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Mixotrophic Growth of *Thiobacillus* A2 on Acetate and Thiosulfate as Growth Limiting Substrates in the Chemostat

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Abstract. During heterotrophic growth on acetate, in batch culture, the autotrophic growth potential of *Thiobacillus* A2, i.e. the capacity to oxidize thiosulfate and to fix carbon dioxide via the Calvin cycle, was completely repressed. The presence of thiosulfate in a batch culture with acetate as the organic substrate partly released the repression of the thiosulfate oxidizing system. Cultivation of the organism in continuous culture at a dilution rate of 0.05 h^{-1} with different concentration ratios of thiosulfate and acetate in the reservoir medium led to mixotrophic growth under dual substrate limitation. Growth on the different mixtures of acetate and thiosulfate yielded upto 30% more cell dry weight than predicted from the growth yields on comparable amounts of these substrates separately. The extent to which the carbon dioxide fixation capacity and the maximum thiosulfate and acetate oxidation capacity are repressed appeared to be a function of the thiosulfate to acetate concentration ratio in the reservoir medium. The results of ^{14}C-acetate assimilation experiments and of gas-analysis demonstrated that the extent to which acetate was assimilated depended also on the substrate ratio in the inflowing medium. Under the different growth conditions surprisingly little variation was found in some tri-carboxylic acid cycle enzyme activities. Cultivation of *T.* A2 at different growth rates with a fixed mixture of thiosulfate (18 mM) and acetate (11 mM) in the medium, showed that dual substrate limitation occured at dilution rates ranging from 0.03–0.20 h^{-1}.

Key words: *Thiobacillus* A2 – Mixotrophic growth – Facultatively chemolithotrophic *Thiobacillus* – Dual substrate limitation – Assimilation efficiency.

Abbreviations: PPO = 2,5-diphenoloxazol, RubPCase = Ribulose-1,5-bisphosphate carboxylase, Tris = tris (hydroxymethyl) aminomethane, EDTA = ethylenediaminetetra-acetic acid

Most studies on the regulation of the metabolism of inorganic and organic compounds in facultative chemolithotrophs have focused on the metabolic features of cultures grown in batch culture. These studies describe many examples of repression of the autotrophic carbon assimilation (London and Rittenberg, 1966; Matin, 1978; Rittenberg, 1969; Schlegel, 1975) and/or chemolithotrophic energy generation (Matin, 1978; Rittenberg, 1969; Schlegel, 1975; Le John et al., 1967) by the presence of organic substrates.

For example in *Nitrobacter agilis* the chemolithotrophic energy generating system (nitrite – oxidase) is partly repressed during growth on acetate in the presence of nitrite (Steinmüller and Bock, 1977). Taylor and Hoare (1971) reported a repression to about 3% of the ribulose-1,5-bisphosphate carboxylase in *Thiobacillus* A2-cells grown in the presence of both thiosulfate and acetate. Matin and Rittenberg (1970) on the other hand showed that in *Thiobacillus intermedius*, grown in a glucose-thiosulfate medium, the organotrophic energy generation was prevented by the presence of the inorganic energy source (= thiosulfate). This is comparable with the situation in *Alcaligenes* (Hydrogenomonas) *eutrophus* H16, where H_2 caused repression of the Entner-Doudoroff pathway enzymes and thus prevented both anabolic and catabolic utilization of fructose (Blackkolb and Schlegel, 1968; Schlegel and Trüper, 1966).

Matin recently reported (Matin, 1978, 1977) that *Thiobacillus novellus* was "hampered" in its growth if both thiosulfate and glucose were present in batch culture. Repression of glucose metabolizing enzymes (Perez and Matin, 1979) and inhibition of glucose transport (Schleiss and Matin, 1979) were reported to be responsible for this phenomenon. Also many heterotrophic organisms show preferential utilization of one organic substrate in the presence of high concentrations of others (Paigen and Williams, 1970; Dijkhuizen et al., 1978). However, numerous reports are available by

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now in which simultaneous utilization of organic substrates is described in carbon and/or energy-limited cultures (Dijkhuizen, 1979; Harder and Dijkhuizen, 1976; Law and Button, 1977; Mateles et al., 1967; Silver and Mateles, 1969). It should be expected that under similar conditions facultatively chemolithotrophic thiobacilli also would be able to make simultaneous use of organic and inorganic carbon and/or energy sources. In a recent review (Matin, 1978) Matin noted that indeed *T. novellus* was able to benefit both from glucose and thiosulfate if both substrates were growth limiting in continuous culture. The aim of our investigations has been to explore how the metabolism of a facultatively chemolithotrophic *Thiobacillus* is organized under mixed substrate conditions. In this study we have focused on the growth of *Thiobacillus* A2 on mixtures of thiosulfate and acetate. *Thiobacillus* A2 is a metabolically versatile organism able to grow autotrophically on thiosulfate and formate (Kelly et al., 1979) and heterotrophically on a great variety of organic substrates (Taylor and Hoare, 1969).

The outcome of this investigation clearly shows that *Thiobacillus* A2 is capable of mixotrophic growth with different mixtures of acetate and thiosulfate as growth limiting substrates in the chemostat at different dilution rates.

Materials and Methods

Organism. The organism used in this study, the facultatively chemolithotrophic *Thiobacillus* A2, has been described previously (Taylor and Hoare, 1969; Gottschal et al., 1979).

Media and Growth Conditions. The composition of the basal medium used in batch and continuous cultivation is the same as described in an earlier publication (Gottschal et al., 1979). This medium contained (% w/v): K_2HPO_4, 0.4; KH_2PO_4, 0.15; $MgSO_4 \cdot 7\ H_2O$, 0.04; NH_4Cl, 0.04; in deionized water, plus 2 ml per liter of a trace elements solution (Vishniac and Santer, 1957). The trace elements solution contained 2.2 g instead of the originally reported 22 g $ZnSO_4 \cdot 7\ H_2O$ per liter. The pH was 7.2. To this basal medium different amounts of Na-thiosulfate and Na-acetate were added according to the description in the experimental section. The medium was sterilized by autoclaving for 30 min at 118°C. Magnesium sulfate plus trace elements were sterilized separately, each in 5% of the volume. The continuous culture equipment was designed as described by Harder et al. (1974) and Kuenen and Veldkamp (1973). The oxygen concentration in the culture was automatically controlled at 50% air saturation. Cultures were maintained at 28°C, at a pH 8.0 by automatic addition of 1 M Na_2CO_3 or 1 M HCl, depending on the substrate used. In gas analysis experiments Na_2CO_3 was replaced by 1 N NaOH.

Yields. After centrifugation at 12,000 × g for 10 min, cells were resuspended in deionized water. The protein concentration in these suspensions was measured using the method described by Lowry (1951). The organic cell carbon content of the suspensions was measured with a total organic carbon analyzer (Beckman). Elemental analysis of the cell material showed that 49% of the cell dry weight was composed of organic carbon, independent of the growth substrate. Thiosulfate concentrations were determined using the method described by Sörbo (1957). The acetate concentration in the reservoir was measured as organic carbon with the total organic carbon analyzer.

Preparation of Cell-Free Extracts. Bacteria were harvested from steady state chemostat cultures and centrifuged at 2°C and 12,000 × g for 15 min. Pellets were washed with 0.1 M Tris-maleate buffer of pH 8.0. The pellets were resuspended in the same buffer or (for RubPcase-assay, see below) in 0.1 M Tris-HCl buffer (pH 8.2, 20 mM $MgCl_2$, 5 mM $NaHCO_3$ and 0.5 mM dithiothreitol). Cell densities in these suspensions were 5 – 20 mg protein per ml. The cell suspensions were sonified at 2°C with an ultrasonic desintegrator (MSE Ltd., London, England), 5 times during 30 s with 30 s intervals. Glass beads (Ballotini, ∅ 0.11 mm) were added to the cell suspensions prior to sonification (1 g beads per 2 ml suspension). After centrifugation at 20,000 × g for 20 min, the supernatant was used for enzyme assays.

Enzyme Assays

Ribulose-1,5-bisphosphate carboxylase (= RubPcase), EC 4.1.1.39. The enzyme was activated by preincubation of the crude cell-free extract for 10 min at room temperature (Lorimer et al., 1977).

The assay mixture (300 µl) consisted of 0.05 – 0.5 mg protein of the crude cell-free extract and the following compounds in µmol: Tris-HCl, 30; $MgCl_2$, 6; dithiothreitol, 0.15, and ^{14}C-labeled $NaHCO_3$, 1.8. The final pH was 8.2. The bicarbonate in the reaction mixture had a specific radioactivity of 0.125 µCi/µmol. After a further preincubation at 28°C during 1 min the reaction was started by the addition of 0.3 µmol of ribulose-1,5-bisphosphate followed by removal of 50 µl quantities at 30, 60, 90 and 120 s, which were transferred into 200 µl quantities of 100% acetic acid at 60°C in scintillation vials. After 20 min 4 ml of a toluene mixture [2 l toluene, 1 l Triton X100 and 10 g PPO (2,5-diphenoloxazol)] was added to the vials. Radioactivity was measured in a liquid scintillation counter (Nuclear Chicago Corp., Mark II, Des Plaines, Ill., U.S.A.). The rate of CO_2-fixation was constant for at least 150 s, and activity was strictly proportional to the amount of crude extract added.

The activity of the following enzymes were determined photometrically in 1 ml reaction mixtures (1 cm cuvettes) at 28°C in a Perkin-Elmer 124 double beam spectrophotometer. Quantities in µmol of the different reagents in the reaction mixture are given below.
Isocitrate dehydrogenase, EC 1.1.1.42 (Kornberg, 1955).
Tris-maleate (pH 8.0), 10; $MgCl_2$, 10; NADP, 0.2; DL-isocitrate, 0.2.
Sulfite-oxidase, (Charles and Suzuki, 1966).
Tris-maleate (pH 8.0), 50; EDTA, 4; $K_3Fe(CN)_6$, 2; Na_2SO_3, 2.
NADH-oxidase, EC 1.6.99.3 (Matin and Rittenberg, 1971).
Tris-maleate (pH 8.0), 10; NADH, 0.1.
2-Oxoglutarate dehydrogenase-complex, (Kaufman, 1955).
Tris-HCl (pH 8.0), 10; Coenzyme A, 0.2; 2-oxoglutarate, 6; NAD, 1.
This assay has been performed anaerobically.
Isocitrate-lyase, EC 4.1.3.1 (Dixon and Kornberg, 1959).
Tris-maleate (pH 8.0), 20; $MgCl_2$, 5; glutathion (reduced), 10; phenyl-hydrazine-HCl, 4; DL-isocitrate, 10.
Malate dehydrogenase, EC 1.1.1.37 (Charles, 1971).
Tris-maleate (pH 8.0), 10; NAD, 1; DL-malate, 100.

Reactions were followed by measuring NAD (P) reduction or NADH oxidation at 340 nm ($\varepsilon = 6.23 \times 10^3 M^{-1} cm^{-1}$), $K_3Fe(CN)_6$ reduction at 420 nm ($\varepsilon = 1.03 \times 10^3\ M^{-1}\ cm^{-1}$) or hydrazone formation at 324 nm ($\varepsilon = 1.7 \times 10^4\ M^{-1}\ cm^{-1}$).

All recordings of enzyme activity were linear for at least 3 min and directly proportional to the amount of extract added.

CO_2-Fixation by Whole Cells. Activity was measured in concentrated cell suspensions (± 1 mg protein/ml) in basal medium at pH 8.0. Six test tubes, each containing 0.1 ml of cell suspension, were incubated for 2 min at 28°C in the presence of thiosulfate (0.5 µmol). Aeration was achieved by blowing a constant stream of air over the liquid surface and by magnetic stirring. The reaction was started by addition of ^{14}C-bicarbonate (0.5 µmol, 0.5 µCi/µmol) simultaneously to all test tubes. At 45 s intervals the reaction was terminated by addition of

200 µmol non-radioactive bicarbonate in 2 ml of a 5 mM potassium phosphate buffer (pH 8.0), and rapid filtration through a 0.45 µm Millipore filter. The filters were dried at 100°C for 3 min and radioactivity was measured in a gas-flow counter (Nuclear-Chicago Corporation, Des Plaines, Ill., U.S.A.). The method was carefully checked for linearity with time. The rates recorded were proportional to the amount of cells added.

Gas Analysis in the Continuous Culture. O_2 consumption and CO_2 production in chemostat cultures were determined (Dijkhuizen et al., 1977) by comparison of the content of these gases in the air entering and leaving the culture. Calculations were made according to Fiechter and von Meyenberg (1968). A Servomex OA 184 analyzer (Servomex Control Ltd., Crowborough, Sussex, England) and an infrared gas analyzer model SB2 (Grubb Parsons; New Castle) were used for O_2 and CO_2 measurements respectively. The gas flow rate through the culture was determined with a volumeter type 1 (Meterfabriek Dordrecht, Holland). Concentration of CO_2 dissolved in the culture fluid was measured with the total organic carbon analyzer.

Miscellaneous Methods. Optical densities were measured using a Hitachi Perkin Elmer 139 spectrophotometer. Maximum oxygen consumption rates of cell suspensions were measured polarographically with a YSI-Biological oxygen Monitor.

Chemicals. Ribulose-1,5-bisphosphate was obtained from Sigma Chemical Co. (St. Louis). ^{14}C-bicarbonate and ^{14}C-acetate were obtained from the Radiochemical Centre, Amersham, England. All other chemicals used were of analytical grade, obtained from commercial sources.

Results

The capacity to oxidize reduced sulfur compounds (e.g. hydrogen sulfide and thiosulfate) appeared to be repressed completely in *Thiobacillus* A2 during growth on organic substrates such as acetate, glycollate, fructose or formate. The first experiment was aimed to investigate whether these enzymes still would be repressed in the presence of a mixture of thiosulfate and organic substrate (e.g. acetate). *T.* A2 was pregrown in an acetate-limited chemostat at a dilution rate (= D) of 0.10 h^{-1} with acetate as the only carbon and energy source. At zero time the addition of acetate medium was stopped and the culture was diluted two-fold with fresh medium containing both thiosulfate and acetate. Final concentrations were 17 mM and 12 mM, respectively, as shown in Fig. 1B. Temperature, pH (8.0) and O_2-tension (50 % air saturation) were kept constant as growth proceeded. As judged from optical density measurements, growth continued without a lag phase, with a specific rate of 0.13 h^{-1} (Fig. 1A). Data on protein and organic carbon in the culture, however, indicated a short lag of approximately half an hour, before growth proceeded. A gradual increase in specific growth rate, upto 0.17 h^{-1} (based on protein) and 0.19 h^{-1} (based on organic carbon) after about 5 h, was indicated by these data. Initially only acetate was utilized for growth. After about 2 h thiosulfate oxidizing capacity (= $Q_{O_2}^{max}$-thiosulfate) could be detected. This was measured polarographically in washed samples. The maximum O_2-consumption rate in the presence of acetate and thiosulfate appeared to be the sum of the rates observed with thiosulfate and acetate separately. The further induction of the thiosulfate oxidizing capacity proceeded relatively slowly until 8 h after the start of the experiment. At this moment, coincident with the exhaustion of acetate, the increase in thiosulfate oxidizing capacity suddenly accelerated markedly. Interestingly the $Q_{O_2}^{max}$-acetate which had been more or less constant, also increased 2 – 3 fold. At this same moment growth stopped. In a further experiment it was shown that this was due to the inability of the cells to fix CO_2. In another batch culture experiment essentially the same growth conditions were

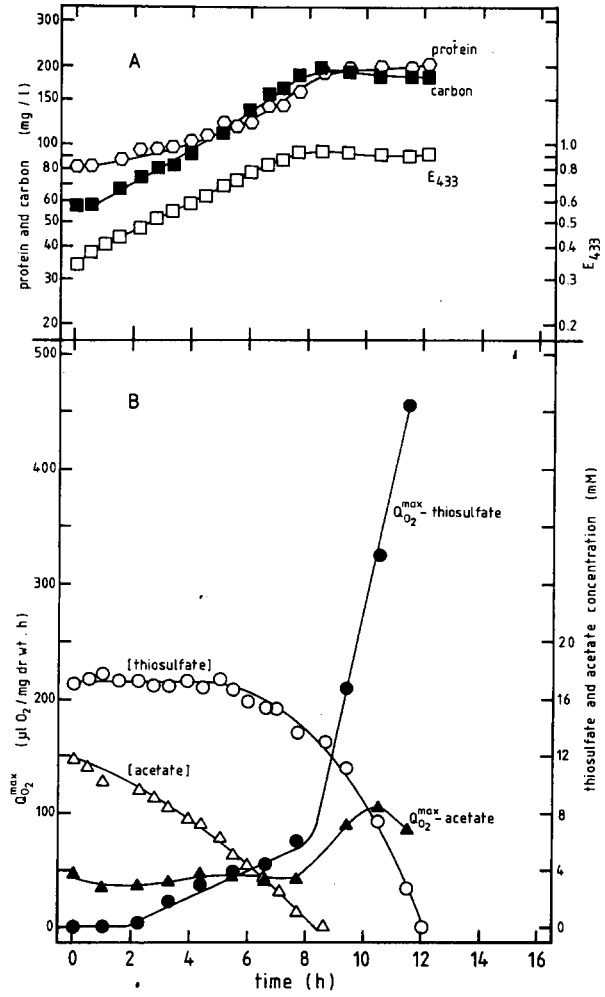

Fig. 1 A and B. Cell density, maximum substrate oxidation capacities ($Q_{O_2}^{max}$) and substrate concentrations during growth of *Thiobacillus* A2 on a mixture of acetate and thiosulfate in batch culture. The inoculum consisted of cells from an acetate-limited continuous culture at a dilution rate of 0.10 h^{-1}. **A** Protein O–O; Organic cell carbon ■——■; Absorbance □——□. **B** $Q_{O_2}^{max}$-thiosulfate ●——●; $Q_{O_2}^{max}$-acetate ▲——▲; Thiosulfate concentration ○——○; Acetate concentration △——△

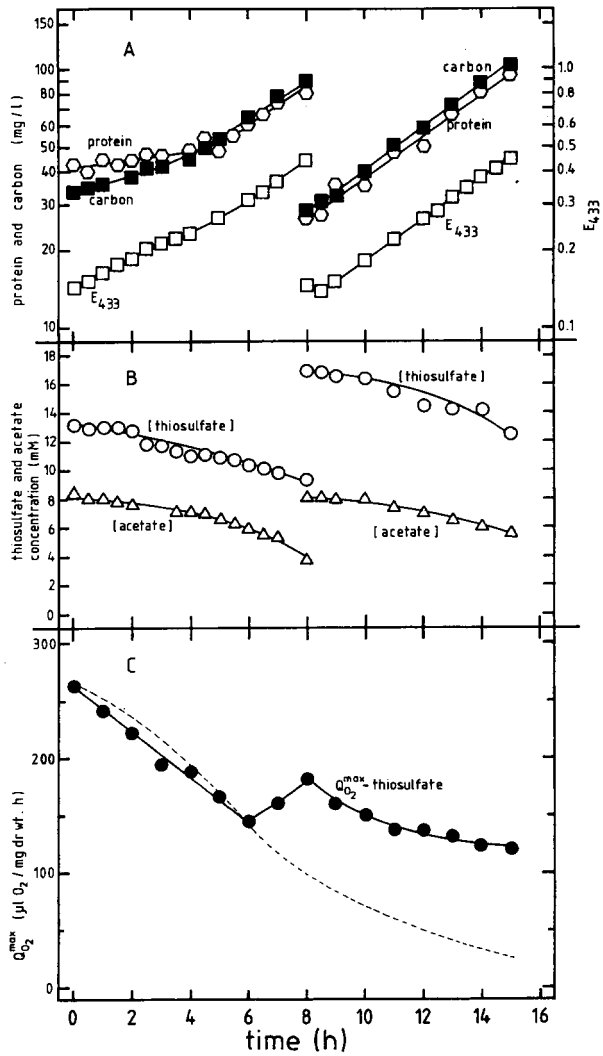

Fig. 2A—C. Cell density, $Q_{O_2}^{max}$-thiosulfate and substrate concentrations during growth of *T.* A2 on a mixture of acetate and thiosulfate in batch culture. The inoculum consisted of autotrophically grown cells from a thiosulfate-limited chemostat culture at a dilution rate of 0.02 h^{-1}. Symbols as in Fig. 1. The dashed line in Fig. 2C represents the predicted decrease in the $Q_{O_2}^{max}$-thiosulfate if the synthesis of this enzyme-system would have been repressed completely during the course of the experiment

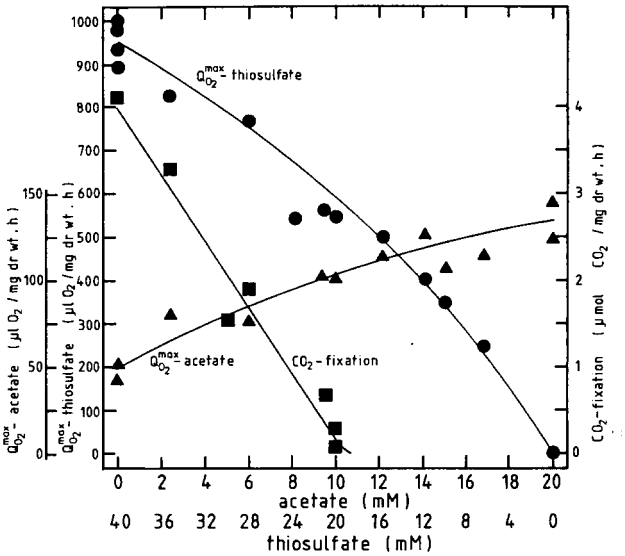

Fig. 3. Carbon dioxide fixation potential and maximum substrate oxidation potentials of whole cells of *T.* A2 as a function of different acetate and thiosulfate concentrations in the reservoir medium of chemostat cultures. Data were obtained with cells from thiosulfate and/or acetate-limited chemostat cultures in steady state at a dilution rate of 0.05 h^{-1}. $Q_{O_2}^{max}$-thiosulfate ●–●; $Q_{O_2}^{max}$-acetate ▲–▲; CO_2-fixation potential ■–■

applied, this time, however, to cells pregrown autotrophically on thiosulfate in continuous culture at a dilution rate of 0.02 h^{-1}. Both acetate and thiosulfate were utilized simultaneously from the onset of the experiment (Fig. 2B). The $Q_{O_2}^{max}$-thiosulfate decreased initially, but after 6 h a slight increase was observed (Fig. 2C). After a second dilution with fresh medium (at 8 h) the $Q_{O_2}^{max}$-thiosulfate decreased again. The observed decrease in thiosulfate oxidizing capacity was compared with the expected decrease if repression of enzyme synthesis were complete (dashed line in Fig. 2C). Such a comparison showed that at relatively high acetate concentrations the enzyme synthesis was completely repressed, whereas at lower acetate concentrations some synthesis occurred. The increase of thiosulfate oxidizing capacity in the presence of still significant concentrations of acetate (4–6 mM) indicated that the degree of repression of the chemolithotrophic energy generating system might be governed by the ratio between the concentration of thiosulfate and acetate. In the next experiments chemostat cultures have been used to study in more detail the effect of mixed substrates on the growth of *T.* A2.

In Fig. 3 the results are shown of a series of experiments in which *T.* A2 was cultivated in continuous culture at a dilution rate of 0.05 h^{-1} with different mixtures of thiosulfate and acetate as growth limiting substrates. The data represent steady-state values i.e. after at least 5 volume changes. Both acetate and thiosulfate were growth-limiting since no detectable quantities were present in the culture and because an increase in concentration of either one of these substrates in the feed caused a proportional increase in cell density. The observed change in CO_2-fixation potential measured in whole cells with excess of thiosulfate und CO_2, ran parallel to the change in activity of ribulose-bisphosphate carboxylase (RubPcase) in cell-free extracts prepared from these cells (compare Fig. 3 with Table 1). The activity of this enzyme measured with $^{14}CO_2$ and excess of ribulose-bisphosphate was about 80% of the CO_2-fixation rate observed in whole

Table 1. Some enzyme activities in crude cell free extracts of *Thiobacillus* A2 from steady state chemostat cultures grown at a dilution rate of 0.05 h^{-1}, limited by different thiosulfate-acetate mixtures

	20 mM Acetate + 0 mM thiosulfate	15 mM Acetate + 10 mM thiosulfate	10 mM Acetate + 20 mM thiosulfate	2 mM Acetate + 36 mM thiosulfate	0 mM Acetate + 40 mM thiosulfate
Isocitrate dehydrogenase	751	286	318	279	325
Malate dehydrogenase	481	243	310	289	239
2-Oxoglutarate dehydrogenase	0.26	0.0	N.D.	0.0	N.D.
Isocitrate lyase	< 1	< 1	< 1	< 1	< 1
Sulfite oxidase	245	312	237	290	298
RubPcase	< 0.2	< 0.2	3.1	63.2	75
NADH-oxidase	6.2	N.D.	11.1	N.D.	N.D.

Activities are given in nmol · min · mg protein
N.D. = Not determined

cells. The maximum potential to oxidize acetate and thiosulfate ($Q_{O_2}^{max}$) was determined polarographically by measuring the O_2-uptake rate by whole cells in the presence of excess of either of these substrates. The results, depicted in Fig. 3, showed that both substrate oxidation potentials were almost directly proportional to the relative concentrations of the respective substrates in the feed. It should be noted that the $Q_{O_2}^{max}$ for thiosulfate was zero if acetate was the sole substrate, whereas acetate could still be respired if only thiosulfate was present in the medium.

It had been reported (Peeters et al., 1970) that in *T.* A2 the TCA-cycle was much more active in heterotrophically grown cells than in autotrophically grown cells. Measurements of some TCA-cycle enzymes in crude cell-free extracts (Table 1) were aimed to investigate whether the gradual change from heterotrophic (acetate) to autotrophic (thiosulfate) growth conditions is reflected in a decreasing TCA-cycle activity. Relatively small changes in enzyme activities were observed. Only malate- and isocitrate-dehydrogenase showed a 2 – 3 fold increased activity in purely heterotrophically compared to mixotrophically or autotrophically grown cells. Isocitrate-lyase was absent in all cell extracts tested. Also in cells grown in batch culture on acetate as the sole substrate this enzyme was not detectable. Activity of this enzyme was easily detected in another organism, *Pseudomonas oxalaticus*, grown on acetate, using the same enzyme assay. A trace of activity of 2-oxoglutarate dehydrogenase could be detected in completely heterotrophically grown cells only. This observation indicated that the rate of acetate oxidation via the TCA-cycle would be by far too low to explain the growth rate of *T.* A2. Thus an alternative pathway for the metabolism of acetate must be available to *T.* A2. The question how acetate is metabolized under such conditions, remains unanswered, as it is with some other organisms lacking

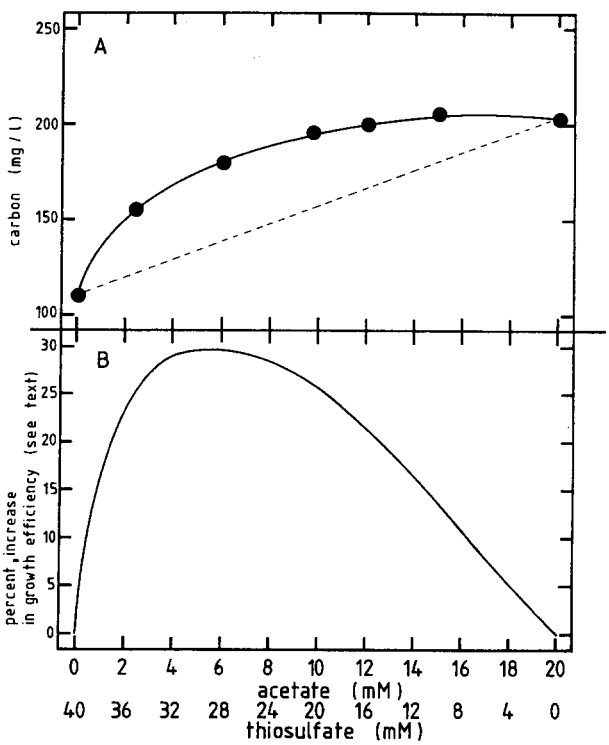

Fig. 4A and B. Organic cell-carbon (●——●) as a function of different acetate and thiosulfate concentrations in the reservoir medium of chemostat cultures. Data were obtained with cells from continuous cultures in steady state at a dilution rate of 0.05 h^{-1}. The dashed line represents the expected cell-density if it were the arithmetical sum of heterotrophic growth on acetate and autotrophic growth on thiosulfate. **B** The difference between "predicted" and observed cell density (Fig. 4A) as a percentage of the "predicted" values

isocitrate-lyase activity during growth on acetate (Quayle, 1975).

Figure 4A shows the results of dry weight measurements (as cell-carbon) of steady state cultures with thiosulfate, acetate or mixtures of these as growth-

Table 2. O_2 consumption and CO_2 production determined by analysis of the spent gas from steady state chemostat cultures of *T.* A2, limited by different mixtures of thiosulfate and acetate. Dilution rate was 0.05 h^{-1}. Organic cell carbon content of the cultures was determined with a total organic carbon analyzer

Medium composition		Culture density	Results of O_2 and CO_2 analysis in steady state cultures				
Acetate mMol organic carbon/l	Thiosulfate mMol/l	Cell-Carbon mMol/l	CO_2 from acetate mMol/l	Percent[a] acetate carbon assimilated	Total O_2-consumption mMol/l	O_2 reduced[b] by thiosulfate mMol/l	Theoretical O_2-[c] consumption in thiosulfate oxidation mMol/l
38.54	0	15.6	22.4	41.8	24.1	1.7	0
37.70	7.6	19.7	18.5	50.9	34.4	15.9	15.2
37.12	15.2	22.1	14.7	60.4	46.2	31.5	30.4
35.80	22.8	24.7	12.5	65.1	58.6	46.1	45.6

[a] Calculated as follows: $\frac{\text{(mmol consumed acetate-carbon)} - \text{(mmol } CO_2 \text{ produced from acetate)}}{\text{mmol consumed acetate-carbon}}$

[b] These figures were obtained by substracting the amount of CO_2 consumed in oxidation of acetate from the total O_2-consumption. The oxygen consumed in the oxidation of acetate was assumed to equal the amount of CO_2 produced (RQ = 1)

[c] A stoichiometry of 2 mol of oxygen was assumed for the complete oxidation of 1 mol of thiosulfate to sulfate

limiting substrates. If the dry weight during growth on the mixed substrates were the result of heterotrophic growth on acetate plus autotrophic growth on thiosulfate this would have been described by the dotted line. The results obtained, as described by the solid line in Fig. 4A, demonstrated that growth under these conditions was much more efficient. In Fig. 4B the relative increase in cell dry weight compared to the predicted dry weight on the basis of heterotrophic and autotrophic growth (dotted line in Fig. 4A) was plotted against the different acetate-thiosulfate mixtures in the feed. Apparently most efficient growth occurred at relatively low concentrations of acetate (2–10 mM) combined with relatively high concentrations of thiosulfate (20–36 mM) in the inflowing medium. Analysis of the amount of O_2 consumed and CO_2 produced, was carried out in cultures of *T.* A2 grown in continuous culture on mixtures of acetate and thiosulfate as growth limiting substrates. This could provide insight into the role of acetate as carbon versus energy source under such conditions. Gases were measured directly in the outflowing air, using an O_2 and CO_2 analyzer. Results of such an analysis are presented in Table 2. The ratio between the thiosulfate and acetate concentrations in the feed were chosen in such a way that no CO_2-fixation would occur (see Fig. 3). A technical difference between this experiment and that shown in Fig. 3 was that different ratios of thiosulfate and acetate were created by increasing the thiosulfate concentration in the feed leaving the acetate concentration practically constant. The data (Table 2) showed that with increasing concentration of thiosulfate an increasing amount of the acetate was assimilated. Apparently the oxidation of thiosulfate provided the energy needed for this elevated extent of assimilation. The amount of oxygen consumed in the oxidation of thiosulfate was very close to that which was theoretically expected (Table 2, last column) if complete oxidation to sulfate was assumed. The theoretically maximum contribution of acetate to the total cell carbon (Fig. 4) expressed as the percentage* of the total amount of acetate consumed was plotted in Fig. 5 together with the percentage actually found by gas analysis. It was clear that up to a concentration of 12 mM acetate and 16 mM thiosulfate all cellular carbon was derived from acetate. This should be expected since no CO_2-fixing capacity was present in cells grown at these relatively high acetate concentrations (see Fig. 3).

More interesting is the physiological state in which CO_2-fixing capacity is available in spite of the presence of enough acetate to provide all cell material. This was the case if a mixture of 8 mM acetate and 24 mM thiosulfate was fed to the continuous culture (compare Figs. 3 and 5). Under these conditions results of gas analysis would be very difficult to interpret, because two processes namely CO_2 production (acetate oxidation) and CO_2 fixation might occur simultaneously. Therefore uniformly ^{14}C-labeled acetate was used in the medium in order to measure the extent of acetate assimilation. After a steady state was reached the specific radioactivity of the cells was compared with that of the acetate in the medium. The same experiment was performed with a mixture of 5 mM acetate and 30 mM thiosulfate. It appeared that 69 % of the acetate

* Calculated as follows: $\frac{\text{(mmol cell carbon)}}{\text{(mmol consumed acetate-carbon)}}$

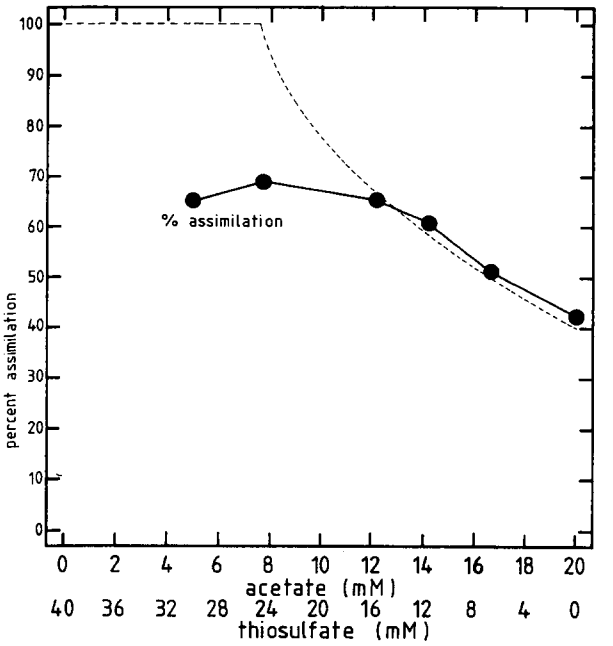

Fig. 5. The amount of acetate assimilated relative to the amount of acetate consumed in steady state chemostat cultures of *Thiobacillus* A2 as a function of different acetate and thiosulfate concentrations in the reservoir medium. The dilution rate was 0.05 h^{-1}. The solid line (●——●) represents values determined by gas analysis (see Table 2) or by ^{14}C-acetate assimilation experiments (see text). The dashed line represents the predicted amount of acetate assimilated if acetate would be preferred to CO_2 as carbon source. This was calculated by dividing the amount of organic cell carbon in steady state by the organic carbon available as acetate in the continuous culture (Fig. 4A)

had been assimilated in the first case and 65% in the second experiment (Fig. 5).

Figure 6 shows the results of experiments in which *T.* A2 was cultivated in continuous culture at various dilution rates. Growth was limited by a mixture of thiosulfate (18 mM) and acetate (11 mM). Steady states could be obtained at dilution rates as high as 0.20 h^{-1}, equal to the maximum specific growth rate on acetate. Only in this case thiosulfate (10.5 mM) and acetate (3.0 mM) could be detected in the culture. Cell yield on this substrate mixture was always higher than the sum of the yields obtained during growth on both substrates separately. The cell yield on acetate at dilution rates between 0.03 h^{-1} and 0.20 h^{-1} was 18.6 – 20.6 (mg dry weight/mmol acetate). The cell yield on thiosulfate at dilution rates between 0.015 h^{-1} and 0.10 h^{-1} was 4.0 – 5.6 as determined by Kuenen (1979). With the acetate-thiosulfate mixture employed, no CO_2-fixation occurred at any dilution rate tested. This allowed direct calculation of the extent of acetate assimilation from the cell-carbon content of the culture and the amount of acetate provided to the chemostat. It

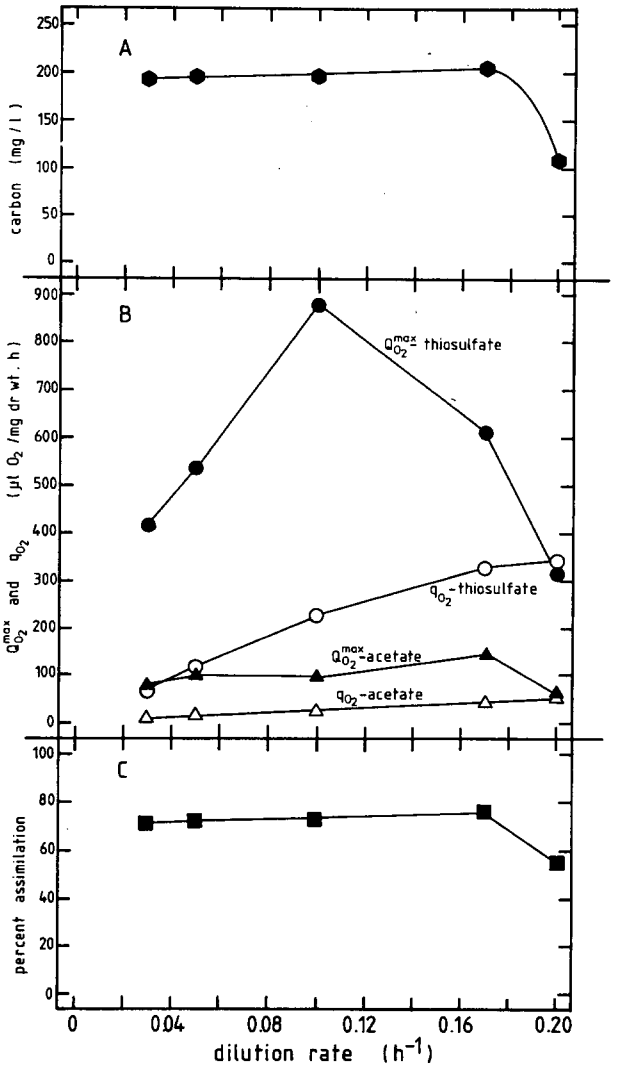

Fig. 6A–C. Cell density, substrate oxidation rates and the extent to which acetate is assimilated as a function of dilution rate in steady state continuous cultures of *T.* A2. The reservoir medium was composed of a fixed mixture of 18 mM thiosulfate and 11 mM acetate. At all dilution rates *T.* A2 was unable to fix $^{14}CO_2$ autotrophically. **A** Organic cell carbon ●——●. **B** $Q_{O_2}^{max}$-thiosulfate ●——●; $Q_{O_2}^{max}$-acetate ▲——▲. Actual substrate oxidation rate in the steady state continuous culture, measured by gas analysis: q_{O_2}-thiosulfate ○——○; q_{O_2}-acetate △——△. **C** Amount of acetate as similated relative to the total amount consumed in the cultures ■——■, calculated as $\frac{\text{organic cell-carbon}}{\text{acetate carbon consumed}}$

was apparent from the result of such a calculation (Fig. 6C) that 70 – 76% of the acetate was assimilated at dilution rates between 0.03 and 0.17 h^{-1}. On the other hand at a dilution rate of 0.20 h^{-1} only 55% of the consumed acetate was assimilated. This is not surprising since less than 50% of the thiosulfate was oxidized at this high dilution rate. In Fig. 6B the

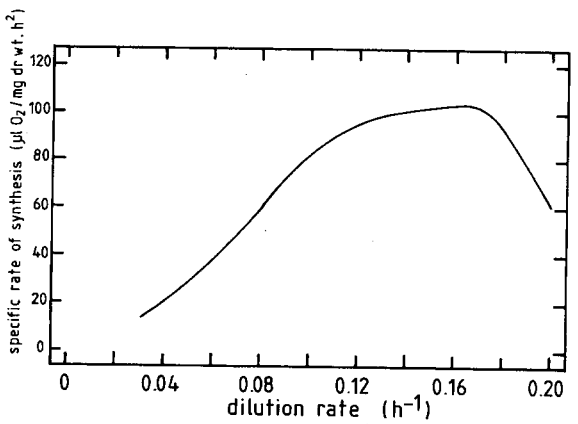

Fig. 7. The specific rate of synthesis of the enzyme system responsible for the thiosulfate oxidizing potential ($= D \cdot Q_{O_2}^{max}$-thiosulfate) as a function of dilution rate. Medium as given for Fig. 6

oxidation potential for both thiosulfate and acetate has been compared with the actual rate of substrate oxidation as determined by direct analysis of O_2 and CO_2 in the inflowing and outflowing air of the culture. In order to discriminate between the oxygen consumed in the oxidation of thiosulfate and acetate, it was assumed that one mol of O_2 is consumed in the oxidation of acetate for each mol of CO_2 produced from acetate. The $Q_{O_2}^{max}$ values for both substrates (Fig. 6B) appeared to be far in excess over those actually "needed" in the culture. Only at a dilution rate of 0.20 h^{-1} the actual oxidation rate equalled the $Q_{O_2}^{max}$ values. Whereas the $Q_{O_2}^{max}$ for acetate did not change very much with the different dilution rates, the $Q_{O_2}^{max}$ for thiosulfate increased considerably with increasing dilution rate up to $D = 0.10\ h^{-1}$. At higher dilution rates a decrease in this thiosulfate-oxidizing potential was observed. Repression of the synthesis of this oxidizing system might have occurred by the relatively high acetate and/or thiosulfate concentrations at these higher dilution rates. Such an explanation would be in agreement with the observed partial repression by acetate in batch cultures (see above). Moreover in batch culture experiments with excess of thiosulfate as the sole substrate (data not shown) much lower $Q_{O_2}^{max}$-thiosulfate values were found than under thiosulfate-limiting conditions. Quite another explanation might be that the rate of synthesis of one (or more) essential component of the thiosulfate-oxidizing system has reached a maximum value at $D = 0.10\ h^{-1}$. This view was supported by the data presented in Fig. 7, in which the specific rate of synthesis of the thiosulfate-oxidizing capacity in steady state ($= D \cdot Q_{O_2}^{max}$-thiosulfate) has been plotted versus dilution rate. This picture shows that the specific synthesis did indeed show only a minor increase between a dilution rate of 0.10 h^{-1} and 0.17 h^{-1}. A rapid decrease in the rate of synthesis was observed at $D > 0.17\ h^{-1}$, which could indicate a higher degree of repression at these dilution rates.

Discussion

Thiobacillus A2 grown in batch culture in the presence of both thiosulfate and acetate utilized these substrates simultaneously. Nevertheless acetate did repress the synthesis of enzymes for thiosulfate oxidation. This is concluded from the following observations. Firstly the rate of induction of thiosulfate oxidizing capacity in the presence of acetate was much slower than after depletion of the acetate (Fig. 1). Secondly, the thiosulfate oxidizing capacity was reduced during mixotrophic growth on thiosulfate plus acetate. These results support earlier findings in cultures of *T.* A2 which showed partial repression of sulfite-oxidase in the presence of acetate (Taylor and Hoare, 1971). Le John et al. (1967) reported complete repression of the thiosulfate oxidizing system in *T. novellus* by glucose at concentrations above 25 mM and partial repression at lower glucose concentrations. More recent experiments with *T. novellus* (Perez and Matin, 1979; Matin, 1978), did show simultaneous utilization of thiosulfate and glucose at glucose concentrations between 5 and 50 mM, though the presence of thiosulfate caused a decrease in growth rate to about 65% of that observed in a glucose medium. In the present experiments no such inhibition was observed since *T.* A2 was able to grow on a mixture of acetate and thiosulfate at a growth rate of 0.19 h^{-1} which is very close to the maximum specific growth rate of 0.22 h^{-1} observed with our strain of *T.* A2 (Gottschal et al., 1979).

Previous reports on continuous culture experiments with mixtures of two or more growth limiting substrates (Harder and Dijkhuizen, 1976) have clearly shown that many diauxic growth phenomena observed so far in batch culture do not occur if the substrate concentrations are kept sufficiently low. Simultaneous substrate utilization probably is rather the rule than the exception under these conditions. Our results have shown that *T.* A2 was able to use the "autotrophic" substrate thiosulfate and the organic substrate acetate simultaneously for growth. Under dual substrate limitation by acetate and thiosulfate in the chemostat the cell-yields of *T.* A2 were up to 30% higher than expected from the sum of the yields observed on the separate substrates (Fig. 4A). Similar yield-increments were found in mixotrophically grown cultures of *T. novellus* (Perez and Matin, 1979) and *Pseudomonas oxalaticus* (Dijkhuizen, 1979). The explanation for the increment in the cell-yield observed during mixotrophic growth probably lies in the sparing effect which assimilation of an organic compound has on the energy-requirements for cell-biosynthesis as compared to the

autotrophic production of cell-material. This hypothesis is supported by the fact that the gradual increment in the cell-yield coincided with the decline and the eventual total loss of the capacity to synthesize cell-material autotrophically (Figs. 3 and 4).

Dijkhuizen (1979) observed a rather sudden rearrangement in the metabolism of mixotrophically grown cultures of *Ps. oxalaticus* under dual limitation of formate and acetate at a dilution rate of 0.10 h^{-1}. The author concluded that at this dilution rate more acetate was assimilated (compared to the dissimilation of it) relative to that at lower dilution rates. Such a sudden changeover in metabolism did not occur in cultures of *T.* A2 at dilution rates between 0.03 h^{-1} and 0.17 h^{-1}. But the extent to which acetate was assimilated did show variations in response to different thiosulfate-acetate ratios in the medium rather than to changes in dilution rate. With increasing thiosulfate-acetate ratios in the medium, acetate assimilation increased from 42 – 70 % of the total amount of acetate consumed (Fig. 5).

From our results it is evident that during growth on different thiosulfate-acetate mixtures in continuous culture not only the assimilatory pathways but also the oxidative enzyme systems are under strict control. This is particularly apparent for the thiosulfate-oxidizing system. As discussed above acetate clearly has a repressing effect on the thiosulfate-oxidizing capacity in batch cultures. Repression, however, cannot be the only regulating factor for this enzyme system. Induction by thiosulfate (or S^{2-}; unpublished results) probably is necessary which is indicated by the fact that cells grown on acetate alone are unable to oxidize thiosulfate, even if cultivated at extremely low dilution rate ($D = 0.005\ h^{-1}$; results not shown) in the chemostat. Furthermore thiosulfate oxidizing capacity appears in cultures growing on acetate, shortly after addition of thiosulfate (Fig. 1). The most likely explanation available at this moment for the observed remarkably precise enzyme regulation (Fig. 3) seems therefore to be a balance between induction (by thiosulfate) and repression (by acetate). This balance must be brought about by the relative concentrations of thiosulfate and acetate in the culture. These are determined not only by the growth characteristics of the organisms but also by the dilution rate and, indirectly, by the thiosulfate-acetate concentration ratio in the reservoir medium. Data on the steady state concentrations of both substrates in the chemostat were not obtained but should be of great importance to further our understanding of the precise regulation.

Apart from the exact physiological mechanism of dual substrate utilization it will be evident that thiobacilli with a mixotrophic potential comparable to that described above for *T.* A2 might benefit at least in two ways from their type of metabolism. Firstly, more efficient growth in terms of cell-yield occurs on mixtures of a reduced sulfur compound and an organic substrate. Secondly, when in the natural environment inorganic sulfur compounds and organic substrates would be available simultaneously, the mixotrophic type of metabolism might allow organisms like *T.* A2 to outcompete specialized organisms such as obligately chemolithotrophic thiobacilli and heterotrophic bacteria.

This particular aspect has been demonstrated to be indeed of considerable competitive advantage to *T.* A2 (Gottschal et al., 1979) and other facultatively chemolithotrophic thiobacilli (Gottschal and Kuenen, in preparation).

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