%)	0.37[e]	54
YE (0.020%)	0.90	37
Vitamin B_{12} + calcium pantothenate	0.58	84
Calcium pantothenate	0.45	120
Thiamine HCl	0.09	
Nicotinic acid	0.13	
Pyridoxine	0.09	
p-Aminobenzoic acid	0.11	
Folic acid	0.11	
Biotin (300 μg)	0.12	
Riboflavin (50 μg)	0.09	
Riboflavin (250 μg)	0.11	
Mixture of vitamins 6 to 13 + vitamin B_{12}	0.54	48
Bicarbonate (0.05%)	0.12	

[a] Inoculum and growth conditions as described in footnote *a* of Table 1.

[b] 250 μg of vitamin per 100 ml of 62 mM methanol medium was added except where mentioned.

[c] Maximum turbidity obtained in 15 to 17 days.

[d] Doubling time values were calculated from the middle range of the growth curves.

[e] Maximum turbidity obtained after 5 days of growth.

Oxidative and enzymatic studies. The oxidative properties of the oxalate-, C_1-compound-, and mixed-substrate-grown whole cells are shown in Table 3. Oxalate-grown cells did not oxidize thiosulfate, whereas formate-grown cells oxidized thiosulfate for 20 to 30 min. Thiosulfate-grown cells oxidized thiosulfate and sulfite rapidly and formate slowly, with lag periods of 10 to 15 min. O+T- and F+T-grown cells, harvested at time periods described in Materials and Methods, oxidized thiosulfate as rapidly as thiosulfate-grown cells for 60 min or more, whereas O+F-grown cells oxidized it in a pattern resembling that of formate-grown cells for only 20 to 30 min. Formamide-grown cells oxidized formamide, formate, and methanol well, and the oxidative pattern with thiosulfate closely resembled that of formate-grown cells. Methanol-grown cells oxidized methanol, formate, and HCHO. The pattern of thiosulfate oxidation by methanol-grown cells resembled that of formate-grown cells.

The enzymatic activities in crude cell ex-

tracts of cells grown on the above-mentioned substrates are shown in Table 4. Glyoxylate carboligase activity was present in oxalate-, O+T-, and O+F-grown (harvested at an early phase of growth, as shown by arrows in Fig. 1)

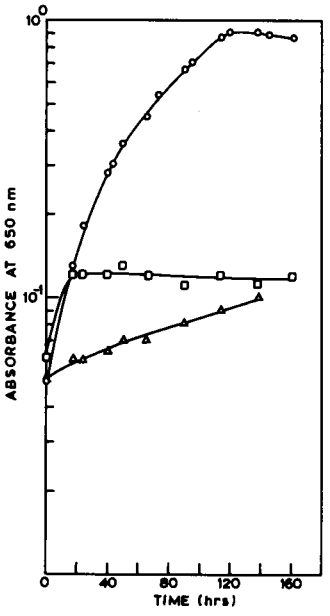

FIG. 2. *Growth pattern of T. novellus on methanol. Symbols:* ○, *62 mM methanol plus 0.02% YE;* □, *0.02% YE alone;* △, *62 mM methanol alone.*

cells at a level of activity ten times higher than that of acetate-grown cells. Hydroxypyruvate reductase activity was not stimulated in oxalate- or C_1-substrate-grown cells as compared with that of acetate-grown cells and, furthermore, was at a low level (<0.1 U/mg of protein). RuBPCase was present at a high level of activity in formate- and formamide-grown cells, similar to that reported in thiosulfate-grown *T. novellus* (28), whereas its activity was low or undetectable in O+T-, O+F-, methanol-, and oxalate-grown cells.

NAD-linked formate dehydrogenase was present in cells grown on all of the substrates except thiosulfate and acetate when assayed spectrophotometrically (Table 4). In thiosulfate-grown cells, its activity was undetectable when assayed manometrically under anaerobic conditions. NAD-linked formate and formaldehyde dehydrogenase in methanol-grown cells were not stimulated by 6 μmol of $MgCl_2$, 6 μmol of glutathione (GSH), and 2 mM KCN, and there was no activity with NADP. Methanol dehydrogenase activity in methanol-grown cells, linked to NAD, NADP, or ferricyanide, was not detected at pH 7.0. A PMS-plus-DCPIP-linked methanol dehydrogenase activity in methanol-grown cells was also absent when tested at pH 7.0 (phosphate buffer) and pH 7.8 [tris(hydroxymethyl)aminomethane-hydrochloride buffer] even upon the addition of 6 μmol of GSH, 6 μmol of $MgCl_2$, and 2 mM

TABLE 3. *Oxidative properties of T. novellus*[a]

Grown on	$Q(O_2)$ (μl of O_2 taken up/h per mg [dry wt])[b]						
	Oxalate	Formate	Thiosulfate	Sulfite	Methanol	Formamide	Formaldehyde
Oxalate	30–71 (3)	30–59 (3)	0–9 (3)	0	7	–	24
Formate	0	21–45 (5)	27–63 (5)	11 (3)	–	–	–
Thiosulfate	0–5 (2)	12–22 (5)	57–80 (5)	54	21	14	–
Formamide	4–8 (2)	18–54 (2)	24–26 (2)	–	13–42 (2)	18–54 (2)	–
Methanol	0	22–39 (4)	27–50 (6)	–	26–58 (5)	20–31 (2)	32 (3)
Oxalate plus formate	30–55 (2)	55 (2)	28 (2)	–	–	–	–
Oxalate plus thiosulfate	47 (2)	47 (2)	37–42 (3)	–	32	16	–
Formate plus thiosulfate	0	46 (2)	80 (2)	–	–	–	–

[a] Cells, grown and harvested as described in the text, were washed three times in 0.025 M sodium phosphate buffer (pH 7.0) and suspended in the same buffer to a final concentration of 3 to 6 mg (dry weight) per ml. The assay system was the same as that described by Chandra and Shethna (8).

[b] 30 μmol of oxalate, formate, thiosulfate, sulfite, and formamide; 50 μmol of methanol; and 100 μmol of formaldehyde were used. The $Q(O_2)$ values were calculated from the O_2 uptake values in the first 0- to 30-min period of oxidation. The endogenous O_2 uptake values were subtracted before calculation of $Q(O_2)$. The number in parentheses indicates the number of determinations, and the range represents the minimum and maximum values of $Q(O_2)$. All of the determinations were identical in cases where no range is given. In cases where no figures are given in parentheses, results were based on a single experiment. –, Not tested.

TABLE 4. *Enzyme activities in T. novellus grown on different carbon sources*

Enzyme	Sp act[a] (U/mg of protein)							
	Oxalate[b]	Formate	Thiosulfate	Methanol	Formamide	Oxalate + thiosulfate	Oxalate + formate	Acetate
Formate dehydrogenase								
NAD linked	0.10	0.14	0	0.05	0.23	0.07	0.01	0
DCPIP linked	0.08	–	–	0.01	–	–	–	–
Formaldehyde dehydrogenase								
NAD linked	0.06	–	–	0.03	–	–	–	–
DCPIP linked	0.01	–	–	0.01	–	–	–	–
Catalase	0.61	–	0.62	0.67	0.69	–	–	–
Oxalyl-CoA decarboxylase	0.08	–	–	–	–	–	–	–
Ribulose bisphosphate carboxylase	0	83.00	32.00	10.00	24.00	2.00	5.90	–
Glyoxylate carboligase[c]	0.33	–	–	–	–	0.33	0.30	0.03
Hydroxypyruvate reductase	0.07	0.01	0.01	0.07	0.06	0.05	0.04	0.05
Serine-glyoxylate aminotransferase	0.01	–	–	–	–	–	–	–
Oxalyl-CoA reductase	0.26	0	0	–	–	–	–	–

[a] Highest values obtained in two or more experiments. –, Not tested.

[b] Growth substrate.

[c] Activities were obtained with 1.8 μmol of thiamine pyrophosphate and 5 μmol of $MgCl_2$ in the reaction mixture. Activities of 0.33 U/mg of protein with thiamine pyrophosphate alone and 0.136 U/mg of protein with $MgCl_2$ alone were obtained in oxalate-grown cells.

KCN. The extracts of methanol-grown cells catalyzed a very weak oxidation of methanol, with a specific activity of 8.0 μl of O_2 consumed per h per mg of protein. This oxidation was not stimulated by 1 μmol of flavin adenine dinucleotide. Catalase activity was present in extracts of oxalate-, thiosulfate-, methanol-, and formamide-grown cells (Table 4).

In formamide-grown cells, attempts to detect formamidase activity by the ammonia nesslerization method failed because of interference of endogenous ammonia in the crude extracts and chemical hydrolysis of formamide under the alkaline conditions of the assay.

DISCUSSION

T. novellus is the first facultative chemolithotroph, and first thiobacillus, known to utilize oxalate and several C_1 compounds (formate, formamide, and methanol; Table 1). It thus behaves as a facultative methylotroph. The only other facultative chemolithotroph shown by Cox and Quayle (15) to similarly utilize C_1 compounds (methanol, methylamine, and formate) is the hydrogen oxidizer *Micrococcus denitrificans*, but it does not utilize oxalate. Utilization of formamide as the sole carbon source is known in a few C_1-compound-metabolizing bacteria (35). Growth of *T. novellus* on methanol (Table 2, Fig. 2) is stimulated by YE or calcium pantothenate; similar observations have been reported for several bacteria (23, 24, 33, 34, 38).

The total cell yield of *T. novellus* on methanol-YE medium was low compared to the theoretical values calculated by Van Dijken and Harder (48) for the ribulose monophosphate pathway on methanol. Also, the low cell yield and slow growth rate on methanol could affect the possible use of *T. novellus* as a source of single-cell protein with methanol.

The enzymes of oxalate and C_1-compound metabolism have been extensively examined by various methods (4, 7, 37). Hence, a rapid screening of organisms for operation of the above pathways is now possible by demonstrating the presence of key enzymes in crude cell extracts. This concept has been discussed by Anthony (4).

Synthesis of cell constituents from oxalate is reported to proceed either by the glycerate pathway, as in *P. oxalaticus*, or by the serine pathway, as in pink-pigmented organisms (7, 10a, 21). The necessary energy for growth on oxalate is derived from a series of catabolic reactions involving the formation of oxalyl-CoA, formyl-CoA, and formate as intermediates (7).

The assimilation of formate can proceed after oxidation of formate to CO_2, which is then fixed by RuBPCase (6). This pathway operates in only four aerobic bacteria, *P. oxalaticus* (6), *Bacterium formoxidans* (43), *Hydrogenomonas eutropha* Z-1 (32), and *Alcaligenes* FOR_1 (10). Alternatively, formate can be assimilated by the serine pathway as in *Pseudomonas* AM2 and AM1 (7, 37) and *Pseudomonas* C (18), where the reduction level of formate is conserved and formyl tetrahydrofolate is formed as an intermediate (37). The energy requirement for autotrophic growth on formate is much higher than for heterotrophic growth on formate by the serine pathway (46). The serine pathway also operates in the assimilation of methanol and methylamine in most organisms (4, 37). Methanol and methane can also be assimilated by the ribulose monophosphate cycle (4, 46). A unique case of aerobic autotrophic assimilation of methanol carbon by *M. denitrificans* has been recently reported (15).

Our investigations on oxalate and C_1-compound metabolism and thiosulfate oxidation in *T. novellus* have revealed the following. Oxalate is not utilized by *T. novellus* autotrophically since RuBPCase is absent in oxalate-grown cells (Table 4). The key enzymes of the glycerate pathway are oxalyl-CoA reductase and glyoxylate carboligase. Oxalyl-CoA reductase is present in oxalate-grown cells but not in formate- or thiosulfate-grown cells. Glyoxylate carboligase is present at 10-fold-higher activity in oxalate-grown cells as compared with that of acetate-grown cells (Table 4). These data indicate heterotrophic utilization of oxalate by the glycerate pathway. Acetate-grown cells were used for comparison because the specific activities of enzymes of the citric acid and glyoxylic acid cycles are reported to be highest in these cells (11). The level of glyoxylate carboligase activity in *T. novellus* is also higher than the level reported in oxalate-grown *P. oxalaticus* (7), where the glycerate pathway of oxalate assimilation has been unequivocally established. Furthermore, hydroxypyruvate reductase was not stimulated in *T. novellus* grown on oxalate compared with that of *T. novellus* grown on other substrates (Table 4), ruling out the operation of the serine pathway. Similar results were reported for *P. oxalaticus* (6, 7). It may be noted that, in organisms in which the serine pathway operates, high hydroxypyruvate reductase activity (>0.4 U/mg of protein) is found (7, 9, 10, 15).

In formate-grown cells RuBPCase was present at a high level of activity (Table 4), similar to that reported in thiosulfate-grown *T. novellus* (28). This indicates autotrophic utilization of formate carbon. The low hydroxypyruvate reductase activity in formate-grown cells (which is not stimulated as compared with that in acetate-grown cells) rules out the serine pathway. *T. novellus* is the first *Thiobacillus* species in which autotrophic growth on formate has been detected. *Thiobacillus* A2 (47) also utilizes formate, but there is no enzymatic or other evidence for autotrophic or heterotrophic growth. (The other four aerobic bacteria shown to be autotrophic on formate were mentioned earlier.)

P. oxalaticus and *T. novellus* closely resemble each other in the pattern of metabolism of oxalate (heterotrophic glycerate pathway) and formate (autotrophic ribulose bisphosphate pathway). However, there are a few interesting differences. (i) The growth rate of *P. oxalaticus* is higher on formate than on oxalate (6, 16), whereas in *T. novellus* heterotrophic growth on oxalate is faster than autotrophic growth on formate. (ii) In *P. oxalaticus*, NAD-linked formate dehydrogenase activity is nearly threefold higher in oxalate-grown cells than in formate-grown cells (6). This is not so in *T. novellus*.

McCarthy and Charles (28) reported that HCO_3^-, sulfite, and sulfite plus HCO_3^- do not "induce" RuBPCase in *T. novellus* and only thiosulfate does so. Our results show that formate can also induce this enzyme in *T. novellus*.

In formamide-grown cells, good RuBPCase activity (Table 4) indicated an autotrophic assimilation of formamide carbon, just as on formate. The presence of NAD-linked formate dehydrogenase indicated that formate is formed as an intermediate during growth on formamide. The fact that the whole cells oxidized formamide points to the presence of formamidase activity, but this enzyme could not be detected

by the assay procedure used. It appears possible that, during growth on formamide, the formamide is converted into formate by the amidase reaction, as reported for other bacteria (14), and subsequently formate induces the enzymes of the autotrophic pathway as well as the NAD-linked formate dehydrogenase.

The data in Table 4 support the hypothesis that, at the early phase of growth on mixed substrates (Fig. 1), the predominant mode of metabolism is heterotrophic by the glycerate pathway, since glyoxylate carboligase activity is as good as that of oxalate-grown cells and RuBPCase activity is low compared with that of formamide- or thiosulfate-grown cells. At the later phases of growth, the metabolism is probably autotrophic or mixotrophic, since growth on mixed substrates continued even after it had stopped in the control flask with oxalate alone. Further studies on the enzymatic levels in the middle and late phases of growth on mixed substrates, using formate-grown cells as the inoculum, would enable comparisons to be made of the metabolism of *T. novellus* and *P. oxalacticus* (6; M. Knight et al., Proc. Soc. Gen. Microbiol. 3:p7, 1975) on mixed substrates.

In methanol-grown cells, the RuBPCase activity was low compared with that of formate-, thiosulfate-, or formamide-grown cells (Table 4). Hydroxypyruvate reductase activity was also not stimulated in methanol-grown cells as compared with cells grown on other substrates. It therefore appears that the major route of methanol assimilation could be heterotrophic by either the ribulose monophosphate pathway or a novel route with only a limited reductive pentose cycle.

We found NAD-linked formate dehydrogenase in oxalate- and C_1-compound-grown *T. novellus* but not in thiosulfate- and acetate-grown cells. Aleem (1) reported formate oxidase activity in thiosulfate-grown *T. novellus*, which also has thiosulfate oxidase activity. In view of Aleem's (1) findings of formate-cytochrome *c* reductase, it would be of interest to investigate whether such systems also occur in oxalate- and C_1-compound-grown *T. novellus* in addition to NAD-linked formate dehydrogenase. Two different systems for formate oxidation have been reported in an oxalate-decomposing *Alcaligenes* species (9).

The first step of methanol dissimilation in microorganisms, i.e., oxidation to HCHO, can be catalyzed by: (i) an NH_4^+-dependent, PMS-plus-DCPIP-linked methanol dehydrogenase, with a pH optimum of 8.5 to 9.0 (22); (ii) an NAD-linked methanol dehydrogenase (22, 30, 41); and (iii) a methanol oxidase coupled with peroxidative activity of catalase (39). Formaldehyde is further oxidized to formate by NAD-linked dehydrogenases, dependently or independently of GSH; or by DCPIP-linked HCHO dehydrogenase; or frequently by the DCPIP-linked methanol dehydrogenase itself (22, 37). Multiple enzymes for HCHO oxidation can occur simultaneously (36). Formate is oxidized by NAD-linked dehydrogenase in most methanol-utilizing bacteria (4, 37).

In methanol-grown *T. novellus*, NAD- or PMS-plus-DCPIP-linked methanol dehydrogenase was undetectable at the pH values tested (see Results). The oxidation of methanol by the extracts, although weak (Results), indicates methanol oxidase activity. The catalase activity in methanol-grown *T. novellus* was not higher than that in oxalate-, thiosulfate-, or formamide-grown cells (Table 4), implying that catalase does not function in methanol oxidation as distinctly as it does in *Candida boidinii* (39), in which the catalase activity was fivefold higher in methanol-grown cells than in glucose- or ethanol-grown cells. Recently, the suggested peroxidative activity of catalase in the in vivo oxidation of methanol in yeasts has been disputed (49). In *T. novellus* it is not certain whether, by modifying the pH conditions or by adding as yet undetermined cofactors, DCPIP-linked methanol dehydrogenase can be detected or whether the oxidase activity can be stimulated. The first step of methanol oxidation needs to be investigated further for final conclusions to be drawn. Formaldehyde is oxidized by both NAD and DCPIP-linked HCHO dehydrogenase independently of GSH in *T. novellus* (Table 4). On the basis of the oxidation of methanol, HCHO, and formate by whole cells (Table 3) and the presence of NAD-linked HCHO and formate dehydrogenase in extracts (Table 4), it is suggested that *T. novellus* oxidizes methanol via HCHO and formate to CO_2.

A unique pattern of thiosulfate oxidation was noted in formate-, methanol-, formamide-, and O+F-grown cells—the oxidation abruptly stopped after 20 to 30 min. In contrast, cells grown on media with thiosulfate, viz., thiosulfate, O+T, and F+T media, oxidized thiosulfate linearly and at a high rate for 60 min or more. These results suggest the requirement of some cofactors for thiosulfate oxidation which are probably synthesized in sufficient concentrations only when thiosulfate is present as an oxidizable substrate. An alternate explanation could be that tetrathionate is formed which is inhibitory to the cells. However, it has been reported that thiosulfate-grown *T. novellus*

does not form tetrathionate as an intermediate during thiosulfate oxidation (12). Le'John et al. (25) have investigated the catabolite repression of thiosulfate-oxidizing enzymes in *T. novellus*. It is difficult to make a direct comparison of our results with their results due to the differences in the growth conditions and assay methods employed.

T. novellus is a facultative chemolithotroph that also utilizes oxalate and C_1 compounds. Therefore, it is an ideal organism for investigating the biochemical basis of facultative autotrophy and regulation of RuBPCase on simple organic C_1 and C_2 compounds, which are at the borderline between inorganic CO_2 and complex organic compounds.

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