Dimethyl Sulphoxide and Dimethyl Sulphide as a Carbon, Sulphur and Energy Source for Growth of *Hyphomicrobium* S

By J. A. M. DE BONT,* J. P. VAN DIJKEN† and W. HARDER‡

Department of Microbiology, Agricultural University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands

(Received 30 March 1981)

A *Hyphomicrobium* sp. capable of growth on dimethyl sulphide and dimethyl sulphoxide was isolated from aerobic enrichment cultures containing dimethyl sulphoxide as the carbon and energy source. Suspensions of cells taken from a dimethyl sulphoxide-limited chemostat oxidized dimethyl sulphide, methanethiol, formaldehyde, formate and thiosulphate. Enzyme studies indicated that the pathway of dimethyl sulphoxide metabolism involves an initial reduction to dimethyl sulphide, which is subsequently oxidized by an NADH-dependent mono-oxygenase to formaldehyde and methanethiol. Further oxidation of methanethiol is by a hydrogen peroxide-producing oxidase, again resulting in the production of formaldehyde. Extracts of dimethyl sulphoxide-grown cells also contained high levels of catalase as well as NAD⁺-dependent formaldehyde and formate dehydrogenases. The organism probably used the serine pathway for growth on dimethyl sulphoxide. This was indicated by the presence of high activities of hydroxypyruvate reductase in dimethyl sulphoxide-grown cells.

INTRODUCTION

Dimethyl sulphoxide (DMSO) is widely used as a solvent and as a consequence is introduced into the biosphere as a pollutant. It is also produced in nature by atmospheric photochemical oxidation of dimethyl sulphide (DMS) (Cox & Sandalls, 1974). DMSO has many interesting pharmacological properties and therefore its effects, fate and metabolism have been studied extensively in plants, animals and man. Although isotope studies with labelled DMSO generally indicated that the compound is metabolized in vivo and that DMS and dimethyl sulphone are metabolic products (Wood, 1971; Jacob & Herschler, 1975), the pathway(s) of DMSO metabolism have so far remained obscure. In a number of prokaryotic and eukaryotic micro-organisms DMSO may function as a terminal electron acceptor and is then reduced to DMS by way of a DMSO reductase (Ando et al., 1957; Yen & Marrs, 1977; Zinder & Brock, 1978a, b). This enzyme has been identified in *Escherichia coli* and uses NADH and, to a lesser extent, NADPH as electron donor (Zinder & Brock, 1978a). In yeasts DMSO reduction is strictly NADPH-dependent and is mediated by a different enzyme (Bamforth, 1980). In certain plants DMSO may serve as a source of sulphur (Smale et al., 1975). A similar observation has been made with *Aerobacter aerogenes*. This organism was also able to metabolize the carbon moieties of DMSO since 80% of the added ¹⁴C-labelled DMSO was recovered as CO₂ (Rammler & Zaffaroni, 1967).

† Permanent address: Department of Microbiology, Delft University of Technology, Julianalaan 67A, 2628 BC Delft, The Netherlands.

‡ Permanent address: Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.
DMS arises chiefly from algal metabolism and is considered to be the major organic sulphur compound to escape from aquatic environments (Lovelock et al., 1972: Bremner & Steele, 1978), but has also been implicated in the volatilization of sulphur from soil (Banwart & Bremner, 1976). Methylated sulphides are also produced in significant quantities by the sulphate cellulose industry which may lead to serious environmental pollution. Direct evidence for the biological oxidation of DMSO or DMS in terrestrial ecosystems is scarce, although several observations indicated the occurrence of such oxidations (see Bremner & Steele, 1978). Also removal of methylated sulphides from waste water has been observed (cf. Sivelä, 1980). Sivelä & Sundman (1975) isolated a Thiobacillus-type organism (designated Thiobacillus MS1) from a biofilter used for odour removal from effluents of a sulphate cellulose mill and demonstrated that this organism was able to metabolize DMS. Subsequent work (Sivelä, 1980) indicated that Thiobacillus MS1 was able to use DMS as an additional carbon and/or energy source when grown in a thiosulphate-limited chemostat. The available evidence suggested that this organism was in fact an obligately chemolithotrophic Thiobacillus sp. in which, during growth on thiosulphate plus DMS media, the Calvin cycle (for CO₂ fixation) and the serine pathway (for formaldehyde assimilation) may function in concert.

Recently, we isolated a Hyphomicrobium sp. from soil which is able to grow on DMS or DMSO as the sole source of carbon, sulphur and energy. The present paper describes the metabolism of these two compounds by this organism.

METHODS

Media and culture conditions. For enrichment, isolation and maintenance of Hyphomicrobium S a medium was used which contained (per litre deionized water): K₂HPO₄, 1.55 g; NaH₂PO₄, 0.85 g; NH₄Cl, 2.0 g; MgCl₂.6H₂O, 0.075 g; (NH₄)₂SO₄, 0.1 g; yeast extract, 0.1 g; DMSO, 1.0 g; and 0.2 ml of a trace element solution as described by Vishniac & Santer (1957). The organism was maintained on agar slopes of this DMSO medium to which Oxoid no. 3 agar (12 g l⁻¹) had been added. Incubation of these slopes was in a desiccator at 30 °C for 2 months; they were then stored at 4 °C. For metabolic studies the organism was grown in a continuous culture using a stirred fermentor with a working volume of 1.5 l at 30 °C and a dilution rate of 0.014 h⁻¹. In these studies the above medium was used from which K₂HPO₄, (NH₄)₂SO₄ and yeast extract were omitted; under these conditions the culture was DMSO-limited. The pH of the chemostat culture was controlled at 6-6 by automatic addition of 1 M-KOH.

Enrichment and isolation procedures. Erlenmeyer flasks (100 ml capacity) each containing 50 ml DMSO medium were inoculated with 1-2 g soil samples and after static incubation for 2 months at 30 °C a few drops of these cultures were transferred to other flasks with the same medium. After an additional 2 months incubation, when substantial visible turbidity had developed, the cultures were streaked on to DMSO agar plates and incubated in a desiccator. Pure cultures were obtained by conventional techniques. The extremely slow growth of the organism that was eventually isolated in pure cultures could only grow on DMS and DMSO, purity of liquid cultures was routinely checked by plating on to nutrient agar and by phase contrast and electron microscopy.

Respiration measurements. Organisms from the continuous culture were harvested by centrifugation at 16000 g for 10 min at 4 °C, washed once with 50 mm-potassium phosphate buffer pH 7-2 and resuspended in the same buffer. Oxygen uptake rates were determined with a Clark-type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instrument Co., Ohio, U.S.A.) at 30 °C, in a reaction mixture of 2.5 ml. Endogenous oxygen uptake was measured for at least 5 min, then the substrate was added and the oxygen uptake was followed for 5 min. Substrate-dependent oxygen uptake is expressed as nmol O₂ min⁻¹ (mg cell protein)⁻¹.

Analytical procedures. DMS, methanethiol (MS) and acetaldehyde were determined with a Becker 417 gas chromatograph fitted with a Porapak R column; the column temperature was 180 °C and the carrier gas was N₂. Under these conditions DMS and ethanethiol (ES) had the same retention times. Utilization or accumulation of DMS, MS, ES and acetaldehyde by washed cell suspensions or cell-free extracts was measured in stopped 13 ml Vacutainer tubes (Becton-Dickinson, France) containing 1 ml reaction mixture. Incubation was at 30 °C in a water-bath shaker. Samples (0.1 ml) for analysis were taken from the gas phase. Complete equilibrium of the above compounds between the water phase and the gas phase was reached within 10 min under the conditions.
Metabolism of dimethyl sulphoxide

employed. Formaldehyde was determined according to Nash (1953), and sulphate according to Dodgson (1961). Protein contents of cell-free extracts and whole cells were determined by the method of Lowry, using bovine serum albumin as a standard.

Preparation of cell-free extracts. Approx. 1 g wet wt cells was suspended in 3 ml 50 mM-potassium phosphate buffer pH 7-2 containing 2 mM-MgCl₂ and disrupted by ultrasonic disintegration with a Branson B-12 sonifier (10 × 15 s at 0 °C). Whole cells and debris were removed by centrifugation at 27000 g for 20 min at 4 °C and the supernatant was used for enzyme assays.

Enzyme assays. All assays were performed at 30 °C. Spectrophotometric assays were performed with a Beckman DB-GT spectrophotometer. Activities are expressed as nmol substrate consumed or product formed min⁻¹ (mg protein)⁻¹ except for catalase activity which is expressed as ΔA₂₅₀ min⁻¹ (mg protein)⁻¹.

(i) DMSO reductase. The reaction mixture (1 ml) contained: potassium phosphate buffer pH 7-2, 50 mM; NADH, 2 mM; DMSO, 10 mM. The reaction was started by the addition of extract (0-4 mg protein). Time-dependent DMS production was followed gas chromatographically as described above; it was strictly dependent on the presence of both extract and NADH. DMSO reductase was also measured spectrophotometrically by way of the DMSO-dependent oxidation of NADH. The reaction mixture was the same as described above except that the NADH concentration was 0-1 mM.

(ii) DMS mono-oxygenase. The reaction mixture (1 ml) contained: potassium phosphate buffer pH 7-2, 50 mM; DMS, 0-05 mM; NADH, 2 mM. The reaction was started by the addition of extract (1 mg protein). Time-dependent disappearance of DMS was followed gas chromatographically as described above. DMS consumption by extracts was strictly dependent on both NADH and oxygen.

(iii) MS oxidase. The reaction mixture (1 ml) contained: potassium phosphate buffer pH 7-2, 50 mM; MS, 0-5 mM. The reaction was started by the addition of extract (0-4 mg protein). The time-dependent MS disappearance, as determined by gas chromatography, was corrected for the rate of MS disappearance in the absence of extract. Both the enzymic and chemical conversion of MS were negligible in the absence of oxygen. The activity of the enzyme was also determined with an oxygen electrode. Assay conditions were the same as described for the gas chromatographic assay. The reaction was started by the addition of MS gas to a final concentration of 0-5 mM.

(iv) D-2-Hydroxyglutarate dehydrogenase. The reaction mixture (1 ml) contained Tris/HCl pH 8-5, 50 mM; NAD⁺, 1 mM; extract, 0-5 mg protein. The reaction was started by the addition of D-2-hydroxyglutarate to a final concentration of 8 mM and the rate of NADH formation was recorded.

(v) Other enzymes. The following enzymes were assayed according to published procedures: catalase according to Lück (1963); NAD⁺-dependent formaldehyde dehydrogenase according to van Dijken et al. (1976); NAD⁺-dependent formate dehydrogenase according to Johnson et al. (1964); hydroxypyruvate reductase according to Blackmore & Quayle (1970) except that 50 mM-potassium phosphate pH 7-0 was used as a buffer.

Electron microscopy. Cells were washed in distilled water, shadowed with platinum and examined in a Philips EM201 electron microscope.

RESULTS

Isolation and properties of Hyphomicrobium S

From enrichment cultures containing DMSO as the carbon and energy source an organism was isolated which, on the basis of its morphology (Fig. 1), was tentatively identified as a Hyphomicrobium sp. It was designated Hyphomicrobium S. In batch cultures without pH control with DMSO as the substrate the organism grew very slowly, probably due to a rapid decrease in the culture pH owing to the production of sulphuric acid from the substrate. This became apparent during growth of the organism in pH-controlled chemostat cultures, where almost stoichiometric production of H₂SO₄ from DMSO was observed, as judged by the alkali consumption. More than 90% of the sulphur present in DMSO (which was the only source of sulphur in the medium) was recovered in the culture supernatant as sulphate. When the organism was grown in pH-controlled batch cultures the doubling time was 32 h.

Hyphomicrobium S is an obligate methylotroph. It can only grow on DMSO and DMS under aerobic conditions and, unlike many other Hyphomicrobium strains (Harder & Attwood, 1978), is unable to grow aerobically or anaerobically (in the presence of nitrate) on any of the following compounds: ethanol, acetate, methanol, formate, trimethylamine N-oxide, trimethylamine, dimethylamine and methylamine. In addition, dimethyl disulphide, dimethyl sulphone, methanesulphonic acid or thiosulphate did not support growth. Growth of
Fig. 1. Micrograph of a shadowed preparation of DMSO-grown *Hyphomicrobium* S. The bar marker represents 2 μm.

Table 1. *Rates of substrate-dependent oxygen uptake by washed suspensions of DMSO-grown Hyphomicrobium S*

The organism was grown in a DMSO-limited chemostat at a dilution rate of 0.014 h⁻¹. The final concentration of the various substrates was 1 mM except for thiosulphate which was 10 mM. Rates of uptake are expressed as nmol O₂ min⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of oxygen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>90</td>
</tr>
<tr>
<td>DMS</td>
<td>118</td>
</tr>
<tr>
<td>MS</td>
<td>44</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>22</td>
</tr>
<tr>
<td>Formate</td>
<td>10</td>
</tr>
<tr>
<td>Thiosulphate</td>
<td>20</td>
</tr>
</tbody>
</table>

Dimethyl sulphone, methanesulphonic acid and methanol were not oxidized.

the organism on MS could not be tested because of the appreciable auto-oxidation of this compound under aerobic conditions.

Washed suspensions of *Hyphomicrobium* S grown in a DMSO-limited chemostat at a dilution rate of 0.014 h⁻¹ oxidized a number of compounds that may be considered potential intermediates in the metabolism of DMSO (Table 1). On the basis of these results it was decided to investigate the possibility that DMSO was metabolized according to:

\[
\text{DMSO} \rightarrow \text{DMS} \rightarrow \text{MS} \rightarrow \text{HCHO} + \text{SO}_4^{2-}
\]

**Excretion of intermediate metabolites during DMSO or DMS metabolism**

A transient accumulation of DMS from DMSO was detected during aerobic incubations of washed organisms grown on DMSO (Fig. 2a). Under anaerobic conditions such an
Metabolism of dimethyl sulphoxide

Fig. 2. Excretion of intermediates during DMSO, DMS or MS metabolism by washed cell suspensions of *Hyphomicrobium* S. The organism was grown in a DMSO-limited chemostat at a dilution rate of 0.014 h⁻¹. Reaction mixtures (1 ml) contained 2.2 mg cell protein. (a) Accumulation of DMS from DMSO (5 μmol) under aerobic (O) and anaerobic (●) conditions; the arrow indicates the time of addition of formaldehyde (1 μmol). (b) Consumption of MS (10 μmol) in the absence (□) and presence (●) of ES (10 μmol). (c) Accumulation of MS from DMS (10 μmol) in the presence of ES (10 μmol). (d) Accumulation of acetaldehyde (▲) from ES (▼, 10 μmol).

accumulation was only detected when a source of reducing power such as formaldehyde was added (arrow) to the incubation mixture. The stoichiometry of DMS formation indicated that 2 μmol DMSO was reduced upon addition of 1 μmol formaldehyde. Washed suspensions of DMSO-grown organisms were also able to oxidize MS (Fig. 2b). The oxidation of this compound was partly inhibited by ES which enabled a demonstration of the accumulation of MS from DMS (Fig. 2c). Unfortunately it was not possible to follow the formation of metabolic products from MS. This was probably due to both the rapid metabolism of these products and the limited sensitivity of the assay for formaldehyde. However, it was possible to demonstrate accumulation of acetaldehyde from ES in washed suspensions of DMSO-grown cells of *Hyphomicrobium* S (Fig. 2d), a reaction which is probably mediated by the enzyme that oxidizes MS. Therefore, it is not unreasonable to postulate that formaldehyde is a product of MS metabolism in *Hyphomicrobium* S. Although the organism metabolized ES and its product acetaldehyde, it was unable to grow in media containing ES as the sole carbon and energy source.

Activities of enzymes in cell-free extracts

The results described above suggest that DMS and MS are intermediates in the metabolism of DMSO by *Hyphomicrobium* S. Interestingly, the oxidation of DMSO is initiated by reduction of this compound to DMS. As in other bacteria able to reduce DMSO, cell-free extracts of *Hyphomicrobium* S contained NADH-dependent DMSO reductase. This enzyme also showed some activity in the presence of NADPH, but this was approximately 20-fold lower than that observed with NADH.
Cell-free extracts of DMSO-grown organisms catalyzed the utilization of DMS in an aerobic reaction mixture containing NADH (Fig. 3a). When NADH was omitted DMS disappearance was not observed and the same result was obtained when NAD$^+$ was substituted for NADH. Under anaerobic conditions DMS was also not metabolized (data not shown). These results indicate that oxidation of DMS is probably mediated by a mono-oxygenase.

MS was also utilized by cell-free extracts of DMSO-grown organisms under aerobic conditions (Fig. 3b), but in this reaction no cofactor was required. In the presence of boiled extract some MS oxidation took place which must be attributed to auto-oxidation. Under anaerobic conditions MS was not utilized, indicating that the enzyme involved is an oxidase. Many such enzymes generate H$_2$O$_2$ during oxidation of their substrate and it is entirely feasible that this is true in the case of MS oxidase since very high catalase activity was detected in cell-free extracts of DMSO-grown cells.

Table 2 summarizes activities of enzymes which are thought to be involved in the metabolism of DMSO. The successive oxidation reactions of DMS and MS most probably lead to the production of formaldehyde. In cell-free extracts production of formaldehyde was detected from MS, but not from DMS because DMS mono-oxygenase activity was very low. This formaldehyde can be either oxidized by NAD$^+$-linked formaldehyde dehydrogenase and NAD$^+$-linked formate dehydrogenase or assimilated via the homoisocitrate lyase serine
Metabolism of dimethyl sulphoxide

pathway as judged by the activities of hydroxypyruvate reductase and D-2-hydroxyglutarate dehydrogenase (Kortstee, 1980).

**DISCUSSION**

On the basis of the results reported above we propose a pathway for the metabolism of DMSO and DMS in *Hyphomicrobium* S as shown in Fig. 4.

The first step in DMSO metabolism is a reduction mediated by DMSO reductase for which, as in a number of other bacteria, NADH is the preferred electron donor (Zinder & Brock, 1978b; Bamforth, 1980). During growth of *Hyphomicrobium* S on DMSO this NADH is probably supplied by the oxidation of formaldehyde which is generated later in the pathway. This was indicated by the finding that DMSO reduction under anaerobic conditions required the presence of formaldehyde (Fig. 2a). Furthermore, the stoichiometry of DMS formation was such that 2 µmol DMSO was reduced upon addition of 1 µmol formaldehyde, which is compatible with the suggested role of NAD⁺-dependent formaldehyde and formate dehydrogenases in the oxidation of formaldehyde to CO₂.

The oxidation of DMS is by way of a mono-oxygenase for which, again, NADH is the preferred electron donor. Although DMS oxidation by cell-free extracts was readily detected (Fig. 3a), the specific activity of the enzyme as measured by NADH-dependent DMS oxidation was very low (Table 2). The enzyme was very unstable; more than 50% of the activity was lost when cell-free extracts were stored in ice for 2 h. These properties are not unusual for bacterial mono-oxygenases and may partly be attributed to suboptimal assay and/or cell disruption procedures. The products of DMS oxidation are most probably MS (Fig. 2c) and formaldehyde.

MS oxidation by cell-free extracts was readily detected, both by measuring the rate of MS

![Fig. 4. Proposed pathway of DMSO metabolism in *Hyphomicrobium* S.](image-url)
disappearance (Fig. 3b) and by measuring MS-dependent oxygen uptake. The reaction was strictly dependent on the presence of oxygen and showed no cofactor requirement. These findings suggested that MS oxidation was probably mediated by an oxidase which produces H$_2$O$_2$. The very high catalase activity in cell-free extracts (Table 2) is fully compatible with this suggestion. Another product of MS oxidation that has been detected is formaldehyde, but it was only possible to obtain qualitative data on this formaldehyde production. Formation of acetaldehyde from ES, which is probably oxidized by the same enzyme (Fig. 2b), has been demonstrated (Fig. 2d). In the present study the route for the oxidation of the sulphur moiety of DMSO to sulphate has not been elucidated. It must be expected, however, that the enzyme systems involved in thiosulphate oxidation (Table 1) would participate in the oxidation of the sulphur-containing product of MS oxidation.

During DMSO metabolism formaldehyde is generated in two successive oxidations. One of these molecules must be oxidized to CO$_2$ in order to supply the reducing power required for the conversion of DMSO into MS. This leaves one molecule of formaldehyde from which metabolic energy can be generated and from which cell material can be formed. In addition, further oxidation to sulphate of the reduced sulphur compound formed during MS oxidation may generate metabolically useful energy. Carbon assimilation in *Hyphomicrobium* is most probably by the homoisocitrate lyase serine pathway (Kortstee, 1980). The evidence for this is based on the high activity of hydroxypyruvate reductase and on the presence of a D-2-hydroxyglutarate dehydrogenase.

*Hyphomicrobium* S has a very limited potential for the metabolism of carbon and energy sources; in fact, it can only utilize two or, possibly, three substrates for growth. Although the organism must be considered an obligate methylotroph, unlike all other obligately methylotrophic *Hyphomicrobium* sp. isolated to date (see Harder & Attwood, 1978), it is unable to grow in the presence of methanol. Furthermore, the organism differs from many other *Hyphomicrobium* strains in that it is unable to utilize nitrate as a terminal electron acceptor (see Harder & Attwood, 1978). This dependency of *Hyphomicrobium* S on aerobic conditions for growth is no doubt a reflection of the nature of the enzymes involved in DMS and MS oxidation.

It is not known to what extent organisms of the type of *Hyphomicrobium* S play any role in the removal of DMSO or DMS from the biosphere. It also remains to be established whether such obligate methylotrophic bacteria are present in biofilters used for the removal of methylated sulphides from paper-mill waste water, along with obligate chemolithotrophic organisms (Sivelå & Sundman, 1975).

It is a pleasure to acknowledge the help of Dr C. van Eykelenburg in the preparation of Fig. 1 and to thank Mr Kolja van Beek for many stimulating discussions.

**REFERENCES**


physiology and biochemistry of hyphomicrobia. Advances in Microbial Physiology 17, 303–359.


