Oxidation of Carbon Disulphide as the Sole Source of Energy for the Autotrophic Growth of *Thiobacillus thioparus* Strain TK-m

By NEIL A. SMITH AND DON P. KELLY*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

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The ability of micro-organisms to grow on carbon disulphide (CS₂) as a sole source of carbon and energy appears to be very limited: none was obtained from enrichment culture and eight *Thiobacillus* species could not use it. *Thiobacillus thioparus* strain TK-m could grow autotrophically on either CS₂ or carbonyl sulphide (COS) as sole substrates. Growth yield on CS₂ was 7.9 ± 0.9 g cell-carbon (mol CS₂)⁻¹, and yields on COS, thiosulphate or thiocyanate were in the range 5.6–6.1. COS was detected as an intermediate during growth on CS₂, and there was quantitative conversion of the sulphur of CS₂ to sulphate during growth. Aerobic oxidation of CS₂ by suspensions of strain TK-m exhibited a *K_s* of 16.5 µM and a *V_{max}* of 524 nmol O₂ consumed min⁻¹ (mg organism-protein)⁻¹. When incubated anaerobically with CS₂, strain TK-m sequentially produced COS and H₂S. CS₂ oxidation is proposed to proceed by its sequential hydrolytic cleavage to COS then H₂S, with release of all the carbon as CO₂, followed by oxidation of the sulphide to sulphate. This oxidation provides all the energy for growth, which is dependent on the autotrophic fixation of CO₂, apparently by means of ribulose biphosphate carboxylase.

INTRODUCTION

There is no significant literature on the microbial breakdown and metabolism of carbon disulphide (CS₂), notwithstanding the fact that it is a geochemically significant atmospheric trace gas with an atmospheric lifetime of 12 d (Khalil & Rasmussen, 1984). Similarly, there is no information on the microbial oxidation of carbonyl sulphide (COS), which is a major intermediate in the oxidative metabolism of CS₂ by rat liver microsomes (Chengelis & Neal, 1987). These two gases occur in the atmosphere at concentrations of 0.07–0.56 parts per billion (Bremner & Steele, 1978), with COS being claimed as the most abundant sulphur gas in the atmosphere, with a lifetime of about 2 years (Khalil & Rasmussen, 1984; Steudler & Peterson, 1984). Other important sources are volcanic emissions of COS (Stoiber *et al.*, 1971), and the release of CS₂ during its industrial use, which is significant because of the known hazard to human health presented by this compound (Sweetnam *et al.*, 1987).

CS₂ and COS are known to have a number of biogenic sources, including the degradation of cysteine, cystine, thiocyanates and other sulphur-containing compounds in soils, manures, decomposing plant material and the marine environment (Bremner & Steele, 1978). They have also been shown to arise from the breakdown of pesticides and are themselves known to have harmful effects on some plants and fungi (Stotzky & Schenk, 1976; Bremner & Steele, 1978). CS₂ is also a natural product in the roots of *Acacia*, in which it may have a fungicidal role (Whitfield *et al.*, 1981). It is also known to be an inhibitor of soil nitrification (Ashworth *et al.*, 1977). The demonstration of metabolism of CS₂ by micro-organisms appears at present to be limited to the observation that some methanogens can use it as a sulphur source for growth (Rajagopal &

Abbreviations: COS, carbonyl sulphide; CS₂, carbon disulphide; CTAB, cetyltrimethylammonium bromide; MT, methanethiol; TOC, total organic carbon.

Daniels, 1986), and a very brief report of its oxidation by *Thiobacillus thiooxidans* (Butler *et al.*, 1969).

We now report some physiological and biochemical studies on the autotrophic, chemolithotrophic growth of *Thiobacillus thioparus* on CS₂ and COS as sole growth substrates.

METHODS

Culture media. The mineral salts medium used for all batch and continuous cultures contained (g l⁻¹): KH₂PO₄, 2.0; K₂HPO₄, 2.0; NH₄Cl, 0.4; Na₂CO₃, 0.4; MgCl₂·6H₂O, 0.2; 3 ml of vitamin mixture (Kanagawa *et al.*, 1982) and 1 ml of trace metal solution (Tuovinen & Kelly, 1973); initial pH 7.1. Substrates were added as described below. Solid medium for plates and slopes was prepared by adding substrate and agar (1.5%, w/v) to the mineral salts medium. *Thiobacillus thioparus* strain TK-m was maintained on agar medium with 20 mM-sodium thiosulphate.

Growth in batch and continuous culture. Batch cultures (50 ml) were grown in 250 ml Quickfit flasks sealed with rubber Suba-seal stoppers. CS₂ (2 mm, unless otherwise stated) was injected into the flasks using a Hamilton syringe. COS was added either as unsterilized gas or in solution in sterile distilled water. Thiosulphate (10–20 mM) and thiocyanate (2–10 mM) were both sterilized in the mineral salts medium.

Radiolabelling experiments to measure the incorporation of ¹⁴C-labelled one-carbon compounds into cells growing on 2 mm-CS₂ or COS were done in sealed flasks as described above. Cultures (50 ml) from which Na₂CO₃ had been omitted were supplemented with either 10 mM-NaH¹⁴CO₃, 10 mM-sodium [¹⁴C]formate or 1 mM-H¹⁴CHO. Samples (2 ml) were filtered through Whatman membrane filters (0.2 µm pore size), washed with water and counted in 10 ml of LKB Optiphase Safe scintillant using a Beckman LS 7000 spectrometer.

Chemostat culture work was done in a 1 litre water-jacketed glass vessel (LH Engineering) containing 750 ml of culture. Cultures were maintained at 30 ± 1 °C, stirred at 750 r.p.m. and aerated at 750 ml min⁻¹ with air. Culture pH was controlled at pH 6.8 by automatic titration with 0.5 M-K₂CO₃. Continuous cultures were established on 7.5–10.0 mM-thiosulphate as the growth-limiting substrate, following inoculation (20%, v/v) with a batch culture grown on 20 mM-thiosulphate and allowing 3 d further batch growth before commencing flow of medium. Some cultures were supplied with both thiosulphate and thiocyanate as dual growth-limiting substrates. Substrates were metered into the vessel separately from the mineral salts medium.

Respiration measurements. Oxygen uptake rates by suspensions of organisms were determined using a Teflon-coated Clark-type Rank oxygen electrode cell linked to a chart recorder. Suspensions (2 ml) were taken either directly from a 10 mM-thiosulphate-limited chemostat (*D* 0.085 h⁻¹) or from a centrifuged, washed and resuspended suspension of organisms previously grown in batch culture on CS₂. Substrate-dependent oxygen uptake was measured at 30 °C, the reaction being initiated by injection of substrate (5–20 µl). Results are expressed as nmol O₂ min⁻¹ (mg protein)⁻¹, corrected for endogenous oxygen uptake.

Determination of transitory intermediates of CS₂ metabolism. Gas chromatography/mass spectrometry (GC/MS) analyses were done using a Kratos mass spectrometer, model MS 25RFA, linked to a Carlo Erba gas chromatograph, model MFC 500, and fitted with a polar capillary column with length, internal diameter and film thickness of 25 m, 0.33 mm and 0.5 µm, respectively. The system used helium gas as carrier, with a flow rate of 1 ml min⁻¹. The temperature of the column was programmed at 50 °C with increments of 20 °C min⁻¹ to a final temperature of 220 °C. The injection and detector temperatures were 240 and 200 °C, respectively. Identification of intermediates was by comparison of the mass spectra produced from the samples with those held on file by the computer, and by comparison with the mass spectra produced from authentic compounds. Samples for GC/MS analysis were prepared as follows. Batch cultures (50 ml) on 2 mm-CS₂ in sealed flasks were sampled (1 ml) periodically throughout growth, and volatile intermediates extracted by vigorous shaking with chloroform (2 ml) in sealed tubes. The organic phase was removed, transferred to a second tube and dried with a few crystals of CaCl₂. This solution was taken for analysis using injection volumes of 0.5–1.0 µl with a split ratio of 100:1.

Gas chromatography (GC) analyses were done using a Pye Unicam Series 204 gas chromatograph linked to a Pye Unicam computing integrator (model PU 4810) and fitted with a Teflon column packed with acetone-washed Porapak QS. Column length and internal diameter were 1 m and 3 mm, respectively. The system was equipped with a flame photometric detector incorporating a sulphur filter permitting light transmission at 349 nm. The injection, column and detector temperatures were 200, 100 and 250 °C, respectively. Gas flow rates for nitrogen (carrier gas), air and hydrogen were 40, 50 and 70 ml min⁻¹, respectively. Analyses were initiated by the injection of 100 µl of headspace gas from cultures. Compounds were identified by comparison of retention times of peaks produced from samples with those obtained from authentic compounds. Under the conditions used, H₂S, COS and CS₂ had retention times of 0.81, 1.1 and 11.3 min, respectively.

Time-dependent disappearance of CS₂ and appearance of H₂S and COS by suspensions incubated aerobically or anaerobically at 30 °C were determined as follows. Triplicate batch cultures (100 ml in 500 ml stoppered flasks) were fed with five additions of 1 mM-CS₂ over a period of 12 d. The cultures were centrifuged at 21 000 *g* for 15 min, washed and the organisms (15 mg protein) resuspended in 10 ml mineral salts medium. The suspension

was divided into two equal portions in 23 ml volume universal bottles sealed with rubber Suba-seals. Each was deoxygenated by bubbling with nitrogen and allowed to equilibrate at 30 °C. Reaction was initiated by injecting 10 µmol of CS₂. CS₂, COS and H₂S were then monitored in the 18 ml of headspace gas in the tubes over a period of 3–4 h. At this time total H₂S production was determined by acidifying the suspensions with 1 M-HCl to drive H₂S from solution and the headspace gas reanalysed. For comparison the experiment was also run under an air atmosphere.

Other analytical methods. Growth of cultures was monitored by measuring optical density (1 cm) at 440 nm in a Unicam SP 1700 UV/VIS spectrophotometer. Samples from chemostat cultures were used to prepare calibration curves relating optical density to biomass in terms of total organic carbon (TOC), protein or dry weight (mg l⁻¹). Dry weights were determined by drying a centrifuged, washed and resuspended suspension of organisms in distilled water to constant weight at 105 °C. Pellets of organisms centrifuged from 4 ml culture samples were assayed for TOC and protein: for TOC, aqueous suspensions of pellets were assayed using a Beckman Total Organic Carbon Analyzer, model 905B; for protein, organisms were dissolved in 0.5 M-NaOH (30 min) and assayed by the Lowry procedure. Sulphate was determined by atomic absorption spectrophotometry: sulphate was precipitated with a standard solution of barium chloride and residual barium in the clear supernatant solution was measured at 553.6 nm (Varian Instrument Manual). Thiosulphate (and thiocyanate) were determined according to Kelly *et al.* (1969). Ribulose-1,5-bisphosphate carboxylase activity was assayed as described by Kelly & Wood (1982), except that Triton X-100 was replaced by cetyltrimethylammonium bromide (CTAB; 0.1%, w/v) as the permeabilizing agent (Leadbeater *et al.*, 1982).

Special chemicals. CS₂ (99.9%) was obtained from Fisons or Aldrich; COS (96+%) was from Aldrich; Na₂¹⁴CO₃, sodium [¹⁴C]formate and H¹⁴CHO were from Amersham. Chloroform used in the GC/MS analysis was of chromatographic grade from Fisons or Aldrich.

RESULTS

Growth of thiobacilli on CS₂. No growth could be obtained on 2 mM-CS₂ of *T. neapolitanus* (DSM 581), *T. versutus* (DSM 582), *T. thioparus* (DSM 505), *T. acidophilus* (DSM 700), *T. thiooxidans* (ATCC 8085), *T. aquaesulis* (DSM 4255, ATCC 43788) or *T. tepidarius* (DSM 3134, ATCC 43215). Slight growth and decrease in pH were seen with *T. ferrooxidans* (DSM 585), but this was not sustained on subculture. Growth was obtained using *T. thioparus* strain TK-m (Kanagawa *et al.*, 1982; Kanagawa & Kelly, 1986), which is also capable of growth on dimethyl sulphide and thiocyanate (Smith & Kelly, 1988).

Growth rate and yield of *T. thioparus* strain TK-m in batch culture on CS₂ and COS, and a comparison with yields on thiosulphate and thiocyanate. In batch culture on 2 mM-CS₂ in sealed flasks, strain TK-m exhibited a doubling time of 30 to 40 h (i.e. a specific growth rate of about 0.02 h⁻¹). Biomass production in these batch cultures was in the range 13.6–17.6 mg bacterial organic carbon per litre of culture, indicating a yield of 7.9 ± 0.9 g cell-carbon (mol CS₂)⁻¹ (from five estimations).

When strain TK-m was grown on COS under similar conditions, a yield of 6.1 ± 0.8 g cell-carbon (mol COS)⁻¹ was obtained.

A thiosulphate (7.5 mM)-limited chemostat culture, at a dilution rate of 0.085 h⁻¹, gave a yield of 5.88 g cell-carbon (mol thiosulphate)⁻¹. Supplementing the substrate feed of this culture with 5.2 mM-thiocyanate resulted in complete oxidation of the thiocyanate (as well as of the thiosulphate) and an increase in the steady state biomass equivalent to a yield of 5.58 g cell-carbon (mol thiocyanate)⁻¹.

From the oxygen requirements for the oxidation of each of these substrates (2 mol mol⁻¹ for COS, thiosulphate and thiocyanate; 4 mol mol⁻¹ for CS₂) the yields would be expected to be similar for the COS, thiosulphate and thiocyanate, as was found. The yield on CS₂ would thus have been expected to fall in the range 11.7 ± 0.5.

Stoichiometry and intermediates of CS₂ metabolism. During growth on CS₂, analysis by GC/MS of the chloroform-extractable volatiles from the culture medium showed a rapid initial formation of a compound indistinguishable from COS. This compound was detectable

Fig. 1. Demonstration by GC/MS of the intermediate formation of COS during the growth of *T. thioparus* strain TK-m on CS₂ (4 mM). Chloroform-extractable components of the culture were analysed as described in Methods. (a) Standard COS; (b) substrate CS₂ standard; (c–g) samples from the culture taken at the times indicated and analysed by GC/MS with chloroform as the internal reference compound.

throughout the culture period of 4 d but remained below the concentration of the residual CS₂ (Fig. 1).

When a suspension (5 ml) of *T. thioparus* TK-m was incubated anaerobically under a headspace of 18 ml nitrogen and given 10 μ mol CS₂, the CS₂ disappeared quite rapidly from the headspace gas (Fig. 2). Its disappearance was paralleled by the formation of COS and H₂S, with further H₂S accumulation apparently occurring at the expense of COS disappearance (Fig. 2). After 380 min anaerobic incubation, the experiment (Fig. 2) was terminated by acidification with HCl to release H₂S from solution. At that time, the amounts (μ mol) of CS₂, COS and H₂S recovered in the headspace were 1.99, 0.41 and 15.4 respectively. This meant that of the 10 μ mol CS₂ added, 8 μ mol were converted quantitatively to COS (2.6%) and H₂S (97.4%). Under aerobic conditions, no such COS or H₂S accumulation was seen in a 200 min incubation, although traces of H₂S (1–6 nmol) were detected, and 49 nmol COS were seen after 20 min, declining to an undetectable level by 63 min.

Fig. 2. Formation of COS (○) and H₂S (▲) from CS₂ (●) during anaerobic incubation with *T. thioparus* strain TK-m. Suspensions were incubated under nitrogen with 10 μmol CS₂ as described in Methods, and the headspace gas was analysed for CS₂, COS and H₂S.

Oxidation of CS₂ and other sulphur compounds by T. thioparus strain TK-m. Using the oxygen electrode cell, the rate of oxidation of CS₂ by CS₂-grown organisms increased over a range of seven concentrations from 128 nmol O₂ consumed min⁻¹ (mg cell-protein)⁻¹ (at 5 μM) to 447 (at 50 μM), with no evidence of substrate inhibition over this concentration range. K_m and V_{max} were computed by the Lineweaver-Burk and Eadie-Hofstee procedures as $K_m = 16.0$ and 16.5 μM, and $V_{max} = 511$ and 524 nmol O₂ min⁻¹ (mg protein)⁻¹, respectively. The Pearson's correlation coefficients for the fit of the data (16 rate values) were 0.973 and 0.871.

The stoichiometry of CS₂ oxidation fell below the expected molar ratio of four O₂ per CS₂ oxidized: the best ratio was 1.5:1.0 at 25 μM-CS₂, with a mean of 1.32 ± 0.13 O₂ per CS₂ for 5–50 μM. This probably indicated incomplete oxidation of sulphide, possibly resulting in sulphur accumulation.

For comparison, the oxidation of inorganic sulphur compounds by organisms taken from a thiosulphate-limited chemostat was assayed. Such organisms did not oxidize CS₂. Sulphide was oxidized by organisms supplied with concentrations between 10 and 1000 μM. The oxidation rate increased from 106 nmol O₂ min⁻¹ (mg cell-protein)⁻¹ at 10 μM to 258 at 100 μM, but above this there was substrate inhibition with the rate at 1 mM falling to 130. Thiosulphate oxidation showed complex kinetics, with a high affinity, low V_{max} phase below 50 μM (resembling the kinetics seen with sulphide), and a low affinity, high V_{max} phase above 50 μM (Table 1). Tetrathionate oxidation resembled the second phase of thiosulphate oxidation, with rate increasing from 144 at 25 μM to 345 at 100 μM, and giving low K_m and high V_{max} values (Table 1).

Growth, sulphate production and assimilation of CO₂ by T. thioparus strain TK-m using CS₂ or COS as sole substrate. When grown on either CS₂ or COS, virtually all the cell-carbon was seen to be derived from CO₂ (Fig. 3). The decline in the amount of ¹⁴CO₂ fixed in the later stages of growth was attributed to dilution of the specific activity of the added H¹⁴CO₃ by the CO₂ produced from the oxidation of the substrates. In the early stages of growth, before this dilution became apparent, at least 90% of the increase in biomass was attributable to CO₂ fixation.

Fig. 3. Growth of *T. thioparus* strain TK-m on CS_2 or COS. (a) $^{14}\text{CO}_2$ fixation during growth of cultures in sealed flasks, as described in Methods, on 2 mM- CS_2 (●) or 2 mM-COS (○). Increase in cell-carbon as TOC and $^{14}\text{CO}_2$ fixation were measured over a 96 h growth period. Data are presented as a differential plot of the two measures of increase in cell-carbon. (b) Increase in biomass (as TOC, ○), fixation of $^{14}\text{CO}_2$ (●) and formation of sulphate (△) by cultures growing on 2 mM- CS_2 . The data presented are mean values from duplicate cultures; deviation from the mean was $\pm 10\%$. Data have been normalized to initial zero values by deducting the starting biomass and sulphate levels in the cultures (4.5 mg TOC l^{-1} and 5.25 mM, respectively).

Table 1. Kinetics of oxidation of inorganic sulphur compounds by *T. thioparus* strain TK-m

All substrates were tested using at least four concentrations over the ranges indicated. Pearson's correlation coefficients for fit of the data to the computed lines were all in the range 0.934 to 0.999.

Substrate	Concentration range (μM)	K_m (μM)		$V_{\max}$ [$\text{nmol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$]	
		Lineweaver-Burk	Eadie-Hofstee	Lineweaver-Burk	Eadie-Hofstee
Sulphide	10–100	18.2	18.5	298	300
Thiosulphate	10–50	14.8	13.8	198	193
	50–500	105	110	464	477
Tetrathionate	25–100	99	86	721	661

Making simultaneous measurements of increase in absorbance, TOC in the organisms, $^{14}\text{CO}_2$ fixation and sulphate production showed these all to be proportionate during growth (Fig. 3b). During the complete oxidation of approximately 2 mM- CS_2 , about 13.9 mg of newly synthesized cell-carbon was produced per litre (at least 90% from CO_2), and 3.6 mM-sulphate was recovered. This represented 90% of the sulphur present in the CS_2 supplied, and showed that complete oxidation of this substrate took place.

Incorporation of ^{14}C -labelled formaldehyde during growth on CS_2 . H^{14}CHO (added at 0.25 mM) was incorporated during growth on CS_2 (2 mM) at a rate that was proportional to increase in biomass, but accounted for only about 5% of the cell-carbon fixed during growth. This would be consistent with its being assimilated only after oxidation to CO_2 .

Ribulose-1,5-bisphosphate carboxylase in *T. thioparus* strain TK-m grown on CS₂. Low levels of this enzyme were detected using the permeabilized whole organisms method. No activity was found if Triton X-100 was used as the permeabilizing agent. Using CTAB-treated cells gave a specific activity of about 3 nmol CO₂ fixed min⁻¹ (mg dry wt)⁻¹, which was equivalent to about 5 nmol min⁻¹ (mg total organism protein)⁻¹. This can be compared with a specific activity, under similar assay conditions, of 45 nmol min⁻¹ (mg dry wt)⁻¹ for organisms grown autotrophically in a thiosulphate-limited chemostat at a dilution rate of 0.08 h⁻¹.

DISCUSSION

Our results serve both as the first detailed study of the microbial breakdown of CS₂, and as a further demonstration of the metabolic versatility of *T. thioparus* strain TK-m. As well as its ability, as a typical *Thiobacillus*, to oxidize thiosulphate, tetrathionate and sulphide, it can grow on dimethyl sulphide as sole energy source (Kanagawa & Kelly, 1986). We have now shown that it can also grow autotrophically on thiocyanate (S=C=N), CS₂ (S=C=S) and COS (O=C=S), the metabolism of each of which requires the scission of a S=C bond. The ability of the organism to attack CS₂ under anaerobic conditions suggests that the first step of aerobic CS₂ metabolism might be its hydrolytic cleavage to COS and H₂S

The COS formed appears to undergo similar hydrolysis to CO₂ and H₂S.

This scheme is in contrast to that proposed for the oxidative degradation of CS₂ by rat liver, which is believed to be initiated by a NADPH-dependent monooxygenase, containing cytochrome P-450 (Chengelis & Neal, 1987). Clearly a monooxygenase cannot be involved in the anaerobic production of COS and H₂S observed by us. The possibility that the normal aerobic metabolism of CS₂ is initiated by an oxygenase, and that the anaerobic behaviour was artefactual, cannot, however, be unequivocally discounted.

The only source of energy available from the dissimilation of CS₂ is clearly the oxidation to sulphate of the H₂S produced from it. CS₂ and COS must therefore be regarded exclusively as chemolithotrophic substrates, in contrast to the methylated sulphides, which can be both chemolithotrophic and chemoorganotrophic energy sources. Growth yields on thiosulphate, thiocyanate and COS were similar, at 5.6–6.1 g cell-carbon mol⁻¹, indicating sulphur oxidation to be the only source of metabolic energy in each case. Yields on CS₂ tended to be lower than expected given its equivalence to two sulphide molecules. This might be attributable in part to the difficulties of dispensing it with high accuracy into culture flasks, and partly a consequence of the very low growth rates observed. If, however, an NAD(P)H-dependent monooxygenase is involved in the initial cleavage of CS₂, the growth yield would be expected to be low, because NAD(P) reduction driven by sulphide oxidation requires energy-dependent electron transport from cytochrome *b* or *c* (Kelly, 1982). This could consume at least one ATP and two of the 14 reducing equivalents available from CS₂ oxidation (via a monooxygenase-dependent pathway) per NAD(P) reduced. If this occurred, one could predict that the yield on CS₂ would only be 1.25 times greater than observed with COS. This would be a value of about 7.6, which compares closely with the observed yield of 7.9 g cell-carbon (mol CS₂)⁻¹.

The mechanism of sulphide oxidation has not been further investigated, but the oxygen electrode data on *K_m* and *V_{max}* values for inorganic sulphur compounds, and the two-phase oxidation kinetics seen with thiosulphate, suggest that tetrathionate is an intermediate in thiosulphate oxidation and these might possibly be intermediates in sulphide oxidation.

Growth on CS₂ was shown to be wholly autotrophic when assayed by ¹⁴CO₂ fixation, but only very low levels of ribulose biphosphate carboxylase have so far been detected. This may reflect both the very low growth rate of the organism on CS₂ and failure to assay under optimum conditions. To date, a sufficient bulk of CS₂-grown organisms has not been obtained for the

preparation of cell-free extracts. Formaldehyde was apparently only assimilated after oxidation to CO_2 , thereby discounting any alternative or unique C_1 -assimilatory pathway.

The ability to oxidize CS_2 appears to be much more restricted among the bacteria studied to date than the capacity for oxidation of methylated sulphides or inorganic sulphur compounds. In that it can only be used as a chemolithotrophic energy source for autotrophic growth, it is perhaps not surprising that few organisms can oxidize it. The possibility that there exist in the natural environment specialist organisms better adapted to use it remains to be explored.

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