Mixotrophic Growth of *Thiobacillus* A2 in Chemostat Culture on Formate and Glucose

By ANN P. WOOD AND DON P. KELLY*

Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL, U.K.

(Received 24 October 1980; revised 16 January 1981)

In aerobic chemostat culture *Thiobacillus* A2-GFI grew autotrophically on formate and heterotrophically on glucose with maximum specific growth rates ($\mu_{\max}$) of 0.21 and 0.33 h⁻¹, respectively. At dilution rates of 0.1 and 0.18 h⁻¹, it grew mixotrophically on formate + glucose mixtures, completely consuming both substrates. Ribulose-1,5-bisphosphate carboxylase and formate dehydrogenase were present at high specific activity in autotrophic and mixotrophic cultures, but were repressed in cultures on glucose alone. A greater proportion of added glucose was assimilated in mixotrophic culture than in heterotrophic culture. Raising the dilution rate of a mixotrophic culture from 0.1 or 0.18 to 0.3 h⁻¹ resulted in washout (with an apparent $\mu_{\max}$ for mixotrophic growth of 0.25 h⁻¹) and the establishment of a culture dependent on glucose for growth. Growth yields on formate and glucose were, respectively, 3.3 and 100 g dry wt (mol substrate consumed)⁻¹. Steady state biomass production in mixotrophic culture indicated additive growth yields. The biomass produced in cultures on formate + glucose at a dilution rate of 0.3 h⁻¹ suggested that growth only occurred on glucose, but organisms still contained high activities of ribulose-1,5-bisphosphate carboxylase and formate dehydrogenase. At a formate:glucose ratio (mM) of 100:1, some formate was oxidized and CO₂ was fixed, but formate was not used when this ratio was 50:5. Formate–glucose mixotrophy benefits *Thiobacillus* A2-GFI when substrates are limited at low growth rates ($<\mu_{\max}$ for formate), but is characterized by a $\mu_{\max}$ below that possible on glucose. Physiological behaviour at high growth rates was influenced by the formate:glucose ratio, resulting under some conditions, at least, in loss of mixotrophy and the establishment of heterotrophic growth.

INTRODUCTION

Recent studies have shown that the capacity for mixotrophy (the simultaneous operation of autotrophic and heterotrophic mechanisms for carbon and energy metabolism) can be of considerable advantage to facultatively mixotrophic thiobacilli in competition with specialist autotrophs or heterotrophs (Gottschal, 1980; Gottschal *et al.*, 1979; Smith & Kelly, 1979). Using chemostat culture methods, mixotrophic growth on acetate + thiosulphate, glucose + thiosulphate or formate + oxalate has been shown to enable simultaneous use of both limiting substrates, the use of both inorganic and organic substrates for energy, and the dual functioning of the Calvin CO₂ fixation cycle and organic substrate assimilation (Matin, 1978; Gottschal & Kuenen, 1980; Dijkhuizen, 1979; Dijkhuizen & Harder, 1979; Smith *et al.*, 1980). We have previously demonstrated thiosulphate + glucose mixotrophy in *Thiobacillus* A2 (Smith *et al.*, 1980) and the wholly autotrophic growth of this organism on formate (Kelly *et al.*, 1979).

The aims of this study were to assess the characteristics of formate–glucose mixotrophy, to estimate to what extent energy from formate oxidation could be used to enhance glucose assimilation, and to determine whether autotrophic formate metabolism could occur in

cultures growing mixotrophically at dilution rates exceeding the maximum specific growth rate of a wholly autotrophic culture.

METHODS

Organism and batch growth conditions. The isolation, culture and maintenance of *Thiobacillus* A2 strain GFI have been described previously (Wood & Kelly, 1977). Inocula for chemostat cultures were grown on 50 mM-sodium formate medium in shaken flasks.

Continuous culture. Cultures (750 ml) were grown at 30 °C in an LH modular type series 500 fermenter (LH Engineering, Slough, Bucks) as described previously (Wood & Kelly, 1980b), with air flow rates of 150 or 250 ml min⁻¹ and an impeller speed of 750 rev. min⁻¹. The cultures were maintained at pH 7.8 by automatic titration with 2 or 5 M-NaOH and alkali consumption was recorded throughout continuous culture. Assembly and preparation of the fermenter and media were as described for the LH CC1500 fermenter (Wood & Kelly, 1979). Formic acid and glucose were sterilized separately, and aseptically added to the media reservoirs when cool. After inoculation (10%, v/v) cultures were allowed to establish by batch growth on 50 mM-sodium formate before continuous culture at $D = 0.1 \text{ h}^{-1}$ was commenced. All samples for growth measurements, radiorespirometry and assays for glucose, formate, [¹⁴C]glucose incorporation and enzymes were removed directly from the fermenter.

Culture purity was monitored by comparison of growth of appropriate serial dilutions of culture samples on thiosulphate, formate, glucose and glucose + formate agars. Equal numbers of colonies were produced on each type of agar.

Growth was monitored by measurement of turbidity using a Pye Unicam SP1700 spectrophotometer. Absorbance at 440 nm was related to dry weight using calibration curves. Steady state growth yields were determined directly by estimation of dry weight of centrifuged organisms after washing with distilled water. Steady state protein contents of cultures were determined by harvesting 2 ml samples of culture, dissolving in 2 ml 0.5 M-NaOH at 100 °C for 10 min, and then estimating protein by the Lowry method. Glucose was assayed by a standard method (Somogyi, 1945). Formate was determined by the formate dehydrogenase assay: since the rate of ferricyanide reduction by crude cell-free extracts was dependent on formate concentration below 2 mM, formate remaining in steady state culture filtrates could be assayed directly.

Radiorespirometry. Cultures (500 ml) grown on glucose or glucose + formate were harvested as described previously (Wood & Kelly, 1978). Radiorespirometry, radiorespirometry substrates, and collection and measurement of ¹⁴CO₂ have been described previously (Wood *et al.*, 1977). ¹⁴C was counted in a Beckman LS-7000 scintillation spectrometer.

Enzyme assays. All enzymes were assayed at 30 °C. Methods for most enzyme assays have been described previously (Wood *et al.*, 1977; Wood & Kelly, 1980a). Pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90) was assayed as described by Reeves *et al.* (1974). Formate dehydrogenase [formate:NAD⁺ oxidoreductase; EC 1.2.1.2] was assayed spectrophotometrically as described by Taylor & Hoare (1969). Specific activity of this enzyme is expressed as nmol K₃Fe(CN)₆ reduced min⁻¹ (mg protein)⁻¹. Ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing); EC 4.1.1.39] was assayed using the method described by Smith *et al.* (1980). Whole cells on membranes were incubated for 15 and 30 min before measurement of fixed ¹⁴CO₂. Cell-free extracts were assayed by sampling at 0.5, 1, 1.5, 2, 3, 4, 5 and 6 min. Specific activity of this enzyme is expressed as nmol CO₂ fixed min⁻¹ (mg protein)⁻¹. Rhodanese [thiosulphate:cyanide sulphurtransferase; EC 2.8.1.1] was assayed in a final volume of 2.5 ml, containing unadjusted Tris, pH 10.6 (50 mM), Na₂S₂O₃ (20 mM), KCN (20 mM) and extract (10 µl). The reaction, initiated with KCN, was stopped after 2, 4 and 6 min by adding 0.2 ml 40% (w/v) formaldehyde. Thiocyanate was determined by adding 1.3 ml 0.4 M-ferric nitrate in 1 M-nitric acid and reading absorbance due to ferric thiocyanate at 470 nm. Activity of this enzyme is expressed as nmol thiocyanate formed min⁻¹ (mg protein)⁻¹. Protein in cell-free extracts was determined by the Lowry method.

Glucose assimilation by chemostat cultures. For continuous labelling of the culture, the normal medium supply was supplemented with [U-¹⁴C]glucose at 400 kBq l⁻¹ (10.75 µCi l⁻¹). Steady state incorporation was measured by filtering 1 ml samples of culture through Sartorius SM11306 (0.45 µm pore size, 25 mm diam.) membranes. Samples (0.1 ml) of culture and of input medium were also monitored. All samples and filters were counted in 10 ml of a water-miscible scintillant (Wood *et al.*, 1977), using a Beckman LS-7000 liquid scintillation spectrometer.

RESULTS

Substrate consumption and biomass production in chemostat cultures under single or dual limitation by formate and glucose

Steady state cultures were established on limiting formate at 50 or 100 mM at a dilution rate of 0.1 h⁻¹. Formate was completely consumed to produce steady state biomass values of

Table 1. Enzymes of autotrophic metabolism in *Thiobacillus A2*-GFI from steady state chemostat culture under autotrophic, mixotrophic and heterotrophic growth conditions

Growth-limiting substrate(s) (concn, mM)	Dilution rate (h ⁻¹)	Volume replacements	Biomass* (g dry wt l ⁻¹)	Enzyme activity† [nmol min ⁻¹ (mg protein) ⁻¹]		
				RuBP carboxylase	Formate dehydrogenase	Rhodanese
Formate (50)	0.10	19.0	0.17	42.5	305.1	343.0
Formate (50) + Glucose (5)	0.10	36.7	0.68	25.5	405.8	492.0
Formate (50) + Glucose (5)	0.30‡	7.9	0.32	29.3	566.0	462.0
Glucose (5)	0.30	17.9	0.49	0	11.7	173.0
Formate (100)	0.10	8.7	0.32	85.0	667.0	570.0
Formate (100) + Glucose (1)	0.10	19.0	0.42	93.5	561.0	936.0
Formate (100) + Glucose (1)	0.18	8.1	0.43	148.3	911.0	1078.0
Formate (100) + Glucose (1)	0.30§	8.6	0.10	—	—	—

—, Not tested.

* Biomass was estimated from absorbance at 440 nm and contained $69.5 \pm 3.6\%$ protein under all conditions (seven determinations, mean and standard deviation).

† Assays performed with extracts prepared in a French press.

‡ At this dilution rate no formate was consumed.

§ At this dilution rate about 50 % of the formate was oxidized.

0.17 and 0.32 g dry wt l⁻¹, respectively (Table 1), indicating a yield of 3.3 g dry wt (mol formate consumed)⁻¹. On switching from 50 mM-formate to a medium containing 50 mM-formate + 5 mM-glucose, both substrates were completely consumed and the steady state biomass increased to 0.68 g l⁻¹ (Table 1). Determination of numbers of viable organisms by making serial dilution plates from such a culture at a steady state biomass of 0.71 g l⁻¹, with no residual formate or glucose, gave counts of $1.74 (\pm 0.23) \times 10^9$, $1.98 (\pm 0.22) \times 10^9$ and $1.85 (\pm 0.10) \times 10^9$ organisms ml⁻¹ on formate, glucose and formate + glucose agars, respectively. Biomass production from glucose alone was about 100 g dry wt (mol glucose)⁻¹, equivalent to 0.5 g l⁻¹ from 5 mM-glucose (Wood & Kelly, 1979). Biomass produced on formate + glucose was thus the sum of that on the two substrates separately. Similarly, biomass production increased from 0.32 g l⁻¹ on 100 mM-formate alone to 0.42 g l⁻¹ on 100 mM-formate + 1 mM-glucose, consistent with additive growth yields on the two substrates (Table 1). Viable counts on formate, thiosulphate and glucose agars were identical for cultures on 100 mM-formate (after 6.5 and 8.6 volume replacements) and on 100 mM-formate + 1 mM-glucose (after 2.9, 5.1, 7.0 and 19 volume replacements).

Maximum specific growth rates ($\mu_{\max}$) of organisms in the steady state cultures were determined from the washout rates (Karagouni & Slater, 1978) following the imposition of a dilution rate of 0.3 or 0.6 h⁻¹, respectively, on cultures limited by 50 mM-formate or 50 mM-formate + 5 mM-glucose, previously grown at a dilution rate of 0.1 h⁻¹. Values for apparent $\mu_{\max}$ were 0.21 and 0.25 h⁻¹, respectively. The value of 0.25 h⁻¹ for the formate + glucose culture was that derived from the initial and final biomass for 80% washout of the culture over 5 h. Inspection of washout kinetics showed the log plot to be a slight curve, with an initial washout rate equivalent to a $\mu_{\max}$ of 0.2 h⁻¹, increasing to a value of about 0.35 h⁻¹ for the final 2 to 3 h. In batch culture on glucose (Wood & Kelly, 1977) *Thiobacillus A2*-GFI had a $\mu_{\max}$ of about 0.37 h⁻¹. In these chemostat experiments it exhibited a $\mu_{\max}$ of 0.33 h⁻¹ on switching a glucose-limited culture from a dilution rate of 0.1 to 0.6 h⁻¹.

Effect of altered dilution rate and other perturbations on the behaviour of mixotrophic cultures under dual limitation by formate and glucose

The apparent $\mu_{\max}$ of 0.25 h⁻¹ for bacteria growing with dual substrate limitation (50 mM-formate + 5 mM-glucose) demonstrated that the culture contained a homogeneous

population of bacteria with a mixotrophic physiology that allowed growth at a rate intermediate between those on formate or glucose alone. Raising the dilution rate of the culture on 50 mM-formate + 5 mM-glucose from 0.1 to 0.3 h⁻¹ resulted in partial washout with subsequent establishment of a population growing at 0.3 h⁻¹ by consuming all available glucose but failing to oxidize any of the 50 mM-formate supplied to the culture. The steady state biomass in this culture was only 0.32 g l⁻¹, indicating a yield of only 64 g (mol glucose)⁻¹ (Table 1).

Raising the dilution rate of the culture on 100 mM-formate + 1 mM-glucose from 0.1 to 0.18 h⁻¹ did not affect the completeness of substrate consumption, and only increased the steady state biomass from 0.42 to 0.43 g l⁻¹. A further increase in dilution rate to 0.3 h⁻¹ resulted in washout to a new steady state biomass of about 0.075 g l⁻¹ with a considerable decrease in formate consumption (to about 50 mM) (Table 1).

Removal of two-thirds of a culture growing on 50 mM-formate + 5 mM-glucose (e.g. for enzyme analysis) sometimes resulted in breakdown of the mixotrophic growth state. Re-establishment of mixotrophy then occurred only after a large number of volume replacements. During this period a pseudo-steady state existed in which only glucose was consumed and alkali consumption for pH control increased to a level exceeding that required solely for neutralization of the input formic acid. This could indicate additional acidic products from glucose oxidation, but organic acid accumulation was not monitored.

Variation of ribulose-1,5-bisphosphate carboxylase and formate dehydrogenase in autotrophic, mixotrophic and heterotrophic cultures

In cultures exhibiting wholly autotrophic growth on limiting formate, high activities of ribulose-1,5-bisphosphate (RuBP) carboxylase and formate dehydrogenase were present (Table 1). Specific activities of both enzymes were greater on 100 mM-formate than on 50 mM-formate, although no explanation for this is obvious. Cultures growing heterotrophically on glucose lacked RuBP carboxylase and had negligible formate dehydrogenase activity (Table 1). At a dilution rate of 0.1 h⁻¹, a steady state mixotrophic culture totally consuming 50 mM-formate + 5 mM-glucose had 60% of the carboxylase activity of the comparable formate-limited culture. RuBP carboxylase activity was greater in cultures limited by 100 mM-formate + 1 mM-glucose at dilution rates of 0.1 or 0.18 h⁻¹ than in a culture at 0.1 h⁻¹ on formate alone (Table 1). At a dilution rate of 0.3 h⁻¹, cultures on 50 mM-formate + 5 mM-glucose consumed all the available glucose but did not metabolize significant amounts of the formate, which remained at about 50 mM in the culture vessel. RuBP carboxylase and formate dehydrogenase were, however, present in the steady state culture at high specific activities (Table 1), even though no significant formate oxidation or CO₂ fixation was occurring.

In the prolonged transitions, described above, before truly mixotrophic steady states occurred, repression of RuBP carboxylase and variation in formate dehydrogenase activities were observed.

Rhodanese was assayed in all growth states; this is an enzyme normally found in *Thiobacillus* A2, whose metabolic function is uncertain, although it may be involved in chemolithotrophic sulphur oxidation (Silver & Kelly, 1976), but is not related directly to autotrophic growth on formate. Its activity was very high in most growth states, but significant depression occurred in glucose-grown cultures (Table 1).

Contribution of glucose to carbon metabolism in mixotrophic and heterotrophic cultures

Growth yields of around 100 g dry wt (mol glucose)⁻¹ (Wood & Kelly, 1979, 1980*b*; Smith *et al.*, 1980) for *Thiobacillus* A2 indicate that up to 60% of available glucose-carbon may be assimilated in heterotrophic cultures. Using medium containing [U-¹⁴C]glucose, steady state incorporation of glucose-carbon was found to be dependent on growth rate. At a

Table 2. *Radiorespirometry of specifically labelled glucose by Thiobacillus A2-GFI from steady state chemostat culture under mixotrophic and heterotrophic growth conditions*

The three steady states were established in succession, preceded by a steady state on formate alone. Total $^{14}\text{CO}_2$ release after 31 min oxidation of 66.6 nmol glucose by 1.75 mg dry wt bacteria in a total volume of 2 ml (Wood *et al.*, 1977) is given; oxidation was complete within this time.

Growth substrate(s) (concn, mM)	Dilution rate (h ⁻¹)	Volume replacements at time of expt	$^{14}\text{CO}_2$ release† (nmol, from 66.6 nmol glucose)				
			C-1	C-2	C-3	C-4	C-6
Formate (50) + Glucose (5)	0.1	11.2	39.7	30.9	34.4	45.0	21.5
		30.4	48.4	28.8	27.9	46.8	19.1
		36.7	47.6	35.1	33.0	45.0	18.8
		Mean	45.2	31.6	31.8	45.6	19.8
Formate (50)* + Glucose (5)	0.3	7.9	42.4	20.0	21.2	34.6	17.4
Glucose (5)	0.3	17.9	39.7	32.7	42.7	65.1	21.6

* Formate was not consumed at this dilution rate.

† Use of the three-pathway equations for calculating the relative contributions of the EM, ED and PP pathways (Wood *et al.*, 1977) gave values of 41% EM, 18% ED and 41% PP for culture on formate + glucose at a dilution rate of 0.1 h⁻¹ and 52% EM, 27% ED and 21% PP on glucose alone. Use of the two-pathway (ED + PP) calculations (Wood & Kelly, 1980 a) indicated 100% ED and 88% ED, 12% PP for cultures on formate + glucose at dilution rates of 0.1 and 0.3 h⁻¹, respectively.

dilution rate of 0.1 h⁻¹ glucose assimilated during dual limitation by 100 mM-formate + 1 mM-glucose was constant at $39.6 \pm 4.5\%$ of that added (results for six samples taken at intervals from 3 to 18 volume replacements after switching to these conditions) but rose to $53.3 \pm 0.3\%$ at a dilution rate of 0.18 h⁻¹ (after 4.6 and 8.1 volume replacements). Increasing the dilution rate to 0.3 h⁻¹ resulted in half (50 mM) the added formate being unused, biomass falling to 0.075 g l⁻¹ and only 30.2% of the added glucose being assimilated, the rest being oxidized.

Mechanisms of glucose metabolism in mixotrophic and heterotrophic cultures

Glucose oxidation was monitored by the radiorespirometric procedures which had previously demonstrated that glucose-grown *Thiobacillus A2* and *A2-GFI* employed the Embden-Meyerhof (EM), Entner-Doudoroff (ED) and pentose phosphate (PP) pathways simultaneously (Wood & Kelly, 1978; Wood *et al.*, 1977). Different patterns of $^{14}\text{CO}_2$ release were observed for *Thiobacillus A2-GFI* grown mixotrophically at a dilution rate of 0.1 h⁻¹ on 50 mM-formate + 5 mM-glucose, grown on the same medium at a dilution rate of 0.3 h⁻¹ (in which only the glucose was metabolized) and grown heterotrophically on 5 mM-glucose alone at a dilution rate of 0.3 h⁻¹ (Table 2). The patterns observed with the heterotrophic and truly mixotrophic cultures resembled those for the three-pathway oxidation system, with very high release of the C-1, C-3 and C-4 atoms of the glucose as CO_2 . The data differ, however, from those we obtained earlier from organisms employing all three pathways, where C-1 release equalled or was significantly less than that from C-3 and was generally half that from C-4 (Wood & Kelly, 1978; Wood *et al.*, 1977). In the present experiments the similar release of C-1 and C-4 and very high C-1 and C-2 release relative to C-3 cannot be used as evidence for the presence of the EM pathway, even though C-3 release considerably exceeded C-6. The combination of high release of C-1, C-2 and C-4 suggests the predominant operation of the ED and PP pathways, the latter producing extensive C-2 release. The pattern seen with the formate + glucose culture at a dilution rate of 0.3 h⁻¹ was typical of the ED (88%) and PP (12%) pathways.

Table 3. *Enzymes of heterotrophic metabolism in Thiobacillus A2-GFI from steady state chemostat culture under autotrophic, mixotrophic and heterotrophic growth conditions*

The four steady states were established in succession. No 1-phosphofructokinase or 6-phosphofructokinase was detected in any of the cultures.

Growth substrate(s) (concn, mM)	Dilution rate (h ⁻¹)	Volume replacements before assay	Enzyme activity† [nmol min ⁻¹ (mg protein) ⁻¹]					NADH oxidase
			FBP aldolase	6-PG dehydratase + KDPG aldolase	KDPG aldolase	FBPase pH 7.0	FBPase pH 8.6	
Formate (50)	0.1	19.0	7.4	1.5–6.0	43.6	3.2	17.8	37.4
Formate (50) + Glucose (5)	0.1	13.6	59.6	1.6–4.8	81.0	11.2	14.7	235.5
		30.4	69.4	10.9–17.4	119.1	3.1	21.7	271.8
		36.7	23.0	7.1–9.0	104.3	6.2	47.8	76.4
Formate (50)* + Glucose (5)	0.3	7.9	35.4	82–95	206	3.2	23.1	232.0
Glucose (5)	0.3	17.9	24.1	3.5	41.9	5.9	16.8	163.2

* Formate was not consumed by this culture.

† Enzyme abbreviations: FBP aldolase, fructose-1,6-bisphosphate aldolase; 6-PG dehydratase, 6-phosphogluconate dehydratase; KDPG aldolase, 6-phospho-2-keto-3-deoxy-D-gluconate aldolase; FBPase, fructose-1,6-bisphosphatase.

Enzymes of carbohydrate metabolism in organisms grown under autotrophic, mixotrophic and heterotrophic conditions

The most important observation was the absence of phosphofructokinases from all cultures. When present in *Thiobacillus* A2, this enzyme is easy to detect and assay in crude extract (Wood *et al.*, 1977; Wood & Kelly, 1980*a*). Its activity was not affected by ATP concentrations at least up to 2.5 ± 1 mM-AMP, was not activated by 1 mM-AMP alone in crude extracts, and showed similar activity at pH 7.0, 8.0 and 9.5 (A. P. Wood, unpublished observations). No activity was detected in the extracts of *Thiobacillus* A2-GFI even with altered ATP concentrations or in the presence of added AMP. Its absence implies the absence of the EM pathway. An alternative to phosphofructokinase for the formation of fructose-1,6-bisphosphate and the operation of a modified EM pathway is the pyrophosphate: fructose-6-phosphate phosphotransferase (Reeves *et al.*, 1974). This enzyme was not detectable in crude extracts. Fructose-1,6-bisphosphate aldolase was present in all cultures (Table 3), but has a role in autotrophic gluconeogenesis as well as the PP pathway. The key enzymes of the ED pathway (6-phosphogluconate dehydratase and 6-phospho-2-keto-3-deoxy-D-gluconate aldolase) were present in all cultures (Table 3) and were particularly active in the formate + glucose culture at a dilution rate of 0.3 h^{-1} , which was deduced to have high ED pathway activity from the radiorespirometric experiments. Fructose-1,6-bisphosphatase activities were similar in formate or glucose cultures (Table 3) but pH 8.6 activity was enhanced in some samples grown under mixotrophic conditions.

DISCUSSION

These experiments demonstrate that *Thiobacillus* A2-GFI exhibits true mixotrophy with dual substrate limitation by formate and glucose, comparable with that previously observed with thiosulphate, CO₂ and glucose (Smith *et al.*, 1980). It is clear from this and other work (Gottschal, 1980; Gottschal & Kuenen, 1980; Dijkhuizen, 1979) that the characteristics of a culture growing mixotrophically may be a function of the particular combination of substrates used, their relative concentrations and the imposed growth rate. With a

formate:glucose ratio of 50:5 (mM) there was partial repression of RuBP carboxylase, but with a ratio of 100:1 at the same dilution rate (0.1 h^{-1}) activity was slightly raised. Autotrophic CO_2 fixation could thus provide a significant part of the total carbon assimilated by cultures growing mixotrophically. In the case of the 100 mM-formate + 1 mM-glucose steady state at dilution rates of 0.1 and 0.18 h^{-1} , respectively, the biomass values of 0.42 and 0.43 g l^{-1} indicate the incorporation of 0.185 and 0.189 g C l^{-1} , as *Thiobacillus A2* contains about 44% (w/w) of its dry weight as carbon (Kelly *et al.*, 1979; Smith *et al.*, 1980). Since 39.6% and 53.3% of the added 1 mM-glucose was assimilated in the two cultures, glucose-carbon contributed 0.029 and 0.038 g C l^{-1} , respectively, the remaining 84% and 80% of the total assimilated carbon being derived from CO_2 . At a dilution rate of 0.3 h^{-1} , the biomass was 0.075 g l^{-1} (equivalent to 0.033 g C l^{-1}) even though the proportion of glucose assimilated was only 30.2%, equal to 0.022 g C l^{-1} , meaning that formate-dependent CO_2 fixation still supplied one-third of the total carbon. Comparing this with carbon assimilation in formate-limited chemostats, where the yield of $3.3 \text{ g (mol formate)}^{-1}$ is equivalent to $1.452 \text{ g C (mol formate)}^{-1}$, the amount of formate-dependent CO_2 fixation in the 100 mM-formate + 1 mM-glucose culture at a dilution rate of 0.3 h^{-1} could not have exceeded $0.22 \text{ g C (mol formate consumed)}^{-1}$, since $50 \text{ mmol formate l}^{-1}$ was consumed in the steady state.

It was also demonstrated by these experiments that even the combination of a considerable molar excess of formate over glucose and a high growth rate (close to formate μ_{max}) did not enable use of energy from formate oxidation for assimilation of all added glucose, half of which was still oxidized.

The retention (and even enhancement of activity) of RuBP carboxylase and formate dehydrogenase in cultures provided with both formate and glucose at dilution rates exceeding μ_{max} for formate alone and too high for complete mixotrophy indicates that formate has an inducing effect for these enzymes. This must be true even under conditions (e.g. 50 mM-formate + 5 mM-glucose at a dilution rate of 0.3 h^{-1}) not allowing significant formate oxidation. The lowered growth yield on glucose observed in this 50 mM-formate + 5 mM-glucose culture could be due in part to the unproductive diversion of energy and carbon from glucose oxidation for synthesis of these redundant enzymes. It is noteworthy that strains of '*Hydrogenomonas*' retain RuBP carboxylase activity even during heterotrophic growth on some substrates (Kelly, 1971). It is also highly probable that the unused formate could at this high concentration have had a significant uncoupling effect on energy conservation during glucose oxidation.

Heterotrophic growth of *Thiobacillus A2*-GFI on glucose in the absence of formate resulted in complete repression of RuBP carboxylase and almost complete loss of formate dehydrogenase. The capacity for mixotrophy on formate and glucose was indicated to be slightly unstable since cultures became temporarily heterotrophic as a consequence of perturbations and sometimes took many generations to recover full mixotrophic potential. Clearly, a balance operated between derepression of RuBP carboxylase and formate dehydrogenase by formate and active repression (and probably inhibition) by glucose or a glucose metabolite. A novel feature observed in this study was the capacity of *Thiobacillus A2*-GFI to metabolize formate in mixotrophic cultures at a dilution rate exceeding the μ_{max} for growth on formate alone.

We thank the Science Research Council for grants supporting this work.

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