Autotrophic Metabolism of Formate by *Thiobacillus* Strain A2

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Thiobacillus A2 grew on formate as its sole source of carbon and energy. Growth was autotrophic: formate and carbon dioxide were equivalent as carbon sources during growth on formate. Carbon dioxide was fixed by the Calvin cycle in formate- or thiosulphate-grown bacteria which contained comparable high specific activities of ribulosebiphosphate carboxylase. Formate incorporated by bacteria growing heterotrophically or on thiosulphate showed more restricted metabolism, particularly providing carbon for purines. Bacteria growing on formate or thiosulphate assimilated acetate but showed disproportionately high incorporation into glutamate, proline, arginine, leucine, pyrimidines and lipid. Growth kinetics on formate were studied using extended cultures held at constant formate concentrations at pH 7.8. Yield was a function of substrate concentration and growth rate, which were linearly related in the range 4 to 40 mM-formate. Formate was an inhibitory substrate at higher concentrations. A computer analysis of the inhibited growth kinetics indicated K_s and K_i values of 26.5 and 187 mM-formate, respectively, and a true μ_{max} of 0.299 h⁻¹.

INTRODUCTION

Thiobacillus A2 (Taylor & Hoare, 1969) is a facultatively autotrophic chemolithotroph capable of autotrophic growth on thiosulphate plus carbon dioxide and heterotrophic growth on a wide range of organic substrates (Kelly, 1973; Wood & Kelly, 1977). Taylor & Hoare (1969) demonstrated the presence of an inducible formate dehydrogenase in the organism and showed that it could grow on formate as sole source of carbon and energy. However, no information was obtained about growth kinetics or yields on formate, or about the mechanism by which formate was metabolized. The latter was of particular interest since the organism was known to use the Benson-Calvin reductive pentose phosphate cycle during autotrophic growth on thiosulphate, and the question to be resolved was whether this mechanism also operated during growth on formate (as in *Pseudomonas oxalaticus* and *Paracoccus denitrificans*) or whether alternative pathways such as those used by methylotrophs were present. We have sought to answer this question and to study growth kinetics using both extended culture (Dijkhuizen & Harder, 1975) and continuous chemostat culture methods.

METHODS

Organism and culture conditions. *Thiobacillus* A2, originally obtained from Dr D. S. Hoare, was maintained on agar slants of thiosulphate medium (Wood & Kelly, 1977) or medium with 0.1 M-sodium formate. For growth experiments involving assimilation of ¹⁴C-labelled substrates, cultures were normally grown in 20 or 50 ml medium in 250 ml Erlenmeyer or 'nephlos' flasks (Wood & Kelly, 1977) shaken at 30 °C. Growth in nephlos flasks was monitored as increase in absorbance at 440 nm using a Unicam SP600 spectrophotometer with a test tube attachment. Growth at controlled pH values and in extended culture (see below)

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was studied using 2 l cultures in an LH Engineering CC1500 fermenter, maintained at 30 °C with aeration (500 ml air min⁻¹) and stirred at 500 rev. min⁻¹. The pH was poised at the desired value by automatic titration with 2 or 10 M-formic acid using an EIL model 91B or 9150 pH controller and glass dual electrode with remote reference electrode. The principle of maintaining pH during growth on formate using formic acid meant that the formate concentration in the culture medium was held at a constant value, and growth of the culture could continue exponentially until some factor other than formate became limiting. Such growth conditions have been called 'extended cultures' (Dijkhuizen & Harder, 1975) and enabled growth rates and yields to be measured over a range of fixed formate concentrations in the culture vessel. Actual 'steady-state' formate concentrations during extended cultures were measured using the procedure of Lang & Lang (1972). Growth in extended cultures was monitored turbidimetrically and by replicate dry weight determinations for each growth condition studied. Viable counting on agar media of serial dilutions from cultures demonstrated that all the organisms growing on formate also formed colonies on media with thiosulphate, glucose or sucrose and were characteristically *Thiobacillus* A2. In chemostat cultures at constant pH, sodium formate was the growth-limiting nutrient.

Incorporation of ¹⁴C-labelled substrates. Assimilation of [¹⁴C]formate, ¹⁴CO₂ or [U-¹⁴C]acetate was monitored using membrane filtration methods (Tuovinen & Kelly, 1973); fractionation, analysis and chromatography of labelled bacteria was done by procedures described previously (Kelly, 1967, 1969, 1970; Eccleston & Kelly, 1973). For growth in the presence of ¹⁴CO₂, flasks were sealed with 'Suba-seal' vaccine stoppers. Short-term incorporation of [¹⁴C]formate or ¹⁴CO₂ was studied using bacterial suspensions harvested from formate-grown cultures, washed and resuspended in 25 µM-K₂HPO₄, pH 7.4. Bacteria (10 ml) were stirred in 50 ml beakers or 50 ml sealed conical flasks for 3 min after addition of 1 ml 0.06 M-sodium formate, then 1.5 ml of either sodium [¹⁴C]formate (75 µCi) or Na₂¹⁴CO₃ (200 µCi) were added, and samples (4 ml) were withdrawn into ethanol (20 ml) at 70 °C after 0.5, 1.5 and 3.0 min. The ethanol-soluble cell constituents were separated chromatographically as described by Quayle & Keech (1959). ¹⁴C-labelled compounds detected by radioautography and identified by their comparative chromatographic mobility were then eluted for further chromatography with appropriate reference compounds.

Assay of ribulosebiphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing); EC 4.1.1.39]. Bacteria were harvested from steady-state chemostat cultures on formate by centrifugation (2 °C, 12000 g, 15 min), washed with 0.1 M-Tris/maleate buffer, pH 8.0, and suspended at 10 to 40 mg ml⁻¹ either in similar buffer or in 0.1 M-Tris/HCl, pH 8.2, containing 20 mM-MgCl₂, 5 mM-NaHCO₃ and 0.5 mM-dithiothreitol. Bacteria were sonicated (5 × 30 s at maximum power) in the presence of 1 g Ballotini glass beads (0.11 mm) per 2 ml suspension using an MSE ultrasonic disintegrator. The extracts used for enzyme assay were the supernates obtained from centrifuging at 20000 g for 20 min. Two assay procedures were used.

(i) *Spectrophotometric method* (London & Rittenberg, 1966). Reaction mixtures (1 ml) at pH 8.6 contained (µmol, unless indicated otherwise): Tris/maleate, 250; MgCl₂, 10; NaHCO₃, 37; glutathione, 5; ATP, 3; NADH, 0.14; phosphoglycerate kinase [ATP:3-phospho-D-glycerate 1-phosphotransferase; EC 2.7.2.3], 12 µg; glyceraldehyde phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD⁺ oxidoreductase (phosphorylating); EC 1.2.1.12], 150 µg; ribulose 1,5-bisphosphate, 0.24; crude extract, 0.02 to 0.2 mg protein. Activity was directly proportional to the added protein concentration and the reaction was linear for at least 6 min. No detectable activity was found if any one of ribulose bisphosphate, phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase, NADH or ATP was omitted.

(ii) *¹⁴CO₂-fixation method.* Reaction mixtures (0.3 ml) at pH 8.2 contained (µmol, unless indicated otherwise): Tris/HCl, 27; MgCl₂, 5.4; dithiothreitol, 0.135; NaH¹⁴CO₃ (0.2 µCi µmol⁻¹), 1.85; ribulose 1,5-bisphosphate, 0.2; crude extract, 0.05 to 0.5 mg protein. Reactions were initiated by addition of ribulose 1,5-bisphosphate and samples (50 µl) were removed after 30, 60, 90 and 120 s into 200 µl acetic acid at 60 °C in scintillation vials. After 20 min at 60 °C, vials were cooled, 4 ml scintillation fluid (toluene, 2 l; Triton-X100, 1 l; diphenyloxazole, 10 g) were added and ¹⁴C was assayed in a Nuclear Chicago scintillation counter. Prior to activity measurements, the extract was activated by incubation for 5 to 10 min at room temperature (18 to 20 °C) as described by Badger & Lorimer (1976). There was a linear relationship between ¹⁴CO₂-fixation and time for at least 120 s under these conditions. Activity was proportional to protein concentration.

Protein contents of crude extracts and bovine serum albumin standards were measured by a microbiuret method (Kuenen & Veldkamp, 1972) or by Lowry's Folin procedure.

Chemicals. All radiochemicals were from The Radiochemical Centre, Amersham. All reagents were of analytical or chromatographic grade. Ribulose 1,5-bisphosphate and ATP were from Sigma; glutathione, NADH, phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase were from Boehringer. The latter enzyme was dialysed before use against 1 l of Tris/maleate buffer for 22 h at 4 °C to remove ammonium ions, which were inhibitory to the carboxylase.

Table 1. *Specific growth rates of Thiobacillus A2 at constant pH values in extended exponential growth with constant formate concentration (25 mM)*

Culture pH	Specific growth rate, μ (h ⁻¹)	Growth yield [g dry wt (mol formate) ⁻¹]
7.2	0.080	2.93
7.6	0.092	3.99
7.8	0.154	3.64
8.0	0.103	4.19
8.4	0.057	3.20

Table 2. *Relationship between formate concentration, specific growth rate and yield in extended exponential cultures of Thiobacillus A2 at pH 7.8*

Constant formate concn (mM)	Specific growth rate, μ (h ⁻¹)	Growth yield [g dry wt (mol formate) ⁻¹]
3.6	0.014	1.50
3.8	0.011	1.40
5.0	0.025	1.75
7.8	0.034	2.44
16.4	0.116	2.94
23.4	0.154	4.26
41.2	0.198	2.40
87.9	0.168	2.51
171.4	0.116	3.59
402.2	0.027	0.99

RESULTS

Growth in batch and extended cultures

Using media initially at pH 8.2, growth occurred in shake flask cultures with $\mu = 0.1 \text{ h}^{-1}$, and the yield was proportional to the initial formate concentration between 20 to 100 mM. The culture pH rose during batch growth in accordance with the equation for sodium formate oxidation:

Constant exponential growth rates and yields were determined for extended cultures (Dijkhuizen & Harder, 1975) controlled at several pH values by automatic titration with formic acid to maintain a constant formate concentration (25 mM) in the culture media (Table 1). The growth rate was maximal at about pH 7.8, while the yield was similar between pH 7.6 and 8.0 but decreased at pH 7.2 and 8.4.

In extended cultures held at several fixed formate concentrations and poised at pH 7.8, the growth rate increased with formate concentration up to about 40 mM; higher concentrations were inhibitory (Table 2). The yield also increased with the increasing growth rate (Table 2). The correlation between increase in yield and increase in growth rate consequent on increased substrate concentration was indicated by the relationship between the reciprocals of yields (Y^{-1}) and the reciprocals of either formate concentration (s^{-1}) or growth rate (μ^{-1}) (Fig. 1). Since this plot formally relates yield and growth rate in the same manner as described for substrate-limited chemostat cultures (Pirt, 1965), the extrapolation of the line (assuming the relationship in Fig. 1 is linear and not curved) to the Y^{-1} axis gives the apparent true growth yield (Y_0) and the slope gives a measure of the maintenance energy requirement (m). Extrapolation of the line drawn in Fig. 1 indicated Y_0 to be 4.2 g dry wt (mol formate)⁻¹ and m to be 7 mmol formate (g dry wt)⁻¹ h⁻¹. Assuming the relationship of Y^{-1} versus μ^{-1} to be linear, Y_0 values were also computed [from $Y^{-1} = m^{-1}\mu^{-1} + Y_0^{-1}$],

Fig. 1

Fig. 1. Growth rates and yields of *Thiobacillus* A2 in extended exponential culture at pH 7.8 over a range of concentrations of sodium formate. Reciprocals of growth yields (Y^{-1}) were plotted against reciprocals of specific growth rate (μ^{-1}) (●) or reciprocals of substrate (formate) concentration (mm^{-1}) (○), using the data from Table 2 for all formate concentrations between 3.6 and 171.4 mM; 87.9 and 171.4 mM-formate were growth rate inhibitory.

Fig. 2

Fig. 2. Incorporation of $[^{14}\text{C}]$ formate into *Thiobacillus* A2 during growth of cultures (10 ml) in 250 ml nephlos flasks with 0.1 M-sodium $[^{14}\text{C}]$ formate ($1 \mu\text{Ci ml}^{-1}$) under different conditions of CO_2 supply. The initial organism density was $90 \mu\text{g ml}^{-1}$. Cultures were incubated under air in a flask closed with a cotton plug (○); under air in a flask sealed with a rubber stopper (×); under air in a flask sealed with a rubber stopper, but with KOH pellets in the well of the flask to absorb gaseous CO_2 (●); under air containing 10% (v/v) CO_2 in a sealed flask (Δ); or in a flask continuously flushed with sterile 5% (v/v) CO_2 in air at 100 ml min^{-1} (▲).

using the first six and seven values of Table 2, as 3.7 and 3.3, respectively, with correlation coefficients of 0.96 and 0.93, respectively. The correlation indicates reasonable fit of the data to a linear relationship, but the possibility that the relation tends to a curve (as may also be the case in chemostat culture; J. G. Kuenen, unpublished work) has not been excluded.

Chemostat culture on limiting formate

Chemostat growth kinetics and their interpretation will be discussed in detail elsewhere (J. C. Gottschal & J. G. Kuenen, unpublished) and are here used for comparative purposes only. Using 100 mM input formate concentration, the steady-state formate concentration was less than 0.3 mM and the value for Y_a could be calculated from Y^{-1} versus D^{-1} and from q_{formate} versus D plots to be between 3.0 and 3.3 g dry wt (mol formate) $^{-1}$.

Equivalence of carbon dioxide and formate-carbon during growth on formate in batch culture

During exponential growth on $[^{14}\text{C}]$ formate in sealed shake-flasks containing air depleted of CO_2 , most newly synthesized *Thiobacillus* A2 carbon was derived from formate (Fig. 2). Using conditions under which CO_2 was available to different extents, the amount of formate-carbon assimilated could be decreased considerably (Fig. 2). In flasks open to normal air, only two-thirds of the cell-carbon came from formate, while continuous purging of the culture with 5% (v/v) CO_2 in air reduced this to less than 10% (Table 3). Under conditions

Table 3. *Effect of availability of carbon dioxide on the growth rate and formate-carbon assimilation by Thiobacillus A2*

Cultures were grown on 0.1 M-sodium [^{14}C]formate as described in the legend to Fig. 2. Formate assimilation is expressed relative to the increase in biomass (as μmol formate-carbon incorporated per mg increase in culture dry weight) and as a percentage of the total amount of carbon fixed (assuming the cell carbon content to be 44% of the dry weight).

Growth condition	Specific growth rate, μ (h^{-1})	Formate assimilated [μmol (mg dry wt^{-1})]	Percentage of cell-carbon from formate
Air	0.096	24.2	65.9
Air, sealed flask	0.084	30.4	83.0
Air, sealed flask, CO_2 absorbed by KOH	0.093	33.2	90.7
10% (v/v) CO_2 in air, sealed flask	0.143	8.1	22.0
5% (v/v) CO_2 in air flushed through flask	0.146	2.8	7.7

Table 4. *Short-term incorporation of ^{14}C -labelled carbon dioxide or formate by suspensions of Thiobacillus A2 previously grown on formate*

^{14}C -labelled carbonate or formate was added to bacterial suspensions (7 mg ml^{-1}) and samples were taken and analysed as described in Methods. The results show the ^{14}C distribution among compounds in the 80% (v/v) ethanol-soluble fraction of the bacteria.

Sample time (min)	^{14}C distribution (% of total ^{14}C on chromatogram)					
	Incorporation from $^{14}\text{CO}_2$			Incorporation from [^{14}C]formate		
	0.5	1.5	3.0	0.5	1.5	3.0
Total ^{14}C (c.p.m.)	38818	82284	97989	22777	74630	220030
No. of labelled compounds	19	29	29	19	31	42
Phosphoglyceric acid	14.6	10.6	3.4	20.9	12.8	4.5
Sugar phosphates	22.2	17.8	22.1	16.2	21.6	14.7
Phosphoenolpyruvate	2.7	1.9	1.0	2.2	0.9	0.2
Glutamic acid	17.6	25.4	17.9	21.0	23.7	12.1
Glutamine	31.1	20.3	7.1	17.5	12.1	8.7
Aspartic acid	0.4	0.6	3.1	2.6	1.4	0.8
Asparagine	1.2	1.1	3.1	2.1	4.7	7.6
Leucine, isoleucine, phenylalanine	1.7	4.5	13.2	3.1	8.4	13.5
Glycollic acid (?)	1.7	2.3	3.6	2.1	4.6	3.8
Alanine	—	—	1.6	—	0.6	1.1
Valine	—	1.0	1.9	—	1.5	4.9
Tyrosine	—	0.1	0.1	0.9	0.3	0.3
Citric acid (?)	—	0.9	2.0	0.3	1.0	2.5
Phosphogluconic acid (?)	—	—	—	4.4	1.2	0.6
Percentage of total ^{14}C recovered in the identified compounds	93.2	86.5	80.1	93.3	94.8	75.3

of enhanced CO_2 supply the growth rate on formate was considerably stimulated (Table 3), consistent with growth being dependent on CO_2 -fixation using energy from formate oxidation.

Short-term incorporation of ^{14}C -labelled formate or carbon dioxide by suspensions oxidizing formate

Suspensions of *Thiobacillus* A2 oxidized unlabelled sodium formate for 3 min before the addition of high specific activity [^{14}C]formate or [^{14}C]carbonate and the distribution of fixed ^{14}C was determined after 0.5, 1.5 and 3.0 min further incubation (Table 4). The pattern of ^{14}C -fixation was essentially the same for both substrates, indicating a common pathway for incorporation. Identification of labelled intermediates was based primarily on their chromatographic mobility in several solvents and we are indebted to Professor V.

Table 5. *Ribulosebisphosphate carboxylase activity in Thiobacillus A2 grown in chemostat culture on formate at several dilution rates*

Dilution rate (h ⁻¹)	Enzyme activity [nmol CO ₂ min ⁻¹ (mg protein) ⁻¹]
0.015	86*
0.025	90†
0.040	60*
0.050	60†
0.080	64*
0.100	76*
0.150	115†

* Spectrophotometric assay method.

† ¹⁴CO₂-assay method.

Moses and Professor J. R. Quayle for assistance in identifying some compounds. 3-Phosphoglycerate exhibited the characteristics of a primary product, suggesting the operation of the Calvin reductive pentose phosphate cycle. Compounds chromatographing as glutamate and glutamine accounted for nearly half of the ¹⁴C incorporated after 0.5 min (Table 4), declining to 20 to 25% after 3 min. This might reflect a large steady-state glutamate/glutamine pool, but the early incorporation of label into these compounds probably indicated the flow of fixed carbon from phosphoglycerate (via pyruvate) to acetate, and (via phosphoenolpyruvate and C₃-carboxylation) to oxaloacetate, followed by citrate synthesis and the formation of glutamate from 2-oxoglutarate by the reactions of the tricarboxylic acid cycle.

Presence of ribulosebisphosphate carboxylase in formate-grown Thiobacillus A2

Ribulosebisphosphate carboxylase activity was readily detectable in extracts of chemostat-grown bacteria using both spectrophotometric and ¹⁴C-assay methods. Using either method with appropriately 'activated' extracts (see Methods), activity was linearly proportional to the extract concentration and was only slightly affected by the dilution rate of the culture (Table 5), being distributed around a mean value of about 80 nmol CO₂ fixed min⁻¹ (mg protein)⁻¹. For comparison, activity in extracts of organisms grown on thiosulphate in the chemostat at *D* = 0.025 to 0.1 h⁻¹ ranged from 50 to 70 nmol CO₂ min⁻¹ (mg protein)⁻¹. The activity in extracts from a culture at *D* = 0.025 h⁻¹ [90 nmol CO₂ min⁻¹ (mg protein)⁻¹] was inhibited by (NH₄)₂SO₄ (72% by 20 mM), K₂HPO₄ (48% by 20 mM) and 6-phosphogluconate (73% by 2 mM). Enzyme activity was undetectable [< 1 nmol CO₂ min⁻¹ (mg protein)⁻¹] in *Thiobacillus A2* grown on fructose or acetate.

Ribulosebisphosphate carboxylase in extracts (culture *D* = 0.025 h⁻¹) exhibited oxygenase activity, yielding 3-phosphoglycerate and 2-phosphoglycollate. Oxygen uptake was dependent on ribulose bisphosphate (0.33 mM) and was inhibited by CO₂: the O₂ consumption with CO₂ at 0.65, 2.0, 3.3 and 5.3 mM was 4, 3, 2.5 and 1.05 nmol min⁻¹ (mg protein)⁻¹.

Comparison of incorporation into amino acids and nucleic acids of ¹⁴C from formate, carbon dioxide or acetate by Thiobacillus A2 growing under a variety of conditions

Batch cultures growing exponentially either autotrophically on thiosulphate or formate, or heterotrophically on acetate, succinate or sucrose, were supplied with the ¹⁴C-labelled compounds and the bacteria were killed and analysed after 30 to 510 min further growth. Gross labelling patterns proved similar for both long-term and short-term labelling. Labelling patterns were similar for ¹⁴C distribution among major cell fractions when all cell-carbon was being derived from the labelled substrate (Table 6), as occurred during growth on [¹⁴C]formate or [¹⁴C]acetate alone or thiosulphate plus ¹⁴CO₂. Disproportionately

Table 6. *Distribution of ^{14}C from ^{14}C -labelled formate, carbonate (CO_2) or acetate assimilated by *Thiobacillus* A2 growing autotrophically or heterotrophically*

Growth substrate ...	^{14}C content of fractions (% of total recovered ^{14}C)									
	Formate		Formate		Thiosulphate + CO_2		Formate		Formate	
Labelled substrate ...	Formate	CO_2	Formate	CO_2	Formate	CO_2	Formate	CO_2	Formate	Sucrose
Cell fraction analysed										
Cold 10% (w/v) TCA *	10.6	6.9	12.7	17.6	29.1	23.2	17.6	18.3	8.3	28.9
70% (v/v) Ethanol	11.2	11.3	28.2	16.7	9.6	8.2	16.7	2.1	6.6	2.2
Ether/ethanol (1:1, v/v)	2.3	1.6	3.3	2.7	3.0	1.4	2.7	0.4	2.0	0.3
Hot 5% (w/v) TCA	11.3	14.6	3.9	6.1	20.0	13.5	6.1	45.7	10.3	47.7
Residue	64.6	65.6	51.9	56.9	38.3	53.7	56.9	33.5	72.8	20.9
(Protein)	(62.6)	—	—	(37.9)	—	—	(37.9)	—	(51.5)	—
(Percentage of residue ^{14}C extracted with hot CHCl_3)	(2.0)	—	—	(33.6)	—	(0.9)	(33.6)	(0.9)	(29.2)	—
Percentage recovery of ^{14}C in fractions	108	94	94	98	93	104	98	85	116	93

* TCA, Trichloroacetic acid.

Table 7. *Distribution of ^{14}C among nucleic acid bases after assimilation of ^{14}C -labelled formate, carbonate (CO_2) or acetate by Thiobacillus A2 growing autotrophically or heterotrophically*

Hydrolysed nucleic acids from hot 5% (w/v) trichloroacetic acid fractions (Table 6) were partitioned by two-dimensional ascending paper chromatography (Eccleston & Kelly, 1973).

Labelled substrate	Growth substrate	^{14}C content of bases (% of total ^{14}C on chromatogram)			
		Guanine	Adenine	Uridylic acid	Cytidylic acid
Formate	Formate	27.4	19.1	18.1	35.4
	Thiosulphate + CO_2	56.8	30.1	2.9	10.2
	Acetate	52.8	39.6	2.7	4.8
	Succinate	55.2	38.6	2.4	3.8
	Sucrose	58.5	39.4	0.6	1.5
CO_2	Formate	17.5	11.4	27.9	43.2
	Thiosulphate + CO_2	19.3	11.6	25.6	43.5
Acetate	Formate	6.0	6.4	35.8	51.8
	Thiosulphate + CO_2	8.7	7.6	32.7	51.0
	Acetate	22.5	17.7	23.1	36.7
	Pyruvate	21.1	17.2	21.8	39.9
	Succinate	27.3	18.8	19.8	34.1
	Glutamate	17.8	19.0	21.4	41.7
	Sucrose	7.9	11.3	31.9	48.9

large-scale incorporation of [^{14}C]formate into nucleic acids [hot 5% (w/v) trichloroacetic acid-soluble fractions] occurred when [^{14}C]formate was assimilated by bacteria using thiosulphate, acetate, succinate or sucrose separately as growth substrate (Table 6). Similarly, heavier labelling of lipid [70% (v/v) ethanol and ether/ethanol (1:1) fractions] by [^{14}C]acetate occurred in organisms growing on formate or thiosulphate than on acetate, with comparable lowering of protein and nucleic acid labelling.

Analysis of nucleic acid fractions (Table 7) demonstrated that all four bases were labelled from [^{14}C]formate and that when formate was the sole carbon source the labelling would be a measure of the concentration of each base in the nucleic acid. Similarly, with bacteria grown on [^{14}C]acetate or $^{14}\text{CO}_2$ plus thiosulphate as sole growth substrates, equivalent labelling of all bases would be expected and was approximated experimentally (Table 7), giving guanine plus cytosine contents (mol %) of the total nucleic acid fractions of 61.8, 59.5 and 62.7 for organisms growing, respectively, on formate, acetate and thiosulphate plus CO_2 . Nucleic acid labelling was similar from $^{14}\text{CO}_2$ by bacteria growing on formate or thiosulphate. [^{14}C]Formate was incorporated principally into the purines by bacteria growing on CO_2 (with thiosulphate oxidation), acetate, succinate or sucrose. Conversely, [^{14}C]acetate was incorporated largely into pyrimidines when added to cultures growing on formate, autotrophically on thiosulphate or on sucrose. Acetate labelling of the bases was similar for bacteria growing on acetate, pyruvate, succinate or glutamate (Table 7).

Chromatography of hydrolysed proteins [hot 5% (w/v) trichloroacetic acid-insoluble residues] from the ^{14}C -labelled bacteria showed incorporation of [^{14}C]formate into most amino acids regardless of the major growth substrate, but preferential incorporation into serine plus glycine and histidine by organisms growing on thiosulphate, acetate, succinate and sucrose (Table 8). Autotrophic organisms (growing on thiosulphate plus CO_2 or formate) incorporated acetate- ^{14}C into all amino acids, but showed marked preferential incorporation into glutamate, proline, arginine and leucine (Table 8), resembling what would be found with obligately lithotrophic thiobacilli (Kelly, 1967, 1971).

Amino acids were separated by two-dimensional descending paper chromatography (Kelly, 1967), with separation of eluted Phe, Leu, Ile using a third solvent (Eccleston & Kelly, 1973).

Growth substrate	¹⁴ C content of amino acids (% of total ¹⁴ C on chromatogram)									
	Formate		Thiosulphate + CO ₂		Acetate		Succinate		Sucrose	
	Formate	CO ₂	Formate	CO ₂	Formate	Acetate	Formate	Acetate	Formate	Sucrose
Labelled substrate	...	...	...	...	...	...	...	...	...	...
Amino acid labelled										
Glutamate	12.8	12.9	32.9	5.4	6.7	26.7	7.3	12.8	11.5	5.9
Aspartate	10.3	9.1	5.9	9.7	12.8	9.4	10.0	9.1	3.7	2.8
Serine + glycine	9.7	8.0	0.9	26.2	12.1	1.6	14.2	6.7	18.1	11.3
Threonine	3.9	—	2.3	2.9	3.8	3.7	3.5	3.5	4.9	—
Alanine	10.3	12.8	1.6	9.8	14.0	3.5	6.3	9.2	1.1	0.5
Tyrosine	4.7	5.5	0.7	3.3	6.1	0.9	3.0	4.6	0.5	—
Valine + methionine	7.6	7.2	3.2	9.1	7.1	2.2	7.0	6.3	6.4	10.8
Phenylalanine	4.3	0.8	0.8	15.3	6.7	1.3	4.1	4.1	5.9	2.4
Leucine	10.5	21.2	13.8	10.9	10.9	14.5	5.4	10.6	5.9	2.4
Isoleucine	4.1	3.3	3.3	4.1	4.1	5.5	4.1	4.1	1.6	1.9
Proline	6.4	7.5	14.8	2.3	3.5	10.1	3.7	6.2	23.5	13.1
Arginine	7.2	8.7	14.9	6.7	5.0	10.4	11.7	6.8	22.9	40.9
Lysine + histidine	6.6	6.5	2.7	7.6	6.4	2.4	28.0	6.8	22.9	40.9
Unknown	1.7	0.7	2.2	1.7	0.7	7.7	—	9.5	—	10.5

Table 9. *Constants obtained by least squares fits of selected kinetic models to growth data of Thiobacillus A2 growing in extended culture on formate*

Function fitted*	$\mu_{\max}$ (h ⁻¹)	K_s (mM)	K_i (mM)	K (mM)	Minimal sum of squared residuals
1	0.662	56.97	57.06		0.00703
2	9.288	1237.00	0.98	267.25	0.00872
3	0.405	33.92	137.85		0.00256
4	1.019	114.50	101.72		0.00275
3a	0.299	26.50	187.00		0.00289

* Equations for these functions are given by Dijkhuizen & Harder (1975) and models 3 and 3a are described in the text.

DISCUSSION

The incorporation patterns of [¹⁴C]formate and ¹⁴CO₂, the presence of high specific activity of ribulosebiphosphate carboxylase and the equivalence of formate-carbon and CO₂ for growth on formate as sole energy substrate all demonstrate that *Thiobacillus* A2 employs the Benson-Calvin reductive pentose phosphate cycle for growth on formate. In this respect it is similar to *Pseudomonas oxalaticus* (Quayle & Keech, 1959), but it is, in addition, capable of chemolithotrophic and autotrophic growth using thiosulphate and CO₂. The specific activity of ribulosebiphosphate carboxylase in thiosulphate-grown autotrophic *Thiobacillus* A2 [50 to 70 nmol CO₂ min⁻¹ (mg protein)⁻¹] (compare to the value of 48.8 shown by Charles & White, 1976) is of the same order as that in formate-grown organisms [60 to 115 nmol CO₂ min⁻¹ (mg protein)⁻¹], and similar to that found in *Thiobacillus novellus* grown on formate or thiosulphate [83 or 32 nmol phosphoglycerate formed min⁻¹ (mg protein)⁻¹, respectively; Chandra & Shethna, 1977]. The carboxylase activity necessary to sustain a growth rate of 0.1 h⁻¹ [i.e. producing 0.1 g organisms containing about 64% (w/w) protein (g organisms)⁻¹ h⁻¹] corresponds to about 96 nmol CO₂ min⁻¹ (mg protein)⁻¹, indicating that the observed values are adequate to explain growth on formate using the Calvin cycle.

Formate incorporated by *Thiobacillus* A2 growing heterotrophically or autotrophically with thiosulphate is assimilated largely as formate and exhibits the more restricted metabolism observed during formate assimilation by a heterotroph such as *Escherichia coli* (Roberts *et al.*, 1955) and by obligate lithotrophs such as *Thiobacillus neapolitanus* (Kelly, 1970) and *Chlorobium thiosulfatophilum* (Kelly, 1974). Thus major derivatives from formate-carbon were adenine, guanine and amino acids metabolically close to formate such as glycine, serine and histidine. Even on thiosulphate, where the Calvin cycle also functions, formate assimilation would appear to proceed at least in large measure by direct incorporation rather than via CO₂. Under heterotrophic conditions, when the carboxylase is repressed, direct incorporation is probably the major route for assimilation. During autotrophic growth on either formate or thiosulphate, the much heavier labelling by [¹⁴C]acetate of glutamate, proline, arginine and leucine relative to aspartate than with acetate-grown organisms (Table 8) indicated a biosynthetic role for the tricarboxylic acid cycle predominantly for the conversion of acetate to glutamate via 2-oxoglutarate, as occurs exclusively in obligate chemolithotrophs (Smith *et al.*, 1967; Kelly, 1971). The reported absence of 2-oxoglutarate dehydrogenase from autotrophic *Thiobacillus* A2 (Peeters *et al.*, 1970) suggests that the observed labelling of aspartate-family amino acids was due to a functional glyoxylate bypass, the enzymes for which are present in autotrophic *Thiobacillus* A2 (Peeters *et al.*, 1970). These experiments and others (Whittenbury & Kelly, 1977) demonstrate the strong regulatory control exhibited by *Thiobacillus* A2 over the selection of pathways for intermediary metabolism dependent on the growth substrate conditions.

Taking the value of true growth yield (Y_q) of 4.2 g dry wt (mol formate)⁻¹ calculated from Fig. 1 on the assumption of a linear relationship between Y^{-1} and μ^{-1} , one can

estimate the energy conservation during formate oxidation as has previously been done for chemolithotrophic thiobacilli (Eccleston & Kelly, 1978; Justin & Kelly, 1978). *Thiobacillus* A2 contains 44% (w/w) carbon on a dry weight basis (A. L. Smith & D. P. Kelly, unpublished data), so the apparent maximum yield of 4.2 represents the fixation of 0.154 mol CO₂ for 1 mol formate oxidized. By the Calvin cycle, this requires 0.462 mol ATP and 0.308 mol NADH. Formate oxidation could reduce 1 mol NAD⁺ per mol formate; so, for each 1 mol formate oxidized, 0.308 mol donates reducing equivalents for NAD⁺ reduction for the observed CO₂-fixation, leaving 0.692 mol to be oxidized through ATP-generating energetic metabolism. The total ATP requirement is of the order 0.462 mol for CO₂-fixation and a further 0.146 mol for biosynthesis from the hexose level (according to Stouthamer, 1973), making 0.608 mol ATP to be synthesized during the oxidation for 0.692 mol formate. If this calculation is a reasonable approximation, a P/O ratio of 0.88 is indicated for energy-yielding formate oxidation. The transport of formic acid into the cell is, however, energy-requiring and in *Paracoccus denitrificans* requires 0.5 ATP per mol formate. Consequently, the oxidation of 0.692 mol formate in the above calculation must be accompanied by the synthesis of at least 1.108 ATP, giving a P/O ratio of 1.6, which is probably indicative of two coupling sites operating in *Thiobacillus* A2 during formate oxidation.

At concentrations above about 40 mM, formate became inhibitory, depressing growth rate in extended cultures and thus making estimation of true K_s and $\mu_{\max}$ difficult. A similar effect in *Pseudomonas oxalaticus* was discussed by Dijkhuizen & Harder (1975), who employed a computer analysis to determine rate constants and analyse the kinetics of the substrate inhibition. An obvious difference between *Thiobacillus* A2 and *P. oxalaticus*, apparent in the growth rate data, was that the K_s for formate in the former was obviously considerably greater than the value of 0.5 to 1.0 mM indicated for *P. oxalaticus* (Dijkhuizen & Harder, 1975). Our data were subjected to non-linear least squares fits for each of the four models listed by Dijkhuizen & Harder (1975), using the University of Warwick Computer Unit Burroughs B6700 computer. This analysis showed great variation among the models and vague estimates within each particular model (Table 9). As found by Dijkhuizen & Harder (1975), model 2 was wholly unsuitable. The most serious criticism, however, was the observation that the computed K_s values were not the formate concentration (s) at which $\mu = \mu_{\max}/2$ as required for Michaelis-Menten kinetics. K_s would have tended to this value for models 1 and 4 if $K_s \ll K_i$ which was true for the *P. oxalaticus* data, but not for *Thiobacillus* A2. Since model 3, i.e. $\mu = \mu_{\max} [\exp(-s/K_i) - \exp(-s/K_s)]$, appeared to fit the data least badly, this was modified, to model 3a, by adding the constraint that K_s must be the value of s at which $\mu = \mu_{\max}/2$, i.e. $0.5 = \exp(-K_s/K_i) - \exp(-1)$; $K_s = -K_i \times \log_e [0.5 + \exp(-1)]$; $K_s = K_i \times 0.141702 \dots$ This considerably reduced the standard errors on the estimated variables and improved the internal estimation of the kinetic parameters being sought (Table 9). K_s and K_i were thus indicated to be 26.5 and 187 mM-formate and true $\mu_{\max}$ (in the absence of inhibitory effects) to be 0.299 h⁻¹. The theoretical maximum observable value for μ became 0.186, occurring when $s = [K_s K_i (\log_e K_i - \log_e K_s)] / (K_i - K_s)$, i.e. at 60.3 mM-formate. In fact the maximum value observed for μ was 0.198 (at 41 mM) between 23 to 87 mM formate (Table 2). This model thus seems consistent with the observed results and gives acceptable estimates of the kinetic constants for substrate-inhibited growth on formate by *Thiobacillus* A2. The apparent K_s is higher than might be deduced from chemostat culture (J. C. Gottschal & J. G. Kuenen, unpublished work) where, at a dilution rate of 0.16 h⁻¹, the formate concentration was below 0.3 mM.

In future work with *Thiobacillus* A2, its reported ability (Taylor & Hoare, 1969) to grow on methanol needs reinvestigation: if it proves capable of growth on methanol, it will be of considerable importance to determine if carbon assimilation from methanol as well as formate proceeds by the Calvin cycle as in *Paracoccus denitrificans* (Cox & Quayle, 1975; van Verseveld & Stouthamer, 1978), or whether it is able to develop the ribulose monophosphate or serine pathway for methanol metabolism as in the methylotrophs (Whitten-

bury & Kelly, 1977; Quayle, 1972). Such a combination of metabolic potential may exist in *T. novellus* (Chandra & Shethna, 1977), although other organisms capable of using both methanol and formate employ either the Calvin cycle (van Verseveld & Stouthamer, 1978) or the serine pathway as in *Pseudomonas* AM1 (Anthony, 1975) for the assimilation of both substrates. Growth on formate by *Thiobacillus* A2 is clearly dependent on the inducible formate dehydrogenase (Taylor & Hoare, 1969). It is not capable of growth on methane or on hydrogen and carbon dioxide (D. P. Kelly, unpublished results), and, unlike *P. oxalaticus* and *T. novellus* (Chandra & Shethna, 1977), it is incapable of growth on oxalate, which is, however, not inhibitory to growth on formate (D. P. Kelly, unpublished work), suggesting its autotrophy on formate to be a specialized property dependent on energy generation via formate dehydrogenation. *Thiobacillus* A2 thus seems to represent an organism spanning heterotrophy and thiosulphate- and formate-dependent autotrophy, but is not apparently capable of methylotrophic or hydrogen-dependent growth. It is thus less remarkably versatile than *T. novellus* (Chandra & Shethna, 1977).

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