

**A Thesis Submitted for the Degree of PhD at the University of Warwick**

**Permanent WRAP URL:**

<http://wrap.warwick.ac.uk/106592/>

**Copyright and reuse:**

This thesis is made available online and is protected by original copyright.

Please scroll down to view the document itself.

Please refer to the repository record for this item for information to help you to cite it.

Our policy information is available from the repository home page.

For more information, please contact the WRAP Team at: [wrap@warwick.ac.uk](mailto:wrap@warwick.ac.uk)

Metabolism of Dimethyl Disulphide, Carbon Disulphide  
and other volatile Sulphur Compounds by  
Chemolithoautotrophic Sulphur Bacteria.

by

Neil A. Smith B.Sc.

---

This thesis is presented for the degree of Doctor of Philosophy  
in the Department of Biological Sciences,  
University of Warwick, Coventry.

August, 1988

---

TO MY FAMILY

## C O N T E N T S

	<u>Page No:</u>
List of Contents	i
List of Figures	VIII
List of Tables	XII
Declaration	XIV
Acknowledgments	XV
Abbreviations	XVI
Summary	XIX

### CHAPTER 1 : INTRODUCTION

1.1. <u>THE OCCURRENCE OF VOLATILE ORGANIC SULPHUR COMPOUNDS AND THEIR CONTRIBUTION TO THE GLOBAL SULPHUR CYCLE.</u>	1
1.1.1 The Sulphur Cycle.	1
1.1.2 The contribution of volatile organic sulphur compounds.	1
1.1.3 Concentrations of volatile organic sulphides in the atmosphere.	5
1.1.4 Concentrations of volatile organic sulphides in aquatic and terrestrial environments and emission rates to the atmosphere.	8
1.1.5 Lifetimes of volatile organic sulphides in the environment.	11
1.1.6 Sources of volatile organic sulphides.	12
1.1.7 Mechanisms of organic sulphide production.	13
1.2. <u>OXIDATION OF INORGANIC SULPHUR.</u>	20
1.2.1 Sulphur oxidising bacteria.	20
1.2.2 Pathways of inorganic sulphur oxidation by thiobacilli.	21

1.2.3	Mechanisms of inorganic sulphur oxidation by thiobacilli.	24
1.2.4	Electron transport, energy coupling and growth yields.	25
1.3.	<u>THE METABOLISM OF ONE-CARBON COMPOUNDS.</u>	31
1.3.1	Dissimilatory methylotrophy.	31
1.3.2	Fixation of carbon dioxide (methylotropic autotrophy).	33
1.3.3	Assimilatory methylotrophy via the serine pathway.	35
1.3.4	Assimilatory methylotrophy via the ribulose monophosphate pathway.	37
1.3.5	Electron transport and energy requirement of the three assimilatory pathways.	37
1.4	<u>MICROBIAL METABOLISM OF VOLATILE AND RELATED NON-VOLATILE ORGANIC SULPHUR COMPOUNDS.</u>	42
1.4.1	As sole source of sulphur during aerobic and anaerobic growth.	43
1.4.2	Anaerobic transformations.	43
1.4.3.	Aerobic transformations.	46
 <u>CHAPTER 2 : MATERIALS AND METHODS.</u>		
2.1.	<u>GROWTH OF ORGANISMS IN BATCH AND CONTINUOUS CULTURE.</u>	55
2.1.1	Culture media.	55
2.1.2	Enrichment cultures.	55
2.1.3	Growth in batch cultures.	57
2.1.4	Radiolabelling experiments.	57
2.1.5	Continuous culture.	58

	<u>Page No:</u>
<b>2.2. <u>ANALYTICAL METHODS AND TECHNIQUES.</u></b>	60
2.2.1 Estimation of biomass.	60
2.2.2 Preparation of methanethiol solutions.	61
2.2.3 Estimation of DMDS.	61
2.2.4 Estimation of DMS.	63
2.2.5 Estimation of formate.	63
2.2.6 Estimation of Formaldehyde.	63
2.2.7 Estimation of sulphate.	64
2.2.8 Estimation of thiosulphate and tetrathionate	64
2.2.9 Estimation of thiocyanate	65
2.2.10 Estimation of sulphur.	65
2.2.11 Estimation of nitrate	66
2.2.12 Estimation of nitrite	66
2.2.13 Measurement of oxygen uptake.	67
2.2.14 Gas chromatography and mass spectrometry.	67
2.2.15 Gas chromatography.	68
2.2.16 GC determination of intermediates of organic sulphide metabolism using incubated cell suspensions.	70
2.2.17 Extraction and purification of DNA from isolates.	71
2.2.18 Determination of mol % guanine plus cytosine.	72
2.2.19 Determination of ubiquinone content.	73
2.2.20 Preparation of cell free extracts.	74
<b>2.3. <u>ENZYME ASSAYS.</u></b>	75
2.3.1 Ribulose 1,5-bisphosphate carboxylase.	75
2.3.2 Hydroxypyruvate reductase.	76
2.3.3 Formate dehydrogenase.	77
2.3.4 Formaldehyde dehydrogenase.	77

	<u>Page No:</u>
2.3.5 Catalase.	77
2.3.6 Methyl mercaptan (methanethiol) oxidase.	78
2.3.7 DMDS 'reductase'.	78
2.4. <u>SPECIAL CHEMICALS.</u>	79

CHAPTER 3 : ENRICHMENT AND ISOLATION OF BACTERIA CAPABLE OF GROWTH ON  
METHYLATED SULPHIDES.

3.1. <u>INTRODUCTION.</u>	80
3.2. <u>ISOLATION AND PURIFICATION.</u>	82
3.2.1 Enrichment cultures.	82
3.2.2 Isolation and purification.	83
3.3. <u>CHARACTERISATION OF STRAINS E1-8</u>	85
3.3.1 Determination of the range of compounds used as growth substrates.	85
3.3.2 Some morphological and physiological characteristics of strains E1-8.	87
3.3.3 Incorporation of $^{14}\text{CO}_2$ into cells.	88
3.4. <u>GROWTH OF OTHER THIOBACILLI ON METHYLATED SULPHIDES.</u>	92
3.5. <u>DISCUSSION</u>	93

CHAPTER 4 : METABOLISM OF METHYLATED SULPHIDES BY STRAIN E6

4.1. <u>INTRODUCTION</u>	97
4.2. <u>PHYSIOLOGICAL CHARACTERISTICS OF STRAIN E6</u>	98
4.2.1 pH spectrum, growth rates and yields, and cellular composition.	98
4.2.2 Effect on growth by increased initial DMDS concentrations.	98
4.2.3 Determination of mol % guanine plus cytosine.	100

	<u>Page No:</u>
4.3. <u>FIXATION OF <math>^{14}\text{CO}_2</math> DURING GROWTH OF STRAIN E6 ON DMDS.</u>	104
4.4. <u>CORRELATION BETWEEN DMDS CONSUMPTION AND SULPHATE PRODUCTION.</u>	106
4.5. <u>GROWTH OF STRAIN E6 IN CHEMOSTAT CULTURE.</u>	107
4.5.1 Under DMDS-limitation.	107
4.5.2 Addition of thiosulphate to a DMDS-limited chemostat.	107
4.5.3 Addition of DMS to a DMDS-limited chemostat.	109
4.6. <u>KINETICS OF SULPHUR AND CARBON OXIDATION.</u>	110
4.6.1 Oxidation rates and affinities for substrates.	110
4.6.2 Stoichiometry for DMDS, MT and sulphide oxidation.	114
4.6.3 Oxidation of other thiols and homologues of DMDS.	116
4.7. <u>INHIBITION OF THIOSULPHATE AND TETRATHIOMATE OXIDATION AND THE DETERMINATION OF UBIQUINONES IN DMS GROWN CELLS.</u>	116
4.7.1 Inhibition by NEM.	116
4.7.2 Inhibition by FCCP.	118
4.7.3 Inhibition by HQNO.	119
4.7.4 Determination of ubiquinones.	120
4.8. <u>DISCUSSION.</u>	121

CHAPTER 5 : THE ENZYMOLOGY OF DIMETHYL DISULPHIDE METABOLISM BY

*Thiobacillus thiooxidans* STRAIN E6.

5.1. <u>INTRODUCTION.</u>	126
5.2. <u>EVIDENCE FOR AN INDUCIBLE DMDS (AND DMS) OXIDATION SYSTEM.</u>	127
5.3. <u>EVIDENCE FOR A DMDS 'REDUCTASE'.</u>	129
5.3.1 Evaluation of the assay methods available.	129
5.3.2 Assay of DMDS 'reductase' activity.	129

	<u>Page No:</u>
5.4. <u>EVIDENCE FOR A METHANETHIOL OXIDASE.</u>	133
5.4.1 Consumption of methanethiol by aerobic and anaerobic suspensions.	133
5.4.2 Kinetics of methanethiol oxidation by a crude cell free extract.	133
5.4.3 The involvement of catalase in methanethiol oxidation.	136
5.5. <u>ENZYMES OF ONE CARBON METABOLISM.</u>	138
5.5.1 Ribulose 1,5-bisphosphate carboxylase.	138
5.5.2 Hydroxypyruvate reductase.	138
5.5.3 Formate and formaldehyde dehydrogenases.	138
5.6. <u>DISCUSSION.</u>	140
 <u>CHAPTER 6 : METABOLISM OF VOLATILE AND INORGANIC SULPHUR COMPOUNDS BY <i>Thiobacillus thiooxidans</i> STRAIN TK-m.</u>	
6.1. <u>INTRODUCTION.</u>	143
6.2. <u>GROWTH OF <i>T. thiooxidans</i> TK-m ON INORGANIC SULPHUR COMPOUNDS IN BATCH CULTURE.</u>	144
6.3. <u>GROWTH OF <i>T. thiooxidans</i> TK-m IN CHEMOSTAT CULTURE.</u>	146
6.3.1 Under thiosulphate-limitation.	146
6.3.2 Addition of thiocyanate to a thiosulphate-limited chemostat.	148
6.3.3 Addition of formate to a thiosulphate-limited chemostat.	148
6.4. <u>KINETICS OF SULPHUR AND ONE-CARBON COMPOUND OXIDATION.</u>	149
6.4.1 Oxidation of inorganic sulphur compounds.	149
6.4.2 Oxidation of one carbon and organic sulphur compounds.	151

	<u>Page No:</u>
6.5. <u>GROWTH ON DMS IN BATCH CULTURE.</u>	154
6.5.1 Growth rates and yields.	154
6.5.2 Co-metabolism of other one-carbon compounds.	154
6.5.3 Incorporation of <sup>14</sup> C-labelled formate and formaldehyde during growth on DMS or sulphide.	155
6.5.4 Further evidence for the proposed metabolism of DMS.	157
6.6. <u>THE AUTOTROPHIC, CHEMOLITHOTROPHIC GROWTH OF F. thioparvus TK-m ON CARBON DISULPHIDE.</u>	165
6.6.1 Growth of thiobacilli on carbon disulphide.	165
6.6.2 Growth rate and yield of <i>F. thioparvus</i> TK-m in batch culture on carbon disulphide and carbonyl sulphide, and a comparison with yields on thiosulphate and thiocyanate.	165
6.6.3 Stoichiometry and intermediates of carbon disulphide metabolism.	166
6.6.4 Oxidation of carbon disulphide.	169
6.6.5 Growth, sulphate production and assimilation of carbon dioxide using carbon disulphide or carbonyl sulphide as sole substrates.	169
6.6.6 Determination of the pathway of carbon assimilation during growth on carbon disulphide.	171
6.7. <u>DISCUSSION</u>	174
<u>CHAPTER 7 : CONCLUDING REMARKS AND SUGGESTIONS FOR FURTHER STUDY.</u>	182
REFERENCES	185

## VIII

LIST OF FIGURES

	<u>Page No:</u>
Figure 1.1 The sulphur cycle.	3
Figure 1.2 Dissimilation of methionine in bacteria.	16
Figure 1.3 A proposed scheme of how several important organic sulphur compounds are transformed in natural environments.	18
Figure 1.4 Proposed schemes for thiosulphate oxidation in thiobacilli.	22
Figure 1.5 Thiosulphate oxidation by the periplasmic multi-enzyme system of <i>Thiobacillus versutus</i> .	26
Figure 1.6 Sulphur compound oxidation by <i>Thiobacillus leptodermus</i> .	26
Figure 1.7 Electron transport and phosphorylation in thiobacilli.	27
Figure 1.8 Electron transport and energy conservation in thiobacilli oxidising thiosulphate.	30
Figure 1.9 Growth of bacteria on reduced C-1 compounds.	32
Figure 1.10 The Calvin cycle.	34
Figure 1.11 The serine pathway.	36
Figure 1.12 The ribulose monophosphate pathway.	38/39
Figure 1.13 A simplified scheme of how methylated sulfur compounds may be transformed in the environment.	47
Figure 1.14 Oxidative carbon disulfide and carbonyl sulfide metabolism in rat liver.	49
Figure 1.15 Generalised scheme for DMSO and DMS oxidation by hyphomicrobia and <i>Thiobacillus thioparus</i> TK-m.	53

Figure 2.1	Absorbance spectrum of DMDS in hexane.	62
Figure 2.2	Elution order and retention times of some volatile organic sulphur compounds following GC analysis.	69
Figure 3.1	Differential plots of $^{14}\text{CO}_2$ incorporation versus culture absorbance ( $A_{440}$ ) for strains E3-E7 during growth on DMDS or DMDS.	90/91
Figure 4.1	Culture absorbance ( $A_{440}$ ) versus TOC, protein and dry weight of <i>F. thioeparus</i> strain E6.	99
Figure 4.2	Effect of initial DMDS concentration on the growth of strain E6 in batch culture.	101
Figure 4.3	Absorbance profiles of purified DNA from strain E6, <i>E. coli</i> and <i>F. tepidarius</i> .	102
Figure 4.4	Correlation between DMDS consumption and sulphate production during growth of strain E6.	101
Figure 4.5	Growth of strain E6 in chemostat culture on DMDS.	108
Figure 4.6	Michaelis-Menton and Lineweaver-Burk plots of DMDS, MT, formate and formaldehyde oxidation by strain E6.	113
Figure 4.7	Inhibition of thiosulphate and tetrathionate oxidation by DMDS grown strain E6 cells in the presence of NEM, FCCP and HQNO.	117
Figure 5.1	Growth of strain E6 on DMDS following growth of inoculum cultures on DMDS and thiosulphate.	128
Figure 5.2	Formation of methanethiol from dimethyl disulphide during anaerobic incubations of whole and CTAB permeabilised cell suspensions.	130

	<u>Page No:</u>
Figure 5.3 Methanethiol metabolism by <i>F. thiooparus</i> strain E6 during aerobic and anaerobic incubations of whole cell suspensions.	134
Figure 5.4 Michaelis-Menton and Lineweaver-Burk plots for MT oxidation by cell free extracts of <i>F. thiooparus</i> strain E6.	135
Figure 5.5 Effect of 3-amino-1,2,4-triazole (AT) on the growth of <i>F. thiooparus</i> strain E6 on DMDS and thiosulphate.	137
Figure 5.6 Fixation of $^{14}\text{CO}_2$ by cell free extracts of DMDS grown <i>F. thiooparus</i> strain E6.	139
Figure 5.7 Proposed mechanism of oxidation of DMDS by <i>F. thiooparus</i> strain E6.	141
Figure 6.1 Growth of <i>F. thiooparus</i> TK-m in a thiosulphate limited chemostat culture.	147
Figure 6.2 Kinetics of sulphide, thiosulphate and tetrathionate oxidation by <i>F. thiooparus</i> TK-m.	152/153
Figure 6.3 Assimilation of $\text{H}^{14}\text{COOH}$ by <i>F. thiooparus</i> strain TK-m during growth on DMS.	156
Figure 6.4 Effect of formaldehyde concentration on its assimilation during growth of <i>F. thiooparus</i> strain TK-m on DMS.	158
Figure 6.5 Effect of increased partial pressure of carbon dioxide on the assimilation of formaldehyde during the growth of <i>F. thiooparus</i> strain TK-m on DMS.	159
Figure 6.6 Evidence for an inducible DMS oxidation system in <i>F. thiooparus</i> strain TK-m.	161/162

- Figure 6.7 Inhibition of DMS and thiosulphate oxidation by *F. thiooparus* strain TK-m in the presence of 3-amino-1,2,4-triazole. 163/164
- Figure 6.8 Demonstration by GC/MS of the intermediate formation of COS during growth of *F. thiooparus* strain TK-m on CS<sub>2</sub>. 167
- Figure 6.9 Formation of COS and H<sub>2</sub>S during anaerobic incubation of *F. thiooparus* strain TK-m. 168
- Figure 6.10 Michaelis-Menton and Lineweaver-Burk plots for the oxidation of CS<sub>2</sub> by *F. thiooparus* strain TK-m. 170
- Figure 6.11 Growth of *F. thiooparus* strain TK-m on CS<sub>2</sub> or COS. 172/173
- Figure 6.12 Hypothetical mechanism of CS<sub>2</sub> oxidation by *F. thiooparus* strain TK-m with the intermediate formation of COS in equilibrium with monothiocarbonate. 178
- Figure 6.13 Hypothetic mechanism of CS<sub>2</sub> oxidation by *F. thiooparus* strain TK-m indicated by yield data. 180

LIST OF TABLES

	<u>Page No:</u>	
Table 1.1	Some sulphur compounds of significance in the natural environment and which are produced and degraded by microbiological processes.	2
Table 1.2	Global release of CO <sub>2</sub> , CS <sub>2</sub> and total sulphur emissions from natural sources (Tg S yr <sup>-1</sup> ).	6
Table 1.3	Concentrations of volatile organic sulphides in the environment.	7
Table 1.4	Annual gaseous sulphur emissions from natural sources.	10
Table 1.5	Volatile sulphur compounds produced by microorganisms.	14
Table 1.6	Yields of thiobacilli and other bacteria growing on inorganic sulphur compounds in chemostat culture.	28
Table 1.7	Theoretical ATP requirements for biomass formation in one-carbon assimilation pathways and molar growth yields on some compounds.	40
Table 3.1	Growth of isolates E1 to E8 on inorganic and organic sulphur compounds.	86
Table 4.1	mol % G + C contents of thiobacilli.	103
Table 4.2	Assimilation of <sup>14</sup> C <sub>2</sub> by strain E6 during growth on DMDS.	105
Table 4.3	Oxidation of one-carbon and sulphur compounds by strain E6 previously grown in chemostat culture on DMDS.	111

## XIII

Table 4.4	Kinetic constants for the oxidation of four substrates by strain E6, previously grown in DMDS-limited chemostat culture.	112
Table 4.5	Oxidation of other thiols and homologues of DMDS by organisms previously grown in a DMDS-limited chemostat culture.	115
Table 5.1	Enzymes potentially involved in methanethiol oxidation by <i>F. thioparus</i> strain E6.	132
Table 6.1	Thiosulphate and thiocyanate consumption and nitrite production during anaerobic growth of <i>F. thioparus</i> TK-m using nitrate as the terminal electron acceptor.	145
Table 6.2	Kinetics of oxidation of inorganic sulphur compounds by <i>F. thioparus</i> strain TK-m.	150

DECLARATION

---

The work contained in this thesis is the result of original research conducted by myself under the supervision of Professor D. P. Kelly. All sources of information have been acknowledged by means of reference.

None of the work contained in this thesis has been submitted for any previous degree.

Neil A. Smith.

ACKNOWLEDGMENTS

---

I would like to sincerely thank my supervisor, Professor D. P. Kelly for his invaluable help, encouragement and tuition over the last three years.

My thanks also to all members, past and present, of Environmental Sciences (now Environmental Microbiology) for lending their knowledge and expertise, especially to Dr. Ann Wood for numerous methods and techniques, Mrs Jane Green and Mrs Dorothy Sanders for their technical assistance, Dr. Chris Marfoot for permission to report some of his previously unpublished data, and to Miss Barbara Pike for tea bags and aspirins. Thanks also to all the above for many hours of stimulating discussion, and for making my time at Warwick so enjoyable.

My thanks also to Mrs June Sharratt for typing the manuscript and to my Mum for producing many of the tables.

Finally, a special thanks to Helen for her patience and support over the years.

## A B B R E V I A T I O N S

---

A <sub>440</sub>	-	Culture absorbance (440 nm)
ADP	-	Adenosine diphosphate
AT	-	3-amino-1,2,4-triazole
ATCC	-	American Type Culture Collection
ATP	-	Adenosine triphosphate
BSA	-	Bovine serum albumen
COS	-	Carbonyl sulphide
cpm	-	counts per minute
CTAB	-	Cetyl trimethylammonium bromide
D	-	Dilution rate
DMDS	-	Dimethyl disulphide
DMPT	-	Dimethyl propiothetin
DMS	-	Dimethyl sulphide
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
DSM	-	Deutsche Sammlung von Microorganismen
EDTA	-	Ethylenediaminetetra acetic acid
FCCP	-	Carbonyl cyanide p-trifluoromethoxyphenylhydrazone
GC	-	Gas chromatography
GC/MS	-	Gas chromatography mass spectrometry
HPR	-	Hydroxypyruvate reductase
HQNO	-	2-heptyl-4-hydroxyquinoline-N-oxide
icl <sup>+</sup>	-	Isocitrate lyase positive
K <sub>a</sub>	-	Substrate specificity constant
K <sub>m</sub>	-	Michaelis constant
M	-	Molar

## XVII

$\mu$	-	Growth rate
$\mu_{max}$	-	Maximum specific growth rate
$\mu Ci$	-	microcurie
$\mu mol$	-	micromole
mM	-	millimolar
mmol	-	millimole
mol	-	mole
mol % G + C	-	mole % guanine plus cytosine
MSA	-	Methanesulphonic acid
MT	-	Methanethiol
NAD(H)	-	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	-	Nicotinamide adenine dinucleotide phosphate (reduced)
NEM	-	N-ethyl maleimide
nmol	-	Nanomole
ppb	-	part per billion
ppm	-	part per million
ppt	-	part per trillion
q	-	Specific rate of substrate consumption
RNA	-	Ribonucleic acid
RNAse	-	Ribonuclease
RuBP	-	Ribulose 1,5-bisphosphate
RuBPCase	-	Ribulose 1,5-bisphosphate carboxylase
SDS	-	Sodium dodecyl sulphate
SSC	-	Standard saline citrate
TLC	-	Thin layer chromatography
Tris	-	Tris (hydroxymethyl) aminomethane
v/v	-	Volume to volume
w/v	-	Weight to volume
Y	-	Growth yield
$Y_{max}$	-	Theoretical maximum (or true) growth yield

"There is, therefore, another matter of the volatile kind which waters called sulphureous commonly contain; viz. a subtile vapour, which it is impossible to obtain in a solid form and imparts to these waters, when present in large quantities, the smell of hepar sulphure, putrid eggs or the scouring of a gun."

Joshua Walker, 1784.  
In "Walker on Waters".

SUMMARY

The isolation of a number of strains of bacteria able to grow on dimethyl disulphide (DMDS) and dimethyl sulphide (DMS) as sole sources of energy is described. The isolates came from diverse habitats including soil, peat, marine mud and a freshwater pond, and were morphologically and physiologically best described as thiobacilli capable of growth as Calvin cycle autotrophs on inorganic sulphur compounds, methylated sulphides or thiocyanate. One isolate (EG) was examined in detail and analysis of its DNA showed a mean mol % G + C content of  $60.5 \pm 1.0$  which is in the normal range of *F. thiooparus*. Substrate oxidation kinetics indicated that methanethiol (MT), sulphide, formaldehyde and formate could be implicated as intermediates in DMDS metabolism. Growth yields in chemostat culture on DMDS indicated that energy conservation was probably coupled to the oxidation of formaldehyde and sulphide (derived from DMDS via MT) to  $CO_2$  and sulphide. Further evidence for the proposed oxidation pathway of DMDS was provided by demonstration of activities of a previously uncharacterised NADH-dependent DMDS 'reductase', MT oxidase, catalase, NAD<sup>+</sup>-dependent formaldehyde and formate dehydrogenases, ribulose 1,5-bisphosphate carboxylase and a sulphide oxidising system. This is the first demonstration of the isolation of organisms into pure culture that are capable of growth on DMDS as sole energy substrate.

*F. thiooparus* strain TK-m was found to be capable of growth on carbon disulphide ( $CS_2$ ) and carbonyl sulphide (COS). During growth on  $CS_2$ , GC/MS analysis of the chloroform-extractable volatiles from the culture medium showed the formation of COS as a transient intermediate in  $CS_2$  metabolism. Anaerobic incubation of cell suspensions with  $CS_2$  also showed the production of hydrogen sulphide ( $H_2S$ ) into the headspace. The proposed pathway of  $CS_2$  metabolism by *F. thiooparus* strain TK-m most likely involved its reductive cleavage to COS and  $H_2S$ , COS then undergoing similar hydrolysis to  $CO_2$  and  $\frac{1}{2}S$ . This serves as the first detailed study of microbial  $CS_2$  metabolism.

CHAPTER ONE

INTRODUCTION

---

1.1. THE OCCURRENCE OF VOLATILE ORGANIC SULPHUR COMPOUNDS  
AND THEIR CONTRIBUTION TO THE GLOBAL SULPHUR CYCLE.

1.1.1. The sulphur cycle.

Sulphur occurs in an extremely diverse range of chemical combinations in the natural environment (Table 1.1). The transformations of both inorganic and organic sulphur compounds brought about by the metabolic activities of living organisms appears to have established a steady state situation in the modern natural environment in which a constant flow of sulphur compounds occurs between anoxic and oxic habitats, the oceans, freshwater, atmosphere and terrestrial ecosystems (Kelly, 1988). The summation of these transformations is termed 'The Sulphur Cycle'.

Although in recent years the sulphur cycle has been the subject of many reviews with numerous models being proposed, the general representation (Fig. 1.1.) is of sulphide oxidation, ultimately to sulphate, occurring in aerobic environments and of sulphate reduction to sulphide, in anaerobic environments (Eriksson, 1963; Krouse & McCready, 1979). This, however, tends to overlook the complicated association of numerous sub-cycles, reservoirs or sinks and sulphur fluxes which exist. Much is now known about the biogeochemical cycling of inorganic sulphur through the environment, even if the mechanisms by which individual transformations occur are not fully understood. However, the role of organic sulphur compounds has been greatly under-rated until fairly recently since accurate detection methods have been unavailable.

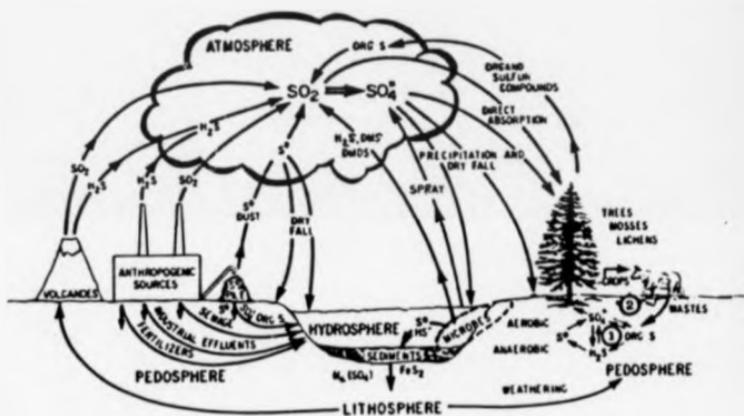
1.1.2. The contribution of volatile organic sulphur compounds.

Until the last decade or so (Bremner & Steele, 1978), hydrogen sulphide ( $H_2S$ ) was regarded as the major biogenic sulphur gas with an estimated

TABLE 1.1 Some sulphur compounds of significance in the natural environment and which are produced and degraded by microbiological processes.

Compound (abbreviation)	Formula	Comment
Hydrogen sulphide	H <sub>2</sub> S	Metabolic intermediate and product of sulphur and sulphate reduction
Dimethyl sulphide (DMS)	(CH <sub>3</sub> ) <sub>2</sub> S	The major volatile metabolic organic sulphur compounds
Dimethyl disulphide	(CH <sub>3</sub> ) <sub>2</sub> S <sub>2</sub>	
Methanethiol	CH <sub>3</sub> SH	Can arise from chemical oxidation of sulphide
Carbon-1 sulphide	CS	
Carbon disulphide	CS <sub>2</sub>	Tetrathionate, S <sub>4</sub> O <sub>6</sub> <sup>2-</sup> is of metabolic significance
Sulphur (S <sup>0</sup> )	S <sub>8</sub>	
Thiosulphate	S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>	Arises largely from combustion of sulphur-containing fuels but sulphite can be oxidised by many organisms
Polythionates	S <sub>x</sub> O <sub>6</sub> <sup>2-</sup>	
Sulphur dioxide	SO <sub>2</sub>	The end-product of many sulphur oxidations
Sulphite	SO <sub>3</sub> <sup>2-</sup>	
Sulphate	SO <sub>4</sub> <sup>2-</sup>	The sulphur amino acids of proteins
Cysteine	H <sub>2</sub> CH-CHNH <sub>2</sub> -COOH	
Methionine	CH <sub>3</sub> -S-CH <sub>2</sub> -CHNH <sub>2</sub> -COOH	Minerals where Mn Fe Cu Co Mo Ni Zn Ag Pb etc In many cases these arose biogenically from metal precipitation by H <sub>2</sub> S produced by bacterial sulphur sulphate and sulphate reduction
Metal sulphides	M <sub>2</sub> S MS MS <sub>2</sub>	
Metal sulphates	MSO <sub>4</sub> M <sub>2</sub> SO <sub>4</sub>	
Thiophene	C <sub>4</sub> H <sub>4</sub> S (heterocyclic ring)	For example, CaSO <sub>4</sub> gypsum which can be reduced by sulphate-reducing bacteria. Some sulphate minerals probably arose from photosynthetic bacterial activity and jarosite minerals can be produced as a result of Fe <sup>2+</sup> oxidation by <i>Thiobacillus ferrooxidans</i>
Butanethiol	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>3</sub> SH	Occurs in numerous compounds, including condensed forms in coal and oil
		Skunks' microbial metabolism not investigated

Figure 1.1 The Sulphur Cycle



global production into the atmosphere from all sources of 142 million tonnes  $S. yr^{-1}$  (Robinson & Robbins, 1970). Total  $H_2S$  production is likely to be much higher than this figure since most of that produced in anoxic environments will subsequently be reoxidised by anaerobic phototrophs, aerobic or denitrifying sulphide-oxidising bacteria or by chemical oxidation (Kelly, 1988). Recent work has tended to undermine the importance of  $H_2S$  as the major source of atmospheric sulphur and it has been shown that some organic sulphides are probably more important. (Lovelock *et al.*, 1972; Rasmussen, 1974).

Volatile organic sulphur compounds are now recognised as having an important role in the biogeochemical cycling of sulphur through the atmosphere. The most important of these are the methylated sulphides: dimethyl sulphide (DMS), dimethyl disulphide (DMDS), methanethiol (MT) as well as carbon disulphide ( $CS_2$ ) and carbonyl sulphide (COS). These compounds are all gases, highly toxic (Ljunggren & Norberg, 1943; Sweetnam *et al.*, 1987; Rodgers *et al.*, 1980; Ashworth *et al.*, 1977; Moubasher *et al.*, 1974) and malodorous (Sivela, 1980; Seluyzhitskii, 1972). In low concentrations DMS is an important odour and flavour component in many foodstuffs including tea, cocoa, beers and milk and many cooked vegetables (Anness, 1981; Anness *et al.*, 1979), but in higher concentrations contributes to spoilage (Keenan & Lindsey, 1968; Bills & Keenan, 1968).

The methylated sulphides appear to have a more significant role, at least in terms of biological turnover, than either  $CS_2$  or COS and the bulk of literature is devoted to these compounds. DMS in particular has been shown to be produced in large amounts in aquatic environments (Andreas & Bernard, 1984) with about half of the estimated total annual global atmospheric

sulphur flux (around 80 million tonnes S) coming from the oceans and about half of this in the form of DMS (Dacey & Wakeham, 1986; Perek *et al.*, 1986).

### 1.1.3. Concentrations of volatile organic sulphides in the atmosphere.

The sulphur content of the atmosphere is comparatively small compared to the hydrosphere and pedosphere and has been estimated at about 1.8 million tonnes (Krouse & McCready, 1979). Atmospheric sulphur arises from both abiotic (anthropogenic and natural) and biotic sources, the former being, by far, the easiest to measure. Biological sulphur fluxes are particularly difficult to estimate since they are so variable with location and season. However, clearly the major sources of atmospheric methylated sulphides are of biotic origin, and whilst  $CS_2$  and COS also arise from similar sources there is also a great input from abiotic sources such as volcanoes (Table 1.2) (Staudler & Peterson, 1984; Krouse & McCready, 1979; Babich & Stotaky, 1978). The abundance of the methylated sulphides is naturally very low (compared to sulphate and sulphur dioxide) thus making accurate estimates of atmospheric concentrations difficult. Since such estimates differ greatly it is not possible to state 'typical' values from the available literature. For example, estimates of DMS concentrations ranged from 30 - 50 ppt (Maroulis & Bandy, 1976) to 1.2 ppb (Lovelock & Maggs, 1976). Other data indicate concentrations of  $CS_2$ , COS and  $H_2S$  to be 0.19, 0.51 (Sandalls & Penkett, 1977) and 6 ppb (Hitchcock, 1976) respectively (Table 1.3).

Low atmospheric concentrations of volatile organic sulphides indicate either that they have little or no role in global atmospheric sulphur pollution or that such measurements just reflect the rate at which these compounds are turned over, possibly producing sulphur dioxide and sulphate. The total amount of sulphur that yearly passes through the atmosphere seems to be

TABLE 1.2 Global release of COS, CS and total sulphur emissions from natural sources ( $\text{Tg S yr}^{-1}$ ).

Source	COS	CS <sub>2</sub>	Total sulphur emissions
Oceans	0.40	0.30	39.2
Wild fires in temperate and boreal forests	0.01	-	-
Inland swamp soils	1.88	2.80	17.4
Salt Marshes	0.12	0.07	1.7
Volcanoes and fumaroles	0.01	-	-
Total	2.42	3.17	58.3

Stuedler and Peterson, 1984.

**TABLE 1.3** Concentrations of volatile organic sulphides in the environment

Compound	Fresh Water ( $\mu\text{g l}^{-1}$ )	Sea Water ( $\text{ng l}^{-1}$ )	Salt Marsh ( $\text{ng l}^{-1}$ )	Air ( $\text{pg l}^{-1}$ )
DMS	70 (a)	97000 (a)	60-3800 (a)	6 (k)
	62 (l)	3.2-84 (j)		120-200 (d)
		7-38 (g)		30-50 (b)
		12 (e)		
		12 (f)		
		120-310 (a)		
		102 (d)		
DMDS	-	14-19 (i)	94-282 (a)	-
MT	-	14-19 (i)	48-144 (a)	-
CS <sub>2</sub>	-	14-19 (i)	76-228 (a)	
		0.5 (f)		190 (c)
CO <sub>S</sub>	-	14-19 (i)	60-180 (a)	510 (c)

\* Hypersaline lake.

- a) Calculated from Wakeham *et al.*, 1984.  
 b) Calculated from Maroulin and Bandy, 1976.  
 c) Calculated from Sandalls and Penkett, 1977.  
 d) Calculated from Andreae and Raemdonck, 1983.  
 e) Calculated from Lovelock *et al.*, 1978.  
 f) Calculated from Lovelock, 1974.  
 g) Calculated from Nguyen *et al.*, 1978.  
 h) Calculated from Franzmann *et al.*, 1987.  
 i) Deprez *et al.*, 1986.  
 j) Andreae and Bernard, 1984.  
 k) Bernard *et al.*, 1982.  
 l) Bechard and Rayburn, 1979.

about 2-10 times as great as industrially released sulphur, 95% of which is in the form of sulphur dioxide (Eriksson, 1963; Robinson & Robbins, 1970; Babick & Stotsky, 1978). However, because of the malodorous nature of the methylated sulphides they can present a great local pollution problem, the odour threshold concentrations of MT, DMS and DMDS being 0.9 - 8.5 ppb, 0.6 - 4.0 ppb and 0.1 - 3.6 ppb respectively (Sivola, 1980). Effluent gases from a sulphate-cellulose mill contained MT, DMS and DMDS at concentrations of 94.0, 16.6 and 21.7 ppm (Sivola, 1975; 1980) and thus greatly exceeded the normal atmospheric concentrations. There are other examples of elevated volatile organic sulphide concentrations (particularly of biological origin) in the air immediately above terrestrial and aquatic environments. It is the biogeochemical processes active in these environments that result in the observed fluxes contributing to atmospheric volatile organic sulphides. These sulphur fluxes are discussed in more detail below.

#### 1.1.4. Concentrations of volatile organic sulphides in aquatic and terrestrial environments and emission rates to the atmosphere.

##### [1] Sea and Freshwater

The literature contains many references to DMS concentrations in aquatic environments, particularly the oceans, reflecting the importance of this compound. There are relatively few references to MT, DMDS, CS<sub>2</sub> or COS. DMS is stated to be insoluble in water (Merck, 1968) but despite its volatility it is now known to be more soluble than was first reported. DMS possesses a dipole moment and saturated solutions at 20°C contain DMS at 1.8% v/v (about 246μM) (Kushlev et al., 1976). The relatively high concentrations of DMS in sea waters compared to those in the atmosphere (Table 1.3) drives a significant ocean-atmosphere flux of DMS (Dacey et al., 1984). The

concentration of DMS in surface waters is proportional to the level of marine primary production and has been estimated on average at between  $0.12 - 84 \text{ ng l}^{-1}$  (Andreae & Barnard, 1984; Andreae, 1985; Lovelock, 1972, 1974; Nguyen, 1978), but the concentration rapidly declines with increasing depth.

These data indicate the importance of surface layer DMS production rather than diffusion from sediments.

A meromictic, hypersaline Antarctic lake contained DMS up to a maximum concentration of  $97 \mu\text{g l}^{-1}$ , this being observed just above the oxic-anoxic interface (Franzmann, 1987). Previous studies of the same water showed that  $\text{COS}$ ,  $\text{CS}_2$ , MT and DMDS were also present but only at concentrations of about  $14 - 19 \text{ ng l}^{-1}$  (Deprez *et al.*, 1986) (Table 1.3). For comparison,  $\text{CS}_2$  at a concentration of  $50 \text{ ng l}^{-1}$  was detected in sea water (Lovelock, 1974) and it was proposed that this compound (and presumably  $\text{COS}$ ) originated from anaerobic mud on the sea bed, this in contrast to DMS (and MT and DMDS) production at the surface (Table 1.3).

Freshwater - atmosphere DMS fluxes will have little effect upon total atmospheric concentrations but high DMS concentrations of about  $62 - 78 \mu\text{g l}^{-1}$  have been detected in freshwater ponds (Bechard & Rayburn, 1979; Wakeham *et al.*, 1984).

Estimates of the total annual ocean-atmosphere DMS-flux are about 39-40 million tonnes S (Andreae & Raesdonck, 1983; Ferek *et al.*, 1986).

#### [41] Inland Soils

Land masses and probably areas of dense vegetation also contribute greatly to volatile organic sulphide fluxes, but there are apparently no reports of their concentrations in such environments. Emission rates of DMS-sulphur

TABLE 1.4 Annual gaseous sulphur emissions from natural sources.

Sulphur Compound (g S m <sup>-2</sup> yr <sup>-1</sup> )	Source		
	Oceanic	Inland non-swamp Soils	Salt Marshes
H <sub>2</sub> S	-	0.097	2.19
DMS	0.105	0.007	1.52
DMDS	-	0.0016	0.34
CS <sub>2</sub>	0.00083	0.0232	0.18
COS	0.0011	0.0156	0.32
<b>Total</b>	<b>0.1069</b>	<b>0.1444</b>	<b>4.55</b>

From Stuedler and Peterson, 1984.

from soils have been estimated at  $11-45 \times 10^{-12} \text{ g. (g dry wt. h)}^{-1}$  corresponding to annual global sulphur emissions of 1.5 - 4.9 million tonnes (Hitchcock, 1975). Emission rates from trees in forest areas ranged from  $2-43 \times 10^{-12} \text{ g. (g dry wt. h)}^{-1}$  (Hitchcock, 1975; Lovelock & Maggs, 1972). Total land emissions of DMS, DMDS, MT,  $\text{CS}_2$  and COS were estimated at  $0.43 \text{ g S (m}^2 \cdot \text{yr)}^{-1}$  (Adams *et al.*, 1981), corresponding to an annual global organic sulphur flux of between 36-110 million tonnes (Grannat *et al.*, 1976; Eriksson, 1963; Adams *et al.*, 1981).

[iii] Inter-tidal and saltmarsh areas

Globally, salt marshes release up to a total of 1.7 million tonnes  $\text{S yr}^{-1}$ , 49% of which is in the form of DMS. Although DMS is the major sulphur species produced in salt marshes, COS and  $\text{H}_2\text{S}$  are the most abundant gases produced from mud flats with average fluxes of about  $0.2 \text{ g S (m}^2 \cdot \text{yr)}^{-1}$  (Aneja *et al.*, 1979). Recent estimates of total global biotic release of volatile organic sulphides, particularly DMS (see above), and the contribution of salt marshes indicate that these areas contribute to only 1.6 - 5.4% of the total global biogenic emissions. However, emission rates of these compounds from salt marshes are higher, per unit area, by one or two orders of magnitude than emissions from either the oceans or inland soils (Table 1.4) (Stuedler & Peterson, 1984; Wakeham *et al.*, 1984; Dacey *et al.*, 1987; Aneja, 1979).

1.1.5. Lifetimes of volatile organic sulphides in the environment.

The lifetime of volatile organic sulphides in the environment (derived from all the above sources) varies quite considerably. For the methylated sulphides it can be measured in terms of hours or days (Andreae & Barnard, 1984) and considering the evolution rates of these compounds, turnover must

be quite phenomenal. In the case of  $CS_2$  and COS, atmospheric lifetimes are about 12 days and 1-2 years respectively, with COS reported as the most abundant sulphur gas in the atmosphere (Khalil & Rasmussen, 1984; Stotsky & Schenk, 1976; Stuedler & Peterson, 1984).

#### 1.1.6. Sources of volatile organic sulphides.

Volatile organic sulphides are produced by animals, plants, fungi, algae and bacteria. DMS and MT are found in the breath of normal human beings (Cooper, 1983), DWDS is an attractant pheromone in hamsters (Singer *et al.*, 1975) and COS has been detected in cattle manure (Elliott & Travis, 1973). DMS, DWDS, MT and  $CS_2$  (in addition to many other volatiles) are produced by plants and crucifers (Lewis & Papavasas, 1970; Whitfield *et al.*, 1981; Stotsky & Schenk, 1976) with the role in plants of preventing the germination of pathogenic fungal spores. MT was also seen to be produced during the growth of *Pentatillium* strain 16 (Sagal & Starkey, 1969). Although small amounts of  $H_2S$ , MT and DWDS have been shown to be produced by blue-green and planktonic algae, the major volatile sulphur compound produced was DMS (Zinder *et al.*, 1977; Bechard & Rayburn, 1974, 1979; Sheridan & Caetenholz, 1968; Jenkins *et al.*, 1967). Undoubtedly the biggest producers of volatile organic sulphides are the bacteria. A whole range of bacteria including *Pseudomonas fluorescens*, *Proteus vulgaris*, *Escherichia coli* and *Clostridium tetani* produce MT, whilst DMS production range of bacteria including *Aerobacter aerogenes*, *Propionibacterium shermanii* (Kadota & Ishida, 1972) and by rumen microorganisms (Salesbury & Herricks, 1975) has also been noted. MT production by coryneform bacteria isolated from dairy and human skin sources and by *Brevibacterium linens* has also been demonstrated (Sharpe *et al.*, 1977; Farchichi *et al.*, 1986). Similarly, several strains of bacteria have been shown to produce DWDS in

pure culture, these included strains of *Lactobacillus*, *Corynebacterium*, *Pseudomonas*, *Alcaligenes* and *Achromobacter* (Tomita et al., 1987).

In addition to the methylated sulphides,  $CS_2$  and COS, microorganisms are known to produce many other potentially important volatile organic sulphur compounds (Table 1.5).

#### 1.1.7. Mechanisms of organic sulphide production.

Methylated sulphides,  $CS_2$  and COS are all evolved from soils as gaseous products of decomposition of organic matter such as animal manure, sewage sludges and plant material, under aerobic and waterlogged conditions (Banwart & Bremner, 1976). Further studies have shown that a major source of these compounds is the catabolism of sulphur containing amino acids. DMS, DMDS and MT were evolved from soils treated with methionine, methionine sulphoxide, methionine sulphone and S-methyl cysteine and  $CS_2$  was evolved when soils were treated with cystine, cysteine, lantionine and djenkolic acid. Only small amounts of DMS and  $CS_2$  (following treatment with homocystine) and COS (with lantionine and djenkolic acid) have been detected (Banwart & Bremner, 1975).

Various bacteria, actinomycetes and filamentous fungi, including many of those mentioned previously (section 1.1.6), can grow on methionine as the only organic substrate and sources of nitrogen and sulphur. Studies of methionine metabolism in bacteria have revealed the mechanism of methylated sulphide production (this being different from the transaminative pathway of methionine degradation in animals (Cooper, 1983)). Methionine was first deaminated to form  $\alpha$ -keto- $\delta$ -methylbutyrate, this then undergoing a demethylation reaction to form  $\alpha$ -keto-butyrate (which was then further

TABLE 1.5 Volatile sulphur compounds produced by microorganisms.

Compound	Formula
Hydrogen sulfide	H <sub>2</sub> S
Sulfur dioxide	SO <sub>2</sub>
Carbon disulfide	CS <sub>2</sub>
Carbonyl sulfide (carbon oxy sulfide)	COS
Methyl mercaptan (methanethiol)	CH <sub>3</sub> SH
Ethyl mercaptan (ethanethiol)	CH <sub>3</sub> CH <sub>2</sub> SH
n-Propyl mercaptan (1-propanethiol)	CH <sub>3</sub> CH <sub>2</sub> CH <sub>2</sub> SH
is-Propyl mercaptan (1-methyl-1-propanethiol)	(CH <sub>3</sub> ) <sub>2</sub> CHSH
Allyl mercaptan (2-propenethiol)	CH <sub>2</sub> =CHCH <sub>2</sub> SH
n-Butyl mercaptan (1-butanethiol)	CH <sub>3</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> SH
is-Butyl mercaptan (2-methyl-1-propanethiol)	(CH <sub>3</sub> ) <sub>2</sub> CHCH <sub>2</sub> SH
Dimethyl sulfide (methylmethane)	CH <sub>3</sub> SCCH <sub>3</sub>
Dimethyl disulfide (methylmethane)	CH <sub>3</sub> SSCH <sub>3</sub>
Dimethyl trisulfide (methylmethane)	CH <sub>3</sub> SSSCH <sub>3</sub>
Ethyl methyl sulfide (methylmethane)	CH <sub>3</sub> CH <sub>2</sub> SCCH <sub>3</sub>
Diethyl sulfide (ethylmethane)	CH <sub>3</sub> CH <sub>2</sub> SCCH <sub>2</sub> CH <sub>3</sub>
Diethyl disulfide (ethylmethane)	CH <sub>3</sub> CH <sub>2</sub> SSCH <sub>2</sub> CH <sub>3</sub>
Dipropyl disulfide (propylmethane)	CH <sub>3</sub> CH <sub>2</sub> CH <sub>2</sub> SSCH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>
Diallyl sulfide (allyl sulfide)	CH <sub>2</sub> =CHCH <sub>2</sub> SCCH=CH <sub>2</sub>
Diallyl disulfide (allyl disulfide)	CH <sub>2</sub> =CHCH <sub>2</sub> SSCH <sub>2</sub> CH=CH <sub>2</sub>
Methyl allyl sulfide	CH <sub>3</sub> SCCH=CH <sub>2</sub>
Allicin (S-allylthio) disulfide	CH <sub>3</sub> CH=CHS <sub>2</sub> CH=CH <sub>2</sub> CH <sub>3</sub>

Brenner and Steele, 1978.

oxidised to carbon dioxide and water) and NT. The production of DMDS was found to be due to the further oxidation of NT (Fig. 1.2). A probable mechanism of DMS production from methionine *via* NT and sulphide is discussed below.

The production of volatile organic sulphides from cysteine is apparently a more complicated process, probably first involving its conversion to methionine, as an intermediate. However, the catabolism of cysteine, *via* 3-mercaptopyruvate, to thiocyanate has been demonstrated (Cooper, 1983), thiocyanate (and isothiocyanate) probably being the source of COS (Munnecke *et al.*, 1962; Sivala, 1980). An enzyme obtained from a strain of *Pseudomonas crociviva* grown on S-methyl cysteine has been shown to catalyse its stoichiometric conversion to NT, ammonia and pyruvate (Kadota & Ishida, 1972). Cysteine desulphydrase enzymes have been demonstrated in a variety of bacteria including *Pseudomonas*, *Corynebacterium*, *Bacillus* and *Citrobacter* species, the function of which is to catalyse cysteine breakdown to yield H<sub>2</sub>S, pyruvate and ammonia (Yamada *et al.*, 1976).

Although the microbial metabolism of sulphur amino acids is likely to be the major source of methylated sulphides in terrestrial environments and that this also undoubtedly occurs in aquatic environments, recent studies have shown other major sources of DMS in marine environments. Dimethyl propiothetin (DMPT) (dimethyl sulphonioacrylate, DMSP) an osmolyte in marine plants, was shown to be decomposed by a *Clostridium* spp. to yield DMS according to the following equation:

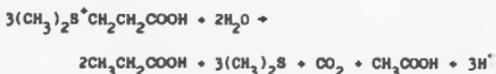
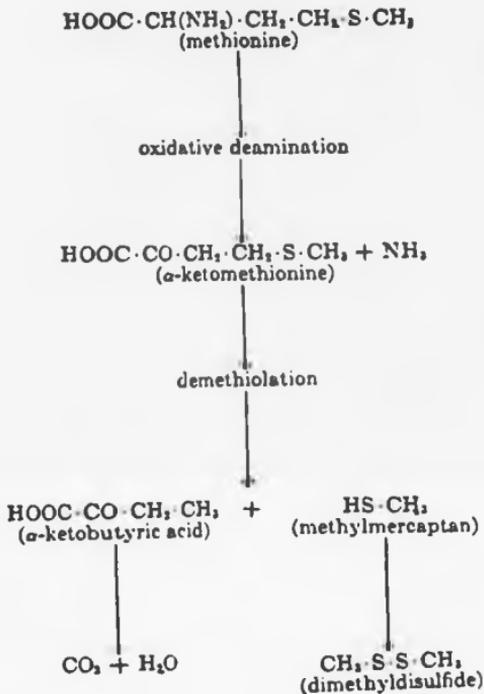


Figure 1.2 Dissimilation of methionine in bacteria.



and a cell free extract of the marine dinoflagellate, *Gyrodinium aureolum* carried out the same reaction (Kadota & Ishida, 1972). In addition to these observations, the phytoplankton *Synechococcus carterus* was shown to produce DMS, via DMPT (Vairavamurthy *et al.*, 1985). In a later study it was observed that the major methylated-sulphur product of DMPT degradation in anoxic salt marsh sediments was DMS, with very little MT produced (Kiene & Capone, 1988). It appears that these anaerobic transformations involving DMPT, acrylate and 3-mercaptopropionate (Fig. 1.3) represent an interesting and important link between sulphide and DMS metabolism. Studies have shown that  $H_2S$  formed from bacterial sulphate reduction can become incorporated into organic matter. A chemical mechanism for the formation of 3-mercaptopropionate has been identified as the abiotic addition of  $H_2S$  to the double bond of acrylate. A natural source of acrylate is DMPT, the enzymatic cleavage of which also yields DMS (Vairavamurthy & Mopper, 1987; Kiene & Taylor, 1988).

Activity of thiol methyl transferase enzymes has been demonstrated in algae, yeasts, protozoa, plants and animals. *Pseudomonas* strain MS isolated from soil, was capable of growth on trimethylsulphonium chloride as its sole source of carbon. During growth on this compound DMS, (plus 5-methyltetrahydrofolate) was formed while the remaining methyl group was incorporated into cell carbon:

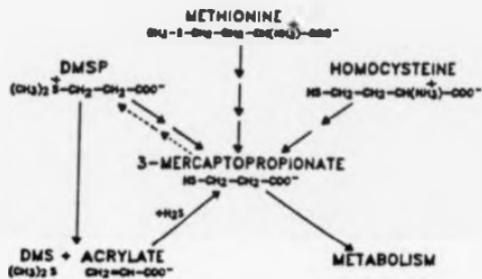


This reaction was catalysed by trimethylsulphonium - tetrahydrofolate methyltransferase which transferred a methyl group from trimethyl sulphonium chloride to tetrahydrofolate (Kadota & Ishida, 1972). More recently similar

LEGEND TO FIGURE 1.3

A proposed scheme of how several important organic sulphur compounds are transformed in natural environments. The central role of 3-mercaptopropionate in these transformations is illustrated. Solid lines represent conversions established in the present work and dotted lines are speculated pathways. Multiple arrows indicate multistep conversions.

Figure 1.3



enzymes have been detected in the majority of heterotrophic bacteria isolated from soil, water, sediment and vegetation. The function of these enzymes is apparently different to the case of *Pseudomonas* MS, being involved in the detoxification of inorganic sulphide by catalysing the transfer of a methyl group, from a suitable methyl donor, to produce MT. Similarly, a second methyl transfer to MT produces DMS. Methyl donors in microorganisms have not yet been sufficiently well defined, but in mammalian systems S-adenosylmethionine has been demonstrated:



Although thiol methyltransferases are common in nature it is not known to what extent they contribute to overall MT and DMS production (Drotar *et al.*, 1987).

## 1.2. OXIDATION OF INORGANIC SULPHUR.

### 1.2.1. Sulphur oxidising bacteria.

The ability to oxidise reduced or partially oxidised inorganic sulphur compounds, at least by some degree, is a property common to many diverse groups of bacteria. Elemental sulphur, sulphide, thiosulphate and polythionates are all oxidised by one or other of: filamentous bacteria like *Beggiatoa*, *Thiothrix* and *Thioploca* (Larkin & Strobl, 1983; Stahl *et al.*, 1987), anaerobic phototrophs like *Chlorobium* and *Chromatium* which use sulphide, sulphur and thiosulphate as electron donors for photosynthesis (Truper & Fischer, 1982), methylotrophic *Hyphomicrobium* which oxidise DMS(O)-sulphur to sulphate (De Bont *et al.*, 1981; Suylen & Kuenen, 1986), extreme thermophiles like the chemolithotrophic *Thermothrix* (Mason *et al.*, 1987) and the acidophilic archaebacteria *Sulfolobus* and *Acidithiobacillus* (Brock *et al.*, 1972), heterotrophs like *Paracoccus denitrificans*, *Aquaspirillum autotrophicum* and some strains of *Pseudomonas* and *Leanobacter* (Friedrich & Mitrenga, 1981; Letti, 1984; Kelly, 1988) and the thiobacilli. Since these bacteria (and undoubtedly many more) are found in diverse aquatic and terrestrial environments they are believed to be responsible for a major part of the biological oxidation of inorganic sulphur in nature.

Since the first observations of their ability to use the energy from inorganic sulphur oxidation to support completely autotrophic growth with no requirement for any organic nutrient, the chemolithotrophic thiobacilli have been the most intensively studied of all the inorganic sulphur oxidisers. The *Thiobacillus* genus contains members whose mole % G + C contents range from 50 - 70, in some cases with zero DNA homology amongst species. There are heterotrophic (Wood & Kelly, 1980; Smith *et al.*, 1980), methylotrophic

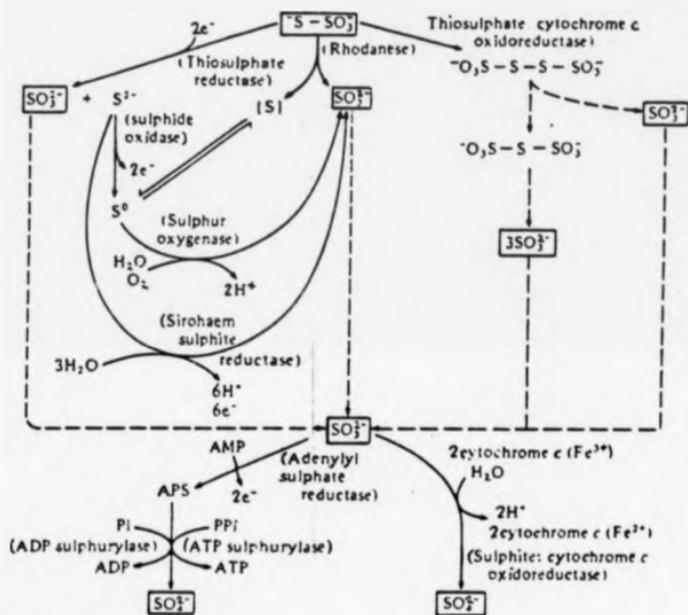
(including 'autotrophic methylotrophs') (Kelly & Wood, 1982, 1984; Kelly *et al.*, 1979) and autotrophic species (Kelly & Tuovinen, 1975; Hampfling & Vishniac, 1967; Kuenen, 1979; Baudaker *et al.*, 1980) as well as facultative and mixotrophic species (Smith *et al.*, 1980; Gottschal & Kuenen, 1980 a,b; Mason & Kelly, 1988). Even among the obligately chemolithotrophic species wide variations are found: temperature optima range from 30 to 43°C and pH optima from 2-7 (Wood & Kelly, 1985, 1986, 1988; Kelly & Harrison, 1988), there is one iron oxidiser: *F. ferrooxidans* (Bounds & Colmer, 1972; Eccleston & Kelly, 1978; Hazell *et al.*, 1986) and one facultative denitrifier: *F. denitrificans* (Timmer-Ten Hoor, 1976, 1981; Justin & Kelly, 1978; Sublette & Sylvester, 1987).

In the past, any non-photosynthetic, Gram-negative rod or coccobacillus with the ability to grow on inorganic sulphur compounds has tended to be conveniently classified as a *Thiobacillus* species. Recent evidence has shown that even between species there are at least two mechanisms by which sulphur can be oxidised and so, irrespective of the various morphological and physiological differences, it remains to be seen whether or not the genus can be more clearly defined.

#### 1.2.2. Pathways of inorganic sulphur oxidation by thiobacilli.

Inorganic sulphur oxidation by thiobacilli has been studied for many years and has been the subject of three recent and comprehensive reviews (Kelly, 1982, 1985, 1988). Despite the wealth of information generated it has not been possible to establish unequivocally the pathway(s) operating, but the latest review (Kelly, 1988) details current knowledge of the processes involved and highlights two distinctive mechanisms existing in two different *Thiobacillus* species (see below). Summarised in Fig. 1.4 are the proposed

Figure 1.4 Enzymes and reactions of thiobacilli that can be amalgamated to produce hypothetical oxidation mechanisms, with thiosulphate as a 'model' substrate.



schemes for thiosulphate oxidation (subsuming mechanisms for sulphide, sulphur, sulphite and polythionate oxidation). There is considerable uncertainty about the fate of the sulphane ( $\bar{S}$ -) atom of thiosulphate ( $\bar{S}\text{-SO}_3$ ) once cleaved by either thiosulphate reductase or rhodanese and the mechanisms of sulphur and sulphide oxidation. It is clear that sulphite ( $\text{SO}_3^{2-}$ ) is produced and there is evidence to suggest that the sulphur oxidising enzyme may be an oxygenase. If this is the case for sulphide and sulphane-sulphur oxidation, some of the oxygen consumed in these oxidations will be incorporated into sulphite and not used in the oxidation of the electron transport chain, thus reducing the available energy yield of the oxidations. The presence or absence of such an oxygenase is thus of great significance in assessing the bioenergetic capabilities of thiobacilli (see below). Polythionates such as tetrathionate ( $\text{S}_4\text{O}_6^{2-}$ ) can be produced from thiosulphate (as an intermediate in thiosulphate oxidation) by a thiosulphate: cytochrome *s* oxidoreductase and can thereafter join the pathway at the sulphite level. Two distinctive pathways for sulphite oxidation to sulphate have been demonstrated: one catalysed by a sulphite: cytochrome *s* oxidoreductase, the other involving the intermediate formation of adenosine phosphosulphate (adenyl sulphate; APS) and known as the 'APS pathway'. The first pathway is commonly found in thiobacilli, the second, if present allows an extra site for ADP or ATP generation by substrate level phosphorylation. In thiobacilli possessing the 'APS pathway', 20% of the total phosphorylation is insensitive to uncouplers of oxidative phosphosulphorylation (Alesh, 1977). Energy (ATP) generation by oxidative phosphosulphorylation is further complicated if a sulphur oxygenase is involved: during aerobic thiosulphate oxidation, two moles of oxygen are consumed per mole of thiosulphate. If an oxygenase functions, only one oxygen could be used for terminal oxidation of the respiratory

chain. With a F/O ratio of one, only two ATP could thus be formed by oxidative phosphorylation for each thiosulphate oxidised. If no oxygenase functions, 4ATP could be produced. If, however, sulphide oxidation allows a P/O ratio of 2, a possible 6ATP could be produced. In short, oxidative phosphorylation is a major (if not only) mechanism of energy conservation in most organisms whilst substrate level phosphorylation (APS pathway) additionally serves only a few (Kelly, 1978, 1982, 1988; Lu, 1983; Lu & Kelly, 1984, 1988 c; Lu *et al.*, 1985). Regardless of variations existing between species, there are several indisputable facts concerning thiosulphate oxidation: the enzyme converting thiosulphate to tetrathionate is present in most thiobacilli; rhodanese appears ubiquitous; and sulphite-oxidising enzymes have been found in all the thiobacilli examined (Kelly, 1988).

### 1.2.3. Mechanisms of inorganic sulphur oxidation by thiobacilli.

Intensive studies of two very different thiobacilli have revealed that they possess different mechanisms of thiosulphate oxidation. These differences and descriptions of the organisms are summarised below.

*F. ferrireducens* (formerly *Thiobacillus* A2) is perhaps the most metabolically versatile of the thiobacilli. It can grow heterotrophically on sugars or acetate (Wood & Kelly, 1980; Gottschal & Kuenen, 1980) or autotrophically on methanol, formate, formaldehyde and methylamine (Kelly & Wood, 1982, 1984; Kelly *et al.*, 1979) or as a chemolithotrophic sulphur oxidiser (Smith *et al.*, 1980). This organism lacks APS reductase and thiosulphate: cytochrome *c* oxidoreductase, it cannot therefore produce tetrathionate from thiosulphate nor metabolise polythionates. Sulphur oxidation occurs in the

periplasmic space between the outer and cytoplasmic membranes. The system comprises a multi-enzyme complex consisting of two enzymes (A and B), two multi-functional cytochromes ( $c_{551}$  and  $c_{552.5}$ ) and a sulphite: cytochrome  $c$  oxidoreductase, but no sulphur oxygenase (Fig. 1.5) (Lu, 1986; Lu & Kelly, 1983 a,b,c, 1984; Lu *et al.*, 1985; Kelly, 1988).

*F. tepidarius* is a moderately thermophilic, obligately chemolithotrophic autotroph (Wood & Kelly, 1985, 1986). It can convert thiosulphate to tetrathionate in the periplasm thus possessing a thiosulphate: cytochrome  $c$  oxidoreductase. Subsequent oxidation of tetrathionate requires interaction with the membrane and possibly transport into the cell. Unlike *F. versutus*, this organism has only one  $c$ -type cytochrome but three membrane bound cytochrome  $b$  components (Fig. 1.6) (Lu & Kelly, 1988 a,b; Kelly, 1988).

#### 1.2.4. Electron transport, energy coupling and growth yields.

Components of respiratory chains in thiobacilli so far identified are cytochromes  $a$ ,  $a_2$ ,  $b$ ,  $c$  and  $c$ , ubiquinones Q8 or Q10 and flavoproteins. They also normally contain succinate and NADH hydrogenases (Fig. 1.7). The oxidation of inorganic sulphur compounds is ultimately by the respiratory chain to oxygen (or nitrate in the case of *F. denitrificans*) as terminal electron acceptor. During thiosulphate oxidation in *F. novellus*, *F. thiooxidans*, *F. neapolitanus* and *F. versutus*, electrons enter the respiratory chain at the level of cytochrome  $c$ , but at the level of flavoprotein or cytochrome  $b$  in *F. denitrificans* and *F. tepidarius*. This suggests that for the latter two organisms, two coupling sites are available for ATP generation by oxidative phosphorylation compared to one site for the majority. This is reflected in the respective yields of these organisms (Table 1.6) (Kelly, 1978, 1982; Lu & Kelly, 1988 c,d.).

LEGEND TO FIGURE 1.5

Thiosulphate oxidation by the periplasmic multi-enzyme system of  
Thiobacillus versutus.

LEGEND TO FIGURE 1.6

Sulphur compound oxidation by Thiobacillus tepidarius

Figure 1.5

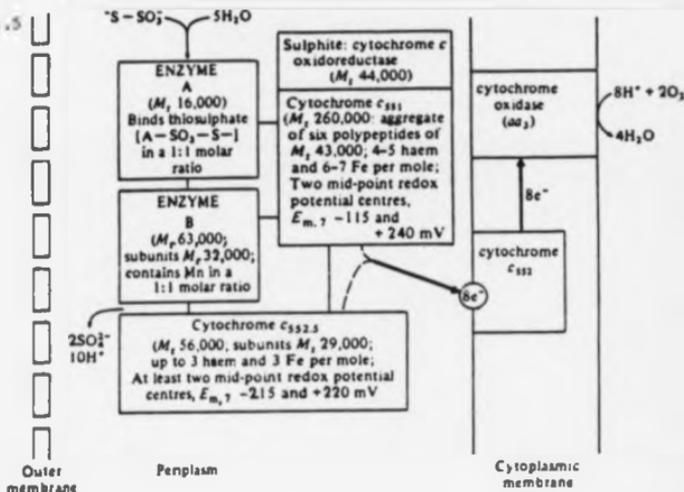
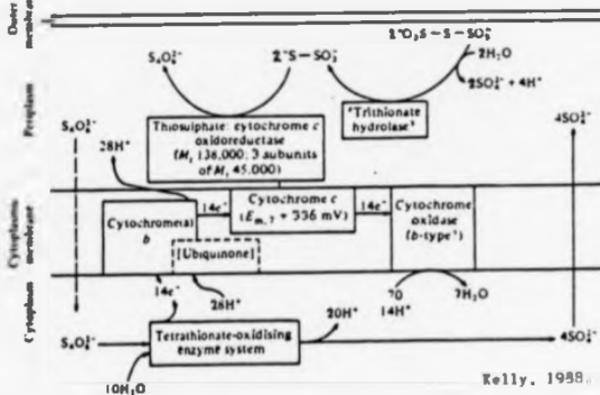


Figure 1.6



Kelly, 1988.

Figure 1.7

Principles of electron transport and phosphorylation in thiobacilli.

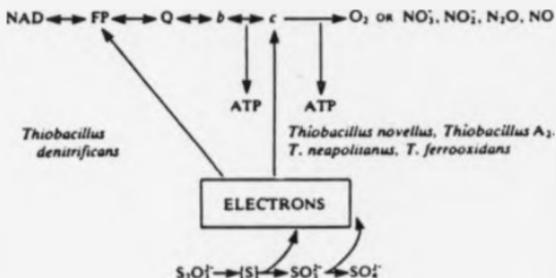


TABLE 1.6 Observed yields (Y) and true growth yields ( $Y^{\max}$ ) of thiobacilli and other bacteria growing on inorganic sulphur compounds in chemostat culture

Data from Kelly (1982) except: a. Wood and Kelly (1986); b. Wood and Kelly (1988); c. Mason and Kelly (1988); d. Friedrich and Mitrenga (1980); e) Mason et al., (1987).

Units of Y and  $Y^{\max}$  are g dry wt organism (mol substrate)<sup>-1</sup>

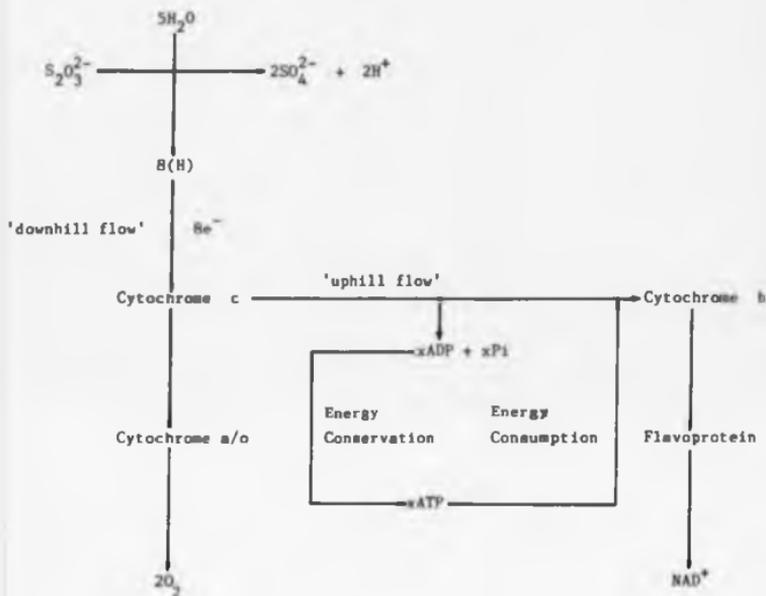
TABLE 1.6

Organism	Substrate	$\gamma_{max}$	Y	D
<u>T. neapolitanus</u>	$S_2O_3$	7.7, 6.3, 6.3 5.5, 8.6. <sup>e</sup>		
	$S_4O_6$	17.2 <sup>e</sup>		
<u>T. versutus</u> (A2)	$S_2O_3$	7.0, 6.4, 8.3 <sup>e</sup>		
<u>T. ferrooxidans</u>	$S_2O_3$	7.5		
	$S_4O_6$	12.2		
<u>T. novellus</u>	$S_2O_3$	7.6	5.7	0.055
<u>T. denitrificans</u>	$S_2O_3$ ( $O_2$ )	14.7		
	$S_2O_3$ ( $NO_3$ )	11.4, 10.0		
	$S^{2-}$ ( $NO_3$ )		9.4	0.03
<u>T. tepidarius</u> <sup>a</sup>	$S_2O_3$	11.0	6.9	0.15
	$S_4O_6$	19.2	14.0	0.15
	$S^{2-}$	9.1	6.9	0.15
<u>T. aquaesulie</u> <sup>b</sup>	$S_2O_3$	11.3	9.3	0.103
	$S_4O_6$		18.7	0.11
<u>T. acidophilus</u> <sup>c</sup>	$S_4O_6$	16.0 <sup>e</sup>	15.6	0.032
<u>T. thiooxidans</u> <sup>e</sup>	$S_2O_3$		6.2	0.034
	$S_4O_6$	12.1		
<u>Thiomicrospira denitrificans</u>	$S_2O_3$ ( $O_2$ )		7.7	0.03
	$S_2O_3$ ( $NO_3$ )		5.2	0.03
	$S^{2-}$ ( $NO_3$ )		5.9	0.03
<u>Th. pelophila</u>	$S_2O_3$ ( $O_2$ )	7.0		
<u>Paracoccus denitrificans</u> <sup>d</sup>	$S_2O_3$ ( $O_2$ )		4.5	(0.14)
<u>Thermothrix thiopara</u> <sup>e</sup>	$S_2O_3$	21.7		
	$S_4O_6$	33.3		

All coupled redox reactions are a 'downhill' flow of reducing potential from more to less reducing reactions. Since the redox values of the probable oxidation steps in the metabolism of inorganic sulphur compounds are not sufficiently electronegative to directly couple the reduction of  $\text{NAD}^+$  or  $\text{NADP}^+$ , the thiobacilli have evolved an energy-dependent 'reversed' electron transport (or 'uphill' flow) for this purpose, which is at the expense of energy conserved during 'downhill' flow (Fig. 1.8). Estimates of the amount of energy required to reduce 1 mole  $\text{NAD(P)}^+$  range between 1-3 moles ATP but can be up to 5 (observed in cell free extracts, not whole cells) depending on whether or not the organism possesses an active adenylate kinase which would re-synthesise a substantial amount of ATP from ADP produced from the reaction of  $\text{NAD}^+$  by ATP-driven reversed electron flow (Kelly, 1978, 1982).

The energy and reducing power generated during the oxidation of inorganic sulphur compounds, irrespective of the actual mechanism involved, is used largely by the chemolithotrophic thiobacilli for the fixation of carbon dioxide by the Calvin cycle, this being their only source of cell carbon. The energy demand of the Calvin cycle is discussed below (see section 1.3.2).

**FIGURE 1.8** Mechanisms of energy conservation and consumption in respiratory chains of thiobacilli oxidising thio sulphate.



Kelly, 1978.

### 1.3. THE METABOLISM OF ONE-CARBON COMPOUNDS.

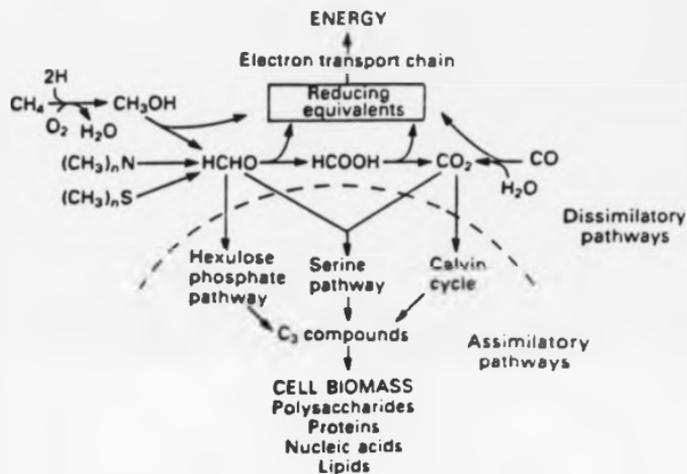
Metabolic transformations of one-carbon compounds can be divided basically into two categories: assimilatory pathways describe mechanisms by which reduced carbon enters cell material and dissimilatory pathways which describe mechanisms of substrate oxidation to yield metabolically useful energy. Only two oxidation products of one-carbon metabolism can be incorporated into cell material, these are formaldehyde and carbon dioxide respectively. Methylootrophs can be divided into three classes according to their physiology: obligate methylootrophs which can grow on only a few reduced one-carbon compounds; 'heterotrophic' methylootrophs<sup>h</sup> which in addition to one-carbon compounds can grow on a variety of multi-carbon compounds; 'autotrophic' methylootrophs which oxidise reduced one-carbon compounds to carbon dioxide and use the energy generated from these oxidations to fix carbon dioxide (Larsen, 1983). It should be noted that organisms capable of growth on one-carbon compound can and have been grouped and categorised under different criteria, for instance classifications based on the 'obligateness' for the one-carbon substrate (Colby *et al.*, 1979; Hanson, 1980).

Biochemically, there are three pathways by which either formaldehyde and/or carbon dioxide is incorporated into cell material by methylootrophs and chemolithoautotrophs (Fig. 1.9), these are briefly described below.

#### 1.3.1. Dissimilatory methylootrophy.

The oxidation of one-carbon compounds (ultimately to carbon dioxide) (Fig. 1.9) can be used to generate energy and reducing equivalents as ATP and NAD(P)H respectively, which are used to fuel carbon dioxide fixation. The oxidation of methane to carbon dioxide involves methanol, formaldehyde

Figure 1.9 Growth of bacteria on reduced  $C_1$  compounds.



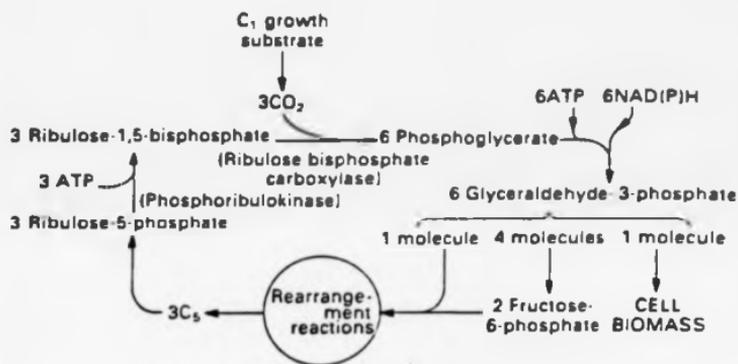
and formate as intermediates, all of which have been detected in one or other of the dissimilatory methylotrophs. Several different formaldehyde and formate dehydrogenases and four methanol dehydrogenases have been detected in methylotrophs, the latter being considerably rarer. Methylated amines and sulphides can be oxidised to formaldehyde by one of two pathways, the first involves dehydrogenases and the second mono-oxygenases (Large, 1983; De Bont *et al.*, 1981; Suylen *et al.*, 1986). Methane oxidation is less widespread even than methanol oxidation and the methane oxidising bacteria (methanotrophs) are obligate. Methanotrophs can be divided into two groups (group I or II) depending upon differences in intracytoplasmic membrane arrangement, carbon assimilation pathways and the type of resting cell produced (Higgins *et al.*, 1981; Galchenko & Andreav, 1984).

### 1.3.2. Fixation of carbon dioxide (methylotrophic autotrophy).

It is a characteristic of chemolithotrophic and methylotrophic autotrophs that the greater part of their cell carbon is obtained by fixation of carbon dioxide by the Calvin (or ribulose bisphosphate or reductive pentose-phosphate) cycle. The fixation of carbon dioxide and its reduction to the level of carbohydrate is a very energy expensive process (Table 1.7). Energy (as ATP) is consumed in the conversion of ribulose monophosphate to bisphosphate by Phosphoribulokinase and (as ATP and NADH) in the conversion of the primary product of carbon dioxide fixation (6-phosphoglycerate) to triose sugars. The other key enzyme of the pathway is ribulose 1,5-bisphosphate carboxylase (Fig.1.10). The stoichiometry of the Calvin cycle is:



Figure 1.10 The Calvin (ribulose 1,5-bisphosphate) cycle of carbon dioxide fixation.



Supplying the Calvin cycle with energy is thus the main energy demand to be met by autotrophs and may account for at least 80% of their total energy requirement for cell biosynthesis (Whittenbury & Kelly, 1977; Kelly, 1978; Large, 1983).

### 1.3.3. Assimilatory methylotrophy via the serine pathway.

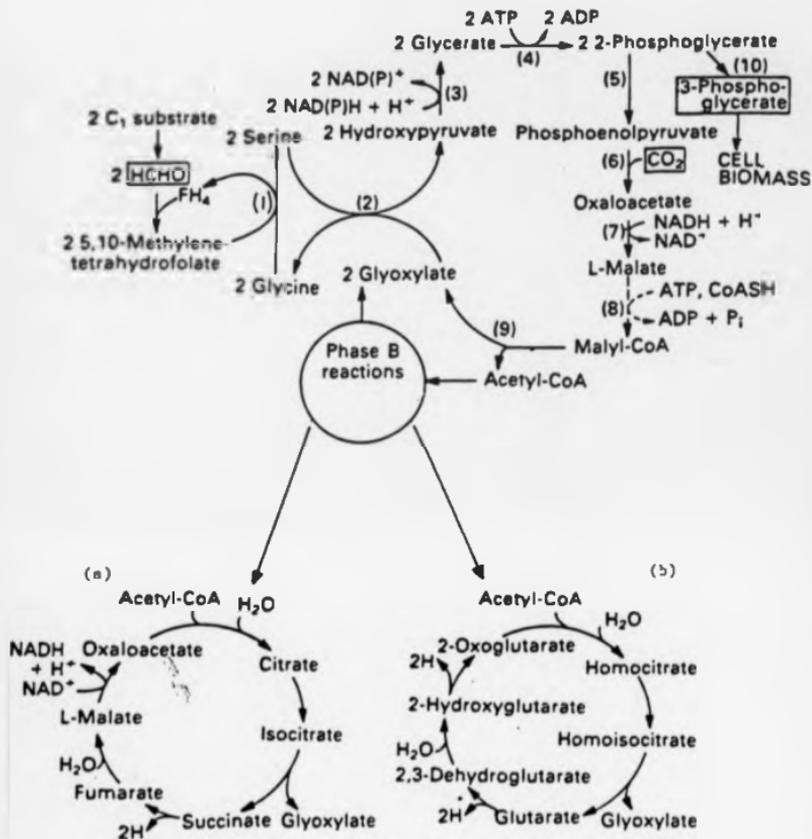
The serine pathway for formaldehyde assimilation (Fig. 1.11) involves the condensation of formaldehyde with glycine (two-carbon unit) and carbon dioxide with phosphoenolpyruvate (three-carbon unit) (Harder *et al.*, 1973) where only 50-70% of the cell carbon comes from formaldehyde. Carbon dioxide is fixed by phosphoenol pyruvate carboxylase (in contrast to the Calvin cycle) (Large, 1983). The three key enzymes of the pathway are hydroxypyruvate reductase, serine hydroxymethyltransferase and serine-glyoxylate aminotransferase. Two variants of the serine pathway exist, differing in the system to convert acetyl-CoA to glyoxylate. The first system (Fig. 1.11a.) involves the enzymes of the tricarboxylic acid cycle and isocitrate lyase (icl) and is known as the icl<sup>+</sup> - serine pathway. In serine pathway methylotrophs which do not possess isocitrate lyase (icl<sup>-</sup>) a second system has been proposed which involves homocitrate and homocitrate lyase (Fig. 1.11b.), although such enzymes have not yet been found. The serine pathway for one-carbon assimilation is favoured by the majority of facultative methylotrophs and type II methanotrophs. (Large & Quayle, 1963; Johnson & Quayle, 1964; Eady & Large, 1968; Battat *et al.*, 1974; Large, 1983).

LEGEND TO FIGURE 1.11

Three phases of the serine pathway: (A), conversion of formaldehyde and  $\text{CO}_2$  into acetyl-coenzyme A; (B), conversion of acetyl-coenzyme A into glycine via glyoxylate; (C), net synthesis of a  $\text{C}_3$  precursor of cell material (3-phosphoglycerate) from glycine and formaldehyde. The enzymes involved are (1), serine hydroxymethyltransferase; (2), serine-glyoxylate aminotransferase; (3), hydroxypyruvate reductase; (4), glycerate kinase; (5), phosphoenolpyruvate hydratase; (6), phosphoenolpyruvate carboxylase; (7), malate dehydrogenase; (8), malyl-coenzyme A synthetase; (9), malyl-coenzyme A lyase; (10), phosphoglycerate mutase.

Alternative mechanisms for phase B of the serine pathway.

Figure 1.11



1.3.4. Assimilatory methylotrophy via the ribulose monophosphate pathway.

Heterotrophic and obligate methylotrophs as well as type I methanotrophs and a few facultative methylotrophs use the ribulose monophosphate pathway (alternatively called the hexulose phosphate, ribose phosphate, allulose phosphate or the Quayle cycle) for the assimilation of formaldehyde. The first step in the pathway is the condensation of formaldehyde with ribulose 5-phosphate to form 3-hexulose 6-phosphate followed by isomerisation of the latter to fructose 6-phosphate. The second step involves cleavage of the hexose phosphate by two alternative routes. By the first route (Fig. 1.12a.) fructose 6-phosphate is cleaved to form dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. By the second route (Fig. 1.12b.) fructose 6-phosphate is converted *via* glucose 6-phosphate into 6-phosphogluconate, which is then cleaved *via* 2-keto-3-deoxy-6-phosphogluconate to glyceraldehyde 3-phosphate and pyruvate. The third stage involves the rearrangement of triose and hexose phosphates to regenerate the ribulose 5-phosphate acceptor. Again there are two possible routes for this, one involving transaldolase the other sedoheptulose biphosphatase (Fig. 1.12c,d.) (Larsen, 1983; Goldberg *et al.*, 1976).

1.3.5. Electron transport and energy requirement of the three assimilation pathways.

Of the three one-carbon assimilation pathways, the ribulose monophosphate pathway is less energy stressful to the cell than the serine pathway, which is less energy demanding than the Calvin cycle (Table 1.7) (Quayle & Ferenci, 1978). Methylotrophs in general are considered to possess only one of the three pathways but recent evidence has suggested that some may possess at least two of the pathways, occupying different roles under varying growth conditions.

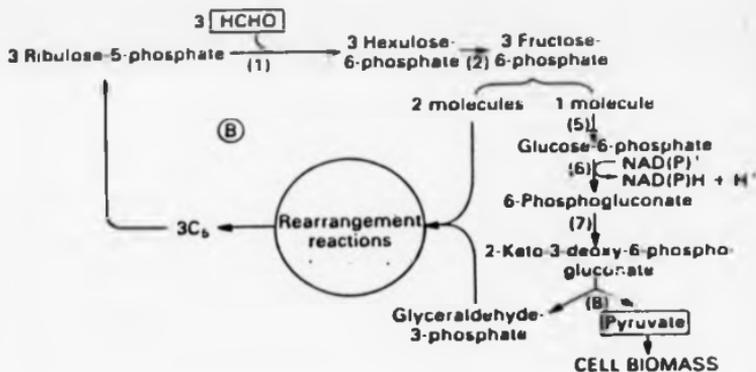
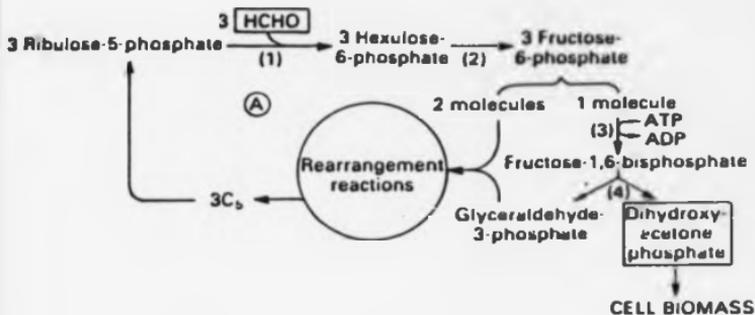
LEGEND TO FIGURE 1.12

The hexulose phosphate pathway of formaldehyde fixation showing the two alternative routes by which hexose molecules may be cleaved. A, the Embden-Meyerhof (fructose biphosphate aldolase) variant.

B, the Entner-Doudoroff (2-keto-3-deoxy-6-phosphogluconate aldolase) variant.

The enzymes involved are: (1), hexulose phosphate synthase; (2), hexulose phosphate isomerase; (3), phosphofruktokinase; (4), fructose biphosphate aldolase; (5), hexose phosphate isomerase; (6), glucose 6-phosphate dehydrogenase; (7), 6-phosphogluconate dehydratase; (8), 2-keto-3-deoxy-6-phosphogluconate aldolase.

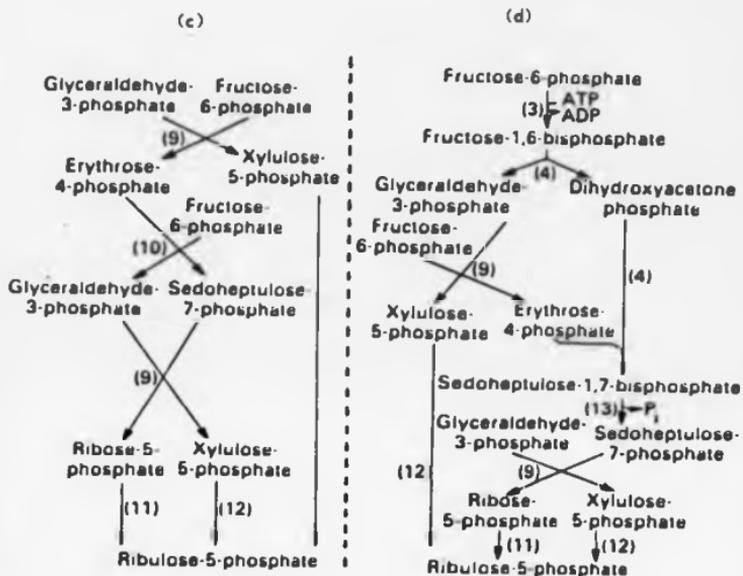
Figure 1.12



LEGEND TO FIGURE 1.12 cont'd

Two alternative routes for the sugar phosphate rearrangement reactions of the hexulose phosphate cycle. (c), involves the enzyme (10), transaldolase; and (d), involves (4), fructose bisphosphate aldolase; (13), sedoheptulose bisphosphatase. Common to both alternatives are (9), tranaketolase; (11), ribose phosphate isomerase and (12), ribulose phosphate 3-epimerase.

Figure 1.12 cont'd.



**TABLE 1.7** Theoretical ATP requirements for biomass formation in one-carbon assimilation pathways and molar growth yields on some compounds

Assimilation Pathway	$y_{ATP}^{max}$	$y^{max}$
Ribulose monophosphate pathway (a)	19.4 - 27.3	15.7 - 17.3 (2)
Serine pathway (b)	12.5	9.8 - 13.1 (2)
Calvin cycle (c)	6.5	(HCHO) 11.0 (3)
		(HCOOH) 3-5 (3)
		(CH <sub>3</sub> OH) 3.5-5.9 (3)
		(CH <sub>3</sub> NH <sub>2</sub> ) 13.0 (3)

$y_{ATP}^{max}$  = Molar growth yield for ATP (g dry wt (mol ATP)<sup>-1</sup>)

$y^{max}$  = Maximum growth yield (g dry wt (mol substrate oxidised)<sup>-1</sup>)

- (1) ATP requirements for biomass formation from methane (a,b) and carbon dioxide (c). (Harder and van Dijken, 1976)
- (2) Growth of *Pseudomonas* spp. and *Methylomonas* spp. on Methanol, methylamine, formaldehyde and formate. (Goldberg *et al.*, 1976)
- (3) Autotrophic growth of *T. versutus* (Kelly and Wood, 1982, 1984; Kelly *et al.*, 1979).

Energy (as ATP) is produced by the reoxidation of NAD(P)H and the prosthetic groups of dehydrogenases reduced during one-carbon oxidation processes. This is by way of electron transport chains and oxidative phosphorylation. Cytochromes  $a$ ,  $a_2$ ,  $b$ ,  $c$  and  $c$  as well as membrane bound and cytoplasmic dehydrogenases have all been identified in methylotropic bacteria, but the actual composition of the chain varies depending on the nature of the one-carbon substrate. For example, cytochrome  $c$  seems to play an essential role in the oxidation of methanol irrespective of the terminal oxidase (i.e. either cytochrome  $a/a_2$  or cytochrome  $c$ ). Other one-carbon substrates such as amines oxidised via dehydrogenases may feed electrons into the chain at the level of cytochrome  $b$  (via a flavoprotein) or via a blue copper-containing protein (amicyanin) which then reduces cytochrome  $c$  (Large, 1983).

1.4. MICROBIAL METABOLISM OF VOLATILE AND RELATED  
NON-VOLATILE ORGANIC SULPHUR COMPOUNDS.

Realisation of the importance of the methylated sulphides and  $CS_2$  and COS to the sulphur cycle has apparently stimulated much research and consequently generated a vast amount of literature describing the various sources of these compounds in the environment. Rather less is known about their biological and/or chemical breakdown. Certainly, chemical oxidation of sulphide to sulphate in sea water is a rapid process (Almgren & Hagstrom, 1974) as is the photooxidation of sulphide (to sulphur dioxide) and DMS (to an organic analogue of sulphate which condenses to form an aerosol) in air. These reactions involving photochemically generated free radicals such as O and OH and resulting in a major loss of both sulphide and DMS from the lower atmosphere (Cox & Sandalla, 1974; Cadle, 1976). It has been shown recently that methanesulphonic acid (MSA) is one of the major oxidation products of DMS in Antarctic waters and has proved far more reliable than measurements of DMS in assessing marine biogenic activity (Saigne & Legrand, 1987). The majority of reports concerning the microbial metabolism of these and related compounds describe 'transformations' or other processes which, although important, are not necessarily linked to energy generating metabolism nor result in their complete turnover. Complete oxidative degradation is apparently restricted to a few aerobic bacteria, the metabolism of these is discussed below. For convenience the microbial metabolism of the methylated sulphides,  $CS_2$ , COS and related compounds is divided into anaerobic and aerobic transformations.

#### 1.4.1. As sole source of sulphur during aerobic and anaerobic growth.

Methanogenic bacteria have long been thought to require fully reduced, inorganic sulphur (i.e. sulphide) as sulphur source (Bhatnagar *et al.*, 1984; Bryant *et al.*, 1971), but recently some organic sulphur compounds at several different oxidation states have been shown to serve as sole sources of sulphur for growth. In addition to inorganic sulphur compounds such as sulphide and thiosulphate, NT, DMS, DMSO and CS<sub>2</sub> supported the growth of *Methanococcus jannaschii*, *Mo. thermoautotrophicus*, *Mo. deltatus* strains ARC and ALH and *Mb. thermoautotrophicum* strains AH and Warburg (Rajagopal & Daniels, 1986).

Similarly, the green alga *Chlorella fusca* strain 211-8b has been shown to grow on more than 100 different sulphur compounds as sulphur sources, including DMS and DMSO (Krauss & Schmidt, 1987).

A heterotrophic bacterium identified as a *Pseudomonas* spp. isolated from activated sludge was found to be capable of using thiocyanate as its sole source of cellular sulphur (and nitrogen) during growth on phenol or succinate (Stafford & Callely, 1969).

Thiourea is generally regarded as toxic to microorganisms since soil nitrification and ammonification are inhibited in its presence, but a *Penicillium* spp. isolated from soil was shown to decompose thiourea during growth on glucose. Sulphate and ammonia were released by the fungus these serving as sole sources of sulphur and nitrogen (Lashen & Starkey, 1970).

#### 1.4.2. Anaerobic transformations.

The production of methane and carbon dioxide from <sup>14</sup>C-labelled NT (and also DMS) during anaerobic incubations of lake sediments was found to be due to biological activity since there was an optimum at 37° but no detectable

activity at 55°C. The stoichiometry of the reaction was as follows:



The resemblance of these sulphur compounds to coenzyme M (2-mercaptoethanesulphonic acid), a methyl group carrier used by methanogenic bacteria, indicated that methanogenic bacteria were probably involved in the anaerobic metabolism of NT and DMS (Zinder & Brock, 1978 a,b).

Subsequent work using specific microbial inhibitors during anaerobic incubations of anoxic salt marsh sediments in the presence of NT, DMS, DMDS and DMSO provided further evidence for the above reactions. The use of 2-bromomethane sulphonic acid (an inhibitor of methanogenesis) indicated that methanogenic bacteria were involved in specific transformation (Kiene & Capone, 1988), and a pure culture of an estuarine methanogen producing NT from DMS has recently been reported (Kiene *et al.*, 1986). The use of sodium molybdate (an inhibitor of sulphate reduction) indicated that sulphate-reducing bacteria could also be involved in similar transformations, but pure cultures have not been obtained. In addition to the mineralisation of methylated sulphides to methane, carbon dioxide and hydrogen sulphide, DMDS was biologically reduced to NT and there was also some evidence to show DMS production from NT, a process probably involving some kind of methyl-transfer reaction (Kiene & Capone, 1988).

DMSO reduction to DMS has been observed in animals (Tiews *et al.*, 1975; Distefano & Borgstedt, 1964), plants (Smale *et al.*, 1975), yeasts (Anness *et al.*, 1979; Bamforth & Anness, 1981) and more importantly in bacteria. Several types of bacteria are capable of anaerobic respiration using DMSO as

a terminal electron acceptor, a property first noted in *Escherichia coli* (Ando *et al.*, 1957; Zinder & Brock, 1978). The redox potential for DMSO reduction to DMS of +160mV (Wood, 1981) lies between those values for fumarate/succinate and nitrate/nitrite (reductions also effected by *E. coli*), and thus the coupling of DMSO reduction to ATP synthesis should present no energetic difficulty. Early biochemical studies of the mechanism of ATP generation included work carried out using a spirillum-type organism (designated strain DL-1) isolated from lake mud, electron transport (using lactate or succinate as electron donors) in this organism apparently involving a *c*-type cytochrome (Zinder & Brock, 1978). Recent enzymological studies of DMSO reduction have revealed that the structure, function and site of dimethyl sulphoxide reductase differs between species of bacteria and between bacteria and yeasts (although in yeasts, DMSO reduction is not linked to energy generating metabolism). In *E. coli* HB101 (Bilous & Weiner, 1985) the photosynthetic bacterium *Rhodospirillum rubrum* (formerly *Rhodospseudomonas capsulata* (McEwen *et al.*, 1985; King *et al.*, 1987) and the yeasts *Saccharomyces cerevisiae* and *S. uvarum* (Anness *et al.*, 1979; Bamforth & Anness, 1981) the preferred electron donors were FADH or NADH, NADH and NADPH respectively. Estimates of  $K_m$  for DMSO reduction were about 170 $\mu$ M, 100 $\mu$ M and 325 $\mu$ M respectively. In both bacteria it appeared that a single enzyme was involved located in the periplasmic space in *Rb. capsulatus* and membrane bound in *E. coli* HB101, the enzyme in *E. coli* HB101 having a requirement for molybdenum as a cofactor. In the case of the yeasts, dimethyl sulphoxide reductase was found to be a multienzyme system (closely resembling methionine sulphoxide reductase) the site of which in the cell was not determined.

Enrichment cultures of anaerobic phototrophic purple bacteria and a pure strain (tentatively identified as a *Thiooysteris* spp.) oxidised up to 10mM DMS

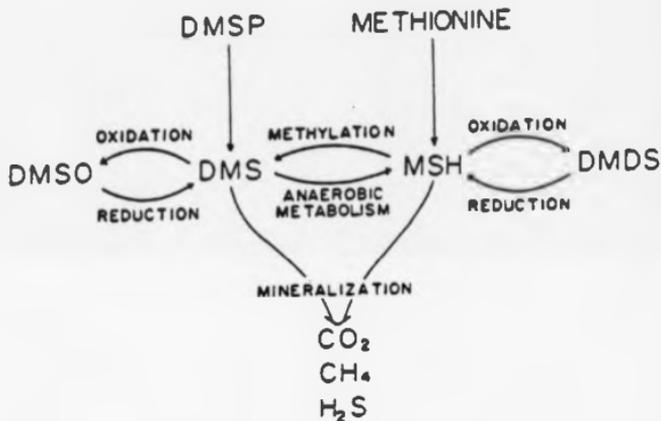
to DMSO, the DMS supporting growth by serving as an electron source for photosynthesis. A determination of the amount of protein produced in cultures and an estimation of the electron balance suggested that the two electrons liberated during the oxidation were quantitatively used to reduce carbon dioxide to biomass (Zayer *et al.*, 1987).

The evidence suggests that in anaerobic environments and in environments of low oxygen tension the inter-conversion of the methylated sulphides from one form to another (which may or may not be directly linked to energy generating metabolism) may have a significant effect upon the cycling of organic sulphur. However, since these transformations are often rapid and do not necessarily result in their degradation or release from such environments it is difficult to estimate their contribution to total biological organic sulphur fluxes (Krouse & McCready, 1979). A summary of some of the anaerobic transformations of methylated sulphides (and some of their probable sources) occurring in the natural environment is given in Fig. 1.13.

#### 1.4.3. Aerobic transformations.

The bacterium responsible for thiocyanate oxidation in sewage effluents first noted by Happold & Key (1937), was later purified and named *Thiobacillus thiocyanoxidans* (Happold *et al.*, 1954). This organism was an obligately autotrophic, chemolithotrophic sulphur oxidiser capable of using thiocyanate as sole sources of energy, carbon and nitrogen. Thiocyanate was first hydrolysed to cyanate and sulphide, cyanate then further hydrolysed to carbon dioxide and ammonia and sulphide oxidised to sulphate. *T. thiocyanoxidans* was later found to be indistinguishable from *T. thioparvus* and despite an isolated report of the anaerobic growth of *T. denitrificans* on thiocyanate (Woolley *et al.*, 1962), its metabolism as a

Figure 1-13 A simplified scheme of how methylated sulphur compounds may be transformed in the environment.



chemolithotrophic substrate is restricted to only a few strains of *F. thioparvus* (Kelly & Harrison, 1988).

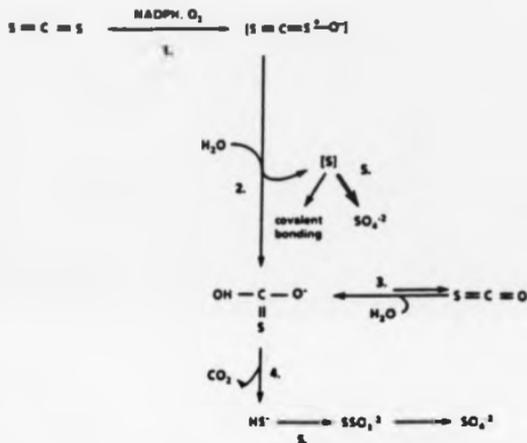
DMS, DMDS, MT, CS<sub>2</sub> and COS have been shown to be absorbed by air-dried and moist soils. It was concluded from experiments with sterilised soils that microorganisms were at least partly responsible for this absorption (Bremner & Banwart, 1976; Bremner & Steele, 1978). The absorption of inorganic sulphur by soils is primarily by chemical and physical means. In contrast, soils absorb organic sulphur gases faster and in greater amounts with increasing molecular weight, absorption of the lower molecular weight and less substituted gases depending upon the build up of an appropriate microbial population (Bohn, 1972). Of the total sulphur present in a forest soil, only 15% was in an inorganic form, the remainder being carbon-bound (including volatile and non-volatile forms). When incubated at 20°C, the organic sulphur was mineralised at rates of 9.2 - 21.0 µg S (g dry wt. wk)<sup>-1</sup> (Strick *et al.*, 1982).

Apart from the observations that CS<sub>2</sub> and COS are probably degraded by soil microorganisms and a brief report of CS<sub>2</sub> oxidation by *F. thiooxidans* (Butler *et al.*, 1969), there are apparently no detailed reports of the microbial metabolism of either CS<sub>2</sub> or COS. The aerobic metabolism of CS<sub>2</sub> has been investigated in isolated rat hepatocytes and liver microsomes, the first step being its oxidation to monothiocarbonate (the hydrated form of COS) and a reactive sulphur species, predominantly by the cytochrome P-450 containing monooxygenase system. Monothiocarbonate was then further metabolised to carbon dioxide and sulphide, a reaction catalysed in part by cytosolic carbonic anhydrase. Both the reactive sulphur species and sulphide were oxidised to non-volatile sulphur compounds, including sulphate, but apparently by different mechanisms (Fig. 1.14) (Chengelis & Neal, 1987).

LEGEND TO FIGURE 1.14

Simplified scheme of oxidative carbon disulphide and carbonyl sulphide metabolism in rat liver. Step 1 is catalysed by the cytochrome P-450 containing monooxygenase. The resulting intermediate breaks down in the presence of water (step 2) producing monothiocarbonate, and reactive sulphur which either binds to microsomal macromolecules or is oxidised to sulphate. Step 3 is the equilibrium reaction between carbonyl sulphide and monothiocarbonate, catalysed by carbonic anhydrase. Step 4 is the conversion of monothiocarbonate to carbon dioxide and hydrogen sulphide ion, which is oxidised (step 5) to thiosulphate and sulphate.

Figure 1.14



As already described, DMSO reduction to DMS is a relatively common property amongst bacteria but the total oxidation of DMSO and DMS to sulphate is apparently a rarer occurrence. Being composed of one or more reduced carbon and sulphur atoms, the methylated sulphides appear to be ideal substrates for methylotrophic and/or chemolithotrophic growth. Prior to my studies, only four obligately aerobic strains of bacteria had been isolated into pure culture which were capable of oxidising DMS and only two of these oxidised DMSO. Perhaps not surprisingly these bacteria are methylotrophs or chemolithotrophs:

[1] *Hyphomicrobium* 8 was isolated from soil samples following aerobic batch enrichment on DMSO and was capable of growth on DMSO and DMS as sole sources of carbon and energy. The maximum specific growth rate on DMSO was  $0.014\text{h}^{-1}$  and suspensions of cells taken from a DMSO-limited chemostat oxidised DMS, MT, formaldehyde, formate and thiosulphate. Enzyme studies indicated that the pathway of DMSO metabolism involved an initial reduction to DMS, which was subsequently oxidised by an NADH-dependent mono-oxygenase to formaldehyde and MT. Further oxidation of MT was by a hydrogen peroxide-producing oxidase, again resulting in the production of formaldehyde. Extracts of DMSO-grown cells contained high levels of catalase and  $\text{NAD}^+$ -dependent formaldehyde and formate dehydrogenases. High levels of hydroxypyruvate reductase indicated that carbon was assimilated by the serine pathway (Fig. 1.11). Only one of the two molecules of formaldehyde produced during DMSO metabolism was assimilated directly, the other being oxidised ultimately to carbon dioxide in order to supply the NADH consumed by the DMSO reductase and DMS mono-oxygenase. Although 90% of DMSO-sulphur was recovered from cultures as sulphate, the sulphur oxidation pathway was not further investigated (De Bont *et al.*, 1981).

[ii] *Hyphomicrobium* EG was isolated by continuous enrichment on DMSO, the purified organism having a high affinity for both DMSO and DMS ( $K_m$  values of 3-6 $\mu$ M), the metabolism of which was similar to that observed in *Hyphomicrobium* S (the major significant difference between the two being that *Hyphomicrobium* S had a maximum specific growth rate of about 0.08h<sup>-1</sup> which enabled much more useful metabolic studies). This organism proved to be an obligate, serine pathway utilising methylotroph, which could also grow on methylated amines (Suylen & Kuenen, 1986). Growth yields observed on DMSO, DMS and methylamine were 16.7, 19.3 and 10.8 g dry weight mol<sup>-1</sup>, respectively. This not only proved that DMSO metabolism was *via* reduction to DMS but also that the organism gained some energy from the oxidation of the sulphur moiety. The addition of sulphide or thiosulphate to methylamine cultures gave increases in biomass corresponding to sulphide- and thiosulphate-dependent yields of 8-10 g dry weight mol<sup>-1</sup>, data which demonstrated the chemolithotrophic potential of the organism (Suylen *et al.*, 1986). Recently the methyl mercaptan (methanethiol) oxidase of *Hyphomicrobium* EG has been purified and studied in some detail. The enzyme was indicated to be a monomer with a molecular weight of 49,000 and represented 8-10% of the total cellular protein. Sulphide was also a substrate for the enzyme with the sulphide-oxidising activity contributing substantially to the total turnover of sulphide (Suylen *et al.*, 1987).

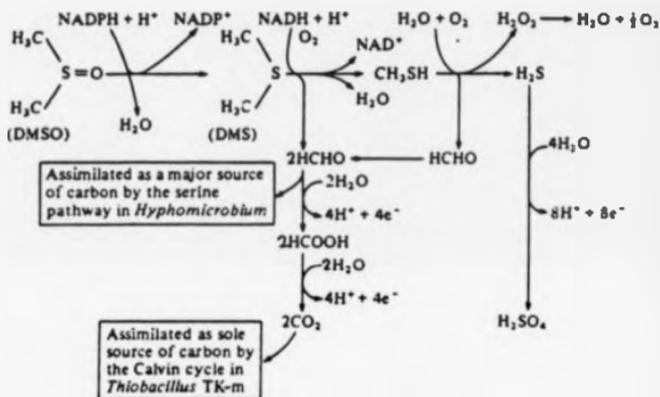
[iii] *Photobacillus* MS1 was isolated from the surface layers of pine bark biofilters used to remove odorous components (including DMS, DMDS and MT) from the effluent gases of a sulphate cellulose mill (Sivela & Sundman, 1975). Energy metabolism of the organism was obligately chemolithotrophic and no growth occurred in the absence of a reduced sulphur compound. Energy derived from the oxidation of thiosulphate was

used to fix carbon dioxide by the Calvin cycle, but carbon dioxide assimilation was repressed when DMS was simultaneously supplied to cultures. During growth on DMS, enzymes for the  $icl^+$ -variant of the serine pathway were induced. Although the methyl groups of DMS were assimilated methylotrophically, energy was provided solely from the oxidation of the sulphur atom (Sivela, 1980).

[iv] *Thiobacillus thiooparus* strain TK-m was derived from *T. thiooparus* strain TK-1, this organism was originally isolated from activated sludge and shown to be involved in the degradation of the pesticide breakdown product O,O-dimethyl phosphorodithioate (Kanagawa *et al.*, 1980; 1982). This organism grew as an autotrophic chemolithotroph on thiosulphate and the  $^{14}C$ -labelled carbon dioxide incorporation pattern also indicated autotrophic growth on DMS. The growth yield of *T. thiooparus* strain TK-m on DMS of 12-13 g cell-carbon  $mol^{-1}$  was consistent with that expected for an autotroph rather than a methylotroph (Fig. 1.15) (Kanagawa & Kelly, 1986).

It appears therefore that there is some metabolic diversity even amongst these few methylated sulphide-users so far described and there are a number of biochemically significant observations which can be made on these systems. *T. thiooparus* strain TK-m, although an obligate chemolithotroph, has been proposed to derive some metabolic energy from the oxidation of the methyl groups of DMS, presumably from the dissimilation of formaldehyde, via formate, to carbon dioxide. This represents an interesting 'methylotrophic', autotrophic mode of metabolism. Energy for the growth of *Thiobacillus* MS1 was derived solely from the oxidation of inorganic or organic sulphur. In the presence of both thiosulphate and DMS, cell carbon was derived preferentially from the assimilation of formaldehyde (via the

**Figure 1.15** Generalised scheme for DMSO and DMS oxidation by hypomicrobia and *Thiobacillus* TK-m.



serine pathway) than from carbon dioxide fixation (via the Calvin cycle), the latter process being more energy demanding. *Hyphomicrobium* EG was obligately methylotrophic, requiring reduced C-1 compounds such as DMS or methylamine for growth. The chemolithotrophic potential of the organism was demonstrated by its ability to oxidise thiosulphate or sulphide (in the presence of a reduced carbon source) the yield data for which indicated that substantial energy was conserved, and presumably used to fuel formaldehyde and carbon dioxide assimilation by the serine pathway. There are only a few other methylotrophs described possessing chemolithotrophic abilities: *F. verustus* and *Paracoccus denitrificans* which can grow autotrophically on methanol (Kelly & Wood, 1982; Cox & Quayle, 1975) and reduced sulphur compounds (Gottschal & Kuenen, 1980; Friedrich & Mitrenga, 1981); *Hyphomicrobium* strain 53-49 which could grow autotrophically on hydrogen (Uebayasi *et al.*, 1981, 1985). Interestingly, a range of other *Hyphomicrobium* species have been isolated which were capable of methylotrophic growth on methylamine, dimethylamine, dimethyl sulphate ( $(\text{CH}_3)_2\text{SO}_4$ ) and monomethyl sulphate ( $\text{CH}_3\text{SO}_4^-$ ), but not on DMSO or DMS (Ohisalba & Kuenzi, 1983). This possibly indicates either an intolerance to reduced sulphur or the absence of an organic/inorganic sulphide oxidising system.

Demonstration of the differences in the aerobic metabolism of the methylated sulphides existing between members of the same genus and between members of different physiological groups of bacteria is of considerable microbiological and biochemical interest. Because of the role of these compounds as possible growth substrates, one might expect therefore that the natural environment harbours a complete spectrum of bacteria with combinations of methylotrophic and chemolithotrophic methylated sulphide oxidising capabilities.

CHAPTER TWO

MATERIALS AND METHODS

---

## 2.1. GROWTH OF ORGANISMS IN BATCH AND CONTINUOUS CULTURE.

### 2.1.1. Culture Media.

The mineral salts medium used for enrichment cultures, and for all batch and continuous cultures of pure isolates contained ( $\text{g l}^{-1}$ ):

$\text{KH}_2\text{PO}_4$ ,	2
$\text{K}_2\text{HPO}_4$ ,	2
$\text{NH}_4\text{Cl}$ ,	0.4
$\text{Na}_2\text{CO}_3$ ,	0.4
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,	0.2
3 ml vitamin mixture (Kanagawa <i>et al.</i> , 1982)	
1 ml trace metal solution (Tuovinen & Kelly, 1973)	

Initially at 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. Pure cultures were maintained on agar medium with 20mM thiosulphate.

### 2.1.2. Enrichment cultures.

The following were added to 200 ml unsterilised mineral salts medium, with or without yeast extract ( $0.1 \text{ g l}^{-1}$ ), in 500 ml bottles:

soil, compost and peat,	20 g air dried weight
manure,	5 g wet weight
marine mud and sediment,	10 g wet weight
water samples were diluted	50 : 50.

The bottles were then sealed with rubber 'subaseal' stoppers. DMS or DMDS were injected through the 'subaseal' using a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) to give an initial concentration of 0.5mM. The bottles were incubated at 30°C with shaking at 100 r.p.m. for three days during which time any fall in pH was noted and cultures re-adjusted to pH

6.8 - 7.0 with NaOH. After three days incubation the contents of the bottles were allowed to settle for 30 minutes after which 40 ml of the upper phase was replaced with 40 ml fresh mineral salts medium. The bottles then being re-stoppered and a further 0.5mM DMS or DMDS injected. This medium replacement procedure was repeated every 3 days until the upper phase became visibly turbid at which point the substrate concentration was progressively raised by 0.5mM steps to a final concentration of 2mM, over a period of two to four months.

When the enrichment cultures were oxidising 2mM substrate in 2-3 days, 25 ml of the upper phase was removed and transferred to sterile 250 ml 'Quickfit' flasks containing 25 ml sterile mineral salts medium. After sealing with 'subseal' stoppers, DMS or DMDS (2mM) were injected. Subculturing was subsequently carried out every 5-7 days (10% v/v inocula).

Isolation and purification of DMS- and DMDS-oxidising organisms was attempted by streaking liquid culture samples onto solid media containing one of the following compounds:

thiosulphate,	20mM;
methylamine or dimethylamine,	20mM;
formate,	20mM;
glucose,	10mM

or onto nutrient agar.

Plates were incubated at 30°C. Single colonies which developed on any of these plates were subsequently transferred back into liquid medium (10 ml) containing 2mM DMS or DMDS. This procedure was repeated several times on isolates which grew successfully until purity was assured. Isolation of pure cultures by this enrichment procedure took up to 10 months.

### 2.1.3. Growth in batch cultures.

Unless otherwise stated, aerobic batch culture experiments were carried out using 50 ml culture volumes in 250 ml 'Quickfit' flasks sealed with rubber 'subaseal' stoppers. Additions of DMS, DMDS or CS<sub>2</sub> (routinely at 2mM) were injected into cultures using a Hamilton syringe. NT and COS were added either as unsterilised gas or in solution, (see section 2.2.2). Elemental sulphur was added to the mineral salts medium as 'flowers' of sulphur (10 g l<sup>-1</sup>). Sulphide was filter sterilised prior to addition to sterile mineral salts medium. To minimise any auto-oxidation of sulphide, several additions of freshly prepared sulphide (1-2mM) were made during growth. The possible auto-oxidation of NT to DMDS in aerobic cultures could not be discounted but appeared to be unimportant. All other substrates were sterilised in the mineral salts medium.

Anaerobic growth of isolates able to use nitrate as the terminal electron acceptor was determined in 100 ml flat bottles completely filled with culture. The mineral salts medium was supplemented with potassium nitrate (1 g l<sup>-1</sup>). The bottles were loosely capped and incubated at 30°C. Cultures were sampled periodically and assayed for the levels of substrate, nitrate and nitrite.

### 2.1.4. Radiolabelling experiments.

Radiolabelling experiments to measure the incorporation of <sup>14</sup>CO<sub>2</sub>, H<sup>14</sup>COOH and H<sup>14</sup>CHO into isolates during growth on DMS, DMDS or CS<sub>2</sub> were carried out using cultures in sealed flasks as described above. Cultures (50 ml), from which Na<sub>2</sub>CO<sub>3</sub> had been omitted were supplemented with either:

- 2.5 or 10mM NaH<sup>14</sup>CO<sub>3</sub> (# approximately 10μCi mmol<sup>-1</sup>)
- 10mM H<sup>14</sup>COONa (# approximately 4μCi mmol<sup>-1</sup>)
- 0.5 or 1mM H<sup>14</sup>CHO (# approximately 50μCi mmol<sup>-1</sup>)

Culture samples (2 ml) were filtered through 25 mm Whatman membrane filters (0.2 $\mu$ m), washed with 2 x 3 ml distilled water and counted in "Optiphase Safe" scintillant (LKB Instruments Limited, Croydon, Surrey, U.K; 10 ml) using a Beckman LS 7000 spectrometer (Beckman Instruments, Inc, Irvine, California, U.S.A.). The specific activities (cpm. $\mu$ mol<sup>-1</sup>) of the labelled compounds used were calculated at the start of each experiment and are given in the appropriate results section.

#### 2.1.5. Continuous culture.

All chemostat culture work was carried out using a 750 ml culture volume in a one litre water-jacketted vessel (LN Engineering, Stoke Poges, Bucks, U.K.). The cultures were maintained at a temperature of 30  $\pm$  1 $^{\circ}$ C with stirring at 750 r.p.m. and aeration with air at 600 ml.min<sup>-1</sup>. pH control, if required, was by automatic titration with 0.5N K<sub>2</sub>CO<sub>3</sub>. Non-volatile growth-limiting substrates such as thiosulphate and thiocyanate (as KSCN) at 20-100mM, were metered separately into the culture vessel at about 1/10th of the rate of the mineral salts medium to give a final input concentration of 2-10mM. Any other additional substrates were again metered separately into the culture vessel.

Several necessary modifications to the conventional chemostat culture system were required when using volatile compounds such as DMS or DMDS as the growth limiting substrates. Silicon rubber tubing was replaced by black butyl or Viton tubing (Watson-Marlow, Falmouth, Cornwall, U.K.) for all connections from the reservoirs to the vessel, since these proved satisfactory in preventing the loss of DMS and DMDS. To minimise any further loss of the growth limiting substrate and given the difficulty in accurate determinations of high concentrations of dissolved DMS or DMDS, volatile substrates were not metered separately into the culture vessel as

described above. Instead, unsterilised (but presumptively sterile) DNS or DWDS was added to the medium reservoir to give a final concentration usually of 2mM. Constant stirring of the medium in the reservoir ensured homogeneous solution of the substrate and prevented the occurrence of any concentration gradients. An air filled balloon was attached to the air inlet to prevent the loss of substrate and to allow for decrease in volume of the residual medium in the reservoir. A 'subseal' port was positioned close to the medium inlet to facilitate sampling of the medium and to enable accurate determination of the input substrate concentration. DNS or DWDS medium was forced into the culture through the aeration tube below the impeller, thus ensuring instant mixing and the prevention of any loss of substrate from the culture. Purity of the culture was determined by periodically plating out samples onto a rich medium on which the isolates could not grow.

Chemostat culture techniques are used to estimate the energy efficiency (growth yield,  $Y$ ) of cultures grown on specific substrates, and have been invaluable in the past to estimate yields of thiobacilli grown on inorganic sulphur compounds where batch culture yield data has been unreliable. Theoretical maximum (or true) growth yield,  $Y^{max}$ , corrected for energy expended in cell maintenance, can be calculated graphically from the following equations (Pirt, 1965, 1975, 1982).

$$\frac{1}{Y} = \frac{1}{Y^{max}} + \frac{m}{\mu} \quad (1)$$

$$\text{or } q = \frac{\mu}{Y^{max}} + m \quad (2)$$

where  $m$  = maintenance coefficient,  $\mu$  = specific growth rate (equal to  $D$ , dilution rate, when the culture is at steady state),  $q$  = specific rate of substrate consumption. From equation (1), it follows that if  $m$  is constant,

the graph of  $\frac{1}{Y}$  versus  $\frac{1}{u}$  will be a straight line with slope  $m$  and intercept  $\frac{1}{Y^{\max}}$  on the ordinate axis. Alternatively,  $Y^{\max}$  and  $m$  may be estimated from the graph of  $q$  versus  $u$  (equation 2). Statistically, equation (1) provides a more accurate representation of the maintenance coefficient than does equation (2), but equation (2) provides a more accurate value of  $Y^{\max}$  than does equation (1), following graph plotting by linear regression analysis (Fieschko & Humphrey, 1984).

## 2.2. ANALYTICAL METHODS AND TECHNIQUES.

### 2.2.1. Estimations of biomass.

Growth of cultures was monitored by measuring absorbance values at 440 nm in a Unicam SP1700 spectrophotometer. Samples from chemostat cultures were used to prepare calibration curves relative to absorbance at 440nm ( $A_{440}$ ) of biomass in terms of ( $\text{mg l}^{-1}$ ): Total organic carbon (TOC), protein or dry weight. Pellets of cells centrifuged in a micro-angle centrifuge (Baird & Tatlock (London) Limited, Romford, Essex, U.K.) from 4 ml culture were assayed for TOC and protein:

TOC was determined in aqueous suspensions of pellets using a Beckman Total Organic Carbon Analyser, Model 905B.

Protein was determined according to Lowry *et al.* (1951), after dissolving the cells in 2 ml 0.5M NaOH for 30 minutes. An alkaline copper reagent was prepared as follows and contained: 98 ml 2% (w/v) sodium carbonate in 0.1M sodium hydroxide, 1 ml 1% (w/v) copper sulphate and 1 ml 2% (w/v) sodium

potassium tartrate. 5 ml of this reagent was then added to 1 ml of the dissolved cell sample, diluted as necessary, mixed well and left at room temperature for 10 minutes. Folin-Ciocalteu reagent was diluted 1.5 parts to 1 part distilled water just before use, and 0.5 ml was added to the samples, mixed and allowed to stand for thirty minutes. Absorbance was measured at 700 nm against a reagent blank. Protein was estimated from a standard curve constructed using bovine serum albumin over a concentration range of 0-300 $\mu\text{gml}^{-1}$ .

Dry weights were determined by drying a centrifuged, washed and re-suspended suspension of cells in distilled water to constant weight at 105°C.

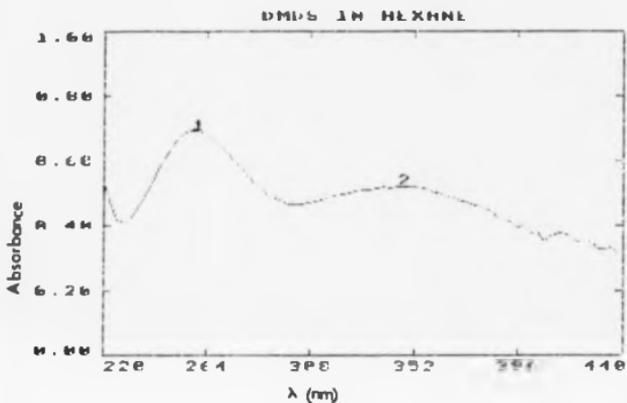
#### 2.2.2. Preparation of methanethiol solutions.

Methanethiol (MT) solutions were prepared as described by Suylen *et al.*, (1986). Because of the tendency of MT to auto-oxidise to form DMDS in aerobic environments all solutions were prepared in deoxygenated distilled water. Distilled water (20 ml) in 'subaseal' stoppered universals was deoxygenated by bubbling with nitrogen gas for 30 minutes. MT gas was then bubbled through for 15 minutes to produce a saturated solution containing about 300mM MT. This was then diluted 1 in 100, the actual concentration being determined by TOC measurements and unless otherwise specified was equivalent to 2.7mM for use.

#### 2.2.3. Estimation of DMDS.

DMDS in aqueous solution (1 ml) was extracted with hexane (2 ml). The DMDS peak height of the upper (hexane) phase was measured at 260 nm against a hexane blank and the DMDS concentration determined by reference to a

Figure 2.1 Absorbance spectrum of DMDS in n-hexane (2ml) following extraction from aqueous solution (2mM).



calibration curve covering a concentration range of 0-6mM. Fig. 2.1 shows the spectrum of DMDS dissolved in hexane.

#### 2.2.4. Estimation of DMS.

DMS in culture samples (0.2 - 0.5 ml) was determined according to the method described by Kanagawa & Kelly, (1986). DMS was extracted with 5 ml 2,2,4 - trimethyl pentane and 2 ml of this solution was mixed with 2 ml 0.2% (w/v) iodine in trimethyl pentane. DMS was estimated from the peak height at 300 nm by reference to a calibration curve covering a DMS concentration range of 0-6mM.

#### 2.2.5. Estimation of formate.

Formate was determined according to Bettat *et al.*, (1974), by noting the change in absorbance at 450 nm when 1 ml of potassium permanganate solution ( $0.5 \text{ g l}^{-1}$ ) was added to 1 ml of sample and incubated for 15 minutes at room temperature. This method was only useable at formate concentrations above  $3 \text{ mg l}^{-1}$ . A change of 1 absorbance unit was equivalent to 150 $\mu\text{g}$  formate in the reaction mixture.

#### 2.2.6. Estimation of formaldehyde.

Formaldehyde was determined according to Houle *et al.*, (1970). To 3 ml of sample in a test tube, 3.3 ml of chromotropic acid reagent was rapidly added to produce good mixing. This reagent was prepared by dissolving 0.33 g chromotropic acid (4,5 - dihydroxy-naphthalene - 2,7 - disulfonic acid, disodium salt) in 100 ml CP grade sulphuric acid. The heat of solution provides the necessary temperature for the colour forming reaction, colour being allowed to develop for 8 minutes. The samples were then cooled and

mixed and the absorbance at 570 nm was measured against a reagent blank. Formaldehyde concentrations were determined by reference to a standard curve covering a concentration range of 0-4  $\text{mg l}^{-1}$ .

#### 2.2.7. Estimations of sulphate.

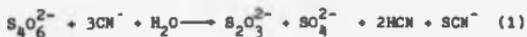
Sulphate in cultures was determined using a Varian atomic absorption spectrophotometer, Series AA-1275 (Varian Techtron Pty Limited, Mulgrave, Victoria, Australia) by measuring residual barium following sulphate precipitation by barium chloride (Varian Manual). Culture samples (2 ml) were spun down in a micro-angle centrifuge for 15 minutes and the supernatants used for sulphate analysis. Supernatants were diluted as necessary and 1% (v/v) HCl added to make the volume up to 5 ml. A stock solution of barium chloride ( $0.2 \text{ g l}^{-1}$ ) was prepared and 5 ml of this added to the samples. After shaking vigorously for a few minutes, the tubes were left overnight for the precipitate to settle. Residual barium was measured in the clear solution using atomic absorption measurements at 553.6 nm. Sulphate was estimated from a standard curve prepared from standard potassium hydrogen sulphate over a concentration range of 0-175  $\text{mg l}^{-1}$ , all dilutions being made in 1% HCl as before.

#### 2.2.8. Estimations of thiosulphate and tetrathionate.

Thiosulphate and tetrathionate were determined colorimetrically according to Kelly *et al.*, (1969). Thiosulphate was determined by adding 4 ml "Koh buffer" (solution containing 100 ml 0.2N  $\text{NaH}_2\text{PO}_4$  and 78 ml 0.2N NaOH) and 5 ml 0.1M KCN to 200  $\mu\text{l}$  of sample in 25 ml volumetric flasks. After mixing, the flasks were allowed to stand for 10 minutes, before rapidly adding and mixing in 1.5 ml 0.1M  $\text{CuSO}_4$  and leaving for a further 5 minutes. Ferric nitrate reagent (3 ml) (1.5M ferric nitrate in 4N  $\text{HClO}_4$ ) was then added,

made up to 25 ml with distilled water and mixed. Thiosulphate was estimated by measuring the absorbance at 460nm against a reagent blank (containing no  $\text{CuSO}_4$ ), where  $1\mu\text{mol Na}_2\text{S}_2\text{O}_3 \equiv 1\mu\text{mol NaSCN} \equiv A_{460} 0.176$ .

Tetrathionate was determined as above except that the addition of  $\text{CuSO}_4$  was omitted. Tetrathionate and thiosulphate react with cyanide according to the following equations:



#### 2.2.9. Estimation of thiocyanate.

Thiocyanate was estimated simply by the addition of 3 ml ferric nitrate reagent to 200-500  $\mu\text{l}$  samples in 25 ml volumetric flasks, made up to 25 ml with distilled water and the absorbance at 460 nm measured as described for the cyanolysis assay of thiosulphate and tetrathionate above.

#### 2.2.10. Estimation of sulphur.

The qualitative determination of sulphur in cultures was carried out as follows: To 5 ml of sample in 25 ml volume flasks was added 15 ml 0.1% (w/v) KCN in acetone solvent (380 ml acetone + 20 ml water), this being shaken and allowed to stand for 2-3 minutes. Flask contents were made

up to 25 ml by the addition of acetone solvent. To 5 ml 0.4% (w/v) ferric chloride in acetone was added 5 ml of the reaction mixture, mixed well, and the absorbance read at 470 nm against a blank taken through the whole procedure, but using 5 ml acetone solvent instead of the sample. For quantitative analysis, standards could be prepared from a solution of 'flowers' of sulphur in acetone.

#### 2.2.11. Estimation of nitrate.

Nitrate was determined according to Caves (1967). Samples (up to 500  $\mu$ l) in 10 ml volumetric flasks were mixed with 1 ml 2% sulphamic acid in order to destroy any interfering nitrite. The flasks were then made up to 10 ml with 5% (v/v) perchloric acid, shaken and the absorbance measured at 210 nm against a perchloric acid blank. Determination of nitrate was made by reference to a calibration curve prepared from potassium nitrate over a range of 0-0.4  $\mu$ mol ml<sup>-1</sup>.

#### 2.2.12. Estimation of nitrite.

Nitrite was determined colorimetrically according to the following method. Samples (up to 500  $\mu$ l) in 10 ml volumetric flasks were mixed with 0.5 ml sulphanilic acid reagent (1.5 g sulphanilic acid, 90 ml glacial acetic acid and 360 ml distilled water) and 0.5 ml Cleves acid reagent (0.6 g Cleves acid or 1-naphthylamine, 7-sulphonic acid, 90 ml glacial acetic acid and 360 ml distilled water). The flask contents were then made up to 10 ml with distilled water. The pink colour was allowed to develop for 20 minutes before measuring the absorbance at 525 nm against a distilled water blank. Nitrite was determined by reference to a calibration curve prepared using sodium nitrite of a concentration range of 0-0.4  $\mu$ mol ml<sup>-1</sup>.

### 2.2.13. Measurement of oxygen uptake.

Oxygen uptake rates were determined using a teflon-coated Clark-type oxygen electrode cell (Rank Bros., Cambridge, U.K.) linked to a chart recorder. When whole cell respiration rates were determined, the reaction chamber contained either 2 ml culture taken directly from a substrate-limited chemostat or 2 ml of a spun, washed and re-suspended suspension from batch cultures. The washing was essential to remove any residual substrate. Suspensions were then saturated with air by bubbling. Substrate-dependent oxygen uptake was determined at 30°C, the reactions being initiated by the injection of substrate (5-20 $\mu$ l). Oxygen uptake by cell free extracts in order to determine a specific activity for methyl mercaptan oxidase is described in detail in section 2.3.6. All results are expressed as  $\mu\text{mol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$  and corrected for endogenous oxygen uptake (this being measured for at least 3 minutes prior to the addition of substrate).

### 2.2.14. Gas chromatography and mass spectrometry. (GC/MS)

GC/MS analysis was carried out in order to identify qualitatively intermediates in the metabolism of some volatile organic sulphur compounds. Analyses were performed using a Kratos mass spectrometer, Model MS 25RFA (Kratos Analytical, Manchester, U.K.) linked to a Carlo Erba gas chromatograph, Model MFC 500 (Fisons Instrumentation, Crawley, Sussex, U.K.) and fitted with a polar capillary column, Model 25 QC3/BP20 (Scientific Glass Engineering (U.K.) Limited, Milton Keynes, U.K.) with length, internal diameter and film thickness of 25 m, 0.33 mm and 0.5 $\mu$  respectively. The system used helium carrier gas with a flow rate through the column of 1 ml  $\text{min}^{-1}$ . The temperature of the column was programmed at 50°C with increments of 20°C  $\text{min}^{-1}$  to a final temperature of 220°C. The injection and detector temperatures were 240°C and 200°C respectively. The mass range available was

18-800 amu and the scan rate was 1 scan  $S^{-1}$ . Identification of intermediates was by comparison of the mass spectra produced from the samples with those held on file by the computer, or by comparison with the mass spectra produced from authentic compounds.

Preparation of samples for GC/MS analysis was as follows: Sealed batch cultures (50 ml) were grown at 30°C on the chosen volatile organic sulphur substrate and sampled periodically throughout the growth cycle. It was possible either to analyse the headspace gases directly simply by removing 100  $\mu$ l of gaseous phase and injecting into the GC/MS, or to analyse dissolved products by extraction with chloroform. If the latter was necessary, 1 ml samples of culture were removed from the flasks and mixed vigorously for several minutes with 2 ml chloroform in sealed tubes. After allowing the two phases to separate, the organic phase was removed and transferred to a second sealed tube. A few crystals of calcium chloride were then added to dry the organic phase before analysis. Injection volumes used were 0.5-1.0  $\mu$ l with a split ratio of 100:1 (i.e. 1/100th of the injected sample was actually delivered onto the column).

#### 2.2.15. Gas chromatography, (GC)

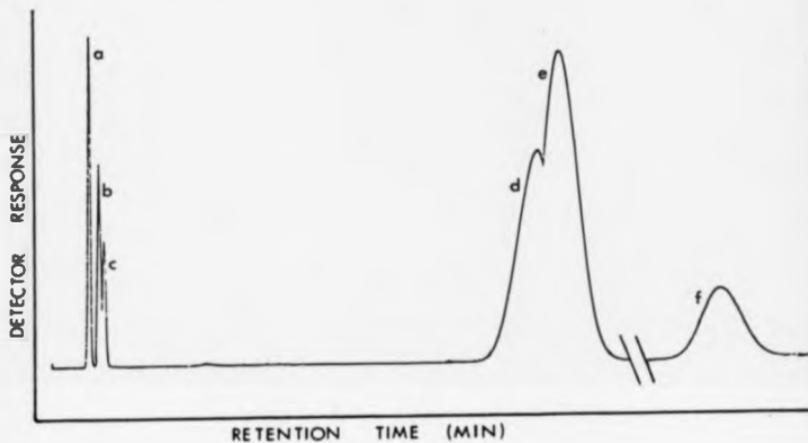
GC analysis was used routinely for the determination of all the volatile organic sulphur compounds used. It proved invaluable in the identification and quantitative determination of headspace gases and was used subsequently to measure the time-dependent (dis)appearance of substrates and intermediates from aerobic and anaerobic incubations of cell suspensions. Analyses were performed using a Pye Unicam Series 204 Gas Chromatograph, (Pye Unicam Limited, Cambridge, U.K.) linked to a Pye Unicam Computing integrator, Model PU 4810, and fitted with a teflon column (and teflon

LEGEND TO FIGURE 2.2

Elution order and retention times of some volatile organic sulphur compounds by GC analysis under the conditions described in the Methods Section.

<u>Peak</u>	<u>Compound</u>	<u>Retention Times (mins.)</u>
a	Hydrogen sulphide	0.81
b	Carbonyl sulphide	1.04
c	Methanethiol	1.17
d	Dimethyl sulphide	11.04
e	Carbon disulphide	11.34
f	Dimethyl disulphide	23.20

Figure 2.2



couplings) 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 (FPD) incorporating a sulphur filter permitting light transmission at 394 nm. The injection, column and detector temperatures used were 200°C, 100°C and 250°C, respectively. Gas flow rates for nitrogen (carrier gas), air and hydrogen were 40 ml min<sup>-1</sup>, 50 ml min<sup>-1</sup> and 70 ml min<sup>-1</sup>, respectively. Analyses were performed by the injection of 100µl headspace gas. Compounds were identified by comparison of retention times of peaks produced from samples with those obtained from authentic compounds, and quantified from the recorded peak area by calculation using freshly prepared standards. Under the conditions employed, retention times and elution order of the important volatile organic sulphur compounds are shown in Fig. 2.2. (See also Banwart & Bremner, 1974; König *et al.*, 1980; Bremner & Banwart, 1974; Headley, 1987; Przyjazny *et al.*, 1983; Merrick-Gass, 1986).

2.2.16. GC Determination of intermediates of organic sulphide metabolism using incubated cell suspensions.

Time-dependent appearance of NT from DMDS was measured by headspace GC analysis during anaerobic incubation of cell suspensions. Culture (200 ml) was taken directly from a DMDS-limited chemostat (1.9µM; D = 0.07 h<sup>-1</sup>), centrifuged at 21,000 g for 15 minutes, washed and re-suspend in 10 ml mineral salts medium. The suspension was then divided into 2 x 5 ml portions, each containing 3.6 µg protein, into universals. The universals were then sealed with 'subaseal' stoppers leaving a headspace of 16-18 ml. One of the cultures was then de-oxygenated by bubbling with nitrogen. Both suspensions were allowed equilibrate at 30°C before the injection of 10µmol DMDS. NT production was monitored during incubation at 30°C, as described

previously. This experiment was also repeated in the presence of 2.5  $\mu$ mol formaldehyde used as a source of reducing power. This same procedure was used to determine the time-dependent disappearance of NT from aerobic and anaerobic incubations of cell suspensions, the reaction initiated by the injection of 5  $\mu$ mol NT.

Time-dependent disappearance of  $CS_2$  and appearance of  $H_2S$  and COS during aerobic and anaerobic incubations of cell suspensions was also measured. Batch cultures (3 x 100 ml) in 500 ml 'Quickfit' flasks were fed with 5 x 1mM additions of  $CS_2$  and grown for 12 days. Cultures were then pooled, centrifuged at 21,000 xg for 15 minutes, washed and re-suspended in 10 ml mineral salts medium. The suspension was divided into 2 x 5 ml portions as before, each containing 7.5 mg protein. Universals were sealed, de-oxygenated and incubated as above. The reaction was initiated by the injection of 10  $\mu$ mol  $CS_2$ . Levels of  $CS_2$ ,  $H_2S$  and COS were then monitored in the headspace gases of both suspensions during an incubation period of 3-4 hours. At the end of the experiment, the total  $H_2S$  production was determined by acidifying the suspensions with 1M HCl to drive all dissolved sulphide into the headspace as  $H_2S$ .

#### 2.2.17. Extraction and purification of DNA from isolates.

Chromosomal DNA was isolated and purified according to Beji *et al.*, (1987). Cells harvested from the chemostat were centrifuged at 17,000 xg for 10 minutes at 4°C and washed with saline-ethylenediaminetetraacetic acid (EDTA) solution (0.15M NaCl, 0.1M EDTA, pH 8.0). After re-centrifugation, the wet weight of cells was determined. Washed cells were then lysed with 0.03M NaOH (10 ml per g original wet wt) for 2 minutes at room temperature before adding 25% w/v sodium dodecyl sulphate (SDS) (1.5 ml per g original wet wt). The action of the alkali was halted by the addition of saline-EDTA solution,

pH 7.0 (35 ml per g original wet wt). The lysate was then centrifuged at 17,000 xg for 5 minutes at 4°C, the supernatant being removed and the viscous phase incubated at 60°C for 30 minutes in the presence of RNase (2.5 mg per g original wet wt). The solution was then treated with proteinase K (0.625 g per g original wet wt) and incubated at 37°C for 15 minutes. Following this incubation, 5M NaCl was added to give a final concentration of 1M. The mixture was then shaken with an equal volume of chloroform: iso-amyl alcohol (24: 1 v/v) for 5 minutes, centrifuged at 5,000 xg for 15 minutes, and the clear lysate precipitated by the addition of an equal volume of cold 95% (v/v) ethanol. The DNA was recovered by spooling on a clean glass rod and then re-dissolved in 20 ml 0.01 xSSC (SSC = 150mM NaCl, 15mM trisodium citrate). After then adding 1M NaCl (to give a final concentration of 0.1M NaCl), DNA was again precipitated by the addition of an equal volume of iso-propyl alcohol. After spooling the purified DNA it was then re-dissolved in a minimum volume of 0.1 xSSC and dialyzed overnight at 4°C against 0.1 xSSC.

#### 2.2.18. Determination of mol % guanine plus cytosine. (mol % G + C)

The mol % G + C ratio of purified DNA was determined by two methods, both based on ultraviolet spectroscopy:

For the method of Fredericq *et al.*, (1961), 1 ml 0.11 M acetic acid, pH 3.0 was placed in 1 ml silica cuvettes, to which was added 50-200µl of purified DNA. The cuvettes were then shaken and the absorbance measured at 260 nm and 280 nm. The mol % G + C was then calculated as follows:

$$\text{ratio} \quad 260/280 \quad = \quad R \quad (1)$$

$$\text{mol \% G + C} = \frac{22400 - 9300 R}{5100 + 11200 R} \times 100 \quad (2)$$

For the method of Ulitzur (1972), the final DNA concentration should correspond to an absorbance at 260 nm of 0.6-0.7. If the absorbance value was too high the DNA solution was diluted accordingly with 0.1 xSSC, if too low it was concentrated by dialysing against a strong sucrose solution. The purity of the DNA sample could be assessed by measuring the absorbance ratios at 260/230 nm and 260/280 nm. Values greater than 1.8 indicate high purity, with little contamination from RNA or protein. Determination of the mol % G + C was performed by measuring the absorbance ratios at 245/270 nm, 240/275 nm and 240/280 nm of the sample DNA and comparing with the same ratios of standard DNA. The standard DNA's used were *E. coli* and *F. tepidarius* which have mol % G + C values of 51.1% and 66.6% respectively (Fig. 4.3). Sample mol % G + C values were calculated as follows:-

$$\frac{(\text{Standard ratio}) - (\text{Test ratio})}{\text{slope}} = x \quad (1)$$

where the slope for 245/270 nm = 0.0047

$$240/275 \text{ nm} = 0.00576$$

$$240/280 \text{ nm} = 0.0076$$

(calculated from the absorbance ratio versus mol % G + C plots of many DNA samples), Ulitzur (1972).

$$\text{Sample mol \% G + C} = (\text{Standard mol \% G + C}) - (x) \quad (2)$$

#### 2.2.19. Determination of the ubiquinone content.

The ubiquinone (UQ) content of isolates was determined based on the method of Di Spirito et al (1983). Approximately 6g wet wt of cells was re-suspended in 15 ml distilled water and passed twice through a French pressure cell (120 MPa). Acetone (180 ml) was then added to the whole pressate and the mixture stirred at 4°C overnight. The mixture was centrifuged at 9,000 xg and 4°C for

20 minutes and the supernatant filtered through a Whatman No.1 filter. A rotary evaporator under vacuum and at 40°C was used to remove the acetone from the yellow filtrate. The remaining material was re-dissolved in 180 ml n-hexane and this was extracted five times (in a separating funnel) with 90 ml distilled water, discarding the aqueous phase on each occasion. The remaining n-hexane fraction was dried by the addition of sodium sulphate, the liquid then being decanted off. Prior to analysis, the n-hexane was 'flash evaporated' at 55°C and the remaining yellow oil re-dissolved in 1-5 ml ethanol.

Thin layer chromatography (TLC) was used to identify the ubiquinones present. The ethanol fraction (10 or 20µl) was spotted onto aluminium sheets (20 x 20 cm) pre-coated with DC-Alufohlen kiesel gel 60 F254 (0.2 mm thick) and using benzene as the solvent, the chromatogram was allowed to run for 50 minutes. After drying, the spots were visible under u.v. light at 254 nm. Ubiquinone standards were run simultaneously with the samples: UQ-8 was obtained from *F. tepidarius* and *F. denitrificans*, UQ-10 was obtained from *F. ferrooxidans* and from the Sigma Chemical Company Limited.

#### 2.2.20. Preparation of cell free extracts.

Cells harvested from a DMDS-limited chemostat (2mM, D = 0.078 h<sup>-1</sup>) were centrifuged at 21,000 xg and 4°C for 15 minutes. Pellets were pooled until 5.4 g wet wt cells had been accumulated. The cells were then re-suspended in 15 ml distilled water and passed twice through a French pressure cell (120 MPa). Whole cells and membranes were separated by centrifuging the particulate suspension at 35,000 xg and 4°C for 30 minutes. The cell free extract was then divided into 2 ml portions before freezing at -20°C until required. The pellets were also retained and frozen. The protein content of the undiluted cell free extract was estimated at 9.6 mg ml<sup>-1</sup>.

### 2.3. ENZYME ASSAYS.

All spectrophotometric assays were performed with a Unicam SP1700 spectrophotometer fitted with a water-jacketted cuvette-housing. All assays, with the exceptions of catalase and MAD(P)<sup>+</sup>-dependent formaldehyde dehydrogenase were performed at 30°C. All assays with the exceptions of ribulose 1,5-bisphosphate carboxylase and DMDS 'reductase' were performed using appropriate dilutions of the cell free extract (see 2.2.20).

#### 2.3.1. Ribulose 1,5-Bisphosphate (RUBP) Carboxylase.

RUBP carboxylase activity was assayed either in permeabilized whole cells or in cell free extracts. The permeabilized whole cell method used was essentially as described by Kelly & Wood (1982), except that the cell permeabilizing agent used was cetyl trimethylammonium bromide (Leadbeater et al., 1982) (0.1% v/v) rather than Triton X-100. Bacteria (0.5-1.0 mg dry weight) were collected on 0.2µm 25 mm Sartorius membrane filters and immersed in 0.4 ml permeabilizing agent in scintillation vials for 20 minutes at 20°C. Vial contents were then activated with [<sup>14</sup>C]-bicarbonate reaction mixture (Kelly & Wood, 1982) at 30°C. The reaction mixture contained a total of 44mM NaHCO<sub>3</sub> at 10µCi [<sup>14</sup>C]ml<sup>-1</sup>. After 10 minutes 0.3 ml 10mM RUBP was added to the vials. Reactions were terminated after a further 15 or 30 minutes incubation by adding 3 ml 5% (v/v) acetic acid in methanol. Zero-time blanks and RUBP-free controls were also assayed. <sup>14</sup>CO<sub>2</sub>-fixation was insignificant in the absence of RUBP. Vial contents were evaporated to dryness at 60°C. The yellow/white solid material was then re-suspended in 1 ml distilled water by shaking at 30°C for 30 minutes. 10 ml "Optiphase Safe" scintillant was added to each vial and <sup>14</sup>C counted as previously described (section 2.1.4.). Specific activity of [<sup>14</sup>C]-bicarbonate was determined by mixing 10µl samples of reaction mixture

with 2 ml absorption reagent (3 parts ethanolamine to 7 parts methoxyethanol) (Wood & Kelly, 1977), and counting in 10 ml scintillant. Reaction rate was linear for at least 30 minutes and specific activities are expressed as  $\text{nmol CO}_2 \text{ fixed. min}^{-1} \text{ (mg protein)}^{-1}$ .

Assay in cell free extracts was performed by adding 200 $\mu$ l extract to 1.8 ml [ $^{14}\text{C}$ ]-bicarbonate reaction mixture in 4 ml test tubes and briefly mixing. The tubes were then incubated at 30°C for 5 minutes before adding RUBP (5 mg in 200  $\mu$ l) and re-mixing. Samples (250 $\mu$ l) were withdrawn between 1-30 minutes incubation and placed into scintillation vials containing 250 $\mu$ l acetic acid. Vial contents were then dried at 60°C. After drying, solid material was re-suspended in 1 ml distilled water before the addition of 10 ml scintillant. Blanks to measure the fixation of  $^{14}\text{CO}_2$  in the absence of RUBP were also run. Specific activities of the label and of the enzyme were calculated as described above.

### 2.3.2. Hydroxypyruvate reductase.

Hydroxypyruvate reductase was assayed according to Kelly & Wood (1984): silica cuvettes contained, in a total of 1 ml, 33 $\mu$ mol potassium phosphate buffer, pH 7.0; NADH or NADPH (13 $\mu$ mol and 12 $\mu$ mol respectively); and 50 $\mu$ l of extract. The reaction was initiated by the addition of 2 $\mu$ mol lithium hydroxypyruvate and the decrease in extinction at 340 nm was measured against a blank containing buffer and extract only. Reaction rates were corrected for the oxidation of NAD(P)H by the extract in the absence of substrate, and specific activities are expressed as  $\text{nmol NAD(P)H oxidised. min}^{-1} \text{ (mg protein)}^{-1}$ . The molar extinction coefficient of NAD(P)H was  $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .

### 2.3.3. Formate dehydrogenase.

Formate dehydrogenase was assayed according to Patel & Hoare (1971). Silica cuvettes containing, in a total volume of 1 ml: 50 $\mu$ mol potassium phosphate buffer, pH 7.0; 1 $\mu$ mol NAD(P)<sup>+</sup>; and 50 $\mu$ l extract. The reaction was initiated by the addition of 10 $\mu$ mol sodium formate and the rate of NAD(P)<sup>+</sup> reduction was followed at 340 nm against a blank containing buffer and extract only. Specific activities are expressed as nmol NAD(P)<sup>+</sup> reduced.min.<sup>-1</sup> (mg protein)<sup>-1</sup>.

### 2.3.4. Formaldehyde dehydrogenase.

NAD(P)<sup>+</sup> - dependent formaldehyde dehydrogenase was assayed according to Stirling & Dalton (1978). The reaction mixture contained, in a total volume of 1.5 ml: 30 $\mu$ mol potassium phosphate buffer, pH 7.2; 1 $\mu$ mol NAD(P)<sup>+</sup>; and 50 $\mu$ l extract. The reaction was initiated by the addition of 3.75 $\mu$ mol formaldehyde after a pre-incubation period of 3 minutes. The rate of NAD(P)<sup>+</sup> reduction was followed at 340 nm against a blank containing buffer and extract only, at 45°C. Specific activities are expressed as nmol NAD(P)<sup>+</sup> reduced .min.<sup>-1</sup> (mg protein)<sup>-1</sup>.

### 2.3.5. Catalase.

Catalase was assayed according to Haywood & Large (1981). 0.1 ml diluted extract was added to 2.9 ml of a solution containing 0.1 ml of a 30% (v/v) solution of H<sub>2</sub>O<sub>2</sub> in 50 ml 50mM potassium phosphate buffer, pH 7.0. Decrease in absorbance was followed and the time taken for A<sub>240</sub> to fall from 0.45 to 0.40 was determined at 25°C. This corresponds to the decomposition of 3.45 $\mu$ mol H<sub>2</sub>O<sub>2</sub> in the 3 ml reaction volume, where activity = 3.45 / [time (min) required], (Sigma catalogue). Specific activity is expressed as nmol H<sub>2</sub>O<sub>2</sub>. min.<sup>-1</sup>. (mg protein)<sup>-1</sup>.

### 2.3.6. Methyl mercaptan (Methanethiol) oxidase

MM(MT) oxidase activity was assayed by  $O_2$  consumption essentially as described by Suylen *et al.*, (1987). The oxygen electrode cell contained, in a total volume of 2 ml: 100 $\mu$ mol potassium phosphate buffer, pH 7.2; and 100 $\mu$ l extract. The reaction was initiated by the injection of 30 nmol MT. MT solutions were prepared as described earlier (see section 2.2.2.) and freshly made immediately before use. Rates of  $O_2$  uptake were measured as previously described (see section 2.2.13) and were proportional to the amount of protein added. Specific activity is expressed as nmol  $O_2$  consumed.  $min^{-1}$ . (mg protein) $^{-1}$  and corrected for any endogenous  $O_2$  uptake.

### 2.3.7. DNDS 'Reductase'

DNDS 'reductase' activity was assayed as the time-dependent appearance of NT according to the following method. A 50 ml volume of culture was taken directly from a DNDS-limited chemostat (2mM, D = 0.078h $^{-1}$ ), centrifuged at 21,000  $g$  for 15 min and re-suspended in 2 ml distilled water. The suspension was then divided into 2 x 1 ml portions (each containing 0.88 mg protein) in universals before sealing with rubber 'subaseal' stoppers and deoxygenating. All further additions were made in deoxygenated solutions. Cells were permeabilised by the addition of 0.5 ml 0.25% (v/v) cetyl trimethylammonium bromide and incubated at 30°C for 20 min. NADH (20 $\mu$ mol) was then added to one universal, the other one acted as the control. Potassium phosphate buffer, pH 7.0 (150 $\mu$ mol in 3 ml) containing dissolved DNDS was the final addition. The final reaction volume of 5 ml contained 2 $\mu$ mol DNDS which left a headspace in the universal of 18 ml. The suspensions were then incubated at 30°C and the time-dependent appearance of NT was measured by GC analysis of the headspace gases (see section 2.2.15.). Specific activity is expressed as nmol NT produced.  $min^{-1}$ . (mg protein) $^{-1}$ , and corrected for any NT production in the absence of NADH.

#### 2.4. SPECIAL CHEMICALS

All chemicals and reagents used routinely were of analytical grade. Methanethiol, carbon disulphide and thiocyanate were obtained from Fisons, PLC; dimethyl sulphide, carbonyl sulphide, ethanethiol, 1-butanethiol, 1-hexanethiol, thiophenol, diethyl disulphide and dipropyl disulphide from the Aldrich Chemical Company Limited; dimethyl disulphide, dimethyl sulphoxide and 3-amino 1,2,4-triazole from the Sigma Chemical Company Limited; 2-mercaptoethanol from BDH Limited; and  $\text{Na}_2^{14}\text{CO}_3$ ,  $\text{NaOO}^{14}\text{CH}$ , and  $\text{H}^{14}\text{CHO}$  from Amersham International Limited. All solvents used for GC/MS and GC work were of chromatographic grade, and were obtained from one of the above sources.

C H A P T E R   T H R E E

ENRICHMENT AND ISOLATION OF BACTERIA CAPABLE OF GROWTH ON

METHYLATED SULPHIDES

---

### 3.1. INTRODUCTION.

The bacteria described previously having the ability to grow on DMS as the sole growth substrate were not isolated in the laboratory from DMS enrichments because of its reported toxicity. All previous attempts to obtain DMS-oxidising strains from DMS enrichments have proved unsuccessful (Suylen & Kuenen, 1986), although 'thiobacillus-like' organisms were isolated on thiosulphate agar from DMS enrichments using acclimatised activated sewage sludge (Kanagawa & Kelly, 1986). *F. thioparus* TK-m was a component of a mixed culture able to degrade a pesticide breakdown product (Kanagawa & Kelly, 1986). *Thiobacillus* NS1 was obtained from biofilters used in the treatment of wood pulping effluents (Sivela, 1980). The hyphomicrobia were obtained originally as DMSO-users (De Bont *et al.*, 1981; Suylen & Kuenen, 1986; Suylen *et al.*, 1986). Although the use of indirect means such as enrichment with DMSO, in order to circumvent the toxicity of DMS, has led to the isolation of DMS-oxidising strains, the isolates described to date apparently have a very restricted metabolism. Consequently there have been no reports of any organisms able to oxidise DMDS as its sole source of energy: *F. thioparus* TK-m (Kanagawa & Kelly, 1986; This study) and *Hyphomicrobium* S (De Bont *et al.*, 1981) cannot use it, although a mixed culture enriched on DMSO could oxidise DMDS but only at 8% of the rate of DMS oxidation (Suylen & Kuenen, 1986). This suggested that the ability to grow on DMDS was not a property necessarily common to DMSO- or DMS-oxidising bacteria and indicated that specific enrichment using DMDS as the sole growth substrate was necessary to detect DMDS-oxidising strains.

In setting up enrichment cultures, it is considered advantageous to use inocula from source materials where the desired organisms are usually

exposed to the enrichment substrate or a compound closely related to it. Selection pressure existing in the natural environment then hopefully ensures the presence of the organisms in the sample. This was certainly the case for the isolation of *Thiobacillus* MS1: the cellulose mill represents a somewhat artificial environment where the local concentrations of methylated sulphides by far exceed those normally found in most other natural habitats. Pine logs from which the bark was stripped and used to pack the biofilters (from which *Thiobacillus* MS1 was obtained) had previously been stored in reservoirs surrounding the cellulose mill. Other bacteria similar to *Thiobacillus* MS1 were readily detectable in samples of mud and water taken from the reservoirs. These organisms had presumably become established by being able to oxidise methylated sulphides during the forty years of operation of the mill (Sivela & Sundman, 1975; Sivela, 1980).

As already discussed, methylated sulphides arise in most if not all natural environments, often with high evolution rates, even though detectable levels are very low. This suggests that it may not be necessary to obtain pre-treated or otherwise 'exotic' source materials, since the indigenous population might contain methylated sulphide degrading strains. It would appear that the main problem in isolating methylated sulphide-users might not be in the choice of source material, but in selecting a suitably low initial substrate concentration to support growth but giving a minimum harmful effect upon the methylated sulphide-users (if present). In this study source materials were collected from a range of natural environments, both terrestrial and aquatic, the only prerequisite being that samples were taken during the summer months when maximum biological activity was likely to be occurring.

### 3.2. ISOLATION AND PURIFICATION.

#### 3.2.1. Enrichment cultures.

The following source materials were used to initiate enrichment cultures for the isolation of DMS- and DMDS- degrading bacteria: E1, commercially available gardener's peat; E2, red clay-soil, Burton upon Trent area; E3, garden compost; E4, cattle manure; E5, marine mud, Plymouth Sound; E6, pond water, Gibbet Hill, University of Warwick campus; E7, moss from a deodorisation unit; E8, marine sediment, Salcombe Bay. The quantities of source materials and the enrichment methods are described fully in the Methods Section (see 2.1.2).

During the early stages of the enrichment procedure, some of the cultures were heavily loaded with organic material and the fall in pH (commonly from pH 7.0 to pH 5.0) which occurred during the three days incubation between subcultures was presumed largely due to the action of the heterotrophic population and the production of organic acids. However, after several replacements of mineral salts medium much of the organic material and consequently most of the heterotrophs had been eliminated. Apart from a small amount of EDTA present in the trace metal solution ( $10 \text{ mg l}^{-1}$ ), the only input of organic material into the cultures was of the substrates themselves.

A second series of enrichment cultures containing yeast extract were established and treated exactly as described previously. The only apparent effect the yeast extract had was to enable the heterotrophs to proliferate. Although these cultures were allowed to run for several weeks they were not used for isolation purposes.

An initial concentration of DMS or DMDS of 0.5mM proved most satisfactory in being sufficiently low to be tolerated by the enrichment cultures but which at the same time gave rise to a detectable increase in biomass as judged by culture turbidity. Complete substrate degradation was ensured, and determined by the lack of any residual odour, before further additions were made. Stepping up the substrate concentration to 2mM did not appear to have any detrimental effect on the cultures.

When the enrichments were oxidising 2mM substrate within 2-3 days, 25ml of the upper phase was removed (after settling) and transferred to sterile 'Quickfit' flasks containing 25 ml sterile mineral salts medium. Subculturing was subsequently carried out every 5-7 days. All enrichments were sustained through successive subculture on 2mM DMS or DMDS, except the soil enrichment (E2), which at this point was discarded. The remaining cultures were sampled at regular intervals to monitor the variety and relative numbers of bacteria sustained and to attempt to isolate the pure or mixed cultures responsible for substrate degradation.

### 3.2.2. Isolation and purification.

Isolation and purification of DMS- and DMDS- degrading strains was attempted by streaking liquid culture samples onto solid media. Considerable difficulty was experienced in obtaining pure cultures from the enrichments: using various heterotrophic media or mineral salts medium with methylamine, dimethylamine or formate resulted in the production of numerous colonies on plates, but these failed to grow when returned to liquid medium with DMS or DMDS as sole substrates. Although there were apparently many types of methylotrophs present, only 2-5 heterotrophic strains were sustained in the enrichments, but these too failed to grow when returned to liquid medium

with DMS or DMDS. All attempts to isolate single colonies from these plates and to reconstitute them as mixed cultures using various combinations of the strains also proved unsuccessful.

Single colony production from enrichments was attempted by inoculating mineral salts agar and incubating at 22°C in sealed jars in an atmosphere containing DMS or DMDS. Tiny colonies were observed at 7-10 days, but again it was not possible to transfer these into liquid culture. The major disadvantage with this method was in determination of the actual substrate concentration available to the cells.

The only isolates of DMS- and DMDS- using bacteria which could be successfully re-grown when transferred back to liquid medium containing DMS or DMDS came from colonies grown on thiosulphate - mineral salts agar. However not all of these isolates grew when transferred, only about 50% were successful. It was not clear whether the remainder failed to grow because either: [i] they took no part in methylated sulphide oxidation and were sustained only as commensals, (ii) they could not catalyse the initial attack on the substrate but could grow on a breakdown product, or [iii] they were capable of growth on either DMS or DMDS but not under the conditions presented to them, i.e. low inoculum size or substrate concentration at 2mM. Even using the successfully re-grown colonies, a lag of between 7-14 days was observed before growth commenced indicating either some inducible mechanism for DMS or DMDS metabolism, or some substrate inhibition effect.

It was concluded that the growth seen on organic nutrient media was of bacteria incapable of growth on methylated sulphides. These bacteria could have been sustained in the enrichments as commensals using excreted products from the DMS- and DMDS- using organisms, which could not themselves grow on the organic media.

Five organisms were initially isolated in pure culture by transfer from thiosulphate agar. These isolates were coded as for the sample sources: E3, E4 and E5 enriched originally on DMS, and E6 and E7 on DMDS. Isolation into pure culture of organisms coded E1 and E8, both enriched on DMS, were later made. All seven isolates were routinely maintained as autotrophic cultures on solid medium containing thiosulphate as the sole energy source, or in liquid culture on DMS or DMDS.

### 3.3. CHARACTERISATION OF STRAINS E1 - 8.

#### 3.3.1. Determination of the range of compounds used as growth substrates.

Strains E1 - 8 were capable of growth on a fairly restricted range of inorganic and organic sulphur compounds (see Table 3.1). None grew on sucrose, fructose, glucose, acetate, citrate or formate (each at 10mM), formaldehyde (0.5mM), methylamine, dimethylamine or trimethylamine (5mM), methanol, dimethyl sulphate, dimethyl sulphoxide, ethane thiol, diethyl sulphide, diethyl sulphate (each at 2mM) or methane. Physiologically, these strains resembled chemolithotrophic thiobacilli in that all grew on thiosulphate and sulphide and some also used elemental sulphur or thiocyanate. In contrast to most thiobacilli all were able to grow on methylated sulphides and COS, but not on CS<sub>2</sub>. Strain E4 alone grew on thiourea and could also use thioacetamide. The surprising feature of this series of growth experiments was that those isolates derived from DMS enrichments could also use DMDS and similarly those derived from DMDS enrichments could also use DMS, although they showed a preference for their enrichment substrate. These organisms are the first to be reported to grow on DMDS as the sole growth substrate in pure culture.

TABLE 3.1 Growth of isolates from enrichment cultures on inorganic and organic compounds.

SUBSTRATE (mM)	Grown after 8 days incubation							
	E1	E3	E4	E5	E6	E7	E8	
Sulphide (2)	+	+	+	+	+	+	+	
Sulphur (10 g l <sup>-1</sup> )	+/-	-	-	-	+	+	+	
Thiosulphate (10)	+	+	+	+	+	+	+	
Tetrathionate (10)	+	-	+	+	+	+	+	
Sulphite (5)	n.t.	+	+	+/-	+	+	n.t.	
Dithionate (10)	n.t.	+/-	+	+/-	+	+	n.t.	
Methanethiol (2)	+	+	+	+	+	+	+	
Dimethyl sulphide (2)	+	+	+	+	+	+	+	
Dimethyl disulphide(2)	+	+	+/-	+	+	+	+	
Diethyl sulphide (2)	-	-	-	-	-	-	-	
Ethaneethiol (2)	-	-	-	-	-	-	-	
Dimethyl sulphoxide(2)	-	-	-	-	-	-	-	
Dimethyl sulphate (2)	-	-	-	-	-	-	-	
Diethyl sulphate (2)	-	-	-	-	-	-	-	
Carbon disulphide(2)	-	-	-	-	-	-	-	
Carbonyl sulphide (2)	+	+	+	+	+	+	+	
Thiocyanate (3)	n.t.	-	+	+	+	+	n.t.	
Thiourea (2)	n.t.	-	+	-	-	-	n.t.	
Thioacetamide(2)	n.t.	-	+	-	-	-	n.t.	
Thioacetic acid (2)	n.t.	-	-	-	-	-	n.t.	
Formate (10)	-	-	-	-	-	-	-	
Formaldehyde (0.5)	-	-	-	-	-	-	-	
Methanol (2)	-	-	-	-	-	-	-	
Methane (2)	-	-	-	-	-	-	-	
Methylamine (5)	-	-	-	-	-	-	-	
Dimethylamine (5)	-	-	-	-	-	-	-	
Trimethylamine (5)	n.t.	-	-	-	-	-	n.t.	
Glucose	-	-	-	-	-	-	-	
Fructose	n.t.	-	-	-	-	-	n.t.	
Sucrose	n.t.	-	-	-	-	-	n.t.	
Acetate	-	-	-	-	-	-	-	
Citrate	n.t.	-	-	-	-	-	n.t.	

\* Growth was scored as : good (+), poor (+/-) or absent (-).

Growth on sulphur was scored by visual inspection of turbidity after allowing sulphur to settle. No growth of isolates E3 and E5 occurred during 3 weeks incubation.

n.t. : not tested

3.3.2. Some morphological and physiological characteristics of strains E1 - 8.

All seven pure cultures were small, motile, Gram negative rods with dimensions of about  $0.5 \times 1-2 \mu\text{m}$ . In liquid batch culture on all substrates that would support their growth (Table 3.1), acid production was observed. For example, using an initial culture pH of 7.1, a typical final pH of 6.3 - 6.4 was observed following the complete consumption of 2mM DWDS or, 5mM DMS of thiosulphate. In the presence of high concentrations of thiosulphate (20-40mM), elemental sulphur was precipitated followed by acid production giving rise to a final pH of about 4.5 - 5.0 at the end of growth.

Weak anaerobic growth and denitrification was observed with all strains on thiosulphate and also, with the exception of strain E3, on thiocyanate. In all cases nitrate was reduced only as far as nitrite. After 9 days incubation of cultures at 30°C using thiosulphate and thiocyanate as sole energy sources, the final culture pH was reduced to pH 6.6 - 6.7. In addition to a visible increase in biomass, there was also evidence of precipitated elemental sulphur in all cultures. Because of this the theoretical stoichiometry of thiosulphate and thiocyanate consumption to nitrate reduced (assuming that thiosulphate and thiocyanate would be completely oxidised to sulphate) of 1 : 8 was never achieved. Typical results were in the order of 1 : 3-4.

Colonies grown on thiosulphate plates were small, with diameters of 1-2 mm and white/yellow in appearance but tended to darken with age. With the exception of strain E4, young colonies (4-5 days) were round with raised centres. With age, the colonies produced by these strains tended to become encrusted with elemental sulphur which gave them a dull, irregular appearance and exaggerated their true size, this was particularly noticeable

with strains E6 and E7 and is characteristic of *F. thiooparus* strains. Strain E4, earlier shown to be capable of growth on thiourea and thioacetamide had the distinctive feature that even in older colonies (10-14 days), they still retained their shiny, smooth surfaces even though precipitated sulphur was in evidence. They also retained their round, convex appearance. These features are typical of *F. neapolitanus* strains. Although mineral salts agar plates incubated in a DMS or DMDS atmosphere failed to yield any successful methylated sulphide users from the enrichment cultures, strains E1 - 8 could be maintained on DMS or DMDS plates where the substrate was added directly to the cooling agar prior to pouring. Because of the volatility of the substrates (where boiling points are close to the setting point of the agar) much of that added was lost. Nevertheless, residual substrate was sufficient to support healthy growth of colonies provided plates were sealed during incubation. Colonies again were about 1-2 mm in diameter, round with raised centres, thus resembling young colonies grown on thiosulphate plates. However, even on aging, colonies remained clear and colourless with no evidence of precipitated sulphur. Strains were not routinely maintained on DMS or DMDS plates because of the difficulties in preparing the plates and containing the malodorous substrates during prolonged incubation.

### 3.3.3. Incorporation of $^{14}\text{CO}_2$ into cells.

Since all strains could be maintained as autotrophs using thiosulphate as the sole energy source, cell carbon was presumably derived by the fixation of  $\text{CO}_2$ . It was then necessary to discover to what extent the strains fixed  $\text{CO}_2$  during growth on either DMS or DMDS in order to determine whether growth on these compounds was autotrophic or otherwise. Using the five originally isolated strains pre-grown on either DMS or DMDS, batch culture experiments

were carried out for the simultaneous measurement of increase in cell carbon and the fixation of  $^{14}\text{CO}_2$ . Strains E3 and E4 were grown on DMS, strains E6 and E7 on DMDS, and E5 on both. Cultures were sampled from time zero up to 79h incubation and measurements of culture absorbance and  $^{14}\text{CO}_2$  incorporation into cells were determined at regular intervals as described in the Methods Section (see 2.1.4). Figs 3.1a,b show differential plots of  $^{14}\text{CO}_2$  incorporation as  $\text{cpm} (\text{ml culture})^{-1}$  versus  $A_{440}$ . During the first 10h of growth of all strains, up to 93% of the cell carbon was derived directly from  $\text{CO}_2$ , indicated by a linear relationship of increased  $^{14}\text{CO}_2$  incorporation and biomass production.

This indicated that growth of these five strains (and presumably E1 and E8) was autotrophic rather than methylotrophic in terms of the pathway of carbon assimilation. As growth proceeded, the increase in  $^{14}\text{CO}_2$  incorporation decreased relative to increase in biomass (Fig. 3.1a,b). This was particularly noticeable when further additions of substrate were made and suggested that DMS and DMDS carbon was probably oxidised at some stage to  $\text{CO}_2$  or to a compound competing with  $^{14}\text{CO}_2$  for assimilation by the bacteria. Competition of this sort would then have the effect of diluting the original specific activity of the label and causing the apparent decrease in its fixation. Figs. 3.1a,b also clearly show that although  $^{14}\text{CO}_2$  is incorporated by strain E5 when grown on DMS and DMDS, its poor growth on DMDS showed it to have a strong preference for growth on DMS, on which it was originally enriched. With the exception of strain E5 grown on DMDS, at the end of growth the cultures had produced between 30.6 and 34.3  $\text{mg l}^{-1}$  of new cell carbon, which was labelled at only 47% to 60% of the original  $^{14}\text{C}$ -bicarbonate specific activity.

LEGEND TO FIGURE 3.1

Differential plots of  $^{14}\text{CO}_2$  fixation versus culture absorbance ( $A_{440}$ ) for strains E3-E7. (a) E3 ( $\blacktriangle$ ), E4 ( $\blacktriangledown$ ) and E5 ( $\blacksquare$ ) growing on 2mM DMS. (b) E5 ( $\triangle$ ), E6 ( $\circ$ ) and E7 ( $\square$ ) growing on 2mM DMS. The specific activity of the labelled  $\text{NaH}^{14}\text{CO}_3$  was  $17957 \text{ cpm} \cdot \mu\text{mol}^{-1}$ .

Figure 3.1a

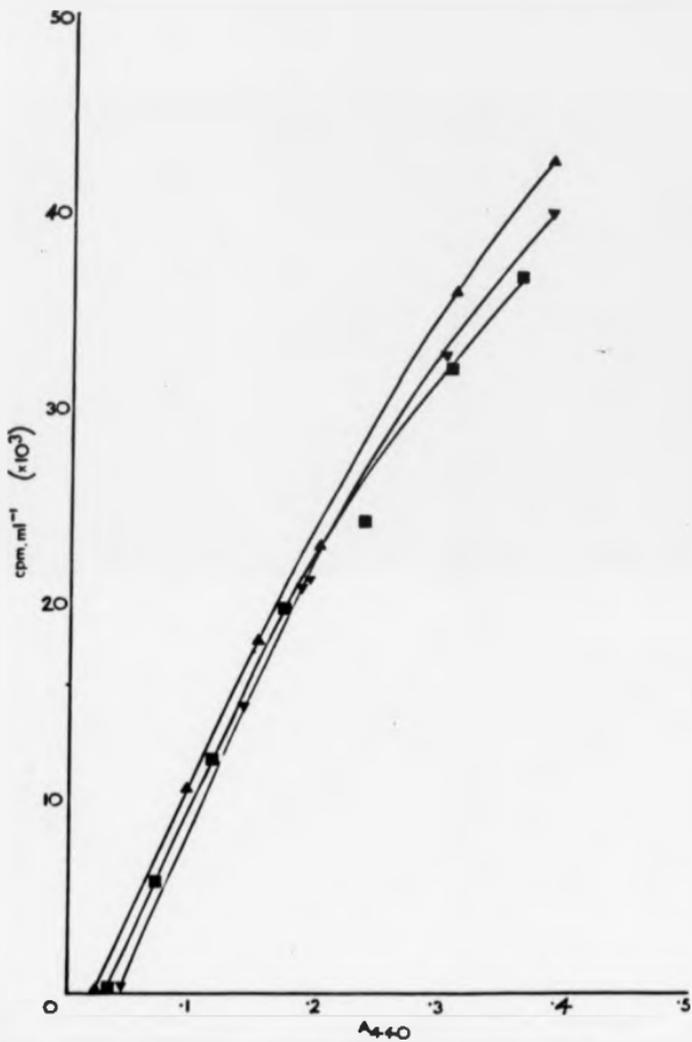
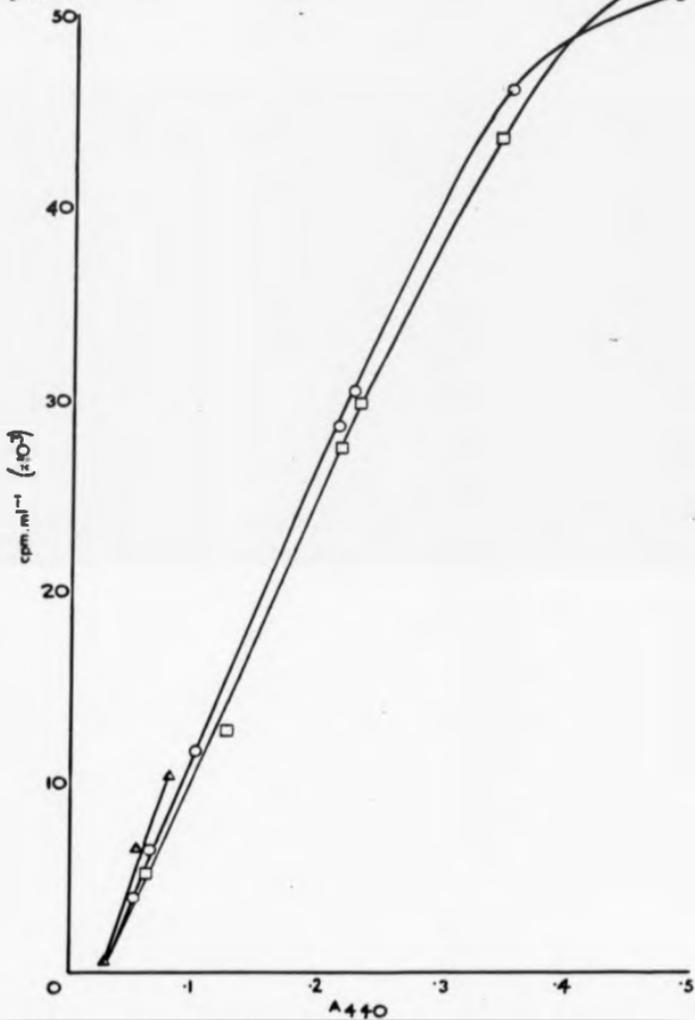


Figure 3.1b



#### 3.4. GROWTH OF OTHER THIOBACILLI ON METHYLATED SULPHIDES.

Some thiobacilli have previously been shown to grow on DMS in pure culture (Sivela & Sundman, 1975; Sivela, 1980; Kanagawa & Kelly, 1986). Although *T. thiooxidans*, *T. neapolitanus*, *T. intermedius* and *T. novellus* have been shown not to use methane thiol as a growth substrate (Sand, 1987), it appeared from the literature that a full screening of other *Thiobacillus* strains for their ability to grow on methylated sulphides had not been performed. Batch culture experiments to determine growth on MT, DMS and DMDS were carried out using a range of available *Thiobacillus* species which included: *T. varautus* (DSM 582), *T. ferrooxidans* (DSM 588), *T. thiooxidans* (ATCC 8085), *T. neapolitanus* (DSM 581), *T. leptodermus* (DSM 3134, ATCC 42215), *T. aquasulfa* (DSM 4255, ATCC 42788), *T. acidophilus* (DSM 700) and *T. thioparvus* (DSM 505). Inoculum cultures were pre-grown on either thiosulphate or tetrathionate. Apart from a very small increase in biomass and fall in pH observed for *T. ferrooxidans* grown on DMS, no growth was seen for any species on either of the three substrates. The growth of *T. ferrooxidans* however, could not be sustained on subculture.

### 3.5. DISCUSSION.

This is the first report of the isolation into pure culture of strains derived from batch enrichments capable of using DMS and DMDS (and also MT) as sole growth substrates. Although such a batch enrichment program proved very time consuming compared to continuous enrichments, it had the advantage of being technically more simple enabling sampling of many source materials simultaneously. Also, by allowing a period of batch growth, slower growing strains would not be lost from the culture if too high a dilution rate were used. Batch and continuous enrichments have however been used successfully in the past to yield DMSO- using bacteria (De Bont *et al* 1981; Suylen & Kuenen, 1986). Both enrichment methods however involve a certain amount of tedious isolation work to yield pure cultures.

Apart from the important isolation of several more strains capable of growth on DMS and MT in addition to those few already described, this is the first reported demonstration of aerobic bacteria capable of growth on DMDS as the sole energy substrate, although *Thiobacillus* NS1 could use DMDS when in a 1 : 1 mixture with DMS (Sivela & Sundman, 1975). The isolation of DMDS-degrading strains from a range of natural environments is of considerable interest, not only in showing how commonly this property occurs, but in indicating that DMDS too is probably an important and ubiquitous intermediate in the biogeochemical cycling of sulphur. Because of the difficulty of obtaining colony development from enrichments using plates incubated in air containing DMS or DMDS, all of the strains isolated have been obtained through their ability to grow as chemolithotrophic autotrophs with thiosulphate as the sole source of energy. No DMS- or DMDS- users were obtained by selection on methylamine medium. Consequently the isolation technique used exerted a strong selective bias towards the detection of

thiosulphate-oxidising 'thiobacillus-like' bacteria, able to use methylated sulphides, like *F. thioparus* TK-m (Kanagawa & Kelly, 1986) and *Thiobacillus* MS1 (Sivella, 1980). The results do not therefore preclude the possibility that DMS- and DMDS- users, able to grow as methylotrophs but unable to grow autotrophically on thiosulphate, were also present in the samples tested. Such organisms might have resembled the DMSO- and DMS- oxidising hyphomicrobia described earlier (De Bont *et al.*, 1981; Suylen & Kuenen, 1986; Suylen *et al.*, 1986), but their presence in the enrichment cultures could only have been proved by an even more exhaustive screening of large numbers of bacteria able to produce colonies on agar media with DMS, DMDS, DMSO or, in some cases, methylamine as substrate (De Bont *et al.*, 1981; Suylen & Kuenen, 1986). The inability of the isolated strains to use DMSO, and the inability of the hyphomicrobia to grow on thiosulphate, and in one case at least on methylamine or DMDS (De Bont *et al.*, 1981) means that conclusive demonstration of the different types of bacteria that might degrade methylated sulphides in specific environments is not simple.

All the strains isolated exhibited a very narrow range of substrates used, although not as restricted as *Hyphomicrobium* S, which used only DMSO and DMS (De Bont *et al.*, 1981). Their ability to grow autotrophically using energy from oxidising sulphide, sulphur, thiosulphate or tetrathionate under aerobic conditions is a characteristic of the *Thiobacillus* genus (Kelly & Harrison, 1988). All strains, except E3, also grew on thiocyanate, which is a property of some *F. thioparus* strains (Kelly & Harrison, 1988) including the strain TK-m which also uses DMS and  $CS_2$ , but not DMDS (Kanagawa & Kelly, 1986; This Study, see Ch. 6). Although all the isolated strains apparently grew on  $CO_2$ , none was capable of growth on  $CS_2$ . This indicates that in order to yield  $CS_2$  degrading strains,  $CS_2$  should have been included as an enrichment substrate. Five of the seven strains apparently grew autotrophically on either DMS or

DMDS determined by their  $^{14}\text{CO}_2$  incorporation patterns, and in this respect resembled *F. thioparvus* TK-m. Colonial morphology of all strains grown on thiosulphate plates were characteristic of thiobacilli and indicated that at least two distinct types were present. Acid production in liquid batch cultures containing high concentrations of thiosulphate was also consistent with that commonly observed with *F. thioparvus* strains. Weak anaerobic growth of all strains on thiosulphate was observed using nitrate as an alternative terminal electron acceptor. In each case nitrate was reduced only as far as nitrite (not to dinitrogen) which is a characteristic distinguishing *F. thioparvus* strains from *F. denitrificans* strains. Neither DMS nor DMDS supported anaerobic growth, possibly indicating some metabolic process with a requirement for molecular oxygen.

At this stage in their characterisation these strains can most probably be assigned to the genus *Thiobacillus*, with the distinctive feature that they are methylated sulphide oxidisers. This conclusion contradicts the statement by Sand (1987) that thiobacilli would not be able to produce sulphuric acid from DMDS, DMS, and NT as a contributory factor in corrosion of concrete sewage pipelines. Although no growth and subsequent acid production by *F. thiooxidans* or *F. neapolitanus* was observed on DMDS, DMS or NT in this study, and hence consistent with those observations of Sand (1987), it is perhaps unwise to make such a generalisation about the whole *Thiobacillus* genus given the diversity of their metabolism (see Kelly & Harrison, 1988).

In a later study the isolation of DMS and DMDS degrading strains was attempted using a different protocol, whereby an organic input was maintained during subculturing. Sample source material was streaked out onto mineral salts agar plates containing either DMS or DMDS. All colonies which developed were similarly subcultured. This method yielded some twenty

heterotrophic isolates, resembling coryneform bacteria, which in addition to DNS, DWDS, acetate and glucose, would also grow on diethyl sulphide, diethyl disulphide, dipropyl sulphide and 1-butane thiol. Some strains also appeared to use  $CS_2$ , but not sulphide. In liquid culture these isolates would only grow on the organic sulphur compounds in the presence of a small amount of yeast extract. None was capable of chemolithoautotrophic growth on any inorganic sulphur compound. (Chris Harfoot, personal communication). The existence of facultative methylotrophs and heterotrophic inorganic sulphur oxidisers is well documented (see Ch. 1), and so clearly one could envisage heterotrophic methylated sulphide degraders as having an important role in the natural environment, and their isolation of considerable interest. In hindsight, although the enrichment procedure used in the present study might have been expected to yield any autotrophic or methylotrophic methylated sulphide degrading strains present in the source material, the lack of any other organic input would have reduced the likelihood of isolating heterotrophic strains. It was tentatively concluded however that the autotrophic strains isolated during this study were at least the major methylated sulphide users in their various habitats.

CHAPTER F O U R

THE METABOLISM OF METHYLATED SULPHIDES BY STRAIN E6

---

#### 4.1. INTRODUCTION.

The isolation of several other strains of bacteria capable of growth on DMDS, in addition to DMS and MT, is of considerable microbiological and biochemical interest. Since these strains are the first of their kind to be reported, the mechanism of DMDS metabolism obviously merits further investigation. Furthermore, these strains strongly resembled autotrophic thiobacilli fixing  $\text{CO}_2$  whilst using inorganic sulphur compounds or methylated sulphides as energy sources. This feature alone distinguishes these strains and also *F. thioparvus* TK-m (Kanagawa & Kelly, 1986) from the DMS(O) oxidising *Hyphomicrobia* (De Bont *et al.*, 1981; Suylen & Kuenen, 1986; Suylen *et al.*, 1986) and *Thiobacillus* MS1 (Sivela & Sundman, 1975; Sivela, 1980) which assimilated DMS-carbon via the serine pathway. With one exception, all strains were capable of growth on thiocyanate. Since thiocyanate oxidising thiobacilli have been described, it would be interesting to establish whether or not there is any relationship between the ability to oxidise thiocyanate and the methylated sulphides.

A more detailed study was made of strain E6 originally isolated from a DMDS enrichment, which grew on all of the methylated sulphides and inorganic sulphur substrates tested.

#### 4.2. PHYSIOLOGICAL CHARACTERISTICS OF STRAIN E6.

##### 4.2.1. pH spectrum, growth rates and yields and cellular composition.

Strain E6 exhibited lags of 5-6 days when transferred from DMDS to DMS (and vice versa) and also when transferred from thiosulphate to DMDS or DMS. This lag however was greatly reduced when cells were transferred from DMDS or DMS to thiosulphate. Cells had a pH optimum of 6.7 - 6.9, although there was very little difference in growth rates ( $\mu = 0.08 - 0.009 \text{ h}^{-1}$ ) on either DMDS (2mM) or thiosulphate (5mM) between initial pH values of 6.2 - 7.2. No growth occurred below pH 5.5 or above pH 8.2. A final pH of 6.3 - 6.4 was observed following the complete consumption of 2mM DMDS or 5mM thiosulphate. The maximum specific growth rate ( $\mu_{\text{max}}$ ) observed during growth of batch cultures on both DMDS (2mM) and thiosulphate (5mM) was about  $0.09 \text{ h}^{-1}$ . From six determinations using cultures grown on 1-4 mM DMDS, the yield was  $13.6 \pm 0.8 \text{ g cell carbon (mole DMDS)}^{-1}$ . Since the addition of DMDS to cultures was by injection of undiluted liquid DMDS, the observed yields were surprisingly reproducible indicated by a low standard error. Similar experiments conducted using cultures grown on 2-10mM thiosulphate gave a yield of  $2.6 \pm 0.4 \text{ g cell carbon (mole thiosulphate)}^{-1}$ .

From the plots of culture absorbance ( $A_{440}$ ) versus TOC, protein and dry weight (Fig. 4.1), ( $A_{440}$ ) of 0.1 was equivalent to 115, 152 and  $244 \text{ mg l}^{-1}$ , respectively, cells therefore containing carbon at 47.1% and protein at 62.3% of the dry weight.

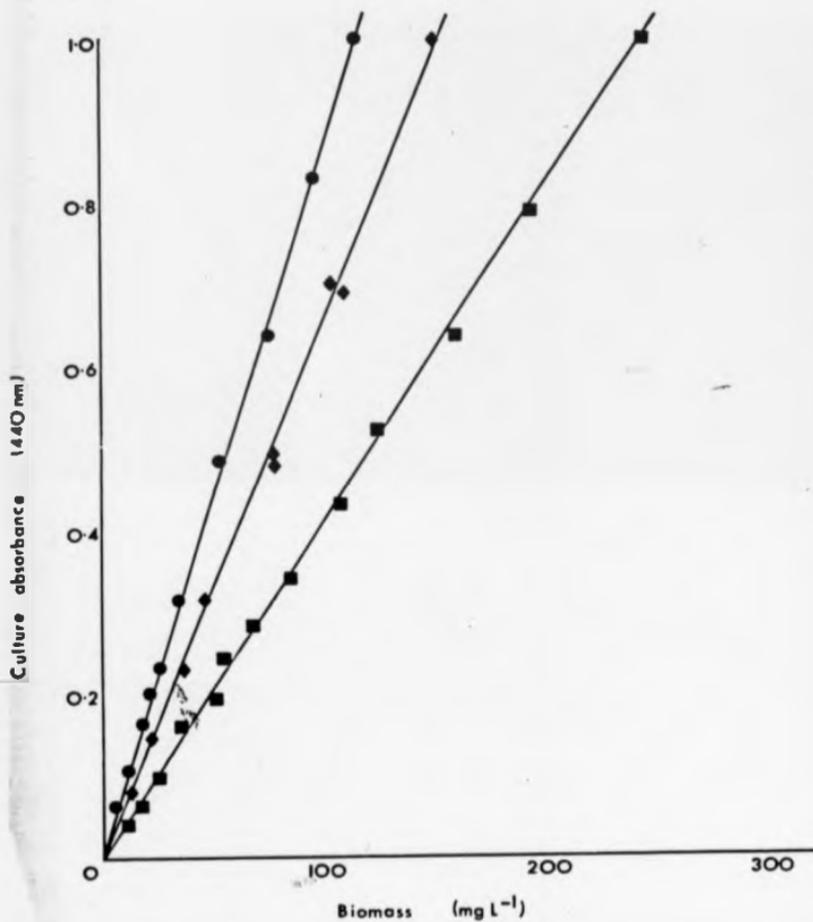
##### 4.2.2. Effect on growth by increased initial DMDS concentrations.

Strain E6 was routinely maintained in liquid medium by batch growth on 2mM DMDS, under the conditions described earlier, simply because this produced a reasonable biomass for easy inoculation upon subculture. Although careful

LEGEND TO FIGURE 4.1

Culture absorbance ( $A_{440}$ ) versus biomass ( $\text{mg l}^{-1}$ ) as total organic carbon (●), protein (◆) and dry weight (■) of T. thioaerius strain E6.

Figure 4.1



consideration was given to the choice of DWDS concentrations used in the enrichment cultures, the optimum concentration for growth of the pure culture had not been determined. Growth in liquid batch culture on DWDS commenced at initial concentrations up to 5mM (which is close to saturation of aqueous solution), but the initial rate of growth was depressed by increased concentrations of DWDS, indicating it to be an inhibitory substrate. Using eight concentrations of DWDS between 0.5 and 5mM, initial specific growth rate (11 - 21 h after inoculation) decreased from 0.087 h<sup>-1</sup> (at 0.5mM) to 0.07 (2mM), 0.053 (4mM) and 0.04 at 5mM DWDS. Final biomass levels were, however, proportioned to the amount of DWDS supplied. This is illustrated in Fig. 4.2.

#### 4.2.3. Determination of mole % Guanine + Cytosine.

The mole % G + C content of purified DNA from strain E6 was determined by two methods (see Section 2.2.18). According to the method of Fredericq *et al* (1961) this was calculated to be 59.9. Similar determinations for purified samples of DNA from *F. tepidarius* and *E. coli* gave figures of 66.6 and 51.1, respectively. For the absorbance ratios method of Ulitzur (1972), mole % G + C determination was calculated by comparison with known standards. For this purpose *F. tepidarius* and *E. coli* DNA was used. Absorbance profiles of all three purified DNA samples is illustrated in Fig. 4.3. Using *F. tepidarius* as the standard the mole % G + C content of strain E6 was calculated at 59.6 (at the ratio of 245/270), 60.2 and 61.4 (240/275), and 62.4 (240/280). Using *E. coli* as standard gave figures of 60.3 (245/270) and 59.6 (240/275). Consequently from these seven estimations using the two procedures, the DNA of strain E6 showed a mean mole % G + C content of 60.5 ± 1.0. This is in the normal range for *F. thioparus* (Kelly & Harrison, 1988). Comparative figures for other thiobacilli are given in Table 4.1.

LEGEND TO FIGURE 4.2

Growth of strain E6 in batch culture on DMDS. The inoculum culture was grown to the end of the log phase on DMDS (2mM):

Growth, as increase in optical density, shown for different initial concentrations (mM) of DMDS: 0.5 ( $\blacktriangle$ ), 1.5 ( $\circ$ ), 2.5 ( $\triangle$ ) and 5.0 ( $\bullet$ ).

LEGEND TO FIGURE 4.4

Growth ( $\blacktriangle$ ), DMDS disappearance ( $\bullet$ ) and sulphate formation ( $\circ$ ) shown for a culture growing on an initial concentration of 2.1 mM-DMDS.

Figure 4.2

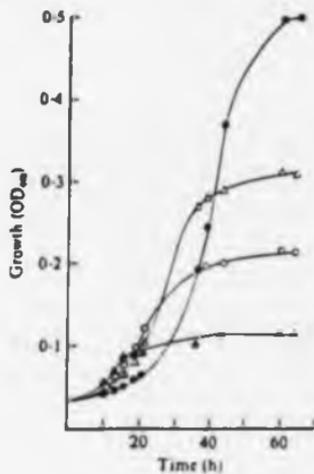
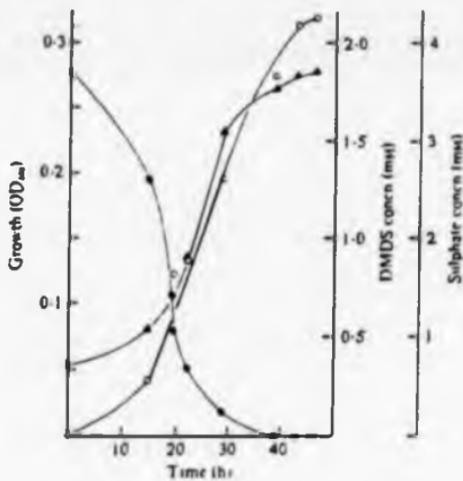


Figure 4.4



LEGEND TO FIGURE 4.3

Absorbance spectra of purified DNA from strain E6 (□).  
E. coli (◇) and T. tepidarius (○). See Methods Section  
for extraction procedures.

Figure 4.3

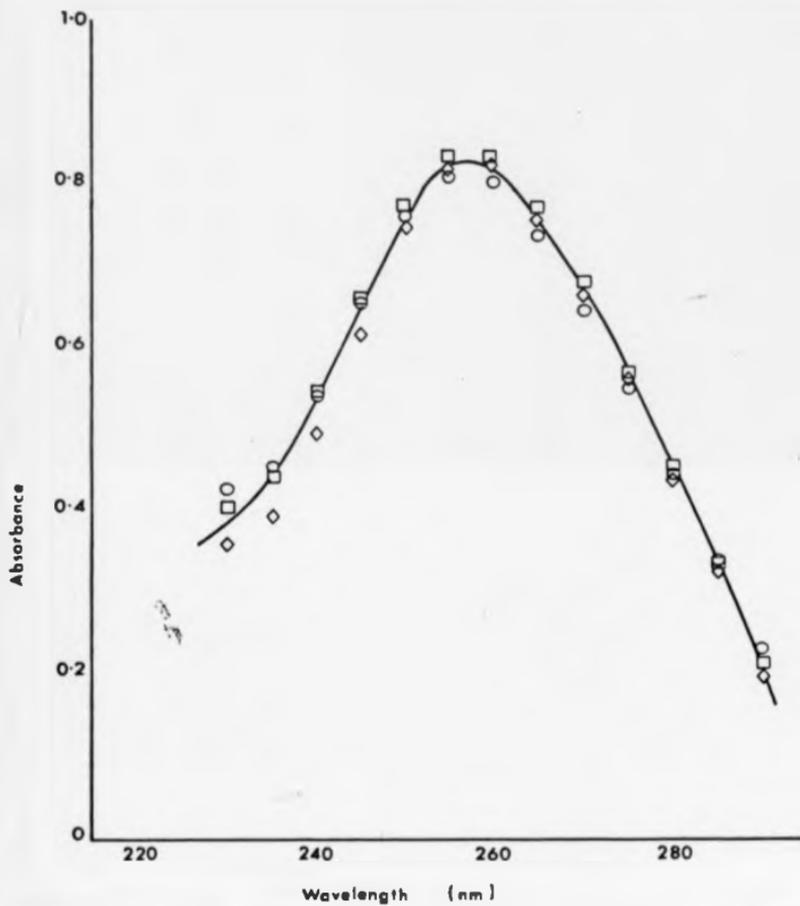


TABLE 4.1 Compilation of mol% G + C of the DNA for Thiobacillus species.

<u>Species</u>	<u>mol% G + C</u>
<u>T. thioparus</u>	61-66
<u>T. neapolitanus</u>	52.3 - 57
<u>T. tepidarius</u>	66.6
<u>T. denitrificans</u>	63 - 67.9
<u>T. aquaesulis</u>	65.7
<u>T. novellus</u>	66 - 68.4
<u>T. versutus</u>	65 - 69.5
<u>T. intermedia</u>	64.8 - 67
<u>T. perometabolis</u>	65 - 67.9
<u>T. delicatus</u>	66 - 67
<u>T. ferrooxidans</u>	53 - 59
<u>T. thiooxidans</u>	51 - 62
<u>T. albertis</u>	61.5
<u>T. acidophilus</u>	60.8 - 64

From Kelly and Harrison, 1988.

### 4.3. FIXATION OF $^{14}\text{CO}_2$ DURING GROWTH OF STRAIN E6 ON DWDS.

From earlier radiolabelling experiments (Chapter 3), the incorporation pattern of  $^{14}\text{CO}_2$  into strain E6 cells suggested that not only was growth on DWDS autotrophic but DWDS- carbon was probably oxidised to  $\text{CO}_2$ , this then being available for fixation. Conclusive proof of the latter could be determined by repeating the experiments and measuring the extent to which different amounts of added label are diluted following the addition of further substrate.

Using cultures previously maintained through successive subculture on DWDS as sole substrate, simultaneous measurement of growth and  $^{14}\text{CO}_2$  fixation showed that initially virtually all the cell carbon was derived from  $\text{CO}_2$  (Table 4.2), but the amount of  $^{14}\text{C}$  fixed relative to increase in biomass decreased during growth. This phenomenon was due to dilution of the added  $^{14}\text{CO}_2$  by oxidation of the DWDS to  $\text{CO}_2$ , and similar results have been reported during the growth of *F. salsoparus* TK-m on DMS (Kanagawa & Kelly, 1986). Assuming complete oxidation to  $\text{CO}_2$  of the 2, 4 and 6mM DWDS added at the times indicated on Table 4.2, the specific activity of the available  $^{14}\text{CO}_2$  could be re-calculated and the actual  $\text{CO}_2$  fixation estimated. This showed that  $\text{CO}_2$  - fixation was probably the only route for carbon assimilation during growth on DWDS (Table 4.2). Repeating this experiment with different initial amounts of  $^{14}\text{CO}_2$  provided further proof: the apparent fixation of  $^{14}\text{CO}_2$  was greatly decreased when 2.5mM rather than 10mM  $^{14}\text{C}$  - bicarbonate was present, but calculating the true specific activities after the presumed complete oxidation of DWDS to  $\text{CO}_2$  indicated that at least 80% of the cell carbon was provided from  $\text{CO}_2$  fixation. These results and the demonstration that the proportion of cell carbon initially obtained from  $\text{CO}_2$  was over 90% (Table 4.2), supports the assumption that DWDS was completely oxidised giving two moles of  $\text{CO}_2$  (mole DWDS consumed)<sup>-1</sup>.

LEGEND TO TABLE 4.2

- \* Indicates time of addition of a further 2 mM-DMDS.
- † Initial specific activity of  $^{14}\text{CO}_2$ , 17957 c.p.m.  $\mu\text{mol}^{-1}$ .
- ‡ Figures in parentheses are values for mg cell-carbon  $\text{l}^{-1}$  obtained from  $^{14}\text{CO}_2$ , calculated using specific activities based on the assumption that all the DMDS present was converted to  $\text{CO}_2$ . The specific activities at 56, 80 and 96h were calculated as 12826, 9976 and 8162 c.p.m.  $(\mu\text{mol CO}_2)^{-1}$  respectively. This overestimates  $^{14}\text{CO}_2$  fixation, as specific activity obviously declines progressively rather than instantaneously for each quantity of DMDS oxidized, but does allow an estimate of the maximum amount of carbon derived directly from  $\text{CO}_2$  fixation.
- § Proportions of total cell-carbon that could be derived from  $\text{CO}_2$  fixation, based on the above assumptions.

**TABLE 4.2** Growth and  $\text{CO}_2$  fixation by strain B6 growing on 2mM DMS  
in batch culture in the presence of 10mM  $\text{NaH}^{14}\text{CO}_3$ .

Time (h)	Optical density (440 nm)	Increase in		$^{14}\text{CO}_2$ fixed†		Apparent proportion of TDC derived from $^{14}\text{CO}_2$
		biomass (mg TOC $\text{l}^{-1}$ )	c.p.m. $\text{ml}^{-1}$	mg cell- carbon $\text{l}^{-1}$		
0	0.027	0	-	-	-	-
8	0.030	0.3	452	0.3	93	
24	0.052	3.3	3890	3.3	79	
32	0.101	12.1	11457	7.7	63	
48	0.213	30.0	28421	19.0	63	
56*	0.227	32.5	30340	20.3 (28.4)‡	62 (87)§	
72	0.348	51.0	46101	30.8	59	
80*	0.352	52.0	43872	29.3 (52.8)‡	56 (101)§	
96	0.483	73.0	51245	34.3 (75.3)‡	47 (103)§	

#### 4.4. CORRELATION BETWEEN DMDS CONSUMPTION AND SULPHATE PRODUCTION.

Growth on 2mM DMDS showed sulphate formation to be correlated with DMDS disappearance and increase in biomass. Growth and sulphate formation continued at a slower rate following the disappearance of dissolved DMDS (Fig. 4.4). Initially, about 87% of the added DMDS was in solution, the remainder being in the vapour phase in the flask. After 28h only 3% of this was left in solution, and biomass had increased to 80% of the final value achieved, but only 61% of the final concentration of sulphate had been produced. At 39h, when dissolved DMDS was no longer detectable, increase in biomass was at 94% of the final level and sulphate production was at least 86% complete (Fig. 4.4). This apparent continuation of growth after disappearance of dissolved DMDS could have been due to partition of DMDS between the liquid and vapour phases and its consumption in the final phase of growth at a rate determined by its solution rate. Additionally, there might have been some accumulation and subsequent use at a slower rate of intermediates from DMDS oxidation.

Within the error of measurement, two moles of sulphate were produced per mole of DMDS supplied, as would be expected if both sulphur atoms of DMDS were oxidised to sulphate. The overall increase in biomass (Fig. 4.4) for the complete consumption of DMDS was  $27 \text{ mg TOC l}^{-1}$ , equivalent to a yield of  $13.5 \text{ g cell carbon (mole DMDS)}^{-1}$  or  $6.75 \text{ g cell carbon (mole sulphate produced)}^{-1}$ . These figures are consistent with yields observed in other batch experiments (see above).

#### 4.5. GROWTH OF STRAIN B6 IN CHEMOSTAT CULTURE.

##### 4.5.1. Under DMDS - limitation.

Steady state cultures were established on 2mM DMDS as the growth limiting substrate at dilution rates between 0.046 and 0.081  $\text{h}^{-1}$ , between which the steady state growth yield (Y) increased from 13.0 to 15.6 g cell carbon (mole DMDS) $^{-1}$ . The true growth yield ( $Y_{\text{max}}$ ) was estimated from plots of 1/Y *versus* 1/D and of the specific rate of DMDS consumption ( $q_{\text{DMDS}}$ ) *versus* D (Fig. 4.5 a,b). DMDS consumption was estimated both from direct assay of the input medium and from the amount of sulphate produced in the steady state culture. For both plots, lines were drawn by linear regression fitting of yield data points derived from calculations based on input DMDS concentration. Good correlation existed between the two estimates of DMDS supplied, and also demonstrated again that DMDS was quantitatively oxidised to sulphate in steady state cultures. No elemental sulphur was produced by the cultures from DMDS, nor were there any detectable levels of formaldehyde or formate.

At steady state there was no odour of DMDS, or of any other volatile intermediate detectable in the effluent air flow. From Fig. 4.5a, extrapolation to zero dilution rate gave a  $q_{\text{DMDS}}$  value (the maintenance coefficient) of 1.1. The same value of  $q_{\text{DMDS}}$  was obtained from the slope of Fig. 4.5b.  $Y_{\text{max}}$  from both plots was about 17g cell carbon (mole DMDS) $^{-1}$ .

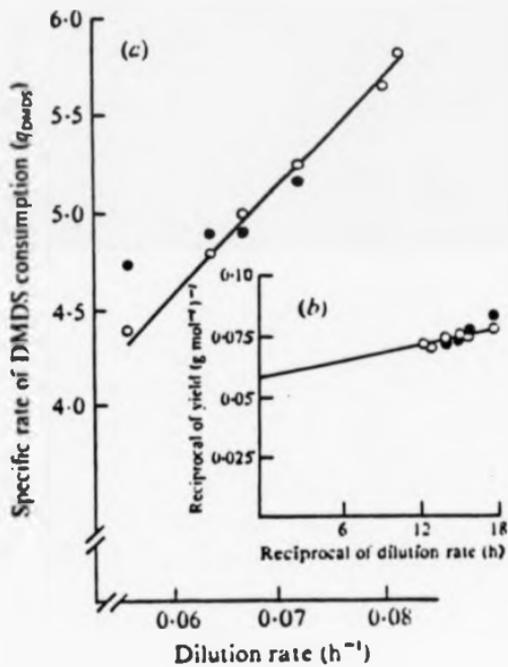
##### 4.5.2. Addition of thiosulphate to a DMDS-limited chemostat.

When a steady state culture ( $D = 0.078 \text{ h}^{-1}$ ) on 1.5 mM DMDS was simultaneously supplied with 2mM thiosulphate, both substrates were completely oxidised. Upon the addition of thiosulphate there was an immediate increase in biomass, the steady state biomass rising from 22.5 to 27.5 mg cell carbon  $\text{l}^{-1}$  and accompanied by a decrease from pH 6.7 to pH 6.3.

LEGEND TO FIGURE 4.5

Growth of Strain E6 in chemostat culture. Steady state cultures on 2 mM-DMS were obtained at several dilution rates. (a) Specific rate of DMS consumption [ $q_{\text{DMS}}$  =  $\mu\text{mol DMS consumed h}^{-1} (\text{g cell-carbon})^{-1}$ ] versus dilution rate. DMS consumption was estimated from both direct measurement of input DMS concentration ( $\circ$ ) and determination of steady state production rates for sulphate ( $\bullet$ ). The line was drawn by linear regression fitting of the ( $\circ$ ) data points. Extrapolation to zero dilution rate gives a  $q_{\text{DMS}}$  value (the maintenance coefficient) of 1.1. (b) Reciprocals of yield [ $\text{g cell-carbon} (\text{mol DMS})^{-1}$ ] versus dilution rate. The slope gives a  $q_{\text{DMS}}$  value (the maintenance coefficient) of 1.1. Symbols are as in (a).

FIGURE 4.5



This indicated a thiosulphate-dependent yield of 2.45 g cell carbon (mole thiosulphate oxidised)<sup>-1</sup>.

#### 4.5.3. Addition of DMS to a DMDS - Limited Chemostat.

Supplementing a DMDS - limited chemostat (1.8mM; D = 0.076 h<sup>-1</sup>) with DMS (1.2mM) resulted in an increase in steady state biomass from 23.0 to 37.2 mg cell carbon l<sup>-1</sup>. This increase, however, was not immediate as in the case of thiosulphate additions. Instead, a lag of about 4 days accompanied by a period of perturbation was observed, followed by a rapid increase in biomass. This data indicated substrate dependent yields of 14.6 g cell carbon (mole DMDS)<sup>-1</sup> and 11.8 g cell carbon (mole DMS)<sup>-1</sup>. The difference in yield on these two substrates of 2.8 g cell carbon corresponds to the energy made available for growth from the oxidation of the additional sulphur atom in DMDS. This is in good agreement with the observed increase in biomass produced by supplying a DMDS-limited chemostat with thiosulphate, since thiosulphate is energetically equivalent to sulphide.

Although several other thiols and homologues of DMDS were oxidised in the oxygen electrode by suspensions of DMDS-grown cells (see 4.6.3) it was not known if any of these compounds could provide energy to stimulate a further increase in biomass when metered separately under DMDS-limitation. With this in mind, preliminary batch culture experiments were carried out to determine the effects of adding ethanethiol to cells growing on DMDS. It was found that even at an ethanethiol concentration of 0.5mM, growth on 2mM DMDS ceased. Similar additions to the chemostat were then considered inadvisable.

#### 4.6. KINETICS OF SULPHUR AND CARBON OXIDATION.

##### 4.6.1. Oxidation rates and affinities for substrates.

Suspensions of strain E6 cells taken from a DMDS-limited chemostat (2mM;  $D = 0.074 \text{ h}^{-1}$ ) showed the concentration dependent oxidation of DMDS, NT, thiosulphate and sulphide, and could also oxidise formate and formaldehyde (Table 4.3). The substrate concentrations indicated are those which gave maximum values for oxygen uptake rate. Oxidation rates (V) were measured using the following substrate concentration ranges (S) : DMDS (2.5 - 30 $\mu\text{M}$ ), NT (1 - 20 $\mu\text{M}$ ), sulphide (10 - 100 $\mu\text{M}$ ), formaldehyde (10 - 500 $\mu\text{M}$ ) and formate (0.5 - 5mM). These data were used to construct Michaelis-Menton and Lineweaver-Burk plots illustrated in Figs. 4.6 1a-d.  $K_S$  and  $V_{\text{max}}$  values estimated from these plots and from plots of V versus V/S and S/V versus S are summarized in Table 4.4. As indicated, strain E6 showed high affinities for DMDS, NT and sulphide with  $K_S$  values of 2.5, 3.2 and 10 $\mu\text{M}$  respectively. Oxidation of these substrates was also very rapid with  $V_{\text{max}}$  values estimated at  $348 \pm 18$ ,  $1003 \pm 60$  and  $267 \pm 10 \text{ nmol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$  respectively. However, much lower affinities were observed for formaldehyde and formate,  $K_S$  values estimated at 84 and 678 $\mu\text{M}$  respectively. However, much lower affinities were observed for formaldehyde and formate,  $K_S$  values estimated at 84 and 678 $\mu\text{M}$  respectively. The lower oxidation rates also observed for formaldehyde and formate ( $76 \pm 10$  and  $35 \pm 0.5 \text{ nmol O}_2 \text{ min}^{-1} [\text{mg protein}]^{-1}$ ) compared to those of DMDS and NT might suggest an accumulation of these compounds in cultures, however, as already discussed this was not the case. DMDS and NT were shown to be inhibitory substrates (Figs. 4.6.a-d), maximum oxygen uptake rates being achieved at low substrate concentrations (20 $\mu\text{M}$  and 7 $\mu\text{M}$  respectively). However, above these concentrations there was no apparent decrease in oxygen uptake rates which might have been expected instead of the plateaus observed. Over the

**TABLE 4.3** Oxidation of one-carbon and sulphur compounds by strain E6 previously grown in chemostat culture on DMS (2mM ; 0.074h<sup>-1</sup>)

Substrate	Concn. ( $\mu$ M)	Oxygen uptake rate* (nmol O <sub>2</sub> min <sup>-1</sup> (mg protein) <sup>-1</sup> )
Sulphide	1000	588
Thiosulphate	500	410
Methanethiol	7	581
Dimethyl disulphide	20	301
Formaldehyde	500	51
Formate	5000	33
Dimethyl sulphide	5-50	0
Methylamine	5-5000	0
Dimethylamine	5-5000	0

\* Corrected for endogenous rate of oxygen uptake  
[6-7 nmol O<sub>2</sub> min<sup>-1</sup> (mg protein)<sup>-1</sup>]

**TABLE 4.4** Kinetic constants for the oxidation of four substrates by strain E6, previously grown in DMS-limited chemostat culture ( $2mM : 0.074 h^{-1}$ )

Substrate	$K_m$ ( $\mu M$ )	$V_{max}$ * [ $nmol O_2 min^{-1} (mg\ protein)^{-1}$ ]
Methanethiol	3.2	1003 +/- 60
Dimethyl disulphide	2.5	348 +/- 18
Formaldehyde	84	76 +/- 10
Formate	678	35 +/- 0.5

\* Values are given as mean (+/- SE) for estimates using plots of  $1/v$  versus  $1/s$ ,  $v$  versus  $v/s$ , and  $s/v$  versus  $s$ .

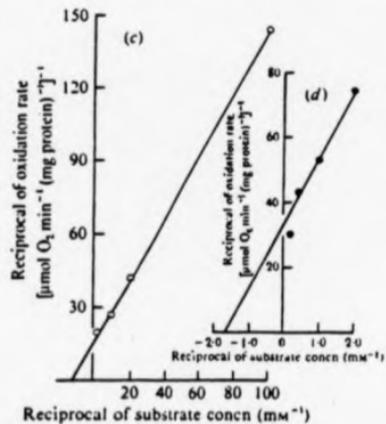
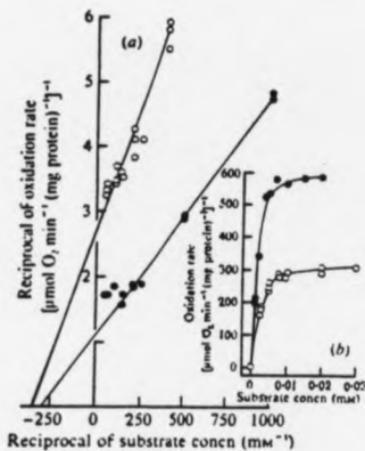
LEGEND TO FIGURE 4.6

Kinetics of oxidation of DMDS, MT, formaldehyde and formate by strain E6,  
taken from substrate-limited chemostat culture on DMDS:

(a), Lineweaver-Burk plot of reciprocals of oxidation rates versus reciprocals  
of substrate concentration for DMDS (○) and MT (●). (b) Oxidation rate  
versus substrate concentration for DMDS (○) and MT (●).

(c) and (d) Lineweaver-Burk plots for formaldehyde (○) and formate (●).

Figure 4.6



concentration ranges used, sulphide, formaldehyde and formate did not appear to be inhibitory.

DMS and methylamine were not oxidised by DMDS-grown cells (Table 4.3), but both DMDS and DMS were oxidised by organisms removed from the chemostat subject to dual substrate limitation (see above). Two weeks after the addition of DMS (1.2mM) to a DMDS-limited chemostat (2mM;  $D = 0.076 \text{ h}^{-1}$ ), DMDS (5 $\mu\text{M}$ ) was oxidised at  $240 \text{ nmol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$  and DMS at 95.5 (5 $\mu\text{M}$ ) and 79 (20 $\mu\text{M}$ ). These figures indicate that even at very low concentrations DMS is also an inhibitory substrate. Using the oxygen uptake rate figures for DMDS and DMS, both at 5 $\mu\text{M}$ , DMS was oxidised at only 40% of the rate of DMDS oxidation.

#### 4.6.2. Stoichiometry for DMDS, NT and Sulphide Oxidation.

Using different amounts of DMDS, the stoichiometry of oxygen uptake was found to be  $6.5 \text{ nmol O}_2 (\text{nmol DMDS})^{-1}$ , which indicated the complete oxidation of DMDS as follows:



Similarly, using different amounts of NT, its complete oxidation approximated to:



The observed stoichiometry for the complete oxidation of sulphide to sulphate was always below the theoretical value of  $2 \text{ nmol O}_2 (\text{nmol S}^{2-})^{-1}$ . Typical values were about 1.4 - 1.7  $\text{nmol} (\text{nmol S}^{2-})^{-1}$ .

TABLE 4.5 Oxidation of other thiols and homologues of methanethiol and dimethyl disulphide by *T. thioparus* strain E5

Substrate	nmol O <sub>2</sub> consumed min <sup>-1</sup> (mg protein) <sup>-1</sup>	% of MT oxidation rate
Ethanethiol (10uM)	78 +/- 6	14.7
1-butanethiol (10uM)	62 +/- 2	11.5
1-hexanethiol (10uM)	57 +/- 2	10.6
2-mercaptoethanol (10uM)	21 +/- 2	3.9
Thiophenol (10uM)	20 +/- 0.5	3.7
Diethyl disulphide (20uM)	73 +/- 6	13.5
Dipropyl disulphide (20uM)	20 +/- 4	3.7
Thiocyanate (50uM)	0	0

(MT oxidation rate at 5uM (100%) was calculated to be  
538 nmol O<sub>2</sub> min<sup>-1</sup>(mg protein)<sup>-1</sup>)

#### 4.6.3. Oxidation of other thiols and homologues of DMDS.

Suspensions of strain E6 cells in the oxygen electrode oxidised NT (5 $\mu$ M) at 538 nmol O<sub>2</sub> min<sup>-1</sup> (mg protein)<sup>-1</sup>, but only oxidised ethanethiol, 1 - butanethiol, 1 - hexanethiol, 2 - mercaptoethanol and thiophenol (each at 10 $\mu$ M) only at 78, 62, 57, 21 and 20 nmol O<sub>2</sub> min<sup>-1</sup> (mg protein)<sup>-1</sup> respectively (Table 4.5). DMDS (20 $\mu$ M) was oxidised at 309 nmol O<sub>2</sub> min<sup>-1</sup> (mg protein)<sup>-1</sup> compared with only 73 and 20 for diethyl disulphide and dipropyl disulphide (both at 20 $\mu$ M) respectively. The low affinities for these compounds indicated that the enzyme systems responsible for DMDS and NT oxidation were very specific.

#### 4.7. INHIBITION OF THIOSULPHATE AND TETRATHIONATE OXIDATION AND THE DEGRADATION OF UBIQUINONES IN MICE CHROMIUM CELLS.

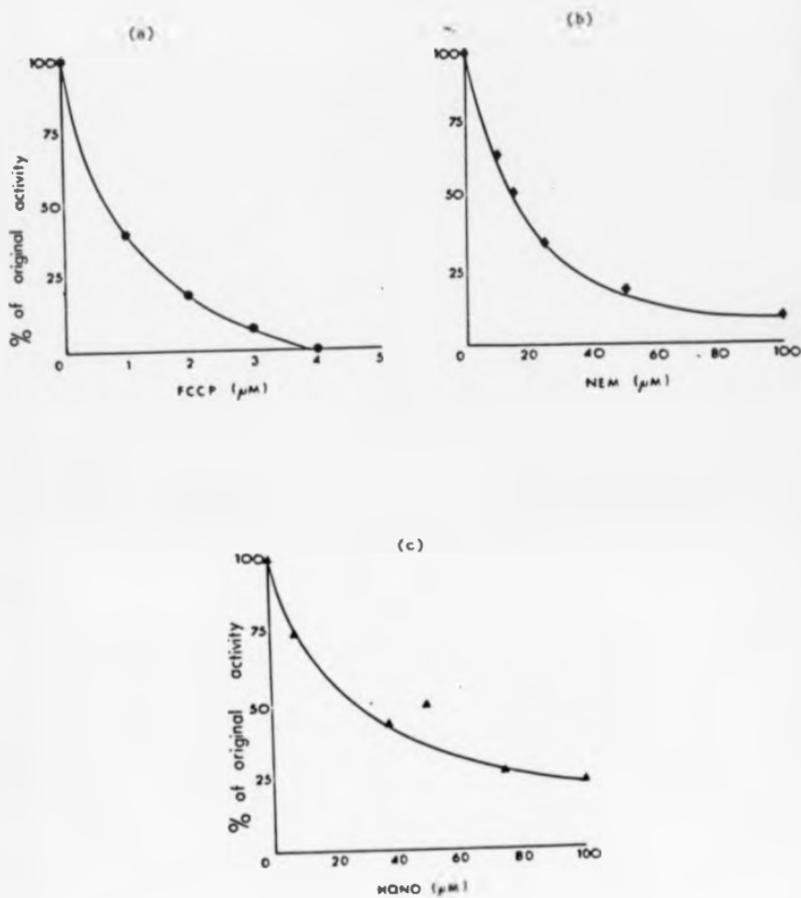
##### 4.7.1. Inhibition by NEM.

Thiol groups on the cell membrane are reported to be necessary for the metabolism of thiosulphate and the use of thiol-binding reagents such as NEM (N-ethyl maleimide) block their oxidation to sulphate by *F. neoopolitana*, *F. ferrooxidans* and *F. varianus* (Trudinger, 1965; Kelly & Tuovinen, 1975). According to Trudinger (1965), tetrathionate is not a true intermediate of thiosulphate oxidation but a secondary product arising from the reaction of an "activated" molecule of thiosulphate with a second free thiosulphate molecule. The effect of NEM is to block the oxidation of "activated" thiosulphate to sulphate. Alternatively, if tetrathionate is a true intermediate of thiosulphate oxidation, the effect of NEM is to block tetrathionate oxidation to sulphate. Whichever mechanism is operating the net effect is the accumulation of tetrathionate from thiosulphate.

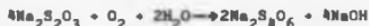
LEGEND TO FIGURE 4.7

Inhibition of thiosulphate (a and b) and tetrathionate (c) oxidation by T. thioparus strain E6 in the presence of NEM (◆), FCCP (●) and HQNO (▲). Cells were taken directly from a DMDS-limited chemostat culture (2 mM;  $D=0.072h^{-1}$ ) and allowed a pre-incubation period of 2-4 mins. in the oxygen electrode cell prior to the addition of substrate.

Figure 4.7



Cells taken directly from a DNDS-limited chemostat (1.8mM; D = 0.072 h<sup>-1</sup>) oxidised thiosulphate (50μM) at a rate of 120 nmol O<sub>2</sub> min<sup>-1</sup> (μg cell protein)<sup>-1</sup>. When thiosulphate was added following a pre-incubation period of 2-4 min. in the presence of NEM (10 - 100μM), the rate of thiosulphate oxidation decreased with increased inhibitor concentration (Fig. 4.7.6a), the oxidation rate being inhibited 50% (I<sub>50</sub>) by 16μM NEM. In addition to decreased oxidation rates in the presence of NEM, the stoichiometry of oxygen uptake also indicated the incomplete oxidation of thiosulphate to sulphate, the amount of oxygen consumed approximating to the following equation:



When NEM (25μM) was added 4 minutes after thiosulphate, there followed an immediate decrease in oxidation rate, oxygen uptake ceasing after a further 9 minutes. The addition of a further 50μM thiosulphate resulted in the immediate re-commencement of oxygen uptake and again indicated only the first phase of thiosulphate oxidation to tetrathionate.

#### 4.7.2. Inhibition by FCCP.

FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) is a potent protonophoric uncoupler in that it abolishes proton gradients and prevents any membrane associated energy metabolism such as ATP synthesis and solute transport. In *F. tepidarius* the oxidation of thiosulphate to tetrathionate occurs in the periplasm and is unaffected by FCCP, but the further oxidation of tetrathionate to sulphate cannot proceed in the presence of FCCP since this prevents transport of tetrathionate to the cytoplasmic side of the cell membrane (Kelly, 1988; Lu & Kelly, 1988 a,b). The net effect of FCCP

therefore is to cause an accumulation of tetrathionate from thiosulphate. In contrast, in *F. varaninus* FCCP has no effect on the complete oxidation of thiosulphate to sulphate (in the periplasm) since tetrathionate is not an intermediate in thiosulphate oxidation. However ATP synthesis linked to electron transport is abolished (Kelly, 1988; Lu & Kelly, 1988 c).

Thiosulphate oxidation rate in DMDS-grown strain E6 cells was inhibited when thiosulphate (50 $\mu$ M) was added after 2-4 minutes pre-incubation with FCCP (1-3 $\mu$ M). A value for  $I_{50}$  of 0.8 $\mu$ M FCCP was observed and FCCP concentrations above 3 $\mu$ M prevented any oxygen uptake (Fig. 4.7a). The stoichiometry of oxygen uptake indicated incomplete oxidation of thiosulphate with the presumed accumulation of tetrathionate. Tetrathionate was not oxidised in the presence of FCCP.

#### 4.7.3. Inhibition by HQNO.

HQNO (2-heptyl-4-hydroxyquinoline-N-oxide) inhibits electron transport between cytochrome *b* and cytochrome *c*. Since the electrons made available from the oxidation of thiosulphate to sulphate (in the case of *F. varaninus*) and from thiosulphate to tetrathionate (*F. sapidarius*) are linked to cytochrome *c*, such oxidations are insensitive to HQNO. In the latter case, further oxidation of tetrathionate to sulphate involves cytochrome *b* and so are sensitive to HQNO (Kelly, 1982; Kelly, 1988; Lu & Kelly, 1988 a,b).

Thiosulphate oxidation by DMDS-grown strain E6 cells was unaffected by HQNO up to 150 $\mu$ M, however 75 $\mu$ M HQNO apparently resulted in a 40% decrease in the oxidation rate of tetrathionate (the uninhibited rate was 106 nmol O<sub>2</sub> mm<sup>-1</sup> (mg cell protein)<sup>-1</sup>) (Fig. 4.7c).

#### 4.7.4. Determination of Ubiquinones.

The ubiquinones extracted from DMDS-grown strain E6 cells were identified by comparison with RF values of known standards following TLC analysis. RF values of 0.22 were calculated for the UQ-10 standards from sigma and *F. ferrooxidans*, and 0.68 for the UQ-8 standards from *F. tepidarius* and *F. dentrificans*. The results from the sample track showed the presence of only one very intense spot which also had an RF value of 0.68. This indicated that the major (if not only) ubiquinone present in strain E6 was UQ-8.

#### 4.8. DISCUSSION.

The maximum specific growth rate exhibited by strain E6 on DMDS or thiosulphate was about  $0.09\text{h}^{-1}$ , which is at least equal to that of *Hyphomicrobium* EG on DMS, and considerably greater than that of *Hyphomicrobium* S (De Bont *et al.*, 1981; Suylen & Kuenen, 1986). Cells were capable of growth over a fairly broad pH range with an optimum of pH 6.7 - 6.9, acid production resulting during batch cultures on all substrates that would support growth. Cells were composed of 47% carbon and 62% protein, figures which are typical for most thiobacilli (Kelly & Harrison, 1988). Purified DNA from strain E6 showed a mole % C + G content of  $60.5 \pm 1.0$ . Since this is in the normal range for *F. thio-parvus* (Kelly & Harrison, 1988), strain E6 was subsequently referred to as *Thiobacillus thio-parvus* strain E6.

Repetition of the  $^{14}\text{C}$ -radiolabelling experiments again showed that carbon assimilation during growth on DMDS was predominantly by  $\text{CO}_2$ -fixation. Not only was DMDS-carbon shown to be oxidised to  $\text{CO}_2$ , it was also possible, from re-calculation of the specific activities of the  $^{14}\text{C}$ -label following dilution, to estimate the stoichiometry of  $\text{CO}_2$  production of 2 moles  $\text{CO}_2$  (mole DMDS consumed) $^{-1}$ . The  $^{14}\text{CO}_2$  incorporation data along with the demonstration that growth on DMDS produced 2 moles sulphate (mole DMDS consumed) $^{-1}$  described the complete oxidation of the DMDS molecule. Further evidence for this was also provided by the stoichiometry of oxygen uptake. Low substrate affinity constants ( $K_s$ ) of  $2.5\mu\text{M}$  and  $3.2\mu\text{M}$  were determined for DMDS and MT respectively. This appears to be consistent with the observation that all strains described to date capable of oxidising one or more of the methylated sulphides have high affinities for their substrate. Reported  $K_s$  values for DMS, using DMS-grown cells are: *F. thio-parvus* TK-m,  $45\mu\text{M}$  (Kanagawa & Kelly, 1986); *Hyphomicrobium* S,  $16\mu\text{M}$ ; *Hyphomicrobium* EG,

48 $\mu$ M (Suylen & Kuenen, 1986), (these latter two figures both fell to 5 $\mu$ M when cells were grown on DMSO). There was however a distinct difference between these strains for the affinities of inorganic sulphur compounds.  $K_s$  values for *F. thioparvus* E6 were about 10 $\mu$ M and 27 $\mu$ M for sulphide and thiosulphate respectively and are in contrast to those observed for *Hyphomicrobium* EG which had  $K_s$  values of 17 $\mu$ M (DMSO-grown) or 22 $\mu$ M (methylamine-grown) and 0.5mM for sulphide and thiosulphate respectively (Suylen *et al.*, 1986). Non-sulphur analogues of DMS and NT such as dimethylamine and methylamine were not oxidised either by DMDS-grown cells (Table 4.3) or under dual substrate limitation with DMDS and DMS. Higher thiols and analogues of DMDS were oxidised (although at a much slower rate) but were unlikely to provide any useful energy for growth. For instance, ethanethiol added to a culture growing on 2mM DMDS caused growth to cease even at very low concentrations. It was not clear however whether this toxic effect upon the DMDS/NT metabolic system was due either to ethanethiol itself or to one of its breakdown products - presumably sulphide and acetaldehyde.

The failure to obtain growth on formaldehyde or formate as sole substrates indicated that there may be some dependence for growth on the presence of an oxidisable sulphur compound. Formate would also only support weak growth of *Hyphomicrobium* EG (Suylen & Kuenen, 1986). The inability to grow on methylamine and dimethylamine (and also formaldehyde) again indicated that DMDS, DMS and NT were not metabolised by any methylotrophic pathway.

The growth yields of thiobacilli on inorganic sulphur compounds depends upon the mechanism of oxidation and hence the amount of energy conserved at each stage. Sensitivity to inhibitors of electron transport and inorganic sulphur compound oxidation (particularly thiosulphate and tetrathionate)

has in the past provided useful data to help establish the differences existing between species and hence the discovery that there are at least two distinct mechanisms for thiosulphate oxidation (Kelly, 1988) (refer to Ch.1). *F. thioparvus* strain E6 showed a thiosulphate-dependent yield comparable to that observed with *F. varausus* at about 2.5 g cell-carbon (mole)<sup>-1</sup> (see below). Consequently one might therefore expect that electron transport is coupled to thiosulphate oxidation exclusively linked through cytochrome *a* with no involvement of cytochrome *b*. Although thiosulphate oxidation was insensitive to HQNO, there was some evidence to show that tetrathionate oxidation was sensitive (Fig. 4.6c), but the reasons for which are not clear. This observation tends to suggest the involvement of cytochrome *b*. If this were the case then a yield comparable to that seen with *F. septidarius* and *F. denitrificans* would be expected due to the extra site available for ATP generation (Wood & Kelly, 1985, 1986; Justin & Kelly, 1978; Kelly, 1982). The possibility that HQNO-sensitive electron transport involving cytochrome *b* but not involving ATP generation occurred seems unlikely but cannot be ruled out. NEM- and FCCP-sensitive thiosulphate oxidation indicated that tetrathionate was an intermediate in thiosulphate oxidation and that the first phase of thiosulphate oxidation occurs in the periplasm. In this respect, the mechanism of thiosulphate oxidation more closely resembled that occurring in *F. septidarius* or *F. neapolitanus* than in *F. varausus* (Trudinger, 1965; Kelly & Tuovinen, 1975; Kelly, 1988).  $I_{50}$  values for FCCP (using thiosulphate as substrate) and HQNO (using tetrathionate) for strain E6 were 0.8 $\mu$ M and 35 $\mu$ M respectively, and were in good agreement with similar figures obtained for *F. septidarius* of 0.05 $\mu$ M and 8 $\mu$ M respectively. Strain E6 however appeared to be far more sensitive to NEM than *F. neapolitanus* (Kelly & Tuovinen, 1975), inhibition of thiosulphate oxidation being observed using micromolar rather than millimolar concentrations.

The structural variation of ubiquinones together with their role in electron transport chains have a diagnostic value in microbial characterisation (Collins & Jones, 1981). The demonstration that UQ-8 was the only detectable ubiquinone present in DMDS-grown cells is also indicative of *F. thioeparus* strains. Since it appears that electrons from the oxidation of organic and inorganic sulphur enter the electron transport chain at the level of cytochrome *c*, UQ-8 is involved, along with cytochrome *b* and flavoproteins, in reverse electron flow before the reduction of  $\text{NAD}^+$  (Kelly, 1982; Inglede, 1982).

The growth yield of *F. thioeparus* E6 in DMDS-limited chemostat culture (at a dilution rate of  $0.078\text{h}^{-1}$ ) was 14.6 g cell-carbon  $(\text{mole DMDS})^{-1}$ . This increased in the presence of DMS by the equivalent of 11.8 g cell-carbon  $(\text{mole DMS})^{-1}$ , the difference between the DMDS- and DMS-dependent yields representing an estimate of the yield derived from the energy made available from the oxidation of the extra sulphur atom (2.8 g cell-carbon  $(\text{mole S}^{2-})^{-1}$ ) of DMDS.

In the presence of thiosulphate the observed stimulation in yield was equivalent to 2.45 g cell-carbon  $(\text{mole thiosulphate})^{-1}$ . Thus, assuming a yield equivalent to this to be due to each of the sulphide groups of DMDS (Kelly & Kuenen, 1984), sulphide oxidation would support 4.9 of the observed 14.6 g cell-carbon  $(\text{mole DMDS})^{-1}$ . This would mean that oxidation of the methyl-groups of DMDS to  $\text{CO}_2$  provided energy for production of 4.85 g cell-carbon  $(\text{mole CO}_2 \text{ produced})^{-1}$ . In view of the fact that the conversion of the methyl-groups to formaldehyde might not result in energy conservation (Suylen *et al.*, 1986), the energy yielding steps would be the oxidation of formaldehyde to formate and formate to  $\text{CO}_2$ . Previous work with *F. parvus* (A2), which can grow autotrophically on formaldehyde and

formate, showed yields (under growth conditions comparable to those used for *F. thiotropus* E6) of about 5 g cell-carbon (mole formaldehyde)<sup>-1</sup> and 2 g cell-carbon (mole formate)<sup>-1</sup> (Kelly *et al.*, 1979; Wood & Kelly, 1981; Kelly & Wood, 1984). The yields observed with *F. thiotropus* E6 are thus wholly consistent with energy being derived simultaneously from the oxidation of formaldehyde and sulphide produced from DMDS, and being used to support autotrophic growth dependent on the Calvin cycle.

C H A P T E R   F I V E

THE ENZYMOLOGY OF DIMETHYL DISULPHIDE METABOLISM

BY THIOBACILLUS THIOPARUS STRAIN E6

---

### 5.1. INTRODUCTION.

The mechanism of DMDS degradation can be inferred from the observations so far. DMS is excluded as an intermediate because: (i) the long lag before growth observed when cultures previously grown on DMS or DMDS were inoculated into media containing the other substrate; and (ii) the failure of DMDS-grown organisms to oxidise DMS in the oxygen electrode, although MT was oxidised very rapidly. This indicated that the initial step in DMDS metabolism might be its reductive cleavage to MT. By analogy with DMS and MT oxidation by hyphomicrobia (De Bont *et al.*, 1981; Suylen *et al.*, 1986), MT was probably oxidised to sulphide and formaldehyde, the latter being oxidised *via* formate to  $\text{CO}_2$ , and the former to sulphate. The ability of DMDS-grown organisms to oxidise formaldehyde, formate and sulphide supports this view. The studies so far have mainly involved physiological observations of whole cells. Conclusive evidence for the DMDS degradation pathway proposed above requires firstly, the identification of at least some of the intermediates involved in the process and secondly, demonstration of the activity of enzymes responsible for catalysing the individual steps of the pathway. The following chapter describes some enzyme analyses with cell free extracts and also includes other whole cell experiments carried out in order to provide further indirect evidence for their involvement.

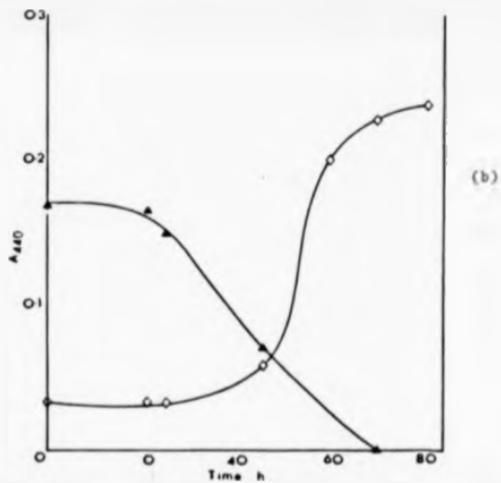
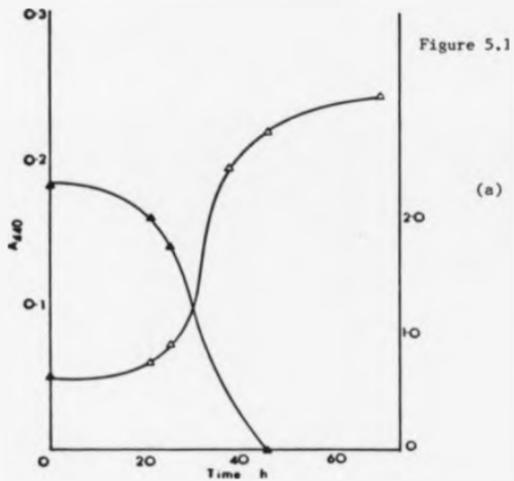
## 5.2. EVIDENCE FOR INDUCIBLE DMDS (AND DMS) OXIDATION SYSTEMS.

Batch cultures of *F. thiooparus* E6 were grown on both of DMDS (2mM) and thiosulphate (10mM). Before the onset of the stationary phase, each of these was then used to inoculate further DMDS and thiosulphate cultures and their growth monitored for up to 80h incubation at 30°C (Figs 5.1a,b). No significant lag was observed for DMDS-grown cells in the DMDS culture (Fig. 5.1a), but only 22% of the added DMDS had been consumed up to 20h incubation. Exponential growth continued up to 45h incubation and this corresponded to the time of complete consumption of dissolved DMDS. There was a further period of continued growth up to 68h which accounted for about 14% of the final biomass achieved, the explanation for this phenomenon has already been discussed in detail (see section 4.3). A lag of 25h was observed following the inoculation of a DMDS culture with thiosulphate grown cells (Fig. 5.1b) and there was no significant consumption of DMDS until growth commenced. There was a much slower onset to the exponential phase than in the previous case, but the observed maximum growth rate was similar in both cases at about  $0.08-0.09h^{-1}$ . There was also a much slower rate of consumption of DMDS, this not complete until 70h incubation. Following the inoculation of thiosulphate cultures with DMDS- and thiosulphate-grown cells, the patterns of growth and consumption of thiosulphate were almost identical in both cases.

On numerous occasions lags were observed following the transfer of *F. thiooparus* E6 from DMDS to DMS (and *vice versa*) and from thiosulphate to DMS, but not from DMS to thiosulphate. More detailed experiments like those described above were, however, not conducted following such transfers, but even so, from these data it appears that the metabolism of DMDS and DMS is *via* different routes, both of which are inducible.

LEGEND TO FIGURE 5.1

Evidence for an inducible DMDS oxidation system. Batch cultures (50 ml) of T. thioarum strain E6 grown on DMDS (2 mM) and thiosulphate (10 mM) were used as inocula (10% v/v) for further DMDS cultures. The rate of DMDS consumption ( $\blacktriangle$ ) and growth on (a) DMDS ( $\triangle$ ) and (b) thiosulphate ( $\diamond$ ) was monitored for up to 70h incubation at 30°C.



### 5.3. EVIDENCE FOR A DMDS REDUCTASE.

#### 5.3.1. Evaluation of the assay methods available.

If the initial attack on DMDS involved its reductive cleavage to MT, the activity of the enzyme responsible for catalysing the reaction could be assayed, at least in theory, by measuring: (i) the rate of disappearance of DMDS, (ii) the rate of appearance of MT, or (iii) the rate of disappearance of a physiological reductant donor - presumably either NADPH or NADH. Repeated attempts to assay DMDS reductase by this latter method using cell free extracts proved unsuccessful. Even when using combinations of different assay conditions: temperature, 25-45°C; pH, 6.0-8.0; DMDS concentration, 0.2-20µM; phosphate buffer, 50-150µmol; there was no increase in the rate of NADPH or NADH disappearance above the respective oxidase levels of 43.5 nmol min<sup>-1</sup> (mg protein)<sup>-1</sup> (Table 5.1). Assay by the time-dependent disappearance of DMDS by GC analysis was considered impractical since DMDS had a long retention time in comparison to the time course required for such experiments. The time-dependent appearance of MT from whole cell incubations provided the best evidence for the existence of a DMDS reductase.

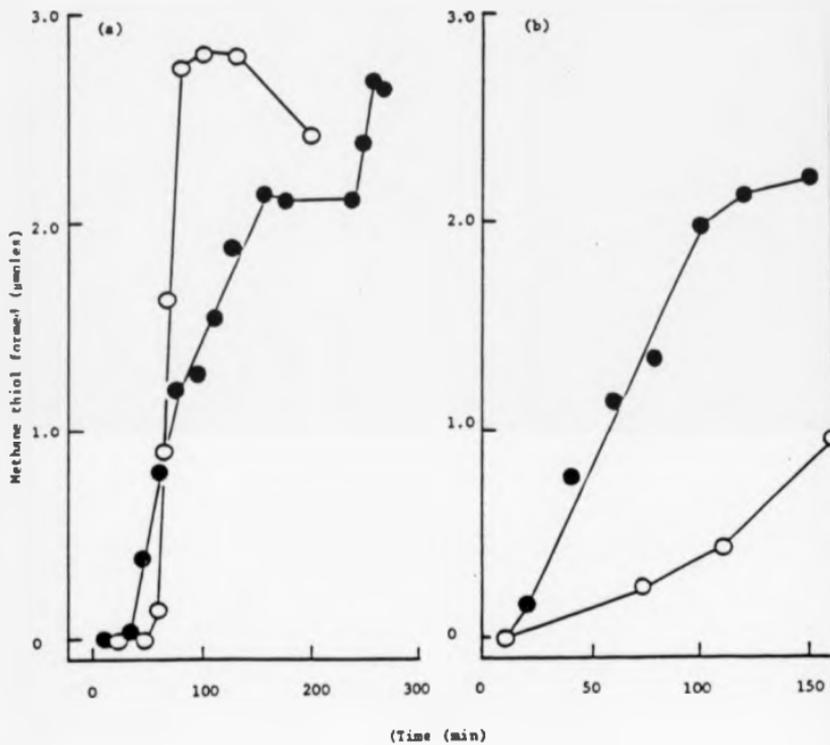
#### 5.3.2. Assay of DMDS reductase activity.

Suspensions of *F. thloporus* E6, harvested from a DMDS-limited chemostat culture, produced methanethiol from DMDS when incubated under anaerobic conditions (Fig. 5.2). There was a significant lag before MT production into the headspace gas commenced, possibly due to equilibration between solution and gas phase. This lag was slightly decreased when formaldehyde was also supplied (as a possible source of reductant), but neither the rate nor extent of MT production was as great in its presence. Adding further

LEGEND TO FIGURE 5.2

Formation of methane thiol from dimethyl disulphide by (a) suspensions of intact organisms and (b) CTAB-treated suspensions of T. thioparus strain E6. (a) DMDS (10 umoles) was added to 5 ml of cell suspension (3.5 mg protein ml<sup>-1</sup>) under a headspace of 18 ml of nitrogen gas. MT in the headspace was measured using suspensions incubated at 30°C in the absence (○) and presence (●) of 0.5 mM HCHO. A further 10 umoles of DMDS was added to the latter at 240 min. (b) A suspension (5 ml) treated with 0.08% (w/v) CTAB was incubated with DMDS (2 umoles) in the absence (○) and presence (●) of 4 mM NADH.

Figure 5.2



DMDS to the formaldehyde supplemented suspension resulted in immediate rapid production of more NT (Fig. 5.2a). In the absence of formaldehyde, the rate of NT production into the headspace was  $35 \text{ nmol min}^{-1} (\text{mg cell protein})^{-1}$ , and the total NT produced was about  $3 \text{ } \mu\text{mol}$ . The observed specific activities of DMDS reductase, along with the specific activities of all the other enzymes assayed, are listed in Table 5.1. The maximum NT that might be produced from the added DMDS was  $20 \text{ } \mu\text{mol}$ . Given the extremely high solubility of NT in water (about  $480\text{mM}$  in water saturated with NT at  $20^\circ\text{C}$ ; Merck, 1968), the amount of DMDS reduced to NT was probably significantly greater than the 15% observed.

Suspensions of organisms permeabilized by CTAB showed a low initial rate of NT production from DMDS, but its formation increased progressively with time (Fig. 5.2b). In the presence of NADH (as a reductant donor) the rate was linear and was initially about six times faster than in its absence (Fig. 5.2b). With NADH, total NT production into the headspace was about  $2.2 \text{ } \mu\text{mol}$  of a possible  $4 \text{ } \mu\text{mol}$ . Again, a significant quantity of NT was probably also retained in solution. The rate of NT formation from DMDS was thus in the range of  $6\text{-}10 \text{ nmol min}^{-1} (\text{mg cell protein})^{-1}$  under these conditions.

TABLE 5.1 Enzymes potentially involved in methanethiol oxidation by  
T. thioferus strain E6

Enzyme	Specific activity in crude extract (nmol min <sup>-1</sup> (mg protein) <sup>-1</sup> )
'DMS reductase'	10-35
Methane thiol oxidase (oxygen uptake rate)	67.0
Formaldehyde dehydrogenase	
NAD <sup>+</sup>	22.4
NADP <sup>+</sup>	1.0
Formate dehydrogenase	
NAD <sup>+</sup>	101.3
NADP <sup>+</sup>	0
NADH oxidase	43.5
NADPH oxidase	43.5
Catalase	3369
Hydroxypyruvate reductase NADH	6.5
NADPH	0
Ribulose 1,5 - biphosphate carboxylase	0

#### 5.4. EVIDENCE FOR A METHANETHIOL (METHYL MERCAPTAN) OXIDASE.

##### 5.4.1. Consumption of methanethiol by aerobic and anaerobic suspensions.

An aerobic suspension of intact *F. thioparus* E6 cells harvested from a DMDS-limited chemostat removed NT from the headspace of a sealed tube during shaking, at 30°C, at a rate of 27.8 nmol min<sup>-1</sup> (mg cell protein)<sup>-1</sup>. This compared with a rate of 6.8 by an anaerobic suspension and 2.1 in the absence of organisms under anaerobic conditions (Fig. 5.3). No hydrogen sulphide was detected (i.e. at less than 0.01 μmol in 15 ml) in the gas phase of either the aerobic or anaerobic incubations.

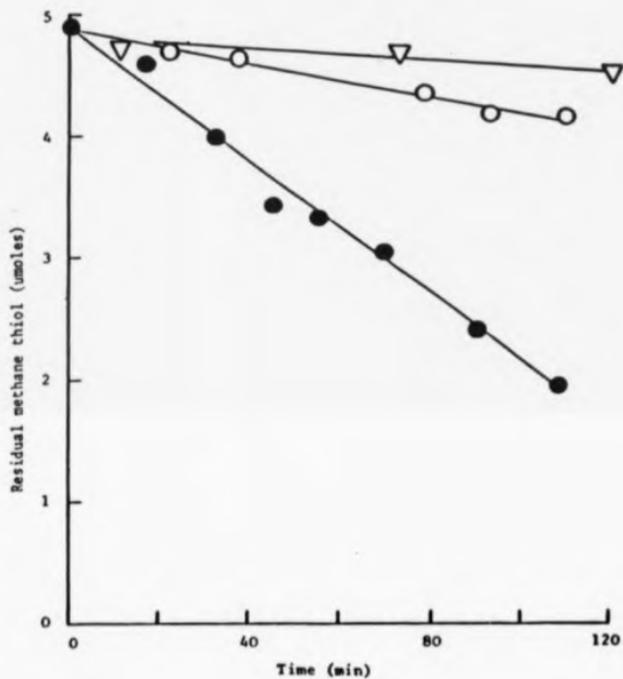
##### 5.4.2. Kinetics of NT oxidation by a crude cell free extract.

In the oxygen electrode, a cell free extract of *F. thioparus* E6 showed the concentration-dependent oxidation of NT illustrated in Fig. 5.4b. Maximum oxygen uptake rate was observed at 10-20 μM NT, NT becoming strongly inhibitory at concentrations greater than 15 μM. These data were used to construct a Lineweaver-Burk plot (Fig. 5.4a),  $K_m$  and  $V_{max}$  values then being computed from this and Eadie-Hofstee plots ( $V$  versus  $V/S$ ) as:  $K_m = 9.7 \pm 1.6 \mu M$  and  $V_{max} = 122 \pm 12 \text{ nmol min}^{-1} (\text{mg extract protein})^{-1}$  respectively. An estimation of the inhibition constant ( $K_i$ ) for NT was made from a Dixon plot (Dixon, 1953) using data points from the inhibitory part of the Lineweaver-Burk plot. Assuming inhibition to be competitive,  $K_i$  was estimated at about 10 μM. NT oxidase in the cell free extract was assayed using 15 μM NT, the observed activity was 67 nmol O<sub>2</sub> consumed min<sup>-1</sup> (mg extract protein)<sup>-1</sup>.

LEGEND TO FIGURE 5.3

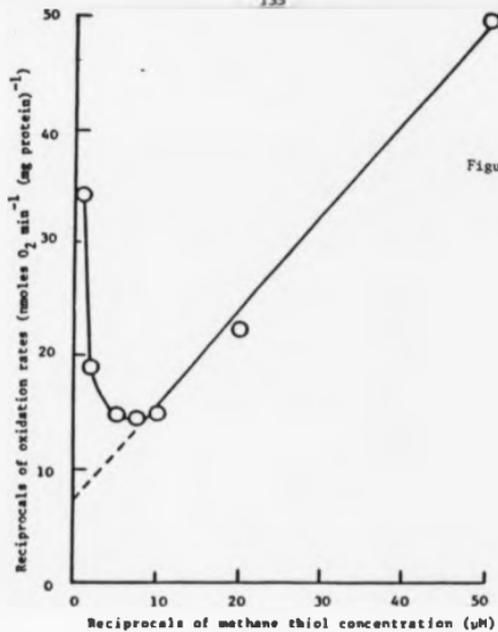
Consumption of MT (4.9 umoles) by suspensions (5 ml, 3.6 mg protein) incubated under 15 ml of air (●) or nitrogen (○) was measured. An anaerobic control with no organisms (▽) was also tested. Rates of disappearance were 27.8 and 6.8 umoles min<sup>-1</sup> (mg protein)<sup>-1</sup> aerobically and anaerobically, respectively.

Figure 5.3

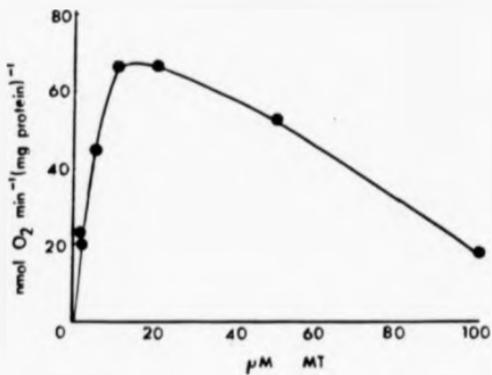


LEGEND TO FIGURE 5.4.

Methane thiol metabolism by extracts and intact suspensions of T. thioparus strain E6. MT oxidation by crude cell-free extract. Oxidation rates for concentrations between 2-100  $\mu$ M are given as (a) Lineweaver-Burk and (b) oxidation rate versus methanethiol concentration plots.



(a)



(b)

#### 5.4.3. The involvement of catalase in methemethiol oxidation.

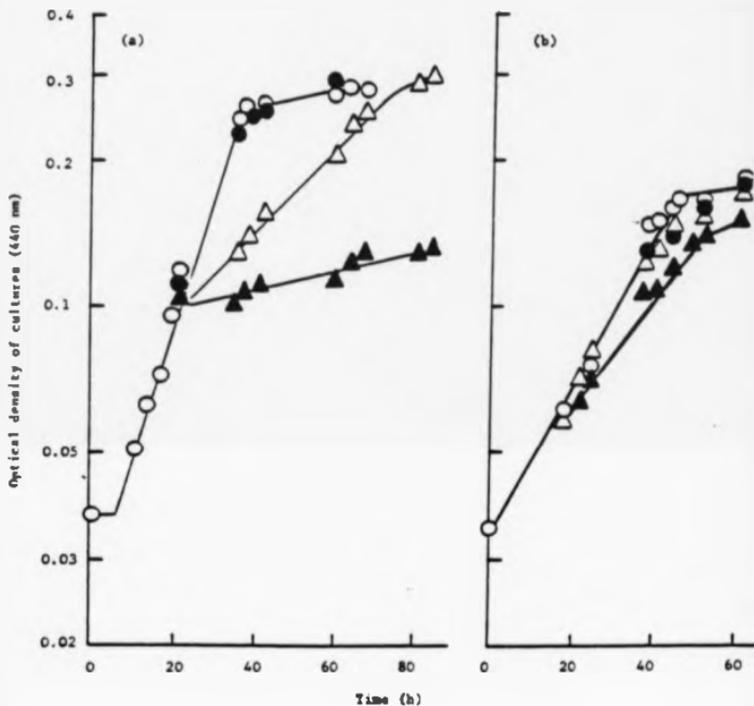
MT oxidation by *Hyphomicrobium* EG was proposed to be by means of a  $H_2O_2$ -producing MT oxidase, and inhibition of growth on DMSO, but not so severely on methylamine, by the catalase inhibitor 3-amino 1,2,4-triazole (AT) (Cohen & Somerson, 1969) provided indirect evidence for this (Suylen *et al.*, 1986). Growth of *F. thioautotrophicus* E6 was also strongly inhibited by AT, but at lower concentrations than required to affect *Hyphomicrobium* EG. Inhibition of growth occurred at AT concentrations of 1mM and above, with complete inhibition observed at 10mM. No inhibition was observed at 0.1mM AT compared with the uninhibited control (Fig. 5.5a). Growth on thiosulphate was relatively insensitive to inhibition by AT, at least over the concentration range used (Fig. 5.5b).

A high catalase activity in the cell free extract of  $3369 \text{ nmol min}^{-1} (\text{mg extract protein})^{-1}$  was observed.

LEGEND TO FIGURE 5.5

Effect of 3-amino-1,2,4-triazole (AT) on the growth of T. thioarvus strain E6 on (a) 2 mM DMDS and (b) 10 mM thiosulphate. Replicate batch cultures in shaken flasks were supplemented with AT at 19 h, to give concentrations of 0 (●), 0.1 mM (○), 1.0 mM (△) and 5 mM (▲).

Figure 5.5



## 5.5. ENZYMES OF ONE-CARBON METABOLISM.

### 5.5.1. Ribulose 1,5-Bisphosphate Carboxylase (RuBPCase).

The Key Calvin cycle enzyme RuBPCase was found to be present in CTAB permeabilised whole cells at a specific activity of 33 nmol CO<sub>2</sub> fixed min<sup>-1</sup> (mg dry weight)<sup>-1</sup>, equivalent to 53 nmol min<sup>-1</sup> (mg cell protein)<sup>-1</sup>. Assay using the cell free extract gave a specific activity of 80 nmol min<sup>-1</sup> (mg extract protein)<sup>-1</sup>, calculated from the initial rate observed in the progress curve (Fig. 5.6).

### 5.5.2. Hydroxypyruvate Reductase (HPR).

The Key serine pathway enzyme HPR was found to be present in the cell free extract at an activity of 6.5 nmol NADH oxidised min<sup>-1</sup> (mg extract protein)<sup>-1</sup>, against a background of 43.5 for NADH oxidation in the absence of hydroxypyruvate. At this level, HPR was not indicated to have any role in primary carbon assimilation. No NADPH-dependent HPR activity was detected.

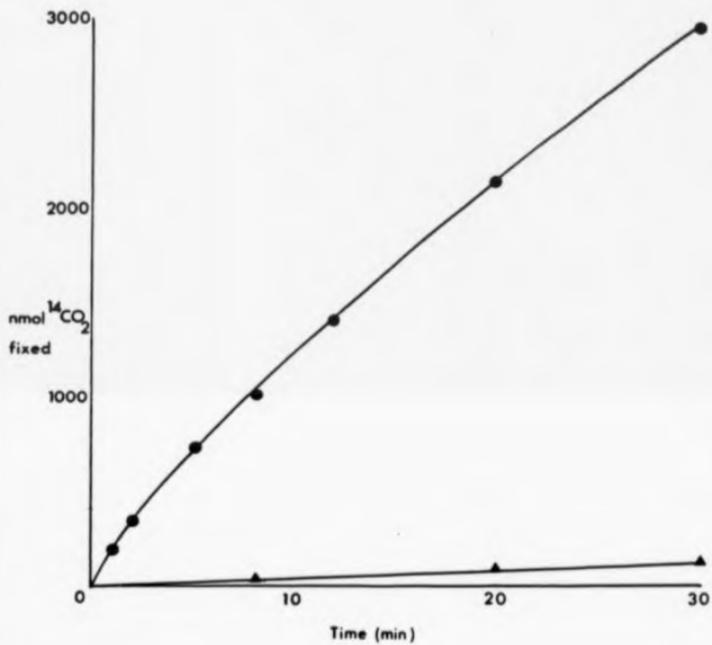
### 5.5.3. Formaldehyde and formate dehydrogenases.

Both formaldehyde and formate dehydrogenase were NAD-dependent, with specific activities of 22.4 and 101.3 nmol min<sup>-1</sup> (mg extract protein)<sup>-1</sup> respectively. No significant activities of NADP-dependent enzymes was detected.

LEGEND TO FIGURE 5.6

Fixation of  $^{14}\text{CO}_2$  by cell free extracts of DMDS-grown T. thioparus strain E6. Plots shown are for data recorded for complete reaction mixture (●) and in the absence of RuBP (▲). The specific activity of the labelled  $\text{NaH}^{14}\text{CO}_3$  was  $17854 \text{ cpm.}\mu\text{mol}^{-1}$ . In each case the reaction mixture (see Section 2.3.1) contained  $0.96 \text{ mg. protein ml}^{-1}$ .

Figure 5.6



### 5.6. DISCUSSION.

These results demonstrate that the oxidation of DMDS by *F. thioparus* E6 proceeds by a pathway that is analogous to that reported for the oxidation of methylated sulphides by hyphomicrobia and *F. thioparus* TK-m. The initial step is novel, and must clearly be the reductive cleavage of DMDS to produce two molecules of MT. This reaction was oxygen-independent, being demonstrable under anaerobic conditions. This 'DMDS reductase' used NADH as a reductant when assayed in permeabilised cells, but was not demonstrated in cell-free extracts. The MT so produced was believed to be oxidised *via* formaldehyde and formate to carbon dioxide and sulphate according to the scheme outlined in Fig. 5.7. Although the MT oxidase was not purified, MT oxidation by crude extracts showed a  $K_m$  of about 10 $\mu$ M, like that reported for the purified enzyme from *Hyphomicrobium* EG. (Suylen *et al.*, 1987). Substrate inhibition by MT was also similar, exhibiting a very low  $K_i$  value.

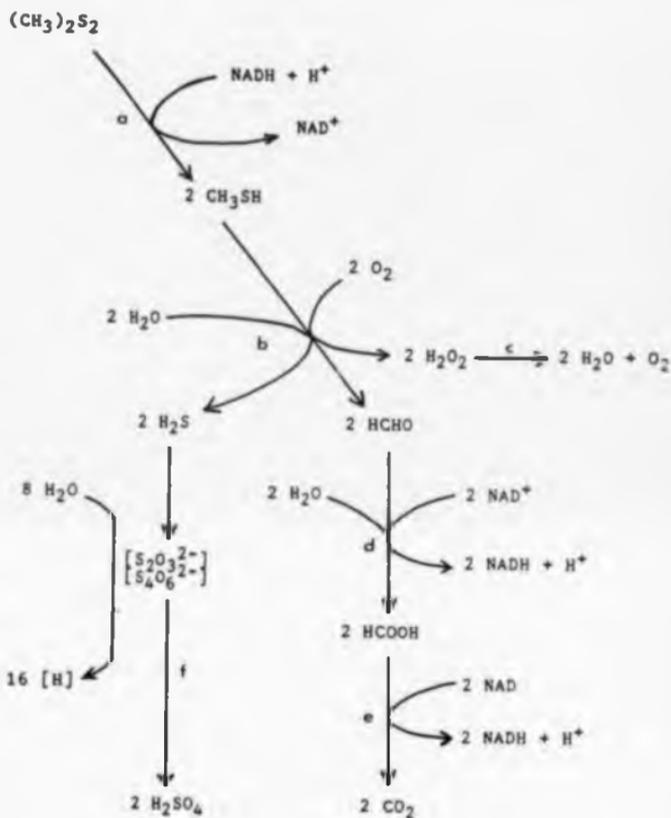
The oxidation of MT, by analogy with the hyphomicrobia (De Bont *et al.*, 1981; Suylen *et al.*, 1987), produced hydrogen peroxide which would have required destruction by catalase. The presence of very high levels of catalase, and the inhibition by the catalase inhibitor 3-amino-1,2,4-triazole of growth on DMDS (but much less so on thiosulphate), both indicate that this step was a central one for MT oxidation. Failure to see hydrogen sulphide production under anaerobic conditions confirmed the strictly oxygen-requiring nature of this step.

The transfer of thiosulphate-grown cells to DMDS cultures (but not *vice versa*) caused a significant lag before the onset of growth, this indicating that at least one step in DMDS metabolism was inducible. The induction of methylotrophic enzymes of the serine pathway, which apparently occurred

LEGEND TO FIGURE 5.7

Proposed mechanism of oxidation of dimethyl disulphide by T. thioarvus strain E6. The steps identified are (a) NADH-stimulated DMDS reductase; (b) methane thiol oxidase, producing  $H_2S$  and hydrogen peroxide; (c) catalase; this is the site of inhibitory action by 3-amino-1,2,4-triazole; (d) NAD-specific formaldehyde dehydrogenase; (e) NAD-specific formate dehydrogenase; (f) sulphide oxidising system, possibly involving thiosulphate and tetrathionate as intermediates and liberating 16 reducing equivalent (from the water used to supply oxygen for sulphate formation), which can be oxidized by four moles of oxygen via the respiratory chain.

Figure 5.7



during transfer of *Thiobacillus* NS1 from thiosulphate to DMS, was discounted due to the low levels of hydroxypyruvate reductase observed (against comparatively high levels of NAD(P)H oxidase levels). The results also confirmed the autotrophic nature of growth on DMDS, with ribulose 1,5-bisphosphate carboxylase as the key assimilatory enzyme. The specific activity of this enzyme in cell-free extracts was almost double that observed with permeabilized whole cells, indicating ample enzyme to support growth at the rate observed.

C H A P T E R   S I X

THE METABOLISM OF VOLATILE AND INORGANIC SULPHUR COMPOUNDS

BY THIOBACILLUS THIOPARUS STRAIN TK-m

---

### 6.1. INTRODUCTION.

In common with *Hyphomicrobium* S (De Bont et al., 1981), *F. thioparus* TK-m is incapable of growth on DMDS, but is capable of growth on DMS (Kanagawa & Kelly, 1986). These observations are in contrast to those made of *F. thioparus* E6 and indeed the other six strains isolated during the enrichment program (Ch.3), which were all capable of growth on both substrates. Such observations tend to support the proposed theory that the initial step(s) at least, of DMS and DMDS metabolism are independent.

During routine testing of the substrate specificity range of *F. thioparus* TK-m, it was discovered that carbon disulphide could support its growth. Considering the importance of this compound in the cycling of sulphur through the environment very little is known about the biogeochemical transformations which it undergoes, and virtually nothing is known concerning its microbial degradation.

The following experiments were designed to further investigate the metabolism of *F. thioparus* TK-m with respect to DMS, and the apparently novel CS<sub>2</sub> degradation.

6.2. GROWTH OF F. THIOPARUS TK-m ON INORGANIC SULPHUR COMPOUNDS  
IN BATCH CULTURE.

*F. thioparus* TK-m was capable of growth on thiosulphate and tetrathionate (both at 20mM), thiocyanate (5mM), sulphide (-2mM) and sulphur (10 g l<sup>-1</sup>), with observed specific growth rates of about 0.08 - 0.09h<sup>-1</sup> for thiosulphate and tetrathionate, 0.06 - 0.07h<sup>-1</sup> for sulphide and thiocyanate and 0.025h<sup>-1</sup> for sulphur. There was apparently little difference in the growth on thiocyanate between concentrations of 0.5 - 5.0mM, although it was observed to be inhibitory at 10mM. This is in contrast to results obtained with *F. thiooxyxidans* which was inhibited by thiocyanate concentrations of greater than 2.5mM (Happold *et al.*, 1954). Growth yields on these compounds were not easily reproducible, but an estimated mean value of 5 ± 1.5 g cell-carbon (mole sulphide equivalent)<sup>-1</sup> was made from at least five determinations on each of the compounds. This figure was higher than one would expect for a *F. thioparus* strain.

*F. thioparus* TK-m was also capable of anaerobic growth on thiosulphate and thiocyanate using nitrate as the terminal electron acceptor, nitrate being reduced only as far as nitrite. Anaerobic cultures initially containing 20mM thiosulphate or 5mM thiocyanate and using thiosulphate grown-cells as inocula (10% v/v) were incubated at 30°C for up to 245 hours. Thiosulphate and thiocyanate consumption and nitrite production were monitored during growth (Table 6.1.). Thiocyanate was more rapidly removed from the cultures than was thiosulphate, being completely consumed at 161 hours incubation. At the end of the experiment 26.2mM and 13.14mM nitrite had been produced in the thiosulphate and thiocyanate cultures respectively, equivalent to 5.8 mol nitrite (mol thiosulphate)<sup>-1</sup> and 2.72 mol nitrite (mol thiocyanate)<sup>-1</sup>. Both of these estimates are below the expected values

**TABLE 6.1** Anaerobic Growth of *T. thioparus* strain TK-m on thiosulphate and thiocyanate using nitrate as the terminal electron acceptor.

TIME (h)	20 mM S <sub>2</sub> O <sub>3</sub>		5 mM KSCN	
	NO <sub>2</sub>	S <sub>2</sub> O <sub>3</sub>	NO <sub>2</sub>	SCN <sup>-</sup>
0	0.01	19.82	0.025	4.83
20	0.20	19.66	0.42	4.55
42	10.60	19.42	1.71	4.52
77	13.38	17.96	2.31	1.76
90	14.85	17.06	5.0	1.59
115	15.71	16.50	7.48	0.94
138	15.81	16.49	10.02	0.53
161	18.94	16.29	13.21	0
186	22.80	15.82	13.14	0
245	26.20	15.31	-	-
Final Culture pH	6.84		7.19	

of 8 mol nitrite (mol thiosulphate or thiocyanate)<sup>-1</sup>. Acid production was observed in the thiosulphate culture, the culture pH falling from pH 7.12 to 6.84 by the end of growth. There was no significant change in the culture pH following growth on thiocyanate, this being consistent with sulphate production balanced by ammonia production from thiocyanate:



### 6.3. GROWTH OF T. THIOPARUS TK-6 IN CHEMOSTAT CULTURE.

#### 6.3.1. Under thiosulphate limitation.

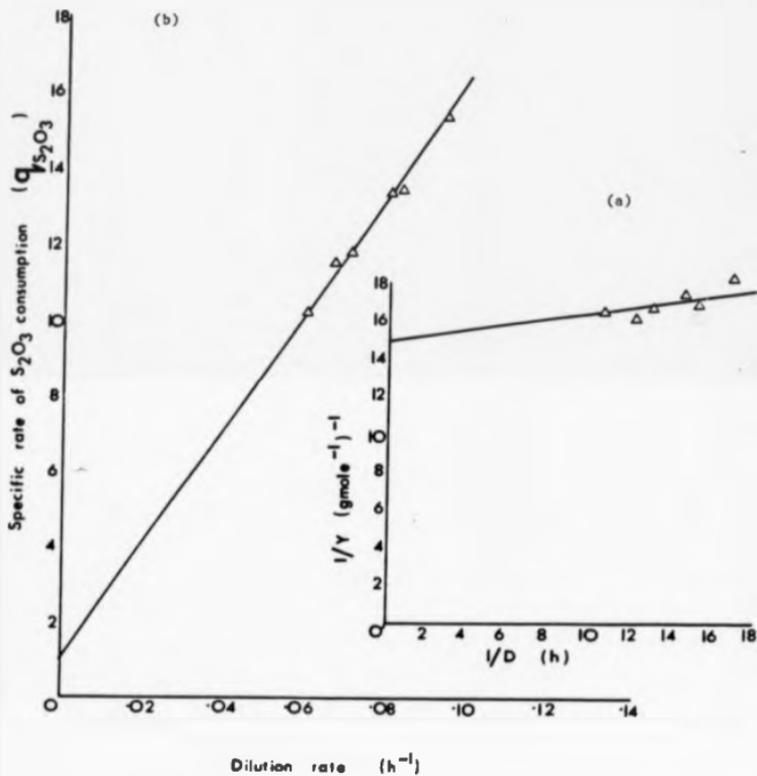
No elemental sulphur was produced from thiosulphate at a concentration of 10mM, but was observed in the culture at concentrations greater than 20mM. At steady state on 10mM thiosulphate and at a dilution rate of 0.076 h<sup>-1</sup>, the culture was shown not to be oxygen-limited by calculation of the theoretical oxygen requirement for complete substrate oxidation. The culture was also shown not to be under carbon dioxide-limitation since the addition of an extra 5% (v/v) CO<sub>2</sub> to the air supply did not cause any stimulation of further biomass production.

Steady state cultures were established on 10mM thiosulphate as the growth limiting substrate at dilution rates between 0.058 and 0.094 h<sup>-1</sup>, between which the steady state growth yield (Y) increased from 5.1 to 6.2 g cell-carbon (mole thiosulphate)<sup>-1</sup>. The true growth yield (Y<sub>max</sub>) was estimated from plots of 1/Y *versus* 1/D and of the specific rate of thiosulphate consumption (q<sub>thiosulphate</sub>) *versus* D (Figs. 6.1a,b). From Fig. 6.1b, extrapolation to zero dilution rate gave a value for the

LEGEND TO FIGURE 6.1

Growth of T. thiooperus strain TK-m in chemostat culture. Steady state cultures on 10mM thiosulphate were obtained at several dilution rates. (a) specific rate of thiosulphate consumption ( $q_{\text{DMDS}} = \text{mmol thiosulphate consumed h}^{-1} (\text{g cell-carbon})^{-1}$ ) versus dilution rate. (b) reciprocal of yield ( $\text{g cell-carbon (mol thiosulphate)}^{-1}$ ) versus dilution rate.

Figure 6.1



maintenance coefficient of 1.2 mmol thiosulphate (g cell-carbon)<sup>-1</sup> h<sup>-1</sup>, reciprocal of the slope gave a value for  $Y_{max}$  of 6.58 g cell-carbon (mole thiosulphate)<sup>-1</sup>. Values for  $q_{thiosulphate}$  and  $Y_{max}$  from Fig. 6.1a were 1.3 and 6.67 respectively.

Cells harvested from the chemostat were assayed for RuBPCase activity. The observed specific activity of 46 nmol CO<sub>2</sub> fixed min<sup>-1</sup> (mg cell protein)<sup>-1</sup> was 39% higher than the maximum values obtained from cells grown in thiosulphate batch cultures.

#### 6.3.2. Addition of thiocyanate to a thiosulphate-limited chemostat.

A steady state culture ( $D = 0.085$  h<sup>-1</sup>) on 7.48mM thiosulphate achieved a level of biomass corresponding to 44 mg l<sup>-1</sup> total cell-carbon, equivalent to a yield of 5.88 g cell-carbon (mole thiosulphate)<sup>-1</sup>. When the same culture was simultaneously supplied with 5.2mM thiocyanate, both substrates were completely oxidised and there followed an immediate increase in biomass to 73 mg l<sup>-1</sup> total cell-carbon. This indicated a thiocyanate-dependent yield of 5.58 g cell-carbon (mole thiocyanate)<sup>-1</sup>. These data not only show that thiosulphate and thiocyanate are co-metabolised by *f. thiotropus* TK-m, but that within the error of measurement, this organism is capable of generating the same amount of energy for growth from the complete oxidation of both substrates.

#### 6.3.3. Addition of formate to a thiosulphate-limited chemostat.

Supplementing a thiosulphate-limited chemostat (9.2mM;  $D = 0.08$  h<sup>-1</sup>) with formate (10mM) caused a temporary period of instability during which time the culture biomass decreased slightly and elemental sulphur was observed.

However, after 24 h the culture returned to its original steady state. Throughout the duration of this experiment the level of residual formate in the culture decreased from 7.3mM (after three days) to 4.5, 3.3 and finally 1.9mM after five, six and ten days respectively. However, there was no apparent stimulation of increase in culture biomass. There were no detectable levels of residual thiosulphate in the culture and the yield remained at about 5.8 g cell carbon (mole thiosulphate consumed)<sup>-1</sup>. Certainly formate was removed (presumably by oxidation) from the culture, but its oxidation was apparently not linked to energy generating metabolism. Under these conditions one might expect to observe up to a 50% increase in biomass since in theory the complete oxidation of formate could yield 2ATP (mole)<sup>-1</sup>, assuming electrons enter the respiratory chain at the level of MAD<sup>+</sup>, compared to 4ATP (mole)<sup>-1</sup> for thiosulphate oxidation (refer to Section 1.2), but clearly this was not the case.

#### 6.4. KINETICS OF SULPHUR AND ONE-CARBON COMPOUND OXIDATION.

##### 6.4.1 Oxidation of inorganic sulphur compounds.

The oxidation of inorganic sulphur compounds by organisms taken directly from a thiosulphate-limited chemostat (10mM; D = 0.08 h<sup>-1</sup>) was assayed. Sulphide was oxidised by organisms supplied with concentrations between 10 and 1000µM (Fig. 6.2a). The oxidation rate increased from 106 nmol oxygen consumed min<sup>-1</sup> (mg cell-protein)<sup>-1</sup> at 10µM to 258 at 100µM, but above this there was substrate inhibition with the rate at 1mM falling to 130. Determinations of K<sub>s</sub> for sulphide oxidation from Lineweaver-Burk and Eadie-Hofstee plots gave values of 18.2 and 18.5µM respectively. Similar determinations of V<sub>max</sub> were 298 and 300 nmol oxygen consumed min<sup>-1</sup> (mg cell-protein)<sup>-1</sup> (Table 6.2).

TABLE 6.2 Kinetics of oxidation of inorganic sulphur compounds by  
T. thiooarus strain TK-m.

Substrate	Concentration range ( $\mu\text{M}$ )	$k_m$ ( $\mu\text{M}$ )		$V_{\text{max}}$ ( $\text{nmol O}_2 \text{ min}^{-1}$ ( $\text{mg protein}^{-1}$ ))	
		L-B*	E-H*	L-B	E-H
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

\* L-B, Lineweaver-Burk; E-H, Eadie-Hofstee.

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

Thiosulphate was oxidised when supplied at concentrations between 10 and 500 $\mu$ M (Fig. 6.2b), the oxidation rate increasing from 81 nmol oxygen consumed  $\text{min}^{-1}$  ( $\text{mg cell protein}^{-1}$ ) at 10 $\mu$ M to 401 at 500 $\mu$ M. Although no substrate inhibition was observed, at least over the concentration range used, thiosulphate oxidation showed complex kinetics. There was a high affinity, low  $V_{\text{max}}$  phase at concentrations below 50 $\mu$ M (resembling the kinetics seen for sulphide oxidation), and a low affinity, high  $V_{\text{max}}$  phase at concentrations above 50 $\mu$ M (Table 6.2).

Tetrathionate oxidation resembled the second phase of thiosulphate oxidation, with rate increasing from 144 nmol oxygen consumed  $\text{min}^{-1}$  ( $\text{mg cell protein}^{-1}$ ) at 25 $\mu$ M to 345 at 100 $\mu$ M (Fig. 6.2c), and giving low  $K_m$  values of 99 and 86 $\mu$ M and high  $V_{\text{max}}$  values of 721 and 661 nmol oxygen consumed  $\text{min}^{-1}$  ( $\text{mg cell protein}^{-1}$ ) respectively. (Table 6.2).

#### 6.4.2. Oxidation of one-carbon and organic sulphur compounds.

Apart from sulphide, thiosulphate and tetrathionate, organisms taken from a thiosulphate-limited chemostat oxidised a very restricted range of other compounds. No oxygen uptake above the endogenous rate was observed with the following substrates: DMS (1-50 $\mu$ M), MT (3-30 $\mu$ M),  $\text{CS}_2$  (10-100 $\mu$ M), thiocyanate (10-1000 $\mu$ M) and methylamine and dimethylamine (100-1000 $\mu$ M). Formate (1-5mM) and formaldehyde (0.1-1.0mM) were slowly oxidised, oxidation rate increasing from 5.0 - 8.3 and 5.2 - 6.6 nmol oxygen consumed  $\text{min}^{-1}$  ( $\text{mg cell protein}^{-1}$ ) respectively, over the concentration ranges used, indicating a very low affinity for these substrates.

Low affinity for formate was also observed even when a thiosulphate-limited chemostat was apparently simultaneously oxidising formate (see Section 6.2.3.), the oxidation rate determined as 11.3 nmol oxygen consumed  $\text{min}^{-1}$  ( $\text{mg cell protein}^{-1}$ ).

LEGEND TO FIGURE 6.2

Kinetics of (a) sulphide (●), (b) thiosulphate (▲) and (c) tetrathionate (◆) oxidation by whole cell suspensions (2ml) of T. thiooerus strain TX-m taken from a thiosulphate limited chemostat (10 mM;  $D=0.08h^{-1}$ ).

Figure 6.2

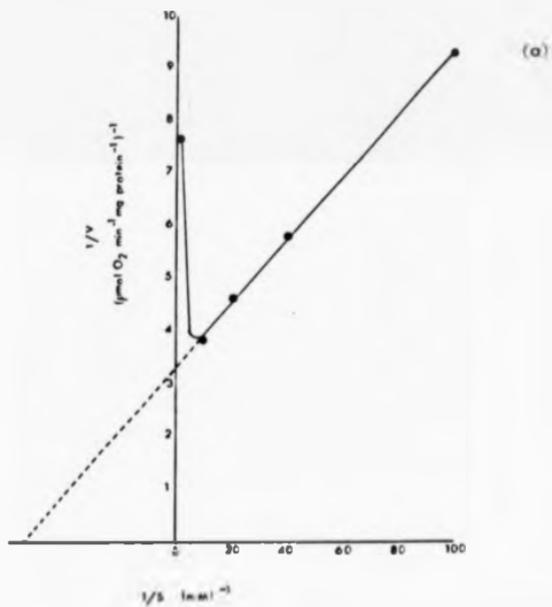
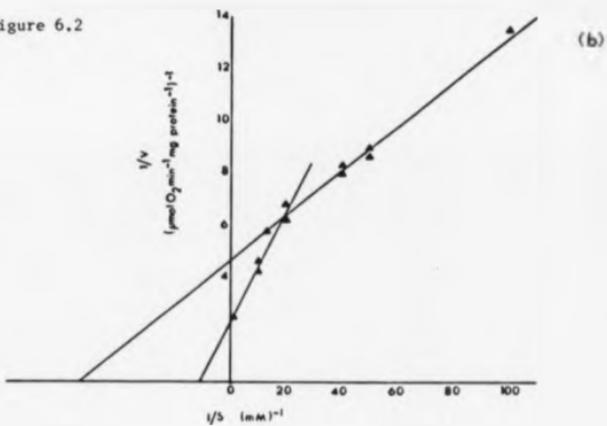
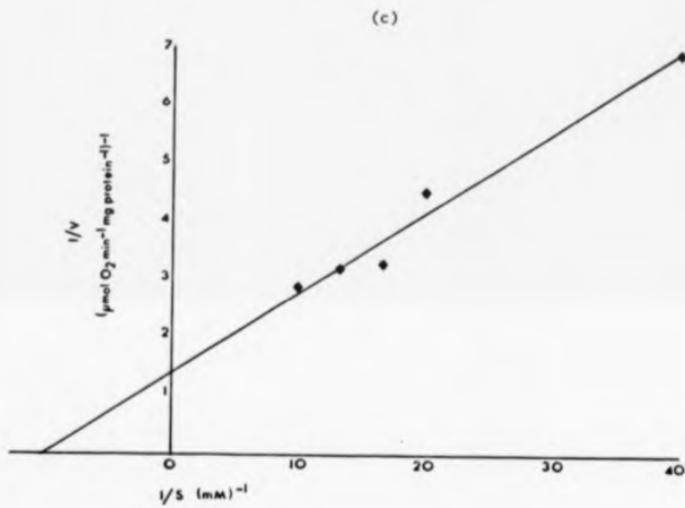


Figure 6.2 (cont'd.)



## 6.5. GROWTH ON DMS IN BATCH CULTURE.

### 6.5.1. Growth rates and yields.

Previous results for the growth of *F. thioparvus* TK-m using DMS as the sole energy substrate had indicated yields of 12-13 g cell carbon (mole DMS consumed)<sup>-1</sup> (Kanagawa & Kelly, 1986). This estimation of yield proved to be satisfactorily reproducible: from five determinations using DMS at 1-4mM, yields of  $12.2 \pm 0.6$  g cell carbon (mole DMS consumed)<sup>-1</sup> were observed. Increasing the initial DMS concentration from 1 to 4mM, caused a decrease in the initial growth rate (similar to that observed during the growth of *F. thioparvus* E6 on DNDS), however, the maximum specific growth rate observed was about  $0.09 \text{ h}^{-1}$ .

Cells harvested from batch cultures following growth on DMS were assayed for RuBPCase activity by the cell permeabilisation method. The observed specific activity from three determinations using CTAB as the permeabilising agent was  $31 \pm 4$  nmol CO<sub>2</sub> fixed min<sup>-1</sup> (mg cell protein)<sup>-1</sup>, which was three to four times higher than when using Triton X-100.

### 6.5.2. Co-metabolism of other one-carbon compounds.

Growth of batch cultures on 2mM DMS was monitored before and after the addition of methanol, formaldehyde, formate and dimethylamine. The latter two substrates were tolerated up to a concentration of 10mM, but methanol and formaldehyde were observed to be inhibitory at concentrations above 2mM and 0.25mM respectively. None of these additional substrates stimulated any increase in yield compared to an unamended control, nor could they alone support growth in the absence of DMS.

6.5.3. Incorporation of  $^{14}\text{C}$ -labelled formate and formaldehyde during growth on DMS or sulphide.

Cultures previously maintained through successive subculture on DMS as sole substrate were used as inocula for radiolabelling experiments carried out in order to simultaneously monitor growth and the incorporation of  $\text{H}^{14}\text{COOH}$ . Cells were grown on either DMS (2mM) or sulphide (1mM) in the presence of 10mM  $\text{H}^{14}\text{COOH}$ . The results demonstrated that although  $\text{H}^{14}\text{COOH}$  was incorporated into cell material during growth on both DMS and sulphide (Fig. 6.3), by far the better incorporation was seen on the former. Comparable growth was observed on sulphide in the absence and presence of an extra 7.5% (v/v)  $\text{CO}_2$ , however, incorporation of  $\text{H}^{14}\text{COOH}$  in the latter case was decreased by about 35%. This suggested that  $\text{H}^{14}\text{COOH}$  was first oxidised to  $^{14}\text{CO}_2$ , this then being in competition with unlabelled  $\text{CO}_2$  for fixation into cell carbon. No incorporation of  $\text{H}^{14}\text{COOH}$  was observed in the absence of either DMS or sulphide.

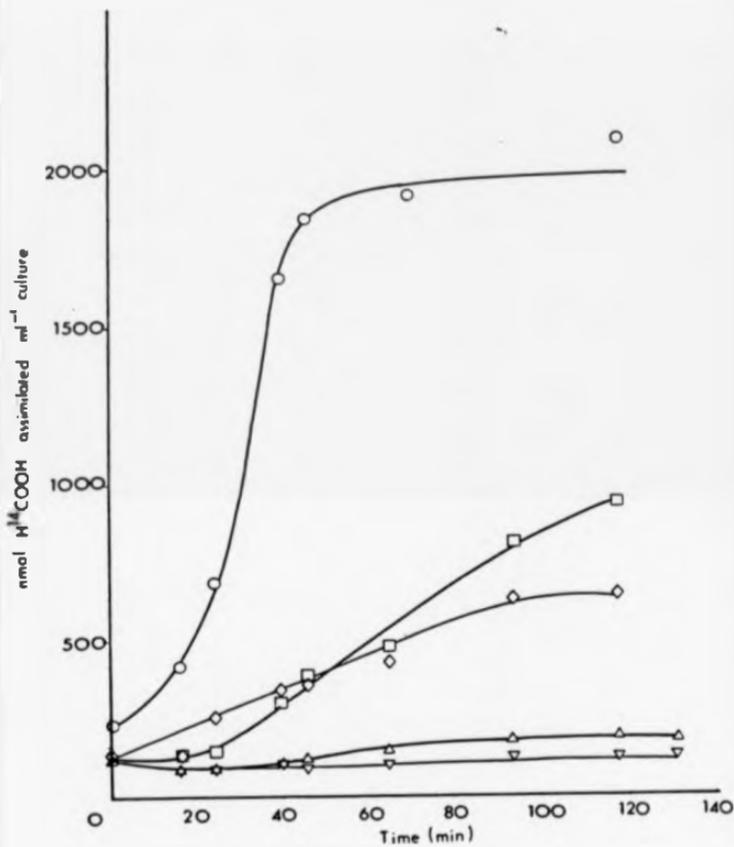
At the end of growth, for those cultures grown on sulphide, sulphide plus  $\text{CO}_2$  and DMS the proportion of cell carbon derived from  $\text{H}^{14}\text{COOH}$  was 84%, 24% and 34% respectively. These figures again illustrate that incorporation of  $\text{H}^{14}\text{COOH}$  is decreased in the presence of increased levels of  $\text{CO}_2$ . By the same argument, this also explains the observed decrease in incorporation when cells were grown on DMS, the additional source of ' $\text{CO}_2$ ' presumably arising from the oxidation of the methyl groups of DMS.

Similar experiments to those carried out above were performed to measure the incorporation of  $\text{H}^{14}\text{CHO}$  into cell material. Previous results had already indicated that formaldehyde was an inhibitory substrate, so preliminary experiments were carried out to determine the most suitable formaldehyde concentration to use. Incorporation of  $\text{H}^{14}\text{CHO}$  was observed

LEGEND TO FIGURE 6.3

Assimilation of  $H^{14}COOH$  by T.thioperus strain TK-m during growth. With the exception of one culture, all were sealed, each containing 10mM  $H^{14}COONa$ . Cultures were grown on : 1mM sulphide ( $\square$ ), 1mM sulphide plus 7.5% v/v  $CO_2$  ( $\diamond$ ), 2mM DMS ( $\circ$ ), formate alone with flask sealed ( $\nabla$ ) and formate alone with flask open ( $\Delta$ ). The specific activity of the labelled  $H^{14}COONa$  was 7090 cpm. $\mu mol^{-1}$ .

Figure 6.3



using initial concentrations of 0.1-1.0mM during growth on 2mM DMS. Although concentrations above 0.1mM appeared to be inhibitory, indicated by the slower initial growth rate, maximum incorporation was observed at 0.25mM (Fig. 6.4a,b). At concentrations greater than 0.25mM, inhibition of growth consequently limited  $H^{14}CHO$  incorporation. Growth on 2mM DMS was completely inhibited by 1mM  $H^{14}CHO$ . At the end of growth the proportion of cell carbon derived from  $H^{14}CHO$  to total cell carbon was considerably lower than that observed for cell carbon derived from  $H^{14}COOH$ . Figures were calculated to be 4.8% (at 0.25mM), 4.0% (at 0.5mM), 1.5% (at 0.1mM) and zero (at 1.0mM). Up to about 46% of the added label (at 0.25mM) was lost from solution, this presumably represents the amount incorporated into cell material plus any unfixed  $^{14}CO_2$  present in the gas phase. In a second series of experiments the incorporation of  $H^{14}CHO$  (at 0.25mM) was measured during growth on 2mM DMS in the presence of increased amounts of unlabelled  $CO_2$ . The level of incorporation decreased significantly in the presence of 2.5 - 10% (v/v)  $CO_2$  (Fig. 6.5), but the patterns of growth and final biomass measurements were similar in each case. At the end of growth the proportion of cell carbon derived from  $H^{14}CHO$  decreased from 4.9% (with no added  $CO_2$ ) to 1.9%, 1.2% and 0.85% in the presence of 2.5, 5.0 and 10% (v/v)  $CO_2$  respectively. These observations again provide evidence to suggest that formaldehyde (as an intermediate in DMS metabolism) is oxidised (via formate) to  $CO_2$ .

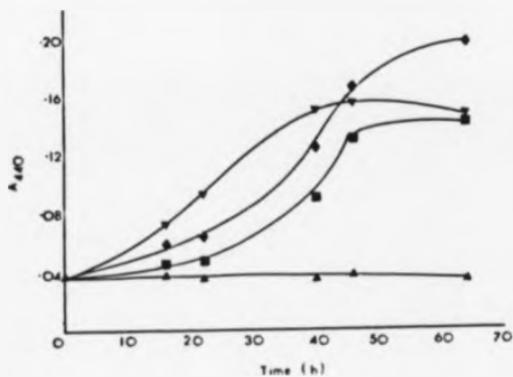
#### 6.5.4. Further evidence for the proposed metabolism of DMS.

Previous results with *F. thioparus* E6 have shown that the mechanism for DMDS (and probably DMS) oxidation was inducible but that the mechanism for sulphide oxidation was constitutive. This was also demonstrated for the growth of *F. thioparus* TK-m on DMS and thio sulphate. Batch cultures were

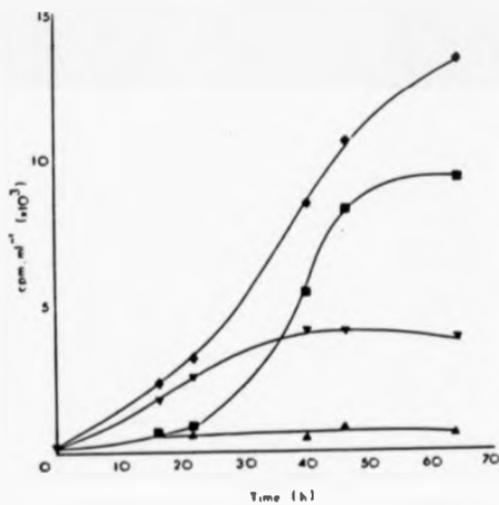
LEGEND TO FIGURE 6.4

Growth of *T. thioparus* strain TK-m on 2 mM DMS in the presence of  $H^{14}CHO$ . (a) Increase in culture absorbance ( $A_{440}$ ) versus time and (b) assimilation of  $H^{14}CHO$  versus time. Sealed batch cultures (50 ml) were supplemented with  $H^{14}CHO$  at 0.1 mM ( $\nabla$ ), 0.25 mM ( $\blacklozenge$ ), 0.5 mM ( $\blacksquare$ ) and 1.00 mM ( $\blacktriangle$ ). The specific activity of the labelled  $H^{14}CHO$  was  $104619 \text{ cpm.}\mu\text{mol}^{-1}$ .

Figure 6.4



(a)

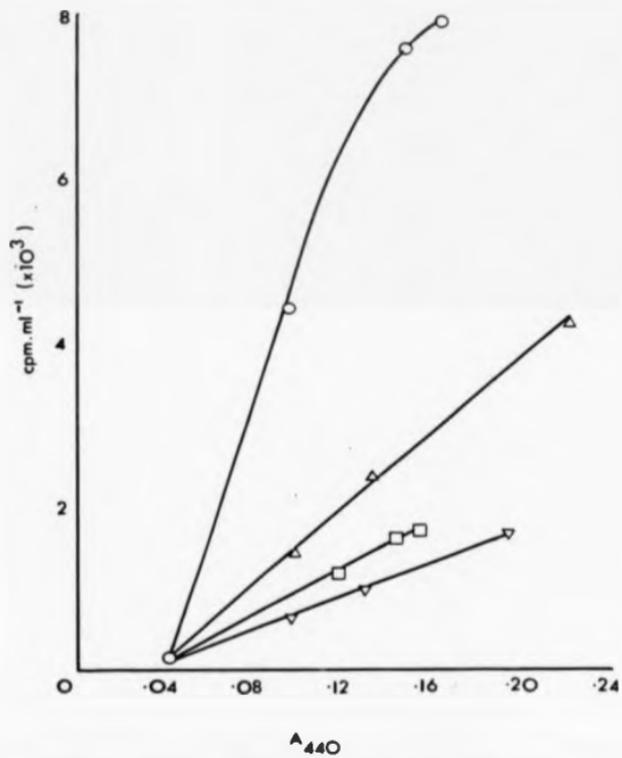


(b)

LEGEND TO FIGURE 6.5

Effect of increased partial pressure of  $\text{CO}_2$  on the assimilation of  $\text{H}^{14}\text{CHO}$  by T. thioparus strain TX-m during growth on 2mM DMS. Sealed cultures (50ml) were supplemented with  $\text{CO}_2$  (% v/v) at 2.5 ( $\Delta$ ), 5 ( $\square$ ) and 10 ( $\nabla$ ), an unsupplemented culture ( $\circ$ ) was also included as a control. The specific activity of the labelled  $\text{H}^{14}\text{CHO}$  was  $104619 \text{ cpm.}\mu\text{mol}^{-1}$ .

Figure 6.5



grown on both DMS (2mM) and thiosulphate (10mM) and before the onset of the stationary phase, each of these was used to inoculate further DMS and thiosulphate cultures. Growth was monitored for up to 70 h incubation at 30°C. The complete consumption of dissolved DMS by DMS-grown cells was observed at 40 h incubation, this corresponding to the onset of the stationary phase. A significantly longer lag phase was observed for the growth of thiosulphate-grown cells on DMS, with the cessation of growth and complete consumption of DMS occurring at 65 h incubation (Figs. 6.6a,b). In the case of both DMS- and thiosulphate-grown cells inoculated into thiosulphate cultures, there followed an almost identical pattern of growth and thiosulphate consumption (Figs. 6.6c,d). In the presence of both DMS and thiosulphate, both substrates were consumed rapidly and at approximately the same rate by DMS-grown cells. Thiosulphate-grown cells rapidly consumed thiosulphate but again there was a lag of about 30 h before DMS consumption proceeded. However, following this lag, DMS was rapidly consumed (Figs. 6.6e,f). Although the initial growth rates observed in these cultures differed quite considerably due to the various transfers, the maximum specific growth rates were similar in each case at about 0.072-0.091 h<sup>-1</sup>.

AT was indicated as an intermediate in DMS metabolism by *F. thioparvus* TK-m, since growth on DMS was severely inhibited in the presence of the catalase inhibitor 3-amino 1,2,4-triazole. The pattern of inhibition more closely resembled that observed with *F. thioparvus* E6 growing on DMDS (see Section 5.4.3) than *Hyphomicrobium* EG growing on DMSO (Suylen *et al.*, 1986). There was apparently no inhibition of growth at an AT concentration of 0.1mM, compared to an uninhibited control, but inhibition was observed at concentrations of 1.0 and 5.0mM. Complete inhibition of growth was observed using 10mM AT. In comparison, growth on thiosulphate was relatively insensitive to AT at least over the concentration range used (Figs. 6.7a,b).

LEGEND TO FIGURE 6.6

Evidence for an inducible DMS oxidation pathway in Thiobacillus thiooxidans strain TK-m. Inoculum cultures were pregrown on DMS (a, c, e) or thiosulphate (b, d, f) before transfer to further cultures containing 2mM DMS (a, b), 10 mM thiosulphate (c, d), or both substrates (e, f). Growth, as culture absorbance ( $A_{440}$ ) ( $\blacktriangle$ ), DMS consumption ( $\blacksquare$ ) and thiosulphate consumption ( $\blacklozenge$ ) were monitored for up to 70h incubation at 30°C.

Figure 6.6

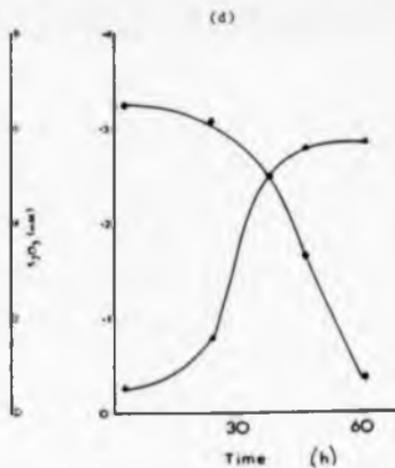
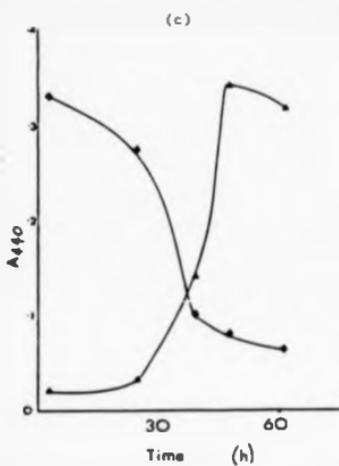
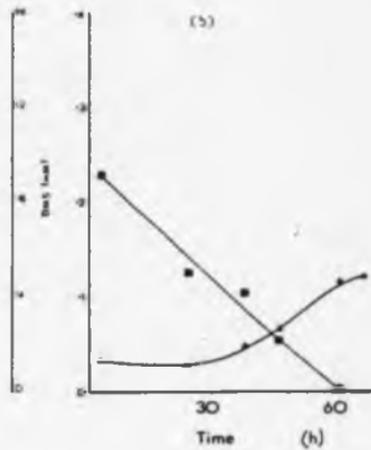
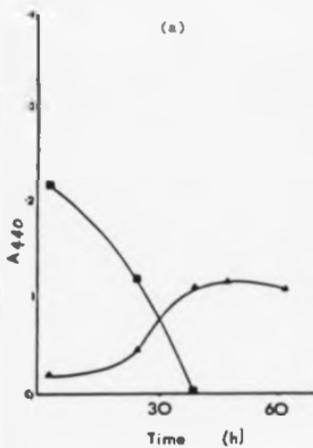
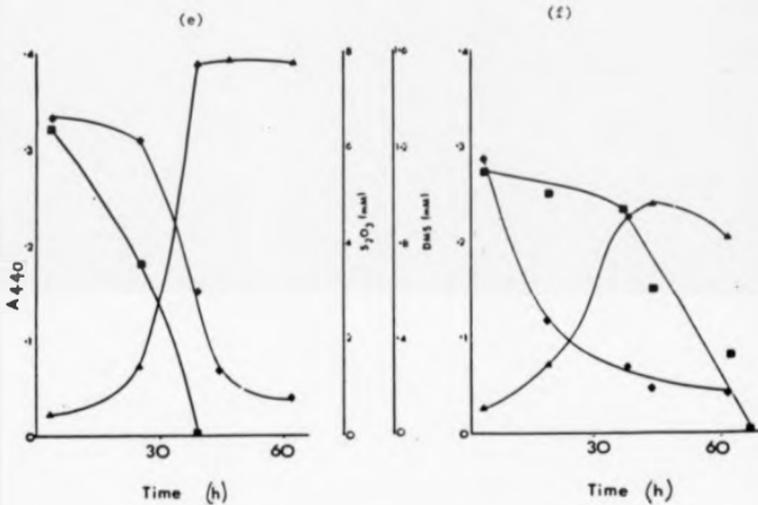


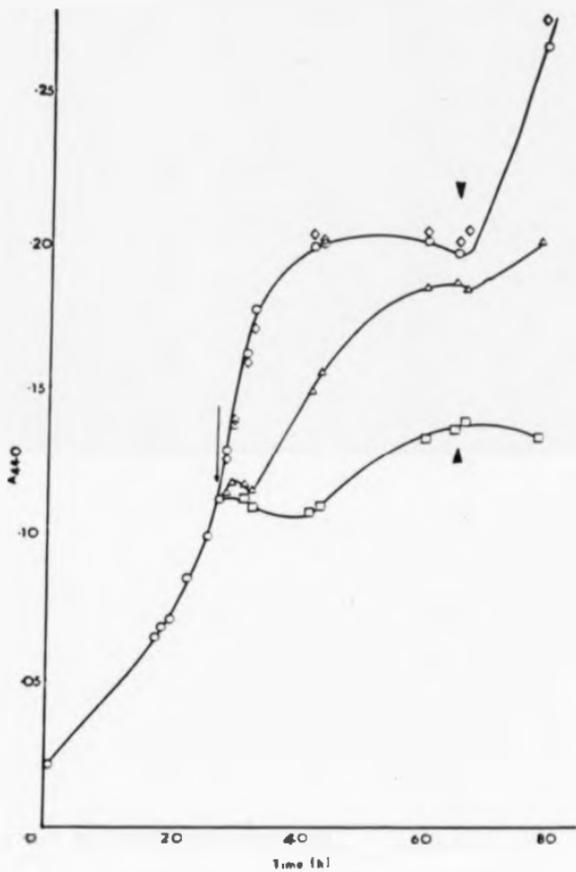
Figure 6.6 cont'd.



LEGEND TO FIGURE 6.7

(a) The effect of the catalase inhibitor 3-amino-1,2,4-triazole on T. thioparus strain TK-m during growth on DMS (2mM) in 50ml sealed cultures. The arrow indicates the time of addition of inhibitor at concentrations of 0.1mM ( $\diamond$ ), 1.0mM ( $\Delta$ ) and 5.0mM ( $\square$ ). Growth of an uninhibited control ( $\circ$ ) was also monitored. Arrow heads indicate time of addition of a further 2mM DMS.

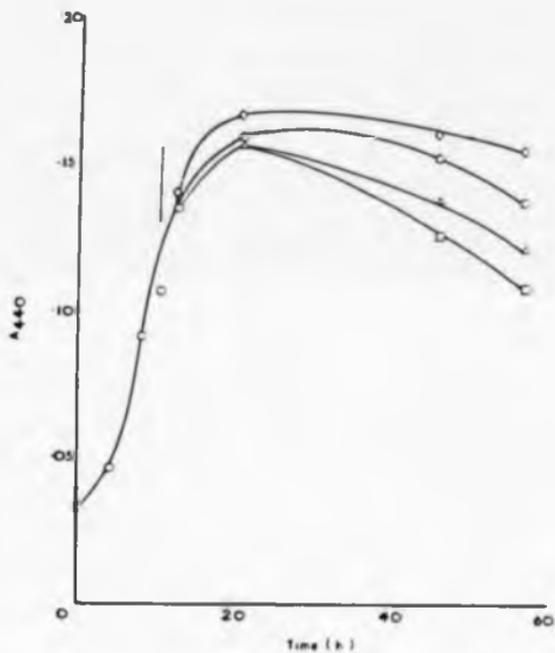
Figure 6.7a



LEGEND TO FIGURE 6.7 (cont'd.)

(b) The effect of the catalase inhibitor 3-amino-1,2,4-triazole on T. thioparus strain TK-m during growth on thiosulphate (10mM) in 50ml sealed cultures. Symbols are as for Fig. 6.7a.

Figure 6.7 b



6.6. THE AUTOTROPHIC, CHEMOLITHOTROPHIC GROWTH OF T. THIOPARUS TK-m ON CARBON DISULPHIDE.

6.6.1. Growth of thiobacilli on carbon disulphide.

No growth could be obtained on 2mM CS<sub>2</sub> of *F. neapolitanus* (DSM 581), *F. versutus* (DSM 582), *F. thioparus* (DSM 505), *F. acidophilus* (DSM 700), *F. thiooxidans* (ATCC 8085), *F. aquasulfae* (DSM 4255, ATCC 43788) or *F. tapidarius* (DSM 3134, ATCC 43215). Slight growth and decrease in pH were seen with *F. ferrooxidans* (DSM 585), but this was not sustained on subculture. Growth was observed using *F. thioparus* strain TK-m (Kanagawa *et al.*, 1982; Kanagawa & Kelly, 1986), which was also capable of growth on DMS and thiocyanate (Smith & Kelly, 1988).

6.6.2. Growth rate and yield of T. thioparus TK-m in batch culture on carbon disulphide and carbonyl sulphide, and a comparison with yields on thiosulphate and thiocyanate.

In batch culture on 2mM CS<sub>2</sub> in sealed flasks, *F. thioparus* strain TK-m exhibited a doubling time of 30-40 hours (i.e. a specific growth rate of about 0.02 h<sup>-1</sup>). Biomass production in these cultures was in the range of 13.6 - 17.6 mg cell carbon per litre of culture, indicating a yield of 7.9 ± 0.9 g cell-carbon (mole carbon disulphide)<sup>-1</sup> (from five estimations). When grown on carbonyl sulphide (COS) under similar conditions, a yield of 6.1 ± 0.8 g cell-carbon (mole COS)<sup>-1</sup> was obtained.

As already reported, a chemostat culture at a dilution rate of 0.085 h<sup>-1</sup> under dual substrate limitation with thiosulphate (7.5mM) and thiocyanate (5.2mM) gave yields equivalent to 5.88 and 5.58 g cell carbon (mole)<sup>-1</sup> respectively. For the oxygen requirements for the oxidation of the above

compounds ( $2 \text{ mol mol}^{-1}$  for COS, thiosulphate and thiocyanate;  $4 \text{ mol mol}^{-1}$  for  $\text{CS}_2$ ) the yields would be expected to be similar for COS, thiosulphate and thiocyanate, as was found. The yield on  $\text{CS}_2$  would thus have been expected to fall in the range  $11.7 \pm 0.5 \text{ g cell-carbon (mole } \text{CS}_2)^{-1}$ .

### 6.6.3. Stoichiometry and intermediates of carbon disulphide metabolism.

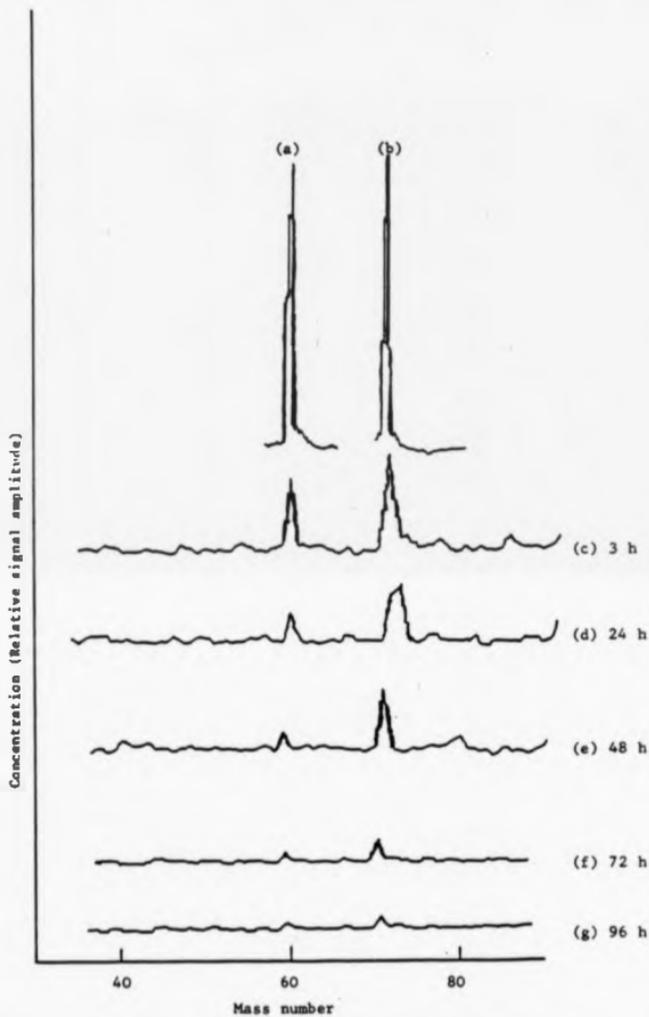
During growth on  $\text{CS}_2$ , analysis by gas chromatography/mass spectrometry of the chloroform-extractable volatiles from the culture medium showed a rapid initial formation of a compound indistinguishable from COS. This compound was detectable throughout the culture period of four days but remained below the concentration of residual  $\text{CS}_2$  (Fig. 6.8).

Triplicate 100 ml batch cultures (in 500 ml stoppered flasks) were fed with five additions of  $1 \text{ mM } \text{CS}_2$  over a period of twelve days. The cultures were centrifuged at  $21,000 \text{ xg}$  for 15 mins, washed and re-suspended in 10 ml mineral salts medium. When a suspension (5 ml;  $7.5 \text{ mg}$  cell protein) of *F. thloparus* TK-m was incubated anaerobically under a headspace of 18 ml nitrogen and given  $10 \mu\text{mol}$  of  $\text{CS}_2$ , the  $\text{CS}_2$  disappeared quite rapidly from the headspace gas when analysed by GC (Fig 6.9). Its disappearance was paralleled by the formation of COS and  $\text{H}_2\text{S}$ , with further  $\text{H}_2\text{S}$  accumulation apparently occurring at the expense of COS disappearance (Fig. 6.9). After 380 mins anaerobic incubation, the experiment was terminated by acidification with hydrochloric acid to release  $\text{H}_2\text{S}$  from solution. At that time, the amounts ( $\mu\text{mol}$ ) of  $\text{CS}_2$ , COS and  $\text{H}_2\text{S}$  recovered in the headspace were 1.99, 0.41 and 15.4 respectively. This meant that of the  $10 \mu\text{mol } \text{CS}_2$  added,  $8 \mu\text{mol}$  was converted quantitatively to COS (2.6%) and  $\text{H}_2\text{S}$  (97.4%). Under aerobic conditions, no such COS or  $\text{H}_2\text{S}$  accumulation was seen in a 200 mins incubation, although traces of  $\text{H}_2\text{S}$  (1-6 nmol) were detected, and 49 nmol COS were seen after 20 mins, declining to an undetectable level by 63 mins.

LEGEND TO FIGURE 6.8

Demonstration by GC/MS of the intermediate formation of carbonyl sulphide during the growth of *T. thioparus* strain TK-m on carbon disulphide (4 mM). Chloroform-extractable components of the culture were analysed as described in the Methods. (a) standard COS; (b) substrate CS<sub>2</sub> standard; (c) - (g) samples from the culture taken at the times indicated and analysed by GC/MS with chloroform as the internal reference compound.

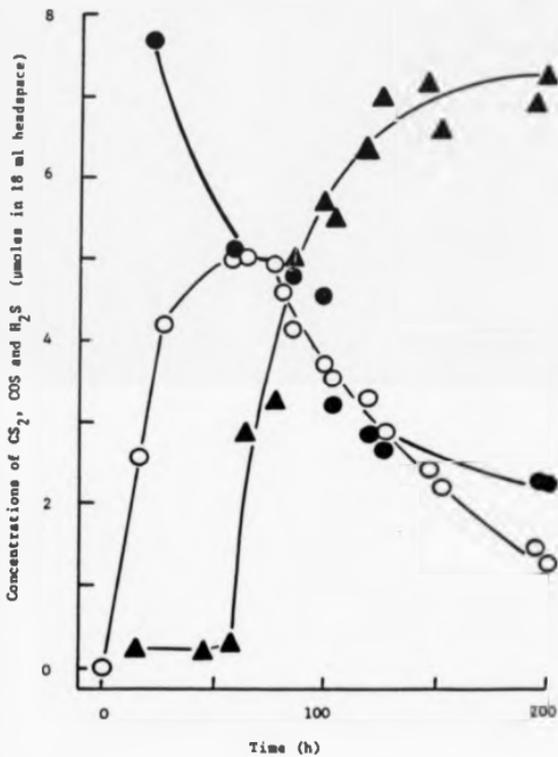
Figure 6.8



LEGEND TO FIGURE 6.9

Formation of carbonyl sulphide (○) and hydrogen sulphide (▲) from carbon disulphide (●) during anaerobic incubation with T. thioparus strain TK-m. Suspensions were incubated under nitrogen with 10 umoles of CS<sub>2</sub> as described in the Methods, and the headspace gas analysed for CS<sub>2</sub>, COS and H<sub>2</sub>S.

Figure 6.9



#### 6.6.4. Oxidation of carbon disulphide.

Although *F. thiooparus* TK- cells taken directly from a thiosulphate-limited chemostat culture did not oxidise CS<sub>2</sub> (see section 6.4.2) in the oxygen electrode, CS<sub>2</sub> oxidation was observed using CS<sub>2</sub>-grown cells. Suspensions of CS<sub>2</sub>-grown cells oxidised a range of seven concentrations of CS<sub>2</sub> (Fig 6.10a). Oxidation rate increased from 128 nmol O<sub>2</sub> consumed min<sup>-1</sup> (mg cell protein)<sup>-1</sup> with 5μM CS<sub>2</sub> to 477 with 50μM, with no evidence of substrate inhibition over this range. From 16 rate values, K<sub>m</sub> and V<sub>max</sub> were determined by Lineweaver-Burk (Fig. 6.10b) and computed by Eadie-Hofstee plots as K<sub>m</sub> = 16.0 and 16.5μM and V<sub>max</sub> = 511 and 524 nmol O<sub>2</sub> consumed min<sup>-1</sup> (mg protein)<sup>-1</sup>, respectively. The Pearson's correlation coefficients for the fit of the data were 0.973 and 0.871.

The stoichiometry of CS<sub>2</sub> oxidation fell below the expected molar ratio of four O<sub>2</sub> per CS<sub>2</sub> oxidised: the best ratio was 1.5 : 1.0 at 25μM CS<sub>2</sub>, with a mean of 1.32 ± 0.13 O<sub>2</sub> per CS<sub>2</sub> for 5-50μM. This probably indicated incomplete oxidation of sulphide, possibly resulting in sulphur accumulation.

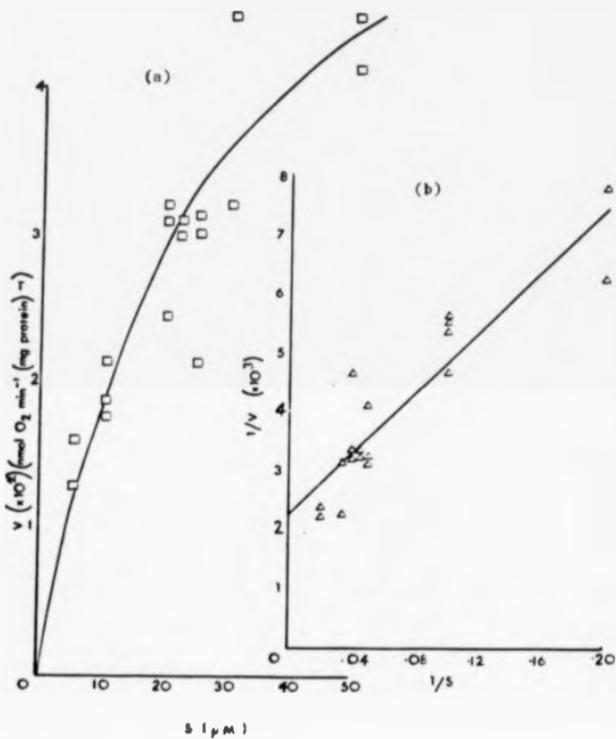
#### 6.6.5. Growth, sulphate production and assimilation of carbon dioxide using carbon disulphide or carbonyl sulphide as sole substrate.

When grown on either CS<sub>2</sub> or COS, virtually all the cell carbon was seen to be derived from carbon dioxide (Fig. 6.11a). The decline in the amount of <sup>14</sup>C<sub>2</sub> fixed in the later stages of growth was attributed to dilution of the specific activity of the added <sup>14</sup>C-bicarbonate by the CO<sub>2</sub> 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 CO<sub>2</sub>-fixation.

LEGEND TO FIGURE 6.10

Kinetics of oxidation of carbon disulphide by T. thioparus strain TK-m. (a) Lineveaver-Burk plot of reciprocals of oxidation rate versus substrate concentration and (b) oxidation rate versus subatrate concentration.

Figure 6.10



Making simultaneous measurements of increase in absorbance, total organic carbon in the organisms,  $^{14}\text{C}$ -carbon dioxide fixation and sulphate production showed these all to be proportionate during growth (Fig. 6.11b). During the complete oxidation of approximately 2mM  $\text{CS}_2$ , about 13.9 mg of newly synthesised organism-carbon was produced per litre (at least 90% from carbon dioxide), and 3.6mM sulphate was recovered. This represented 90% of the sulphur present in the  $\text{CS}_2$  supplied, and showed that complete oxidation of the substrate took place.

6.6.6. Determination of the pathway of carbon assimilation during growth on carbon disulphide.

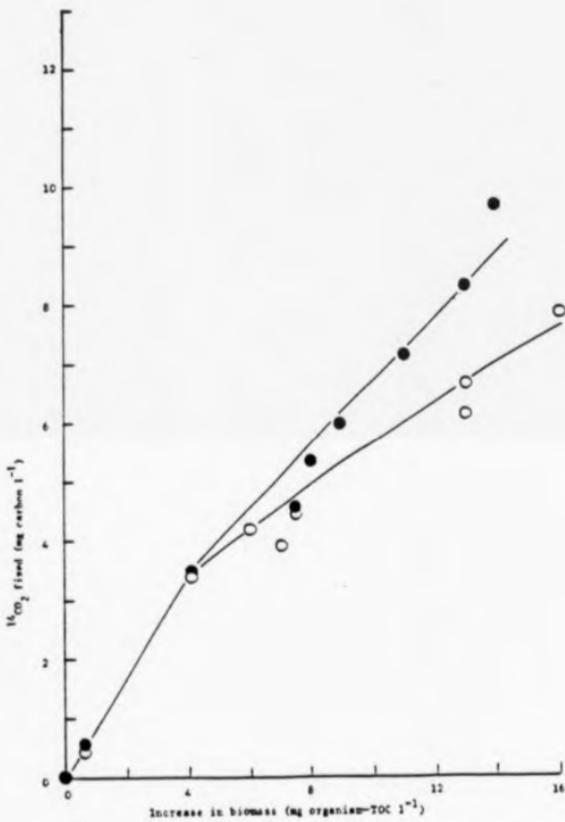
$\text{H}^{14}\text{CHO}$  (added at 0.25mM) was incorporated during growth on  $\text{CS}_2$  (2mM) at a rate that was proportional to increase in biomass, but accounted for only about 5% of the cell-carbon fixed during growth. This would be consistent with its being assimilated only after oxidation to carbon dioxide.

Low levels of ribulose 1,5-bisphosphate carboxylase were detected using the permeabilized whole cell procedure. No activity was found if Triton X-100 was used as the permeabilising agent. Using CTAB-treated cells gave a specific activity of about 3 nmol  $\text{CO}_2$  fixed  $\text{min}^{-1}$  ( $\text{mg dry wt}^{-1}$ ), which was equivalent to about 5 nmol  $\text{min}^{-1}$  ( $\text{mg total protein}^{-1}$ ). This can be compared with a specific activity, under similar assay conditions, of 46 nmol  $\text{min}^{-1}$  ( $\text{mg cell protein}^{-1}$ ) for organisms grown autotrophically in a thiosulphate-limited chemostat at a dilution rate of 0.08  $\text{h}^{-1}$ .

LEGEND TO FIGURE 6.11

Growth of T. thioparus strain TK-m on carbon disulphide or carbonyl sulphide. (a)  $^{14}\text{CO}_2$ -fixation during growth of cultures in sealed flasks, as described in the Methods, on 2 mM  $\text{CS}_2$  (●) or 2 mM  $\text{COS}$  (○). Increase in organism-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 organism-carbon.

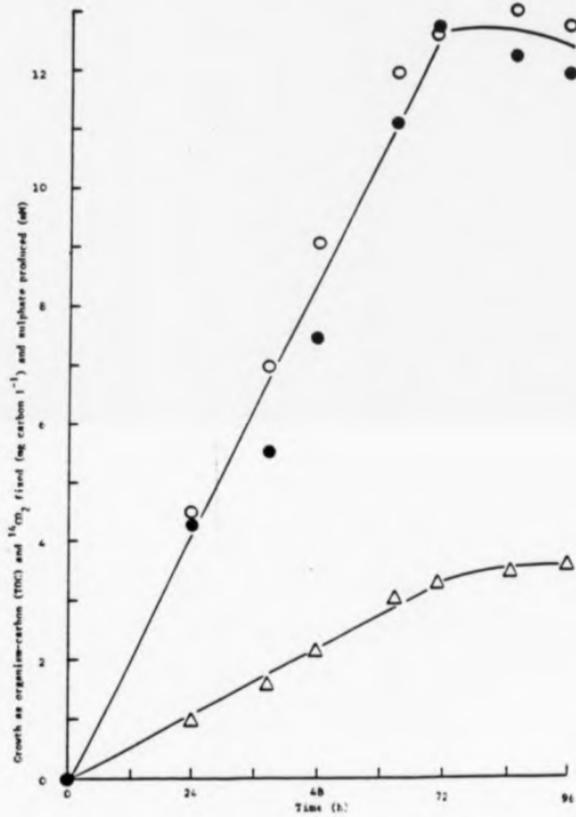
Figure 6.11a



LEGEND TO FIGURE 6.11 (cont'd.)

(b) Increase in biomass (as TOC, ○), fixation of  $^{14}\text{CO}_2$  (●) and formation of sulphate (Δ) by cultures growing on 2 mM  $\text{CS}_2$ . 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  $\text{l}^{-1}$  and 5.25 mM, respectively).

Figure 6.11b



## 6.7. DISCUSSION.

*F. thiotropus* TK-m was previously shown to grow on DMS as the sole source of energy and it was proposed that the energy generated from the simultaneous oxidation of sulphur and carbon from DMS supported autotrophic growth (Kanagawa & Kelly, 1986). It has been possible during this study not only to reproduce the original  $^{14}\text{CO}_2$  incorporation patterns, but also to demonstrate the presence of ribulose 1,5-bisphosphate carboxylase in DMS-grown cells at an activity sufficient to support autotrophic growth. Further radiolabelling experiments demonstrated that formaldehyde and formate were likely intermediates in DMS metabolism both of which were oxidised to carbon dioxide. Likewise methanethiol was also proposed to be an intermediate since growth on DMS (but not on thiosulphate) was inhibited in the presence of the catalase inhibitor 3-amino 1,2,4-triazole, catalase being necessary to remove hydrogen peroxide produced by the methanethiol oxidase (Suylen *et al.*, 1986; Smith & Kelly, 1988).

In common with DMS metabolism by *F. thiotropus* E6, DMS metabolism by *F. thiotropus* TK-m was inducible, with a lag of about 3-4 days observed when thiosulphate-grown cells were transferred to DMS cultures. The same long lag was not observed when DMS-grown cells were transferred to thiosulphate cultures. Further evidence for an inducible DMS oxidation system was provided by oxygen uptake data: neither DMS nor NT were oxidised by thiosulphate grown cells.

Although the growth yields of *F. thiotropus* TK-m and *F. thiotropus* E6 on DMS were comparable at about 12 g cell-carbon mol<sup>-1</sup>, there appeared to be an important distinction between the two: since the growth yield on thiosulphate of *F. thiotropus* TK-m was approximately twice that of *F. thiotropus* E6, considerably less energy must be derived from DMS-carbon

oxidation. It is possible that in this organism formaldehyde and formate oxidation were for some reason not linked efficiently to energy generation, but generate reducing power presumably to fuel the Calvin cycle. This could then possibly explain the observed low oxidation rates of formaldehyde and formate and why there was apparently no stimulation in yield when formate was added to a thiosulphate-limited chemostat even though formate was removed from the culture. Clearly,  $^{14}\text{C}$ -labelled formaldehyde and formate were assimilated into cell carbon following oxidation to carbon dioxide.

Autotrophic, chemolithotrophic growth of *F. thioparvus* TK-m was observed on a range of inorganic sulphur compounds which normally support the growth of most other thiobacilli (Kelly, 1982, 1988; Kelly & Harrison, 1988). The mechanism of sulphide oxidation was not 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, suggested that tetrathionate was an intermediate in thiosulphate oxidation and these might probably be intermediates in sulphide oxidation. Thiocyanate was shown to support aerobic and anaerobic growth of *F. thioparvus* TK-m, the yield data indicating that the complete oxidation of one mole of thiocyanate yielded as much energy for growth as the oxidation of one mole of thiosulphate. During anaerobic growth on thiosulphate and thiocyanate the failure to observe the stoichiometric appearance of nitrite and disappearance of substrate probably arise as a consequence of either:

- [i] the incomplete oxidation of substrate possible only as far as sulphur,
- [ii] the possibility that some aerobic oxidation took place, or
- [iii] growth was inhibited to some extent due to the build up of nitrite which is toxic to some thiobacilli; (Woolley *et al.*, 1962).

Except for its ability to grow autotrophically on DMS, *F. thioparus* TK-m resembles most other obligately chemolithotrophic thiobacilli. However, it possesses several features which distinguish it from other thiobacilli, including the other DMS-oxidisers isolated during this study. Firstly, it was not capable of growth on DMDS nor was DMDS oxidised by DMS grown cells. The nature of the initial attack on DMS was not further investigated, but by analogy with DMS oxidation by hyphomicrobia, the initial reaction could be catalysed by a DMS mono-oxygenase (De Bont *et al.*, 1981; Suylen & Kuenen, 1986; Suylen *et al.*, 1986). This observation also adds further support to the theory that the enzymes catalysing the initial attack on DMS and DMDS are independent, even if the enzyme system for the complete degradation of methanethiol (as an intermediate) is common to both substrates. Secondly, the chemostat true growth yield on thiosulphate of about 6.6 g cell-carbon (mole)<sup>-1</sup> was higher than one would expect for a *F. thioparus* strain and is more consistent with yields observed with *F. denitrificans* (Timmer-Ten Hoer, 1981; Kelly, 1982) and *F. tepidarius* (Wood & Kelly, 1985, 1986). *F. thioparus* TK-m was capable of anaerobic growth on thiosulphate and thiocyanate using nitrate as the terminal electron acceptor. Nitrate was reduced only as far as nitrite (not dinitrogen), this being a characteristic distinguishing *F. thioparus* strains from *F. denitrificans* strains (Woolley *et al.*, 1962). The ability to grow on thiocyanate (aerobically or anaerobically) is characteristic of some but not all *F. thioparus* strains (Kelly & Harrison, 1988). Thirdly, *F. thioparus* TK-m was capable of growth on carbon disulphide. Apart from a very brief report of its oxidation by *F. thiooxidans* (Butler *et al.*, 1969), virtually nothing is known about microbial CS<sub>2</sub> metabolism.

The results obtained have demonstrated that *F. thioparus* TK-m grows autotrophically on thiocyanate (S = C = N), carbon disulphide (S = C = S) and carbonyl sulphide (O = C = S), the metabolism of each of which requires

the scission of a carbon-sulphur double bond. The ability of the organism to attack carbon disulphide under anaerobic conditions suggests that the first step of aerobic carbon disulphide metabolism might be its hydrolytic cleavage to carbonyl sulphide and hydrogen sulphide:



The carbonyl sulphide appears to undergo similar hydrolysis to carbon dioxide and hydrogen sulphide:

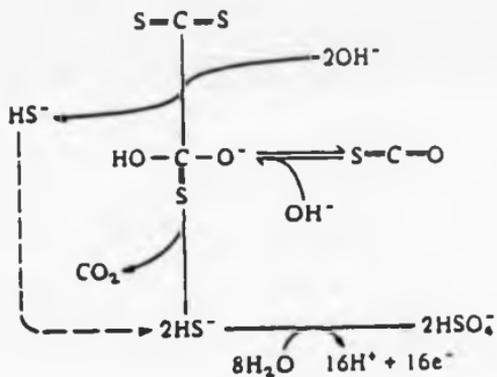


This scheme is in contrast to that proposed for the oxidative degradation of  $\text{CS}_2$  by rat liver hepatocytes and microsomes, which is believed to be initiated by a NADPH-dependent monooxygenase containing cytochrome P-450 (Chengelis & Neel, 1987). Clearly a monooxygenase cannot be involved in the anaerobic production of  $\text{COS}$  and  $\text{H}_2\text{S}$  observed during the incubation of cell suspensions. The possibility that the normal aerobic metabolism of  $\text{CS}_2$  is initiated by an oxygenase, and the anaerobic behaviour was artefactual, cannot, however, be unequivocally discounted.

The only source of energy available from the dissimilation of carbon disulphide is clearly the oxidation to sulphate of the hydrogen sulphide produced from it. Carbon disulphide and carbonyl sulphide, must therefore be regarded exclusively as chemolithotrophic substrates, in contrast to the methylated sulphides, which can be both chemolitho- and chemocorgano-trophic energy sources. Growth yields on thiosulphate, thiocyanate and carbonyl sulphide were similar, at 5.6 - 6.1 g cell-carbon  $\text{mol}^{-1}$ , indicating sulphur oxidation to be the only source of metabolic energy in each case. Yields on

FIGURE 6.12

Hypothetical mechanism for carbon disulphide oxidation by *Thiobacillus* TK-m based on data obtained from aerobic and anaerobic incubations of cell suspensions.



carbon disulphide 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  $CS_2$ , 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  $CS_2$  oxidation (via a monooxygenase-dependent pathway) per NAD(P) reduced (Fig. 6.13). If this occurred, one could predict that the yield on  $CS_2$  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 (mole  $CS_2$ )<sup>-1</sup>.

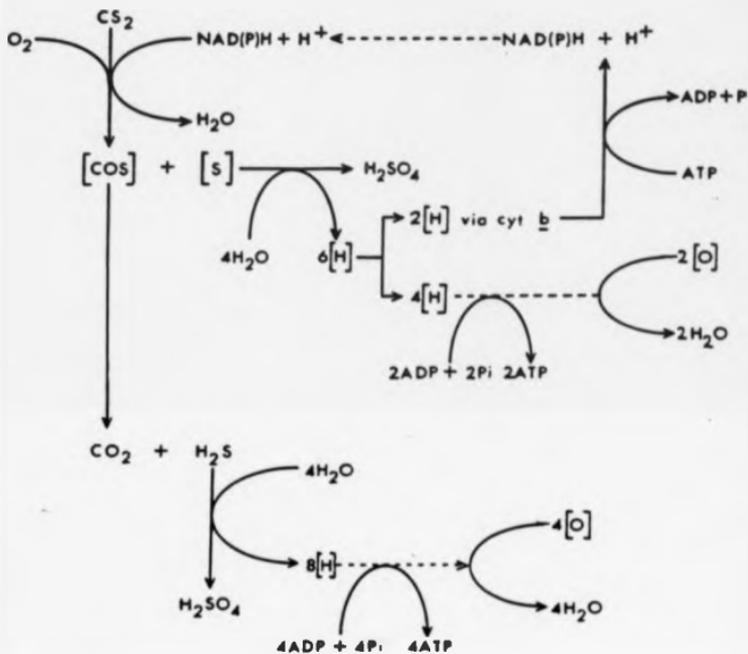
Growth on carbon disulphide was shown to be wholly autotrophic when assayed by <sup>14</sup>C<sub>2</sub> fixation, but only very low levels of ribulose 1,5-bisphosphate carboxylase have been detected. This may reflect both the very low growth rate of the organism on  $CS_2$  and failure to assay under optimum conditions. A sufficient bulk of  $CS_2$ -grown organisms was not achieved in order to prepare cell-free extracts for further enzyme studies. Formaldehyde was apparently only assimilated after oxidation to carbon dioxide, thereby discounting any alternative or unique C<sub>1</sub>-assimilatory pathway.

The ability to oxidise carbon disulphide appears to be much more restricted among the bacteria studied to date than the capacity for oxidation of the methylated sulphides or inorganic sulphur compounds. No carbon disulphide oxidising strains were detected in any of the enrichment cultures (Cb.3), and neither was  $CS_2$  capable of supporting growth of the seven methylated sulphide oxidisers isolated nor any of the range of other available

LEGEND TO FIGURE 6.13

Proposed mechanism of carbon disulphide oxidation by T. thioparus strain TK-m, suggested by yield date of organisms grown on carbon disulphide, carbonyl sulphide, thiosulphate and thiocyanate.

Figure 6.13



thiobacilli. In that it can only be used as a chemolithotrophic energy source for autotrophic growth, it is perhaps not surprising that few organisms can oxidise it. The possibility that there exist in the natural environment specialist organisms better adapted to use it remains to be explored.

As well as its ability, as a typical *Thiobacillus*, to oxidise thiosulphate, tetrathionate and sulphide, *F. thioparvus* TK-m can also grow on DMS as its sole substrate. These results serve both as the first detailed study of the microbial breakdown of  $CS_2$ , and as a further demonstration of the metabolic versatility of the organism.

CHAPTER SEVEN

CONCLUDING REMARKS AND SUGGESTIONS FOR FURTHER STUDY

---

7. CONCLUDING REMARKS AND SUGGESTIONS FOR FURTHER STUDY

Undoubtedly, low molecular weight, volatile organic sulphur compounds like the methylated sulphides,  $CS_2$  and COS play an important role in the cycling of sulphur through the biosphere and there is a growing list of known biological and, to a lesser extent, chemical mechanisms by which they are produced and degraded. Microbial degradation of these and related compounds in specific environments have been shown to have a significant role in their turnover. The isolation of several more strains of bacteria capable of growth on DMS, and demonstrated for the first time on DMDS, adds to those few strains already described. The surprising feature is their ability to grow autotrophically on an organic compound - a mode of metabolism which is still relatively uncommon. Although it appears that  $CS_2$  and COS are restricted to being chemolithotrophic substrates, it is easy to imagine that methylotrophs and facultative heterotrophs could play a more significant role in methylated sulphide degradation and certainly this is an area worthy of further investigation.

On several occasions it was noted that on transfer of some DM(D)S oxidising strains following prolonged subculture on thiosulphate to further DMS or DMDS cultures, they failed to grow even when cells appeared healthy at the time of transfer and inoculum sizes were large. It was suggested that this effect might be observed if the genes coding for the enzymes of methylated sulphide metabolism were plasmid borne and the plasmids had been lost during prolonged growth on thiosulphate. The genes coding for enzymes of inorganic sulphur oxidation in thiobacilli would obviously be expected to be chromosomal. Plasmid curing experiments using DMS or DMDS grown cells were not conducted but would have been worthwhile. There is apparently a very restricted literature on the genetics of thiobacilli and most of this

concerns organisms of commercial interest such as strains of *F. ferrooxidans* (Mac *et al.*, 1980; Martin *et al.*, 1981; Holmes *et al.*, 1984) and an isolated report of the characterisation of mutants of *F. varus* deficient in autotrophic metabolism (Kraczkiewicz-Dowjat & Kelly, 1985).

There is a whole range of other, larger naturally occurring volatile and non-volatile organic sulphur compounds such as higher thiols, allyl and alkyl sulphides (Sparnins *et al.*, 1988) and thiophene derivatives, the microbial metabolism, of which, have not been investigated to any great extent. Thiophene derivatives are common in nature and their microbial degradation has in the past been the subject of several studies (Amphlett & Callery, 1969; Cripps, 1973; Zurrer *et al.*, 1987; Kanagawa & Kelly, 1987). Thiophene degradation as part of the desulphurisation of coal and oil has also been of commercial interest (Kodama, 1977; Kurita *et al.*, 1971; Ensley, 1984; Kohler *et al.*, 1984; Monticello & Finnerty, 1985). It is not clear whether the organisms capable of degrading thiophene derivatives also oxidise the sulphur atom or if it is excreted to be later oxidised by some other organism. As far as higher thiols are concerned it is reasonable to speculate that their carbon skeletons are substrates for growth of heterotrophic microorganisms. If such organisms are also unable to oxidise the sulphur atom(s) of these compounds, then they too will be excreted, possibly as  $H_2S$  or  $HS^-$  as occurs during sulphur amino acid catabolism, and again provides substrates for other organisms. There is therefore considerable scope amongst the bacteria for the concerted action of mixed populations to completely degrade the larger organic sulphur compounds and perhaps this too would be a worthwhile course of study rather than attempting to isolate pure cultures.

PUBLICATIONS AND PRESENTATIONS

- SMITH, M.A. & KELLY, D.P. (1988). Autotrophic growth of chemolithotrophic sulphur bacteria on dimethyl disulphide, dimethyl sulphide, methanethiol, carbon disulphide and thiocyanate. The nitrogen and sulphur cycles, Society for General Microbiology (110th meeting), Southampton.
- SMITH, M.A. & KELLY, D.P. (1988). Isolation and physiological characterisation of autotrophic sulphur bacteria oxidising dimethyl disulphide as sole source of energy. *J. Gen. Microbiol.* 134, 1407-1417.
- SMITH, M.A. & KELLY, D.P. (1988). Mechanism of oxidation of dimethyl disulphide by *Thiobacillus thiooparus* strain E6. *J. Gen. Microbiol.* (accepted for publication).
- SMITH, M.A. & KELLY, D.P. (1988) Oxidation of carbon disulphide as the sole source of energy for the autotrophic growth of *Thiobacillus thiooparus* strain TK-m. *J. Gen. Microbiol.* (accepted for publication).

R E F E R E N C E S

---

- ADAMS, D.F. & FARWELL S.O. (1981). Biogenic sulfur source strengths. *Environ. Sci. Technol.* 15, 1493-1498.
- ALLEN, M.I.E. (1977). Coupling of energy with electron transfer reactions in chemolithotrophic bacteria. In: *Microbial Energetics* (Haddock, B.A. and Hamilton, W.A., Eds.), 351-381. *Soc. Gen. Microbiol.*, University press, Cambridge.
- ALNGREN, T. & HAGSTROM, I. (1974). The oxidation rate of sulphide in seawater. *Water Res.* 8, 395-400.
- ANDO, H., KUMAGAI, M., KARASHINADA, T. & IIDA, M. (1957). Diagnostic use of dimethyl sulfoxide reduction test within Enterobacteriaceae. *Japan J. Microbiol.* 1, 335-338.
- ANDREAE, M.O. (1980). Dimethyl sulphoxide in marine and freshwater. *Limnol. Oceanogr.* 25, 1054-1063.
- ANDREAE, M.O. (1983). Dimethyl sulphide in the surface ocean and the marine atmosphere: a global view. *Science*, 221, 744-747.
- ANDREAE, M.O. (1985). Dimethyl sulfide in the water column and the sediment of the Peru upwelling area. *Limnol. Oceanogr.* 30, 1208-1218.
- ANDREAE, M.O. & BARNARD, W.B. (1984). The marine chemistry of dimethyl sulfide. *Mar. Chem.* 14, 267-269.

- ANILJA, V.P., OVERTON, J.R., CUPITT, L.T., DURHAM, J.L. & WILSON, W.E. (1979). Direct measurements of emission rates of some atmospheric biogenic sulphur compounds. *Tellus*, 31, 174-178.
- ANNES, B.J. (1981). The determination of dimethyl sulfoxide in aqueous solution. *J. Sci. Food Agr.* 32, 353-358.
- ANNES, B.J., BANFORTH, C.W. & MAINWRIGHT, T. (1979). The measurement of dimethyl sulfoxide in barley and malt and its reduction to dimethyl sulfide by yeast. *J. Inst. Brew.* 85, 346-349.
- ASHMORTH, J., BRIGGS, G.G., EVANS, A.A. & NATULA, J. (1977). Inhibition of nitrification by nitrapyrin, carbon disulphide and trithiocarbonate. *J. Sci. Food Agr.* 28, 673-683.
- BABICH, H. & STOTZKY, G. (1978) Atmospheric sulfur compounds and microbes. *Environ. Res.* 15, 513-531.
- BANFORTH, C.W. & ANNES, B.J. (1981). The role of dimethyl sulphoxide reductase in the formation of dimethyl sulphide during fermentations. *J. Inst. Brew.* 87, 30-34.
- BANWART, W.L. & BREDDER, J.M. (1974). Gas chromatographic identification of sulphur gases in soil atmospheres. *Soil Biol. Biochem.* 6, 113-115.
- BANWART, W.L. & BREDDER, J.M. (1975). Formation of volatile sulfur compounds by microbial decomposition of sulfur containing amino acids in soil. *Soil Biol. Biochem.* 7, 359-364.

- BAMMART, W.L. & BREKNER, J.M. (1976). Evolution of volatile sulfur compounds from soils treated with sulfur containing organic materials. *Soil Biol. Biochem.* 8, 439-443.
- BARNARD, W.R., ANDREAE, M.O., MATKINS, W.E., BINGEMER, H. & GEORGIU, H.W. (1982). The flux of dimethylsulfide from oceans to the atmosphere. *J. Geophys. Res.* 87, 8787-8793.
- BATTAT, E., GOLDBERG, I. & MATELES, R.I. (1974). Growth of *Pseudomonas* C on C-1 compounds: continuous culture. *Appl. Microbiol.* 28, 906-911.
- BECHARD, N.J. & RAYBURN, W.R. (1974). Emissions of volatile sulfur compounds from freshwater algae. *J. Phycol.* 10, 10-14.
- BECHARD, N.J. & RAYBURN, W.R. (1979). Volatile organic sulfides from freshwater algae. *J. Phycol.* 15, 379-383.
- BELI, A., IZARD, D., GAVINI, F. & LECLERC, M. (1987). A rapid chemical procedure for isolation and purification of chromosomal DNA from Gram-negative bacilli. *Anal. Biochem.* 162, 18-23.
- BHATHNAGAR, L., KENRIQUET, M., ZEIKUS, J.G. & AUBERT, J.P. (1984). Utilisation of mercapto-2-ethanol as medium reductant for determination of the metabolic response of methanogens towards inorganic sulphur compounds. *FEMS Microbiol. Lett.* 22, 155-158.
- BILLS, D.D. & KEENAN, T.W. (1968). Dimethyl sulfide and its precursor in sweetcorn. *J. Agr. Food Chem.* 16, 643-645.

- BILOUS, P.T. & WEINER, J.H. (1985). Dimethyl sulfoxide reductase activity by anaerobically grown *E. coli* HB101. *J. Bacteriol.* 162, 1151-1155.
- BOEN, H. (1972). Soil absorption of air pollutants. *J. Environ. Qual.* 1, 372-377.
- BOUNDS, H.C. & COLMER, A.R. (1972). Comparison of the kinetics of thiosulphate oxidation by three iron oxidisers. *Can. J. Microbiol.* 18, 735-740.
- BRENNER, J.M. & BANMART, W.L. (1974). Identifying volatile S compounds by gas chromatography. *Sulphur Inst. J.* 10, 6-9.
- BRENNER, J.M. & STEELE, C.G. (1978). Role of microorganisms in the atmospheric sulfur cycle. In: *Advances in Microbial Ecology* 2, 155-201. M. Alexander (Ed.), Plenum Press. New York. London.
- BROCK, T.D., BROCK, K.M., BELLY, R.T. & WEISS, R.L. (1972). *Sulfolobus*: A new genus of sulfur-oxidising bacteria at low pH and high temperature. *Arch. Microbiol.* 84, 54-68.
- BRYANT, M.P., TZENG, S.F., ROBINSON, I.M. & JOINER, A.E. (1971). Nutrient requirements of methanogenic bacteria. *Adv. Chem. Ser.* 105, 23-40.
- BUTLER, R.G., ROTHSCCHILD, B. & KELLER, J.R. (1969). Metabolism of carbon disulphide by *Thiobacillus thiooxidans*. *Bacteriological Proceedings*, P64, American Society for Microbiology.

- CADLE, E.D. (1967). The photo-oxidation of hydrogen sulfide and dimethyl sulfide in air. *Atmos. Environ.* 10, 417-419.
- CAMSE, P.A. (1967). The determination of nitrate in soil solutions by ultraviolet spectrophotometry. *Analyst* 92, 311-315.
- CHEMELIS, C.P. & NEAL, E.A. (1987). Oxidative metabolism of carbon disulphide by isolated rat hepatocytes and microsomes. *Biochem. Pharmacol.* 36, 363-368.
- COHEN, G. & SOMERSON, M.L. (1969). Catalase - aminotriazole method for measuring secretion of hydrogen peroxide by microorganisms. *J. Bacteriol.* 98, 543-546.
- COLBY, J., DALTON, M. & WHITTENBURY, R. (1979). Biological and biochemical aspects of microbial growth on C1 compounds. *Ann. Rev. Microbiol.* 33, 481-517.
- COLLINS, M.D. & JONES, D. (1981). Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiol. Rev.* 45, 316-354.
- COOPER, A.J.L. (1983). Biochemistry of sulfur containing amino acids. *Ann. Rev. Biochem.* 52, 187-222.
- COX, P.A. & SANDALLS, F.J. (1974). The photooxidation of hydrogen sulphide and dimethyl sulphide in air. *Atmos. Environ.* 8, 1269-1281.

- DACEY, J.W.H., KING, G.M. & WAKEMAN, S.G. (1987). Factors controlling emission of dimethyl sulfide from salt marshes. *Nature*, 330, 643-645.
- DACEY, J.W.H., WAKEMAN, S.G. & HOWES, B.L. (1984). Henry's Law Constants for dimethyl sulfide in freshwater and seawater. *Geophys. Res. Lett.* 11, 991-994.
- DE BONT, J.A.M., VAN DIJKEN, J.F. & HARDER, W. (1981). Dimethyl sulfoxide and dimethyl sulfide as a carbon, sulphur and energy source for growth of *Hyphomicrobium* S. *J. Gen. Microbiol.* 127, 315-323.
- DEPREZ, P.P., FRANZMANN, P.D. & BURTON, M.B. (1986). Determination of reduced sulphur gases in Antarctic lakes and seawater by gas chromatography after solid adsorbent pre-concentration. *J. Chromatogr.* 362, 9-21.
- DISPIRITO, A.A., MSIAO-TSU LOH, W. & TUOVINEN, O.H. (1983). A novel method for the isolation of bacterial quinones and its application to appraise the ubiquinone composition of *Photobacillus ferrooxidans*. *Arch. Microbiol.* 135, 77-80.
- DISTEFANO, V. & BORGSTEDT, M.H. (1964). Reduction of dimethyl sulfoxide to dimethyl sulfide in the cat. *Science* 144, 1137-1138.
- DIXON, M. (1953). The determination of enzyme inhibitor constants. *Biochem. J.* 55, 170-171.

- DROTAR, A., BURTON, G.A., TAVERNIER, J.E. & FALL, R. (1987).  
Widespread occurrence of bacterial thiol-methyl transferases and  
biogenic emission of methylated sulfur gases. *Appl. Env.  
Micro.* 53, 1626-1631.
- BADY, R.B. & LARKE, P.J. (1988). Purification and properties of an amine dehydrogenase from *Pseudomonas*  
*AH 1* and its role in growth on methylamine. *Biochem. J.* 108, 243-255.
- BECLESTON, M. & KELLY, D.P. (1978). Oxidation kinetics and chemostat  
growth kinetics of *F. farinosus* on tetrathionate and  
thiosulphate. *J. Bacteriol.* 134, 718-727.
- ELLIOT, L.F. & TRAVIS, T.A. (1973). Detection of carbonyl sulfide and  
other gases emanating from beef cattle manure. *Soil Sci.  
Soc. Amer. Proc.* 37, 700-702.
- ENSLEY, B.D. (1984). Microbial metabolism of condensed thiophenes.  
In: *Microbial degradation of organic compounds* (Gibson, T.D.  
Ed.). Marcel Dekker, inc. New York, Basel.
- ERIKSSON, E. (1963). The yearly circulation of sulfur in nature.  
*J. Geophys. Res.* 68, 4001-4008.
- FERCHICHI, M., HEMME, D. & BOUILLANNE, C. (1986). Influence of oxygen  
and pH on methanethiol production from L-methionine by  
*Brevibacterium linens* CNRZ 918. *Appl. Environ. Microbiol.* 51,  
725-729.
- FERREK, R.J., CHATFIELD, R.B. & ANDREAK, M.O. (1986). Vertical  
distribution of dimethyl sulfide in the marine atmosphere.  
*Nature.* 320, 514-516.

- FIESCHKO, J. & HUMPHREY, A.E. (1984). Statistical analysis in the estimation of maintenance and true growth yield co-efficients. *Biotechnol. Bioeng.* 26, 394-396.
- FRANZMANN, P.D., DEPREZ, P., BURTON, H.B. & van den HOFF, J. (1987). Limnology of Organic Lake, Antarctica, a meromictic lake that contains high concentrations of dimethyl sulphide. *Aust. J. Mar. Freshw. Res.* 38, (in press).
- FREDERIOQ, E., OTH, A. & FONTAINE, P. (1961). The ultra violet spectrum of deoxyribonucleic acids and their constituents. *J. Mol. Biol.* 3, 11-17.
- FRIEDRICH, C.G. & MITRENGA, G. (1981). Oxidation of thiosulfate by *Paracoccus denitrificans* and other hydrogen bacteria. *FEMS Microbiol. Lett.* 10, 209-212.
- GALCHENKO, V.F. & ANDRREEV, L.V. (1984). Taxonomy of obligate methanotrophs. In: *Microbial growth on C1 compounds*. (Crawford, R.L. & Hanson, R.S., Eds.), ASM, Washington.
- GHISALBA, G. & KUENZI, W. (1983). Biodegradation and utilisation of monomethyl sulfate by specialised methylotrophs. *Experientia.* 39, 1257-1263.
- GOLDBERG, I., ROCK, J.B., BEN-BASSAT, A. & MATELES, R.I. (1976). Bacterial yields on methanol, methylamine, formaldehyde and formate. *Biotechnol. Bioeng.* 18, 1657-1668.

- GOTTSCHAL, J.C. & KUENEN, J.G. (1980a). Mixotrophic growth of *Thiobacillus* A2 on acetate and thiosulphate as growth-limiting substrates in the chemostat. *Arch. Microbiol.* 126, 33-42.
- GOTTSCHAL, J.C. & KUENEN, J.G. (1980b). Selective enrichment of facultatively chemolithotrophic thiobacilli and related organisms in continuous culture. *FEMS Microbiol. Lett.* 7, 241-247.
- GRANNAT, L., RODHE, M. & HALLBERG, B.O. (1976). In: Nitrogen, phosphorus and sulphur - Global cycles: scope 7 (SVENSSON, B.M. & ODERLUND, S., Eds.). *Ecol. Bull.* 89-134.
- HALL, W.R. & BERK, R.S. (1967). Microbial growth on mercaptosuccinic acid. *Can. J. Microbiol.* 13, 515-518.
- HANSON, R.S. (1980). Ecology and diversity of methylotrophic organisms. *Adv. Appl. Microbiol.* 26, 3-39.
- HAPPOLD, F.C., JOHNSTONE, K.I. & ROGERS, H.I. (1954). The isolation and characterisation of an organism oxidising thiocyanate. *J. Gen. Microbiol.* 10, 261-266.
- HAPPOLD, F.C. & KEY, A. (1937). The bacterial purification of gas-works liquors II. The biological oxidation of ammonium thiocyanate. *Biochem. J.* 31, 1323-1329

- HARDER, W., ATTWOOD, N.M. & QUAYLE, J.R. (1973). Methanol assimilation by *Hyphomicrobium* sp. J.Gen. Microbiol. 78, 155-163.
- HARDER, W. & VAN DIJKEN, J.P. (1976). Theoretical considerations on the relation between energy production and growth of methane-utilising bacteria. In: Symposium on microbial production and utilisation of gases. (Schlegel, G., Gottschalk, G. & Pfennig, N., Eds.), 403-418. E. Goltze Verlag, Göttingen.
- HAYWOOD, G.W. & LARGE, P.J. (1981). Microbial oxidation of amines. Distribution, purification and properties of two primary-amine oxidases from the yeast *Candida boidinii* grown on amines as sole nitrogen source. Biochem. J. 199, 187-201.
- HAZEU, W., BIJLOVELD, W., GROTHENDIJS, J.T.C., KAKES, E. & KUENEN, J.G. (1986). Kinetics and energetics of reduced sulphur oxidation by chemostat cultures of *Thiobacillus ferrooxidans*. Antonie van Leeuwenhoek 52, 507-578.
- HEADLEY, J.V. (1987). GC/MS identification of organosulphur compounds in environmental samples. Biomed. Environ. Mass Spectrom. 13, 275-280.
- HEMPFLING, W.P. & VISHNIAC, W. (1967). Yield coefficients of *T. neapolitanus* in continuous culture. J. Bacteriol. 93, 874-878.
- HIGGINS, I.J., BEST, D.J., HAMMOND, R.C. & SCOTT, D. (1981). Methane oxidising microorganisms. Microbiol. Revs. 45, 556-590.

- HITCHCOCK, D.R. (1975). Dimethyl sulfide emissions to the global atmosphere. In: Chemosphere No.3. 137-138. Pergamon Press.
- HITCHCOCK, D.R. (1976). Atmospheric sulphates from biological sources. J. Air Pollut. Control Assoc. 26, 210-215.
- MOLNES, D.S., LOBOS, J.H., BOPP, L.H. & WELCH, G.C. (1984). Cloning of a *Thiobacillus ferrooxidans* plasmid in *Escherichia coli*. J. Bacteriol. 157, 324-326.
- MOORE, A.T.T. (1976). Energetic aspects of the metabolism of reduced sulphur compounds in *F. denitrificans*. Antonie van Leeuwenhoek. 42, 483-492.
- MOORE, A.T.T. (1981). Cell yield and bioenergetics of *Thiomicrospira denitrificans* compared to *F. denitrificans*. Antonie van Leeuwenhoek. 47, 231-243.
- HOWE, R.J., LONG, D.E. & SMETTER, D. (1976). A simpler optimised colorimetric method for formaldehyde. Anal. Lett. 3, 401-409.
- INGLEDEW, J.W. (1982). *Thiobacillus ferrooxidans*. The bioenergetics of an acidophilic chemolithotroph. Biochem. Biophys. Acta. 683, 89-117.
- JENKINS, D., REDSKER, L.L. & THOMAS, J.S. (1967). Odorous compounds in natural waters. Some sulfur compounds associated with blue-green algae. Environ. Sci. Technol. 1, 731-735.

- JOHNSON, J.L. (1985). Determination of DNA base composition. In: methods in Microbiology. 18. G. Gottschalk (Ed.), Academic press.
- JOHNSON, F.A. & QUAYLE, JR. (1974). Oxidation of methanol, formaldehyde and formate by methanol grown *Pseudomonas* AN 1. Biochem. J. 93, 281-285.
- JUSTIN, F. & KELLY, D.P. (1978). Growth kinetics of *Thiobacillus denitrificans* in anaerobic and aerobic chemostat cultures. J. Gen. Microbiol. 107, 123-130.
- KADOTA, H. & ISHIDA, Y. (1977). Production of volatile sulfur compounds by microorganisms. Annu. Rev. Microbiol. 26, 127-138.
- KANAGAMA, T., DAZAI, M. & FUKUOKA, S. (1982). Degradation of O, O-dimethyl phosphorodithioate by *Thiobacillus thioparvus* TK-1 and *Pseudomonas* AK-2. Agric. Biol. Chem. 46, 2571-2578.
- KANAGAMA, T., DAZAI, M. & TAKAHARA, Y. (1980). Degradation of O, O-dimethyl phosphorodithioate by activated sludge. Agric. Biol. Chem. 44, 2631-2635.
- KANAGAMA, T. & KELLY, D.P. (1986). Breakdown of dimethyl sulfide by mixed cultures and by *Thiobacillus thioparvus*. FEMS Microbiol. Letts. 33, 13-19.
- KANAGAMA, T. & KELLY, D.P. (1987). Degradation of substituted thiophenes by bacteria isolated from activated sludge. Microb. Ecol. 13, 47-57.

- KEMMAN, T.W. & LINDSAY, R.C. (1968). Evidence for a dimethyl sulfide precursor in milk. *J. Dairy Sci.* 51, 112-114.
- KELLY, D.P. (1970). Thin layer chromatography of inorganic sulphur compounds. *J. Chromatogr.* 51, 343-345.
- KELLY, D.P. (1978). Bioenergetics of chemolithotrophic bacteria. In: *Companion to microbiology*, 363-386. Bull, A.T. & Meadows, P.M. (Eds.), Longman, London.
- KELLY, D.P. (1979). Autotrophic metabolism of formate by *Thiobacillus* strain A2. *J. Gen. Microbiol.* 114, 1-13.
- KELLY, D.P. (1982). Biochemistry of the chemolithotrophic oxidation of inorganic sulphur. *Phil. Trans. R. Soc. Lond. B.* 298, 499-528.
- KELLY, D.P. (1985). Physiology of the thiobacilli: elucidating the sulphur oxidation pathway. *Microbiol. Sci.* 2, 105-109.
- KELLY, D.P. (1988). Oxidation of sulphur compounds. In: *The Nitrogen and Sulphur Cycles*, Cole, J.A. & Ferguson, S. (Eds). Society for General Microbiology, *Symposium* 82, 65-98.
- KELLY, D.P., CHAMBERS, L.A. & TRUDINGER, P.A. (1969). Cyanolysis and spectrophotometric estimation of trithionate in mixtures with thiosulphate and tetrathionate. *Anal. Chem.* 41, 898-901.

- KELLY, D.P. & HARRISON, A.P. (1988). *Thiobacillus*. In: Bergey's Manual of Systematic Bacteriology, Vol 3 (in press).  
Bryant, M.P., Pfennig, N. & Staley, J.T. (Eds.). Baltimore, Williams and Wilkins.
- KELLY, D.P. & TUOVINEN, O.H. (1975). Metabolism of inorganic sulphur compounds by *Thiobacillus ferrooxidans* and some comparative studies on *Thiobacillus* A2 and *T. neapolitanus*. Plant soil 43, 77-93.
- KELLY, D.P. & WOOD, A.P. (1982). Autotrophic growth of *Thiobacillus* A2 on methanol. FEMS Microbiol. Letts. 15, 229-233.
- KELLY, D.P. & WOOD, A.P. (1984). Potential for methylotrophic autotrophy in *Thiobacillus versutus* (*Thiobacillus* sp. strain A2). In: Microbial growth on C-1 compounds, 324-329. Crawford, R.L. & Hanson, R.S. (Eds.). American Society for Microbiology, Washington, D.C.
- KHALIL, M.A. & RASMUSSEN, R.A. (1984). Global sources, lifetimes and mass balances of carbonyl sulphide (COS) and carbon disulphide in the earth's atmosphere. Atmos. Environ. 18, 1805-1813.
- KIENE, R.P. & CAPORE, D.G. (1988). Microbial transformation of methylated sulfur compounds in anoxic salt marsh sediments. Microb. Ecol. 15, 275-291.

- KIENE, R.P., OREMLAND, R.S., CATENA, A., MILLER, L.W. & CAPONE, D.G. (1986). Metabolism of reduced methylated sulphur compounds by anaerobic sediments and a pure culture of an estuarine methanogen. *Appl. Environ. Microbiol.* 52, 1037-1045.
- KIENE, R.P. & TAYLOR, B.F. (1988). Biotransformations of organosulphur compounds in sediments via 3-mercaptopropionate. *Nature* 332, 148-150.
- KING, G.P., RICHARDSON, D.J., JACKSON, J.B. & FERGUSON, S.J. (1987). Dimethyl sulphoxide and trimethylamine-N-oxide as bacterial electron transport acceptors: Use of nuclear magnetic resonance to assay and characterise the reductase system in *Rhodospirillum rubrum*. *Arch. Microbiol.* 149, 47-51.
- KODAMA, K. (1977). Induction of dibenzothiophene oxidation by *Pseudomonas putida*. *Agric. Biol. Chem.* 41, 1193-1196.
- KOHLER, W., GRENZ, I.L., SCHICHT, B. & ECKART, V. (1984). Microbial desulphurisation of petroleum and heavy petroleum fractions 4. Anaerobic degradation of organic sulphur compounds of petroleum. *Zbl. Mikrobiol.* 139, 239-247.
- KONIG, W.A., LUDWIG, K., SIEVERS, S., STOLTING, K.H. & GUNTHER, W. (1980). Identification of volatile organic sulfur compounds in municipal sewage systems by GC/MS. *J. High Resolut. Chromatogr. Chromatogr. Commun.* 3, 415-416.

- KRACZKIEWICZ-DOMJAT, A. & KELLY, D.F. (1985). Isolation and partial characterisation of mutants of *F. varium* deficient in autotrophic metabolism. *Microbiol.* 88, 185-189.
- KRAUS, F. & SCHMIDT, A. (1987). Sulphur sources for growth of *Chlorococcum fusca* and their influence on key enzymes of sulphur metabolism. *J. Gen. Microbiol.* 133, 1209-1219.
- KROUSE, E.R. & McCREADY, R.G.L. (1979). Biogeochemical cycling of sulphur. In: *Studies in Environmental Science* 3, biogeochemical cycling of mineral-forming elements. P.A. Trudinger & D.J. Swaine (Eds). Elsevier Scientific Publication Company.
- KUEHN, J.G. (1979). Growth yields and "maintenance energy requirement" in *Thiobacillus* species under energy limitation. *Arch. Microbiol.* 122, 183-188.
- KURITA, S., ENDO, T., NAKAMURA, H., YAGI, T. & TIMAYA, N. (1971). Decomposition of some organic sulphur compounds in petroleum by anaerobic bacteria. *J. Gen. Appl. Microbiol.* 17, 185-198.
- KUSHELEV, Y.V., LYBMAN, N.Y., MAZANCO, A.F., ZEMLYANSKII, V.A. & BOIKOV, I.G. (1976). A few properties of solutions of dimethyl sulfide and dimethyl sulfoxide in water. *Sov. Chem. Ind.* 10, 775.
- LARGE, P.J. (1983). Physiology and Biochemistry of methylophilic bacteria. In: *Methylophilicity and methanogenesis. Aspects of microbiology* 8, (Cole, J.A., Knowles, C.J. & Schlessinger, D., Eds.), 25-57.

- LARGE, P.J. & QUAYLE, J.R. (1963). Microbial growth on C-1 Compounds 5: Enzyme activities in extracts of *Pseudomonas* AM 1. *Biochem. J.* 81, 386-396.
- LARKIN, J.N. & STROHL, W.R. (1983). *Beggiatoa, Thiobacillus and Thioploca*. *Annu. Rev. Microbiol.* 37, 341-367.
- LASHEN, E.S. & STARKEY, R.L. (1970). Decomposition of thioureas by a *Pentothidium* species and soil and sewage sludge microflora. *J. Gen. Microbiol.* 64, 139-150.
- LEADBEATER, L., SIEBERT, K., SCHOBERT, P. & BOWIEN, B. (1982). Relationship between activities and protein levels of ribulose biphosphate carboxylase and phosphoribulokinase in *Alcaligenes faecalis*. *FEMS Microbiol Letts.* 14, 263-266.
- LEITL, A. (1984). Soil heterotrophic bacteria in transformations of inorganic sulphur. *Folia Microbiol.* 29, 131-137.
- LEWIS, J.A. & PAPAIVIZAS, G.C. (1970). Evolution of volatile sulfur-containing compounds from decomposition of crucifers in soils. *Soil. Biol. Biochem.* 2, 239-246.
- LJUNGGREN, G. & NORBERG, B.O. (1943). On the effect of toxicity of dimethyl sulfide, dimethyl disulfide and methyl mercaptan. *Acta. Physiol. Scand.* 5, 248-255.
- LOVELLOCK, J.E. (1974).  $CS_2$  and the natural sulphur cycle. *Nature* 248, 625-626.

- LOVELOCK, J.E., MAOQS, R.G. & RASMUSSEN, R.A. (1972). Atmospheric dimethyl sulphide and the natural sulphur cycle. *Nature* 237, 452-453.
- LU, W.P. (1983). Enzymology and energetics of thiosulphate oxidation by *Thiobacillus* A2. Ph.D. thesis.
- LU, W.P. & KELLY, D.P. (1984). Properties and role of sulfite: cytochrome c oxidoreductase purified from *T. versutus* (A2). *J. Gen. Microbiol.* 130, 1683-1692.
- LU, W.P. & KELLY, D.P. (1988 a). Kinetics and energetic aspects of inorganic sulphur compound oxidation by *Thiobacillus tepidarius*. *J. Gen. Microbiol.* 134, 865-876.
- LU, W.P. & KELLY, D.P. (1988 b). Cellular location and partial purification of the 'thiosulphate-oxidising enzyme' and 'trithionate hydrolase' from *Thiobacillus tepidarius*. *J. Gen. Microbiol.* 138, 877-885.
- LU, W.P. & KELLY, D.P. (1988 c). Chemolithotrophic ATP synthesis and NAD(P)<sup>+</sup> reduction in *Thiobacillus tepidarius* and *T. versutus*. *Arch. Microbiol.* 149, 303-307.
- LU, W.P. & KELLY, D.P. (1988 d). Respiration-driven proton translocation in *Thiobacillus versutus* and the role of the periplasmic thiosulphate-oxidising enzyme system. *Arch. Microbiol.* 149, 297-302.

- LU, W.P., SMOBODA, E.E.P. & KELLY, D.P. (1985). Properties of thiosulphate-oxidising multi-enzyme system from *F. varansus*. *Biochim. Biophys. Acta* 828, 116-122.
- MAROULIS, P.J. & BANDY, A.R. (1976). Estimate of the contribution of biologically produced dimethyl sulfide to the global sulfur cycle. *Science* 196, 247-248.
- MARTIN, P.A.W., DUGAN, P.R. & TUOVINEN, O.H. (1981). Plasmid DNA in acidophilic, chemolithotrophic thiobacilli. *Can. J. Microbiol.* 27, 850-853.
- MASON, J. & KELLY, D.P. (1988). Mixotrophic and autotrophic growth of *Thiobacillus acidophilus* on tetrathionate. *Arch. Microbiol.* 149, 317-323.
- MASON, J., KELLY D.P. & WOOD, A.P. (1987). Chemolithotrophic and autotrophic growth of *Thermotrix thioopara* and some thiobacilli on thiosulphate and some polythionates, and a re-assessment of the growth yields of *Ths. thioopara* in chemostat culture. *J. Gen. Microbiol.* 133, 1249-1256.
- MAO, N.W.H., DUGAN, P.R., MARTIN, P.A.W. & TUOVINEN, O.H. (1980). Plasmid DNA in chemoorganotrophic *Thiobacillus ferrooxidans* and *T. acidophilus*. *FEMS Microbiol Letts.* 8, 121-125.

- McEWAN, A.G., WETZSTEIN, H.G., FERGUSON, S.J. & JACKSON, J.B. (1985).  
Periplasmic location of the terminal reductase in  
trimethylamine-N-oxide and dimethyl sulfoxide respiration in the  
photosynthetic bacterium *Rhodospseudomonas capsulata*.  
*Biochim. Biophys. Acta.* 806, 410-417.
- MERCK (1968). The Merck Index (Stecher, F.G. Ed.) 8th Edition. Rahway,  
Merck & Co.
- MERRICK-GASS, M.T. (1986). Analysis of trace sulfur gases in the  
atmosphere and in natural gas by gas chromatography using a flame  
photometric detector. Gas chromatography brief, Hewlett Packard.
- MONTICELLO, D.J. & FINNERTY, W.R. (1985). Microbial desulphurisation  
of fossil fuels. *Ann. Rev. Microbiol.* 39, 371-389.
- MOUBASHER, A.M., ELMAGHY, M.A. & ABDEL-HAFEZ, S.I. (1974). Effect of  
fumigation of three grains with formalin and carbon disulphide on  
the grain-borne fungi. *Bull. Fac. Sci., Assiut Univ.* 3, 13-27.
- MUNNECKE, D.E., DOMSCH, J.H. & ECKERT, J.W. (1962). Fungicidal activity  
of air passed through columns of soil treated with fungicides.  
*Phytopath.* 52, 1298-
- NASH, T. (1953). The colorimetric estimation of formaldehyde by means of  
the Hantzsch reaction. *Biochem. J.* 55, 416-421.
- NGUYEN, B.C., A., BONSANG, B. & LAMBERT, G. (1978).  
Re-evaluation of the role of dimethyl sulphide in the sulphur  
budget. *Nature* 275, 637-639.

- PATEL, H.N. & HOARE, D.S. (1971). Physiological studies of methane and methanol oxidising bacteria: oxidation of C-1 compounds by *Methyloccoccus capsulatus* J. Bacteriol. 107, 187-192.
- PIRT, S.J. (1982). Maintenance energy: a general model for energy-limited and energy sufficient growth. Arch. Microbiol. 133, 300-302.
- PIRT, S.J. (1975). Principles of microbes and cell cultivation. Blackwell Scientific Publications, Oxford, Edinburgh, Melbourne.
- PRZYJAZNY, A., JANICKI, W., CHRZANOWSKI, W. & STASZEWSKI, E. (1983). Headspace GC determination of distribution co-efficients of selected organosulphur compounds and their dependence on some parameters. J. Chromatogr. 280, 249-260.
- QUAYLE, J.R. & FERENCZI, T. (1978). Evolutionary aspects of autotrophy. Microbiol. Revs. 42, 251-275.
- RAJAGOPAL, B.S. & DANIELS, L. (1986). Investigation of mercaptans, organic sulfides, and inorganic sulfur compounds as sulfur sources for the growth of methanogenic bacteria. Curr. Microbiol. 14, 137-144.
- RASMUSSEN, R.A. (1974). Emission of biogenic hydrogen sulfide. Tellus 26, 254-260.
- ROBINSON, E. & ROBBINS, R.C. (1970). Gaseous sulfur pollutants from urban and natural sources. J. Air Pollut. Cont. Assoc. 20, 233-235.

- RODGERS, G.A., ASHWORTH, J. & WALKER, M. (1980). Recovery of nitrifier populations from inhibition by nitrapyrin or  $CS_2$ . Zbl. Bakt. II Abt. 135, 477-483.
- SAIGNE, C. & LEGRANDE, N. (1987). Measurement of methanesulphonic acid in Antarctic ice. Nature 330, 240-242.
- SALSBURY, R.L. & MERRICKS, D.L. (1975). Production of methanethiol and dimethyl sulphide by rumen microorganisms. Plant Soil 43, 191-209.
- SAND, W. (1987). Importance of hydrogen sulphide, thiosulphate and methyl mercaptan for growth of *Shibbolethia* during simulation of concrete corrosion. Appl. Env. Microbiol. 53, 1645-1648.
- SANDALLS, F.J. & PENKETT, S.A. (1977). Measurements of carbonyl sulphide and carbon disulphide in the atmosphere. Atmos. Environ. 11, 197-199.
- SECAL, W. & STARKEY, R.L. (1969). Microbial decomposition of methionine and identity of the resulting sulfur products. J. Bacteriol. 98, 908-913.
- SELYUZITSKII, G.B. (1972). Experimental data used to determine the maximum permissible concentration of methyl mercaptan, dimethyl sulphide and dimethyl disulphide in the air of the production area of paper and pulp plants. Gig. Tr. Prof. Zabol. 16, 46-47.

- SHARPE, N.E., LAM, B.A. & PHILLIPS, B.A. (1977). Methanethiol production by coryneform bacteria: Strains from dairy and human skin sources and *Brevibacterium linum*. *J. Gen. Microbiol.* 101, 345-349.
- SHERIDAN, R.P. & CASTENHOLZ, R.W. (1968). Production of hydrogen sulfide by thermophilic blue-green algae. *Nature* 217, 1063-1064.
- SINGER, A.G., AGOSTA, W.C., O'CONNELL, R.J., PFAFFMANN, C., BOWEN, D.V. & FIELD, F.H. (1975). Dimethyl disulphide: an attractant pheromone in hamster vaginal secretion. *Science* 191, 948-950.
- SIVELA, S.K. (1980). Dimethyl sulphide as a growth substrate for an obligately chemolithotrophic *Thiobacillus*. *Commentationes physico-mathematicae dissertationes, Helsinki* 1, 1-69.
- SIVELA, S.K. & SUNDMAN, V. (1975). Demonstration of *Thiobacillus*-type bacteria which utilize methyl sulphides. *Arch. Microbiol.* 103, 303-304.
- SLIWINSKI, R.A. & DOTY, D.M. (1958). Determination of micro-quantities of methyl mercaptan in gamma-irradiated meat. *J. Agr. Food Chem.* 6, 41-44.
- SLY, L.I., BLACKALL, L.L., KRAAT, P.C., TIAN-SHEN, T. & SANGHOBOL, V. (1986). The use of second derivative plots for the determination of mol % guanine plus cytosine of DNA by the thermal denaturation method. *Journal of Microbiological Methods* 5, 139-156.

- SMALE, B.C., LASATER, N.J. & MERTER, J.T. (1975). Fate and metabolism of DMSO in agricultural crops. *Am. N.Y. Acad. Sci.* 243, 228-236.
- SMITH, A.L., KELLY, D.P. & WOOD, A.P. (1980). Metabolism of *Thiobacillus* A2 grown under autotrophic, mixotrophic and heterotrophic conditions in chemostat culture. *J. Gen. Microbiol.* 121, 127-138.
- SMITH, M.A. & KELLY, D.P. (1988). Isolation and physiological characterisation of autotrophic sulphur bacteria oxidising dimethyl disulphide as sole source of energy. *J. Gen. Microbiol.* 134, 1407-1417.
- SPARMINS, V.L., BARANY, G. & MATTENBERG, L.W. (1988). Effects of organosulfur compounds from garlic and onions on benzo[a]pyrene-induced neoplasia and glutathione S-transferase activity in the mouse. *Carcinogenesis* 9, 131-134.
- STAFFORD, D.A. & CALLELY, A.G. (1969). The utilisation of thiocyanate by a heterotrophic bacterium. *J. Gen. Microbiol.* 55, 285-289.
- STAHL, D.A., LANE, D.J., OLSEN, G.J., MELLER, D.J., SCHMIDT, T.M. & PAGE, M.B. (1987). Phylogenic analysis of certain sulphide-oxidising and related morphologically conspicuous bacteria by 5S ribonucleic acid sequences. *Int. J. Syst. Bacteriol* 37, 116-122.
- STEDLER, P.A. & PETERSON, B.J. (1984). Contribution of gaseous sulphur from salt marshes to the global sulfur cycle. *Nature* 311, 455-457.

- STIRLING, B.I. & DALTON, H. (1978). Purification and properties of an NAD(P)<sup>+</sup>-linked formaldehyde dehydrogenase from *Methyloccoccus capsulatus* (Bath). *J. Gen. Microbiol.* 107, 19-29.
- STOIBER, G., LROGTT, D.C., KEMKINS, T.F., MURRMANN, R.P. & ROSE, W.I. (1971). Organic compounds in volcanic gas from Santiaguito volcano, Guatemala. *Bull. Geo. Soc. Amer.* 82, 2299-
- STOTZKY, G. & SCHENK, S. (1976). Volatile organic compounds and microorganisms. *CRC Crit. Rev. Microbiol.* 4, 333-371.
- STRICK, J.E., SCHINDLER, S.C., DAVID, M.B., MITCHELL, W.J. & NAKAS, J.P. (1982). Importance of organic sulfur constituents and microbial activity to sulfur transformations in an Adirondack forest soil. *North East Environ. Sci.* 1, 161-169.
- SUBLETTE, K.L. & SYLVESTER, N.D. (1987). Oxidation of hydrogen sulfide by *Thiobacillus denitrificans*: desulfurisation of natural gas. *Biotech. Bioeng.* 29, 249-257.
- SUYLEN, G.N.H. & KUENEN, J.G. (1986). Chemostat enrichment and isolation of *Hyphomicrobium* EG, a dimethyl sulfide oxidising methylotroph and re-evaluation of *Thiobacillus* MS1. *Antonie van Leeuwenhoek* 52, 281-293.
- SUYLEN, G.N.H., LARGE, P.J., VAN DIJKEN, J.P. & KUENEN, J.G. (1987). Methyl mercaptan oxidase, a key enzyme in the metabolism of methylated sulphur compounds by *Hyphomicrobium* EG. *J. Gen. Microbiol.* 133, 2989-2997.

- SUYLEN, G.M.H., STEFESS, G.C. & KUENEN, J.C. (1986). Chemolithotrophic potential of a *Hyphomicrobium* species capable of growth on methylated sulphur compounds. *Arch. Microbiol.* 146, 192-198.
- SURETHAM, P.M., TAYLOR, S.W.C. & ELMOOD, P.C. (1987). Exposure to carbon disulphide and ischaemic heart disease in a viscose rayon factory. *Br. J. Ind. Med.* 44, 220-227.
- TIMS, J., SHARNER, E., HARRE, M. & FLOG, L. (1975). Metabolism and excretion of dimethyl sulfoxide in cows and calves after topical and parenteral application. *Ann. N.Y. Acad. Sci.* 243, 139-150.
- TRABE-TORRE, A. (1981). Cell yield and biogenesis of *Thiospirillum denitrificans* compared with *Thiobacillus denitrificans*. *Antonie Van Leeuwenhoek* 47, 237-243.
- TOKITA, B., IMOU, H., CHAYA, K., MAKIURA, A., HANAMURA, H. & UENO, K. (1987). Identification of dimethyl disulfide-forming bacteria isolated from activated sludge. *Appl. Environ. Microbiol.* 53, 1541-1547.
- TRUDINGER, P.A. (1965). Effect of thiol-binding reagents on the metabolism of thiosulphate and tetrathionate by *Thiobacillus neapolitanus*. *J. Bacteriol.* 89, 617-625.
- TRUPER, H.G. & FISCHER, U. (1982). Anaerobic oxidation of sulphur compounds as electron donors for bacterial photosynthesis. *Phil. Trans. R. Soc. Lond.* B298, 529-542.
- TUOVINEN, O.H. & KELLY, D.P. (1973). Studies on the growth of *Thiobacillus ferrooxidans*. *Archiv für Mikrobiologie* 88, 285-298.

- UEBAYASI, M., KAMAJIURA, S., TOMIZUKA, H. & KAMIBAYASHI, A. (1985).  
Comparison of two key enzymes of *Hyphomicrobium* sp. 53-49 under  
various growth conditions: Aerobic, denitrifying and autotrophic.  
*Agric. Biol. Chem.* 59, 1799-1807.
- UEBAYASI, M., TOMIZUKA, H., KAMIBAYASHI, A. & TOMOMIURA, K. (1981).  
Autotrophic growth of a *Hyphomicrobium* sp. and its hydrogenase  
activity. *Agric. Biol. Chem.* 55, 1783-1790.
- ULITZUR, S. (1972). Rapid determination of DNA base composition by  
ultraviolet spectroscopy. *Biochim. Biophys. Acta* 272, 1-11.
- VAIRAVAMURTHY, A., ANDREAE, M.O. & IVERSON, B.L. (1985). Biosynthesis of  
dimethyl sulfide and dimethyl propiothetin by *Eymenomonas*  
*oertzenae* in relation to sulfur source and salinity variations.  
*Limnol. Oceanogr.* 30, 59-70.
- VAIRAVAMURTHY, A. & WOPPER, K. (1987). Geochemical formation of  
organosulphur compounds (thiols) by addition of  $H_2S$  to  
sedimentary organic matter. *Nature* 329, 623-625.
- van VERSEVELD, H.W., CHESBRO, W.R., BRASTER, N. & STOUTHAMER, A.M. (1984).  
Eubacteria have three growth modes keyed to nutrient flow:  
consequences for the concept of maintenance and maximal growth  
yield. *Arch. Microbiol.* 137, 176-184.
- WAKEMAN, S.G., HOWES, B.L. & DALEY, J.W.H. (1984). Dimethyl sulfide in a  
stratified coastal salt pond. *Nature* 310, 770-772.

- WHITFIELD, F.B., SHRA, S.R., WILLEMS, K.T. & SHAW, K.T. (1981). Volatile components from roots of *Acacia pulchella* R.Br. and their effect on *Phytophthora cinnamomi* Rands. Aust. J. Bot. 29, 195-208.
- WHITTENBURY, R. & KELLY, D.P. (1977). Autotrophy: A conceptual phoenix. In: Microbial Energetics (Haddock, B.A. & Hamilton, W.A., Eds.) 121-149. Soc. Gen. Microbiol. University Press, Cambridge.
- WOOD, A.P. & KELLY, D.P. (1980). Carbohydrate degradation pathways in *Thiobacillus* A2 grown on various sugars. J. Gen. Microbiol. 120, 333-345.
- WOOD, A.P. & KELLY, D.P. (1985). Physiological characteristics of a new thermophilic obligately chemolithotrophic *Thiobacillus* species, *T. tepidarius*. Int. J. Syst. Bacteriol. 35, 434-437.
- WOOD, A.P. & KELLY, D.P. (1986). Chemolithotrophic metabolism of the newly-isolated moderately thermophilic, obligately autotrophic *T. tepidarius*. Arch. Microbiol. 144, 71-77.
- WOOD, A.P. & KELLY, D.P. (1988). Isolation and physiological characterisation of *Thiobacillus aquasulfis* sp. nov. a novel facultatively autotrophic moderate thermophile. Arch. Microbiol. 149, 339-343.
- WOOLLEY, D., JONES, G.L. & MAPPOLD, F.C. (1962). Some metabolic differences between *Thiobacillus thioparvus*, *T. denitrificans* and *T. thiocyanatidans*. J. Gen. Microbiol. 29, 311-316.
- YAMADA, H., KUNAGAI, H. & OHKISHI, H. (1976). Process for producing L-cysteine or its derivatives. United States Patent, No. 3,974,031.

- YANOVER, S.B. & MAUMERKO, M.P. (1983). Effect of carbon disulphide emulsion on nitrifying soil capacity. *Mikrobiol. Zh.* 45, 21-23.
- YUJATT, J.B. (1954). Studies on the metabolism of *Thiobacillus thiooxyanoxidans*. *J. Gen. Microbiol.* 11, 139-149.
- ZEYER, J., KICHER, P., WAKEMAN, S.G. & SCHWARZENBACH, B.P. (1987). Oxidation of dimethyl sulfide to dimethyl sulfoxide by phototrophic purple bacteria. *Appl. Environ. Microbiol.* 53, 2026-2032.
- ZINDER, S.H. & BROCK, T.D. (1978 a). Dimethyl sulfoxide as an electron acceptor for anaerobic growth. *Arch. Microbiol.* 116, 35-40.
- ZINDER, S.H. & BROCK, T.D. (1978 b). Production of methane and carbon dioxide from methanethiol and dimethyl sulfide by anaerobic lake sediments. *Nature* 273, 226-228.
- ZINDER, S.H. & BROCK, T.D. (1978 c). Methane, carbon dioxide and hydrogen sulfide production from the terminal methyl group of methionine by anaerobic lake sediments. *Appl. Environ. Microbiol.* 35, 344-352.
- ZINDER, S.H., DOEMEL, W.M. & BROCK, T.D. (1977). Production of volatile sulfur compounds during the decomposition of algal mats. *Appl. Environ. Microbiol.* 34, 859-860.
- ZURREN, D., COOK, A.M. & LEISINGER, T. (1987). Microbial desulfonation of substituted naphthalenesulfonic acids and benzenesulfonic acids. *Appl. Environ. Microbiol.* 53, 1459-1463.

THE BRITISH LIBRARY DOCUMENT SUPPLY CENTRE

TITLE

Metabolism of Dimethyl Disulphide, Carbon Disulphide

and other volatile Sulphur Compounds by

Chemolithoautotrophic Sulphur Bacteria.

AUTHOR

Neil A. Smith

INSTITUTION  
and DATE

University of Warwick,

1985

Attention is drawn to the fact that the copyright of this thesis rests with its author.

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no information derived from it may be published without the author's prior written consent.

THE BRITISH LIBRARY  
DOCUMENT SUPPLY CENTRE

Boston Spa, Wetherby  
West Yorkshire  
United Kingdom



20

REDUCTION X

CAMERA 2.

D92021