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COLOURLESS SULFUR BACTERIA AND THEIR ROLE IN THE SULFUR CYCLE*

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SUMMARY

The bacteria belonging to the families of the Thiobacteriaceae, Beggiatoaceae and Achromatiaceae are commonly called the colourless sulfur bacteria. While their ability to oxidize reduced inorganic sulfur compounds has clearly been established, it is still not known whether all these organisms can derive metabolically useful energy from these oxidations.

During the last decades research has mainly focussed on the genus Thiobacillus. Bacteria belonging to this genus can oxidize a variety of reduced inorganic sulfur compounds and detailed information is available on the biochemistry and physiology of these energy-yielding reactions. The thiobacilli, most of which can synthesize all cell material from CO_2, possess a well-regulated metabolic machinery with high biosynthetic capacities, which is essentially similar to that of other procaryotic organisms.

Although the qualitative role of colourless sulfur bacteria in the sulfur cycle is well documented, quantitative data are virtually absent. Activities of colourless sulfur bacteria in nature must be related to direct and indirect parameters, such as: the rate of oxidation of (S^{35}) sulfur compounds, the rate of $C^{14}O_2$-fixation, the rate of acid production and numbers and growth rates of the bacteria. However, chemical reactions and similar activities of heterotrophic organisms mask the activities of the colourless sulfur bacteria to various extents, depending on the condition of the natural environment. This interference is minimal in regions where high temperature and/or low pH allow the development of a dominant population of colourless sulfur bacteria, such as hot acid sulfur springs, sulfide ores, sulfur deposits and some acid soils.

The oxidation of inorganic sulfur compounds is carried out by a spectrum of sulfur-oxidizing organisms which includes: 1) obligately chemolithotrophic organisms 2) mixotrophs 3) chemolithotrophic heterotrophs 4) heterotrophs which do not gain energy from the oxidation of sulfur compounds but benefit in other ways from this reaction, and 5) heterotrophs which do not benefit from the oxidation of sulfur compounds. The spectrum is completed by a hypothetical group of heterotrophic organisms, which may have a symbiotic relationship with thiobacilli and related bacteria. Such heterotrophs may stimulate the growth of colourless sulfur bacteria and thereby contribute to the oxidation of sulfur compounds.

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Future research should focus in the first place on obtaining and studying pure cultures of many of the colourless sulfur bacteria. In the second place, studies on the physiological and ecological aspects of mixed cultures of colourless sulfur bacteria and heterotrophs may add to a better understanding of the role of the colourless sulfur bacteria in the sulfur cycle.

INTRODUCTION

The name colourless sulfur bacteria was originally used by Winogradski [99] to designate procaryotic organisms which could grow autotrophically at the expense of oxidizable inorganic sulfur compounds, which served as the energy source. At the present state of our knowledge this group of bacteria should be described, rather than defined, as procaryotic organisms which can or are believed to be able to derive metabolically useful energy from the oxidation of reduced inorganic sulfur compounds.

Colourless sulfur bacteria play an important role in the sulfur cycle (Fig. 1) [3 49 69 75 102]. Sulfide, which originates from anaerobic

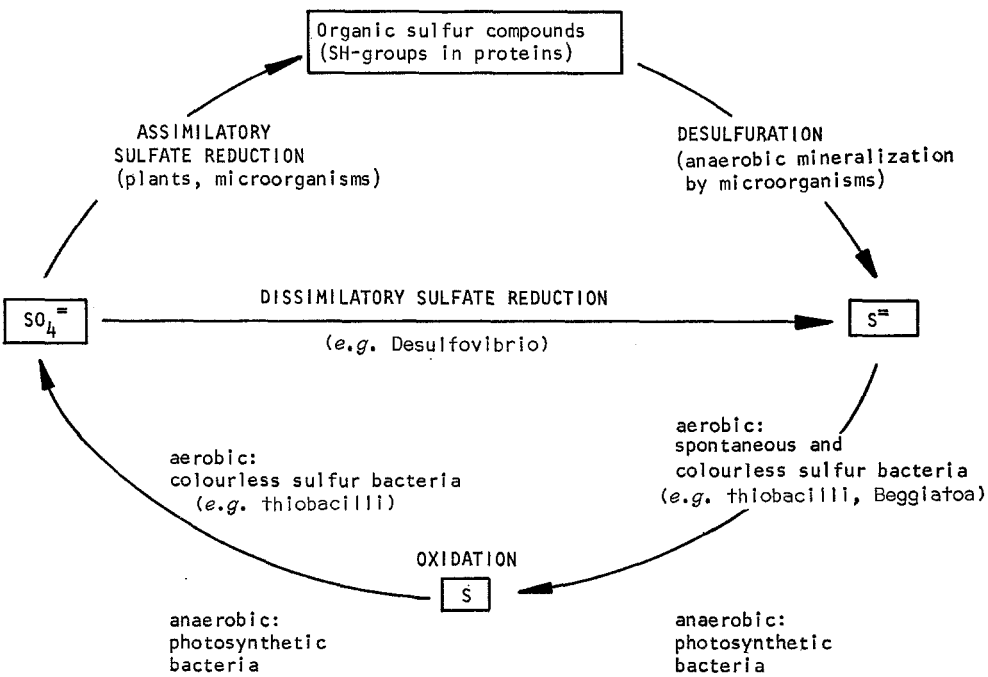

Fig. 1. The sulfur cycle.

sulfate reduction and from decaying organic matter is recycled to sulfate under both aerobic and anaerobic conditions. The anaerobic oxidation of sulfide and other reduced sulfur compounds is effected by the photosynthetic bacteria: this subject has been dealt with in another paper in this symposium. Under aerobic conditions sulfide is oxidized both chemically and biologically to sulfate. In nature a variety of reduced inorganic sulfur compounds (*e.g.* sulfur) occur as intermediates between sulfide and sulfate. As these compounds are oxidized only very slowly by a direct chemical reaction with oxygen, it is obvious that biological oxidation must play an important role in the recycling of reduced sulfur compounds under aerobic conditions. During evolution procaryotic organisms have evolved, which are specialized in such oxidations and which are known as the colourless sulfur bacteria.

GENERAL DESCRIPTION AND SOME PHYSIOLOGICAL ASPECTS OF THE COLOURLESS SULFUR BACTERIA

The colourless sulfur bacteria are divided into three families, the Thiobacteriaceae, the Beggiatoaceae, and the Achromatiaceae. The genera which belong to the different families are shown in Table 1. The taxonomic value of this division is small from a phylogenetic point of view. For example it has been argued that the Beggiatoaceae should be considered as colourless blue green algae [12 68]. Also, the genera of the family of the Thiobacteriaceae seem to have no phylogenetic but only physiological relations [33]; the same holds for organisms grouped in the genus Thiobacillus [47].

Many of the colourless sulfur bacteria can directly be recognized under the microscope and therefore had been described and named

TABLE 1

The colourless sulfur bacteria

Thiobacteriaceae	Beggiatoaceae	Achromatiaceae
Thiobacterium	Beggiatoa	Achromatium
Macromonas	Thiospirillopsis	
Thiovulum	Thioploca	
Thiospira	Thiothrix	
Thiobacillus	Thiodendron	
Thiomicrospira		
Sulfolobus		

before 1900 [3 99]. As these observations were made on crude cultures and attempts to cultivate these organisms have very often failed, very little is yet known on the physiology of many of these bacteria. In some cases, the failure to cultivate these organisms may be due to the very narrow zones of sulfide and oxygen in which these organisms thrive [68]. Such conditions have proved to be extremely difficult to reproduce in the laboratory. However, the recent development and successful application of simple devices to create oxygen-sulfide gradients for the growth of colourless sulfur bacteria seems promising [15 68].

The morphology and physiology of the somewhat better known families and species among the colourless sulfur bacteria will be discussed very briefly in the following paragraphs. For pictures and photographs of the various organisms the reader is referred to the literature [3 11 20 47 71 73 99].

Beggiatoa was first studied in detail by Winogradski [99]. Morphologically it is a (colourless) blue green alga related to Oscillatoria. The flexible trichomes move by gliding. Typical for Beggiatoa is the presence of sulfur droplets in the cells. The sulfur is formed during the oxidation of sulfide and can be oxidized further to sulfuric acid [14 99]. From his work with Beggiatoa Winogradski developed the concept of the 'inorgoxidant' (in other words the obligate autotroph) but except an early report of Keil [39] and a relatively recent report [44], investigations have failed to demonstrate autotrophic species of Beggiatoa. Most species known thus far are heterotrophic organisms [23 71], the growth of which can be stimulated by the presence of sulfide. Evidence has been obtained [14 85] that Beggiatoa lacks catalase and therefore may form the toxic hydrogen peroxide intracellularly during growth on organic substrates. The hydrogen peroxide could be removed by a non-enzymic reaction with sulfide, which would explain how Beggiatoa would benefit from the oxidation of sulfide. It seems unlikely that the intracellular sulfur would also react spontaneously with hydrogen peroxide. Therefore, it seems reasonable to assume that Beggiatoa possesses an enzyme system that oxidizes sulfur. It remains to be investigated whether Beggiatoa possesses the ability to oxidize the intracellular sulfur only to dispose of this compound or perhaps also to obtain energy from this reaction.

In appearance, Thiothrix is very similar to Beggiatoa [99]. One

end of the non-motile trichome is attached to surfaces or solid particles. The trichomes usually are arranged in rosettes. Thiothrix is believed to have a growth cycle similar to that of the genus Leucothrix [31]: swarming cells can develop on the tips of the trichomes, move away by gliding and attach again to solid particles to form a new rosette of trichomes. Autotrophic growth of Thiothrix was reported as early as 1912 [39], but has never been confirmed. The role of sulfide in the metabolism of this organism may be similar to that suggested for Beggiatoa, *i.e.* to remove the toxic hydrogen peroxide during growth on organic substrates [85].

Achromatium is one of the least-known colourless sulfur bacteria. Virtually nothing is known on the physiology of this bacterium or the role of sulfide in its metabolism. Typical for Achromatium is its enormous size (40 μ in diameter) and the usual presence of large calcium carbonate inclusions in the cells [6]. Bavendamm [3] observed that, at relatively high oxygen concentrations, this organism contained a few intracellular sulfur particles and many carbonate inclusions, while the reverse was true at low oxygen concentrations. Perhaps the calcium carbonate particles serve as an internal device for pH control [6].

Thiovulum was discovered in 1913 [30]. It forms mats of cells in a very narrow zone of low oxygen- and sulfide concentrations. Motile cells are characterized by a jerky, rolling movement. Although cells have been cultivated in an artificial sulfide gradient system [68], they have never been studied in pure culture. Experiments with partially purified cultures [100] indicate that in Thiovulum sulfide can serve as the energy source for CO_2-fixation.

Sulfolobus acidocaldarius was recently discovered by Brock *et al.*[11]. It is a very small (diameter 1 μ), irregularly shaped, thermophilic organism, which occurs in acid hot springs all over the world. Its pH-optimum is 2–3 and growth occurs from 55–85°C. The organism can grow not only autotrophically on sulfur as the energy source, but also mixotrophically (*i.e.* on sulfur as the energy source and on organic compounds which may act as both energy and carbon sources) and heterotrophically on a variety of organic compounds. Sulfolobus has a special cell envelope, which is apparently very well suited to stand the hot acid of its natural environment.

Thiomicrospira pelophila was isolated in our laboratory [47]. It is a very thin spirillum-shaped organism with physiological charac-

teristics which are very similar to those of *Thiobacillus thioparus* (see below). However, *Tms. pelophila* has a much higher tolerance to sulfide than *T. thioparus*.

Thiobacillus is the best known genus within the group of colourless sulfur bacteria. Most thiobacilli described thus far are pseudomonads in appearance; but nonmotile rods and coccoid organisms are also included in this genus [36]. The listing of organisms as thiobacilli is based rather on physiological than on morphological criteria. Thiobacilli have in common their ability to derive energy from the oxidation of reduced inorganic sulfur compounds. For specific and detailed information the reader is referred to the literature, in particular to several excellent reviews [41 66 70 95].

With the exception of *T. perometabolis* [53] and probably *T. trautweinii* [79], the thiobacilli can grow autotrophically. They possess a reductive ribulosediphosphate cycle (Calvin cycle) for the fixation of CO_2 [41]. The CO_2 fixing enzyme, ribulosediphosphatecarboxylase, of at least one Thiobacillus seems to be located in polyhedral inclusions in the cell. These membrane-bound inclusions, which have also been observed in several other species, have been designated as carboxysomes [73].

Two major physiological groups exist within the genus Thiobacillus. Firstly the obligate chemolithotrophs, which are dependent on the oxidation of reduced inorganic sulfur compounds to obtain energy and use CO_2 as the major carbon source under all growth conditions [48 66]. Secondly the mixotrophs ('facultative autotrophs'), which cannot only grow autotrophically, but also mixotrophically and heterotrophically [66].

It hardly needs to be stated that the biosynthetic capacity of thiobacilli is large since CO_2 can serve as the sole carbon source for the synthesis of cell material [41]. Regulation of enzyme activity takes place by modulation of existing enzymes [21 27 41 81]. Induction and repression of enzymes has been shown to occur in mixotrophic thiobacilli. Classical examples are the repression of autotrophic enzymes under heterotrophic growth conditions and *vice versa* [51 54 55 62 83].

The biochemical explanation for the inability of the obligate chemolithotrophs to grow heterotrophically is still unknown. This inability has been related to the absence of an operative citric acid cycle and glyoxylic acid cycle [40 74]. Among several other

TABLE 2

Oxidations of inorganic sulfur compounds effected by thiobacilli and other colourless sulfur bacteria*

1	$H_2S + 2\,O_2$	$\rightarrow H_2SO_4$
2	$2\,H_2S + O_2$	$\rightarrow 2\,S^0 + 2\,H_2O$
3	$2\,S^0 + 3\,O_2 + 2\,H_2O$	$\rightarrow 2\,H_2SO_4$
4	$Na_2S_2O_3 + 2\,O_2 + H_2O$	$\rightarrow Na_2SO_4 + H_2SO_4$
5	$4\,Na_2S_2O_3 + O_2 + 2\,H_2O$	$\rightarrow 2\,Na_2S_4O_6 + 4\,NaOH$
6	$2\,Na_2S_4O_6 + 7\,O_2 + 6\,H_2O$	$\rightarrow 2\,Na_2SO_4 + 6\,H_2SO_4$
7	$2\,KSCN + 4\,O_2 + 4\,H_2O$	$\rightarrow (NH_4)_2SO_4 + K_2SO_4 + 2\,CO_2$
8	$5\,H_2S + 8\,KNO_3$	$\rightarrow 4\,K_2SO_4 + H_2SO_4 + 4\,N_2 + 4\,H_2O$
9	$5\,S^0 + 6\,KNO_3 + 2\,H_2O$	$\rightarrow 3\,K_2SO_4 + 2\,H_2SO_4 + 3\,N_2$
10	$5\,Na_2S_2O_3 + 8\,NaNO_3 + H_2O$	$\rightarrow 9\,Na_2SO_4 + H_2SO_4 + 4\,N_2$

* Adapted from Starkey [80].

interesting explanations, which have been reviewed by Rittenberg [66 67] and Kelly [41] are: 1. a limited transport of organic molecules [41 48]; 2. the production of toxic products during heterotrophic metabolism which would result in growth inhibition [7]; 3. the inability of the metabolic machinery of obligate chemolithotrophs to switch from chemolithotrophic to heterotrophic metabolism [61]. Umbreit and his coworkers [8 59] have challenged all these hypotheses by reporting heterotrophic growth of obligate chemolithotrophs. But this has recently led to quite some controversy, since other investigators have not been able to reproduce these results [55]. It must be concluded that at the present state of our knowledge, a clear explanation of obligate chemolithotrophy, cannot be given.

The oxidations of reduced inorganic sulfur compounds carried out by thiobacilli and other colourless sulfur bacteria are given in Table 2.

When oxygen is not available or when the oxygen concentration is low, nitrate may serve as the terminal electron acceptor in several of these oxidation processes [32 101]. All reactions are known to be effected by thiobacilli. Sulfide is also oxidized by Beggiatoa, Thiothrix [99], Achromatium [3] and Thiovulum [100], and probably by most other colourless sulfur bacteria.

T. thioparus and *T. neapolitanus* catalyze reactions (3)–(7), and (3)–(6), respectively, at neutral pH [42 80]. In a similar pH range *T. denitrificans* can carry out such oxidations not only with oxygen but also with nitrate as the terminal electron acceptor (8)–(10).

T. thioparus can grow anaerobically in the presence of nitrate to some extent [32 101], but produces nitrite which inhibits its growth. *T. ferrooxidans* (which is equivalent to *Ferrobacillus ferrooxidans* [43]) and *T. thiooxidans*, which both thrive under acidic conditions down to pH 1–2, can oxidize not only sulfur (3), but also thiosulfate and tetrathionate [80 88]. In addition, *T. ferrooxidans* is able to oxidize ferrous ions, and also pyrite, according to the following reaction sequences [80]:

$$2FeS_2 + 2H_2O + 7O_2 \rightarrow 2FeSO_4 + 2H_2SO_4$$
$$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O$$
$$Fe_2(SO_4)_3 + FeS_2 \rightarrow 3FeSO_4 + 2S$$
$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$$

It has been shown that the first reaction does not occur only chemically but can also be brought about by *T. ferrooxidans* [88].

All the Thiobacillus species mentioned so far are considered to be obligate chemolithotrophs, though perhaps some strains of *T. ferrooxidans* are mixotrophs [72]. Several truly mixotrophic thiobacilli have been described [52 79 82], which have pH ranges somewhere between 3–9, and carry out oxidations of sulfur compounds which are essentially similar to those carried out by the obligate chemolithotrophic thiobacilli. *Thiobacillus A2*, which is capable of using nitrate as terminal electron acceptor, is the mixotrophic counterpart of *T. denitrificans* [82 84].

The biochemical pathway of the oxidation of sulfur compounds by thiobacilli has been the subject of several reviews [42 70 87]. A brief summary of the hypothetical pathways for the oxidation of thiosulfate, sulfur and sulfide is given in Fig. 2. A characteristic of the oxidation process of thiosulfate is the involvement of organic sulfhydryl groups (RS^-), which can bind with an intermediate oxidation product of thiosulfate to give a sulfenyl thiosulfate ($RSSSO_3^-$) which is then hydrolyzed to a polysulfide (RSS^-). The polysulfide is also formed by a direct reaction of the sulfhydryl-group with sulfur or by the reaction of the oxidized sulfhydryl-groups (RSSR) with sulfide. Polythionates, which are often found in cultures of thiobacilli [70], would be formed from the sulfenyl-thiosulfate as a side product rather than as intermediates. The common product of the oxidation of the polysulfides is sulfite,

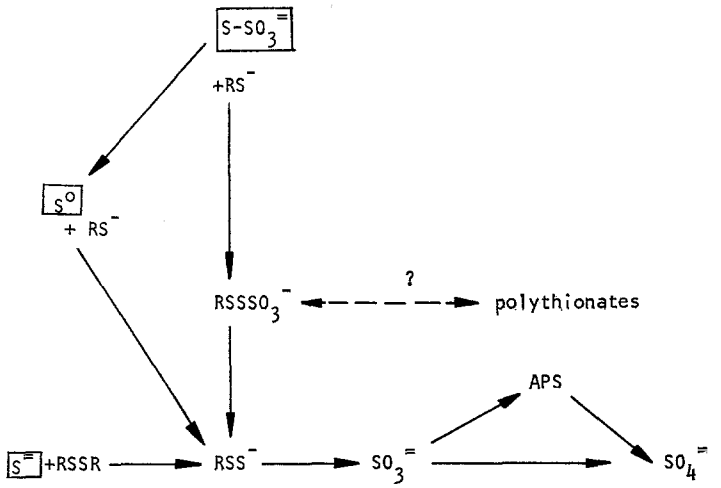

Fig. 2. Hypothetical pathways of the oxidation of sulfur compounds by thiobacilli.

which has been shown to be oxidized to sulfate either directly by a sulfide-cytochrome c oxidoreductase [16], or via the adenosine-5'-phosphosulfate-(APS) route [63].

Concluding this section it can be summarized that on one hand our knowledge on the physiology of thiobacilli has developed satisfactorily, although the biochemical basis of obligate chemolithotrophy and the pathways of the oxidation of sulfur compounds need further investigation. On the other hand, there is a great need for research to modernize our antique knowledge of many of the other colourless sulfur bacteria.

OCCURRENCE, DISTRIBUTION AND ROLE OF THE COLOURLESS SULFUR BACTERIA IN NATURE

In nature the colourless sulfur bacteria can be found almost everywhere. The character of the environment will determine which types will be found. In Fig. 3 the different habitats of the colourless sulfur bacteria have been divided into exceptional (outer circle) and moderate (inner circle) environments. Next to the given areas, the names are indicated of some of the colourless sulfur bacteria which occur under the given conditions. As exceptional environ-

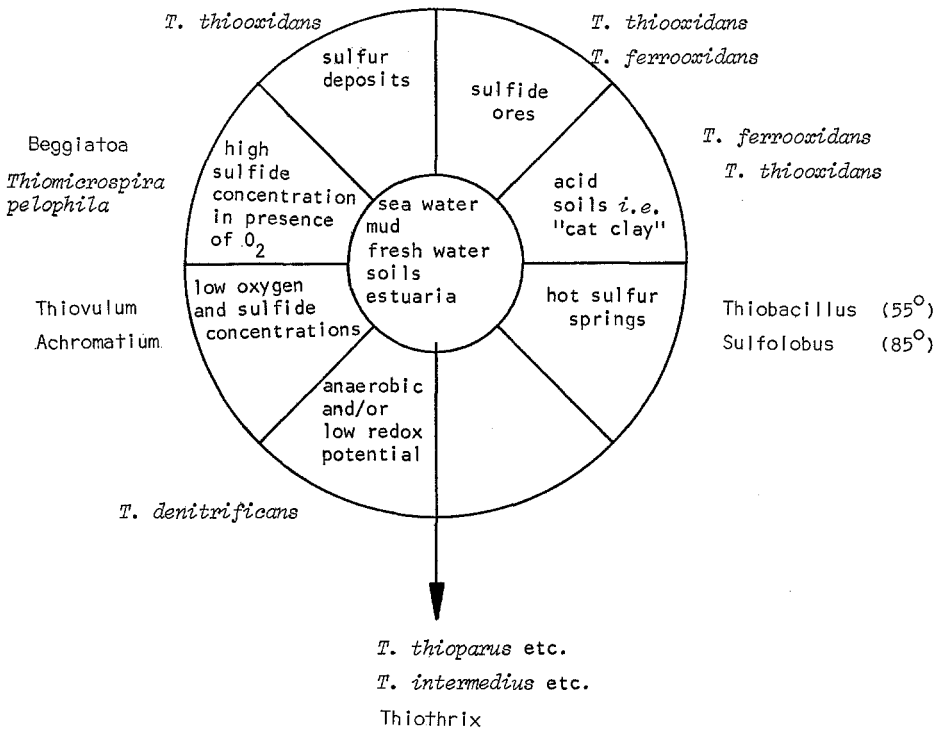

Fig. 3. Occurrence of colourless sulfur bacteria in 'exceptional' and 'moderate' environments.

ments, in the first place, are considered sulfur deposits where *T. thiooxidans* [75] can be found, and sulfide ores, which are an excellent substrate for *T. ferrooxidans*, and *T. thiooxidans* [75 80 88] which may develop as a secondary population. In many cases, if water and oxygen are available in sufficient amounts, the pH of these deposits can become very low as a result of massive development of these acidophilic thiobacilli [4 75 88]. *T. ferrooxidans* and *T. thiooxidans* also occur in high numbers in acid soil (*i.e.* cat clay in empoldered lands or areas which have been flooded temporarily [5 29 65]).

Another enrichment of colourless sulfur bacteria (Fig. 3) can be found in hot sulfur springs [10]. Particularly interesting are the acid hot springs (temperatures 70–85°C) where Sulfolobus occurs in impressive numbers and activities [56 57]. In the temperature range between 40–55°C (Fig. 3) most thermophilic thiobacilli or thiosulfate oxidizing rods are found [24 98 103]. Aerobic environments

with relatively high sulfide concentrations are also listed as exceptional. For example Beggiatoa [3 35 39 46] is sometimes encountered as a veil between anaerobic mud with a high sulfide content and well-oxygenated water, and Thiomicrospira is relatively abundant in pockets on the surface of tidal mud flats which show high concentrations of sulfide [45]. Very specialized groups of organisms like Thiovulum [68] can be found in narrow zones where low oxygen and sulfide concentrations coexist. The same is perhaps true for Achromatium [69], the development of which appears to be dependent also on the presence of high concentrations of calcium salts [3]. Finally, conditions suited for the growth of *T. denitrificans* are also considered exceptional *i.e.* anaerobic conditions or low oxygen concentrations in the presence of nitrate.

The inner circle represents the moderate environments which prevail in nature, like most fresh and sea water, muds, estuaries, etc. The arrow points to the kinds of colourless sulfur bacteria which usually develop under such moderate conditions in nature: most thiobacilli [95], and perhaps also Thiothrix [3 22 39]. Thiothrix usually occurs, attached to particles and surfaces, in streaming or turbulent waters which contain both oxygen and sulfide. Within these moderate environments many confined areas will exist which should be considered as exceptional, for instance because of a high local production of sulfide.

In the study of the role and function of colourless sulfur bacteria in the sulfur cycle two basic questions have to be answered. In the first place, what is the qualitative role of each of these organisms in the oxidation of sulfur compounds. And in the second place, to what extent do the colourless sulfur bacteria contribute to the total oxidation of sulfur compounds. In the previous section it has been indicated that the first question has been investigated in considerable detail for thiobacilli, but not for the other colourless sulfur bacteria. The second question faces and challenges the ecologist and has proved to be extremely difficult to answer [9]. It clearly is beyond the scope of this paper to discuss the general problems in quantitative estimation of activities of bacteria in nature and therefore only some specific difficulties encountered with colourless sulfur bacteria will be discussed with particular reference to the thiobacilli.

In only a very few cases it is possible to measure directly the

activity of colourless sulfur bacteria in nature. More commonly one has to rely on indirect measurements of parameters which may be related to this activity, *e.g.* numbers of bacteria; decrease of pH due to the production of sulfuric acid; the rate of sulfide, sulfur or thiosulfate oxidation or product formation (involving tracer methods with radioactive sulfur compounds), and the rate of fixation of $C^{14}O_2$ as a measure for chemolithotrophic activities. However, all these methods have very serious limitations and drawbacks as will be emphasized below.

Numbers of thiobacilli have been determined by several investigators [4 24 58 75 90]. Apart from the fact that numbers of bacteria can only serve as circumstantial evidence for their activities, the know-how of the enumeration of bacteria in general is very limited. For example, evidence has been obtained that completely different counting procedures may be necessary for one particular organism, depending on the growth limiting substrate in nature. It was found that thiosulfate-limited continuous cultures of *T. pelophila* yielded 90–95 per cent viable cells on thiosulfate agar. However, when iron was the growth limiting factor in a similar culture, no colonies developed on the same agar [45]. Also, it is known that the high concentration of salts in the media which are commonly used can be inhibitory (for example thiosulfate [46] and phosphate [2]). Furthermore the redox potential may be very critical [50 75] and sometimes vitamins must be added [47]. Even types of agar or membrane filters may be critical [89]. Therefore it can be concluded that low counts of thiobacilli and other sulfur oxidizers are not necessarily representative for their number in nature.

When the rate of oxidation of reduced sulfur compounds is studied as an index of activity of colourless sulfur bacteria, the separation of chemical and biological oxidation is a very serious problem. Inorganic reduced sulfur compounds are relatively reactive [70]. In particular sulfide and sulfite, but also thiosulfate, may react spontaneously with oxygen, and so the necessity of appropriate controls is obvious. However, such controls will have limited value since biological and chemical oxidation may very well compete. This may be illustrated by the studies of Cline and Richards [19] and Chen and Morris [17], who showed that sulfide and oxygen, when reacting at low concentrations (0.05–1 m*M*), yielded predominantly thiosulfate (20–40 per cent) and sulfate (40

per cent). Some sulfite was also found (10 per cent), but hardly any sulfur. Similar observations were made by Sorokin [76]. Chen and Morris [18] developed a model for this oxidation process as is shown in Fig. 4. They observed that the initial rate of oxidation of sulfide was low. One of the first reaction products, namely traces of sulfur, combined with sulfide to form polysulfides which catalyzed the oxidation of sulfide. In other words the reaction is autocatalytic. The spontaneous reaction is highly influenced by factors as pH and trace metals [17 19].

One important conclusion from this work is that thiosulfate is an important reaction product of the sulfide oxidation process and therefore may be an important substrate in nature. In fact, thiosulfate has been detected near the thermocline of the Black Sea [77 92]. The other, more speculative conclusion which may be drawn is that bacteria may influence, and perhaps even control, the rate of chemical oxidation of sulfide, for example by removing the polysulfides from the system. This would result in a decrease in the rate of spontaneous oxidation of sulfide. If this hypothesis is true, controls for abiogenic oxidation would be an artifact, and consequently estimates of biological activities would become too low. On the other hand, the reverse may be true if bacteria would rapidly oxidize sulfide to sulfur, and thereby catalyze the spontaneous reaction of oxygen with sulfide [18]. It is a common observation that mixtures of high concentrations of sulfide ($\gg$ 1mM) and oxygen yield sulfur as one of the main oxidation products [17 70]. This reaction is greatly influenced by pH and by the presence of traces of divalent metal ions and some other factors [18]. In nature,

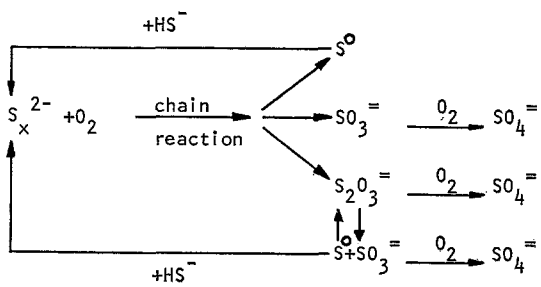

Fig. 4. Hypothetical reactions occurring during the chemical oxidation of sulfide by oxygen. (after Chen and Morris [18]).

gradients of oxygen and sulfide concentrations can be observed. The concentrations of these compounds will depend on the slope of the gradients, the rate of production of sulfide, the rate of diffusion of sulfide and oxygen, the presence of salts, *etc.* However, the simultaneous presence of both oxygen and sulfide at concentrations above 1 m*M* is exceptional in natural habitats [49]. In most fresh water environments far lower sulfide concentrations will be found and so spontaneous production of sulfur in 'moderate' environments certainly must be considered as an exception. This is in contrast with an 'exceptional' environment like acid hot sulfur springs where the sulfide is considered to be converted completely to sulfur by a chemical reaction [56].

Still another difficulty in the study of these organisms is that sulfur compounds are also oxidized by a large variety of heterotrophic bacteria which may mask the activity of the colourless sulfur bacteria [26 96]. Investigators have tried to escape this dilemma by measuring not only sulfur oxidation but also CO_2 fixation as a measure of growth [77 78]. However, it is known that a number of colourless sulfur bacteria may not use CO_2 as the main carbon source (*i.e.* mixotrophs), and it is equally well-known that heterotrophic bacteria incorporate CO_2 up to 3 to 6 per cent of their total cell carbon. Therefore, if in a given environment 10^8 heterotrophic bacteria and 10^6 'autotrophic' thiobacilli are growing at the same rate, the heterotrophic CO_2 fixation will be 3–6 times higher than that of the thiobacilli. It is obvious that a correction for the CO_2 fixation by heterotrophic bacteria (their growth rate being unknown) will be extremely hard to make.

In spite of these difficulties the activities of colourless sulfur bacteria have been determined in some cases. In general it can be said that such estimations have been possible only in exceptional environments when pH, or pH and temperature, and the character of the substrate were selective enough to allow development of dominant populations of chemolithotrophic bacteria, and to rule out major non-specific oxidations, *i.e.* chemical oxidations, or oxidations by heterotrophic bacteria. In the following paragraphs a few examples of such estimates will be discussed.

The role and importance of thiobacilli in the oxidation of sulfur compounds in numerous sulfur deposits has been discussed by Sokolova and Karavaiko [75]. They have clearly established:

1). no oxidation of sulfur ores without bacteria, 2). high numbers of *T. thiooxidans*, up to 1 million per gram ore, 3). very low pH often correlated with high numbers of *T. thiooxidans*, and 4). oxygen required for this process. Surprisingly, no report is made of attempts to isolate heterotrophs able to oxidize sulfur. However, their presence may be unlikely since it is known that most microorganisms cannot thrive and mostly do not survive at such low pH.

Although the real rate of oxidation of sulfur compounds by *T. thiooxidans* was never measured, and therefore conclusive evidence is still lacking, the data provided can be taken as cumulative evidence for the crucial role of *T. thiooxidans* in the oxidation of these sulfur ores. In the same sense the involvement of *T. ferrooxidans*, often in combination with *T. thiooxidans*, in the oxidation of sulfide ores (pyrite) at low pH has clearly been established [88]. In such extreme environments, CO_2 fixation in combination with sulfur or sulfide oxidation are appropriate processes to measure the activities of chemolithotrophic bacteria. As has been shown recently by Brock and coworkers [4 24 56] the use of refined techniques with labeled $C^{14}O_2$ and S^{35} allow extensive exploration of parameters which influence the activities of chemolithotrophs in their 'extreme' environments.

A very simple natural system was studied by Moser *et al.*[56], namely hot acid sulfur springs. Hydrogen sulfide escapes from the deeper layer of the sulfur pools and is oxidized spontaneously by oxygen to elemental sulfur. The sulfur is further oxidized to sulfuric acid by Sulfolobus. *Sulfolobus acidocaldarius* was found in almost pure culture and in high constant numbers in such springs. By using labeled S° the sulfur oxidized per day was shown to be 67 and 190 g per m^2 per day for the Moose Pool and Sulfur Caldron, respectively (Table 3). These quantities represent 1/15 and 1/23 of the total amounts of sulfur assayed in the respective pools. In related experiments [57] the growth rates of steady state populations of Sulfolobus *in situ* were estimated from the dilution rates of the pools. The calculated minimum doubling times, required to maintain the steady state population of Sulfolobus, averaged from 10 to 30 hours. Similar growth rates have been found in the laboratory. However, the calculated doubling times of Sulfolobus in the two larger pools (Moose Pool, Sulfur Caldron) was much lower, 28 to 35 days. Interestingly, the sulfide oxidation rate in these pools

TABLE 3

Properties of Moose Pool and Sulfur Caldron and rates of S^0 oxidation

Site	Temperature (°C)	pH	S^0 (mg/ml)	Sulfolobus (cells/ml)	Surface area (m^2)	Volume (m^3)	S^0 oxidation: g sq.m^{-1} day^{-1}	S^0 oxidation: kg day^{-1}
Moose Pool	72–80.5	1.59	1.02	2.5×10^7	300	300	67	20
Sulfur Caldron	65–67.5	1.53	1.09	7.4×10^7	100	400	190	19

* Adapted from Mosser *et al.*, 1973 [56].

was the highest of all the *S. acidocaldarius*-containing springs examined. As pointed out by the authors [57] the actual growth rate of Sulfolobus may be much higher if the death rate of Sulfolobus is high. High oxidation rates of sulfur do not, however, require high growth rates. The efficiency of growth on sulfur compounds may vary greatly dependent on the growth-limiting substrate. This has been shown to occur, for example, in thiosulfate- and carbon dioxide-limited continuous cultures of *T. neapolitanus* and *T. pelophila* [48].

Thus, the experiments on the hot acid sulfur springs clearly represent an unique example where growth, numbers and activities of a sulfur bacterium can be related. Such simple systems may have little relevance to the average natural situation but research in this field seems to be most valuable since such model systems allow us to test techniques and to investigate principles which may help to understand more complicated systems. An example of such a more complicate system is the chemocline of the Black Sea, which has been studied by Sorokin [77] and Tuttle and Jannasch [92]. (The term 'chemocline' is used to designate the intermediate layer between the aerobic and anaerobic phase of the Black Sea.)

Figure 5 shows a typical pattern of variation in relevant parameters as a function of the depth in the Black Sea [77]. Interesting is the area of the chemocline where sulfide and oxygen coexist, over a layer of 30–40 meter. This coexistence is the result of the steady state equilibrium which is due to O_2 diffusing downwards and H_2S diffusing upwards. In the chemocline the rate of $C^{14}O_2$ fixation was maximal. Correction for heterothropic CO_2 fixation was made by measuring CO_2 fixation at the higher levels where no bacterial sulfide oxidation could be demonstrated, but such a correction seems hardly appropriate since the activities of hetero-

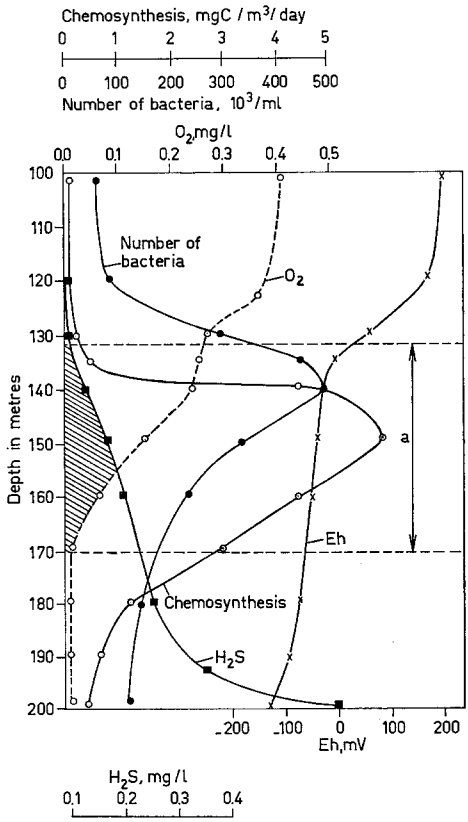

Fig. 5. The vertical distribution of O_2, H_2S, Eh, chemosynthesis and the total number of bacteria in the intermediate layer of the Black Sea at station 1308; a, the layer of coexistence of H_2S and O_2 (after Sorokin [77]).

trophic bacteria may be very different at different depths. Nevertheless it is very probable that the $C^{14}O_2$ is fixed by thiobacilli which thereby oxidize the sulfide in the chemocline. However, several investigators [77 91 92] failed to isolate appreciable numbers of true thiobacilli, or any other thiosulfate-oxidizing bacteria from this environment. To date, no unequivocal evidence exists for the presence of any true thiobacilli in the Black Sea.

Other investigators (*i.e.* Ivanov, see Ref. [77]) showed that the oxidation rate of sulfide, as measured by sulfide disappearance was almost similar in both samples and sterile controls which led

TABLE 4

The rate of H_2S^{35}-oxidation above and in the chemocline of a region (Station 1315) of the Black Sea*

Depth (m)	E_H (mV)	Conditions of oxidations of sulfide**	$S_2O_3^=$ total S	$SO_4^=$ total S	$S^=$ oxidized to $SO_4^=$ by bacteria per day µmol/l
120	+210	chem	35	34	
		chem + biol	31	33	0.19
150	− 15	chem	8	8	
		chem + biol	3	17.5	0.71
180	− 90	chem	0.8	0.6	
		chem + biol	0.7	1.6	0.21

* Adapted from Sorokin [77].

** Samples were incubated with ('chem') and without ('chem + biol') chloroform.

to the conclusion that bacteria were not important in the oxidation of sulfide in the chemocline.

This view has been challenged by Sorokin [77] who realized that thiosulfate measurements should be carried out since this compound could be an important intermediate in the chemical oxidation of sulfide [76]. He added S^{35}-sulfide to samples and controls and followed the oxidation of this compound to sulfur, thiosulfate and $SO_4^=$. At different depths the percentages of sulfur, thiosulfate and $SO_4^=$ relative to the total sulfur were measured after incubation for approximately 36 hours with and without chloroform. Some of the results are summarized in Table 4. In the controls, the percentages of sulfate and thiosulfate formed during oxidation of sulfide were almost equal at all depths. However, when bacteria participated (chem. + biol.), the percentages of thiosulfate and sulfate formed were very different in the region of coexistence of oxygen and sulfide (150–180 m), whereas these percentages were equal again at 120 m. This indicates that at the higher levels the bacteria did not participate significantly in the oxidation of sulfide.

It was concluded that in the region between 150 and 180 m the thiosulfate formed had been oxidized by bacteria [77]. This is not necessarily true since the bacteria may interfere with the formation of thiosulfate by interfering with the chemical oxidation process of sulfide as has been pointed out in a preceding paragraph.

Sorokin [77] also measured chemosynthetic activities by the $C^{14}O_2$ method. A close relation was observed between the rate of sulfide oxidation by the bacteria and the chemosynthesis. The efficiency with which the energy of sulfide oxidation was used for CO_2 fixation by the natural bacterial population seemed unexpectedly high, namely 20 to 40 per cent. However, in these calculations it was assumed that the bacteria were not interfering with the chemical oxidation, and thus used thiosulfate only. As pointed out above the bacteria may actually oxidize the sulfide too, which would strongly reduce the efficiency of CO_2 fixation. Another explanation, as suggested by Sorokin, is CO_2 fixation by methane oxidizing bacteria which were present in the chemocline; bacteria which assimilate methanol or methane via the serine pathway may fix up to 30 per cent of the total cell carbon from CO_2 [64]. Still another possibility is that under natural conditions the thiobacilli can utilize sulfide or thiosulfate much more efficiently than usually assumed resulting in a higher CO_2 fixation. There are some indications to support this explanation which will be discussed in the last section of this paper.

Our knowledge of the role of colourless sulfur bacteria in soil is, again, mostly limited to thiobacilli. For example the oxidation of pyrite in empoldered or flooded land sometimes results in very low pH values [5 29 65], which is due to a combined action of *T. ferrooxidans* and *T. thiooxidans* [80]. This action sometimes leads to the formation of cat clay (acid soils, in which clay particles are cemented together by basic ferric sulfate formed during the oxidation of pyrite) [80]. It is also known that addition of sulfur, sometimes used as a fertilizer, very often induces in the soil a rapid response of thiobacilli [13 25]. This response depends, of course, on many factors as temperature, moisture, organic compounds, salts, pH, *etc.*[1 42]. On the other hand, oxidation of sulfur in soil may not always be carried out by thiobacilli, but by a variety of heterothrophic micro-organisms, particularly at neutral pH[26]. In a recent study [96] high numbers of heterotrophic bacteria, which could oxidize sulfur or thiosulfate to sulfate, were isolated from sulfur-oxidizing soils. Since the number of thiobacilli in such soils was relatively small, it was concluded that the oxidation of sulfur in such soils is carried out mainly by heterotrophs.

The possibility of recognizing some of the colourless sulfur bacteria

directly under the microscope has been exploited by B. B. Jørgensen (personal communication). He studied the sulfur cycle of an estuarine lake in Denmark. This lake is severely polluted and occasionally large parts turn anaerobic because of overproduction of sulfide. In the upper 5 cm of the silt of this lake (10–15 m below the water surface) high numbers of at least two species of Beggiatoa were observed. The total mass of the Beggiatoa population, which was directly estimated from their dimensions and numbers, amounted to approximately 400 to 600 $\mu g/cm^3$. At present nothing is known about the activities of the Beggiatoa. However, it is interesting to realize that the total surface-area of the Beggiatoa/cm^3 would be equivalent to that of 2 to 5.10^7 thiobacilli. One might speculate that such a surface may possess a significant sulfide-oxidizing capacity and, therefore, may play a crucial role in the oxidation of sulfide in these muds.

In environments like the silt of the estuarine lake, mentioned above, Beggiatoa is very often found in thin veils on top of the mud where these organisms are able to maintain, and at the same time exploit a steep oxygen and sulfide gradient between the anaerobic mud and the aerobic waterlayer. It is sometimes not realized that Beggiatoa and colourless sulfur bacteria in general have in this way an extremely important ecological function in shielding the aerobic from the anaerobic phase.

Thus, biological oxidation of sulfide in a certain system may proceed at the same rate as chemical oxidation, but this does not necessarily lead to the conclusion that biological oxidation is unimportant in such a system [97].

Summarizing this section it can be stated that in certain exceptional environments, low pH (sometimes combined with high temperature) has made it possible to relate the oxidation of sulfur compounds exclusively to the activity of *T. thiooxidans*, *T. ferrooxidans* or Sulfolobus.

Our knowledge of the ecology of the thiobacilli and other colourless sulfur bacteria in moderate environments is very limited. Although the ecological techniques to determine activities of bacteria are still very primitive, use of techniques for measuring oxidation of S^{35}-sulfur compounds and for uptake of $C^{14}O_2$ seems promising [4 24 56 57]. Furthermore specific techniques to measure activities and numbers of colourless sulfur bacteria need to be developed.

THE SPECTRUM OF ORGANISMS INVOLVED IN THE OXIDATION OF SULFUR COMPOUNDS

Colourless sulfur bacteria are not all equally versatile. There appears to be a complete spectrum from obligate chemolithotrophs to heterotrophs as is shown in Fig. 6. In the top segment of the circle the obligate chemolithotrophs are shown. Organic materials seem not to play a significant role in their metabolism, although it has recently been shown [48] that organic compounds can stimulate the growth of such organisms to a limited extent. The mixotrophs have a very flexible metabolism which allows them to grow either heterophically or autotrophically. We know that the maximum specific growth rate of mixotrophic thiobacilli under autotrophic conditions is significantly lower than that of obligately chemolithotrophic thiobacilli (0.1 versus 0.4 h^{-1}) [48], while their maximum specific growth rate under heterotrophic conditions is much lower than that of common pseudomonads (0.5 versus 0.8 h^{-1}). Even under mixotrophic conditions the mixotroph may be out-grown

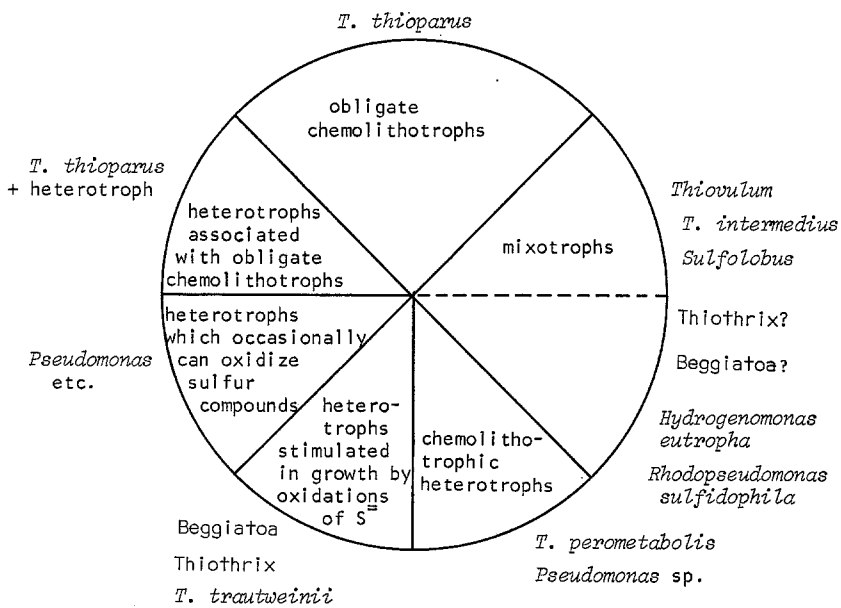

Fig. 6. The spectrum of organisms involved in the aerobic metabolism of inorganic reduced sulfur compounds.

by a mixture of obligate chemolithotrophs and heterotrophs and it seems therefore that *only* under conditions where autotrophic and heterotrophic conditions alternate, the mixotroph will have a definite advantage over the two other types of bacteria. This prediction is currently being investigated in our laboratory.

The next organisms in the spectrum are mixotrophic organisms which do not belong to the colourless sulfur bacteria but have been included as a curiosity. For example *Hydrogenomonas eutropha* [55] and *Rhodopseudomonas sulfidophila* [28] can oxidize thiosulfate (under aerobic conditions) and can perhaps grow autotrophically (in the dark) on some sulfur compounds.

The next in sequence is *T. perometabolis* which can derive energy from the oxidation of sulfur compounds but is unable to grow autotrophically [53]. This organism has been characterized as chemolithotrophic heterotroph [66]. In this group should also be included some marine pseudomonads, since addition of thiosulfate to the heterotrophic growth medium causes impressive increases in cell yield [93 94].

In the next segment organisms are listed, which cannot derive energy from the oxidation of reduced sulfur compounds, but benefit in other ways from this oxidation process. For example, as mentioned in a previous paragraph, some *Beggiatoa* species are catalase negative, and can dispose of the toxic intracellular hydrogen peroxide by reaction of this compound with hydrogen sulfide [14 85].

The following group of organisms in the spectrum are the heterotrophs which can simply oxidize sulfur compounds but seem not to benefit from this oxidation [86].

The circle is finally closed with a group of organisms which may live in association with obligate chemolithotrophs and may stimulate their growth and, in turn, live from excretion products of the obligate chemolithotrophs. In this way these heterotrophs would contribute indirectly to the oxidation of sulfur compounds.

This last group was created because of the following observations: 1). *T. thiooxidans* may excrete into the medium 20 per cent of the total $C^{14}O_2$ fixed [37] and, under certain conditions *T. neapolitanus* may excrete up to 50 per cent. (D. P. Kelly, personal communication). 2). Autotrophic cultures of Thiobacillus or Thiomicrospira in completely inorganic media can become contaminated with high numbers of heterotrophs (up to one heterotroph per 10 thiobacil-

li) [46 60]. As such cultures are highly viable, it is likely that the heterotrophs grow on excretion products rather than on lysed cells. 3). There are old observations on heterotrophs stimulating the growth rate of thiobacilli (G. W. Harmsen, personal communication) but these observations have never been proved. 4). More recently, it has been reported that *T. thioparus* can grow in anaerobic thiosulfate medium with nitrate, in association with heterotrophic bacteria. *T. thioparus* produces nitrite from the available nitrate, and the heterotrophic bacteria reduce the nitrite further to nitrogen gas [38 84]. It must be assumed that the heterotrophs are able to grow on some excretion products or lysed cells of *T. thioparus*.

Recently a mixed culture of *T. thioparus* and heterotrophs has been obtained from natural samples by selection in continuous culture [34], performed in an anaerobic thiosulfate limited chemostat with nitrate as the terminal electron acceptor (Anje Timmerten Hoor, personal communication). Interestingly, the mixed culture produced almost twice as much protein/litre as a pure culture of *T. denitrificans*. This result may be explained by assuming growth of heterotrophs on excretion products of *T. thioparus*. However, the impressive increase in yield would require *T. thioparus* to excrete enormous quantities of organic compounds into the medium. As this may seem unlikely, another explanation may be that a symbiotic relationship between *T. thioparus* and the heterotrophs allows the autotroph to utilize the available energy source much more efficiently. In the light of these considerations the apparently high efficiency of 'chemosynthesis' as observed by Sorokin [77] might be explained accordingly.

From the above considerations it may be concluded that an understanding of the role of colourless sulfur bacteria in the sulfur cycle will not only require extensive investigation of colourless sulfur bacteria themselves but also of the other organisms of the spectrum just described. Therefore it will be important that research should focus on the physiological and ecological aspects of both pure and mixed cultures.

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REFERENCES

1 Attoe, O. J. and Olsen, R. A., Factors affecting rate of oxidation in soils of elemental sulfur and that added in rock phosphate-sulfur fusions. Soil Sci. **101**, 317–325 (1966).

2 Baalsrud, K. and Baalsrud, K. S., Studies on *Thiobacillus denitrificans*. Arch. Mikrobiol. **20**, 34–62 (1954).

3 Bavendamm, W., Die farblosen und roten Schwefelbakterien. *In* Pflanzenforschung, Heft 2, pp. 1–157. (Ed. R. Kolkwitz) Gustav Fischer Verlag, Jena (1924).

4 Belly, R. T. and Brock, T. D., Ecology of iron oxidizing bacteria in pyrite materials associated with coal. J. Bacteriol. **117**, 726–732 (1974).

5 Bloomfield, C., The oxidation of iron sulphides in soils in relation to the formation of acid sulphate soils, and of ochre deposits in field drains. J. Soil Sci. **23**, 1–16 (1972).

6 Boer, W. E. de, Rivière, J. W. M. la, and Schmidt, K., Some properties of *Achromatium oxaliferum*. Antonie van Leeuwenhoek J. Microbiol. Serol. **37**, 553–563 (1971).

7 Borichewski, R. M., Keto acids as growth-limiting factors in autotrophic growth of *Thiobacillus thiooxidans*. J. Bacteriol. **93**, 597–599 (1967).

8 Borichewski, R. M. and Umbreit, W. W., Growth of *Thiobacillus thiooxidans* on glucose. Arch. Biochem. Biophys. **116**, 97–102 (1966).

9 Brock, T. D., Principles of microbial ecology, Prentice-Hall Inc., Englewood Cliffs, New Jersey (1966).

10 Brock, T. D., Brock, M. L., Bott, T. L., and Edwards, M. R., Microbial life at 90°C; the sulfur bacteria of Boulder springs. J. Bacteriol. **107**, 303–314 (1971).

11 Brock, T. D., Brock, K. M., Belly, R. T. and Weiss, R. L., Sulfolobus: A new genus of sulfur oxidizing bacterial living at low pH, and high temperature. Arch. Mikrobiol. **84**, 54–68 (1972).

12 Buchanan, R. E., *In* Bergey's Manual of Determinative Bacteriology, 7th Ed., pp. 837–844. (Eds. R. S. Breed, E. G. D. Murray and N. R. Smith.) The Williams and Wilkins Comp., Baltimore (1957).

13 Burns, G. R., Oxidation of sulphur in soils. The Sulphur Institute, Washington and London, Technical Bulletin No. 13, pp. 1–41 (1967).

14 Burton, S. D. and Morita, R. Y., Effect of catalase and cultural conditions on growth of *Beggiatoa*. J. Bacteriol. **88**, 1755–1761 (1964).

15 Caldwell, D. E. and Caldwell, S. J., The response of littoral communities of bacteria to variations in sulfide and thiosulfate. Bacteriol. Proc. G239 (1974).

16 Charles, A. M. and Suzuki, I., Sulfite oxidase of a facultative autotroph, *Thiobacillus novellus*. Biochem. Biophys. Research Commun. **19**, 686–690 (1965).

17 Chen, K. Y. and Morris, J. C., Oxidation of aqueous sulfide by O_2:1. general characteristics and catalytic influences. *In* Adv. in Water Pollution Research, **11**, pp. 111–32/1 – 111–32/16 (Ed. S. H. Jenkins) Pergamon Press, San Francisco (1972).

18 Chen, K. Y. and Morris, J. C., Kinetics of oxidation of aqueous sulfide by O_2. Environm. Sci. Technol. **6**, 529–537 (1972).

19 Cline, J. D. and Richards, F. A., Oxygenation of hydrogen sulfide in seawater at constant salinity, temperature and pH. Environm. Sci. Technol. **3**, 838–843 (1969).

20 Collins, V. G., Isolation, cultivation and maintenance of autotrophs. *In* Methods in Microbiology, **3B**, pp. 1–52. (Ed. J. R. Norris and D. W. Ribbons) Academic Press, London, New York (1969).

21 Cornish, A. S. and Johnson, E. J., Regulation of pyruvate kinase from *Thiobacillus neapolitanus*. Arch. Biochem. Biophys. **142**, 584–590 (1971).

22 Farquhar, G. J. and Boyle, W. C., Control of Thiothrix in activated sludge. J. Water Pollution Control Fed. **44**, 14–24 (1972).

23 Faust, L. and Wolfe, R. S., Enrichment and cultivation of *Beggiatoa alba*. J. Bacteriol. **81**, 99–106 (1961).
24 Fliermans, C. B. and Brock, T. D., Ecology of sulfur-oxidizing bacteria in hot acid soils. J. Bacteriol. **111**, 343–350 (1972).
25 Gleen, H. and Quastel, J. H., Sulphur metabolism in soil. Appl. Microbiol. **1**, 70–77 (1953).
26 Guitonneau, G. and Keiling, J., L'évolution et la solubilisation du soufre élémentaire dans la terre arable. Ann. agron. NS **2**, 690–725 (1932).
27 Hampton, M. L. and Hanson, R. S., Regulation of isocitrate dehydrogenase from *Thiobacillus thiooxidans* and *Pseudomonas fluorescens*. Biochem. Biophys. Research Commun. **36**, 296–305 (1969).
28 Hansen, T. A., Sulfide als electrondonor voor Rhodospirillaceae. Dissertation, University of Groningen (1974).
29 Hart, M. G., Sulphur oxidation in tidal mangrove soils of Sierra Leone. Plant and Soil **11**, 215–236 (1959).
30 Hinze, G., Beitrage zur Kenntnis der farblosen Schwefelbakterien. Ber. Deutsch. Bot. Ges. **31**, 189–202 (1913).
31 Howard, R. and Stanier, R. Y., The genera Leucothrix and Thiothrix. Bacteriol. Rev. **19**, 49–64 (1955).
32 Hutchinson, M., Johnstone, K. I. and White, D., Taxonomy of the genus Thiobacillus: the outcome of numerical taxonomy applied to the group as a whole. J. Gen. Microbiol. **57**, 397–410 (1969).
33 Janke, A. and Breed, R. S., *In* Bergey's Manual of determinative bacteriology, 7th Ed., pp. 78–88, 83–84 (Eds. R. S. Breed, E. G. D. Murray and N. R. Smith) The Williams and Wilkins Comp., Baltimore (1957).
34 Jannasch, H. W., Enrichment of aquatic bacteria in continuous culture. Arch. Mikrobiol. **59**, 165–173 (1967).
35 Jørgensen, B. B. and Fenchel, T., The sulfur cycle of a marine sediment model system. Marine Biology **24**, 189–201 (1974).
36 Johnson, C. and Vishniac, W., Chemoautotrophic bacteria. *In* Handbook of Microbiology, pp. 5–15 (Eds. A. I. Laskin and H. A. Lechevalier) CRC Press, Cleveland (1972).
37 Karavaiko, G. I. and Pivovarova, T. A., Oxidation of elemental sulfur by *Thiobacillus thiooxidans*. Mikrobiologiya **42**, 345–350 (1973) (Engl. transl.).
38 Karavaiko, G. I., Shchetinina, E. V., Pivovarova, T. A. and Mubarakova, K. Y., Denitrifying bacteria isolated from deposits of sulfide ores. Mikrobiologiya **42**, 109–114 (1973) (Engl. transl.).
39 Keil, F., Beitrage zur Physiologie der farblosen Schwefelbakterien. Beitr. Biol. Pflanz. **11**, 335–372 (1912).
40 Kelly, D. P., The incorporation of acetate by the chemoautotroph *Thiobacillus neapolitanus* strain C. Arch. Mikrobiol. **58**, 99–116 (1967).
41 Kelly, D. P., Autotrophy: Concepts of lithotrophic bacteria and their organic metabolism. Ann. Rev. Microbiol. **25**, 177–210 (1971).
42 Kelly, D. P., Transformations of sulphur and its compounds in soils. Intern. Symp. Sulphur in Agriculture (1970). Annale agronomique, Numéro hors série 217–232 (1972).
43 Kelly, D. P. and Tuovinen, O. H., Recommendation that the names *Ferrobacillus ferrooxidans* Leathen and Braley and *F. sulfoxidans* Kinsel be recognized as synonyms of *Thiobacillus ferrooxidans* Temple and Colmer. Intern. J. Syst. Bacteriol. **22**, 170–172 (1972).
44 Kowalik, U. and Pringsheim, E. G., The oxidation of hydrogen sulfide by Beggiatoa. Am. J. Botany **53**, 801–806 (1966).
45 Kuenen, J. G., Een studie van kleurloze zwavelbacteriën uit het Groninger Wad. Dissertation, University of Groningen (1972).

46 Kuenen, J. G., unpublished results.
47 Kuenen, J. G. and Veldkamp, H., *Thiomicrospira pelophila*, nov. gen., nov. sp., a new obligately chemolithotrophic colourless sulfur bacterium. Antonie van Leeuwenhoek J. Microbiol. Serol. **38**, 241–256 (1972).
48 Kuenen, J. G. and Veldkamp, H., Effects of organic compounds on growth of chemostat cultures of *Thiomicrospira pelophila*, *Thiobacillus thioparus* and *Thiobacillus neapolitanus*. Arch. Mikrobiol. **94**, 173–190 (1973).
49 Kuznetsov, S. I., Die Rolle der Mikroorganismen im Stoffkreislauf der Seen. (Transl. 1959) (Ed. A. Pochman) VEB Deutscher Verlag der Wissenschaften, Berlin (1952).
50 Kuznetsov, S. I. and Sokolova, G. A., Contributions to the physiology of *Thiobacillus thioparus*. Mikrobiologiya **29**, 131–134 (1960) (Engl. transl.).
51 Léjohn, H. B., Van Caeseele, L. and Lees, H., Catabolite repression in the facultative chemoautotroph *Thiobacillus novellus*. J. Bacteriol. **94**, 1484–1491 (1967).
52 London, J., *Thiobacillus intermedius* nov. sp. A novel type of facultative autotroph. Arch. Mikrobiol. **46**, 329–337 (1963).
53 London, J. and Rittenberg, S. C., *Thiobacillus perometabolis* nov. sp., a non autotrophic Thiobacillus. Arch. Mikrobiol. **59**, 218–225 (1967).
54 Matin A. and Rittenberg, S. C., Regulation of glucose metabolism in *Thiobacillus intermedius*. J. Bacteriol. **104**, 239–246 (1970).
55 Matin, A. and Rittenberg, S. C., Enzymes of carbohydrate metabolism in *Thiobacillus* species. J. Bacteriol. **107**, 179–186 (1971).
56 Mosser, J. L., Mosser, A. G., and Brock, T. D., Bacterial origin of sulfuric acid in geothermal habitats. Science **179**, 1323–1324 (1973).
57 Mosser, J. L., Bohlool, B. B., and Brock, T. D., Growth rates of *Sulfolobus acidocaldarius* in nature. J. Bacteriol. **118**, 1075–1081 (1974).
58 Niemelä, S. I. and Tuovinen, O. H., Acidophilic Thiobacilli in the River Sirppujoki. J. Gen. Microbiol. **73**, 23–28 (1972).
59 Pan, P. C. and Umbreit, W. W., Growth of obligate autotrophic bacteria on glucose in a continuous flow-through apparatus. J. Bacteriol. **109**, 1149–1155 (1972).
60 Pan, P. C. and Umbreit, W. W., Growth of mixed cultures of autotrophic and heterotrophic organisms. Can J. Microbiol. **18**, 153–156 (1972).
61 Pearce, J., Leach, C. K. and Carr, N. G., The incomplete tricarboxylic acid cycle in the blue-green alga *Anabaena variabilis*. J. Gen Microbiol. **55**, 371–378 (1969).
62 Peeters, T., Liu, M. S. and Aleem, M. I. H., The tricarboxylic acid cycle in *Thiobacillus denitrificans* and *Thiobacillus A2*. J. Gen. Microbiol. **64**, 29–35 (1970).
63 Peck, H. D., Symposium on metabolism of inorganic compounds. V. Comparative metabolism of inorganic sulfur compounds in microorganisms. Bacteriol. Rev. **26**, 67–94 (1962).
64 Quayle, J. R., The metabolism of one carbon compounds. Adv. Microbiol. Physiol. **7**, 119–203 (1972).
65 Quispel, A., Harmsen, G. W. and Otzen, D., Contribution to the chemical and bacteriological oxidation of pyrite in soil. Plant and Soil **4**, 43–55 (1952).
66 Rittenberg, S. C., The roles of exogenous organic matter in the physiology of chemolithotrophic bacteria. Adv. Micr. Physiol. **3**, 159–196 (1969).
67 Rittenberg, S. C., The obligate autotroph – the demise of a concept. Antonie van Leeuwenhoek, J. Microbiol. Serol. **38**, 457–478 (1972).
68 Rivière, J. W. M. la, Enrichment of colourless sulfur bacteria. Zbl. Bakt. Abt. I., Orig. Suppl. **1**, 17–27 (1965).
69 Rivière, J. W. M. la, The microbial sulfur cycle and some of its implications for the geochemistry of sulfur isotopes. Geolog. Rundschau **55**, 568–582 (1966).

70 Roy, A. B. and Trudinger, P. A., The biochemistry of inorganic compounds of sulphur. Cambridge University Press, London, New York (1970).
71 Scotten, H. L. and Stokes, J. L., Isolation and properties of Beggiatoa. Arch. Mikrobiol. **42**, 353–368 (1962).
72 Shafia, F. and Wilkinson, R. F., Growth of *Ferrobacillus ferrooxidans* on organic matter. J. Bacteriol. **97**, 256–260 (1969).
73 Shively, J. M., Ball, F., Brown, D. H. and Saunders, R. E., Functional organelles in prokaryotes. Science **182**, 584–586 (1973).
74 Smith, A. J., London, J., and Stanier, R. Y., Biochemical basis of obligate autotrophy in blue-green algae and thiobacilli. J. Bacteriol. **94**, 972–983 (1967).
75 Sokolova, G. A. and Karavaiko, G. I., Physiology and geochemical activity of Thiobacilli. Israel Programme for Scientific Translations, Jerusalem (1968).
76 Sorokin, Y. I., The mechanism of chemical and biological oxidation of sodium, calcium and iron sulfides. Mikrobiologiya **39**, 220–224 (1970) (Engl. transl.).
77 Sorokin, Y. I., The bacterial population and the process of hydrogen sulfide oxidation in the Black Sea. J. Cons. Intern. Explor. Mer **34**, 423–454 (1972).
78 Sorokin, Y. I. and Kadota, H., Techniques for the assessment of microbial production and decomposition in fresh water. IBP Handbook **23** (1972).
79 Starkey, R. L., Isolation of some bacteria which oxidize thiosulfate. Soil Sci. **39**, 197–219 (1935).
80 Starkey, R. L., Oxidation and reduction of sulfur compounds in soils. Soil Sci. **101**, 297–306 (1966).
81 Taylor, B. F., Regulation of citrate synthase activity in strictly and facultatively autotrophic thiobacilli. Biochem. Biophys. Research Commun. **40**, 957–963 (1970).
82 Taylor, B. F. and Hoare, D. S., New facultative Thiobacillus and a reevaluation of the heterotrophic potential of *Thiobacillus novellus*. J. Bacteriol. **100**, 487–497 (1969).
83 Taylor, B. F. and Hoare, D. S., *Thiobacillus denitrificans* as an obligate chemolithotroph. II. Cell suspensions and enzymic studies. Arch. Mikrobiol. **80**, 262–276 (1971).
84 Taylor, B. F., Hoare, D. S., and Hoare, S. L., *Thiobacillus denitrificans* as an obligate chemolithotroph. Isolation and growth studies. Arch. Mikrobiol. **78**, 193–204 (1971).
85 Tredway, J. V. and Burton, S. D., Morphological examination of Beggiatoa and Thiothrix obtained from bacterial mats on the surface of solid waste bales deposited in the continental shelf. Bacteriol. Proc. G105 (1974).
86 Trudinger, P. A., Metabolism of thiosulfate and tetrathionate by heterotrophic bacteria from soil. J. Bacteriol. **93**, 550–559 (1967).
87 Trudinger, P. A., The metabolism of inorganic sulphur compounds by thiobacilli. Rev. Pure and Appl. Chem. **17**, 1–24 (1967).
88 Tuovinen, O. H. and Kelly, D. P., Biology of *Thiobacillus ferrooxidans* in relation to the microbiological leaching of sulphide ores. Z. allg. Mikrobiol. **12**, 311–346 (1973).
89 Tuovinen, O. H. and Kelly, D. P., Studies on the growth of *Thiobacillus ferrooxidans*. I. Use of membrane filters and ferrous iron agar to determine viable numbers and comparison with $^{14}CO_2$-fixation and iron oxidation as measures of growth. Arch. Mikrobiol. **88**, 285–298 (1973).
90 Tuttle, J. H., Dugen, P. R., MacMillan, C. B. and Randles, C. I., Microbial dissimilatory sulfur cycle in acid mine water. J. Bacteriol. **97**, 594–602 (1969).
91 Tuttle, J. H. and Jannasch, H. W., Occurrence and types of Thiobacillus-like bacteria in the sea. Limnol. Oceanogr. **17**, 532–543 (1972).

92 Tuttle, J. H. and Jannasch, H. W., Sulfide and thiosulfate-oxidizing bacteria in anoxic marine basins. Marine Biol. **20**, 64–70 (1973).
93 Tuttle, J. H., Holmes, P. E. and Jannasch, H. W., Growth stimulation of marine pseudomonas by thiosulfate. Bacteriol. Proc. G238 (1974).
94 Tuttle, J. H., Holmes, P. E. and Jannasch, H. W., Growth rate stimulation of marine pseudomonads by thiosulfate. Arch. Mikrobiol. **99**, 1–14 (1974).
95 Vishniac, W. and Santer, M., The thiobacilli. Bacteriol. Rev. **21**, 195–213 (1957).
96 Vitolins, M. I. and Swaby, R. J., Activity of sulphur oxidizing micro-organisms in some Australian soils. Australian J. Soil Research **7**, 171–183 (1969).
97 Water Pollution Research, Technical paper no. 11. Dep. of Scientific and Industrial Res., H. M. Stationery Office, London, pp. 188–194, 247–277 (1964).
98 Williams, R. A. D. and Hoare, D. S., Physiology of a new facultatively autotrophic thermophilic Thiobacillus, J. Gen. Microbiol. **50**, 555–566 (1972).
99 Winogradski, S., Contribution à la morphologie et à la physiologie des sulfobactéries (1888). *In* Microbiologie du Sol, Oeuvres Complètes, Masson et Cie., Paris, pp. 83–126 (1949).
100 Wirsen, C. O., Gonye, E. R., and Jannasch, H. W., Physiological and morphological studies on Thiovulum sps. Bacteriol. Proc. G237 (1974).
101 Woolley, D., Jones, G. L., and Happold, F. C., Some metabolic differences between *Thiobacillus thioparus*, *T. denitrificans* and *T. thiocyanooxidans*. J. Gen. Microbiol. **29**, 311–316 (1962).
102 Zajic, J. E., Microbial biogeochemistry. Academic Press, New York, London (1969).
103 Zavarzin, G. A. and Zhilina, T. N., Thione bacteria from thermal springs. Mikrobiologiya **33**, 753–758 (1964) (Engl. transl.).