Metabolism of *Thiobacillus* A2 Grown Under Autotrophic, Mixotrophic and Heterotrophic Conditions in Chemostat Culture

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Thiobacillus A2 was grown in chemostat culture under four distinct types of substrate limitation: chemolithoautotrophically with limitation by thiosulphate or CO₂; heterotrophically with limitation by glucose; and mixotrophically with dual limitation by both thiosulphate and glucose. Under mixotrophic conditions energy was obtained from the oxidation of both thiosulphate and glucose, and carbon was derived both from CO₂ fixation by the Calvin cycle and from glucose. Ribulosebiphosphate carboxylase (RuBP carboxylase) activity was negligible and chemolithotrophic thiosulphate oxidation and autotrophic CO₂ fixation were apparently repressed in bacteria which had been grown heterotrophically. Conversely, under autotrophic conditions the ability to oxidize glucose was repressed. Growth yields from mixotrophic cultures were the sum of those obtained under single substrate limitation. Intermediate activities of RuBP carboxylase were detected in mixotrophic cultures, but more glucose was assimilated mixotrophically than heterotrophically. Glucose was metabolized by the Entner–Doudoroff (85 to 90 %) and pentose phosphate (10 to 15 %) pathways under both heterotrophic and mixotrophic conditions, with slight involvement also of the Embden–Meyerhof pathway (<9 %) heterotrophically. RuBP carboxylase activity in autotrophic cultures was enhanced four- or fivefold by CO₂ limitation. Repression of RuBP carboxylase activity and thiosulphate-oxidizing ability during the transition from autotrophy to heterotrophy and the activities of carbohydrate-metabolizing enzymes in autotrophic, heterotrophic and mixotrophic cultures are described.

INTRODUCTION

Facultatively autotrophic thiobacilli, such as *Thiobacillus novellus*, *T. intermedius* and *Thiobacillus* A2 (Starkey, 1935; London, 1963; Taylor & Hoare, 1969) which are capable of growth either as heterotrophs or as chemolithotrophic autotrophs in the absence of organic matter, have been known for many years, but their ecological importance and capacities for mixotrophic growth have only been recognized quite recently (Rittenberg, 1969, 1972; Whittenbury & Kelly, 1977; Matin, 1978; Gottschal *et al.*, 1979; Smith & Kelly, 1979). The ability of facultative thiobacilli to ‘switch off’ chemolithoautotrophic metabolism when cultured heterotrophically is well known (Vishniac & Trudinger, 1962; Aleem & Huang, 1965; Taylor & Hoare, 1969; Kelly, 1971; Matin, 1978) and the view developed that autotrophic and heterotrophic systems tended not to operate simultaneously in facultative autotrophs (Whittenbury & Kelly, 1977). This is true of *T. novellus* and *Thiobacillus* A2 in which autotrophic systems are repressed during batch culture on organic substrates (Schlegel, 1975). This is, however, not the case with some other facultative autotrophs (Kelly, 1971) as was demonstrated by the isolation of *T. intermedius*, which can grow mixotrophically, simultaneously using thiosulphate oxidation for energy generation and glucose for carbon (London & Rittenberg, 1966; Matin & Rittenberg, 1970; Matin, 1978). Such metabolism

in *T. intermedius* is the result of mutual antagonism between glucose- and thiosulphate-using systems (Matin & Rittenberg, 1970; Matin, 1978), resulting essentially in chemolithotrophic heterotrophy (Whittenbury & Kelly, 1977). True mixotrophy should allow simultaneous operation of energy generation from the oxidation of both an inorganic sulphur compound and an organic substrate and the simultaneous assimilation of both CO₂ (by the Calvin cycle) and organic carbon. Such mixotrophy in thiobacilli probably occurs only under conditions of low nutrient concentration and was presumed to occur in chemostat cultures of *Thiobacillus* A2 and *T. novellus* under dual limitation by glucose and thiosulphate (Matin, 1978; Smith & Kelly, 1979), or acetate and thiosulphate (Gottschal *et al.*, 1979).

Conditions in a substrate-limited continuous culture are probably more representative of many natural environments than those in a nutrient-sufficient mixotrophic batch culture, and are thus the conditions under which true mixotrophy should be examined. There are several examples of the concomitant use of 'autotrophic' and 'heterotrophic' substrates by facultative autotrophs (Matin, 1978; Dijkhuizen, 1979; Dijkhuizen & Harder, 1979; Gottschal *et al.*, 1979; Smith & Kelly, 1979), but this report, and a concurrent study of Gottschal & Kuenen (1980), are the first to prove the occurrence of mixotrophy as defined above, i.e. simultaneous use of both substrates for lithotrophic and organotrophic energy generation and autotrophic and heterotrophic carbon metabolism. It is not known to what extent there is regulation to 'partition' energy generation from inorganic or organic sources and carbon for biosynthesis from glucose or CO₂ under mixed-substrate limitation.

This paper reports the occurrence and kinetics of true mixotrophy in *Thiobacillus* A2.

METHODS

Abbreviations. The following abbreviations are used: μCi is employed as the unit of radioactivity and is equivalent to 37 kilobecquerels (kBq); RuBP, ribulose 1,5-bisphosphate; PEP, phosphoenolpyruvate; butyl-PBD, 2-(4'-*tert*-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole; KDPG, 2-keto-3-deoxy-6-phosphogluconate.

Organism and culture conditions. *Thiobacillus* strain A2 (Taylor & Hoare, 1969) was maintained on thiosulphate agar medium (Wood & Kelly, 1977) and grown in shake-flask and chemostat culture as previously described (Kelly *et al.*, 1979; Smith & Kelly, 1979) using media containing 1.5 g KH₂PO₄ l⁻¹ and 7.9 g Na₂HPO₄·2H₂O l⁻¹ and adjusted to pH 7.6 with NaOH. For investigation of CO₂-limitation in chemostat culture, the culture pH was maintained at pH 7.6 by automatic titration with 3.2 M-KOH prepared in freshly distilled water and kept free of CO₂ by means of soda-lime traps. Cultures (750 ml) were stirred at 750 rev. min⁻¹ and the CO₂ supply to the chemostat was varied by altering the CO₂ content and flow rate of the air plus CO₂ mixtures passing through the culture. Culture purity was routinely monitored by examining growth on nutrient agar. Samples from mixotrophic cultures gave equal numbers of colonies on thiosulphate agar and glucose agar.

Analytical procedures. Biomass, glucose, sulphur compounds and rates of thiosulphate oxidation were determined as described previously (Smith & Kelly, 1979).

Cell volume and total number determinations. Samples from the chemostat were immediately diluted with 'Isoton' (Coulter Electronics, Harpenden, Herts) that had been prefiltered through a 0.45 μm pore-size membrane. Numbers of organisms and mean cell volumes were measured with a Coulter counter using latex beads of known diameter as volume standards.

Glucose assimilation by chemostat cultures. This was determined by two methods. (i) *Pulse labelling*: very high specific activity [U-¹⁴C]glucose was injected to give 13.3 $\mu\text{Ci l}^{-1}$ and duplicate samples (2 ml) were filtered through 0.45 μm pore-size Sartorius membranes at intervals up to 60 min. (ii) *Continuous labelling*: the normal medium supply was supplemented with [U-¹⁴C]glucose (5 $\mu\text{Ci l}^{-1}$) and steady state incorporation was measured using filtered samples (3 ml) as before. Filters and filtrates were counted in 10 ml of a water-miscible scintillant (Wood *et al.*, 1977) using a Packard 2425 liquid scintillation spectrometer.

Thiosulphate oxidation and ¹⁴CO₂ fixation by organisms from chemostat culture. Warburg flasks at 30 °C contained *Thiobacillus* A2 (0.3 to 4.0 mg protein in a total of 2 ml) harvested from the culture and resuspended in thiosulphate-free medium. Test flasks received NaH¹⁴CO₃ (10 μmol , 2 μCi) and Na₂S₂O₃ (6 μmol) or glucose (5.1 μmol). When oxygen consumption was complete, 1.0 ml samples were mixed with 2 ml 10% (v/v) acetic acid in ethanol. Samples (0.5 ml) of the mixture were evaporated to dryness in scintillation vials, 10 ml 0.6% (w/v) butyl-PBD in methanol/toluene (1:3, by vol.) was added and the ¹⁴C radioactivity in

non-volatile products was determined. Problems were encountered in these experiments because, when oxidation rates were high, the concentration of dissolved CO_2 limited CO_2 fixation coupled to thiosulphate oxidation. This resulted in the amount of CO_2 fixation being a function of the oxidation rate rather than proportional to the amount of thiosulphate oxidized. The organism concentration, bicarbonate supply and oxidation rates were therefore adjusted so that the rate of CO_2 fixation was not limited by any external factors. Estimation of CO_2 fixation coupled to glucose oxidation was complicated by the progressive dilution of the added $^{14}\text{CO}_2$ by unlabelled CO_2 produced from glucose. By the end of the experiments reported, this dilution had decreased the initial specific activity by no more than 20 to 30 %. In the Results, $^{14}\text{CO}_2$ fixation during glucose oxidation is presented as a range indicated by the initial and final specific activities.

Enzyme assays. (i) *Carboxylating enzymes* were routinely assayed by procedures based on those of Glover & Morris (1978) using whole organisms. (The resulting activities were compared with those derived from standard assays using cell-free extracts.) Organisms in samples (1 to 6 ml) from chemostats were filtered on to 0.45 μm pore-size Sartorius membranes (25 mm diam.) to give 0.2 to 0.32 mg protein per membrane. Filters were placed in scintillation vials (20 ml capacity) and incubated at 20 °C for 20 min with 0.4 ml 5 % (v/v) aqueous Triton X-100 prior to carboxylase assay. Incubation with 10 or 15 % (v/v) Triton X-100 was also initially tested, but the activity of RuBP carboxylase was approximately 26 % higher when only 5 % (v/v) was used. RuBP carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing); EC 4.1.1.39] was assayed in triplicate in scintillation vials containing membranes treated with Triton X-100. Assay mixture (0.9 ml) containing (mM): Tris/HCl (pH 8.0), 78; reduced glutathione, 2.1; MgCl_2 , 25; $\text{NaH}^{14}\text{CO}_3$, 44 (165 $\mu\text{Ci mmol}^{-1}$), was added to each vial. After 'activation' at 22 °C for 10 min, the reaction was initiated by adding 0.3 ml 10 mM-RuBP and terminated after a further 60 min by adding 3 ml 5 % (v/v) acetic acid in methanol. Zero-time and RuBP-free controls were included. The mixture was dried in the vial, 10 ml butyl-PBD in methanol/toluene was added and ^{14}C radioactivity was counted 3 to 4 d later. This delay was allowed because counts increased for 40 to 50 h following scintillant addition (due to removal of ^{14}C from vial walls) and were then stable indefinitely. The rate of $^{14}\text{CO}_2$ fixation in the RuBP carboxylase assay was constant for at least 60 and 90 min, respectively, with 0.45 and 0.3 mg protein per membrane. Maximum activity was observed with 1.8 mM-RuBP under these conditions. Similar rates of $^{14}\text{CO}_2$ fixation were detected with 2.8 or 3.7 mM-RuBP, but with 0.3 or 1.0 mM-RuBP the rates were, respectively, 25 % and 60 % of the maximum. The activity at the routine assay temperature of 22 °C was 45 % of that at 30 °C.

PEP carboxylase [orthophosphate:oxaloacetate carboxy-lyase (phosphorylating); EC 4.1.1.31] was assayed in vials as above. Assay mixture (1 ml) containing (mM): Tris/ H_2SO_4 (pH 8.0), 70; reduced glutathione, 2.1; MgSO_4 , 21.4; $\text{NaH}^{14}\text{CO}_3$, 40 (165 $\mu\text{Ci mmol}^{-1}$); sodium glutamate, 8; acetyl-coenzyme A, 0.32, was added to each vial. After 10 min preincubation, the reaction was initiated by adding 0.2 ml 92 mM-PEP and terminated as for RuBP carboxylase. Acetyl-CoA (lithium salt; Sigma) increased activity fivefold when supplied at 0.2 mM and above, but no increase in activity was produced by adding aspartate aminotransferase. Chloride ions were excluded from the assay because PEP carboxylase is known to be inhibited by them in *Escherichia coli* W (Izui *et al.*, 1970). Comparably low activities were, however, found in thiosulphate-grown *Thiobacillus A2* using either chloride or sulphate buffers.

Pyruvate carboxylase [pyruvate:carbon-dioxide ligase (ADP-forming); EC 6.4.1.1] was assayed as above using a reaction mixture (1 ml) containing (mM): Tris/HCl (pH 8.0), 70; reduced glutathione, 2.1; MgCl_2 , 21.4; $\text{NaH}^{14}\text{CO}_3$, 40 (165 $\mu\text{Ci mmol}^{-1}$); sodium glutamate, 8; acetyl-coenzyme A, 0.32; ATP, 3.2. After preincubation for 10 min, 0.2 ml 80 mM-sodium pyruvate was added and incubation was continued as for RuBP carboxylase.

For comparison with the whole-cell assay method for carboxylases, cell-free extracts were also prepared. Samples (100 to 200 ml) from chemostats were centrifuged at 20000 *g*, organisms were washed with Tris/ H_2SO_4 buffer (pH 8.0) and resuspended to about 10 mg dry wt ml^{-1} in buffer containing 0.5 mM-dithiothreitol. Samples (2 ml) were mixed with 1 g Ballotini glass beads (0.11 mm diam.) and disrupted ultrasonically at 0 to 2 °C for five successive 30 s periods using maximum power in an MSE ultrasonic disintegrator. Alternatively, suspensions were passed twice through a French pressure cell at 140 MPa at 2 to 5 °C. Debris was removed from crude extracts by centrifuging at 38000 *g* for 20 min.

Carboxylases were assayed in scintillation vials in the same final assay volumes as used for the membrane filter method, using about 3 mg crude extract protein per assay in a procedure based on that of Kelly *et al.* (1979). After activation and initiation of the reaction with RuBP, samples (50 μl) were removed after 30, 60, 90, 120, 240 and 600 s and mixed with 3 ml 5 % (v/v) acetic acid in methanol. Vial contents were dried and counted as above. The rate of $^{14}\text{CO}_2$ fixation was constant for at least 120 s (Kelly *et al.*, 1979) and was unaffected by the addition of 0.4 ml 5 % (v/v) Triton X-100 as used for the membrane filter assay. Using separate preparations, specific activities [$\text{nmol } ^{14}\text{CO}_2 \text{ fixed min}^{-1} (\text{mg protein})^{-1}$] at 22 °C were, respectively, 34.7 and 10.5 for French press and Triton treatments, and 25.2 and 7.1 for ultrasonic disruption and Triton treatments. The Triton X-100 assay procedure thus gave about 30 % of the activity detectable in the cell-free

extracts. At 30 °C, activities in cell-free extracts were 56 to 78 nmol $^{14}\text{CO}_2$ fixed min^{-1} (mg protein) $^{-1}$, i.e. about twice those at 22 °C.

(ii) *Enzymes of carbohydrate metabolism* were assayed in crude cell-free extracts prepared as described previously (Wood *et al.*, 1977). All enzymes were assayed using a Unicam SP1700 recording spectrophotometer. ϵ_{340} for NADH and NADPH was taken as $6.21 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$. Fructose-1,6-bisphosphatase [D-fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11] was assayed by the method of Johnson & MacElroy (1973). 6-Phosphofructokinase (EC 2.7.1.11) was assayed by the method of Baumann & Baumann (1975). Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) was assayed as described by Tabita & Lundgren (1971), and 6-phosphogluconate dehydratase (EC 4.2.1.12) and KDPG aldolase (EC 4.1.2.14) were assayed by measuring the rates of pyruvate production from 6-phosphogluconate using the spectrophotometric method described by Wood *et al.* (1977). Assays in which rates of NADH oxidation were determined were supplemented with 1 to 2 mM-KCN to prevent interference by NADH oxidase activity. NADH oxidase was assayed as described by Wood *et al.* (1977).

Activities of all enzymes are expressed as nmol substrate converted min^{-1} (mg protein) $^{-1}$. Protein was determined by the Lowry method.

Radiorespirometry. Radiorespirometric methods, labelled substrates and collection and measurement of $^{14}\text{CO}_2$ have been described previously (Wood *et al.*, 1977). ^{14}C was counted in Packard 2425 and Beckman LS-7000 scintillation counters.

Elemental analysis. Chemostat-grown *Thiobacillus* A2 was harvested, washed and dried at 105 °C before determining C, H and N in duplicate or triplicate using a Perkin-Elmer elemental analyser.

Materials. Biochemicals were obtained from Sigma. For RuBP carboxylase assay, tetrasodium ribulose bisphosphate was the preferred substrate. However, for some assays the dibarium salt was used: barium was removed by treatment in aqueous solution with Dowex 50W-X8 resin (H^+ form) and the solution was adjusted to pH 7.0 with 10 M-NaOH.

RESULTS

Biomass production in chemostat culture

Thiobacillus A2 could be maintained in steady state chemostat culture at a dilution rate of 0.08 h^{-1} under three distinct growth regimes: chemolithotrophically with thiosulphate oxidation as the sole energy source and CO_2 as the sole carbon source for autotrophic growth; heterotrophically on glucose; or mixotrophically on thiosulphate plus glucose. The steady state biomass concentration was a function of the limiting substrate concentration and growth yields [g dry wt (g mol substrate consumed) $^{-1}$] were 5.9 for thiosulphate and 109.9 for glucose, in agreement with previous observations (Kuenen, 1979; Smith & Kelly, 1979; Wood & Kelly, 1979). Cultures supplied with both thiosulphate and glucose consumed both substrates completely. Biomass concentrations (g dry wt l^{-1}) during limitation by thiosulphate (50 mM), glucose (2.3 mM) and a mixture of the two were 0.294, 0.256 and 0.560, respectively. Under these conditions, dual limitation thus produced additive growth yields. Elemental analysis of organisms from cultures subject to limitation by thiosulphate, glucose, or thiosulphate plus glucose, respectively, gave the following values (% w/dry wt): carbon, 44.4, 44.8, 42.8; hydrogen, 6.5, 6.4, 6.5; nitrogen, 12.7, 11.6, 11.9. These values are similar to the composition reported earlier for thiosulphate- and formate-grown *Thiobacillus* A2 (Kuenen, 1979).

*Effect of CO_2 limitation on *Thiobacillus* A2 growing on thiosulphate in chemostat culture*

Either thiosulphate or CO_2 could be made the limiting substrate during autotrophic growth by varying the CO_2 content of the air passed through steady state cultures at constant stirring rates (Table 1). Steady state dissolved CO_2 concentrations or rates of dissolution of CO_2 were not estimated. When the proportion of CO_2 in the gas phase was decreased from 1.63 to 0.03 % (v/v) the culture became CO_2 -limited and 71 % of the thiosulphate was unoxidized: the growth yield fell and the specific activity of RuBP carboxylase increased fourfold (Table 1). The yield and RuBP carboxylase activity returned to their original levels when the CO_2 concentration was increased to 0.49 % (v/v). CO_2 at 0.15 % (v/v) was also limiting, although prolonged culture resulted in an increased yield, greater thiosulphate

Table 1. *Effect of CO₂ supply on Thiobacillus A2 growing chemolithotrophically on thiosulphate in chemostat culture*

The culture volume was 750 ml, the dilution rate was 0.08 h⁻¹ and the concentration of thiosulphate in the feed was 50 mM.

CO ₂ in gas phase (% v/v)	Rate of gas flow (ml air + CO ₂ min ⁻¹)	Culture volume replacements under given conditions*	Total CO ₂ supplied (μg C ml ⁻¹)	Total cell C produced (μg C ml ⁻¹)	Residual thiosulphate (μmol ml ⁻¹)	Yield†	RuBP carboxylase activity‡
Series 1							
1.63	220	9.6 ^a	1910	129	0	5.8 (2.6)	12.6
0.03	200	5.9 ^b	32	21	35.5	3.3 (1.5)	53.4
0.49	220	7.6 ^b	571	108	0	4.9 (2.2)	12.7
Series 2							
0.15	205	6.1 ^c	167	56	16.2	3.7 (1.6)	14.2
0.15	205	29.1 ^d	167	111	5.3	5.6 (2.5)	24.0
0.08	505	11.1 ^b	215	53	17.5	3.7 (1.6)	22.3

* a, Following prior batch culture; b, following previous steady state; c, following 0.03 % (v/v) CO₂; d, continuation of c.

† Expressed as g dry wt (mol thiosulphate consumed)⁻¹ and, in parentheses, as g cell C (mol thiosulphate consumed)⁻¹, calculated assuming that organisms contain 44.4 % (w/dry wt) carbon.

‡ Assayed using Triton X-100-treated organisms on membrane filters: activities are expressed as nmol CO₂ fixed min⁻¹ (mg protein)⁻¹; organisms contained 61.4 ± s.d. 9.2 (6) % (w/dry wt) protein.

consumption and a rise in RuBP carboxylase activity to a level intermediate between that with 0.03 and 0.49 % CO₂. The subsequent reduction of the CO₂ concentration to 0.08 % (v/v) resulted in a reduced yield and thiosulphate consumption without a significant increase in RuBP carboxylase activity even after 11 culture volume replacements (Table 1). The rate of ¹⁴CO₂ fixation by Triton X-100-treated bacteria in the absence of added substrate was 0.07 to 0.3 nmol CO₂ fixed min⁻¹ (mg protein)⁻¹. Fixation when supplemented with PEP did not exceed 0.06 nmol CO₂ min⁻¹ (mg protein)⁻¹ with samples from either CO₂-limited or thiosulphate-limited cultures, indicating no significant PEP carboxylase activity. Pyruvate carboxylase activity was about 0.9 nmol CO₂ min⁻¹ (mg protein)⁻¹ in bacteria grown with 0.15 % (v/v) CO₂.

Transition of Thiobacillus A2 in chemostat culture from autotrophic growth with thiosulphate to heterotrophic growth on glucose

A number of morphological and physiological changes occurred during the transition from thiosulphate-limited autotrophic growth to glucose-limited growth (Fig. 1; Table 2). The biomass produced with 2.33 mM-glucose was about 87 % of that produced with 50 mM-thiosulphate. Total cell numbers fell to about 67 % of the autotrophic level while the mean cell volume increased (Fig. 1a). The relationship between dry weight and culture absorbance remained the same, the latter falling to about 83 % of the autotrophic value (Fig. 1b). The ability of the bacteria to oxidize thiosulphate declined, the specific rate of thiosulphate oxidation falling about 28 % and 53 % after 1 and 2 volume replacements and by 95 % after about 6 volume replacements (Fig. 1c). The capacity to couple CO₂ fixation efficiently to thiosulphate oxidation declined more gradually, although the rate was obviously dependent on the rate of thiosulphate oxidation. The efficiency of coupling had declined by 30 % after 2 volume replacements and by only about 75 % after 6 volume replacements (Fig. 1c). Clearly, the capacity for chemolithoautotrophy was not immediately repressed when the thiosulphate supply was removed, since a more rapid loss of thiosulphate-oxidizing capacity would then be expected. RuBP carboxylase activity fell more than 70 % and 96 % in 2 and

Fig. 1. Changes accompanying the transition of *Thiobacillus* A2 from autotrophic to heterotrophic growth. A steady state chemostat culture ($D = 0.08 \text{ h}^{-1}$) growing with a limiting supply of 50 mM-thiosulphate was switched at time 0 to a medium containing only limiting 2.33 mM-glucose. Changes in the following parameters were measured: (a) total cell number (Δ) and mean cell volume ($\blacktriangle$); (b) absorbance at 440 nm ($\blacksquare$); (c) thiosulphate oxidation activity (Warburg assay) ($\circ$) and ability to fix $^{14}\text{CO}_2$ during thiosulphate oxidation (Warburg assay) ($\bullet$).

4 volume replacements, respectively (Table 2), indicating immediate cessation of its synthesis following transfer to glucose medium. The activity of PEP carboxylase increased slightly after the switch to glucose but reached relatively high activity only between 15 and 44 volume replacements (Table 2). The possibility that this was due to mutant selection has not been tested.

CO₂ fixation by Thiobacillus A2 during steady state autotrophic, mixotrophic or heterotrophic growth

In a thiosulphate-limited culture with excess CO_2 , RuBP carboxylase activity remained constant at about $10.3 \text{ nmol CO}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$ (using the whole-cell 22°C assay). This activity declined to about $7.8 \text{ nmol CO}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$ after 3.7 or even 9.6 volume replacements in the mixotrophic culture in which both thiosulphate (50 mM) and glucose (2.33 mM) were totally consumed (Table 3), indicating the stable operation of both autotrophic and heterotrophic metabolism. After prolonged culture with glucose, RuBP carboxylase declined to an insignificant activity. PEP carboxylase was effectively absent from autotrophic and mixotrophic cultures, but significant activities of pyruvate carboxylase were detected (Table 3). Pyruvate carboxylase was essentially absent from glucose-grown cultures, although these had PEP carboxylase activity similar to the activity of pyruvate carboxylase in autotrophic or mixotrophic cultures. Different anaplerotic CO_2 -fixing mechanisms thus occurred autotrophically and heterotrophically. Organisms harvested from glucose steady states (13 volume replacements after switching from thiosulphate) or thio-

Table 2. Changes in activities of ribulosebisphosphate and phosphoenolpyruvate carboxylases during transition of *Thiobacillus* A2 from autotrophic to heterotrophic growth

A steady state chemostat culture ($D = 0.08 \text{ h}^{-1}$) growing with a limiting supply of 50 mM-thiosulphate was switched at time 0 to a medium containing only limiting 2.33 mM-glucose. Assays were performed using Triton X-100-treated organisms on membrane filters.

Volume replacements after time 0	Enzyme activities [nmol CO_2 fixed min^{-1} (mg protein) $^{-1}$]	
	RuBP carboxylase	PEP carboxylase
0	9.4	0.1
1.9	2.7	0.6
3.8	0.4	0.2
5.7	0.9	—
7.7	0.7	0.9
14.8	0.6	0.7
43.6	0.2	5.8

Table 3. Ribulosebisphosphate, phosphoenolpyruvate and pyruvate carboxylase activities in *Thiobacillus* A2 maintained in steady state chemostat culture under autotrophic, mixotrophic and heterotrophic growth conditions

All cultures were aerated at 220 ml min^{-1} (750 ml culture vol.) $^{-1}$ with air containing 0.48 % (v/v) CO_2 ; the dilution rate was 0.08 h^{-1} . Samples for enzyme analysis were taken after at least 5 volume replacements for cultures growing with thiosulphate, after 3.7, 7.3 and 9.6 volume replacements with thiosulphate plus glucose and after more than 30 volume replacements with glucose. All assays were performed at 22°C using Triton X-100-treated organisms on membrane filters.

Limiting substrate(s) (mM)	Enzyme activities* [nmol CO_2 fixed min^{-1} (mg protein) $^{-1}$]		
	RuBP carboxylase	PEP carboxylase	Pyruvate carboxylase
Thiosulphate (50)	10.26 ± 0.88 (6)	0.07 (2)	3.31
Thiosulphate (50) + glucose (2.33)	7.79 ± 1.97 (3)	0.30†	$4.04 \pm$
Glucose (2.33)	0.38 ± 0.30 (2)	5.84	0.80

* Average activities $\pm$ standard error; values in parentheses indicate the number of determinations made using successive samples from steady state cultures.

† After 9.6 volume replacements.

‡ After 7.3 volume replacements.

sulphate steady states (5.8 volume replacements after switching from glucose) fixed very little $^{14}\text{CO}_2$ when resuspended in substrate-free medium (see Methods). Glucose-grown bacteria would oxidize glucose (2.33 mM) essentially without a lag in Warburg flasks, consuming $1.7 \mu\text{l O}_2 \text{ min}^{-1}$ (mg protein) $^{-1}$ and coupled this to the fixation of 3.2 to 4.4 nmol CO_2 ($\mu\text{mol O}_2$ consumed) $^{-1}$. In contrast, bacteria from cultures grown on thiosulphate oxidized thiosulphate immediately [$8.8 \mu\text{l O}_2 \text{ min}^{-1}$ (mg protein) $^{-1}$], fixing 48.9 nmol CO_2 ($\mu\text{mol O}_2$) $^{-1}$, but oxidized glucose only after a 4 h lag [$1.8 \mu\text{l O}_2 \text{ min}^{-1}$ (mg protein) $^{-1}$] and fixed 14.9 to 19.4 nmol CO_2 ($\mu\text{mol O}_2$) $^{-1}$. CO_2 fixation by both autotrophic and heterotrophic cultures could thus apparently be supported by glucose oxidation. Bacteria from mixotrophic cultures oxidized thiosulphate without a lag [$7.7 \mu\text{l O}_2 \text{ min}^{-1}$ (mg protein) $^{-1}$] and fixed 35.5 nmol CO_2 ($\mu\text{mol O}_2$) $^{-1}$. For comparison, *T. neapolitanus* harvested from chemostat culture on thiosulphate oxidized thiosulphate at $42.1 \mu\text{l O}_2 \text{ min}^{-1}$ (mg protein) $^{-1}$ and fixed 31.5 nmol CO_2 ($\mu\text{mol O}_2$) $^{-1}$, i.e. only 64 % of that fixed by thiosulphate-grown *Thiobacillus* A2. It is interesting to note that the lower efficiency of

Table 4. *Enzymes of carbohydrate metabolism in Thiobacillus A2 maintained in steady state chemostat culture under autotrophic, mixotrophic and heterotrophic growth conditions*

Growth conditions were as in Table 3. Samples were taken for enzyme analysis after 8.1, 7.6 and 11.5 volume replacements, respectively, for cultures growing on thiosulphate, thiosulphate plus glucose and glucose. All enzymes were assayed in cell-free extracts prepared with a French press.

Enzyme	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]		
	Limiting substrate(s):		
	Thiosulphate	Thiosulphate + glucose	Glucose
6-Phosphofructokinase	ND	0.0-6	ND
Fructose-1,6-bisphosphate aldolase	5.9	8.0	8.7
6-Phosphogluconate dehydratase + KDPG aldolase	ND	44.2	51.0
Fructose-1,6-bisphosphatase:			
pH 7.0	26.0	2.6	0.25
pH 8.6	50.8	24.0	3.3
NADH oxidase	10.6	163.2	81.7

ND, Not detectable.

Table 5. *Estimation of relative contribution of glucose-oxidizing pathways for [¹⁴C]glucose catabolism by mixotrophic and heterotrophic Thiobacillus A2 in chemostat culture*

Culture conditions* (see Table 3)	Total cumulative ¹⁴ CO ₂ release (nmol, from 66.6 nmol glucose) from glucose labelled in:					
	C-1	C-2	C-3	C-4	C-5	C-6
Mixotrophic	42.5	20.0	16.1	35.7	17.2	14.4
Heterotrophic ^a	43.1	18.2	14.0	33.2	—	13.2
Heterotrophic ^b	38.3	20.7	22.0	28.9	10.0	18.2

	Relative contribution (%) of pathways		
	Embden-Meyerhof	Entner-Doudoroff	Pentose phosphate
Mixotrophic	0	89.8	10.2
Heterotrophic ^a	0	85.1	14.9
Heterotrophic ^b	0 (9†)	85.9	14.1

—, Not determined.

* a, 11.5 volume replacements following autotrophic culture; b, 15 volume replacements following autotrophic culture. a and b represent independently established cultures.

† A small contribution from the Embden-Meyerhof pathway is indicated by the small excess (3.8 nmol, from 66.6 nmol glucose) of CO₂ released from C-3 compared with C-6, and a small peak of C-3 release at 0.5 to 4 min in the time course of the radiorespirometry experiment. This would indicate a maximum contribution of about 9% by this pathway to the total glucose oxidation.

T. neapolitanus in this assay is comparable with its lower growth yield (67% of the *Thiobacillus A2* value) in the chemostat at a dilution rate of 0.08 h⁻¹ (Smith & Kelly, 1979).

Glucose metabolism in autotrophic, mixotrophic and heterotrophic cultures

Enzymes important in glucose oxidation and gluconeogenesis in *Thiobacillus A2* were assayed in bacteria from all three growth modes (Table 4). As previously shown with batch cultures (Wood *et al.*, 1977), fructose-1,6-bisphosphate aldolase activity was detected in thiosulphate- and glucose-grown bacteria and was found to be of similar activity in bacteria from mixotrophic culture. Phosphofructokinase (essential if the complete Embden-Meyerhof pathway were present in the bacteria) was virtually absent under all three conditions. The

Fig. 2. Glucose assimilation by *Thiobacillus* A2 during the transition from mixotrophic to heterotrophic growth. A steady state chemostat culture ($D = 0.08 \text{ h}^{-1}$) growing on $[^{14}\text{C}]$ glucose (2.33 mM) plus thiosulphate (50 mM) was switched at time 0 to a supply of $[^{14}\text{C}]$ glucose alone and changes in absorbance at 440 nm (●) and the percentage of the $[^{14}\text{C}]$ glucose converted into cell carbon (○) were monitored.

key enzyme pair of the Entner–Doudoroff pathway (6-phosphogluconate dehydratase and KDPG aldolase) were absent from autotrophic cultures but present in mixotrophic and heterotrophic cultures. As previously shown (Wood *et al.*, 1977), separate fructose-1,6-bisphosphatase activities were detectable at pH 7.0 and 8.6. Both activities were very low in heterotrophic cultures, but were higher in autotrophic and mixotrophic ones, although the ratio of activity at pH 8.6 to that at pH 7.0 was much greater in the mixotrophic culture (Table 4). ‘NADH oxidase’ activity was low in autotrophic organisms but greatly increased when glucose was also being metabolized (Table 4).

The mechanisms employed for glucose oxidation during mixotrophic and heterotrophic growth were estimated radiorespirometrically. In both cases $^{14}\text{CO}_2$ was produced most rapidly and extensively from the C-1 and C-4 atoms of glucose, with C-1 exceeding C-4 (Table 5). This indicated the predominant operation of the Entner–Doudoroff and pentose phosphate pathways [using the procedures and calculation methods described by Wood & Kelly (1979) and Wood *et al.* (1977)]. This observation is consistent with the high activities of Entner–Doudoroff pathway enzymes and low activity of phosphofructokinase in mixotrophic and heterotrophic cultures. Slightly different radiorespirometry patterns were obtained with bacteria from two glucose steady states 11.5 and 15 volume replacements after growth with thiosulphate. In the first there was no evidence of any Embden–Meyerhof pathway activity, but in the other the rate of C-3 release gave a peak early in the oxidation and the amount of C-3 released was cumulatively greater than that of C-6 (Table 5). The rate of C-4 release also peaked earlier than that of C-1 and the cumulative amount of C-4 released exceeded C-1 for the first 5 min of oxidation. These observations indicated that there was a small contribution by the Embden–Meyerhof pathway at least early in the oxidation. Using the final cumulative values for $^{14}\text{CO}_2$ production during complete oxidation of the added glucose (Table 5), and the standard two-pathway equation (Wood & Kelly, 1979), the Entner–Doudoroff pathway accounted for 85 to 90 % of glucose oxidation, with the pentose phosphate pathway effecting the rest.

Glucose assimilation in mixotrophic and heterotrophic chemostat culture ($D = 0.08 \text{ h}^{-1}$)

A steady state mixotrophic culture with glucose and thiosulphate was established using [^{14}C]glucose and the steady state rate of conversion of ^{14}C to fixed cell carbon was measured (Fig. 2). Under these conditions 51 % of the input glucose was assimilated. This culture was then switched to a medium containing only [^{14}C]glucose (time 0 on Fig. 2) and a new steady state was established. During the transition (4 to 5 volume replacements; Fig. 2), the biomass concentration declined by about 50 %. The proportion of [^{14}C]glucose assimilated fell to about 38 % within 1 volume replacement. In separate experiments, in which mixotrophic and heterotrophic steady state cultures were pulse-labelled with [^{14}C]glucose, 62 % and 42 %, respectively, of the added glucose was assimilated.

DISCUSSION

These experiments demonstrate that, as in batch culture, *Thiobacillus* A2 in chemostat culture lost the ability to metabolize glucose when grown autotrophically and, conversely, progressively lost the ability to oxidize thiosulphate and fix CO_2 by means of the Calvin cycle when maintained on glucose. In contrast, when subjected to dual limitation by both thiosulphate and glucose the organism behaved truly mixotrophically, totally consuming both substrates and obtaining carbon by both the Calvin cycle and the assimilation of glucose. A number of deductions concerning the interactions of autotrophic and heterotrophic physiological processes can be made from the results. In thiosulphate-limited autotrophic cultures, RuBP carboxylase activity was about 10.3 (22°C) or 22.9 (30°C) $\text{nmol CO}_2 \text{ fixed min}^{-1} (\text{mg protein})^{-1}$ when assayed by the Triton X-100 method, compared with 35.5 (22°C) or 79.1 (30°C) when crude extracts prepared in the French press or by ultrasonic disruption were assayed. These are similar to previously reported values (Charles & White, 1976; Kelly *et al.*, 1979) and are adequate to support growth at the dilution rate of 0.08 h^{-1} . CO_2 could be made the limiting substrate (Table 1) resulting in incomplete thiosulphate oxidation and a four- or fivefold increase in RuBP carboxylase activity. The yield also decreased, indicating that the energy-coupling efficiency from thiosulphate oxidation decreased under CO_2 -limitation. An explanation of this could in part be due to 'uncoupled' oxidation of some thiosulphate, as was reported for *T. neapolitanus* (Kuenen & Veldkamp, 1973). This possibility also suggests that with 0.49% (v/v) CO_2 (Table 1), the limiting substrate could still be CO_2 , since the yield was 15 % lower than with 1.63% (v/v) CO_2 . Prolonged cultivation with 0.15% (v/v) CO_2 (after switching from 0.03%) resulted in eventual recovery to the control growth yield accompanied by an approximate doubling in RuBP carboxylase activity (Table 1). Similar variation in RuBP carboxylase in response to CO_2 -limitation has previously been shown in *T. neapolitanus* (Kuenen & Veldkamp, 1973). Supplementary CO_2 fixation occurred by pyruvate carboxylation in autotrophic and mixotrophic cultures but by PEP carboxylation in glucose-limited cultures (Table 3).

Major changes accompanying the transitions via mixotrophy from autotrophy to heterotrophy were the loss of RuBP carboxylase and pyruvate carboxylase and increase in PEP carboxylase activities (Tables 2 and 3); a decrease in fructose-bisphosphatase activity (required for autotrophic gluconeogenesis); and the development of the Entner-Doudoroff pathway for glucose oxidation (Tables 4 and 5). During both mixotrophic and heterotrophic growth, glucose was dissimilated predominantly by the Entner-Doudoroff and pentose phosphate pathways. Phosphofructokinase was essentially absent under all three growth conditions (Table 4) indicating that the Embden-Meyerhof pathway played little role under any of these conditions of substrate supply and growth rate. This is consistent with earlier observations with glucose-limited cultures (Wood & Kelly, 1979).

Under the one set of mixotrophic conditions used, the culture retained the ability to oxidize thiosulphate for energy generation and had about 76 % of the RuBP carboxylase

activity of autotrophic thiosulphate-limited cultures. The additive growth yields observed with dual mixotrophic substrate-limitation could suggest that mixotrophy is simply additive functioning of autotrophic and heterotrophic activities. This was demonstrated to be an over-simplification, since more glucose was assimilated mixotrophically than in glucose-limited culture. This indicates that glucose was also assimilated at the expense of chemolithotrophic energy, and consequently slightly less CO₂ fixation was supported by thiosulphate oxidation during mixotrophic than during autotrophic growth. The contribution of CO₂ to mixotrophic growth was reduced below the autotrophic level by an amount similar to the reduction in RuBP carboxylase activity.

These studies have confirmed that true mixotrophy occurs in *Thiobacillus* A2 with glucose and thiosulphate as dual limiting substrates in the chemostat.

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