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Effects of Organic Compounds on Growth of Chemostat Cultures of *Thiomicrospira pelophila*, *Thiobacillus thioparus* and *Thiobacillus neapolitanus*

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Summary. Cultures of *Thiomicrospira pelophila*, *Thiobacillus thioparus* and *Thiobacillus neapolitanus* were grown in thiosulfate-limited chemostats in a mineral-thiosulfate medium with and without organic supplements. Acetate, succinate and mixtures of amino acids increased the dry weight by 12—24% and the protein by 11—38%. Addition of both acetate and succinate had a cumulative effect. Saccharose, glucose, fructose, ribose, glycerol, glycerate, pyruvate, lactate or malate were without effect. The increase in dry weight of *T. neapolitanus* by ^{14}C-acetate was directly related to the relative contribution of this compound to the total cell carbon.

In CO_2-limited cultures of *T. neapolitanus* the effects of acetate on dry weight and protein were similar to those found in thiosulfate-limited cultures. In CO_2-limited cultures of *T. pelophila* a combination of acetate and succinate caused an increase in dry weight of 27% and of 50% in protein, the increase in protein being twice as high as in thiosulfate-limited cultures.

There were no measurable differences in the activities of ribulosediphosphate carboxylase (RudPcase) in cell free extracts obtained from thiosulfate- or CO_2-limited cultures of *T. pelophila* or *T. neapolitanus* grown in the presence or absence of organic compounds. In *T. pelophila* the RudPcase activity was almost constant at all growth rates tested, and independent of the type of growth-limitation. For *T. neapolitanus* the specific RudPcase activity varied slightly with the growth rate. In CO_2-limited cultures the activity was three times that found in thiosulfate-limited cultures, thus showing that the RudPcase activity can be influenced by nutritional conditions.

For many years most of the thiobacilli and nitrifying bacteria have been considered to be obligate autotrophs. Such bacteria were believed to be strictly dependent on carbon dioxide as the carbon source and a reduced inorganic compound as the energy source. It has now become apparent that this concept is no longer tenable. Many organic compounds have been shown to be incorporated by growing cultures of these bacteria. Recently, it has been proposed to designate such organisms as obligate chemolithotrophs (Rittenberg, 1969). Obligately chemolithotrophic bacteria are obligately dependent on a reduced inorganic compound as the energy source.

Although organic compounds can be taken up by growing cells of obligate chemolithotrophs, the extent of incorporation is apparently limited. The highest values reported are 10—25% of the total cell carbon for ^{14}C-acetate (Kelly, 1967; Smith *et al.*, 1967; Taylor *et al.*, 1971; Kuenen, 1972), and 10% for succinate (Smith *et al.*, 1967). In spite of this significant uptake, neither the growth rate nor the cell yield was affected by these compounds (Kelly, 1967; Smith *et al.*, 1967; Saxena and Vishniac, 1970; Taylor and Hoare, 1971). Such an increase might be expected since the incorporation of organic compounds leads to a relatively lower contribution of carbon dioxide to the total cell carbon. Since the CO_2-fixation process has a high energy requirement, one would expect the inclusion of organic compounds in the inorganic medium to have an energy sparing effect and therefore to increase the cell yield. A similar suggestion has been made by Rittenberg (1969).

There are several reasons to believe that the failure to detect a cell yield increase might be due to the exclusive use of batch cultures. Firstly, in batch culture the energy source and all other growth requirements are present in excess. Conceivably the energy sparing effect might not occur under such conditions because of an insufficient coupling between energy generation and biosynthesis. Secondly, the increases in cell yield caused by organic compounds could be too low to be detectable in batch culture. This is especially true for thiosulfate-grown cultures of obligately chemolithotrophic thiobacilli in which sulfur formation, due to excess thiosulfate, very often occurs (Trudinger, 1967). This may cause considerable variations in growth yield data.

Since these drawbacks of batch cultures are not encountered in an open culture system, the effect of organic compounds on growth yield of obligately chemolithotrophic thiobacilli and related organisms was studied in thiosulfate-limited chemostats. When such a chemostat culture is in a steady state, no sulfur is formed. An additional advantage is that cultivation in the chemostat results in reproducible steady states which make this cultivation method suited for quantitative measurements. In a later stage of this study, CO_2-limited cultures were also studied.

Mixotrophic thiobacilli (Rittenberg, 1969) display up to 98% repression of one of the key enzymes of the Calvin cycle, ribulosediphosphate carboxylase (RudPcase), when organic compounds are added to autotrophically grown cultures (Aleem and Huang, 1965; London and Rittenberg, 1966; Tabita and Lundgren, 1971; Taylor and Hoare, 1971). Very little is known of the effect of organic compounds on the level of this enzyme in obligate chemolithotrophs. Recently, Taylor and Hoare (1971) reported a decrease of 25% in the RudPcase activity in cell free extracts of *Thiobacillus denitrificans* grown in the presence of 20 mM acetate. To investigate whether similar effects occur under the influence

of organic compounds in continuous culture, the levels of RudPcase were measured in the obligately chemolithotrophic *Thiomicrospira pelophila* (Kuenen and Veldkamp, 1972), a marine *Thiobacillus thioparus* and *Thiobacillus neapolitanus*. *T. pelophila* was recently isolated from marine mud and appeared to have a physiology which is strikingly similar to that of *T. thioparus* (Kuenen and Veldkamp, 1972). Incorporation experiments with ^{14}C-acetate showed that *T. pelophila* shares with the obligately chemolithotrophic thiobacilli the lack of an operative citric acid and glyoxylic acid cycle (Smith *et al.*, 1967; Kuenen, 1972).

Materials and Methods

Organisms. Experiments were carried out with a vitamin B_{12}-dependent strain of *Thiomicrospira pelophila* (Kuenen and Veldkamp, 1972) a marine strain of *Thiobacillus thioparus* (Kuenen and Veldkamp, 1972), and *Thiobacillus neapolitanus* (strain X), which was kindly provided by Dr. A. Matin. *T. pelophila* and *T. thioparus* were maintained as described earlier (Kuenen and Veldkamp, 1972). *Thiobacillus neapolitanus* was maintained on plates and in a liquid medium (described below).

Media. Concentrations of dissolved compounds are expressed as the percentage weight/volume unless otherwise stated. The basal minerals medium used for the cultivation of the marine *T. pelophila* and *T. thioparus* contained (%) : NaCl, 2.5; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7\ H_2O$, 0.15; $CaCl_2$, 0.03; K_2HPO_4, 0.05 in deionized water, plus 0.2 ml per liter of a trace elements mixture (Vishniac and Santer, 1957). For the cultivation of *T. pelophila*, vitamin B_{12} was added to a final concentration of 15 µg/l. For *T. thioparus*, 0.03% K_2HPO_4 and 0.02% $KH_2PO_4 \cdot 3\ H_2O$ were used instead of 0.05% K_2HPO_4. Phosphate was sterilized separately. For batch cultivation the medium contained 0.8% $Na_2S_2O_3 \cdot 5H_2O$ and was sterilized by autoclaving for 20 min at 120° C.

The basal medium for cultivation of *T. neapolitanus* contained (%) : NH_4Cl, 0.1; $MgSO_4 \cdot 7\ H_2O$, 0.15; K_2HPO_4, 0.5; KH_2PO_4, 0.5 and deionized water. Of the trace element mixture 2 ml were added per liter of medium. Liquid media contained 1% $Na_2S_2O_3 \cdot 5\ H_2O$, unless otherwise stated. Solid media contained 1% agar (Difco) and 0.8% $Na_2S_2O_3 \cdot 5\ H_2O$. The media were sterilized as described above.

Media for Continuous Culture. The medium used in the chemostat experiments consisted of basal medinm and 0.8% $Na_2S_2O_3 \cdot 5\ H_2O$ for *T. pelophila* and *T. thioparus* and of basal medium and 1% $Na_2S_2O_3 \cdot 5\ H_2O$ for *T. neapolitanus*. Thiosulfate was the growth limiting factor. Increasing or lowering the thiosulfate concentration, proportionally increased or lowered the total dry weight and protein content of steady state cultures. Organic compounds were added to the media in the concentrations indicated in the text. The medium was sterilized by filtration through a membrane filter with a pore size of 0.15 µm (Sartorius, Göttingen, West Germany). In addition to the controls prescribed by the manufacturer, the filtration procedure was checked, after processing the thiosulfate medium, by filtering 500 ml of a rich medium (YAG, see below) through the filter. This YAG medium was incubated at 30° C for at least 14 days. If growth occurred in this medium, cultures grown in the corresponding thiosulfate medium were discarded. The YAG medium contained (%): Sodium lactate or sodium acetate, 0.1; glucose, 0.2; yeast extract, 0.3; NaCl, 1.5; tap water, pH 7.0.

Contamination by Other Bacteria. All cultures were frequently checked for contaminants. Chemostat cultures were checked in each new steady state. Impurities in

the cultures of *T. pelophila* could be detected microscopically because of its unusual morphology (Kuenen and Veldkamp, 1972). In addition, cultures were streaked on thiosulfate agar plates and inoculated in liquid YAG medium. Contamination was rare. When it occurred, the cultures were discarded.

Batch Cultivation. The bacteria were grown in Erlenmeyer flasks at 25—28°C on a rotary shaker, as described by Kuenen and Veldkamp (1972); 200 ml—500 ml of such cultures were used as an inoculum for the chemostats with working volume of 1.5 liter.

Continuous Culture under Thiosulfate Limitation. Chemostat experiments were carried out in the equipment as described by Harder and Veldkamp (1967). The dissolved oxygen was monitored by an oxygen probe (New Brunswick, New Jersey, U.S.A.) and maintained between 40—60% air saturation. The culture was automatically neutralized to pH 6.8—7.0 with 2M Na_2CO_3. At all dilution rates tested, the thiosulfate was quantitatively converted to sulfuric acid, as calculated from the Na_2CO_3 used for the neutralization of the sulfuric acid formed. This was confirmed by testing for thiosulfate in rapidly cooled and filtered steady state samples. Both the iodometric titration and the assay according to Sörbo (1957) were negative, indicating that the steady state concentration of thiosulfate (s) was less than 1% of the original concentration (s_r) in the reservoir.

Continuous Culture under CO_2-Limitation. The equipment and medium used was the same as used for thiosulfate limited cultivation. A second metering pump (Vario Perspex 12000, LKB Produkter AB, Bromma, Sweden), connected to the growth vessel, was used for the addition of 40 mM Na_2CO_3. The cultures were neutralized by the addition of CO_2-free 2M NaOH, and aerated with a mixture of nitrogen and oxygen gas in a ratio of 4:1. It was possible to vary the initial concentration of HCO_3^- (s_r) in the culture by changing the ratio of the volumes delivered by the medium pump and by the carbonate pump. Keeping the dilution rate at 0.1 hr^{-1}, the ratio was set in such a way that the dry weight of the culture in the steady state was approximately half of that to be expected if the growth had been limited by the thiosulfate present in the resulting medium. Changes in the ratio of the volumes delivered by the two pumps resulted in a proportional increase or decrease in the dry weight showing that CO_2 was the growth limiting factor. During the steady state approximately 80% of the thiosulfate was oxidized as estimated from the NaOH and Na_2CO_3 used.

Check for Dead Cell Material in CO_2-Limited Cultures. The enumeration of cells of *T. pelophila* turned out to be irreproducible (Kuenen, 1972). Therefore, cultures were examined for evidence of dead cells by measuring the E_{260} and E_{280} in the culture fluid obtained from cultures grown at $D = 0.1$ hr^{-1} ($1/3\ \mu_{max}$). Since thiosulfate shows a considerable absorption at 260 nm, and CO_2-limited cultures contained about 20% of the original concentration of thiosulfate, a correction was made for the thiosulfate present. The corrected E_{260} was never over 0.1 and the E_{280} was negligible, indicating that the number of non-viable cells in the culture was insignificant.

Uptake of 2-^{14}C-Acetate in Thiosulfate-Limited Chemostat Cultures of T. neapolitanus. The medium used consisted of the basal medium plus 0.9% $Na_2S_2O_3 \cdot 5\ H_2O$. The concentration of 2-^{14}C-acetate was 1 mM, of a specific radioactivity of 66 μC/mM. The medium was sterilized by filtration as described above. The acetate was autoclaved separately in concentrated solution. The equipment was essentially the same as described by Harder and Veldkamp (1967); however, the fermentor vessel was replaced by an all-glass vessel of a working volume of 200 ml (Veldkamp and Kuenen, 1973). An LKB pump (Vario Perspex 12000, LKB Produkter AB, Bromma, Sweden) was used. The experiment was started by the addition of the radioactive acetate in

equal concentrations to the medium reservoir and the culture, after a steady state had been established in the inorganic medium. At different time intervals, 2 ml samples were taken from the culture and filtered through a membrane filter (Millipore Corp., Bedford, Mass., U.S.A., 0.22 μm pore size). The filtrates were kept for radioactivity measurements. The filters were washed three times with 10 ml ice cold 0.1 M potassium phosphate buffer, pH 6.8, containing 50 mMoles cold acetate per liter. The filters were transferred to scintillation vials and dried at 105° C for 20 min. Culture samples of 2 ml, taken at the same time intervals, were mixed with 10 ml 0.25 M perchloric acid (PCA) and, after intermittent mixing for 20 min at 4—5° C, filtered through membrane filters (Millipore, pore size 0.45 micron). These filters were washed three times with 10 ml ice cold 0.25 M PCA, and excess liquid was removed with filter paper. The filters were dried as described above. Filters were counted in toluene mixture containing: toluene, 1 liter; 2,5-diphenyloxazol (PPO), 5 g. Samples of 0.1 ml were taken from the culture filtrate, the total culture and from the inflowing medium and transferred to scintillation vials containing dioxane mixture consisting of: dioxane, 1 liter; naphtalene, 100 g; PPO, 7 g. Radioactivity was measured in a liquid scintillation counter (Nuclear Chicago Corp., Mark I, Des Plaines, Ill., U.S.A.). Counting data were converted to disintegrations per minute using appropriate quenching curves. Samples for protein estimation were taken from the steady state culture growing in thiosulfate medium and from the same medium with acetate. At the beginning and at the end of the experiment the culture was checked for contaminating bacteria as described above.

Dry Weight and Protein. Dry weight and protein were determined as described by Kuenen and Veldkamp (1972). Samples were taken from steady state cultures.

Steady States and Reproducibility. A steady state was considered to have been reached after at least 5 volume changes, and when, in addition, the E_{430} of the culture had been constant for 2 volume changes. The error in the average of duplicate determinations of dry weight and protein of the steady state cultures was never more than $\pm$ 3%. To verify the reproducibility of the technique, the standard deviation was calculated from dry weight and protein determinations of 6 and 5 identical and independent steady states, respectively. The standard deviation was 2% for both assays.

Cell Free Extracts. Bacteria were harvested from steady state continuous cultures and centrifuged at 2° C and 12000 g for 40 min. Pellets were washed with 0.1 M TRIS [tris(hydroxy methyl) aminomethane] maleate buffer. For *T. pelophila* the buffer contained in addition 1.5% NaCl, and the pH was 7.5. For *T. neapolitanus* the pH was 7.0. The cell pellets were resuspended in the same buffer to a concentration of 20—40 mg dry weight per ml and sonified at 2° C with an ultrasonic desintegrator (MSE Ltd., London, England). Cells of *T. pelophila* and *T. neapolitanus* were treated 5 times for 30 sec. Glass beads were added to the cell suspension of *T. neapolitanus* prior to sonification (1 g beads per 2 ml suspension). Crude extract was the supernatant prepared from the sonified suspension by centrifuging at 20000 g for 20 min. RudPcase was assayed in these crude extracts.

Assay of Ribulose-1,5-Diphosphate Carboxylase [3-Phospho-D-Glycerate Carboxy-Lyase (Dimerizing), EC 4.1.1.39]. The spectrophotometric method described by London and Rittenberg (1966) was used. The reaction mixture (1 ml) contained 0.02—0.2 mg of protein of the crude extract in buffer and the following compounds in μmoles: TRIS-maleate, 250; $MgCl_2$, 10; $NaHCO_3$, 37; glutathione, 5; ATP, 3; NADH, 0.14; phosphoglycerate kinase (ATP: 3-phospho-D-glycerate-1-phosphotransferase EC 2.7.2.3) 12 μg; glyceraldehyde phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) EC 1.2.1.12) 150 μg; ribulose 1,5 diphosphate, 0.24. The activity was measured in a Cary 14

recording spectrophotometer (Applied Physics Corp., Monrovia, Calif., U.S.A.). The activity was directly proportional to the concentration of the crude cell free extract and the reaction was linear for at least 6 min. The accuracy of the assay was approximately 5%. The reaction could be started by adding either the ribulosediphosphate or the cell free extract with essentially the same result. Adding ribulose diphosphate to the reaction mixture containing cell free extract of *T. pelophila* grown in CO_2-limited culture did, however, not result in a linear reaction. Therefore, in this case the reaction was started by adding cell free extract. Frozen extracts (−40° C) did not show any significant decrease in RudPcase activity during the first 14 days. To make comparisons between the activity of the RudPcase from different cell free extracts as valid as possible, assays were always carried out with fresh extract and an extract which previously had been assayed for RudPcase activity.

Chemicals. All chemicals used were of analytical grade. Ribulose-1,5 diphosphate and ATP were from Sigma (St. Louis, Missouri, U.S.A.). 3-Phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase, NADH and glutathione were from Boehringer (Mannheim, West Germany) and 2-^{14}C-sodium acetate was purchased from the Radiochemical Centre (Amersham, England).

Results

To study the effect of acetate on cell yield and on cellular protein *Thiomicrospira pelophila* was grown in a thiosulfate-limited chemostat in a minerals thiosulfate medium. Dry weight and protein per volume of culture were determined at different dilution rates (= growth rates). As can be seen from Fig. 1, the dry weight showed an increase with increasing dilution rate (D), whereas the reverse was true for the protein. When 1 mM acetate was included in the minerals thiosulfate medium, a definite increase in dry weight and protein averaging from 12—22% could be measured at all dilution rates. Increasing the acetate concentration in the growth medium did not increase the dry weight any further. This was shown by growing *T. pelophila* at a fixed growth rate of 0.1 hr^{-1} in the presence of varying concentrations of acetate in the growth medium. The data are presented in Fig. 2 and show a typical saturation curve. To show that the stimulatory effect of acetate on cell yield and cellular protein was a general phenomenon, similar experiments were carried out with a marine strain of *Thiobacillus thioparus* and with *Thiobacillus neapolitanus*. As can be seen from Tables 1 and 2, the increases in dry weight and protein were similar to those found for *T. pelophila*.

It seemed likely that the apparently limited increase in dry weight and protein caused by acetate was due to a limited use of acetate as a carbon source. Such a limited contribution from acetate had been found in batch cultures of *T. neapolitanus* (Kelly, 1967), *T. thioparus* (Smith *et al.*, 1967) and *T. pelophila* (Kuenen, 1972). To investigate this possibility, the contribution of acetate carbon to the total cell carbon was measured with 2-^{14}C-acetate. For this purpose *T. neapolitanus* was grown in a minerals medium plus 0.9% thiosulfate as the growth limiting factor at a

Table 1. Effect of 1 mM acetate on dry weight and protein of thiosulfate-limited chemostat cultures of *Thiobacillus thioparus*

Dilution rate	Dry weight (mg/l)			Protein (mg/l)		
	T	T+A	% increase	T	T+A	% increase
0.10	152	172	14	83	97	17
0.30	171	206	20	87	119	37

T = minerals medium + 0.8% thiosulfate. T+A = minerals medium + 0.8% thiosulfate + 1 mM acetate.

Table 2. Effect of acetate on dry weight and protein of thiosulfate-limited chemostat cultures of *Thiobacillus neapolitanus* grown in a minerals medium + 1.0% thiosulfate

Organic substrate added	Dilution rate	Dry weight		Protein	
		mg/l	% increase	mg/l	% increase
none	0.10	141		80	
1 mM acetate	0.10	159	12	89	11
2 mM acetate	0.10	168	19	89	11
none	0.27	186		—	

dilution rate of 0.1 hr^{-1}. When a steady state in autotrophic medium had been established, acetate was added to both the culture and the reservoir at a concentration of 1 mM and of equal specific radioactivity (66 μC/mM). The uptake of radioactivity into cell material was followed until a new steady state had been reached. Fig. 3 shows the incorporation of radioactivity expressed as micromoles of ^{14}C-acetate in intact cells and in high molecular weight cell material precipitated with 0.25 M perchloric acid (PCA). It appeared that a saturation level was established in approximately 5 volume changes. During the transition to the new steady state, the protein content of the culture was increased by 12% (from 73 mg/liter to 82 mg/liter) which is similar to the value given in Table 2. In the new steady state the culture fluid contained 75% of the original radioactivity. Since approximately 25% of the acetate was found in the cells, it was concluded that virtually no acetate had been converted to carbon dioxide. The contribution of ^{14}C-acetate to the total cell carbon was 8—10% assuming that the protein content of the cells was 55% of the dry weight (calculated from Table 2) and that the carbon content was between 40% and 50% of the dry weight. If one assumes that the incorporated acetate has been converted to cell material of the same carbon content an increase in dry weight between 8—10% should be

expected. This calculated value compares favorably with the 12% increase which was actually found.

T. pelophila, the marine *T. thioparus* and *T. neapolitanus* lack an operative citric acid cycle and glyoxylic acid cycle (Kelly, 1967; Smith *et al.*, 1967; Kuenen, 1972). As a consequence, the acetate supplied in the growth medium can not enter into macromolecules *via* succinate or malate. Therefore, addition of such a compound to the minerals-thio-

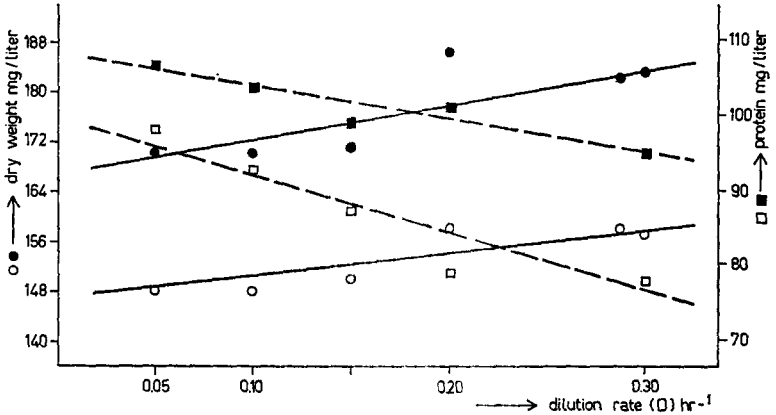

Fig. 1. Effect of 1 mM acetate on dry weight and protein of thiosulfate-limited chemostat cultures of *Thiomicrospira pelophila* grown at different dilution rates (D). ○ Dry weight in minerals medium + 0.8% thiosulfate; □ protein in minerals medium + 0.8% thiosulfate; ● dry weight in minerals medium + 0.8% thiosulfate + 1 mM acetate; ■ protein in minerals medium + 0.8% thiosulfate + 1 mM acetate

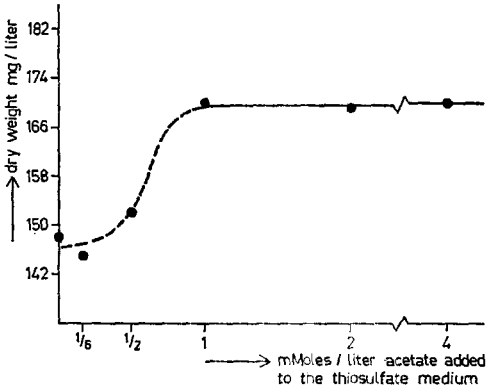

Fig. 2. Effect of increasing concentrations of acetate in the thiosulfate medium on dry weight of thiosulfate-limited chemostat cultures of *Thiomicrospira pelophila* grown in a minerals medium + 0.8% thiosulfate at a dilution rate (D) of 0.1 hr^{-1}

sulfate-acetate medium was expected to cause an additional increase in cellular dry weight and protein. As can be seen from Tables 3 and 4, acetate and succinate had a concerted effect which approximately equaled the sum of the increases caused by the single compounds. Malate showed no effect at all in any of these cultures. Succinate did not cause any increase of cell yield in *T. neapolitanus*. This is consistent with the very low uptake of this compound in batch cultures (Kelly, 1967).

Although a considerable increase in dry weight and protein could be obtained, the contribution of organic compounds to cell material apparently was limited. It was attempted to further increase the cell yield

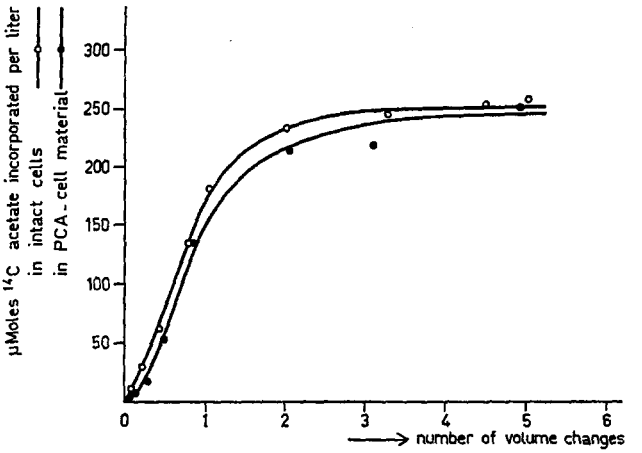

Fig. 3. Incorporation of ^{14}C-acetate in intact cells and PCA precipitable cell material as a function of the number of volume changes; thiosulfate-limited chemostat culture of *Thiobacillus neapolitanus* grown in a minerals medium + 0.9% thiosulfate at a dilution rate (D) of 0.1 hr^{-1}

Table 3. Effect of acetate and succinate on dry weight and protein of thiosulfate-limited chemostat cultures of *Thiomicrospira pelophila* grown in a minerals medium + 0.8% thiosulfate at a dilution rate of 0.1 hr^{-1}

Organic substrate added	Dry weight		Protein	
	mg/l	% increase	mg/l	% increase
none[a]	148		93	
1 mM acetate[a]	170	15	104	12
4 mM acetate[b]	169	15	—	
1 mM succinate	167	13	104	12
1 mM acetate + 1 mM succinate	183	24	118	27
4 mM acetate + 4 mM succinate	183	24	117	26

[a] Data taken from Fig. 1. [b] Data taken from Fig. 2.

Table 4. Effect of acetate and succinate on dry weight and protein of thiosulfate-limited chemostat cultures of *Thiobacillus thioparus* grown in a minerals medium + 0.8% thiosulfate at a dilution rate of 0.1 hr^{-1}

Organic substrate added	Dry weight		Protein	
	mg/l	% increase	mg/l	% increase
none[a]	152		83	
1 mM acetate[a]	172	14	97	17
1 mM acetate + 1 mM succinate	184	21	115	38

[a] Data taken from Table 1.

Table 5. Effect of organic compounds on dry weight and protein of thiosulfate-limited chemostat cultures of *Thiomicrospira pelophila* grown in a minerals medium + 0.8% thiosulfate at a dilution rate of 0.1 hr^{-1}

Organic substrate added	Dry weight		Protein	
	mg/l	% increase	mg/l	% increase
1 mM acetate + 1 mM succinate[a]	183	24	118	27
1 mM acetate + 1 mM glycerol	181	22	107	15
1 mM acetate + 20 mM glucose	179	21	101	9
1 mM aspartate + 1 mM glutamate	167	13	—	
0.1% casaminoacids	171	16	107	15

[a] Data taken from Table 3.

and protein of *T. pelophila* by adding mixtures of precursors or early products of the Calvin cycle to the minerals thiosulfate medium. However, no effect of saccharose (1—20 mM), fructose (1—20 mM), glucose (1—20 mM), ribose (2—20 mM), glycerol (1 mM), glycerate (1 mM), pyruvate (1 mM), or lactate (1 mM) could be observed when added as either the sole organic compound or in combination with acetate and succinate. A mixture of amino acids such as casamino acids (supplemented with tryptophane) gave an increase of 15% in dry weight (Table 5). Aspartate (1 mM) plus glutamate (1 mM) showed a similar effect (Table 5). The highest increase in dry weight obtained was a 35% increase at a dilution rate of 0.1 hr^{-1} in a preliminary experiment using a mixture of 0.1% caseine hydrolysate plus (1 mM) of acetate, succinate, aspartate, glutamate and glycine.

In recent reports on enzyme levels of obligate chemolithotrophs or related organisms, much emphasis has been placed upon the observed lack of response of enzyme levels to the presence of exogenous organic materials (Pearce *et al.*, 1969; Peeters *et al.*, 1970; Taylor and Hoare, 1971). These observations were made on microorganisms grown in batch culture

Table 6. Activity of ribulosediphosphate carboxylase in cell free extracts of thiosulfate-limited cultures of *Thiomicrospira pelophila* grown in the presence or absence of acetate and succinate

Dilution rate hr^{-1}	Medium	Spec. RudPcase activity nmoles/min/mg protein
0.025	T	14
0.05	T	13
0.10	T	13
0.10	T+A+S	13
0.20	T	13

T = minerals thiosulfate medium; T+A+S = minerals thiosulfate medium + 4 mM acetate + 4 mM succinate.

Table 7. Activity of ribulosediphosphate carboxylase of thiosulfate-limited chemostat cultures of *Thiobacillus neapolitanus* grown in the presence or absence of acetate

Dilution rate hr^{-1}	Medium	Spec. RudPcase activity nmoles/min/mg protein
0.10	T	50
0.10	T+A	46
0.27	T	36

T = minerals thiosulfate medium; T+A = minerals thiosulfate medium + 2 mM acetate.

where increases in cell yields and protein never have been reported. It was conceivable that organic compounds would influence enzyme levels under conditions where quantitative differences in dry weight and protein readily could be measured. As stated in the introduction, in mixotrophic thiobacilli the autotrophic levels of ribulosediphosphate carboxylase (RudPcase) activity are strongly repressed in the presence of organic compounds. Therefore, this enzyme was chosen for further study. The levels of RudPcase were measured at a dilution rate (D) of 0.1 hr^{-1} in the presence or absence of either acetate plus succinate or acetate in thiosulfate-limited cultures of *T. pelophila* and *T. neapolitanus*, respectively. It appears from Tables 6 and 7 that the exogenously supplied organic compounds did not significantly influence the levels of RudPcase activity. When further investigating this phenomenon, it appeared that in *T. pelophila* the activity of this enzyme was also almost independent of the growth rate over a wide range (Table 6), whereas in *T. neapolitanus* a slight (25%) decrease in activity of the enzyme could be measured at increased growth rate (Table 7). The values of RudPcase activity presented in each table are comparable since mixed cell free extracts showed activities which were the sum of the separate activities.

In the energy-limited (thiosulfate) chemostat cultures, the contribution of organic compounds to the total cell carbon might have been limited by the available energy. For example, the available energy might have limited the transport or metabolism of exogenous organic compounds. At the same time the unchanged level of RudPcase indicated that the Calvin cycle might not be repressed by the exogenous organic compounds, resulting in a competition for ATP between the CO_2 fixing system and the metabolic pathways involved in the conversion of the exogenous organic compounds. If this assumption was correct, one might expect a higher contribution of organic compounds to the total dry weight and protein in CO_2-limited cultures. Under such conditions the energy will be present in excess and in addition the CO_2 fixing machinery of the cells will be limited by the available CO_2.

To investigate this hypothesis, *T. pelophila* and *T. neapolitanus* were grown under CO_2-limitation in a chemostat at a dilution rate of 0.1 hr^{-1} (see "Materials and Methods"). Although thiosulfate was present in excess under these conditions, there was no detectable formation of sulfur. In thiosulfate-containing batch cultures of *T. pelophila*, large amounts of sulfur are always precipitated (Kuenen and Veldkamp, 1972). Cells of *T. pelophila* and *T. neapolitanus* grown under CO_2-limitation formed long chains of cells and showed many involuted forms. Some lysed cells were also observed. However, the absorption at 260 and 280 nm of the culture fluid was invariably low, indicating that the number of dead cells was negligible. The results of the experiments are summarized in Table 8. The dry weight under CO_2-limitation was about half that obtained under thiosulfate limitation because of experimental design (see "Materials and Methods").

The CO_2-limited cultures of *T. pelophila* showed an increase of 27% in dry weight and a 50% increase in cellular protein when acetate and succinate were included in the minerals thiosulfate medium. Under thiosulfate limitation these figures were 24% and 26%, respectively. Therefore, the increase in protein observed upon addition of acetate and succinate under CO_2 limitation was twice that observed under thiosulfate limitation. However, the increase in dry weight was similar under both limitations. Thus, acetate and succinate changed the mean cell composition of *T. pelophila*.

The increase resulting from the addition of acetate to the CO_2-limited cultures of *T. neapolitanus* was virtually the same as found under thiosulfate limitation. The conclusion from these experiments is that in *T. neapolitanus* the uptake of acetate apparently is not limited by the available energy or by the fact that RudPcase levels are not repressed. An explanation for the results with *T. pelophila* cultures is less evident. The relative high increase in cell protein may indicate that the available

Table 8. Dry weight and protein of thiosulfate- or CO_2-limited cultures of *Thiomicrospira pelophila* and *Thiobacillus neapolitanus*, in the presence or absence of organic compounds, grown at a dilution rate of 0.1 hr^{-1}

Organism	Growth limiting substrate	Dry weight mg/l				Protein mg/l			
		T	T+A	T+A+S	% increase	T	T+A	T+A+S	% increase
Thiomicrospira pelophila [a]	thiosulfate	148		183	24	93		117	26
Thiomicrospira pelophila	CO_2	68		86	27	30		45	50
Thiobacillus neapolitanus [b]	thiosulfate	141	168		19	80	89		11
Thiobacillus neapolitanus	CO_2	60	70		17	34	39		15

[a] Data taken from Table 3. [b] Data taken from Table 2.

T = minerals thiosulfate medium; T+A = minerals thiosulfate medium + 2 mM acetate; T+A+S = minerals thiosulfate medium + 4 mM acetate + 4 mM succinate.

Table 9. Activity of ribulosediphosphate carboxylase in thiosulfate or CO_2-limited chemostat cultures of *Thiomicrospira pelophila* grown in the presence or absence of acetate and succinate at a dilution rate of 0.1 hr^{-1}

Medium	Growth limiting substrate	Spec. RudPcase activity nmoles/min/mg protein
T[a]	thiosulfate	13
T	CO_2	13
T+A+S	CO_2	16

[a] Data taken from Table 6.

T = minerals thiosulfate medium; T+A+S = minerals thiosulfate medium + 4 mM acetate + 4 mM succinate.

Table 10. Activity of ribulosediphosphate carboxylase in thiosulfate- or CO_2- limited chemostat cultures of *Thiobacillus neapolitanus* grown in the presence or absence of acetate at a dilution rate of 0.1 hr^{-1}

Medium	Growth limiting substrate	Spec. RudPcase activity nmoles/min/mg protein
T[a]	thiosulfate	50
T	CO_2	141
T+A	CO_2	153

[a] Data taken from Table 7.

T = minerals thiosulfate medium; T+A = minerals thiosulfate medium + 2 mM acetate.

energy can indeed be a limiting factor in the uptake and incorporation of organic compounds in thiosulfate-limited cultures, as discussed above.

Measurements of RudPcase activities under conditions of CO_2-limitation are summarized in Table 9 and Table 10. Even growth limitation by CO_2 did not change the RudPcase activity of *T. pelophila*. In T. *neapolitanus*, however, the CO_2-limitation caused a three-fold increase in the activity of this enzyme. The presence of organic compounds in the medium did not grossly influence the RudPcase activity in either *T. pelophila* or *T. neapolitanus*.

Discussion

The main purpose of this investigation was to show that organic compounds are not only taken up by cultures of obligately chemolithotrophic bacteria like *Thiomicrospira pelophila, Thiobacillus thioparus*

and *Thiobacillus neapolitanus* but also cause an increase in the total cell yield as compared to cultures grown in a purely mineral medium. The experiments clearly show that a variety of organic compounds can cause such an increase. This is apparently due to an energy-sparing effect in the thiosulfate-limited chemostat cultures, and to a carbon-sparing effect in CO_2-limited cultures.

It seems likely that in the past these effects have escaped detection because the increases are small and therefore need a reproducible cultivation method to be measured.

By using ^{14}C-acetate it was shown for *T. neapolitanus* (Fig. 3) that the increase in dry weight caused by this compound was directly related to the relative contribution of acetate to the total cell carbon. It seems reasonable to assume that the same applies to the increases caused by organic compounds in *T. pelophila* and the marine *T. thioparus*. If this is true, the total amount of CO_2 fixed per volume of culture in autotrophic medium is approximately equal to the total amount of CO_2 fixed in the presence of organic compounds. This view is consistent with the fact that the observed increases were very limited. A strong reduction in the total amount of CO_2 fixed would result in a much larger energy sparing effect, and thus in a larger increase in dry weight, since the CO_2 fixing process has an energy requirement which is almost two times higher than that needed for all other cell functions (Hempfling and Vishniac, 1967).

The addition of a number of organic compounds with 3, 5 or 6 carbon atoms to a thiosulfate-limited culture of *T. pelophila* did not result in any measurable increase in cell yield. This is either due to the fact that these compounds cannot be taken up or converted to cell material to any extent. A very limited uptake of some of these compounds in batch culture is mentioned in several literature reports. ^{14}C-fructose was taken up to 2.8% of the total cell carbon in *Thiobacillus thiooxidans*. In *Thiobacillus denitrificans* glucose and glycerol were taken up to 0.7% only (Smith *et al.*, 1967; Taylor *et al.*, 1971). Increases in cellular dry weight of this magnitude would not have been detectable in our measurements since the reproducibility of these was approximately $\pm$ 3% (see "Materials and Methods"). The absence of any effect of glucose in a concentration as high as 20 mM is difficult to reconcile with the recent finding that several strains of "obligately chemolithotrophic" thiobacilli and other chemolithotrophs can be grown on glucose in a dialysis culture (Pan and Umbreit, 1972). It is likely that the low concentration of thiosulfate present in the chemostat would eliminate the possible occurrence of diauxic effects, such as have been found in batch culture of the mixotrophic *Thiobacillus novellus* (LéJohn *et al.*, 1967). In *Escherichia coli* cultures grown in a chemostat at "moderate" dilution rates, diauxie of glucose and lactose does not occur (Silver and Mateles, 1969).

The protein content of the cells (calculated as a percentage of the dry weight) grown under different conditions appeared to vary considerably, depending on the growth rate, on the type of growth limitation and even on the presence or absence of organic compounds. For example, in CO_2-limited cultures of *T. pelophila* the protein content was 44% in autotrophic medium. The percentage changed to 52% in the presence of acetate and succinate (Table 8). Under thiosulfate limitation, at a dilution rate of 0.1 hr^{-1}, the percentage was 62—64% (Fig. 1), decreasing to 50% at a dilution rate of 0.3 hr^{-1}. In *T. thioparus* the protein content of the cells changed from 56% to 62% when acetate plus succinate was added to the minerals thiosulfate medium (Table 4). At present we have no explanation for these phenomena. Analysis of the cell composition of the bacteria grown under different conditions will be carried out to further investigate this problem.

Our experiments have confirmed earlier reports (Taylor and Hoare, 1971) that the RudPcase activity in cell free extracts of obligate chemolithotrophs is independent of the presence of exogenous organic compounds. No change in the level of this enzyme could be demonstrated either in thiosulfate- or in CO_2-limited cultures.

It has been suggested that one of the explanations for obligate "autotrophy" might be the essential inability of the obligate autotrophs to control the synthesis of enzymes on the transcription level (Pearce *et al.*, 1969). In *T. neapolitanus* nutritional conditions, i.e. CO_2-concentration, can influence the level of RudPcase, since the CO_2-limitation caused a 3-fold increase in the activity of this enzyme, clearly demonstrating that this organism is able to regulate one of the key enzymes of its metabolism.

As already pointed out in an earlier report (Kuenen and Veldkamp, 1972), the uptake of organic compounds by organisms like *T. pelophila*, *T. thioparus* and *T. neapolitanus* is probably of ecological significance since in the natural habitat of these bacteria organic compounds generally are available.

The metabolic machinery of the obligately chemolithotrophic thiobacilli and *T. pelophila* appears to be much less flexible than that of the mixotrophic thiobacilli (Rittenberg, 1969; Kelly, 1971). However, the maximum specific growth rate of the obligate chemolithotrophs in inorganic growth media is much higher than that of the mixotrophs (>0.3 hr^{-1} versus < 0.05 hr^{-1}). Apparantly the price to be paid for this advantage is the loss of the heterotrophic potential. The ubiquitous presence of large numbers of obligately chemolithotrophic thiobacilli in natural environments indicates that this loss is worthwhile.

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