# OXIDATION OF INORGANIC SULFUR COMPOUNDS

BY

*THIOBACILLUS THIOOXIDANS*

BY

CHIU-WING PETER CHAN

A Thesis
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Architecture ..... 0729
Art History ..... 0377
Cinema ..... 0900
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Fine Arts ..... 0357
Information Science ..... 0723
Journalism ..... 0391
Library Science ..... 0399
Mass Communications ..... 0708
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Speech Communication ..... 0459
Theater ..... 0465

### EDUCATION
General ..... 0515
Administration ..... 0514
Adult and Continuing ..... 0516
Agricultural ..... 0517
Art ..... 0273
Bilingual and Multicultural ..... 0282
Business ..... 0688
Community College ..... 0275
Curriculum and Instruction ..... 0727
Early Childhood ..... 0518
Elementary ..... 0524
Finance ..... 0277
Guidance and Counseling ..... 0519
Health ..... 0680
Higher ..... 0745
History of ..... 0520
Home Economics ..... 0278
Industrial ..... 0521
Language and Literature ..... 0279
Mathematics ..... 0280
Music ..... 0522
Philosophy of ..... 0998
Physical ..... 0523

Psychology ..... 0525
Reading ..... 0535
Religious ..... 0527
Sciences ..... 0714
Secondary ..... 0533
Social Sciences ..... 0534
Sociology of ..... 0340
Special ..... 0529
Teacher Training ..... 0530
Technology ..... 0710
Tests and Measurements ..... 0288
Vocational ..... 0747

### LANGUAGE, LITERATURE AND LINGUISTICS
Language
- General ..... 0679
- Ancient ..... 0289
- Linguistics ..... 0290
- Modern ..... 0291

Literature
- General ..... 0401
- Classical ..... 0294
- Comparative ..... 0295
- Medieval ..... 0297
- Modern ..... 0298
- African ..... 0316
- American ..... 0591
- Asian ..... 0305
- Canadian (English) ..... 0352
- Canadian (French) ..... 0355
- English ..... 0593
- Germanic ..... 0311
- Latin American ..... 0312
- Middle Eastern ..... 0315
- Romance ..... 0313
- Slavic and East European ..... 0314

### PHILOSOPHY, RELIGION AND THEOLOGY
Philosophy ..... 0422
Religion
- General ..... 0318
- Biblical Studies ..... 0321
- Clergy ..... 0319
- History of ..... 0320
- Philosophy of ..... 0322

Theology ..... 0469

### SOCIAL SCIENCES
American Studies ..... 0323
Anthropology
- Archaeology ..... 0324
- Cultural ..... 0326
- Physical ..... 0327

Business Administration
- General ..... 0310
- Accounting ..... 0272
- Banking ..... 0770
- Management ..... 0454
- Marketing ..... 0338

Canadian Studies ..... 0385
Economics
- General ..... 0501
- Agricultural ..... 0503
- Commerce-Business ..... 0505
- Finance ..... 0508
- History ..... 0509
- Labor ..... 0510
- Theory ..... 0511

Folklore ..... 0358
Geography ..... 0366
Gerontology ..... 0351
History
- General ..... 0578
- Ancient ..... 0579
- Medieval ..... 0581
- Modern ..... 0582
- Black ..... 0328
- African ..... 0331
- Asia, Australia and Oceania ..... 0332
- Canadian ..... 0334
- European ..... 0335
- Latin American ..... 0336
- Middle Eastern ..... 0333
- United States ..... 0337

History of Science ..... 0585
Law ..... 0398
Political Science
- General ..... 0615
- International Law and Relations ..... 0616
- Public Administration ..... 0617

Recreation ..... 0814
Social Work ..... 0452
Sociology
- General ..... 0626
- Criminology and Penology ..... 0627
- Demography ..... 0938
- Ethnic and Racial Studies ..... 0631
- Individual and Family Studies ..... 0628
- Industrial and Labor Relations ..... 0629
- Public and Social Welfare ..... 0630
- Social Structure and Development ..... 0700
- Theory and Methods ..... 0344

Transportation ..... 0709
Urban and Regional Planning ..... 0999
Women's Studies ..... 0453

# THE SCIENCES AND ENGINEERING

### BIOLOGICAL SCIENCES
Agriculture
- General ..... 0473
- Agronomy ..... 0285
- Animal Culture and Nutrition ..... 0475
- Animal Pathology ..... 0476
- Food Science and Technology ..... 0359
- Forestry and Wildlife ..... 0478
- Plant Culture ..... 0479
- Plant Pathology ..... 0480
- Plant Physiology ..... 0817
- Range Management ..... 0777
- Wood Technology ..... 0746

Biology
- General ..... 0306
- Anatomy ..... 0287
- Biostatistics ..... 0308
- Botany ..... 0309
- Cell ..... 0379
- Ecology ..... 0329
- Entomology ..... 0353
- Genetics ..... 0369
- Limnology ..... 0793
- Microbiology ..... 0410
- Molecular ..... 0307
- Neuroscience ..... 0317
- Oceanography ..... 0416
- Physiology ..... 0433
- Radiation ..... 0821
- Veterinary Science ..... 0778
- Zoology ..... 0472

Biophysics
- General ..... 0786
- Medical ..... 0760

### EARTH SCIENCES
Biogeochemistry ..... 0425
Geochemistry ..... 0996

Geodesy ..... 0370
Geology ..... 0372
Geophysics ..... 0373
Hydrology ..... 0388
Mineralogy ..... 0411
Paleobotany ..... 0345
Paleoecology ..... 0426
Paleontology ..... 0418
Paleozoology ..... 0985
Palynology ..... 0427
Physical Geography ..... 0368
Physical Oceanography ..... 0415

### HEALTH AND ENVIRONMENTAL SCIENCES
Environmental Sciences ..... 0768
Health Sciences
- General ..... 0566
- Audiology ..... 0300
- Chemotherapy ..... 0992
- Dentistry ..... 0567
- Education ..... 0350
- Hospital Management ..... 0769
- Human Development ..... 0758
- Immunology ..... 0982
- Medicine and Surgery ..... 0564
- Mental Health ..... 0347
- Nursing ..... 0569
- Nutrition ..... 0570
- Obstetrics and Gynecology ..... 0380
- Occupational Health and Therapy ..... 0354
- Ophthalmology ..... 0381
- Pathology ..... 0571
- Pharmacology ..... 0419
- Pharmacy ..... 0572
- Physical Therapy ..... 0382
- Public Health ..... 0573
- Radiology ..... 0574
- Recreation ..... 0575
- Speech Pathology ..... 0460
- Toxicology ..... 0383

Home Economics ..... 0386

### PHYSICAL SCIENCES
**Pure Sciences**
Chemistry
- General ..... 0485
- Agricultural ..... 0749
- Analytical ..... 0486
- Biochemistry ..... 0487
- Inorganic ..... 0488
- Nuclear ..... 0738
- Organic ..... 0490
- Pharmaceutical ..... 0491
- Physical ..... 0494
- Polymer ..... 0495
- Radiation ..... 0754

Mathematics ..... 0405
Physics
- General ..... 0605
- Acoustics ..... 0986
- Astronomy and Astrophysics ..... 0606
- Atmospheric Science ..... 0608
- Atomic ..... 0748
- Electronics and Electricity ..... 0607
- Elementary Particles and High Energy ..... 0798
- Fluid and Plasma ..... 0759
- Molecular ..... 0609
- Nuclear ..... 0610
- Optics ..... 0752
- Radiation ..... 0756
- Solid State ..... 0611

Statistics ..... 0463

**Applied Sciences**
Applied Mechanics ..... 0346
Computer Science ..... 0984

Engineering
- General ..... 0537
- Aerospace ..... 0538
- Agricultural ..... 0539
- Automotive ..... 0540
- Biomedical ..... 0541
- Chemical ..... 0542
- Civil ..... 0543
- Electronics and Electrical ..... 0544
- Heat and Thermodynamics ..... 0348
- Hydraulic ..... 0545
- Industrial ..... 0546
- Marine ..... 0547
- Materials Science ..... 0794
- Mechanical ..... 0548
- Metallurgy ..... 0743
- Mining ..... 0551
- Nuclear ..... 0552
- Packaging ..... 0549
- Petroleum ..... 0765
- Sanitary and Municipal ..... 0554
- System Science ..... 0790

Geotechnology ..... 0428
Operations Research ..... 0796
Plastics Technology ..... 0795
Textile Technology ..... 0994

### PSYCHOLOGY
General ..... 0621
Behavioral ..... 0384
Clinical ..... 0622
Developmental ..... 0620
Experimental ..... 0623
Industrial ..... 0624
Personality ..... 0625
Physiological ..... 0989
Psychobiology ..... 0349
Psychometrics ..... 0632
Social ..... 0451

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### COMMUNICATIONS ET LES ARTS
Architecture ..... 0729
Beaux-arts ..... 0357
Bibliothéconomie ..... 0399
Cinéma ..... 0900
Communication verbale ..... 0459
Communications ..... 0708
Danse ..... 0378
Histoire de l'art ..... 0377
Journalisme ..... 0391
Musique ..... 0413
Sciences de l'information ..... 0723
Théâtre ..... 0465

### ÉDUCATION
Généralités ..... 515
Administration ..... 0514
Art ..... 0273
Collèges communautaires ..... 0275
Commerce ..... 0688
Économie domestique ..... 0278
Éducation permanente ..... 0516
Éducation préscolaire ..... 0518
Éducation sanitaire ..... 0680
Enseignement agricole ..... 0517
Enseignement bilingue et multiculturel ..... 0282
Enseignement industriel ..... 0521
Enseignement primaire. ..... 0524
Enseignement professionnel ..... 0747
Enseignement religieux ..... 0527
Enseignement secondaire ..... 0533
Enseignement spécial ..... 0529
Enseignement supérieur ..... 0745
Évaluation ..... 0288
Finances ..... 0277
Formation des enseignants ..... 0530
Histoire de l'éducation ..... 0520
Langues et littérature ..... 0279
Lecture ..... 0535
Mathématiques ..... 0280
Musique ..... 0522
Orientation et consultation ..... 0519
Philosophie de l'éducation ..... 0998
Physique ..... 0523
Programmes d'études et enseignement ..... 0727
Psychologie ..... 0525
Sciences ..... 0714
Sciences sociales ..... 0534
Sociologie de l'éducation ..... 0340
Technologie ..... 0710

### LANGUE, LITTÉRATURE ET LINGUISTIQUE
Langues
- Généralités ..... 0679
- Anciennes ..... 0289
- Linguistique ..... 0290
- Modernes ..... 0291

Littérature
- Généralités ..... 0401
- Anciennes ..... 0294
- Comparée ..... 0295
- Médiévale ..... 0297
- Moderne ..... 0298
- Africaine ..... 0316
- Américaine ..... 0591
- Anglaise ..... 0593
- Asiatique ..... 0305
- Canadienne (Anglaise) ..... 0352
- Canadienne (Française) ..... 0355
- Germanique ..... 0311
- Latino-américaine ..... 0312
- Moyen-orientale ..... 0315
- Romane ..... 0313
- Slave et est-européenne ..... 0314

### PHILOSOPHIE, RELIGION ET THÉOLOGIE
Philosophie ..... 0422
Religion
- Généralités ..... 0318
- Clergé ..... 0319
- Études bibliques ..... 0321
- Histoire des religions ..... 0320
- Philosophie de la religion ..... 0322

Théologie ..... 0469

### SCIENCES SOCIALES
Anthropologie
- Archéologie ..... 0324
- Culturelle ..... 0326
- Physique ..... 0327

Droit ..... 0398
Économie
- Généralités ..... 0501
- Commerce-Affaires ..... 0505
- Économie agricole ..... 0503
- Économie du travail ..... 0510
- Finances ..... 0508
- Histoire ..... 0509
- Théorie ..... 0511

Études américaines ..... 0323
Études canadiennes ..... 0385
Études féministes ..... 0453
Folklore ..... 0358
Géographie ..... 0366
Gérontologie ..... 0351
Gestion des affaires
- Généralités ..... 0310
- Administration ..... 0454
- Banques ..... 0770
- Comptabilité ..... 0272
- Marketing ..... 0338

Histoire
- Histoire générale ..... 0578
- Ancienne ..... 0579
- Médiévale ..... 0581
- Moderne ..... 0582
- Histoire des noirs ..... 0328
- Africaine ..... 0331
- Canadienne ..... 0334
- États-Unis ..... 0337
- Européenne ..... 0335
- Moyen-orientale ..... 0333
- Latino-américaine ..... 0336
- Asie, Australie et Océanie ..... 0332

Histoire des sciences ..... 0585
Loisirs ..... 0814
Planification urbaine et régionale ..... 0999
Science politique
- Généralités ..... 0615
- Administration publique ..... 0617
- Droit et relations internationales ..... 0616

Sociologie
- Généralités ..... 0626
- Aide et bien-être social ..... 0630
- Criminologie et établissements pénitentiaires ..... 0627
- Démographie ..... 0938
- Études de l' individu et de la famille ..... 0628
- Études des relations interethniques et des relations raciales ..... 0631
- Structure et développement social ..... 0700
- Théorie et méthodes. ..... 0344
- Travail et relations industrielles ..... 0629

Transports ..... 0709
Travail social ..... 0452

# SCIENCES ET INGÉNIERIE

### SCIENCES BIOLOGIQUES
Agriculture
- Généralités ..... 0473
- Agronomie. ..... 0285
- Alimentation et technologie alimentaire ..... 0359
- Culture ..... 0479
- Élevage et alimentation ..... 0475
- Exploitation des pâturages ..... 0777
- Pathologie animale ..... 0476
- Pathologie végétale ..... 0480
- Physiologie végétale ..... 0817
- Sylviculture et faune ..... 0478
- Technologie du bois ..... 0746

Biologie
- Généralités ..... 0306
- Anatomie ..... 0287
- Biologie (Statistiques) ..... 0308
- Biologie moléculaire ..... 0307
- Botanique ..... 0309
- Cellule ..... 0379
- Écologie ..... 0329
- Entomologie ..... 0353
- Génétique ..... 0369
- Limnologie ..... 0793
- Microbiologie ..... 0410
- Neurologie ..... 0317
- Océanographie ..... 0416
- Physiologie ..... 0433
- Radiation ..... 0821
- Science vétérinaire ..... 0778
- Zoologie ..... 0472

Biophysique
- Généralités ..... 0786
- Medicale ..... 0760

### SCIENCES DE LA TERRE
Biogéochimie ..... 0425
Géochimie ..... 0996
Géodésie ..... 0370
Géographie physique ..... 0368
Géologie ..... 0372
Géophysique ..... 0373
Hydrologie ..... 0388
Minéralogie ..... 0411
Océanographie physique ..... 0415
Paléobotanique ..... 0345
Paléoécologie ..... 0426
Paléontologie ..... 0418
Paléozoologie ..... 0985
Palynologie ..... 0427

### SCIENCES DE LA SANTÉ ET DE L'ENVIRONNEMENT
Économie domestique ..... 0386
Sciences de l'environnement ..... 0768
Sciences de la santé
- Généralités ..... 0566
- Administration des hôpitaux ..... 0769
- Alimentation et nutrition ..... 0570
- Audiologie ..... 0300
- Chimiothérapie ..... 0992
- Dentisterie ..... 0567
- Développement humain ..... 0758
- Enseignement ..... 0350
- Immunologie ..... 0982
- Loisirs ..... 0575
- Médecine du travail et thérapie ..... 0354
- Médecine et chirurgie ..... 0564
- Obstétrique et gynécologie ..... 0380
- Ophtalmologie ..... 0381
- Orthophonie ..... 0460
- Pathologie ..... 0571
- Pharmacie ..... 0572
- Pharmacologie ..... 0419
- Physiothérapie ..... 0382
- Radiologie ..... 0574
- Santé mentale ..... 0347
- Santé publique ..... 0573
- Soins infirmiers ..... 0569
- Toxicologie ..... 0383

### SCIENCES PHYSIQUES
#### Sciences Pures
Chimie
- Généralités ..... 0485
- Biochimie ..... 487
- Chimie agricole ..... 0749
- Chimie analytique ..... 0486
- Chimie minérale ..... 0488
- Chimie nucléaire ..... 0738
- Chimie organique ..... 0490
- Chimie pharmaceutique ..... 0491
- Physique ..... 0494
- PolymÈres ..... 0495
- Radiation ..... 0754

Mathématiques ..... 0405
Physique
- Généralités ..... 0605
- Acoustique ..... 0986
- Astronomie et astrophysique ..... 0606
- Électronique et électricité ..... 0607
- Fluides et plasma ..... 0759
- Météorologie ..... 0608
- Optique ..... 0752
- Particules (Physique nucléaire) ..... 0798
- Physique atomique ..... 0748
- Physique de l'état solide ..... 0611
- Physique moléculaire ..... 0609
- Physique nucléaire ..... 0610
- Radiation ..... 0756

Statistiques ..... 0463

#### Sciences Appliqués Et Technologie
Informatique ..... 0984
Ingénierie
- Généralités ..... 0537
- Agricole ..... 0539
- Automobile ..... 0540
- Biomédicale ..... 0541
- Chaleur et thermodynamique ..... 0348
- Conditionnement (Emballage) ..... 0549
- Génie aérospatial ..... 0538
- Génie chimique ..... 0542
- Génie civil ..... 0543
- Génie électronique et électrique ..... 0544
- Génie industriel ..... 0546
- Génie mécanique ..... 0548
- Génie nucléaire ..... 0552
- Ingénierie des systèmes ..... 0790
- Mécanique navale ..... 0547
- Métallurgie ..... 0743
- Science des matériaux ..... 0794
- Technique du pétrole ..... 0765
- Technique minière ..... 0551
- Techniques sanitaires et municipales ..... 0554
- Technologie hydraulique ..... 0545

Mécanique appliquée ..... 0346
Géotechnologie ..... 0428
Matières plastiques (Technologie) ..... 0795
Recherche opérationnelle ..... 0796
Textiles et tissus (Technologie) ..... 0794

### PSYCHOLOGIE
Généralités ..... 0621
Personnalité ..... 0625
Psychobiologie ..... 0349
Psychologie clinique ..... 0622
Psychologie du comportement ..... 0384
Psychologie du développement ..... 0620
Psychologie expérimentale ..... 0623
Psychologie industrielle ..... 0624
Psychologie physiologique ..... 0989
Psychologie sociale ..... 0451
Psychométrie ..... 0632

# OXIDATION OF INORGANIC SULFUR COMPOUNDS BY *THIOBACILLUS THIOOXIDANS*

BY

CHIU-WING PETER CHAN

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

TABLE OF CONTENTS

*ABSTRACT*

The oxidation of elemental sulfur by *Thiobacillus thiooxidans* was studied with and without inhibitors, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and N-ethylmaleimide (NEM), for stoichiometry of substrate, total amount of oxygen consumed and oxidation intermediates. Sulfur-oxidizing enzyme was inhibited by NEM. The inhibition by NEM was time-dependent. Elemental sulfur was stoichiometrically oxidized to sulfite by *T. thiooxidans* in the presence of HQNO at wide range of pH from 2.5 to 9.0 with equimolar oxygen consumption. At an acidic pH, sulfur oxidation was inhibited by accumulating sulfite. The amount of sulfite accumulated at pH 6.5 was proportional to the amount of cells. When the cells were oxidizing sulfur in the presence of reduced glutathione (GSH) or sulfite, there was thiosulfate formation at a pH near or above neutrality. Cell-free extracts oxidized sulfur in the presence of GSH producing both sulfite and thiosulfate.

A sensitive and quantitative analytical method for determinating elemental sulfur in a biological system was developed. Elemental sulfur was determined after extraction with petroleum ether by cyanolysis and ferric thiocyanate color formation in acetone. The method was successfully applied to show that sulfide was oxidized by *T. thiooxidans* to elemental sulfur nearly stoichiometrically when further oxidation of elemental sulfur was inhibited by N-ethylmaleimide. Kinetic studies of sulfide oxidation were also investigated.

Oxidation of thiosulfate by *T. thiooxidans* grown on sulfur was studied in intact cells and cell-free extracts. Thiosulfate was oxidized to tetrathionate by cells treated with

N-ethylmaleimide (NEM) at a pH optimum of 2.3. Cell-free extracts also oxidized thiosulfate at the same pH optimum. Tetrathionate oxidation by *T. thiooxidans* was completely inhibited by NEM. The pH optimum of the tetrathionate oxidation was 3.0. Both thiosulfate and tetrathionate were oxidized to sulfite level in the presence of HQNO. The cells with or without NEM-treatment showed two $K_m$ for thiosulfate while the cell-free system showed only one $K_m$. The $K_m$ for thiosulfate generally increased with increasing pH. A soluble thiosulfate-oxidizing enzyme system with an optimum pH around 2 was extracted from intact cells at pH 2.5 in the presence of 1 M ammonium sulfate by passage through a French pressure cell. A native cytochrome c which was reduced by thiosulfate at pH 2.5, and a compound which reduced horse heart cytochrome c at a neutral pH but required sulfite at an acidic pH were also found in this acidic extracts.

*ACKNOWLEDGEMENTS*

The author gives his utmost thanks to God for His provision and guidance over the past five years. From 1989 to 1992, the author went through the valley in his research studies conscientiously trying to purify the sulfur-oxidizing enzyme from *T. thiooxidans* into the wee hours of the morning but all in vain. During this dark period, the author has been fortunate to have had the prayers, support and encouragement from family and many friends. The author's parents and brother gave a lot of practical and personal support, making numerous long-distance telephone calls from Hong Kong and Japan to say: "Hey, Peter, don't give up! You can do it!" These few words and their beliefs in his abilities gave the author strength and courage to keep going and move forward.

The author also thanks his best friend, Joannes Wong, who spent a lot of time having coffee with him at the campus cafeteria till midnight. The "Greenhouse" was their favorite meeting place where the author shared many of his frustrations, struggles and tears. Their interaction also helped the author to maintain an awareness of ordinary life outside the laboratory. The author would like to take this opportunity to acknowledge Dr. Philip & Mrs. Emily Ng. Not only were they spiritual mentors of the author, but they also provided an educated and friendly ear on many occasions whether at Church, in the warmth of their home, or over the phone, and taught the author about the meaning of life and how to handle depression and stress in his research studies.

The research did not have much progress until the early summer of 1992. The author found that intact cells of *T. thiooxidans* behave somewhat differently after being

washed with distilled water. One of his colleague, Mr. Travis Takeuchi, named the author's growing cell culture - "Sick Cells". Because of these "sick cells", the author was able to develop a good model system using NEM-treated cells to investigate the thiosulfate oxidative pathway, and finally to solubilize the thiosulfate-oxidizing enzyme system from the intact cells. God does indeed answer prayers according to His time. The author thanks all the members of the Winnipeg Chinese Alliance Church for their prayers and concerns, and appreciates Dr. Clement Yeung for his sense of humor. The author also thanks his wife, Ashley for her help and encouragment. In fact, this project would not be finished without her constructive criticism and insights. Ashley is a unique source of ideas and advice.

Last but not least, the author wishes to express his gratitude to his supervisor, Dr. Isamu Suzuki for his expert advice, concerns and financial support during the course of this project. In addition the author thanks all his fellow students and all advisory committee members (Dr. Ronald Lyric; Dr. Peter Maeba and Dr. Jan Oleszkiewicz) for their encouragement, interest and friendship.

To

My Mom (SHEUNG WONG) and Dad (YIU-FAI CHAN)

for their support and encouragement

from

'Kindergarten Text Books'

to

'Ph.D Dissertation'

*LIST OF TABLES*

**PART ONE: SULFUR OXIDATION**



**PART TWO: SULFIDE OXIDATION**

## PART THREE: THIOSULFATE OXIDATION

*LIST OF FIGURES*

**PART ONE: SULFUR OXIDATION**



**PART TWO: SULFIDE OXIDATION**



**PART THREE: THIOSULFATE OXIDATION**

## *LIST OF ABBREVIATIONS*

| | |
|---|---|
| cyt | -cytochrome |
| Fig | -figure |
| GSH | -reduced glutathione |
| hr | -hour |
| $K_m$ | -the Michaelis constant as the substrate concentration at which the velocity is half of the maximum velocity |
| $K_i$ | -the Michaelis constant as the inhibitor concentration at which the velocity is reduced to half of the maximum velocity by the inhibitor |
| $\mu$mol | -micromole |
| $\mu$m | -micrometer |
| nmol | -nanomole |
| mM | -millimolar |
| mL | -millilitre |
| min | -minute |
| $V_{max}$ | -the maximum velocity with the enzyme concentration and the conditions used |
| Vol | -volume |

## *PREFACE*

Chapters in this dissertation are written as papers for submission to scientific journals. Parts one and two have been published (Chan and Suzuki 1993. Can. J. Microbiol. 39: 1166-1168 and Suzuki et al 1993. *In:* Biohydrometallurgy Technologies. *Edited* by Torma, A.E. J.E. Wey and V. L. Lakshmanan. The Minerals, Metals & Materials Society, Warrendale, Pa. Vol. 1, pp.109-116). The data described in part three has been accepted for publication (Chan and Suzuki 1994. Thiosulfate oxidation by sulfur-grown *Thiobacillus thiooxidans* cells, cell-free extracts and thiosulfate-oxidizing enzyme. Canadian Journal of Microbiology.)

## *GENERAL INTRODUCTION*

Thiobacilli are chemoautrophic microorganisms which are capable of oxidizing reduced inorganic sulfur compounds such as sulfur, sulfide and thiosulfate to sulfate. Energy derived from the oxidation of reduced sulfur compounds can be used for assimilating atmospheric carbon dioxide so as to synthesize cellular materials. Thiobacilli are further classified as obligate, facultative and mixotrophic depending upon the extent of utilization of organic compounds as a source of nutrition and energy. In addition, thiobacilli are also simply divided into two different major groups, acidophilic and neutrophilic thiobacilli, according to their growth pH.

*T. thiooxidans* belongs to the group of acidophilic thiobacilli. This bacterium is an obligate chemoautotroph which can derive its energy through the oxidation of elemental sulfur to sulfuric acid. In spite of the apparent simplicity of the nutritional requirements of *T. thiooxidans*, the organism is known to be very complex and sophisticated physiologically and biochemically. In addition, *T. thiooxidans* is one of the well-known slow growing bacteria with low cell yields, with a generation time of about 8 hr and a cell yield of 400 mg/L after 7 days (Suzuki *et al.* 1989). Although *T. thiooxidans* has been extensively studied and characterized over the last three decades, there is still no clear understanding on the oxidative mechanism of inorganic sulfur compounds: elemental sulfur, sulfide, thiosulfate and tetrathionate. Earlier investigations with intact cells, cell-free extracts or purified individual enzymes have been able to elucidate only basic outlines of the mechanism of inorganic sulfur compound oxidation. Most recent studies on the oxidation of sulfur compounds have been focused on other

closely related species, *T. ferrooxidans* and *T. acidophilus*. It is probable that *T. ferrooxidans* is the principal organism involved in microbial metal leaching in industrial applications. The cell yield of *T. acidophilus*, a facultative acidophile, is much higher than *T. thiooxidans*, an important factor for enzyme purification. Despite its low cell yield and slow growth, *T. thiooxidans* is the best obligate acidophile model to study the metabolism of different reduced inorganic sulfur compounds. The organism can derive energy for growth only through the oxidation of these compounds. *T. ferrooxidans* can use ferrous ions and *T. acidophilus* can use organic compounds. It is known that *T. thiooxidans* is one of the key microorganisms in the cause of concrete corrosion and acid mine drainage problems. These are important issues in environmental research today.

The objective of this dissertation has been an attempt to dissect the oxidative pathways of the metabolism of elemental sulfur, sulfide, thiosulfate and tetrathionate by measuring the oxidative intermediates, stoichiometry of substrates and total oxygen consumption in the acidophilic sulfur oxidizer, *T. thiooxidans*, using two different kinds of metabolic inhibitors. They are N-ethylmaleimide (NEM) and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO). NEM is a sulfhydryl-binding inhibitor which specifically binds to the sulfhydryl groups of the enzyme protein thus inhibiting the oxidation of sulfur. Sulfite oxidation is inhibited by HQNO, an inhibitor of cyt b-$c_1$ complex in the electron transport system. Finally, a hypothetical model of elemental sulfur, sulfide, thiosulfate and tetrathionate oxidation is proposed.

## *REVIEW Of LITERATURE*

### Historical background of the genus *Thiobacillus*

Winogradsky established the fundamental principle of chemolithotrophic autotrophy in bacteria when he published reports on sulfur-oxidizing bacteria in 1888. Chemoautotrophs, by his definition, were organisms capable of synthesizing all their organic compounds needed for growth without the aid of sunlight. They could derive all their energy from the oxidation of reduced or incompletely oxidized inorganic compounds. Atmospheric carbon dioxide alone would be used as the carbon source, and organic compounds would be utilized partly if at all. There has been much progress in solving the mysteries of autotrophy since Winogradsky discovered this phenomenon. Subsequent work by Nathansohn on the *Beggiatoa* species led to the discovery and naming of the genus *Thiobacillus* in 1904 by Beijerinck (Paine 1987). The first sulfur-oxidizing autotrophic bacterium studied by Nathansonhn and Beijernick was named *Thiobacillus thioparus* in accordance with its most characteristic property of forming a pellicle of elemental sulfur (thium=sulfur, para=to produce). *T. thioparus* was the type species of the *Thiobacillus*. The bacteria in this family were characterized on their ability to derive their energy from the oxidation of elemental sulfur and inorganic sulfur compounds. The individual members of this family were distinguished from each other on the basis of their obligation to use carbon dioxide as the sole carbon source (obligate or facultative autotroph), their utilization of atmospheric oxygen as the terminal electron acceptor, their tolerance to pH, and the variety of their inorganic energy sources. The members of the genus *Thiobacillus* were small (0.5 by 1.3 $\mu$m), Gram-negative,

generally motile, non-sporulating rod-shape bacteria which inhabited a wide variety of marine, freshwater and terrestrial environments. All were capable of autotrophic growth and most of them oxidized elemental sulfur, sulfide ,thiosulfate and tetrathionate to sulfate.

**Discovery and physiology of *Thiobacillus thiooxidans***

The first isolation of *T. thiooxidans* in pure culture and study of its morphology was carried out by Waksman and Joffe in 1922. This bacterium was found to be an obligate chemoautroph deriving its energy and reducing power for growth from the oxidation of elemental sulfur and synthesizing all the cellular carbon by assimilation of atmospheric carbon dioxide. Starkey (1925) further studied and characterized this bacterium in more detail. He also developed different growth media which are still used today. One of the growth media, Starkey medium 1, was used in this entire research project.

Classification of thiobacilli into species is based on physiological properties, different DNA nucleotide composition, and fatty acids composition of the cell wall. *T. thiooxidans* is classified to the group named acidophilic thiobacilli. Among the group members, *Thiobacillus acidophilus* and *Thiobacillus ferrooxidans* belong to this group. *T. ferrooxidans* is a well-documented species which is important in microbial metal leaching. The difference between *T. ferrooxidans* and *T. thiooxidans* is that the former one can derive energy from the oxidation of ferrous ion while *T. acidophilus* is a facultative acidophilic sulfur oxidizer. Morever *T. thiooxidans* is unique in its ability to

withstand extremely acidic conditions at a pH less than 1.0. The bacterium is a small rod-shaped cell, Gram-negative, motile by means of a single polar flagellum, non-spore-forming and a strict aerobe. It lives freely in the soil or in water. The optimum pH range for growth is from 2.0 to 3.5 and optimum temperature range for growth is around 28 to 30 °C. Of all the thiobacilli studied to date, *T. thiooxidans* has the lowest recorded DNA G+C content: 52.0-53.0 mole% (Karavaiko 1988). *T. ferrooxidans* and *T. acidophilus* have higher values, 55.0-57.4 and 62.9-63.2 mole% respectively. On the basis of this, as well as its membrane lipid composition, it has been classified as a class III thiobacillus. To date, *T. thiooxidans* is the only organism to occupy this class (Vishniac 1974).

**Oxidation of elemental sulfur, sulfide, thiosulfate and tetrathionate oxidation by *T. thiooxidans***

One of the requirements for a bacterial species to be included in the genus *Thiobacillus* is its ability of obtaining energy from the oxidation of **elemental sulfur** and other reduced inorganic sulfur compounds. Although the exact pathway for the oxidation of reduced sulfur compounds by these organisms remains in doubt and different species of thiobacilli have different pathways, a generalized scheme for the oxidation of different inorganic sulfur compounds was described in a review article written by Suzuki *et al.* (1994) where the oxidation sequence ($S \rightarrow SO_3^{2-} \rightarrow SO_4^{2-}$) forms the main pathway. Most of the enzymes mentioned in the scheme have been either isolated or identified in *T. thiooxidans* (Bhella 1981; Lizama 1986). A similar sulfur oxidation pathway with a

requirement for $Fe^{3+}$ had been proposed by a group of Japanese researchers (Sugio *et al.* 1975). Under anaerobic conditions, *T. ferrooxidans* mediated sulfur oxidation with ferric ion as the electron acceptor. This novel mechanism involved ferric ion-reducing and ferrous ion-oxidizing components. Two new enzymes, sulfur:ferric ion oxidoreductase and sulfite:ferric ion oxidoreductase, had been isolated from iron-grown *T. ferrooxidans* (Sugio *et al.* 1988). Both had a pH optimum of 6 and were localized in the periplasmic space of the cells.

Elemental sulfur most commonly exists as a circular octet and is cleaved by nucleophilic agents, which may be a sulfhydryl-containing organic compound or a sulfhydryl group on the surface of the cell (Suzuki and Silver 1966). This results in a bound linear polysulfide of which the terminal sulfur atom can be oxidized by the sulfur-oxidizing enzyme (Suzuki 1965a and 1965b; Suzuki and Silver 1966; Silver and Lungdren 1968). The sulfite moiety thus formed can be hydrolyzed from the rest of the molecule, enabling the next sulfur atom to be oxygenated. Sulfite is shown to be the initial end product of this oxygenation reaction through being trapped with formaldehyde (Suzuki and Silver 1966; Silver and Lundgren 1968) or by an active sulfite-oxidizing system (Charles and Suzuki 1966a and 1966b). Despite the presence of the sulfur and sulfite-oxidizing systems in *T. thiooxidans* and intensive investigations into the many aspects of sulfur metabolism in this organism over the past thirty years, the oxidative pathway of elemental sulfur in this organism is still highly controversial. This controversy is probably due to the reactive nature of the inorganic sulfur intermediates which makes the direct demonstration of a partial oxidation reaction difficult.

*T. thiooxidans* is normally found in sulfur and sulfide deposits, acid waters and springs (Pivovarova and Golovacheva 1985). Earlier studies of sulfide oxidation in *T. thiooxidans* with both intact cells and crude extracts led to the hypothesis that elemental sulfur, thiosulfate and polythionates were the intermediate products during oxidation of sulfide to sulfate (Parker and Prisk 1953; Vishniac and Santer 1957; and London and Rittenberg 1964). The initial series of reaction in the sulfide oxidation appears to be cell membrane-associated resulting in the formation of a linear polymeric chain of sulfur atoms bound to a membrane lipoprotein fraction. This process is mediated by a copper-containing protein, cytochromes and ubiquinone and is oxygen-dependent in *T. concretivorus*, *T. thioparus* and *T. thiooxidans* (Moriarity and Nicholas 1969 and 1970). This membrane-bound linear polysulfide can then be oxidized to sulfite, possibly in a mechanism similar to that catalyzed by the sulfur-oxidizing enzyme. Although it has been suggested that the reaction process occurs extracytoplasmically, the site of oxidation of sulfide to elemental sulfur by sulfide oxidase has neither been demonstrated nor isolated to date.

Depending on the species of *Thiobacillus*, three different, but not necessarily mutually exclusive mechanisms of thiosulfate oxidation were observed. The first of these mechanisms involved the formation of elemental sulfur and sulfate. But, it was difficult to obtain a satisfactory stoichiometry between the disappearance of thiosulfate and the formation of elemental sulfur and sulfate. The second proposed mechanism involved polythionates as intermediate compounds since tetrathionate was readily detected in growing cell cultures. The formation of tetrathionate in the oxidation process is

catalyzed by the thiosulfate-oxidizing enzyme with ferricyanide and cytochrome c as electron acceptors (Aleem 1965; Lyric and Suzuki 1970a; Silver and Lundgren 1968; Trudinger 1961a and 1961b). Further oxidation of tetrathionate had been postulated to involve the scission of the sulfite groups with intermediate formation of trithionate and thiosulfate (Roy and Trudinger 1970; Sinha and Walden 1966). Although the detection of polythionates in the culture medium seemed to support this postulate, it should be understood that polythionates could be formed spontaneously from reduced sulfur compounds (Kelly 1982). More recently, researchers in Kuenen's laboratory were able to isolate and purify trithionate hydrolase and thiosulfate dehydrogenase from *T. acidophilus* (Pronk *et al.* 1990; Meulenberg *et al.* 1992 and Meulenberg *et al.* 1993a). The third observed mechanism proposed that the thiosulfate was cleaved by the action of rhodanese into colloidal sulfur and sulfite which were then oxidized to sulfate via the sulfur- and sulfite-oxidizing systems to sulfate (Suzuki 1974).

Although many thiobacilli species are known to oxidize thiosulfate and the metabolic mechanisms are well documented for *T. ferrooxidans* (Silver and Lundgren 1968), *T. thioparus* (Lyric and Suzuki 1970a, 1970b and 1970c), *T. novellus* (Oh and Suzuki 1977a and 1977b), *T. versutus* (Lu 1986) and *T. acidophilus* (Kuenen *et al.* 1993), little information is yet available on the mechanism of thiosulfate oxidation in *T. thiooxidans*. In earlier studies, Tano *et al.* (1968) found that thiosulfate was oxidized to tetrathionate and an unknown oxidized sulfur compound by a cell-free particulate preparation of *T. thiooxidans*. Goodman and Ralph (1980) described a heat output in microcalorimetric studies characterized by three maxima during thiosulfate oxidation by

*T. thiooxidans*. Due to the fact that thiosulfate was exhausted after the second peak, they assumed a transient accumulation of polythionate. In spite of the evidence presented above to support the polythionate pathway, the proposal still suffers from a lack of direct confirmation from experimental evidence and it is still not clear how this pathway involving polythionates as intermediates operates for the overall oxidation of thiosulfate to sulfate. In addition, the key enzyme in this polythionate pathway-thiosulfate:ferricyanide oxidoreductase (thiosulfate oxidizing enzyme)-has neither been demonstrated nor isolated in *T. thiooxidans*.

Resting cell suspensions of acidophilic thiobacilli had been shown to oxidize **tetrathionate** in *T. thiooxidans* (London and Rittenberg 1964; Okuzumi 1966), in *T. ferrooxidans* (Sinha and Walden 1966; Eccleston and Kelly 1978) and in *T. acidophilus* (Hazeu *et al.* 1988; Meulenberg *et al.* 1993). London and Rittenberg (1964) had demonstrated the appearance and utilization of polythionates during thiosulfate oxidation with dialyzed Norit A-treated extracts of *T. thiooxidans*, and Okuzumi (1964) reported that cell-free extracts of *T. thiooxidans* catalyzed the formation of trithionate and pentathionate from tetrathionate. However, doubt exists with regard to the formation of trithionate and pentathionate. It is not known whether these compounds arise from the physiological activities of these bacteria, or whether they arise through a non-biological nucleophilic displacement reaction (Parker and Kharasch 1959; Foss 1961). Recently, tetrathionate hydrolase was studied in cell-free extracts of *T. acidophilus* by Meulenberg *et al.* (1993b). This enzyme catalyzed the stoichiometric conversion of tetrathionate to thiosulfate, sulfur and two protons presumably accompanying the sulfate formation; and

the pH optimum of the enzyme activity was 3.0. To date, the mechanism of tetrathionate oxidation in *T. thiooxidans* is still not very clear. Suzuki *et al.* (1994) suggested that tetrathionate oxidation involved both the sulfane (-S-) and sulfonic acid ($-SO_3H$) portions through the sulfur- and sulfite- oxidizing systems respectively. The oxidation via that pathway can be described by the equation: $Na_2S_4O_6 + 3½O_2 + H_2O \rightarrow Na_2SO_4 + 3H_2SO_4$, i.e. each of the four sulfur atoms of tetrathionate is oxidized to sulfate. However, no evidence exists to support the stoichiometric oxidation of tetrathionate in *T. thiooxidans*.

## REFERENCES


1. Aleem, M.I.H 1965. Mechanism of thiosulfate oxidation by *Thiobacillus ferrooxidans* Biochim. Biophys. Acta **128**: 510-521.

2. Bhella, R.S. 1981. Purification and characterization of sulfur oxidizing enzyme of *Thiobacillus thiooxidans*. M.sc. thesis. University of Manitoba, Winnipeg.

3. Charles, A.M. and I. Suzuki. 1966a. Mechanism of thiosulfate oxidation by *Thiobacillus novellus*. Biochim. Biophys. Acta **128**: 510-512.

4. Charles, A.M. and I. Suzuki. 1966b. Purification and properties of sulfite:cytochrome c oxidoreductase from *Thiobacillus novellus*. Biochim. Biophys. Acta **128**: 522-534.

5. Eccleston, M and D. Kelly. 1978. Oxidation kinetics and chemostat growth kinetics of *Thiobacillus ferrooxidans* on tetrathionate and thiosulfate. J. Bacteriol. **134**: 718-727.

6. Foss, O. 1961. Ionic scission of the sulfur-sulfur bond. *In*: Organic sulfur compounds. *Edited* by N. Kharasch, vol. 1. Pergamon Press, New York. p.83-96.

7. Goodman, A., and B.J. Ralph. 1980. A microcalorimetric study of the metabolic activity of two thiobacillus species. *In*: Trudinger, P.A., M.R. Walter, and B.J. Ralph (eds) Biogeochemistry of ancient and modern environments. Springer, Berlin Heidelberg New York pp.477-483.

8. Hazeu, W., H. Batenburg-van der Vegte., P. Bos., R. K. van der Pas., and J.G. Kuenen. 1988. The production and utilization of intermediary elemental sulfur durinf the oxidation of reduced sulfur compounds by *Thiobacillus ferrooxidans*. Arch. Microbiol. **150**: 574-579.

9. Karavaiko, G.I. 1988. Microorganisms and their significance for biogeotechnology of metals. *In*: Biotechnology of metals *Edited* by Karavaiko, G.I., G. Rossi, A.D. Agate, S.N. Groudev and Z.A. Avakyan Moscow p.18-19.

10. Kelly, D.P. 1982. Biochemistry of the chemolithotrophic oxidation of inorganic sulfur. Phil. Trans. R. Soc. Lond. Ser. B. **298**: 499-528.

11. Kuenen, J. G., J.T. Pronk., W. Hazeu, R. Meulenberg and P. Ros. 1993.

A Review of Bioenergetics and Enzymology of Sulfur Compound Oxidation by Acidophilic Thiobacilli. *In*: Biohydrometallurgical Technologies. *Edited* by Torma, A.E., J.E. Wey and V.L. lakshmanan. The Minerals Metals & Materials Society. Warrendale, Pa. Vol II pp. 487-494.

12. Lizama, H.M. 1986. A study of enzymes involved in the sulfur oxidation of *Thiobacillus thiooxidans* M.sc. thesis. University of Manitoba, Winnipeg.

13. London, J. and S.C. Rittenberg. 1964. Path of sulfur in sulfide and thiosulfate oxidation by thiobacilli. Proc. Natl. Acad. Sci. (U.S.A.) **52**: 1183-1190.

14. Lu, W.P. 1986. A periplasmic location for the thiosulfate-oxidizing multi-enzyme system from *Thiobacillus versutus*. FEMS Microbiol. **34**: 313-317.

15. Lyric, R.M. and I. Suzuki. 1970a. Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus* I. Survey of enzyme and properties of sulfite cytochrome c oxidoreductase. Can. J. Biochem. **48**: 334-343.

16. Lyric, R.M. and I. Suzuki. 1970b. Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus* II. Properties of adenosine-5'-phosphate reductase. Can. J. Biochem. **48**: 344-354.

17. Lyric, R.M. and I. Suzuki. 1970c. Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus* III. Properties of thiosulfate-oxidizing enzyme and proposed pathway of thiosulfate oxidation. Can. J. Biochem. **48**: 355-363.

18. Meulenberg, R., J.T. Pronk., J. Frank, W. Hazeu., P. Bos and J.G. Kuenen. 1992. Purification and partial characterization of a thermostable trithionate hydrolase from the acidophilic sulfur oxidizer *Thiobacillus acidophilus*. Eur. J. Biochem. **209**: 367-374.

19. Meulenberg, R., J.T. Pronk., W. Hazeu, J.P. Van Dijken., J. Frank, P. Bos and J.G. Kuenen. 1993a. Purification and partial characterization of thiosulfate dehydrogenase from *Thiobacillus acidophilus*. J. Gen. Microbiol. **139**: 2033-2039.

20. Meulenberg, R., E.J. Scheer., J.T. Pronk., W. Hazeu., P. Bos and J.G. Kuenen. 1993. Metabolism of tetrathionate in *Thiobacillus acidophilus*.

FEMS. Microbiol. Lett. **112**: 167-172.

21. Moritarty, D.J.W. and D.J.D. Nicholas. 1969. Enzymaic sulfide oxidation by *Thiobacillus concretivorus*. Biochim. Biophys. Acta. **184**: 114-123.

22. Moritarty, D.J.W. and D.J.D. Nicholas. 1970. Products of sulfide oxidation in extracts of *Thiobacillus concretivorus*. Biochim. Biophys. Acta. **197**: 143-151.

23. Oh, J.K. and I. Suzuki. 1977a. Isolation and characterization of a membrane-associated thiosulfate-oxidizing system of *Thiobacillus novellus*. J. Gen. Microbiol. **99**: 397-412.

24. Oh, J. K. and I. Suzuki. 1977b. Resolution of a membrane-associated thiosulfate-oxidizing complex of *Thiobacillus novellus*. J. Gen. Microbiol. **99**: 413-423.

25. Okuzumi, M. 1964. Studies on biochemistry of the thiobacilli Part IX. Reduction of trithionate by *Thiobacillus thiooxidans*. Agr. Biol. Chem. **30**: 713-716.

26. Okuzumi, M. 1966. Studies on biochemistry of the thiobacilli Part VIII. Dismutation of tetrathionate by *Thiobacillus thiooxidans*. Agr. Biol. Chem. **30**: 313-318.

27. Paine, P.J. 1988. Historic and geographic overview of acid mine drainage. *In*: Proceeding: Acid Mine Drainage Seminar/worshop, Halifax, Nova Scotia 23, 24, 25, 26 March, 1987. *Edited* by Biakeman, B., J. Scott, M. Guilcher, J. ferguson and J. Worgan (Ottawa : Environment Canada, 1987) p.1-45.

28. Parker, A.J. and N. Kharasch. 1959. The scission of the sulfur-sulfur bond. Chem. Rev. **59**: 583-628.

29. Parker, C.D. and J. Prisk. 1953. The oxidation of inorganic compounds of sulfur by various sulfur bacteria. J. Gen. Microbiol. **8**: 244-268.

30. Pivovarova, T.A. and R.S. Golovacheva. 1985. Microorganisms important for hydrometallurgy: cytology, physiology and biochemistry. *In*: Biogeotechnology of metals. *Edited* by G.I. Karavaiko and S.N. Groudev, Moscow. p.38.

31. Pronk, J.T., R. Meulenberg., W. Hazeu., P. Bos and J.G. Kuenen. 1990.

Oxidation of reduced inorganic sulfur compounds by acidophilic thiobacilli. FEMS. Microbiol. Rev. **75**: 293-306.

32. Roy, A.B. and P.A. Trudinger. 1970. Oxidation of inorganic sulfur compounds by microorganisms and plants. *In*: The Biochemistry of inorganic compounds of sulfur. Cambridge University Press, London. p.207-250.

33. Silver, M and D.G. Lundgren. 1968. The thiosulfate-oxidizing enzyme of *Ferrobacillus ferrooxidans* (*Thiobacillus ferrooxidans*). Can. J. Biochem. **46**: 1215-1220.

34. Sinha, D.B. and C.C. Walden. 1966. Formation of polythionates and their interelationships during oxidation of thiosulfate by *Thiobacillus ferrooxidans*. Can. J. Microbiol. **12**: 1014-1054.

35. Starkey, R. L. 1925. Concerning the physiology of *Thiobacillus thiooxidans*, an autotrophic bacterium oxidizing sulfur under acid condition. J. Bacteriol. **10**: 135-163.

36. Sugio, T., C. Domatsu., O, Munakata., T. Tano and K. Imai. 1975. Role of a ferric ion-reducing system in sulfur oxidation of *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **49**: 1401-1406.

37. Sugio, T., T. Katagiri., M. Moriyama., Y.I. Zhen., K. Inagaki and T. Tano. 1988. Existence of a new sulfite oxidase which utilizes ferric ions as an electron acceptor in *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **54**: 153-157.

38. Suzuki, I. 1965a. Oxidation of elemental sulfur by an enzyme system of *Thiobacillus thiooxidans*. Biochim. Biophys. Acta. **104**: 359-371.

39. Suzuki, I. 1965b. Incorportation of atmospheric oxygen into thiosulfate by sulfur-oxidizing enzyme of *Thiobacillus thiooxidans*. Biochim. Biophys. Acta. **110**: 97-101.

40. Suzuki, I. and M. Silver. 1966. The initial products and properties of the sulfur oxidizing enzyme of Thiobacilli. Biochim. Biophys. Acta. **122**: 22-23.

41. Suzuki, I. 1974. Mechanism of inorganic oxidation and energy coupling. Annu. Rev. Microbiol. **28**: 85-101.

42. Suzuki, I., J.K. Oh., P.D. Tackaberry., H.M. Lizama and T.L.

Takeuchi. 1989. Determination of activity parameters in *Thiobacillus ferrooxidans* strains as criteria for minerals leaching efficiency (UP-M-589) A final report submitted to CANMET Serial no 2340-6-9126/01-ST and DSS File no 24ST-23440-6-9126 p.25.

43. Suzuki, I., C.W. Chan and T.L. Takeuchi. 1994. Oxidation of inorganic sulfur compounds by thiobacilli. *In*: Environmental Geochemistry of sulfide oxidation. *Edited* by Alpers, C.N. and D.W. Blowes. Amer. Chem. Soci. Washington D.C. pp. 60-67.

44. Tano, T., H. Asano and K. Imai. 1968. Physiological studies on thiobacilli. Part III The oxidation of thiosulfate by particulate fraction of *Thiobacillus thiooxidans*. Agr. Biol. Chem. **32**: 140-143.

45. Trudinger, P.A. 1961a. Thiosulfate oxidation and cytochromes in *Thiobacillus X* 1. Fractionation of bacterial extracts and properties of cytochromes. Biochem. J. **78**: 673-680.

46. Trudinger, P.A. 1961b. Thiosulfate oxidation and cytochromes in *Thiobacillus X* 2. thiosulfate-oxidizing enzyme. Biochem. J. **78**: 680-686.

47. Vishniac, W. and M. Santer. 1957. The thiobacilli. Bacteriol. Rev. **21**: 175-213.

48. Vishniac, W.V. 1974. In Bergey's Manual of Determinative Bacteriolgy, 8th ed. Eds. Buchanan and Gibbons, N.E. Williams and Wilkins Co., Baltimore p.459.

49. Waksman, S.A. and J.S. Joffe. 1922. Microorganisms concerned in the oxidation of sulfur in the soil II *Thiobacillus thiooxidans*, a new sulfur-oxidizing organism isolated from the soil. J. Bacteriol. **7**: 239-256.

# PART ONE

## Sulfur oxidation by *Thiobacillus thiooxidans*.

## ABSTRACT

The oxidation of elemental sulfur by *Thiobacillus thiooxidans* was studied with and without inhibitors, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and N-ethylmaleimide (NEM), for stoichiometry of substrate, total amount of oxygen consumed and oxidation intermediates. Sulfur-oxidizing enzyme was inhibited by NEM. The inhibition by NEM was time-dependent. Elemental sulfur was stoichiometrically oxidized to sulfite by *T. thiooxidans* in the presence of HQNO at wide range of pH from 2.5 to 9.0 with equimolar oxygen consumption. At an acidic pH, sulfur oxidation was inhibited by accumulating sulfite. The amount of sulfite accumulated at pH 6.5 was proportional to the amount of cells. When the cells were oxidizing sulfur in the presence of reduced glutathione (GSH) or sulfite, there was thiosulfate formation at a pH near or above neutrality. Cell-free extracts oxidized sulfur in the presence of GSH producing both sulfite and thiosulfate.

# INTRODUCTION

The first isolation of *Thiobacillus thiooxidans* in pure culture and the study of its morphology was carried out by Waksman and Joffe in 1922. This bacterium was found to be an obligate chemoautotroph deriving its energy and reducing power for growth from the oxidation of elemental sulfur and synthesizing all the cellular carbon by assimilation of atmospheric $CO_2$. The oxidation of sulfur to sulfuric acid by *T. thiooxidans* with sulfite as the key intermediate was proposed by Suzuki (1974) on the basis of the sulfur-oxidizing and sulfite-oxidizing enzyme systems: $S^0 + O_2 + H_2O \rightarrow H_2SO_3$ and $H_2SO_3 + \frac{1}{2}O_2 \rightarrow H_2SO_4$. Despite the presence of sulfur- and sulfite-oxidizing enzyme systems and intensive investigations into many aspects of sulfur metabolism in this organism over the past thirty years, the mechanism of elemental sulfur oxidation in this organism is still controversial. This controversy is probably due to the reactive nature of inorganic sulfur intermediates, which makes the direct demonstration of a partial oxidation reaction difficult. Fortunately quantitative sulfur oxidation to sulfite can be successfully demonstrated in *T. thiooxidans* in the presence of 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), an inhibitor of sulfite oxidation; that is, the two oxidative steps can be uncoupled from each other.

This report presents direct evidence that sulfite is the oxidation product of elemental sulfur oxidation in *T. thiooxidans* when the further reaction of sulfite is inhibited. Both intact cells and cell-free extracts of *T. thiooxidans* are used for this demonstration.

# MATERIALS AND METHODS

## *Organism*

*T. thiooxidans* (ATCC 8085) was grown on elemental sulfur for 5 days at 28°C with 2.5% (vol/vol) inoculum in Starkey's medium 1, with the following composition (per litre): 0.3 g $(NH_4)_2SO_4$, 3.5 g $KH_2PO_4$, 0.5 g $MgSO_4.7H_20$, 0.25 g $CaCl_2$ and 18 mg $FeSO_4.7H_20$ per liter and adjusted to pH 2.3 with sulfuric acid. One liter of medium was placed in a 2.8-L Fernbach flask and after autoclaving and inoculation, 50 g of powdered sulfur (BDH, precipitated sulfur) was spread evenly on the surface. The flask was incubated without shaking. After growth, sulfur was removed by filtration through a Whatman No. 1 filter paper under suction and cells were collected by centrifugation at 12,000 × *g* for 20 min. Cells were washed two times with, and suspended in, distilled water (adjusted to pH 3 with $H_2SO_4$) at a concentration of 20 mg wet cells per mL.

## *Preparation of Sulfur Suspension*

The colloidal sulfur suspension used as substrate for the sulfur-oxidizing enzyme was prepared as follows: 32 g of precipitated sulfur (BDH; low in iron) was suspended in 100 mL of distilled water containing 0.05% Tween-80. After vigorous stirring for 2 hours, the sulfur suspension was stored at 4°C.

## *Preparation of Cell-Free Extracts*

Harvested cells of *T. thiooxidans* were washed once with 0.5 M Tris-HCl buffer (pH 7.8), suspended at a cell concentration of 20 mg/mL in the same buffer and stirred

overnight at 4°C. The cell suspension was centrifuged for 20 min at 12,000 x *g*, and the pellet reconstituted as a 15% (W/V) suspension in 0.5 M Tris-HCl buffer (pH 7.8). This cell suspension was passed three times through a French pressure cell at 110 MPa and then centrifuged for 20 min at 48,000 x *g* to remove the bulk of cell debris. The supernatant was termed as crude "cell-free extracts". The fraction of the cell-free extracts was centrifuged for 1½ hours at 105,000 x *g*. After ultracentrifugation of the cell-free extracts, a brownish supernatant was obtained. Selective protein precipitation using ammonium sulfate was carried out as described by Schleif and Wensink (1981). After ammonium sulfate precipitation and centrifugation, the pellet was resuspended in 0.1 M sodium phosphate buffer pH 6.5. The suspended fraction was dialyzed against the sample phosphate buffer at 4°C overnight in order to remove the ammonium sulfate.

***Sulfite Determination***

Sulfite formed was determined by using the pararosaniline method according to West and Gaeke (1956) after removal of cells and sulfur by centrifugation.

***Thiosulfate Determination***

The reaction was stopped by the addition of 0.1 mL of 1 M cadium acetate. After centrifugation to remove sulfur, protein and glutathione (GSH), the supernatant was used for the colorimetric determination of thiosulfate by the method as described by Sörbo (1957). To 1.2 mL sample containing 0.01 to 1.0 $\mu$mol of thiosulfate, the following reagents were added in this order: 2.0 mL of 0.2 N $NH_4OH$, 1.2 mL of water,

0.5 mL of 0.1 M KCN, 0.3 mL of 0.1 M $CuCl_2$, and 0.5 mL of ferric nitrate reagent (20% $Fe(NO_3)_3$ in 3 N $HNO_3$). After 15 min of incubation, the optical density was measured in a Klett-Summersion colorimeter with a No. 42 blue filter. A blank reading was obtained by adding to KCN, $CuCl_2$ followed by ferric nitrate reagent and the sample containing $NH_4OH$. The values were read from a standard curve obtained by using known amounts of thiosulfate.

### *Assay for Sulfur Oxidation*

#### *Intact Cells*

Sulfur oxidation was assayed by measurement of oxygen consumption at 25°C in a thermostated vessel equipped with a Teflon-covered Clark oxygen electrode (a Gilson Oxygraph). Unless otherwise indicated, the reaction mixture for sulfur oxidation contained the following in a total volume of 1.2 mL: 50 mM phosphate buffer, various amounts of wet cells and 30 mg $S^0$. When the stoichiometry of sulfur oxidation was studied, powdered sulfur was dissolved in dimethylsulfoxide (DMSO) and microliter volumes were injected to start the reaction (Fig. 1).

#### *Cell-Free Extracts*

The reaction mixture was the same as described in intact cells except that 6 $\mu$mol GSH was present and the pH was 6.5. The reaction was started by the addition of GSH (0.1 M solution) to the rest of the reaction mixture. Enzymatic sulfur oxidation rates were always corrected for non-enzymatic oxidation rates of GSH and sulfur.

*Protein Determination*

Protein contents of all the fractions were determined by the colorimetric method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as the reference protein.

*Centrifugations*

Low speed centrifugations were performed in a Sorvall superspeed RC-2B refrigerated centrifuge at 4 °C. The ultracentrifugation of cell-free extracts was carried out in a Beckman L3-50 refrigerated ultracentrifuge (4°C) using a 60 Ti rotor at 105,000 x *g* for 2 hours to yield the supernatant and pellet fractions.

# RESULTS

## *Intact Cells*

### Effect of pH

*T. thiooxidans* oxidized powdered sulfur at a wide range of pH values as shown in Table 1 and HQNO inhibited the oxidation more strongly at lower pH's. There was no sulfite accumulation in the absence of HQNO, but in its presence sulfite accumulated in equimolar quantities to the amount of $O_2$ consumed. When the amount of sulfur as substrate was limited, the molar ratio of sulfur added : $O_2$ used : sulfite formed was 1:1:1, agreeing with the equation : $S^0 + O_2 + H_2O \rightarrow H_2SO_3$ at pH 7.0 in the presence of HQNO (Fig. 1). At pH 2.3, the oxidation was incomplete and the $O_2$ consumption rate decreased progressively due to the inhibition by accumulated sulfite (Fig. 1). Therefore at this pH, the amount of sulfite recovery was lower. In the absence of HQNO, very little sulfite accumulated and the amount of $O_2$ consumed was approximately 1.5 times the amount of sulfur oxidized in moles which is in agreement with the complete oxidation of sulfur to sulfate: $S^0 + 1½O_2 + H_2O \rightarrow H_2SO_4$.

### Effect of reduced glutathione

Reduced glutathione is strictly required in sulfur oxidation in cell-free extracts of *T. thiooxidans* (Suzuki 1965). Intact cells oxidized elemental sulfur without GSH (Table 1), nevertheless GSH increased the rate of $O_2$ consumption (Table 2). Thiosulfate accumulated only at pH above 7 in the presence of reduced glutathione during sulfur oxidation by *T. thiooxidans* (Table 2). The amount of thiosulfate accumulation was

higher than that of the reaction mixture without cells. Sulfite determination was hampered by the interference due to the chemical reaction between sulfur and GSH at higher pH ranges. Therefore the values in Table 2 cannot be considered as sulfite formed.

**Effect of sulfite**

Some thiosulfate formation was observed from sulfur and sulfite ($S^0 + H_2SO_3 \rightarrow H_2S_2O_3$) in the presence of *T. thiooxidans* at a pH above neutrality (Table 3). Sulfite inhibited the sulfur oxidation at acidic pH values as expected.

**Effect of different amounts of wet cells**

Sulfur oxidation rate increased linearly with increasing concentrations of cells (Table 4). In the presence of HQNO, the amount of sulfite accumulated during sulfur oxidation was also proportional to the amount of *T. thiooxidans* added.

*Cell-Free Systems*

**Effect of different amounts of cell-free extracts**

The rate of sulfur oxidation in the presence of GSH increased linearly with increasing amounts of cell-free extracts (Table 5). Both sulfite and thiosulfate were found in the reaction mixtures and the combined amounts were often close to the amounts of $O_2$ consumed.

**Effect of N-ethylmaleimide (NEM)**

The sufur-oxidizing enzyme was inhibited by sulfhydryl-binding reagent, N-ethylmaleimide (NEM). The inhibition of this enzyme was time-dependent (Table 6). The formation of thiosulfate as well as the consumption of $O_2$ decreased rapidly after 15 minutes of preincubation. The inhibition was nearly complete after 60 minutes of preincubation.

## DISCUSSION

*Thiobacillus thiooxidans* oxidized elemental sulfur equally well either at an acidic pH, which was required for growth, or at a neutral pH, at which no growth was possible (Table 1). Various researchers reported 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) or antimycin A to be a strong inhibitor of sulfite oxidation in cell-free systems of thiobacilli (Adams *et al.* 1971; Kodama *et al.* 1970; Takakuwa 1976 and Tano *et al.* 1982). The oxidation of elemental sulfur by *T. thiooxidans* was inhibited by HQNO more strongly under acidic conditions (Table 1 and Fig. 1). Since sulfite was inhibitory to sulfur oxidation only at an acidic pH (Table 3), the inhibition of sulfur oxidation by HQNO was thought to be at the level of sulfite oxidation. The oxidation of sulfur in the presence of HQNO would be expected to slow down as the concentration of sulfite increased with time during sulfur oxidation under acidic conditions. The results shown in Fig. 1 and Table 1 agreed with this interpretation.

Although no thiosulfate accumulated during the oxidation of sulfur alone by *T. thiooxidans* in Table 1, these cells accumulated thiosulfate when sulfur was oxidized in the presence of GSH (Table 2) or sulfite (Table 3) at a pH near or above neutrality. Since the rate of thiosulfate formation was faster than the chemical rate (Table 2), the cells must have catalyzed the condensation reaction: $S^0 + H_2SO_3 \rightarrow H_2S_2O_3$. Imai *et al.* (1962) reported such a reaction with *T. thiooxidans* and crude extracts. The combined amounts of sulfite and thiosulfate often exceeded the amounts of oxygen consumed indicating the overestimation of sulfite due to interference of GSH in the reaction system (Table 2). Cell-free extracts of *T. thiooxidans* oxidized powdered sulfur in the presence

of reduced glutathione and the rate of oxidation increased linearly with increasing amounts of cell-free extracts (Table 5). The results support the interpretation that cell-free extracts, in the presence of GSH, oxidized sulfur to sulfite and also formed thiosulfate from sulfur and sulfite. NEM inhibits the oxidation of sulfur by *T. thiooxidans* (Kodama and Mori 1968). Our experiments confirmed their results (Table 6). A sulfur-oxidizing enzyme of *Desulfurolobus ambivalens*, a thermophilic archaebacterium, had been found to be inhibited by sulfhydryl-binding reagents (Kletzin 1989). Inhibition of the sulfur-oxidizing enzyme by NEM (Table 6) suggests that the site of NEM binding may be located on the sulfur-oxidizing enzyme.

The direct demonstration of elemental sulfur oxidation to sulfite in *T. thiooxidans* establishes the central role of sulfite in inorganic sulfur metabolism. The results reported in this paper support the pathway of sulfur oxidation in *Thiobacillus thiooxidans* as: $S + O_2 + H_2O \rightarrow H_2SO_3$ as well as the formation of thiosulfate by a secondary reaction: $S + SO_3^{2-} \rightarrow S_2SO_3^{2-}$ especially at a pH near or above neutrality.

## REFERENCES


1. Adams, C.A., G.M. Warnes and D.J.D. Nicholas. 1971. A sulfite-dependent nitrate reductase from *Thiobacillus denitrificans*. Biochim. Biophys. Acta. **235**: 398-406.

2. Imai, K., K.M. Okuzumi, and H. Katagiri. 1962. Pathways of sulfur oxidation by *Thiobacillus thiooxidans*. Koso Kagaku Shimpoziumu **17**: 132-143.

3. Kletzin, A. 1989. Coupled enzymatic production of sulfite, thiosulfate and hydrogen sulfide from sulfur: Purification and properties of a sulfur oxygenase reductase from the facultatively anaerobic archaebacterium *Desulfurolobus ambivalens*. J. Bacteriol. **171**: 1638-1643.

4. Kodama, A and T. Mori. 1968. Studies on the metabolism of a sulfur-oxidizing bacterium V. comparative studies on sulfur and sulfite oxidizing systems of *Thiobacillus thiooxidans*. Plant & Cell Physiol. **9**: 725-734.

5. Kodama, A., T. Kodama and T. Mori. 1970. Studies on the metabolism of a sulfur-oxidizing bacterium. VII. Oxidation of sulfite by a cell-free extract of *Thiobacillus thiooxidans*. Plant & Cell Physiol. **11**: 701-711.

6. Lowry, O.M., N.I. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the folin reagent. J. Biol. Chem. **193**: 265-275.

7. Schleif, R.F. and P.W. Wensink. 1981. Practical Methods in Molecular Biology. Springer Verlag, New York. p.62.

8. Sörbo, B. 1957. A colorimetric method for the determination of thiosulfate. Biochim. Biophys. Acta. **23**: 412-416.

9. Suzuki, I. 1965. Oxidation of elemental sulfur by an enzyme system of *Thiobacillus thiooxidans*. Biochim. Biophys. Acta. **104**: 359-371.

10. Suzuki, I. 1974. Mechanisms of inorganic oxidation and energy coupling. Annu. Rev. Microbiol. **28**: 85-101.

11. Takakuwa, S. 1976. Studies on the metabolism of a sulfur-oxidizing bacterium. IX. Electron transfer and the terminal oxidase system in sulfite oxidation by *Thiobacillus thiooxidans*. Plants & Cell Physiol. **17**: 103-110.

12. Tano, T., T. Ito, H. Takesue, T. Sugio and K. Imai. 1982. B-type cytochrome, an electron carrier in the sulfite-oxidation system of *Thiobacillus thiooxidans*. J. Ferment. Technol. **60**: 181-187.

13. Waksman, S.A. and J.S. Joffe. 1992. Microorganisms concerned in the oxidation of sulfur in the soil. II. *Thiobacillus thiooxidans*, a new sulfur-oxidizing organism isolated from the soil. J. Bacteriol. **7**: 239-256.

14. West, P.W. and G.C. Gaeke 1956. Fixation of sulfur dioxide as disulfitomercurate (II) and subsequent colorimetric estimation. Anal. Chem. **28**: 1816-1819.

# TABLES

Table 1. Effect of pH and HQNO on sulfur oxidation by *T. thiooxidans*[a].

| | pH | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2.5 | 3.0 | 4.0 | 5.0 | 6.0 | 6.5 | 7.5 | 8.0 | 8.5 | 9.0 |
| **- HQNO** | | | | | | | | | | |
| $O_2$ consumed (nmol) | 95 | 115 | 113 | 115 | 120 | 130 | 110 | 130 | 90 | 95 |
| Sulfite formed (nmol) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Thiosulfate formed (nmol) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rate (nmol $O_2$/min) | 19.2 | 23.1 | 22.5 | 22.7 | 24.0 | 25.7 | 22.0 | 26.2 | 18.0 | 19.2 |
| **+ HQNO** | | | | | | | | | | |
| $O_2$ consumed (nmol) | 13 | 31 | 52 | 63 | 71 | 83 | 74 | 60 | 56 | 49 |
| Sulfite formed (nmol) | 13 | 30 | 65 | 74 | 88 | 80 | 70 | 63 | 50 | 38 |
| Thiosulfate formed (nmol) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rate (nmol $O_2$/min) | 4.0 | 7.0 | 12.9 | 16.0 | 17.7 | 19.6 | 16.4 | 14.3 | 14.0 | 11.5 |

[a] 1.2 mL reaction mixtures containing 50 mM potassium phosphate buffer, 1 mg wet cells, 30 mg sulfur were followed in a Gilson oxygraph at 25 °C for 5 min. When indicated, 10 $\mu$g HQNO was present.

Table 2. **Effect of pH on sulfur oxidation by *T. thiooxidans* in the presence of reduced glutathione[a].**

| | pH | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2.5 | 3.0 | 4.0 | 5.0 | 6.0 | 6.5 | 7.5 | 8.0 | 8.5 | 9.0 |
| **− Wet cells** | | | | | | | | | | |
| $O_2$ consumed (nmol) | 8 | 8 | 8 | 8 | 8 | 8 | 36 | 57 | 45 | 59 |
| Sulfite found[b] (nmol) | 0 | 0 | 0 | 0 | 5 | 72 | 220 | 295 | 305 | 315 |
| Thiosulfate formed (nmol) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 15 | 15 |
| Rate (nmol $O_2$/min) | 2.4 | 2.4 | 2.4 | 2.4 | 2.4 | 3.1 | 9.75 | 12.5 | 11.5 | 14.5 |
| **+ Wet cells** | | | | | | | | | | |
| $O_2$ consumed (nmol) | 240 | 239 | 244 | 252 | 255 | 271 | 257 | 208 | 219 | 208 |
| Sulfite found[b] (nmol) | 0 | 0 | 0 | 0 | 0 | 40 | 170 | 288 | 210 | 190 |
| Thiosulfate formed (nmol) | 0 | 0 | 0 | 0 | 0 | 15 | 50 | 90 | 90 | 90 |
| Rate (nmol $O_2$/min) | 55.5 | 55.5 | 58.5 | 58.5 | 54.0 | 64.0 | 57.0 | 46.0 | 50.0 | 54.0 |

[a] 1.2 mL reaction mixtures containing 50 mM potassium phosphate buffer, 1 mg wet cells, 6 $\mu$mol reduced glutathione (GSH) and 30 mg sulfur were followed in a Gilson oxygraph at 25 °C for 5 min.

[b] GSH reduced chemically with elemental sulfur near and above neutral pH ranges producing compounds (possibly polysulfides) which reacted with pararosaniline giving false high sulfite values.

Table 3. Effect of pH on sulfur oxidation by *T. thiooxidans* in the presence of sulfite[a].

| | pH | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2.5 | 3.0 | 4.0 | 5.0 | 6.0 | 6.5 | 7.5 | 8.0 | 8.5 | 9.0 |
| **- Wet cells** | | | | | | | | | | |
| $O_2$ consumed (nmol) | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Sulfite found (nmol) | 295 | 270 | 290 | 290 | 263 | 275 | 213 | 245 | 225 | 210 |
| Thiosulfate formed (nmol) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rate (nmol $O_2$/min) | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| **+ Wet cells** | | | | | | | | | | |
| $O_2$ consumed (nmol) | 6 | 8 | 32 | 114 | 244 | 276 | 118 | 98 | 88 | 68 |
| Sulfite found (nmol) | 262 | 260 | 310 | 245 | 245 | 275 | 225 | 205 | 205 | 195 |
| Thiosulfate formed (nmol) | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 38 | 50 | 55 |
| Rate (nmol $O_2$/min) | 1.6 | 0.7 | 7.9 | 30.0 | 50.8 | 50.4 | 29.0 | 24.0 | 24.0 | 20.2 |

[a] 1.2 mL reaction mixtures containing 50 mM potassium phosphate buffer, 1 mg wet cells, 0.3 $\mu$mol $SO_3^{2-}$ and 30 mg sulfur were followed in a Gilson Oxygraph at 25 °C for 5 min.

Table 4. Effect of different amounts of *T. thiooxidans* on sulfur oxidation[a].

| | Amount of wet cells (mg) | | | | | |
|---|---|---|---|---|---|---|
| | 0.0 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| − HQNO | | | | | | |
| $O_2$ consumed (nmol) | 0.0 | 74 | 148 | 214 | 265 | 296 |
| Sulfite formed (nmol) | 0.0 | 21 | 21 | 20 | 20 | 20 |
| Rate (nmol $O_2$/min) | 0.0 | 17.2 | 31.8 | 49.5 | 58.0 | 70.5 |
| + HQNO | | | | | | |
| $O_2$ consumed (nmol) | 0.0 | 56 | 100 | 146 | 168 | 184 |
| Sulfite formed (nmol) | 0.0 | 65 | 115 | 180 | 210 | 275 |
| Rate (nmol $O_2$/min) | 0.0 | 12.9 | 22.2 | 29.6 | 37.2 | 39.7 |

[a] 1.2 mL reaction mixtures containing 50 mM potassium phosphate buffer at pH 6.5, different amounts of wet cells, 30 mg sulfur in a Gilson Oxygraph at 25 °C for 5 min. HQNO (10 $\mu$g) was present when indicated.

Table 5. **Effect of different amounts of cell-free extracts of *T. thiooxidans* on sulfur oxidation[a].**

| | Amount of cell-free extracts (mg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 0.24 | 0.48 | 0.96 | 1.2 | 1.44 | 1.92 | 2.40 |
| Sulfite found (nmol) | 50 | 75 | 107 | 144 | 151 | 175 | 200 | 200 |
| Sulfite formed[b] (nmol) | 0 | 25 | 57 | 94 | 101 | 125 | 150 | 150 |
| Thiosulfate formed (nmol) | 0 | 0 | 13 | 56 | 94 | 106 | 113 | 153 |
| $O_2$ consumed (nmol) | 80 | 50 | 76 | 122 | 133 | 156 | 203 | 271 |
| Rate (nmol $O_2$/min) | 9 | 5.8 | 8.2 | 14.6 | 16.2 | 18.8 | 24.6 | 31.6 |

[a] 1.2 mL reaction mixtures containing 0.1 M sodium phosphate buffer at pH 6.5, amount of cell-free extracts protein as shown, 6 $\mu$mol GSH, 30 mg sulfur were followed in a Gilson Oxygraph at 25 °C for 10 min.

[b] Sulfite formed was calculated by substracting the sulfite value found in the chemical system from those found in the presence of cell-free extracts.

Table 6. **Effect of N-ethylmaleimide on sulfur oxidation by the ammonium sulfate fraction of *T. thiooxidans* extracts[a].**

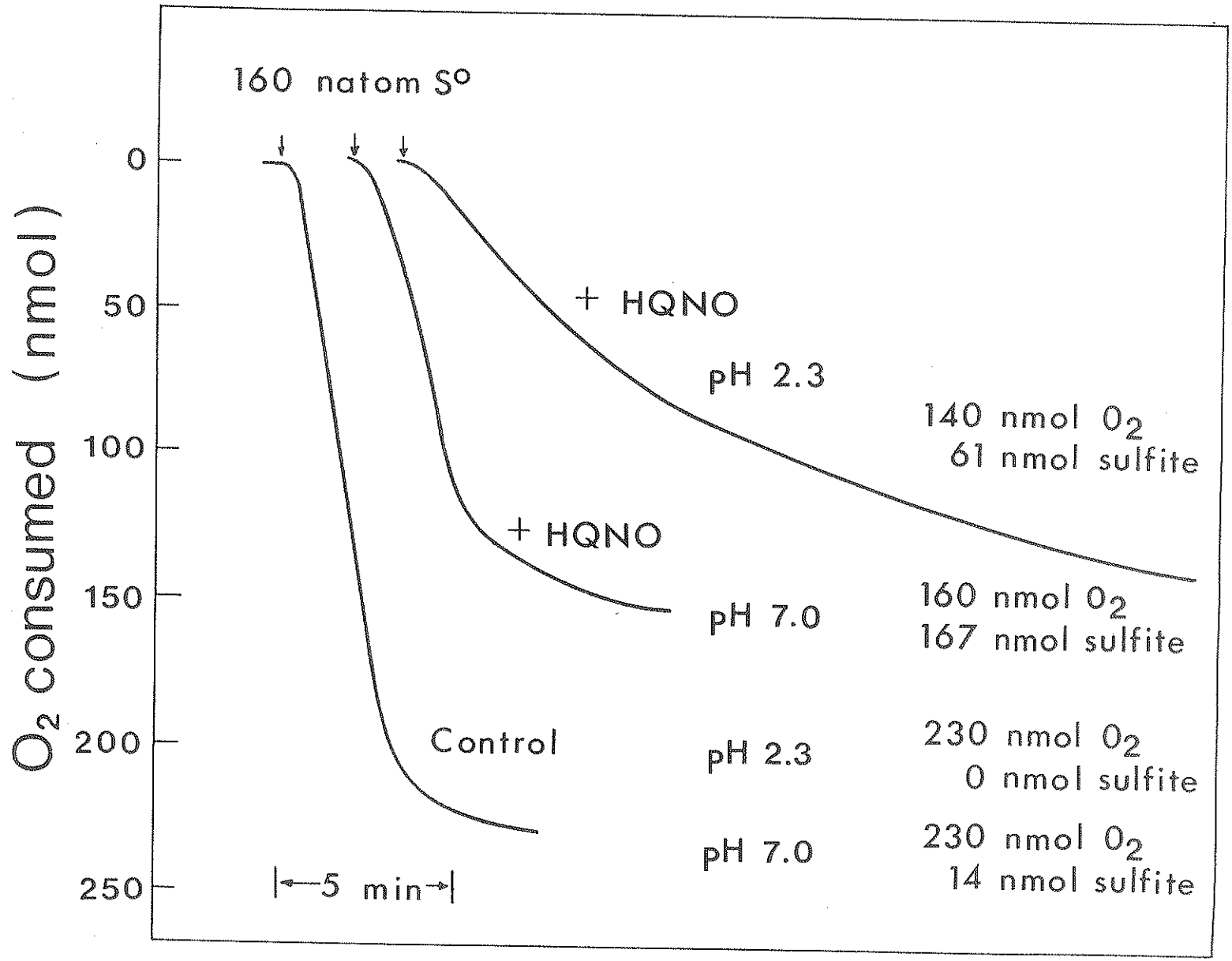
| | | Preincubation time (min) | | | | |
|---|---|---|---|---|---|---|
| | No NEM control | 15 | 30 | 45 | 60 | 90 |
| **- 80% $(NH_4)_2SO_4$ ppt.** | | | | | | |
| Thiosulfate formed (nmol) | 12 | 12 | 10 | 10 | 10 | 10 |
| $O_2$ consumed (nmol) | 20 | 20 | 20 | 20 | 20 | 20 |
| Rate (nmol $O_2$/min) | 2.8 | 2.8 | 2.8 | 2.8 | 2.8 | 2.8 |
| **+ 80% $(NH_4)_2SO_4$ ppt.** | | | | | | |
| Thiosulfate formed (nmol) | 163 | 45 | 12 | 12 | 12 | 12 |
| $O_2$ consumed (nmol) | 200 | 75 | 40 | 30 | 30 | 30 |
| Rate (nmol $O_2$/min) | 23.7 | 7.6 | 4.7 | 4.1 | 3.6 | 3.6 |

[a] The reaction mixture was the same as that described in Table 5 except that the 80% ammonium sulfate precipitate (50 $\mu$L, 0.8 mg protein ) containing the sulfur-oxidizing enzyme was preincubated with 0.5 $\mu$L of 0.1 M NEM.

# FIGURES

Figure 1. Effect of HQNO on the $O_2$ consumption and sulfite formation in the sulfur oxidation by *T. thiooxidans*.

The $O_2$ consumption was followed in a Gilson Oxygraph at 25 °C with 1.2 mL reaction mixtures: 50 mM potassium phosphate buffer (pH 2.3 or 7.0), 4 mg wet cells, 20 $\mu$g HQNO when indicated. The reaction was initiated with the addition of 20 $\mu$L DMSO solution (5.1 $\mu$g = 160 natom sulfur) as indicated by an arrow.


160 natom S°
0
50
100
150
200
250
$O_2$ consumed (nmol)
+ HQNO
pH 2.3
140 nmol $O_2$
61 nmol sulfite
+ HQNO
pH 7.0
160 nmol $O_2$
167 nmol sulfite
Control
pH 2.3
230 nmol $O_2$
0 nmol sulfite
pH 7.0
230 nmol $O_2$
14 nmol sulfite
5 min

# PART TWO

**Quantitative extraction and determination of elemental sulfur and stoichiometric oxidation of sulfide to elemental sulfur by *Thiobacillus thiooxidans*.**

## ABSTRACT

A sensitive and quantitative analytical method for the determination of elemental sulfur in a biological system was developed. Elemental sulfur was determined after extraction with petroleum ether by cyanolysis and ferric thiocyanate color formation in acetone. The method was successfully applied to show that sulfide was oxidized by *Thiobacillus thiooxidans* to elemental sulfur nearly stoichiometrically when further oxidation of elemental sulfur was inhibited by N-ethylmaleimide. Kinetic studies of sulfide oxidation were also investigated.

## INTRODUCTION

Sulfide oxidation in acidophilic thiobacilli has received little or no attention because of experimental problems relating to the high rate of spontaneous oxidation of sulfide in combination with its low solubility at low pH values (Pronk *et al.* 1990). Perhaps for these reasons, publications on sulfide oxidation by acidophilic thiobacilli are few and far between. To date, the exact mechanism of sulfide oxidation in acidophilic thiobacilli has not yet been completely elucidated.

Earlier studies related to sulfide oxidation in *Thiobacillus thiooxidans* with both intact cells and crude extracts led to the hypothesis that elemental sulfur, thiosulfate and polythionates were the intermediate products during oxidation of sulfide to sulfate (Parker and Prisk 1953; Vishniac and Santer 1957; London and Rittenberg 1964; Roy and Trudinger 1970). A sulfide-oxidizing system from *T. thiooxidans* extracts was isolated by Moriarty and Nicholas (1969, 1970 a and b) which was associated with the cell envelope. The $K_m$ of this membrane-bound sulfide-oxidizing system was 2 $\mu$M. Recently, it has been shown that sulfur was formed during oxidation of sulfide by *T. ferrooxidans* (Hazeu *et al.* 1988 and Pronk *et al.* 1990). At present, it is generally agreed that oxidation of sulfide is a two-stage process. Sulfide is probably oxidized to sulfate through polysulfide and sulfite as intermediates. Sulfide loses two electrons through the action of sulfide oxidase which is probably a copper-containing protein, and the resulting sulfur atoms are polymerized. Polysulfide formed during the first stage is oxidized to sulfate. Thus the mechanism of sulfide oxidation in acidophilic thiobacilli is analogous to that for the oxidation of colloidal sulfur (polysulfide) or elemental sulfur

once sulfide is converted to the oxidation level of sulfur. This bound sulfur polymer may be subsequently oxidized to sulfate by sulfur-oxidizing enzyme and sulfite oxidase via a mechanism similar to those described (Suzuki 1974; Pivovarova and Golovacheva 1985; Kuenen *et al.* 1993 and Suzuki *et al.* 1994).

In the above proposed pathway of sulfide oxidation, sulfur is therefore one of the main presumed intermediates. The precise measurement of elemental sulfur in biological systems is difficult (Roy and Trudinger 1970), and this still hampers the complete mass balance studies of inorganic sulfur metabolism (Perry *et al.* 1993). Roy and Trudinger (1970) suggested the possibility of adapting methods developed for non-biological materials to biological systems. We have adapted one such method (Bartlett and Skoog 1954) involving ferric thiocyanate color formation, to determine minute quantities of elemental sulfur after extraction with petroleum ether from aqueous biological reaction mixtures. This method is applied to study the oxidation of sulfide to elemental sulfur in *T. thiooxidans* when further oxidation of elemental sulfur is inhibited by N-ethylmaleimide (NEM). NEM was found to inhibit sulfur oxidation in *T. thiooxidans* (Kodama and Mori 1968).

In addition, 2-*n*-heptyl-4-hyroxyquinoline *N*-oxide (HQNO) is also used during the studies to provide further stoichiometric evidence for each step of the oxidation in order to establish the proposed pathway of sulfur and sulfite oxidation: $H_2S \rightarrow S \rightarrow H_2SO_3 \rightarrow H_2SO_4$ (Suzuki 1974). HQNO, an inhibitor of electron transfer at a b-type cytochrome in *T. thiooxidans* (Tano *et al.* 1982) was shown to specifically inhibit sulfite oxidation in *T. thiooxidans* (Suzuki *et al.* 1992).

Since few kinetic data are available on the aerobic biooxidation of sulfide at extremely low pH values in acidophilic thiobacilli, kinetic investigations of sulfide oxidation at different pH's were therefore studied in NEM-treated *T. thiooxidans*. Such data are of particular interest in the elimination of toxic hydrogen sulfide gas in industrial applications (Plas *et al.* 1992).

# MATERIALS AND METHODS

## *Organism*

*T. thiooxidans* (ATCC 8085) was grown on elemental sulfur for 5 days at 28°C with 2.5% (vol/vol) inoculum in Starkey's medium 1, with the following composition (per litre): 0.3 g $(NH_4)_2SO_4$, 3.5 g $KH_2PO_4$, 0.5 g $MgSO_4.7H_20$, 0.25 g $CaCl_2$ and 18 mg $FeSO_4.7H_20$ per liter and adjusted to pH 2.3 with sulfuric acid. One liter of medium was placed in a 2.8-L Fernbach flask and after autoclaving and inoculation, 50 g of powdered sulfur (BDH, precipitated sulfur) was spread evenly on the surface. The flask was incubated without shaking. After growth, sulfur was removed by filtration through a Whatman No. 1 filter paper under suction and cells were collected by centrifugation at 12,000 × *g* for 20 min. Cells were washed two times with, and suspended in, distilled water (adjusted to pH 3 with $H_2SO_4$) at a concentration of 20 mg wet cells per mL.

## *Preparation of "NEM-treated" Cells*

A suspension of *T. thiooxidans* (5 mL) at a concentration of 20 mg wet cells per mL in water (adjusted to pH 3 with $H_2SO_4$) was mixed with 1 mL of 0.1 M NEM solution, stirred for 15 min at room temperature, and centrifuged at 6000 × *g* for 10 min. The pellet was resupended in 5 mL of water (adjusted to pH 3 with $H_2SO_4$) and termed as "NEM-treated" *T. thiooxidans*.

## *Sulfide Oxidation*

Sodium sulfide solutions were prepared from water-cleaned crystals of $Na_2S.9H_2O$

under nitrogen atmosphere in tightly stoppered bottles. For short-term experiments, $O_2$ consumption was measured polarographically at 25°C in a Gilson Oxygraph with a Clark electrode using 1.2 mL reaction mixture. The reaction mixture in a total volume of 1.2 mL contained 1 mg wet (with or without NEM-treatment) cells of *T. thiooxidans* in 50 mM potassium phosphate (pH 2.3 or pH 6.5). The reaction was started by a microsyringe injection of sodium sulfide solution (0.2 M or 2 mM). HQNO, when indicated, was present at 20 $\mu$g in the 1.2 mL reaction mixture.

***Elemental Sulfur Determination***

Most of the reagents for the determination of elemental sulfur were prepared according to Bartlett and Skoog (1954). Acetone solvent was prepared by diluting 50 mL distilled water with acetone to 1 L. Sodium cyanide solution (0.1 g NaCN in 100 mL acetone solvent) and ferric chloride solution (0.4 g $FeCl_3.6H_2O$ in 100 mL acetone solvent) were prepared following their instructions. A standard sulfur solution was prepared either by dissolving 5 mg elemental sulfur (BDH, precipitated sulfur) in 10 mL petroleum ether (petroleum spirit, AnalaR, boiling point range 80-100°C, BDH Chemicals) or by dissolving 6.4 mg elemental sulfur in 10 mL dimethyl sulfoxide (DMSO) (Fisher Scientific Company; certified by the American Chemical Society). The amount of sulfur is expressed as micromoles per 32 $\mu$g sulfur, although strictly speaking, the units should be called microatoms, since the sulfur likely exists as $S_8$.

Elemental sulfur in the reaction mixture of bacterial sulfide oxidation (1.2 mL) was extracted with 2 mL petroleum ether by 15-20 s of vigorous vortex mixing

(Scientific Products S8220 deluxe mixer, maximum speed 60 Hz)) in a 25-mL Corex centrifuge tube with a screw cap (Corning 8446). The bottom aqueous layer, which was turbid due to bacteria, became clear after mixing and many small bubbles appeared in the top petroleum ether layer. The top layer became clear after centrifugation at 6,000 $\times$ *g* for 10 min, and 0.5 mL of the top layer was carefully removed for sulfur determination.

The top-layer sample was mixed with 1.5 mL of the sodium cyanide solution and the total volume was brought to 2.5 mL with the acetone solvent. After 2 min of the cyanolysis reaction, 0.5 mL of the mixture was withdrawn and mixed with 1 mL of the ferric chloride solution. The absorbance at 464 nm was measured within 10 min in a Hewlett Packard 8452A diode array spectrophotometer with a 1-cm light path cuvette against a blank prepared from 1.2 mL 50 mM potassium phosphate buffer by the same petroleum ether extraction, cyanolysis, and ferric chloride treatment as the experimental sample.

The standard sulfur solution in DMSO was used to prepare standard samples containing 0.1, 0.2, 0.3 and 0.5 $\mu$mol elemental sulfur in 1.2 mL 50 mM potassium phosphate buffer (pH 2.3 or pH 6.5). The sulfur came out of solution immediately upon addition to the aqueous buffer, producing slight turbidity, which disappeared after petroleum ether addition and vortex mixing. The experimental samples were treated in the same way as the reaction mixtures for sulfur extraction, cyanolysis and color development. The absorbance (A) at 464 nm increased linearly with the amount of sulfur and approximately followed the formula: A = (0.2 $\pm$ 0.01) $\times$ amount of sulfur (in

$\mu$mol) with fresh reagents.

### *Sulfite Determination*

Sulfite formed was determined by using the pararosaniline method according to West and Gaeke (1956) after removal of cells and sulfur by centrifugation.

# RESULTS

*Sulfur Extraction and Determination*

The original method of Bartlett and Skoog (1954) was scaled down to one tenth sample volume (0.5 mL) except that the volume of ferric chloride solution was reduced to only one fifth as described in Materials and Methods. The standard curve obtained with 2.5, 5.0, 7.5 and 10 $\mu$g elemental sulfur in 0.5 mL petroleum ether was linear (data not shown).

A series of samples containing known quantities of elemental sulfur in 1.2 mL 50 mM potassium phosphate buffer at pH 2.3 or pH 6.5 were extracted with petroleum ether by vigorous shaking in a vortex and the extracted sulfur in the petroleum ether phase was analyzed as described in Materials and Methods. The results shown in Fig. 1 indicate a linear relationship between the amount of sulfur and absorbance values. The absorbance values were identical to those values in the standard curve for sulfur obtained without extraction described above after correction for the dilution factor of 4. The petroleum ether extraction of elemental sulfur from an aqueous phase was considered to be nearly 100%.

The extraction efficiency of sulfur was also confirmed with 10 $\mu$L of the standard sulfur solution in petroleum ether (5 $\mu$g sulfur) in a Corex tube which was evaporated to dryness with a stream of $N_2$ gas. The addition of 1.2 mL 50 mM potassium phosphate buffer (pH 2.3) and petroleum ether extraction followed. The recovery was again close to 100%.

*Interferences*

Possible interference by other inorganic sulfur compounds on the elemental sulfur determination was studied at pH 2.3 and 7.0 (Table 1). At either pH, the interference is not serious considering the instability of sodium sulfide in air ($S^{2-} \rightarrow S^0$) and of thiosulfate at an acidic pH ($SSO_3^{2-} \rightarrow S^0 + SO_3^{2-}$), and a possible reaction of sulfite and sulfur at a neutral pH ($SO_3^{2-} + S^0 \rightarrow SSO_3^{2-}$). Table 2 shows that the presence of 1 mg wet cells of *T. thiooxidans* has no effect on the extraction efficiency of sulfur at 0.1 to 0.4 $\mu$mol in 1.2 mL.

Hydrophilic sulfur prepared from thiosulfate by acidification according to Roy and Trudinger (1970) could not be extracted appreciably with petroleum ether (data not shown). Hydrophilic sulfur is considered to be a form of elemental sulfur produced under certain conditions by sulfur-oxidizing bacteria that consists of long-chain polythionates (Steudel 1989) soluble only in polar organic solvents (e.g. acetone) and in water.

***Sulfide Oxidation by T. thiooxidans***

*T. thiooxidans* oxidize sulfide in three distinct stages of $O_2$ consumption at either pH 2.3 or 6.5 (Fig. 2). An initial rapid phase followed by a slower phase and finally by a very slow phase to complete the consumption of approximately two moles of $O_2$ for every mole of sulfide: $H_2S + 2O_2 \rightarrow H_2SO_4$. N-ethylmaleimide (NEM), which inhibited sulfur oxidation completely, reduced $O_2$ consumption with sulfide at either pH 2.3 or 6.5 to half a mole per mole of $Na_2S$, essentially leaving only the initial rapid phase intact and

eliminating the second and third phases (Fig. 2). The three phases were more clearly seen in Fig. 3. Cells were pre-treated with NEM in order to avoid the loss of sulfide due to chemical reaction with NEM. The total $O_2$ consumption with different amounts of sulfide as substrate for oxidation by NEM-treated *T. thiooxidans* at different pH values are shown in Table 3. The $O_2$ consumption (0.5 mol $O_2$ /mol of sulfide) agreed with the oxidation of sulfide to sulfur due to the inhibition of sulfur oxidation ($H_2S + ½O_2 \rightarrow S^0 + H_2O$). The optimum pH of sulfide oxidation with NEM-treated *T. thiooxidans* is shown in Fig. 4. The optimum pH of sulfide oxidation was 5.0. The kinetic constants ($K_m$ and $V_{max}$) at different pH of sulfide oxidation were obtained from the results in Fig. 5 and are summarized in Table 4. The $K_m$ values were between 8 and 43 $\mu$M over the range of pH from 2.5 to 7.5. These low values indicate that the sulfide-oxidizing system in *T. thiooxidans* has a high affinity to sulfide, especially at pH 7.0. This value is similar to the $K_m$ values reported by Moriarty and Nicholas (1969), 2 $\mu$M sulfide at pH 7.0.

The stoichiometry of sulfide oxidation to sulfur was established by extracting elemental sulfur with petroleum ether and analyzing the quantity as shown in Table 5. No sulfur was detected in the control experiments, but the reaction mixtures with NEM-treated cells contained approximately one mole of sulfur per mole of sulfide oxidized. The stoichiometry was confirmed with different amounts of sulfide as substrate for oxidation by NEM-treated cells (Table 5). The results agreed with the expected stoichiometry of the oxidation of sulfide to elemental sulfur with consumption of half a mole of $O_2$ per mole of sulfide.

The effect of HQNO on sulfide oxidation by *T. thiooxidans* is shown in Table 6 and Fig. 3. Sulfite accumulated in the reaction mixtures. The stoichiometry of $O_2$ consumption is about one and a half mole of oxygen consumed per mole of sulfide when the oxidation of sulfite is inhibited with HQNO. The stoichiometry of sulfite accumulated and sulfide is one mole sulfite per one mole sulfide.

## DISCUSSION

The original colorimetric method of Bartlett and Skoog (1954) was developed for the determination of elemental sulfur in hydrocarbons. Fliermans and Brock (1973) applied the method for soil samples from which sulfur was first extracted with carbon disulfide, a known solvent for elemental sulfur, for 12 hrs, before evaporation of carbon disulfide and dissociation of elemental sulfur in petroleum ether for the determination. Hazeu *et al.* (1988) applied a similar method, but used acetone for the overnight extraction of elemental sulfur from centrifuged cell pellets. The results presented in this paper show that the petroleum ether extraction of elemental sulfur directly from an aqueous phase and the measurement of extracted sulfur by the colorimetric procedure forms a sensitive and reliable analytical method for the quantitative determination of elemental sulfur in biological or abiological reaction systems. This method is applicable in any aqueous system and the procedures are simple and fast. There is no interference by other inorganic sulfur compounds including polythionates. This method was successfully applied to show that sulfide was oxidized by *T. thiooxidans* to elemental sulfur nearly stoichiometrically when further oxidation of elemental sulfur was inhibited by N-ethylmaleimide (Table 5).

Acidophilic thiobacilli are believed to oxidize sulfide to sulfate with sulfur and sulfite as intermediates: $S^{2-} \rightarrow S^0 \rightarrow SO_3^{2-} \rightarrow SO_4^{2-}$ (Suzuki 1974). *T. thiooxidans* oxidize elemental sulfur to sulfite when the oxidation of sulfite is inhibited with 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (Suzuki *et al.* 1992). In short-term Oxygraph experiments, *T. thiooxidans* in the presence of HQNO oxidized sulfide to sulfite. The results show

that sulfide is oxidized to sulfite with one and a half mole of $O_2$ consumed per mole of sulfide (Table 6). In the presence of HQNO at pH 2.3 the initial rapid phase was unaffected but the second phase was progressively inhibited with a considerable amount of $H_2S$ (43%) being recovered as sulfite. At pH 7.0, HQNO did not inhibit either the initial phase or second phase, but only decreased the total $O_2$ consumption. As much as 84% of $H_2S$ was recovered as sulfite. These results are in general agreement with the interpretation that the initial rapid phase of $O_2$ consumption in Fig. 2 corresponds to the oxidation stage: $H_2S + ½O_2 \rightarrow S + H_2O$ and the second slightly slower phase to the oxidation stage: $S + O_2 + H_2O \rightarrow H_2SO_3$ and the last slow phase to the final phase: $H_2SO_3 + ½O_2 \rightarrow H_2SO_4$. HQNO inhibition of the second phase at pH 2.3 is fully expected from the inhibitory effect of accumulated sulfite on sulfur oxidation (Suzuki *et al.* 1992). These phases are probably overlapping in time, with some sulfur and sulfite oxidations taking place in the initial phase also.

The optimum pH of the sulfide oxidation was 5. The low $K_m$ values indicate that this sulfide-oxidizing enzyme system in the cells has a high affinity for the substrate and therefore, lower sulfide concentrations than those used in Fig. 5 are necessary to obtain more accurate $K_m$ and $V_{max}$ values at different pH values.

The results in this paper not only establish the stoichiometric oxidation of sulfide to sulfur in *T. thiooxidans* when further oxidation of sulfur is inhibited by NEM, but also a further oxidation to sulfite (in the presence of HQNO) before its final oxidation to sulfate (without inhibitor).

## REFERENCES


1. Bartlett, J.K. and D.A. Skoog. 1954. Colorimetric determination of elemental sulfur in hydrocarbons. Anal. Chem. **26**: 1008-1011.

2. Fliermans, C.B. and T.D. Brock. 1973. Assay of elemental sulfur in soil. Soil Sci. **115**: 120-122.

3. Hazeu, W., W.H. Batenburg-van der Vegte, P. Bos, R.K. Van der Pas and J.G. Kuenen. 1988. The production and utilization of intermediary elemental sulfur during the oxidation of reduced sulfur compounds by *Thiobacillus ferrooxidans*. Arch. Mikrobiol. **150**: 574-579.

4. Kodama, A and T. Mori. 1968. Studies on the metabolism of a sulfur-oxidizing bacterium V. comparative studies on sulfur and sulfite oxidizing systems of *Thiobacillus thiooxidans*. Plant & Cell Physiol. **9**: 725-734.

5. Kuenen, J.G., J.T. Pronk, W. Hazeu, R. Meulenberg and P. Bos 1993 A review of bioenergetics and enzymology of sulfur compound oxidation by acidophilic thiobacilli. *In* Biohydrometallurgical Technologies. *Edited* by Torma, A.E., M.L. Apel and C.L. Brierley. The Minerals, Metals & Materials Society.

6. London, J. and S.C. Rittenberg. 1964. Path of sulfur in sulfide and thiosulfate oxidation by thiobacilli. Proc. Natl. Acad. Sci. (U.S.A.) **52**: 1183-1190.

7. Moriarty, D.J.W. and D.J.D. Nicholas. 1969. Enzymatic sulfide oxidation by *Thiobacillus concretivorus*. Biochim. Biophys. Acta. **184**: 114-123.

8. Moriarty, D.J.W. and D.J.D. Nicholas. 1970a. Products of sulfide oxidation in extracts of *Thiobacillus concretivorus*. Biochim. Biophys. Acta. **197**: 143-151.

9. Moriarty, D.J.W. and D.J.D. Nicholas. 1970b. Electron transfer during sulphide and sulfite oxidation by *Thiobacillus concretivorus*. Biochim. Biophys. Acta. **216**: 130-138.

10. Parker, C.D. and J. Prisk. 1953. The oxidation of inorganic compounds of sulfur by various sulfur bacteria. J. Gen. Microbiol. **8**: 244-268.

11. Perry, K.A., J.E. Kostka, G.W. Luther III and K.H. Nealson. 1993.

Mediation of sulfur speciation by a black sea facultative anaerobe. Science. **259**: 801-803.

12. Pivovarova, T.A. and R.S. Golovacheva. 1985. Microorganisms important for hydrometallurgy, cytology, physiology and biochemistry. *In*: Biogeotechnology of metals, Karavaiko, G.I., and S.N. Groudev. (eds), Centre of international projects, G.K.N.T., Moscow p.27.

13. Plas, C., H. Harant, D. Danner, E. Jelinek, K. Wimmer, P. Holubar and R. Braun. 1992. Ratio of biological and chemical oxidation during the aerobic elemination of sulphide by colourless sulphur bacteria. Appl. Microbiol. Biotechnol. **36**: 817-822.

14. Pronk, J.T., R. Meulenberg, W. Hazeu, P. Bos and J.G. Kuenen. 1990. Oxidation of reduced inorganic sulphur compounds by acidophilic thiobacilli. FEMS. Microbiol. Rev. **75**: 293-306.

15. Roy, A.B. and P.A. Trudinger. 1970. The biochemistry of inorganic compounds of sulfur. Cambridge University Press, London.

16. Steudel, R. 1989. On the nature of the "elemental sulfur" ($S^0$) produced by sulfur-oxidizing bacteria - a model for $S^0$ globules, p. 289-303. *In* Schlegel, H.G. and B. Bowien. (ed.), Autotrophic Bacteria. Science Tech. Publishers, Madison, WI.

17. Suzuki, I. 1974. Mechanisms of inorganic oxidation and energy coupling. Annu. Rev. Microbiol. **28**: 85-101.

18. Suzuki, I., C.W. Chan, and T.T. Takeuchi. 1992. Oxidation of Elemental Sulfur to Sulfite by *Thiobacillus thiooxidans* cells. Appl. and Environ. Microbiol. **58**: 3767-3769.

19. Suzuki, I., C.W. Chan and T.L. Takeuchi. 1994. Oxidation of inorganic sulfur compounds by thiobacilli. *In:* Environmental Geochemistry of sulfide Oxidation. *Edited* by Alpers, C.N. and D.W. Blowes. Amer. Chem. Soc., Washington D.C. pp.60-67.

20. Tano, T., T. Ito, H. Takesue, T. Sugio and K. Imai. 1982 B-type cytochrome, an electron carrier in the sulfite-oxidation system of *Thiobacillus thiooxidans*. J. Ferment. Technol. **60**: 181-187.

21. Vishniac, W. and M. Santer. 1957. The thiobacilli. Bacteriol. Rev. **21**: 175-213.

22. West, P.W., and G.C. Gaeke. 1956. Fixation of sulfur dioxide as disulfitomercurate (II) and subsequent colorimetric estimation. Anal. Chem. **28**: 1816-1819.

# TABLES

Table 1. **The effect of inorganic sulfur compounds on the extraction and determination of elemental sulfur.**

| Compound[a] | Amount ($\mu$mol) | Absorbance at 464 nm | | | |
|---|---|---|---|---|---|
| | | pH 2.3 | | pH 7.0 | |
| | | $-S^0$ | $+S^0$ | $-S^0$ | $+S^0$ |
| $S^0$ | 0.20 | - | 0.040 | - | 0.046 |
| $Na_2S$ | 0.20 | 0.007 | 0.044 | 0.005 | 0.042 |
| $Na_2SO_3$ | 0.20 | 0.000 | 0.039 | 0.003 | 0.036 |
| $Na_2S_2O_3$ | 1.00 | 0.008 | 0.037 | 0.002 | 0.043 |
| $Na_2S_4O_6$ | 1.00 | 0.002 | 0.044 | 0.002 | 0.043 |

[a] Compound was present in 1.2 mL of 50 mM potassium phosphate buffer at pH 2.3 or 7.0 with or without 0.2 $\mu$mol elemental sulphur. Addition of petroleum ether, capping, and vortexing started immediately after the addition of these compounds to minimize loss or interaction with sulfur.

Table 2. **The effect of bacterial cells on the extraction and determination of elemental sulfur.**

| Sulfur[a] ($\mu$mol) | Absorbance at 460 nm | |
|---|---|---|
| | - Cells | + Cells |
| 0.0 | - | 0.000 |
| 0.1 | 0.018 | 0.020 |
| 0.2 | 0.041 | 0.040 |
| 0.3 | 0.060 | 0.057 |
| 0.5 | 0.094 | 0.099 |

[a] Different amounts of sulfur were present in 1.2 mL of 50 mM potassium phosphate (pH 2.3) with and without 1 mg wet cells. Extraction of sulfur was carried out immediately after cell addition to minimize the oxidation.

Table 3. **The effect of pH on the total oxygen consumption in sulfide oxidation by *T. thiooxidans*[a].**

| $Na_2S$ ($\mu$mol) | pH | | | | | |
|---|---|---|---|---|---|---|
| | 2.5 | 3.5 | 5.0 | 6.5 | 7.0 | 7.5 |
| 0.1 | 0.036 | 0.058 | 0.056 | 0.062 | 0.064 | 0.068 |
| 0.2 | 0.096 | 0.106 | 0.104 | 0.110 | 0.120 | 0.122 |
| 0.3 | 0.148 | 0.136 | 0.138 | 0.168 | 0.160 | 0.152 |
| 0.4 | 0.190 | 0.200 | 0.19 | 0.194 | 0.196 | 0.234 |

[a] Assay conditions are described in Materials and Methods. $O_2$ used is shown in $\mu$mol.

Table 4. **Summary of kinetic constants of sulfide oxidation by NEM-treated *T. thiooxidans* at different pH[a].**

| pH | $K_m$ (mM) | $V_{max}$ (nmol $O_2$ /min) |
|---|---|---|
| 2.5 | 0.043 | 55.83 |
| 3.5 | 0.027 | 63.90 |
| 5.0 | 0.033 | 71.96 |
| 6.5 | 0.013 | 40.08 |
| 7.0 | 0.008 | 17.02 |
| 7.5 | 0.019 | 9.81 |

[a] Assay conditions are described in Materials and Methods. Experimental data from Fig. 5 were used to obtain $K_m$ and $V_{max}$ values.

Table 5. Stoichiometry of sulfide oxidation by NEM-treated *T. thiooxidans*[a].

| NEM | $Na_2S$ ($\mu$mol) | pH 2.3 | | pH 6.5 | |
|---|---|---|---|---|---|
| | | $S^0$ formed ($\mu$mol) | $O_2$ used ($\mu$mol) | $S^0$ formed ($\mu$mol) | $O_2$ used ($\mu$mol) |
| – | 0.10 | 0.00 | 0.196 | 0.00 | 0.190 |
| + | 0.10 | 0.10 | 0.056 | 0.09 | 0.054 |
| + | 0.20 | 0.19 | 0.094 | 0.21 | 0.112 |
| + | 0.30 | 0.29 | 0.142 | 0.30 | 0.152 |
| + | 0.40 | 0.38 | 0.188 | 0.37 | 0.190 |

[a] The conditions were the same as in Fig. 2 except that the amount of $Na_2S$ was varied. The reaction time was 5 min for NEM-treated cells and 15 min for untreated cells.

Table 6. **Effect of HQNO on sulfide oxidation by *T. thiooxidans*[a].**

| | pH 2.3 | | pH 7.0 | |
|---|---|---|---|---|
| | -HQNO | +HQNO | -HQNO | +HQNO |
| $O_2$ consumed ($\mu$mol) | 0.190 | 0.135 | 0.170 | 0.135 |
| Sulfite formed ($\mu$mol) | 0.004 | 0.043 | 0.030 | 0.084 |
| Reaction time (min) | 5.0 | 8.0 | 4.0 | 4.0 |

[a] Reaction conditions were described in Materials and Methods except that 20 $\mu$g HQNO and 0.1 $\mu$mol of $Na_2S$ were used as substrate.

# FIGURES

Figure 1. The calibration curve of sulfur extraction.

A sulfur solution in DMSO (20 mM) was used to prepared standard sulfur samples in 1.2 mL 50 mM potassium phosphate buffer (pH 2.3) for petroleum ether extraction, cyanolysis and color development as described in Materials and Methods. The results were identical at pH 6.5.

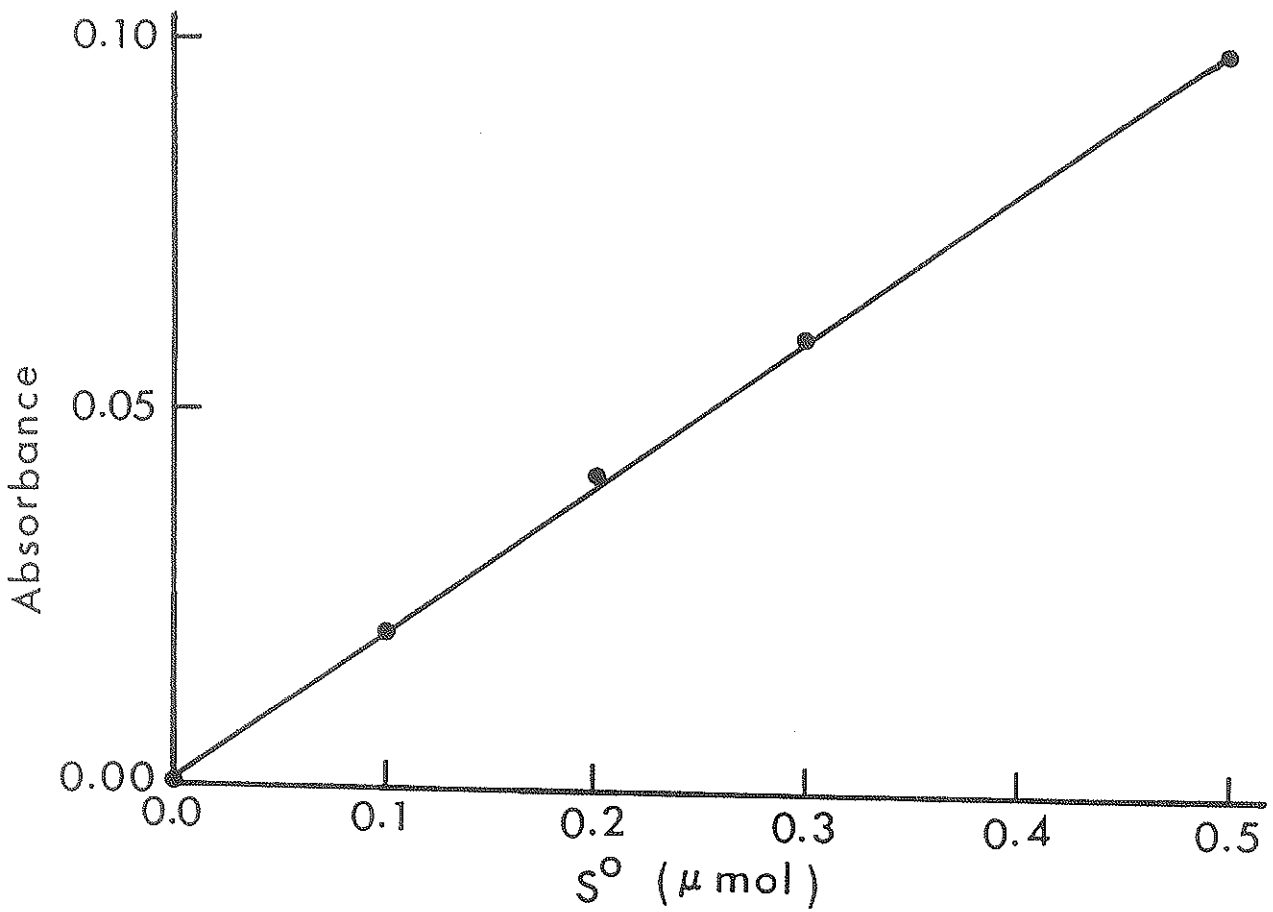

0.10
0.05
0.00
Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
S° (μ mol)

Figure 2. **Effect of *T. thiooxidans* with and without NEM-treatment on the $O_2$ consumption of sulfide oxidation.**

**The $O_2$ consumption was followed in a Gilson Oxygraph at 25°C with 1.2 mL reaction mixtures: 50 mM potassium phosphate buffer (pH 2.3 or 6.5), 1 mg wet cells. The reaction was initiated with the addition of 0.1 $\mu$mol $Na_2S$ solution as indicated by an arrow.**

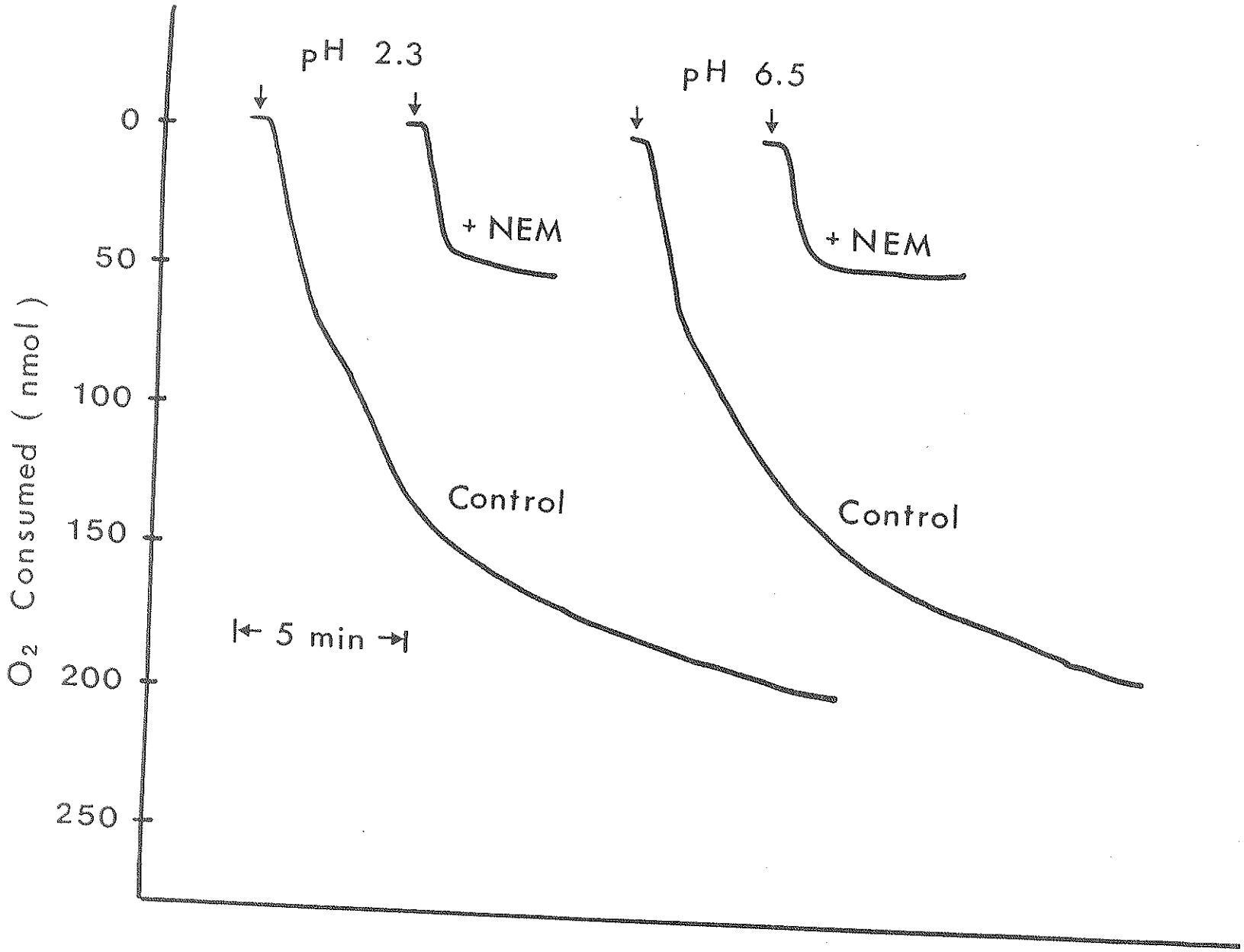

pH 2.3
pH 6.5
+ NEM
+ NEM
Control
Control
0
50
100
150
200
250
$O_2$ Consumed ( nmol )
5 min

Figure 3. Effect of NEM and HQNO on the $O_2$ consumption in sulfide oxidation by *T. thiooxidans*.

The $O_2$ consumption was followed in a Gilson Oxygraph at 25 °C with 1.2 mL reaction mixtures: 50 mM potassium phosphate buffer (pH 2.3 or 7.0), 1 mg wet cells, and 20 $\mu$g HQNO or 1 $\mu$mol NEM was present when indicated. The reaction was started with the addition of 0.5 $\mu$L of 0.2 M $Na_2S$ (anaerobic) as indicated by an arrow.

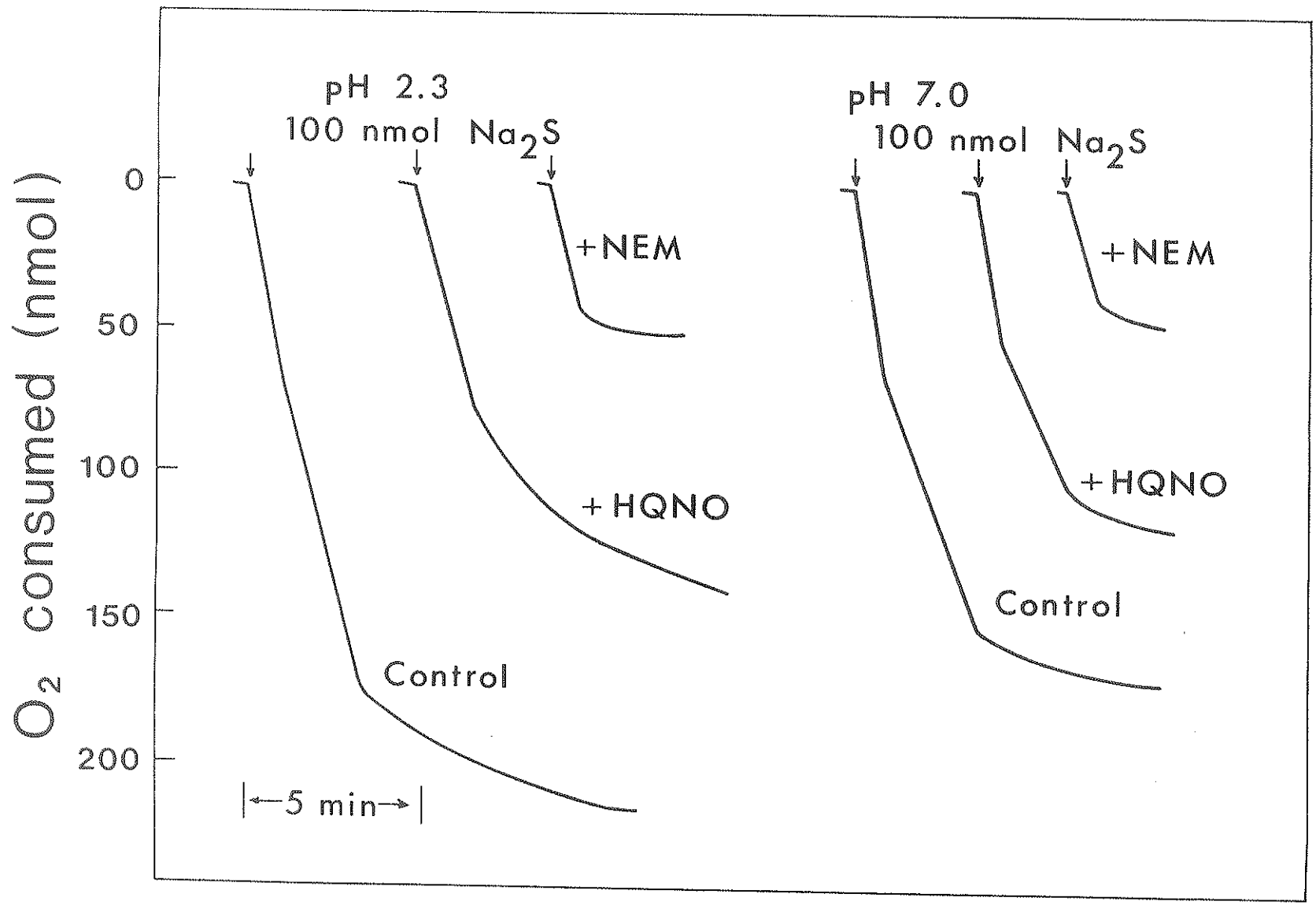

pH 2.3
100 nmol Na2S
+NEM
+HQNO
Control
pH 7.0
100 nmol Na2S
+NEM
+HQNO
Control
O2 consumed (nmol)
0
50
100
150
200
5 min

Figure 4. Effect of pH on sulfide oxidation by NEM-treated *T. thiooxidans*.

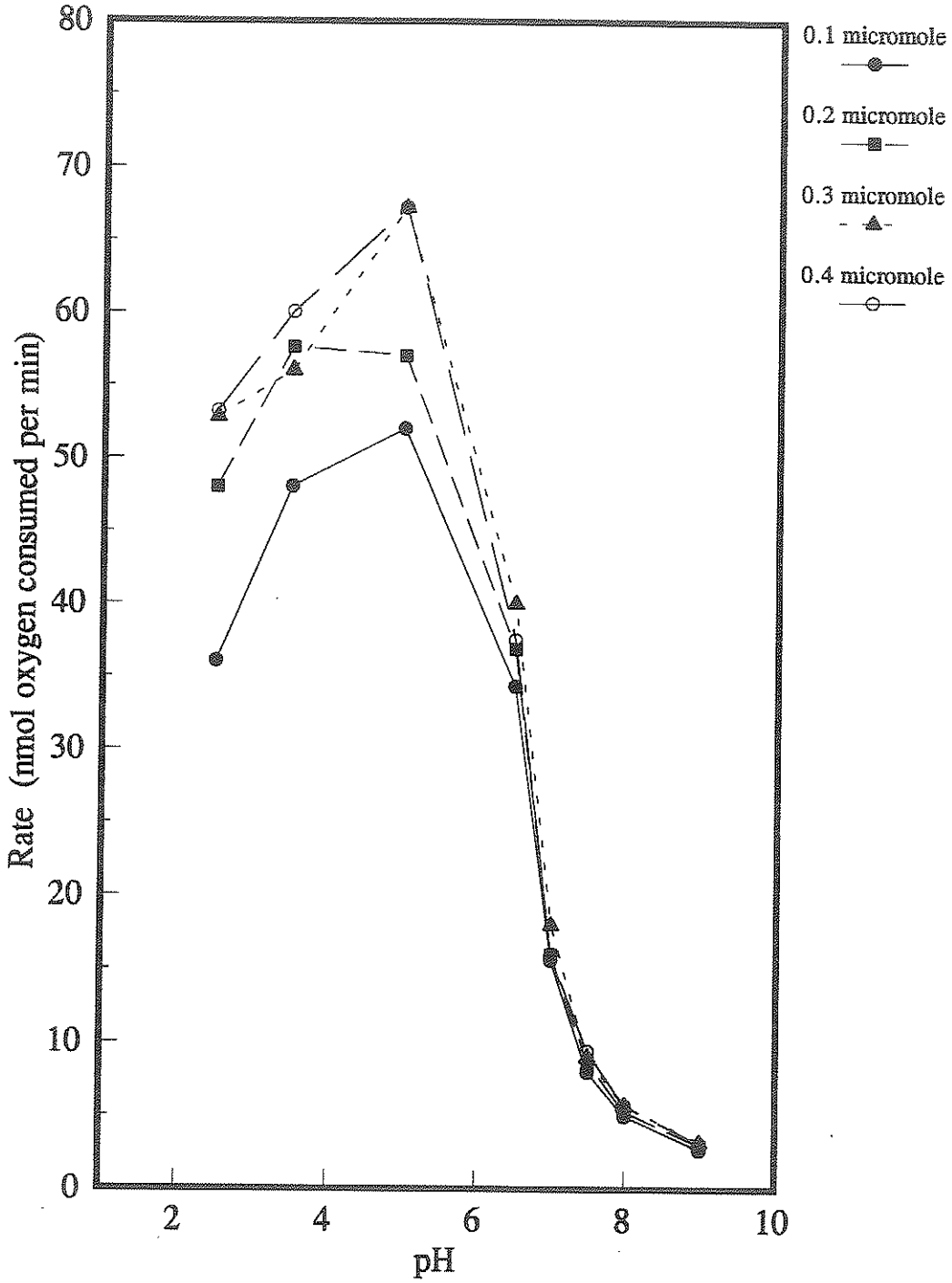
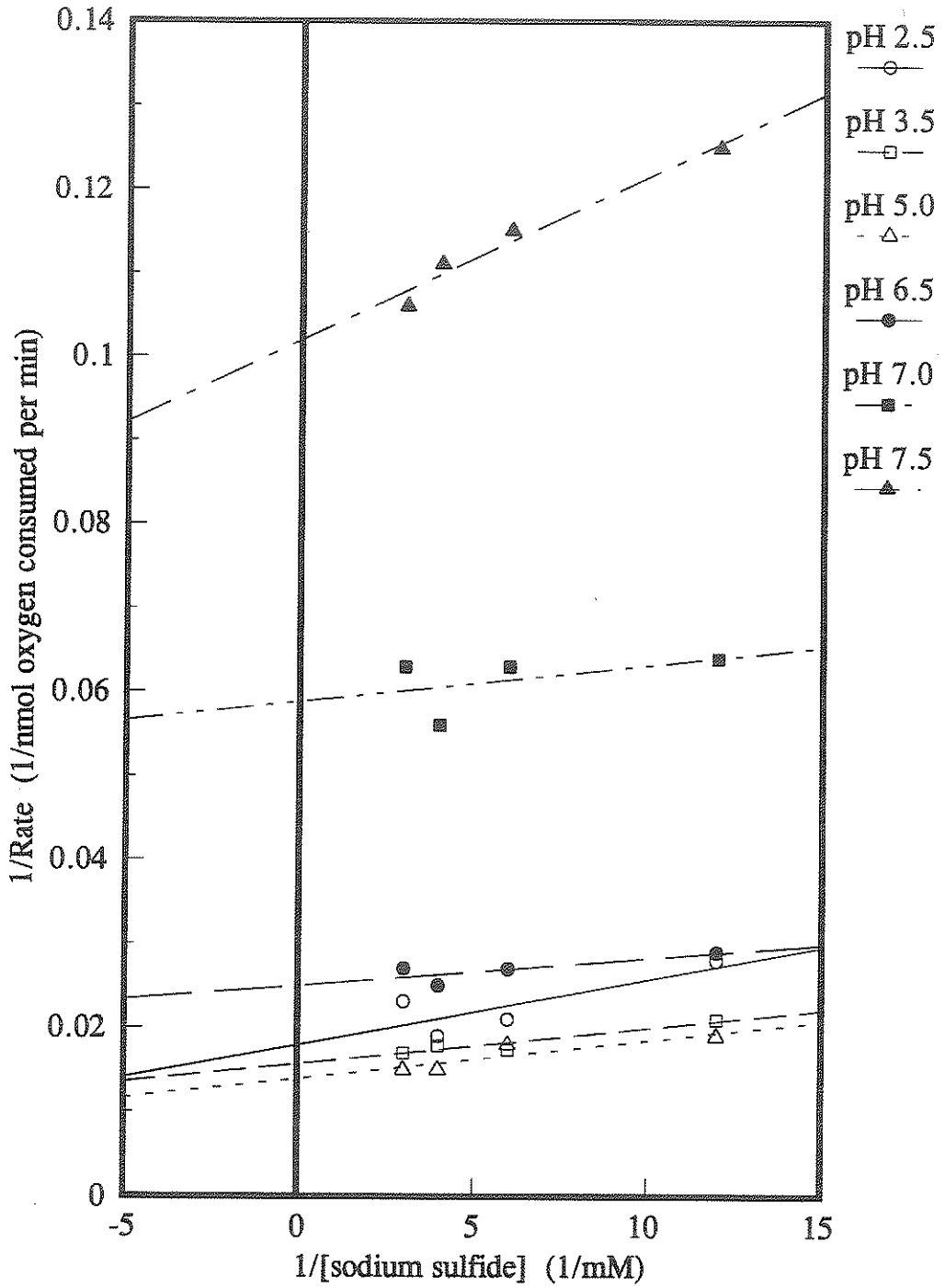

80
70
60
50
40
30
20
10
0
Rate (nmol oxygen consumed per min)
2
4
6
8
10
pH
0.1 micromole
0.2 micromole
0.3 micromole
0.4 micromole

Figure 5. Double reciprocal plot of sulfide oxidation by NEM-treated *T. thiooxidans*.


0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1/Rate (1/nmol oxygen consumed per min)
-5
0
5
10
15
1/[sodium sulfide] (1/mM)
pH 2.5
pH 3.5
pH 5.0
pH 6.5
pH 7.0
pH 7.5

# PART THREE

## Thiosulfate oxidation by sulfur-grown *Thiobacillus thiooxidans*, cell-free extracts and thiosulfate-oxidizing enzyme.

## ABSTRACT

Oxidation of thiosulfate by *Thiobacillus thiooxidans* grown on sulfur was studied in intact cells and cell-free extracts. Thiosulfate was oxidized to tetrathionate by cells treated with N-ethylmaleimide (NEM) at a pH optimum of 2.3. Cell-free extracts also oxidized thiosulfate at the same pH optimum. Tetrathionate oxidation by *T. thiooxidans* was completely inhibited by NEM. The pH optimum of the tetrathionate oxidation was 3.0. Both thiosulfate and tetrathionate were oxidized to sulfite level in the presence of HQNO. The cells with or without NEM-treatment showed two $K_m$ for thiosulfate while the cell-free system showed only one $K_m$. The $K_m$ for thiosulfate generally increased with increasing pH. A soluble thiosulfate-oxidizing enzyme system with an optimum pH around 2 was extracted from intact cells at pH 2.5 in the presence of 1 M ammonium sulfate by passage through a French pressure cell. A native cytochrome c which was reduced by thiosulfate at pH 2.5, and a compound which reduced horse heart cytochrome c at a neutral pH but required sulfite at an acidic pH were also found in these acidic extracts.

## INTRODUCTION

Thiosulfate is oxidized by all species of Thiobacillus. The present knowledge of the metabolic pathway of thiosulfate in *Thiobacillus thiooxidans* is very limited because the oxidative process occurs at an acidic pH. Below pH 4, thiosulfate rapidly decomposes in a complex manner, yielding different products according to the acidity of the solution. Decomposition of thiosulfate involves nucleophilic displacement of sulfite ions resulting in the formation of sulfur and bisulfite. Therefore, it is difficult to determine whether the cells are oxidizing thiosulfate or one of its decomposition products.

Most of the reported studies on the metabolism of thiosulfate concentrated on neutrophilic thiobacilli. Three different but not mutually exclusive mechanisms of thiosulfate oxidation were observed depending on the species of *Thiobacillus*. The first of these mechanisms involved the formation of elemental sulfur and sulfate but it was difficult to obtain satisfactory stoichiometry between the disappearance of thiosulfate and the formation of elemental sulfur and sulfate. The second proposed mechanism involved polythionates as intermediate compounds since tetrathionate was readily detected in growing cultures. The third observed mechanism proposed that the thiosulfate was cleaved to colloidal sulfur and sulfite which were then oxidized to sulfate via the sulfur-oxidizing and sulfite-oxidizing systems. Initially, the polythionate pathway was the preferred model but since then, considerable evidence accumulated in support of the first and third mechanisms. As more and more studies were done in this area, it became more evident that these pathways might not be necessarily mutually exclusive.

Little information is yet available on the mechanism of thiosulfate oxidation in acidophilic thiobacilli. In early studies with *Thiobacillus ferrooxidans*, Sinha and Walden (1966) reported that thiosulfate in the presence of thiol-binding reagents was oxidized to sulfate and trithionate through tetrathionate as an intermediate which was identified from chromatographic analysis. Tano *et al.* (1968) found that thiosulfate was oxidized to tetrathionate and an unknown oxidized sulfur compound by a cell-free particulate preparation of *T. thiooxidans*. Goodman and Ralph (1980) described a heat output in microcalorimetric studies characterized by three maxima during thiosulfate oxidation by *T. thiooxidans*. Due to the fact that thiosulfate was exhausted after the second maximum, they assumed a transient accumulation of polythionates. A thiosulfate-oxidizing enzyme was purified about 250-fold from sulfur-grown *T. ferrooxidans* by Silver and Lundgren (1968). The stoichiometry of the reaction was shown to be

$$2Na_2S_2O_3 + 2Na_3Fe(CN)_6 \rightarrow Na_2S_4O_6 + 2Na_4Fe(CN)_6 \qquad \text{[Equation 1]}$$

with an optimum pH of 5.0 in acetate buffer, and $K_m$ of $9x10^{-4}$ M for thiosulfate. Recently, this kind of ferricyanide-dependent thiosulfate-oxidizing enzyme was also purified to homogeneity from cell-free extracts of acidophilic sulfur oxidizer, *Thiobacillus acidophilus* by Meulenberg *et al.* (1993a). In the presence of ferricyanide, the optimum pH for the enzyme activity was found to be 3. Recently, Shihari *et al.* (1993) proposed that thiosulfate was the alternate substrate for unattached growing *T. ferrooxidans* in the medium growing on elemental sulfur. Chemotactic response of *T. ferrooxidans* to thiosulfate was also observed (Chakraborty *et al.* 1992). Thus, the physiological role of thiosulfate metabolism in acidophilic thiobacilli becomes

increasingly more important.

*T. thiooxidans* grows well on elemental sulfur under acidic conditions producing sulfuric acid. The sulfur-grown cells can oxidize sulfide to sulfate with sulfur and sulfite as possible intermediates (Suzuki *et al.* 1994). These intermediates accumulate when their oxidation is inhibited, thus sulfide is oxidized to sulfur when the oxidation of sulfur is inhibited by N-ethylmaleimide (NEM) (Chan and Suzuki 1993) and sulfur is oxidized to sulfite when the oxidation of sulfite is inhibited by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) (Suzuki *et al.* 1992). Sulfite accumulation is sometimes observed even in the absence of inhibitors in *T. ferrooxidans* during sulfur oxidation (Sugio *et al.* 1987). The accumulation of sulfite could lead to the formation of thiosulfate in the presence of sulfur ($S^0 + SO_3^{2-} \rightarrow S_2O_3^{2-}$) (Imai *et al.* 1962; Suzuki and Silver 1966) and in fact, thiosulfate has been detected in the medium of *T. ferrooxidans* growing on elemental sulfur (Shihari *et al.* 1993).

The above groups of researchers (Sinha and Walden 1966; Silver and Lundgren 1968; Tano *et al.* 1968; Goodman and Ralph 1980 and Meulenberg *et al.* 1993a) had identified tetrathionate as an intermediate in the metabolism of thiosulfate by acidophilic thiobacilli, and various routes had been suggested for the tetrathionate pathway depending on the species of acidophilic thiobacilli. The first one involved the action of tetrathionate hydrolase which catalyzed the stoichiometric conversion of tetrathionate to thiosulfate, sulfur and two protons and presumably sulfate in the cell-free extract of *T. acidophilus* (Meulenberg *et al.* 1993b). The second one, suggested by Suzuki *et al.* (1994), proposed that tetrathionate oxidation involved the oxidation of both sulfane (-S-) and sulfonic acid

($-SO_3H$) portions by the enzymes involved in sulfur- and sulfite oxidizing systems. So far, there is little information on the oxidation of tetrathionate by *T. thiooxidans* in the literature. London and Rittenberg (1964) found that tetrathionate was oxidized completely to sulfate with an oxygen consumption of 3½ μmole $O_2$ per μmole tetrathionate by *T. thiooxidans* extract. Furthermore working with crude extracts of *T. thiooxidans*, Okuzumi (1966) reported that tetrathionate was first dismutated to trithionate and pentathionate, and then further oxidized to elemental sulfur and sulfate.

The purpose of this study is to dissect the oxidative pathway of thiosulfate metabolism in *T. thiooxidans* using two different metabolic inhibitors: NEM and HQNO. Characterization of thiosulfate metabolism in *T. thiooxidans* involved the use of intact cells, cell-free extracts and a soluble fraction extracted from the cells at pH 2.5 in the presence of 1 M ammonium sulfate by passage through a French pressure cell according to the unusual acid treatment protocol of Meulenberg *et al.* (1992). Since tetrathionate can possibly be formed from thiosulfate either by autooxidation or via chemical reactions with intracellular ions, autooxidation of thiosulfate and chemical reaction of thiosulfate with ferric ions are undertaken for comparatives studies.

## MATERIALS AND METHODS

### *Organisms and Growth Conditions*

**Tt-1 (*T. thiooxidans*, ATCC 8085), SM4-HEC-$S^0$ (*T. ferrooxidans*, a mine isolate, Lizama and Suzuki 1988) and Tf-2-$S^0$ (*T. ferrooxidans*, ATCC 19859)**

The organisms were grown on elemental sulfur for 5 days at 28°C with 2.5% (vol/vol) inoculum in Starkey's medium 1: 0.3 g $(NH_4)_2SO_4$, 3.5 g $KH_2PO_4$, 0.5 g $MgSO_4.7H_2O$, 0.25 g $CaCl_2$ and 18 mg $FeSO_4.7H_2O$ per litre and adjusted to pH 2.3 with sulfuric acid. One litre of medium was placed in a 2.8-litre Fernbach flask and after autoclaving and inoculation, 50 g of powdered sulfur (BDH, precipitated sulfur) were spread evenly on the surface. The flask was incubated without shaking. After growth, sulfur was removed by filtration through a Whatman 1 filter paper under suction and cells were collected by centrifugation at 12,000 x *g* for 20 min. The colors of the pellet of Tt-1, SM4-HEC-$S^0$ and Tf-2-$S^0$ were milky-white, grayish-white and yellow respectivity. Cells were washed two times with and then suspended in distilled water (pH 3 with $H_2SO_4$) at a concentration of 20 mg wet cells per mL.

### *Thiobacillus thioparus* (DMS 505)

The culture of *T. thioparus* (DMS 505) was grown on Starkey's medium 2. The medium composition was: 10 g $Na_2S_2O_3.5H_2O$, 4.0 g $K_2HPO_4$, 4.0 g $KH_2PO_4$, 0.3 g $(NH_4)_2SO_4$, 0.014 g $CaCl_2$, 0.05 g $MgSO_4$, 0.014 g $MnSO_4.H_2O$, 0.02 g $FeCl_3.6H_2O$, in 1 litre distilled water. A solution of $Na_2S_2O_3.5H_2O$ (10.0 g) was autoclaved separately and added aseptically just prior to inoculation. The maintenance of the stock culture, the

harvesting of cells and the removal of elemental sulfur were as described previously by Lyric and Suzuki (1970). The color of the cell pellet was pink. Cells were washed two times with and suspended in distilled water without pH adjustment at a concentration of 20 mg wet cells per mL.

*Preparation of NEM-treated Tt-1, SM4-HEC-S°, Tf-2-S° and T. thioparus Cells*

Five milliliters of each thiobacillus cell type, at a concentration of 20 mg wet cells per mL in distilled water, were mixed with 1 mL of 0.1M NEM solution, stirred for 15 min at room temperature and centrifuged at 6,000 x *g* for 10 min. The pellets of the cells were resuspended in 5 mL of distilled water (pH 3 with $H_2SO_4$) except that of *T. thioparus* which was resuspended in distilled water with no pH adjustment. These cells were termed as "NEM-treated" thiobacillus cells.

*Preparation of the Acidic Extracts of T. thiooxidans*

The procedure described for trithionate hydrolase isolation (Meulenberg et al. 1992) was followed. Harvested cells of *T. thiooxidans* were washed and resuspended in a buffer containing 25 mM potassium phosphate and 1.0 M ammonium sulfate at pH 2.5 at a concentration of 600 mg wet cells/mL. The cell suspension was passed three times through a French pressure cell at 110 MPa and centrifuged for 20 min at 48,000 x *g* to remove the bulk of cell debris. The supernatant was further centrifuged for 120 min at 131,000 x *g*. The resulting reddish-brown supernatant was termed "acidic extracts".

*Preparation of Cell-Free Extracts of T. thiooxidans*

Harvested cells of *T. thiooxidans* were washed once with 0.5 M Tris-HCl buffer (pH 7.8), suspended at a cell concentration of 20 mg/mL in the same buffer and stirred overnight at 4°C. This eliminated the necessity of the treatment of the cells with ion-exchange resins as was done by Suzuki (1965) to facilitate rupture of *T. thiooxidans*. The cell suspension was centrifuged for 20 min at 12,000 x *g*, and the pellet reconstituted as a 15% (W/V) suspension in 0.5 M Tris-HCl buffer (pH 7.8). This cell suspension was passed three times through a French pressure cell at 110 MPa and then centrifuged for 20 min at 48,000 x *g* to remove the bulk of cell debris. The supernatant was dialyzed against distilled water to remove the buffer before its use as a cell-free extracts for thiosulfate oxidation. This supernatant was also centrifuged for 120 min at 131,000 x *g* to separate a soluble brownish fraction from a particulate fraction. This brownish fraction was named "spinco supernatant". Selective protein precipitation using ammonium sulfate was carried out as described by Schleif and Wensink (1981). After ammonium sulfate precipitation and centrifugation, the pellet was resuspended in 0.1 M pH 6.5 sodium phosphate buffer. The suspended sample was dialyzed against the same phosphate buffer at 4°C overnight in order to remove the ammonium sulfate.

*Colorimetric Determination of Polythionate*

**Cold cyanolysis**

Tetrathionate was determined colorimetrically after cyanolysis by the method of Sörbo (1958). To 1.2 mL reaction mixture, 2 mL of 0.2 N $NH_4OH$ and 0.5 mL of 0.1

M KCN were added. After 30 min incubation for cold cyanolysis at room temperature, the mixture was centrifuged to remove cells. To the supernatant, 0.5 mL of ferric nitrate reagent [10% $Fe(NO_3)_3.9H_2O$ in 13% $HNO_3$] was added to the final reaction mixture. After an equilibrium time of 15 min, the intensity of color was determined at 460 nm against a control cuvette containing all the reaction components except for the substrate and cells and then compared to a standard curve prepared with different known amounts of thiocyanate.

**Hot cyanolysis**

The procedure was described by Wood (1982). A reaction mixture (1 mL) was added to the solution containing 2.0 mL of 0.2 N $NH_4OH$ and 0.7 mL of 0.1 M KCN and heated in a boiling water bath for 30 min. After cooling and centrifugation, 0.5 mL of ferric nitrate reagent [10% $Fe(NO_3)_3$ in 13% $HNO_3$] was added for color development. The absorbance was determined at 460 nm against an unheated blank containing all the reaction components except the substrate and cells and then compared to a standard curve prepared with different known amounts of thiocyanate.

*Assay of Thiosulfate and Tetrathionate Oxidation Activity*

Thiosulfate oxidation by cells and cell-free extracts was followed by measuring the consumption of oxygen in a Gilson Oxygraph with a Clark Oxygen Electrode at 25°C. The reaction mixture in a total volume of 1.2 mL contained 6 mg wet cells or 8 mg protein (cell-free extracts), 50 mM potassium phosphate buffer of various pH values and various amounts of thiosulfate (1 $\mu$mol for the standard assay). The oxidation rate

was expressed as nmol $O_2$ consumed per min. Tetrathionate oxidation was followed in the same manner except that thiosulfate was replaced with the amount of tetrathionate indicated.

*Assay of Thiosulfate:Ferricyanide Oxidoreductase Activity*

Thiosulfate:ferricyanide oxidoreductase was assayed by the reduction of ferricyanide in a modification of the method by Trudinger (1961). The reaction mixture in a total volume of 1.0 mL contained, unless otherwise indicated, 50 $\mu$mol potassium phosphate buffer (pH 6.0), 1 $\mu$mol potassium ferricyanide, 5 $\mu$mol sodium thiosulfate and various amounts of acidic extract. With a standard amount of acidic extract (0.1 mL) the final pH of the reaction mixture was 4.3. The pH study was always expressed as the final pH. The reaction was started by the addition of thiosulfate, and the absorbance change at 420 nm was followed in a Hewlett Parkard 8452A diode array spectrophotometer with a 1-cm ligh path cuvette. The initial linear rate of $\Delta A_{420nm}$ per min was used as activity. The reduction of native cytochrome c was followed either at 416 nm or 550 nm.

***Sulfite Determination***

A 1.2 mL sample of the reaction mixture was withdrawn and centrifuged at 6,000 x *g* for 10 min, and the concentration of sulfite in the supernatant solution thus obtained was determined spectrophotometrically by the pararosaniline method (West and Gaeke 1956). A solution of 0.1 M $Na_2SO_3$ was prepared in 50 mM disodium EDTA.

*Protein Determination*

Protein was determined routinely as described by Lane (1957), following the absorptions at 280 nm and 260 nm, but the results were periodically confirmed with the method of Bradford (1976).

*Assay of the Chemical Reaction of Thiosulfate with Ferric Ion*

Chemical oxidation of thiosulfate with and without ferric ion was studied at various concentrations of thiosulfate and ferric ion at pH 1.8, 2.3 and 3.0 in 0.1 M $\beta$-alanine-$H_2SO_4$ buffer in a total volume of 2 mL. The fixed concentration of reactant was 1 mM for either thiosulfate or ferric ion when the other reactant concentration was varied. Samples were taken for tetrathionate (cold cyanolysis) or ferrous ion determination.

*Ferrous Ion Determination*

Ferrous ions were determined colorimetrically by a modification of the O-phenanthroline method (Suzuki *et al.* 1990). 1 mL of 0.1 M O-phenanthroline was added to 0.2 mL reaction mixture. The amount of ferrous ion was determined by measuring the absorbance at 550 nm against the sample buffer and then compared to a standard curve prepared with different known amounts of ferrous ion.

*Centrifugations*

Low speed centrifugation was carried out using a Sorvall Superspeed RC-2B

refrigerated centrifuge at 4°C. High speed centrifugation was carried out at 4°C using a Beckman model L, type B ultracentrifuge using a Ti 60 fixed angle rotor.

# RESULTS

## *Thiosulfate Oxidation by T. thiooxidans*

### Effect of N-ethylmaleimide (NEM) on sulfur oxidation

The oxidation of elemental sulfur by *T. thiooxidans* is known to be inhibited by NEM (Kodama and Mori 1968). During the purification of the $S^0$-oxidizing enzyme from *T. thiooxidans*, the enzyme was found to be inhibited by NEM, a sulfhydryl-binding inhibitor. The effect of NEM on the enzyme activity was time-dependent (Table 1).

### Effect of NEM on the thiosulfate oxidation of different sulfur-grown cells

Different strains of $S^0$-grown thiobacilli cells including *T. thiooxidans* were pre-treated with NEM in order to avoid the loss of thiosulfate due to chemical reaction with NEM during the oxidation. The treatment protocol is described in Materials and Methods. Different amounts of thiosulfate were used to study the stoichiometry of thiosulfate oxidation. Polythionate (tetrathionate) accumulated during the oxidation by these NEM-pretreated cells of all three different strains of $S^0$-grown thiobacilli under acidic conditions. They were *T. thiooxidans* (Tables 2 and 3), SM4-HEC-$S^0$ (Table 4) and Tf-2-$S^0$ (Table 5). According to the data from the total oxygen uptake and amount of polythionate accumulated, the stoichiometry of the reaction is suggested to be

$$2S_2O_3^{2-} + \tfrac{1}{2}O_2 + H_2O \rightarrow S_4O_6^{2-} + 2OH^-. \qquad \text{[Equation 2]}$$

The same result was also obtained with the NEM-treated *T. thioparus* which were grown in thiosulfate at neutral pH (Table 6). After cyanolysis of the reaction mixtures, the amounts of thiocyanate increased linearly with increasing thiosulfate concentrations, but

the values in Table 2 were consistently higher than those expected from the equation 2. The amounts of thiocyanate were identical after the hot cyanolysis (Table 3). Since the differences from the theoretical values were constant (85-95 nmol, Table 2), the oxidation of thiosulfate may have somehow increased the amount of cyanide-labile endogenous sulfur released from cells (from 120 to 205-215 nmol). This interpretation is in agreement with experiments on sulfur-grown *T. ferrooxidans* treated with NEM (Tables 4 and 5), where cells had much smaller amounts of cyanide-labile sulfur (15-34 nmols per 6 mg wet cells) and the oxidation of thiosulfate resulted in the thiocyanate equivalent to 80% of the theoretical tetrathionate amount. In all the experiments, the amount of total oxygen consumed was close to the value expected from the equation 2.

**Effect of HQNO on the thiosulfate oxidation by different sulfur-grown cells**

Suzuki *et al.* (1993) reported that the oxidation of sulfite was inhibited with 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) in *T. thiooxidans* metabolizing elemental sulfur which resulted in sulfite accumulation. *T. thiooxidans* and two $S^0$-grown *T. ferrooxidans* (SM4-HEC-$S^0$ and Tf-2-$S^0$) were used to study the effect of HQNO in the oxidation of thiosulfate. All three strains accumulated sulfite from thiosulfate in the presence of HQNO (Table 7). After preincubation for 10 min with 50 $\mu$g HQNO, the cells oxidized 100 nmol $Na_2S_2O_3$ consuming only 100 nmol $O_2$ instead of 200 nmol $O_2$ in the absence of HQNO. There was no sulfite accumulation without HQNO. The NEM-treated *T. thiooxidans* oxidized thiosulfate to tetrathionate with or without HQNO (Tables 8 and 2). The NEM-treated cells consumed only a quarter mole of $O_2$ per mole

of thiosulfate in agreement with the equation 2. The amounts of sulfite accumulated and oxygen consumed (Table 7) indicate that both sulfur atoms of thiosulfate were converted to sulfite by *T. thiooxidans* in the presence of HQNO ($S_2O_3^{2-} + O_2 + H_2O \rightarrow 2SO_3^{2-} + 2H^+$) and to sulfate in the absence of HQNO ($S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$. Although sulfite recovery was low with SM4-HEC-$S^0$, the two sulfur-grown *T. ferrooxidans* strains produced similar results to those of *T. thiooxidans*.

### *Tetrathionate Oxidation by T. thiooxidans*

#### Effect of pH on tetrathionate oxidation

The optimum pH of tetrathionate oxidation was 3.0 which was slight higher than that of thiosulfate oxidation (Table 9).

#### Effect of NEM and HQNO on tetrathionate oxidation

Tetrathionate was completely oxidized to sulfate in the absence of inhibitors and the $O_2$ uptake results (Table 10) agreed with the equation: $H_2S_4O_6 + 3½O_2 + 3H_2O \rightarrow 4H_2SO_4$. The oxidation of tetrathionate, similar to sulfur oxidation, was totally inhibited by NEM (Table 10). Tetrathionate was oxidized to sulfite level in the presence of HQNO. The total oxygen consumption of the tetrathionate oxidation in the presence of HQNO agreed with the equation: $H_2S_4O_6 + 1½O_2 + 3H_2O \rightarrow 4H_2SO_3$.

#### Stoichiometry of tetrathionate oxidation in the presence of HQNO by *T. thiooxidans*

A stoichiometric study of tetrathionate oxidation to sulfite in the presence of

HQNO is shown in Table 11. The results are in reasonable agreement with the theoretical values, i.e., the ratio of tetrathionate : $O_2$ : sulfite is 1 : 1½ : 4. Recovery of the sulfite was somewhat lower and $O_2$ uptake was higher than the theoretical values. This is possibly due to less than 100% purity of sodium tetrathionate used as substrate and the high endogenous $O_2$ uptake caused by high cell concentration (6 mg wet cells/mL). The solution of sodium tetrathionate (Aldrich) contained some sulfur which was removed by centrifugation. The supernatant which was used as substrate was assayed for tetrathionate by cold cyanolysis. A value obtained (80% purity) agrees with the amounts of sulfite formed in Table 11.

### *Characterization of NEM-treated T. thiooxidans on Thiosulfate Oxidation*

#### Effect of pH

The effect of pH is shown in Fig. 1 and it is clear that thiosulfate is oxidized only at very acidic conditions with an optimum at pH 2.3.

#### Effect of cell concentration

The rate of thiosulfate oxidation increased linearly with increasing cell concentrations (0-10 mg NEM-treated wet cells) at pH 2.3 (Fig. 2).

#### Effect of sulfite

The NEM-treated *T. thiooxidans* were preincubated with different amounts of sulfite for 5 min at pH 2.3 before the thiosulfate oxidation was started. As shown in

Fig. 3, sulfite inhibited the oxidation of thiosulfate ($K_i$ around 2 mM sulfite).

### *Thiosulfate Oxidation by Cell-Free Extracts of T. thiooxidans*

#### Effect of pH

The optimum pH for the cell-free extracts of thiosulfate oxidation was also 2.3 (Fig. 4). Stoichiometric studies of the total oxygen consumption were carried out at three different pH with different amounts of thiosulfate. The results are summarized in Table 12. The cell-free extracts of *T. thiooxidans* oxidized thiosulfate to tetrathionate with one quarter mole of $O_2$ per mole of thiosulfate which is in agreement with the results with NEM-treated cells (Tables 2 and 3).

### *Kinetic Studies of T. thiooxidans*

#### NEM-treated *T. thiooxidans*

The effect of thiosulfate concentrations on the oxygen consumption rates of NEM-treated cells at different pH values were studied (Figs. 5-18). The kinetic constants are summarized in Table 13. The reaction did not follow a simple saturation kinetics with increasing thiosulfate concentrations and often showed characteristics of two active sites or two enzymes catalyzing the reaction with two different $K_m$ and $V_{max}$ values. The double reciprocal data points were fitted by the approximation method of Segel (1975) to obtain $K_m$ and $V_{max}$ values from the biphasic plots according to the equation:

$$V = \frac{[S]\ Vmax1}{Km1 + [s]} + \frac{[S]\ Vmax2}{Km2 + [S]}$$

Two reactions, $V_1$ and $V_2$ were considered to take place. $V_1$ had a lower $K_{m1}$ and $V_{max1}$, and $V_2$ the higher $K_{m2}$ and $V_{max2}$. At pH 1.8, $V_1$ disappeared. $V_{max1}$ stayed constant around 17-22 nmol $O_2$ consumed per min from pH 2.0 to pH 3.0, then dropped to 4.1 at pH 4.5. $V_{max2}$ stayed constant around 60-70 nmol $O_2$ consumed per min from pH 1.8 to pH 3.0, then dropped to 15.2 at pH 4.5. $K_{m1}$ stayed low between 0.04-0.17 mM from pH 2.0 to pH 4.5. $K_{m2}$ was 2 to 5 mM between pH 2 and 4 but was lowest at 0.9 mM at pH 1.8. The velocity plots of thiosulfate oxidation at different pH with NEM-treated *T. thiooxidans* are shown in Fig. 5 to Fig. 11. The double reciprocal plots of thiosulfate oxidation at different pH are shown in Figs. 12 to 18, where single lines are drawn to show the deviation of data points from simple saturation kinetics.

**Untreated *T. thiooxidans***

Untreated cells oxidized thiosulfate with similar complex kinetics of two $K_m$ and $V_{max}$ values (Figs. 5-18 and Table 14). $V_{max1}$ stayed constant around 10-20 nmol $O_2$ consumed per min from pH 1.8 to pH 4.5. $V_{max2}$ stayed constant around 30-60 nmol $O_2$ consumed per min from pH 1.8 to pH 3.0, then dropped to 10 at pH 4.0. $K_{m1}$ stayed low between 0.03-0.15 mM from pH 1.8 to pH 4.5. $K_{m2}$ was higher at around 0.5 (pH 1.8-3.5) which increased to 5 mM at pH 4.5. The velocity plots of thiosulfate oxidation at different pH with untreated *T. thiooxidans* are shown in Fig. 5 to Fig. 11. The double reciprocal plots of thiosulfate oxidation at different pH are shown in Fig. 12 to Fig. 18.

### Cell-free extracts of *T. thiooxidans*

The kinetic constants of thiosulfate oxidation by cell-free extracts at different pH are summarized in Table 15. The double reciprocal plot is shown in Fig. 19. This plot is different from that of the intact cells in that the plot is linear, while the intact cells with and without NEM treatment show "biphasic" kinetics.

## *Chemical Reactions of Thiosulfate Under Acidic Conditions*

### Kinetic studies of chemical reaction of thiosulfate with ferric ion

Thiosulfate is known to be unstable under acidic conditions, therefore chemical reactions of thiosulfate were studied in the presence and absence of ferric ion as oxidant. Three different pH values were used to study the kinetics of the chemical oxidation of thiosulfate with ferric ion ($2S_2O_3^{2-} + 2Fe^{3+} \rightarrow S_4O_6^{2-} + 2Fe^{2+}$). They were 1.8, 2.3 and 3.0 respectively.

### Based on ferrous ion formation

The velocity plots of the reactions of different amounts of ferric ion with a fixed amount of thiosulfate at different pH are shown in Fig. 21. The time course plots of the reactions of a fixed amount of ferric ion with different amounts of thiosulfate at pH 1.8, pH 2.3 and pH 3.0 are shown in Fig. 22, Fig. 23 and Fig. 24, respectively. The time course plots of the reactions of a fixed amount of thiosulfate with different amounts of ferric ion at pH 1.8, 2.3, and pH 3.0 are shown in Fig. 25, Fig. 26 and Fig. 27, respectively. The double reciprocal plots of either different amounts of ferric ion with

a fixed amount of thiosulfate or different amounts of thiosulfate with a fixed amount of ferric ion at pH 1.8, pH 2.3 and pH 3.0 show linear lines intercepting the origin, a characteristic of a chemical non-enzymatic reaction (plots not shown).

**Based on thiocyanate ($SCN^-$) formation**

The velocity plot of different amounts of thiosulfate with a fixed amount of ferric ion at different pH is shown in Fig. 28. The values in Fig. 20 and Fig. 28 agree with the stoichiometry expected of two ferrous iron for every thiocyanate. The velocity plot of different amounts of ferric ion with a fixed amount of thiosulfate at different pH is shown in Fig. 29. The time course reaction of a fixed amount of ferric ion added to different amounts of thiosulfate at pH 1.8, pH 2.3 and pH 3.0 are shown in Fig. 30, Fig. 31 and Fig. 32, respectively. The time course reaction of a fixed amount of thiosulfate added to different amounts of ferric ion at pH 1.8, 2.3, and pH 3.0 are shown in Fig. 33, Fig. 34 and Fig. 35, respectively. The double reciprocal plots of either different amounts of ferric ion with a fixed amount of thiosulfate or different amounts of thiosulfate with a fixed amount of ferric ion at pH 1.8, pH 2.3 and pH 3.0 show linear lines intercepting the origin, a characteristic of a chemical non-enzymatic reaction (plots not shown).

**Autooxidation of thiosulfate under acidic conditions**

The chemical reaction of different amounts of thiosulfate in 0.1 M $\beta$-alanine-$H_2SO_4$ buffer at pH 1.8, pH 2.3 and pH 3.0 in the absence of ferric ion is shown in Fig.

36, Fig. 37 and Fig. 38 respectively. The formation of polythionate increased rapidly only if thiosulfate was incubated for more than 30 min at either pH 1.8 and pH 2.3. At pH 3.0, however, the formation of polythionate remained low even when thiosulfate was incubated for more than 30 min.

### *Thiosulfate Oxidation by the Acidic Extracts of T. thiooxidans*

#### Thiosulfate:ferricyanide oxidoreductase activity and other components in the acidic extracts

It had been shown that thiosulfate was oxidized to tetrathionate and an unknown oxidized sulfur compound in a cell-free particulate preparation of *T. thiooxidans* (Tano *et al.* 1968). In an attempt to understand the mechanism of the thiosulfate oxidation, a soluble fraction was required for further investigation. A thiosulfate:ferricyanide oxidoreductase activity was successfully solubilized from intact cells by passing the acidic ammonium sulfate suspension through a French pressure cell as described in Materials and Methods. Interestingly, an acid-soluble cytochrome was also identified in the extracts. This c-type cytochrome was reduced by thiosulfate only at an acidic pH as shown in Table 16 and at pH 2.5, it was almost fully reduced by thiosulfate. This reducing activity was totally destroyed in the boiled extracts (Table 16) but the thiosulfate:ferricyanide oxidoreductase activity was still present (Table 19). The fully reduced cytochrome had typical c-type absorption peaks at 416, 520 and 550 nm (data not shown) which is slightly different from the soluble cytochrome c-552 purified from *T. thiooxidans* (Takakuwa 1975).

The acidic extracts contained a component which reduced yeast (*Saccharomyces cerevisiae*) or horse heart cytochrome c slowly at an acidic pH, but faster at a pH near or above neutrality (Table 17). Thiosulfate increased the rate of reduction only slightly (data not shown) but sulfite increased the rate and extent of reduction. The total extent of horse heart cytochrome c reduction was proportional to the amount of acidic extracts, i.e., $\Delta A_{550nm}$ was doubled when twice as much acidic extracts was used. At an acidic pH, the sulfite effect was stronger. Thus when sulfite was present, the horse heart cytochrome c reduction was almost instantaneous at a pH below 6, but slow at pH 8, the pH where the reduction by the acidic extracts was faster in the absence of sulfite. This reducing activity was not destroyed by boiling of the acidic extracts but was destroyed by the preincubation with EDTA or NEM at pH 7.0 (Table 17). NEM failed to destroy the activity at pH 4.3 where the sulfite effect was stronger (Table 17).

Chromatography of the acidic extracts (1 mL) through a column of Sephadex G-25 (9 mL) or G-50 (20 mL) at pH 2.5 separated the thiosulfate:ferricyanide oxidoreductase activity associated with the native cytochrome c (which was always excluded from the gel) from the horse heart cytochrome c-reducing activity. Therefore the latter is not a component of the former. The acidic extracts also contained some iron, $Fe^{2+}$ and $Fe^{3+}$, which was separated from the native cytochrome c fraction in G-50.

Chromatography of 1 mL acidic extracts at pH 2.5 (25 mM potassium phosphate) on a Sepharose CL-6B column (bed volume 20 mL, void volume 14.4 mL) also separated the native cytochrome c-containing thiosulfate:ferricyanide oxidoreductase activity (elution volume 18.0 mL) from the horse heart cytochrome c reducing activity (elution

volume 22.0 mL). The native cytochrome c was still reduced upon the addition of thiosulfate, but not by sulfite; and the thiosulfate:ferricyanide oxidoreductase activity ($\Delta A_{420}$/min/mg-0.085) was now totally by boiling.

## Characterization of the Thiosulfate:ferricyanide Oxidoreductase in the Acidic Extracts

### Effect of pH

The optimum pH for the thiosulfate:ferricyanide oxidoreductase activity was around pH 2.0 (Fig. 39) although the chemical reduction of ferricyanide by thiosulfate became considerable at the very acidic pH range. The standard assay was, therefore, carried out at pH 4.3.

### Effect of sulfite

The enzyme was inhibited by sulfite (Table 18), which was in agreement with the enzyme from other thiobacilli (Lyric and Suzuki 1970; Meulenberg *et al.* 1993a). Preincubation increased the extent of enzyme inhibition similar to the report for the *T. thioparus* enzyme (Lyric and Suzuki 1970), but the inhibition was much less at pH 2.0 than at pH 4.3 (Table 18).

### Substrate specificity of the thiosulfate oxidation

Table 19 shows that the reduction of ferricyanide by the acidic extracts of *T. thiooxidans* accurred only in the presence of thiosulfate and that sulfite (with or without

AMP), trithionate, or tetrathionate could not replace thiosulfate as the substrate (Table 19).

### Heat stability

Surprisingly, the enzyme activity was not destroyed by boiling (Table 19), since the enzyme from *T. acidophilus* lost 50% of its activity in two hours at 30°C (Meulenberg *et al.* 1993a). However, the activity became more heat-labile after chromatography on a Sepharose CL-6B column losing more than 80% of activity by boiling for 15 min (data not shown).

### Stoichiometry of the thiosulfate oxidation

The amount of ferricyanide reduced was twice the amount of thiocyanate formed in agreement with the stoichiometry: $2S_2O_3^{2-} + 2Fe(CN)_6^{3-} \rightarrow S_4O_6^{2-} + 2Fe(CN)_6^{4-}$. The results are summarized in Table 20.

### Effect of different amounts of acidic extracts

The initial rate of ferricyanide reduction increased with increasing amounts (0.1-1 mg protein) of the acidic extracts (Table 21). The apparent $K_m$ for thiosulfate was 4-5 mM at either pH 4.3 or 2.0 (data not shown).

## DISCUSSION

Thiosulfate is found widely in nature and its metabolism by thiobacilli has been studied extensively (Kelly 1982). Although its eventual oxidation to sulfate is obvious, the pathway of oxidation has been controversial (Pronk *et al.* 1990). Thiosulfate is easily oxidized to tetrathionate by the thiosulfate-oxidizing enzyme system, first established by Trudinger in *T. neapolitanus* (Trudinger 1961) and later confirmed in other thiobacilli (Silver and Lundgren 1968; Lyric and Suzuki 1970; Lu and Kelly 1988; Meulenberg *et al.* 1993a). Further metabolism of tetrathionate has been studied in some thiobacilli (Pronk *et al.* 1990). In *T. versutus* grown in a chemostat with thiosulfate limitation, thiosulfate is oxidized to sulfate without any accumulation of intermediates (Lu 1986). Sulfite is an intermediate if thiosulfate is cleaved to sulfur and sulfite (Suzuki 1974). Sulfite accumulation from thiosulfate has been demonstrated in *T. novellus* (DeLey and Van Poucke 1961), and the results in this paper support the ability of *T. thiooxidans* to convert both sulfane sulfur and sulfonate sulfur to sulfite when a further oxidation of sulfite is inhibited by HQNO (Tables 7 and 8). In the presence of HQNO, thiosulfate is oxidized to sulfite with a stoichiometry in accordance to the equation: $H_2S_2O_3 + O_2 + H_2O \rightarrow 2H_2SO_3$. The pH optimum of tetrathionate oxidation in *T. thiooxidans* is 3.0 (Table 9) indicating that the process is likely to occur extracytoplasmically, probably in the periplasm. Since tetrathionate oxidation is completely inhibited by NEM (Table 10), the reaction must involve sulfhydryl groups similar to the oxidation of sulfur. Tetrathionate is similarly oxidized to sulfite in the presence of HQNO stoichiometrically according to the equation: $H_2S_4O_6 + 1½O_2 + 3H_2O \rightarrow 4H_2SO_3$. This result supports the

mechanism suggested by Suzuki *et al.* (1994) that tetrathionate oxidation in *T. thiooxidans* involves the oxidation of both sulfane (-S-) and sulfonic acid ($-SO_3H$) portions by the sulfur- and sulfite-oxidizing systems respectively. In the absence of HQNO, both thiosulfate and tetrathionate are oxidized to sulfate with corresponding increase in oxygen consumption.

The time course of thiosulfate oxidation in *T. thiooxidans* is biphasic, with a rapid initial oxidation rate of a quarter mole oxygen consumed per mole of thiosulfate, followed by a lag, and then a semi-linear rate to completion. In the presence of NEM, the second phase of the thiosulfate oxidation is inhibited. The NEM-treated *T. thiooxidans* which is not able to oxidize sulfur (Chan and Suzuki 1993; Suzuki *et al.* 1993) oxidize thiosulfate stoichiometrically to tetrathionate under acidic conditions (Tables 2 and 3). Tetrathionate also accumulates in other NEM-treated thiobacilli species (Tables 4 and 5), and *T. neapolitanus* also oxidizes thiosulfate to tetrathionate at pH 7 in the presence of NEM (Trudinger 1965). The cell-free extracts of *T. thiooxidans* oxidize thiosulfate with the same $O_2$ stoichiometry and pH optimum (Table 12).

The effect of thiosulfate concentrations on the $O_2$ consumption rate of NEM-treated and -untreated *T. thiooxidans* produce biphasic double reciprocal plots with two $K_m$ values similar to that reported in *T. ferrooxidans* (Eccleston and Kelly 1978). The plots are analyzed by the approximation method of Segel (1975) to obtain the $K_{m1}$ (low) and $K_{m2}$ (high) values and corresponding maximal velocities, $V_{max1}$ (low) and $V_{max2}$ (high) (Tables 13 and 14). The pH has a profound effect on these values which increase with increasing pH (Tables 13 and 14). The low $K_{m1}$ value of 30-40 $\mu$M at pH 2.5 is in the

same concentration range as those reported for thiosulfate-limited chemostat cultures of *T. ferrooxidans* (Hazeu *et al.* 1986) whose $K_m$ values also increased with increasing pH. The higher $K_m$ values ($K_{m2}$) are close to the values reported by other workers (e.g. Eccleston and Kelly 1978) and also increase with pH (Tables 13 and 14). The cell-free system shows a simple kinetics with one $K_m$ which increases with increasing pH (Table 15). These values are much higher than those of $K_{m1}$ indicating a possibility that some uptake or transport system operates on the cell to increase the affinity for thiosulfate to the $K_{m1}$ level.

A possible formation of tetrathionate may occur either by the autooxidation of thiosulfate at acidic pH or via chemical reaction of the thiosulfate with intracellular metal ions such as ferric ion. Double reciprocal plots of the chemical reactions of thiosulfate with ferric ions are linear, crossing the origin which are unlike enzymatic reactions (plots not shown). Autooxidation of the thiosulfate solutions does not produce tetrathionate for at least 30 min between a pH range of 1.8 to 3.0 (Fig. 36 to 38). Formation of tetrathionate by chemical reactions of thiosulfate with ferric ions are much slower compared to that of NEM-treated and -untreated *T. thiooxidans*, and other thiobacilli cells. Using 1 mM thiosulfate, the rate of tetrathionate formation at pH 2.3 is approximately 0.1 mM per min with 1mM ferric ion (Fig. 29). In Table 2, 6 mg wet cells of *T. thiooxidans* oxidized 1 $\mu$mol thiosulfate at the rate of 10 nmol oxygen per min or 20 nmol tetrathionate per min. The amount of total iron ($Fe^{2+}$ + $Fe^{3+}$) in the acidic extracts is estimated to be 10 nmol $Fe^{3+}$ per 6 mg wet cells. This amount of $Fe^{3+}$ according to Fig. 29 will chemically oxidize thiosulfate at the rate of 0.1 × 0.01 =

0.001 μmol or 1 nmol tetrathionate per min. The above comparative studies indicate that the formation of tetrathionate is not due to the autooxidation of thiosulfate at acidic pH nor a chemical reaction of the thiosulfate with intracellular metal ions but is catalyzed by a thiosulfate-oxidizing enzyme system in *T. thiooxidans*.

Thiosulfate is oxidized by sulfur-grown *T. thiooxidans* and cell-free extracts only at an acidic pH around 2.5, where thiosulfate is chemically unstable (Roy and Trudinger 1970). Therefore, the growth of *T. thiooxidans* on thiosulfate is normally achieved only at a higher pH of 4.5 (Barton and Shively 1968) and 5.0 (Nakamura *et al.* 1990). The rate of thiosulfate oxidation by the sulfur-grown cells at pH 2.3 in Table 1 (10 nmol $O_2$ consumed per min per 6 mg wet cells) corresponds only to 10 to 20% of the rate for sulfur oxidation in $O_2$ consumption. Thiosulfate oxidation by the thiosulfate-grown cells (Nakamura *et al.* 1990) has an activity optimum around pH 5.0 and the rate is more than ten times that of sulfur-grown cells. Since *T. thiooxidans* require a long lag period before the start of growth on thiosulfate when the inoculum is grown on sulfur (Nakamura *et al.* 1990), a new thiosulfate-oxidizing system has to be induced. Thus, the thiosulfate oxidation system in sulfur-grown *T. thiooxidans* must play a different role.

The acidic extracts contain a thiosulfate:ferricyanide oxidoreductase which resembled the enzyme recently purified from *T. acidophilus* grown mixotrophically on thiosulfate and glucose (Meulenberg *et al.* 1993b). This thiosulfate-oxidizing enzyme system has an acidic optimum pH which suggests a periplasmic location as was proposed for the *T. acidophilus* enzyme. The inhibition of this enzyme activity by sulfite (Table 18) is similar to that of the thiosulfate-oxidizing enzymes found in *T. thioparus* and *T.*

*acidophilus*, supporting the role of a thiosulfate oxidative pathway in the metabolism of sulfur. During the oxidation of sulfur, sulfite may be produced slightly faster than the rate of sulfite oxidation, and this accumulated sulfite condenses with sulfur to form thiosulfate which is then oxidized to tetrathionate by the thiosulfate:ferricyanide oxidoreductase. Thiosulfate oxidation to tetrathionate may serve as a means of raising the cellular pH, which will increase the rate of sulfite oxidation to sulfate (Takeuchi and Suzuki 1994) or allow the cells to buffer temporarily the proton excretion from sulfuric acid production (Wentzien *et al.* 1994). A more detailed study of the thiosulfate:ferricyanide oxidoreductase in *T. thiooxidans* is hampered by its low activity (1% of *T. acidophilus* enzyme in the extract) and a low growth yield (200 mg wet cells/L).

Unlike the enzyme from *T. neapolitanus* (Trudinger 1961), *T. thioparus* (Lyric and Suzuki 1970), *T. tepidarius* (Lu and Kelly 1988), and *T. ferrooxidans* (Silver and Lundgren 1968), this thiosulfate-oxidizing enyzme in *T. thiooxidans* is associated with a c-type cytochrome, similar to the *T. acidophilus* enzyme. This native c-type cytochrome is reduced by thiosulfate (Table 16) which is similar to the reduction of a soluble native cytochrome c by the *T. neapolitanus* enzyme. Its acidic optimal pH range agrees with most reports, particularly with the *T. acidophilus* enzyme.

By Sepharose CL-6B chromatography, a heat-stable activity component is separated from the fraction containing the native cytochrome c and the heat-labile thiosulfate:ferricyanide oxidoreductase. This component which reduces horse heart cytochrome c (Table 17) may contain a metal since the reduction is EDTA inhibited, and

also thiol or sulfide groups since it is NEM inhibited. It is speculated that the component is a heat-stable, acid stable, iron-sulfur or iron-sulfide complex of unknown structure which can slowly reduce horse heart cytochrome c at a neutral pH, but requires sulfite at an acidic pH to form a strongly reducing compound which instantaneously reduces the horse heart cytochrome c.

## REFERENCES


1. Barton, L.L. and J.M. Shively. 1968. Thiosulfate oxidation by *Thiobacillus thiooxidans* ATCC 8085. J. Bacteriol. **95**: 720.

2. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

3. Chakraborty, R. and P. Roy. 1992. Chemotaxis of chemolithotrophic *Thiobacillus ferrooxidans* toward thiosulfate. FEMS Microbiology Letters **98**: 9-12.

4. Chan, C.W. and I. Suzuki. 1993. Quantitative extraction and determination of elemental sulfur and stoichiometric oxidation of sulfide to elemental sulfur by *Thiobacillus thiooxidans*. Can. J. Microbiol. **39**: 1166-1168.

5. DeLey, J. and M. Van Pouck. 1961. The formation of sulfite during the oxidation of thiosulfate by *Thiobacillus novellus*. Biochim. Biophys. Acta. **50**: 371-373.

6. Eccleston, M. and D.P. Kelly. 1978. Oxidation kinetics of *Thiobacillus ferrooxidans* on tetrathionate and thiosulfate. J. Bacteriol. **134**: 718-728.

7. Goodman, A. and B.J. Ralph. 1980. A microcalorimetric study of the metabolic activity of two thiobacillus species. *In*: Trudinger, P.A., M.R. Walter and B.J. Ralph (eds). Biogeochemistry of ancient and modern environments. Springer, Berlin Heidelberg New York pp 477-483.

8. Hazeu, W., W. Bijleveld, J.T.C. Grotenhuis, E. Kakes and J.G. Kuenen. 1986. Kinetics and energetics of reduced sulfur oxidation by chemostat cultures of *Thiobacillus ferrooxidans*. Antonie van Leeuwenhoek **52**: 507-518.

9. Imai, K., M. Okuzumi and H. Katagiri. 1962. The pathways of sulfur oxidation by *Thiobacillus thiooxidans*. Koso Kagaku Shimpojiumu **17**: 132-143.

10. Kelly, D.P. 1982. Biochemistry of the chemolithotrophic oxidation of inorganic sulfur. Phil. Trans. R. Soc. (London) **B298**: 499-528.

11. Kodama, A. and T. Mori. 1968. Studies on the metabolism of a sulfur-oxidizing bacterium V. comparative studies on sulfur and sulfite oxidizing systems of *Thiobacillus thiooxidans*. Plant & Cell Physiol. **9**: 725-734.

12. Lane, E. 1957. Spectrophotometric and turibidmetric methods for measuring proteins. *In*: Methods in Enzymology Vol. III. *Edited* by Colowick, S.P and N.O. Kaplan, Academic Press, New York, pp. 447-454.

13. Lizama, H.M. and I. Suzuki. 1988 Bacterial leaching of a sulfide ore by *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*. I. shake flask studies. Biotechnol. Bioeng. **32**: 110-116.

14. London, J. and S.C. Rittenberg. 1964. Path of sulfur in sulfide and thiosulfate oxidation by thiobacilli. Proc. Natl. Acad. Sci. (U.S.A.) **52**: 1183-1190.

15. Lu, W.P. 1986. A periplasmic location for the thiosulfate-oxidizing multi-enzyme system from *Thiobacillus versutus*. FEMS. Microbiol. Lett. **34**: 313-317.

16. Lu, W.P. and D.P. Kelly. 1988. Cellular location and partial purification of the 'thiosulfate-oxidizing enzyme' and 'trithionate hydrolyase' from *Thiobacillus tepidarius*. J. Gen. Microbiol. **134**: 877-885.

17. Lyric, R.M. and I. Suzuki. 1970. Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. III. Properties of thiosulfate-oxidizing enzyme and proposed pathway of thiosulfate oxidation. Can. J. Biochem. **48**: 355-363.

18. Meulenberg, R., J.T. Pronk, J. Frank, W. Hazeu, P. Bos and J.G. Kuenen. 1992. Purification and partial characterization of a thermostable trithionate hydrolase from the acidophilic sulfur oxidizer *Thiobacillus acidophilus*. Eur. J. Biochem. **209**: 367-374.

19. Meulenberg, R., J.T. Pronk, W. Hazeu, J.P. Van Dijken, J. Frank, P. Bos and J.G. Kuenen. 1993a. Purification and partial characterization of thiosulphate dehydrogenase from *Thiobacillus acidophilus*. J. Gen. Microbiol. **139**: 2033-2039.

20. Meulenberg, R., E.J. Scheer., J.T. Pronk., W. Hazeu., P. Bos. and J.G. Kuenen. 1993b. Metabolism of tetrathionate in *Thiobacillus acidophilus*. FEMS. Microbiol. Letters **112**: 167-172.

21. Nakamura, K., H. Miki and Y. Amano. 1990. Cell growth and accumulation of *Thiobacillus thiooxidans* S3 in a pH-controlled thiosulfate medium. J. Gen. Appl. Microbiol. **36**: 369-376.

22. Okuzumi, M. 1966. Studies on biochemistry of the thiobacilli part VIII. Dismutation of tetrathionate by *Thiobacillus thiooxidans*. Agr. Biol. Chem. **30**: 313-318.

23. Pronk, J.T., R. Meulenberg, W. Hazeu, P. Bos and J.G. Kuenen. 1990. Oxidation of reduced inorganic sulfur compounds by acidophilic thiobacilli. FEMS. Microbiol. Rev. **75**: 293-306.

24. Roy, A.B. and P.A. Trudinger. 1970. The biochemistry of inorganic compounds of sulfur. Cambridge University Press, London, U.K.

25. Segel, I.H. 1975. Enzyme kinetics. John Wiley & Sons, New York.

26. Schleif, R.F. and P.W. Wensink. 1981. Practical Methods in Molecular Biology. Springer Verlag, New York. p. 62.

27. Shihari, S.R. Bhavaraju, J.M. Modak, R. Kumar and K.S. Gandhi. 1993. Dissolution of sulphur particles by *Thiobacillus ferrooxidans*: substrate for unattached cells. Biotechnol. Bioeng. **41**: 612-616.

28. Silver, M. and D.G. Lundgren. 1968. The thiosulfate-oxidizing enzyme of *Ferrobacillus ferrooxidans* (*Thiobacillus ferrooxidans*). Can. J. Biochem. **46**: 1215-1220.

29. Sinha, D.B. and C.C. Walden. 1966. Formation of polythionates and their interrelationships during oxidation of thiosulfate by *Thiobacillus ferrooxidans*. Can. J. Microbiol. **12**: 1041-1056.

30. Sörbo, B. 1958. On the formation of thiosulfate from inorganic sulfide by liver tissues and heme compounds. Biochim. Biophys. Acta. **27**: 324-329.

31. Sugio, T., M. Noguchi and T. Tano. 1987. Detoxification of sulfite produced during the oxidation of elemental sulfur by *Thiobacillus ferrooxidans*. Agr. Biol. Chem. **51**: 1431-1433.

32 Suzuki, I. 1965. Oxidation of elemental sulfur by an enzyme system of *Thiobacillus thiooxidans*. Biochim. Biophys. Acta. **104**: 359-371.

33. Suzuki, I. 1974. Mechanism of inorganic oxidation and energy coupling.

Annu. Rev. Microbiol. **28**: 85-101.

34. Suzuki, I., C.W. Chan and T.L. Takeuchi. 1992. Oxidation of elemental sulfur to sulfite by *Thiobacillus thiooxidans* cells. App. Environ. Microbiol. **58**: 3767-3769.

35. Suzuki, I., C.W. Chan and T.L. Takeuchi. 1994. Oxidation of inorganic sulfur compounds by thiobacilli. *In*: Environmental Geochemistry of Sulfide Oxidation. *Edited* by Alpers, C.N. and D.W. Blowes. Amer. Chem. Soc., Washington D.C. pp. 60-67.

36. Suzuki, I., C.W. Chan, R. Vilar and T.L. Takeuchi. 1993. Sulfur and sulfide oxidation by *Thiobacillus thiooxidans*. *In*: Biohydrometallurgical Technologies. *Edited* by Torma, A.E. J.E. Wey and V.L. Lakshmanan The Minerals, Metals & Materials Society, Warrendale, Pa. Vol. 1, pp. 109-116.

37. Suzuki, I. and M. Silver. 1966. The initial product and properties of the sulfur-oxidizing enzyme of thiobacilli. Biochim. Biophys. Acta. **122**: 22-23.

38. Suzuki, I., T.L. Takeuchi, T.D. Yuthasastrakosol and J.K. Oh. 1990. Ferrous iron and sulfur oxidation and ferric iron reduction activities of *Thiobacillus ferrooxidans* are affected by growth on ferrous iron, sulfur, or a sulfide ore. Appl. Environ. Microbiol. **56**: 1620-1626.

39. Takeuchi, T.L. and I. Suzuki. 1994. Effect of pH on sulfite oxidation by *Thiobacillus thiooxidans* cells with sulfurous acid or sulfur dioxide as a possible substrate. J. Bacteriol. **176**: 913-916.

40. Takakuwa, S. 1975. Purification and some properties of cytochrome c-552 from a sulfur-oxidizing bacterium, *Thiobacillus thiooxidans*. J. Biochem. **78**: 181-185.

41. Tano, T., H. Asano and K. Imai. 1968. Physiological studies on thiobacilli. Part III. The oxidation of thiosulfate by particulate fraction of *Thiobacillus thiooxidans*. Agr. Biol. Chem. **32**: 140-143.

42. Trudinger, P.A. 1961. Thiosulfate oxidation and cytochromes in *Thiobacillus X* 2. thiosulfate-oxidizing enzyme. Biochem. J. **78**: 680-686.

43. Trudinger, P.A. 1965. Effect of thiol-binding reagents on the metabolism of thiosulfate and tetrathionate by *Thiobacillus neapolitanus*. J. Bacteriol.

89: 617-625.

44. Wentzien, S., W. Sand, A. Albertsen and R. Steudel. 1994. Thiosulfate and tetrathionate degradation as well as biofilm generation by *Thiobacillus intermedius* and *Thiobacillus versutus* studied by microcalorimetry, HPLC, and ion-pair chromatography. Arch. Microbiol. **161**: 116-125.

45. West, P.W. and G.C. Gaeke. 1956. Fixation of sulfur dioxide as disulfitomercurate (II) and subsequent colorimetric estimation. Anal. Chem. **28**: 1816-1819.

46. Wood, J.L. 1987. Sulfane sulfur. *In*: Methods in Enzymology Vol. 143. *Edited* by Jacoby, W.B. and O.W. Griffith Academic Press, New York. pp.25-29.

# TABLES

Table 1. **Effect of N-ethylmaleimide on $S^0$-oxidizing activity in *T. thiooxidans*[a].**

| Time | Enzyme activity (nmol $O_2$ consumed/min) | Control | Inhibition (%) |
|---|---|---|---|
| No NEM control | 15.6 | 3.43 | 0.00 |
| 10 mins | 11.54 | 3.00 | 29.82 |
| 1 hr | 5.22 | 3.00 | 81.76 |
| 2 hrs | 3.43 | 2.57 | 92.90 |
| 2½ hrs | 2.67 | 2.88 | 100.0 |
| 24 hrs | 5.25 | 5.25 | 100.0 |

[a] 0.1 mL of 80% ammonium sulfate precipitate containing 3.5 mg of enzyme protein was incubated with 5 $\mu$L of 0.1 M NEM at different time intervals. The sample was added to 0.9 mL of 0.1 M sodium phosphate buffer at pH 6.5, 90 $\mu$L elemental sulfur (32 g/100 mL) and 6 $\mu$mol GSH. The oxygen uptake was measured by a Clarke Electrode. Control values were obtained for non-enzymatic chemical oxidation rates. The unit is nmol $O_2$ consumed/min.

Table 2. Oxidation of thiosulfate by NEM-treated *T. thiooxidans*[a].

| | $SCN^-$ ($\mu$mol) | $SCN^-$ accumulated ($\mu$mol) | Total $O_2$ uptake ($\mu$mol) | Rate (nmol $O_2$ consumed/ min) |
|---|---|---|---|---|
| Cell alone | 0.120 | - | - | - |
| 0.2 $\mu$mol $Na_2S_2O_3$ | 0.310 | 0.190 | 0.058 | 8.0 |
| 0.4 $\mu$mol $Na_2S_2O_3$ | 0.410 | 0.290 | 0.096 | 8.8 |
| 0.6 $\mu$mol $Na_2S_2O_3$ | 0.505 | 0.385 | 0.128 | 9.6 |
| 0.8 $\mu$mol $Na_2S_2O_3$ | 0.615 | 0.495 | 0.170 | 10.0 |
| 1.0 $\mu$mol $Na_2S_2O_3$ | 0.715 | 0.595 | 0.210 | 10.0 |

[a] The $O_2$ consumption was followed in a Gilson Oxygraph at 25°C with 1.2 mL reaction mixture of 50 mM potassium phosphate buffer at pH 2.3, 6 mg wet cells and various amounts of $Na_2S_2O_3$. Polythionate was measured according to Sörbo (1958).

Table 3. Oxidation of thiosulfate by NEM-treated *T. thiooxidans*[a].

| | $SCN^-$ ($\mu$mol) | $SCN^-$ accumulated ($\mu$mol) | Total $O_2$ uptake ($\mu$mol) | Rate (nmol $O_2$ consumed/ min) |
|---|---|---|---|---|
| Cell Alone | 0.095 | - | - | - |
| 0.2 $\mu$mol $Na_2S_2O_3$ | 0.275 | 0.180 | 0.054 | 9.6 |
| 0.4 $\mu$mol $Na_2S_2O_3$ | 0.340 | 0.245 | 0.096 | 10.4 |
| 0.6 $\mu$mol $Na_2S_2O_3$ | 0.440 | 0.345 | 0.126 | 10.0 |
| 0.8 $\mu$mol $Na_2S_2O_3$ | 0.593 | 0.498 | 0.190 | 10.0 |
| 1.0 $\mu$mol $Na_2S_2O_3$ | 0.693 | 0.598 | 0.226 | 11.6 |

[a] The $O_2$ consumption was followed in a Gilson Oxygraph at 25°C with 1.2 mL reaction mixture of 50 mM potassium phosphate buffer at pH 2.3, 6 mg wet cells and various amounts of $Na_2S_2O_3$. Polythionate was measured according to Wood (1982).

Table 4. Oxidation of thiosulfate by NEM-treated SM4-HEC-$S^{0a}$

| | $SCN^-$ ($\mu$mol) | $SCN^-$ accumulated ($\mu$mol) | Total $O_2$ uptake ($\mu$mol) | Rate (nmol $O_2$ consumed/ min) |
|---|---|---|---|---|
| Cell alone | 0.034 | - | - | - |
| 0.2 $\mu$mol $Na_2S_2O_3$ | 0.115 | 0.081 | 0.052 | 18.0 |
| 0.4 $\mu$mol $Na_2S_2O_3$ | 0.195 | 0.161 | 0.100 | 23.2 |
| 0.6 $\mu$mol $Na_2S_2O_3$ | 0.272 | 0.238 | 0.136 | 24.0 |
| 0.8 $\mu$mol $Na_2S_2O_3$ | 0.356 | 0.322 | 0.180 | 26.8 |
| 1.0 $\mu$mol $Na_2S_2O_3$ | 0.449 | 0.415 | 0.230 | 27.2 |

[a] The $O_2$ consumption was followed in a Gilson Oxygraph at 25°C with 1.2 mL reaction mixture of 50 mM potassium phosphate buffer at pH 2.3, 6 mg wet cells and various amounts of $Na_2S_2O_3$. Polythionate was measured according to Sörbo (1958).

Table 5. Oxidation of thiosulfate by NEM-treated Tf-2-$S^{0a}$.

| | $SCN^-$ ($\mu$mol) | $SCN^-$ accumulated ($\mu$mol) | Total $O_2$ uptake ($\mu$mol) | Rate (nmol $O_2$ consumed/ min) |
|---|---|---|---|---|
| Cell alone | 0.015 | - | - | - |
| 0.2 $\mu$mol $Na_2S_2O_3$ | 0.079 | 0.064 | 0.050 | 26.0 |
| 0.4 $\mu$mol $Na_2S_2O_3$ | 0.147 | 0.132 | 0.085 | 32.0 |
| 0.6 $\mu$mol $Na_2S_2O_3$ | 0.236 | 0.221 | 0.130 | 33.0 |
| 0.8 $\mu$mol $Na_2S_2O_3$ | 0.323 | 0.308 | 0.180 | 34.0 |
| 1.0 $\mu$mol $Na_2S_2O_3$ | 0.393 | 0.378 | 0.230 | 38.0 |

[a] The $O_2$ consumption was followed in a Gilson Oxygraph at 25°C with 1.2 mL reaction mixture of 50 mM potassium phosphate buffer at pH 2.3, 6 mg wet cells and various amounts of $Na_2S_2O_3$. Polythionate was measured according to Sörbo (1958).

Table 6. Oxidation of thiosulfate by NEM-treated *T. thioparus*[a].

| | $SCN^-$ ($\mu$mol) | $SCN^-$ accumulated ($\mu$mol) | Total $O_2$ uptake ($\mu$mol) | Rate (nmol $O_2$ consumed/min) |
|---|---|---|---|---|
| Cell alone | 0.024 | - | - | - |
| 0.2 $\mu$mol $Na_2S_2O_3$ | 0.120 | 0.096 | 0.048 | 9.2 |
| 0.4 $\mu$mol $Na_2S_2O_3$ | 0.256 | 0.232 | 0.096 | 11.2 |
| 0.6 $\mu$mol $Na_2S_2O_3$ | 0.418 | 0.394 | 0.150 | 12.0 |
| 0.8 $\mu$mol $Na_2S_2O_3$ | 0.587 | 0.563 | 0.198 | 12.4 |
| 1.0 $\mu$mol $Na_2S_2O_3$ | 0.709 | 0.685 | 0.250 | 12.4 |

[a] The $O_2$ consumption was followed in a Gilson Oxygraph at 25°C with 1.2 mL reaction mixture of 50 mM potassium phosphate buffer at pH 7.0, 6 mg wet cells and various amounts of $Na_2S_2O_3$. Polythionate was measured according to Sörbo (1958).

Table 7. **Effect of HQNO on the thiosulfate oxidation by different sulfur-grown thiobacilli cells at pH 2.3[a].**

| | $SO_3^{2-}$ accumulated ($\mu$mol) | Total $O_2$ uptake ($\mu$mol) | Rate (nmol $O_2$ consumed/min) |
|---|---|---|---|
| Tt-1 | | | |
| -HQNO | 0.001 | 0.200 | 24.4 |
| +HQNO | 0.195 | 0.100 | 12.4 |
| +HQNO[b] | 0.137 | 0.110 | 18.8 |
| Tf-2-$S^0$ | | | |
| -HQNO | 0.003 | 0.196 | 27.6 |
| +HQNO | 0.178 | 0.120 | 8.0 |
| SM4-HEC-$S^0$ | | | |
| -HQNO | 0.002 | 0.190 | 16.8 |
| +HQNO | 0.072 | 0.106 | 8.8 |

[a] The $O_2$ consumption was followed in a Gilson Oxygraph at 25°C with 1.2 mL reaction mixture of 50 mM potassium phosphate buffer at pH 2.3, 6 mg wet cells and 50 $\mu$g HQNO. The reaction was initiated with the addition of 0.1 $\mu$mol $Na_2S_2O_3$ after 10 min preincubation with HQNO. The rate of the first phase of thiosulfate oxidation is measured.

[b] The conditions were the same as described above except that Starkey's medium 1 was used.

Table 8. **Effect of HQNO on the thiosulfate oxidation by NEM-treated *T. thiooxidans* at pH 2.3[a].**

| $Na_2S_2O_3$ ($\mu$mol) | $S^0$ accumulated ($\mu$mol) | $SCN^-$ formed ($\mu$mol) | Total $O_2$ uptake ($\mu$mol) |
|---|---|---|---|
| 0.4 | 0.03 | 0.25 | 0.125 |

[a] The reaction mixture was the same as in Table 7. $S^0$ was determinated as described in Part two.

Table 9. **Effect of pH on tetrathionate oxidation by *T. thiooxidans*[a].**

| pH | Reaction rate (nmol $O_2$/min) | Total $O_2$ uptake (nmol) |
|---|---|---|
| 1.8 | 6.0 | 150 |
| 2.0 | 10.8 | 160 |
| 2.3 | 12.4 | 160 |
| 2.5 | 13.6 | 160 |
| 3.0 | 15.2 | 160 |
| 3.5 | 13.0 | 160 |
| 4.0 | 8.0 | 160 |
| 4.5 | 7.6 | incomplete oxidation |
| 5.0 | 5.2 | incomplete oxidation |

[a] The $O_2$ consumption was followed in a Gilson Oxygraph at 25°C with 1.2 mL reaction mixtures: 50 mM potassium phosphate buffer, 6 mg wet cells and 50 nmol $Na_2S_4O_6$. The reaction was initiated with the addition of $Na_2S_4O_6$.

Table 10. **Effect of NEM, HQNO and different amounts of tetrathionate on tetrathionate oxidation of *T. thiooxidans*[a].**

| Amount of tetrathionate (nmol) | Rate (nmol $O_2$/min) | Total $O_2$ uptake (nmol $O_2$/min) |
|---|---|---|
| 10 | 8.0 | 44 |
| 20 | 14.4 | 75 |
| 30 | 17.2 | 108 |
| 40 | 18.4 | 132 |
| 50 | 18.6 | 178 |
| 60 | 20.6 | 198 |
| 70 | 21.4 | 232 |
| 80 | 20.4 | 255 |
| 80[b] | - | - |
| 80[c] | 12.0 | 136 |

[a] The $O_2$ consumption was followed in a Gilson Oxygraph at 25°C with 1.2 mL reaction mixtures: 50 mM potassium phosphate buffer (pH 2.3), 6 mg wet cells and various amounts of $Na_2S_4O_6$.

[b] The reaction mixture remained the same except NEM-treated cells were used.

[c] The reaction was initiated with the addition of 80 nmol tetrathionate after 10 min preincubation of the reaction mixture with 20 $\mu$g HQNO.

Table 11. Stoichiometry of tetrathionate oxidation *T. thiooxidans*[a].

| | Amount of $Na_2S_4O_6$ (nmol) | Rate (nmol $O_2$/min) | Total $O_2$ uptake (nmol $O_2$) | sulfite found (nmol) |
|---|---|---|---|---|
| + HQNO | | | | |
| | 20 | 6.8 | 54 | 76 |
| | 40 | 7.2 | 78 | 130 |
| | 60 | 8.0 | 112 | 199 |
| - HQNO | | | | |
| | 40 | 8 | 136 | 0 |
| | 60 | 12.4 | 188 | 0 |

[a] The assay conditions are the same as described in Table 10. Sulfite was determined by the pararosaniline method (West and Gaeke 1956).

Table 12. **Thiosulfate oxidation of cell-free extracts of *T. thiooxidans* at different pH.**

| $Na_2S_2O_3$ added ($\mu$mol) | 2.0 | | 2.3 | | 2.5 | |
|---|---|---|---|---|---|---|
| | Total $O_2$ uptake[a] | Rate[b] | Total $O_2$ uptake[a] | Rate[b] | Total $O_2$ uptake[a] | Rate[b] |
| 0.10 | 0.03 | 3.2 | 0.03 | 3.2 | 0.038 | 3.8 |
| 0.25 | 0.07 | 4.8 | 0.08 | 7.2 | 0.074 | 7.8 |
| 0.50 | 0.13 | 9.6 | 0.12 | 12.8 | 0.138 | 12.0 |
| 1.00 | 0.23 | 10.0 | 0.25 | 16.4 | 0.200 | 13.2 |

[a] The unit is $\mu$mol.

[b] The unit is nmol $O_2$ consumed/min.

Table 13. **Kinetic constants for thiosulfate oxidation by NEM-treated *T. thiooxidans* at various pH values.**

| pH | $K_{m1}$ | $K_{m2}$ | $V_{max1}$ | $V_{max2}$ |
|---|---|---|---|---|
| 1.8 | --- | 0.9 | --- | 66.7 |
| 2.0 | 0.11 | 1.7 | 16.9 | 67.0 |
| 2.5 | 0.04 | 3.6 | 19.1 | 66.7 |
| 3.0 | 0.07 | 2.0 | 21.4 | 57.1 |
| 3.5 | 0.08 | 4.3 | 7.9 | 32.6 |
| 4.0 | 0.51 | 5.0 | 11.6 | 20.0 |
| 4.5 | 0.17 | 20.0 | 4.1 | 15.2 |

Table 14. Kinetic constants for thiosulfate oxidation by *T. thiooxidans* at various pH values.

| pH | $K_{m1}$ | $K_{m2}$ | $V_{max1}$ | $V_{max2}$ |
|---|---|---|---|---|
| 1.8 | 0.05 | 0.50 | 14.0 | 38.0 |
| 2.0 | 0.08 | 0.65 | 17.5 | 48.0 |
| 2.5 | 0.03 | 0.55 | 12.0 | 56.0 |
| 3.0 | 0.05 | 0.60 | 20.0 | 35.0 |
| 3.5 | 0.08 | 0.60 | 20.0 | 16.0 |
| 4.0 | 0.10 | 2.50 | 15.0 | 10.0 |
| 4.5 | 0.15 | 5.00 | 10.5 | 10.5 |

Table 15. **Kinetic constants for thiosulfate oxidation by cell-free extracts of *T. thiooxidans* at various pH values.**

| pH | $K_m$ (mM) | $V_{max}$ (nmol $O_2$ consumed/min) |
|---|---|---|
| 1.8 | 1.19 | 16.23 |
| 2.0 | 0.26 | 12.84 |
| 2.3 | 0.46 | 21.58 |
| 2.5 | 0.35 | 19.96 |
| 3.0 | 4.75 | 27.31 |

Table 16. **Effect of pH on the reduction of *T. thiooxidans* cytochrome c with thiosulfate[a].**

| Sample | pH | Rate of reduction ($\Delta A_{416nm}$/min) | Total in 4 min: $\Delta A_{416nm}$ | Total in 4 min: % reduction |
|---|---|---|---|---|
| Acidic extracts | | | | |
| | 2.5 | 0.155 | 0.031 | 97 |
| | 3.2 | 0.005 | 0.016 | 50 |
| | 6.5 | 0.003 | 0.011 | 34 |
| | 7.0 | 0.000 | 0.000 | 0 |
| + Dithionite | 3.2 | instant | 0.032 | 100 |
| Boiled extracts | 2.5 | 0.000 | 0.000 | 0 |
| + Dithionite | 2.5 | instant | 0.020 | 100 |

[a] The reaction mixture (final pH as indicated) in a total volume of 1 mL contained 200 $\mu$L acidic extracts (4 mg protein) with or without boiling (15 min) and 50 mM potassium phosphate buffer. The reaction was started with the addition of 1 $\mu$mol $Na_2S_2O_3$ or dithionite (when indicated) and the change in absorbance at 416 nm was determined as the initial rate and the total change.

Table 17. **Reduction of horse heart cytochrome c by the acidic extracts of *T. thiooxidans*.**

| pH | Acidic extracts ($\mu$L) | Additions | $\Delta A_{550nm}$ | Time |
|---|---|---|---|---|
| 6.0 | 10 | None | 0.02 | 6 min |
| | | 0.5 $\mu$mol $Na_2SO_3$ | 0.02 | Instant |
| | | 20 $\mu$L extracts | 0.05 | Instant |
| | | 30 $\mu$L extracts | 0.07 | Instant |
| 6.0 | 20 | none | 0.03 | 6 min |
| | | 0.5 $\mu$mol $Na_2SO_3$ | 0.04 | Instant |
| 6.0 | 50 | 0.5 $\mu$mol $Na_2SO_3$ | 0.15 | Instant |
| 8.0 | 50 | none | 0.09 | 2 min |
| | | 0.5 $\mu$mol $Na_2SO_3$ | 0.05 | 30 min |
| 7.0 | 40 | 0.5 $\mu$mol $Na_2SO_3$ | 0.113 | 1.3 min |
| 7.0 | 40 | 0.5 $\mu$mol EDTA | | |
| | | 0.5 $\mu$mol $Na_2SO_3$ | 0.035 | 13 min |
| 7.0 | 40 | 5 $\mu$mol NEM | | |
| | | 0.5 $\mu$mol $Na_2SO_3$ | 0.003 | 1.5 min |
| 4.3 | 100 | 0.5 $\mu$mol $Na_2SO_3$ | 0.130 | instant |
| 4.3 | 100 | 10 $\mu$mol NEM | | |
| | | 0.5 $\mu$mol $Na_2SO_3$ | 0.130 | Instant |

[a] The reaction mixture in a total volume of 1 mL (pH as indicated) contained 50 mM potassium phosphate buffer, 10 $\mu$mol horse heart cytochrome c, and varied amounts of acidic extracts (20 mg protein/mL) to start the reaction. Additions were made in the sequence shown and the reduction of horse heart cytochrome c followed at 550 nm ($\Delta A_{550nm}$) and time required for the reduction were recorded after each addition of $Na_2SO_3$ or acidic extracts. EDTA was preincubated for 15 min and NEM for 2½ hrs with the acidic extracts before the addition of horse heart cytochrome c and sulfite.

Table 18. **Inhibition of thiosulfate:ferricyanide oxidoreductase in the acidic extracts of *T. thiooxidans* by sulfite[a].**

| pH | $Na_2SO_3$ (mM) | Preincubation | $\Delta A_{420nm}$/min | Activity (%) |
|---|---|---|---|---|
| 4.3 | 0.0 | | 0.121 | 100 |
| | 1.0 | - | 0.041 | 34 |
| 4.3 | 0.0 | | 0.160 | 100 |
| | 1.0 | + | 0.014 | 9 |
| 2.0 | 0.0 | | 0.212 | 100 |
| | 0.5 | + | 0.126 | 59 |

[a] The reaction mixture (1 mL, final pH as indicated) contained 50 mM potassium phosphate buffer, 0.1 mL acidic extracts (2 mg protein), 1 $\mu$mol potassium ferricyanide, and 5 $\mu$mol $Na_2S_2O_3$. Preincubation of the acidic extracts with $Na_2SO_3$ was 15 min.

Table 19. **Thiosulfate:ferricyanide oxidoreductase activity in the acidic extracts of *T. thiooxidans*[a].**

| Substrate | Concentration (mM) | Rate ($\Delta A_{420\ nm}$/min) |
|---|---|---|
| $Na_2S_2O_3$ | 2 | 0.040 |
| | 5 | 0.094 |
| | 5 | 0.072[b] |
| $Na_2SO_3$ + AMP | 2 | 0.000 |
| | 1 | 0.000 |
| $Na_2S_3O_6$ | 2 | 0.000 |
| $Na_2S_4O_6$ | 2 | 0.000 |

[a] The absorbance change at 420 nm ($\Delta A_{420nm}$) was followed at room temperature with 1 mL reaction mixture of 50 mM potassium phosphate buffer, 0.1 mL acidic extracts (2 mg protein), 1 $\mu$mol potassium ferricyanide and substrate (to start the reaction) as indicated. The pH was 4.3.

[b] Boiled acidic extracts (15 min boiling) was used.

Table 20. **Stoichiometric studies of ferricyanide reduction by thiosulfate:ferricyanide oxidoreductase in the acidic extracts of *T. thiooxidans*[a].**

| $S_2O_3$ ($\mu$mol) | Ferricyanide reduced ($\mu$mol) | Thiocyanate ($SCN^-$) formed ($\mu$mol) |
|---|---|---|
| 0.0 | 0.00 | 0.00 |
| 2.5 | 0.68 | 0.25 |
| 5.0 | 0.82 | 0.36 |
| 10.0 | 0.92 | 0.53 |

[a] The reaction mixture contained 50 mM potassium phosphate buffer at pH 6.0, 0.1 mL acidic extracts (2 mg protein), 1 $\mu$mol potassium ferricyanide, and various amounts of $Na_2S_2O_3$. The reaction was initiated with the addition of thiosulfate. After 90 min, polythionate was measured after cyanolysis as thiocyanate ($SCN^-$) according to Sörbo (1958). The values were corrected for chemical reactions without acidic extracts.

Table 21. **Effect of different amounts of acidic extracts of *T. thiooxidans* on ferricyanide reduction[a].**

| Amount of extracts (mg) | Total rate ($\Delta A_{420nm}$/min) | Chemical rate ($\Delta A_{420nm}$/min) | Rate difference ($\Delta A_{420nm}$/min) |
|---|---|---|---|
| 0.1 | 0.0881 | 0.0306 | 0.0575 |
| 0.2 | 0.1102 | 0.0246 | 0.0856 |
| 0.4 | 0.1374 | 0.0182 | 0.1192 |
| 0.6 | 0.1673 | 0.0215 | 0.1458 |
| 0.8 | 0.1726 | 0.0204 | 0.1522 |
| 1.0 | 0.2016 | 0.0202 | 0.1814 |

[a] The reaction mixture contained 50 mM potassium phosphate buffer at pH 2.0, 1 $\mu$mol potassium ferricyanide and various amounts of acidic extracts. The reaction was initiated with the addition of 5 $\mu$mol $Na_2S_2O_3$.

# FIGURES

Figure 1. **Effect of pH on thiosulfate oxidation by NEM-treated *T. thiooxidans*.**

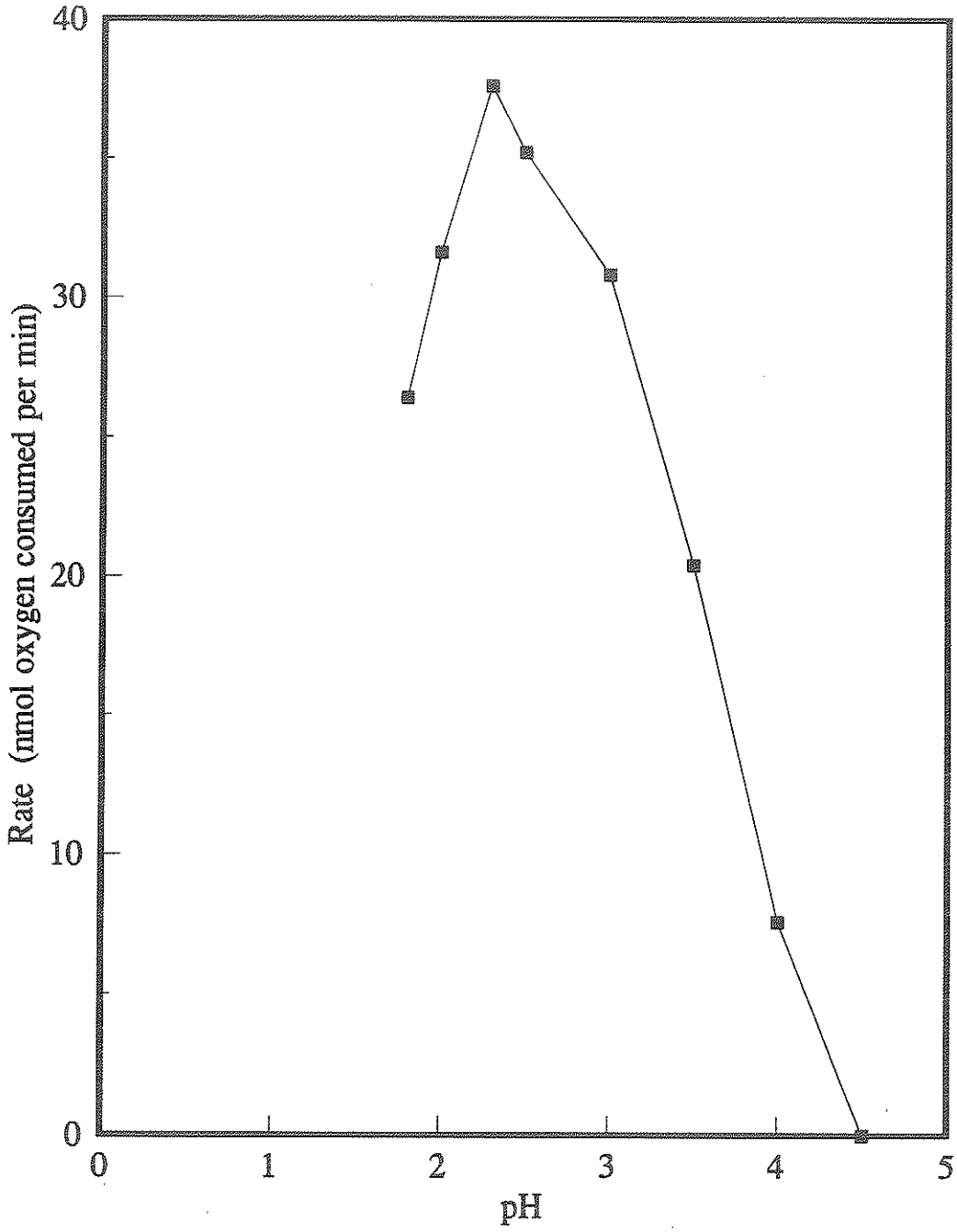

40
30
20
10
0
Rate (nmol oxygen consumed per min)
0
1
2
3
4
5
pH

Figure 2. Effect of cell concentration on thiosulfate oxidation by NEM-treated *T. thiooxidans*.

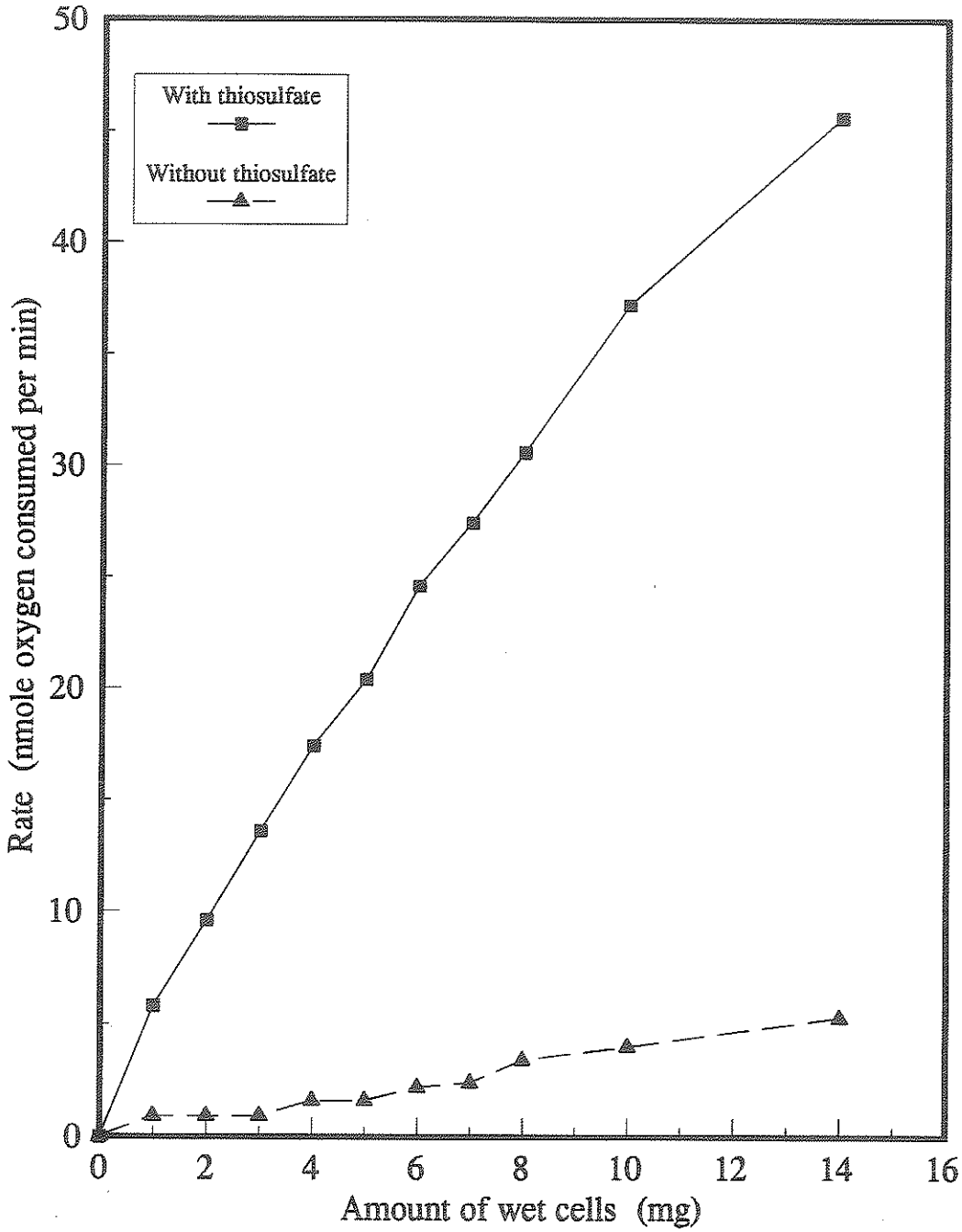

With thiosulfate
Without thiosulfate
Rate (nmole oxygen consumed per min)
Amount of wet cells (mg)
0
10
20
30
40
50
0
2
4
6
8
10
12
14
16

Figure 3. Effect of sulfite on the thiosulfate oxidation by NEM-treated *T. thiooxidans*.

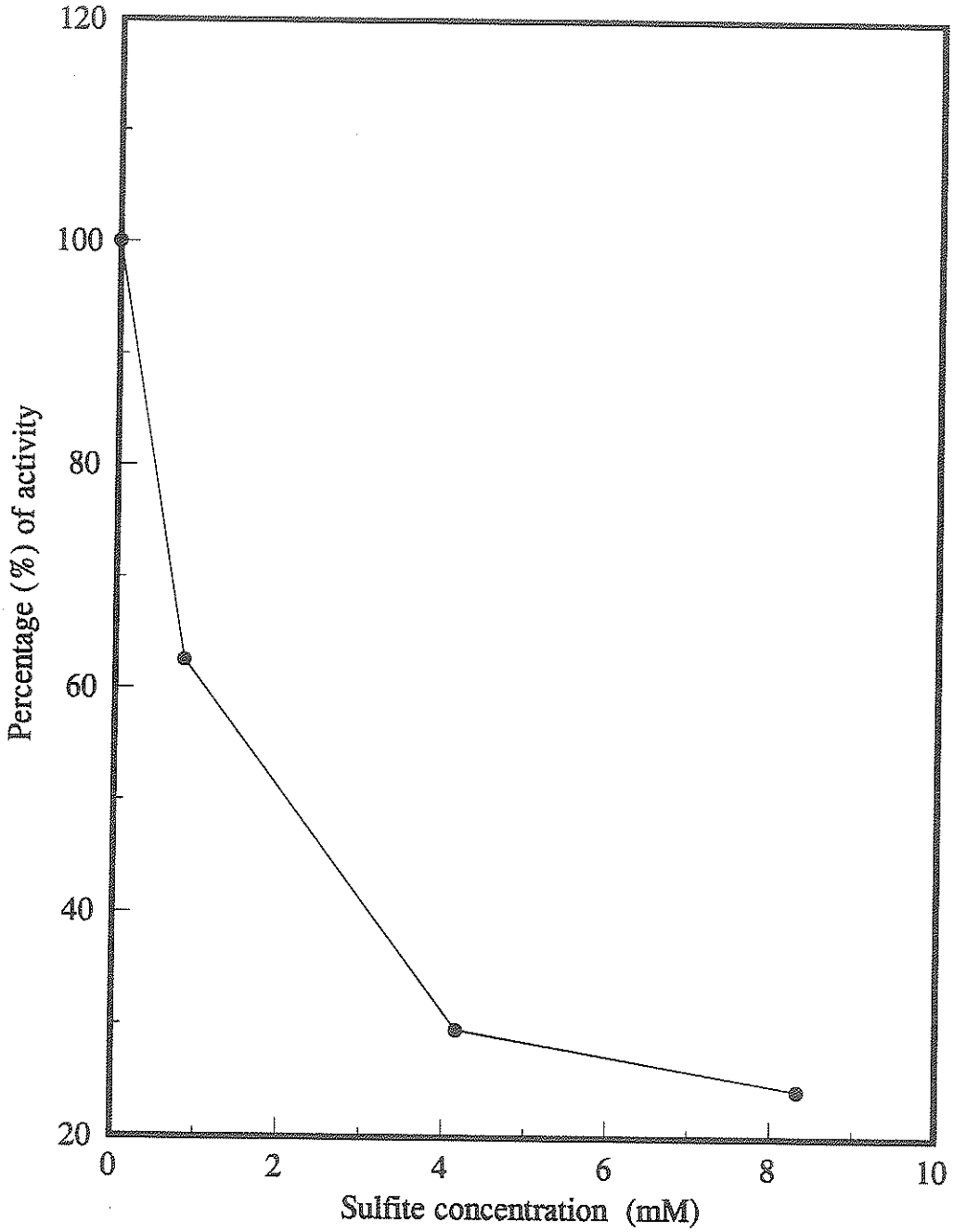

120
100
80
60
40
20
Percentage (%) of activity
0
2
4
6
8
10
Sulfite concentration (mM)

Figure 4. Effect of pH on thiosulfate oxidation by cell-free extracts of *T. thiooxidans*.

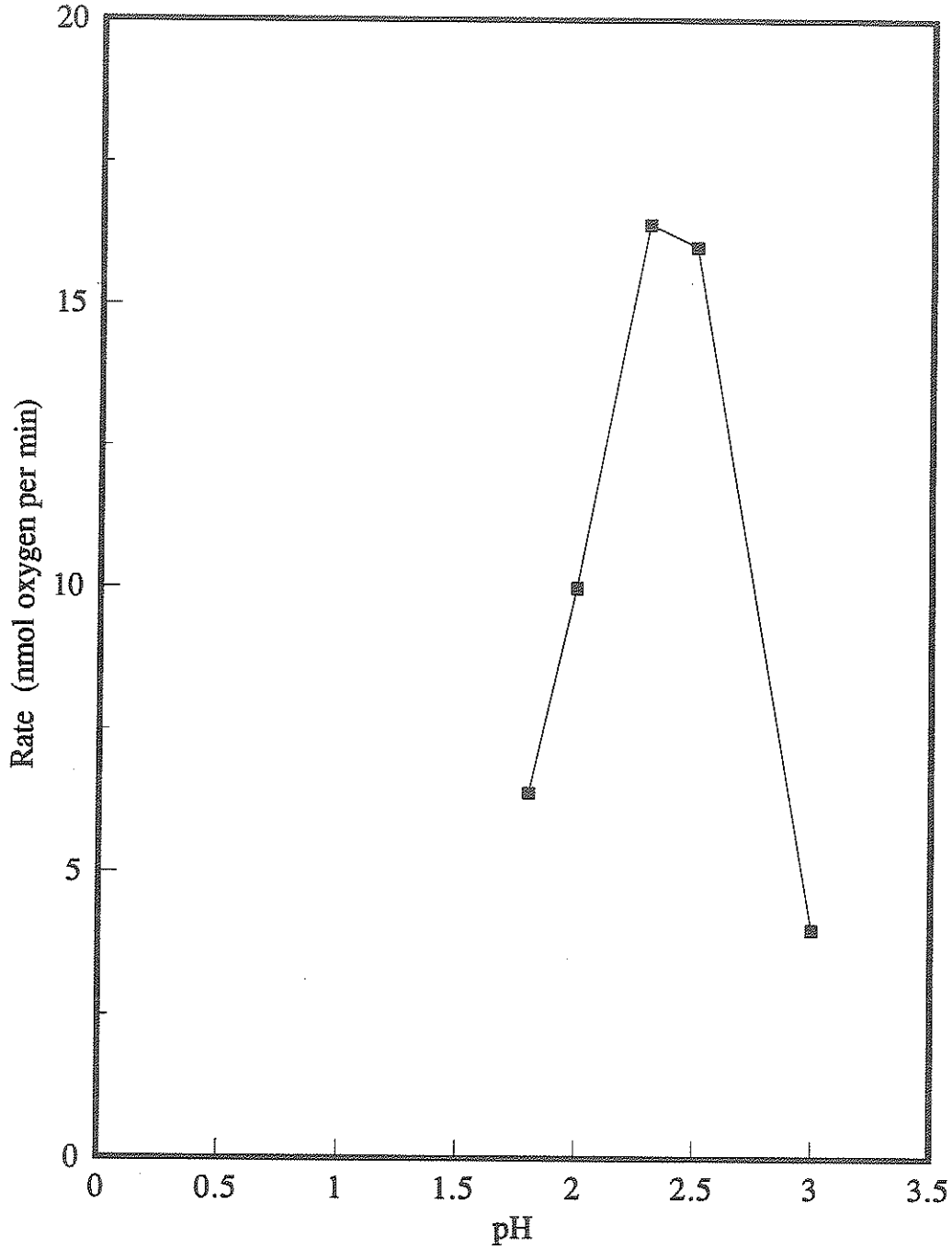

20
15
10
5
0
Rate (nmol oxygen per min)
0
0.5
1
1.5
2
2.5
3
3.5
pH

Figure 5. Velocity plot of thiosulfate oxidation at pH 1.8 by *T. thiooxidans* with and without NEM-treatment.

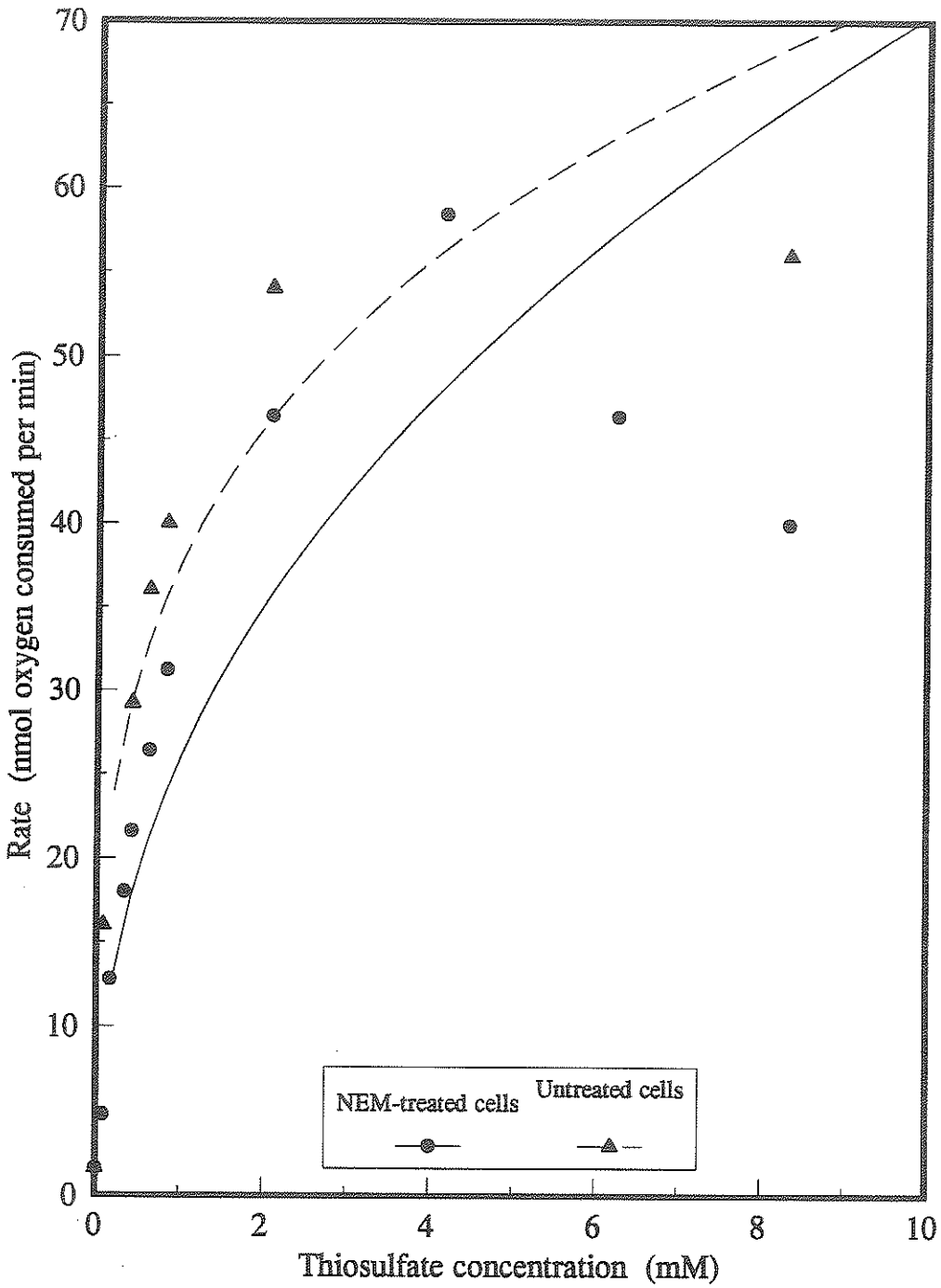

70
60
50
40
30
20
10
0
Rate (nmol oxygen consumed per min)
0
2
4
6
8
10
Thiosulfate concentration (mM)
NEM-treated cells
Untreated cells

Figure 6. Velocity plot of thiosulfate oxidation at pH 2.0 by *T. thiooxidans* with and without NEM-treatment.

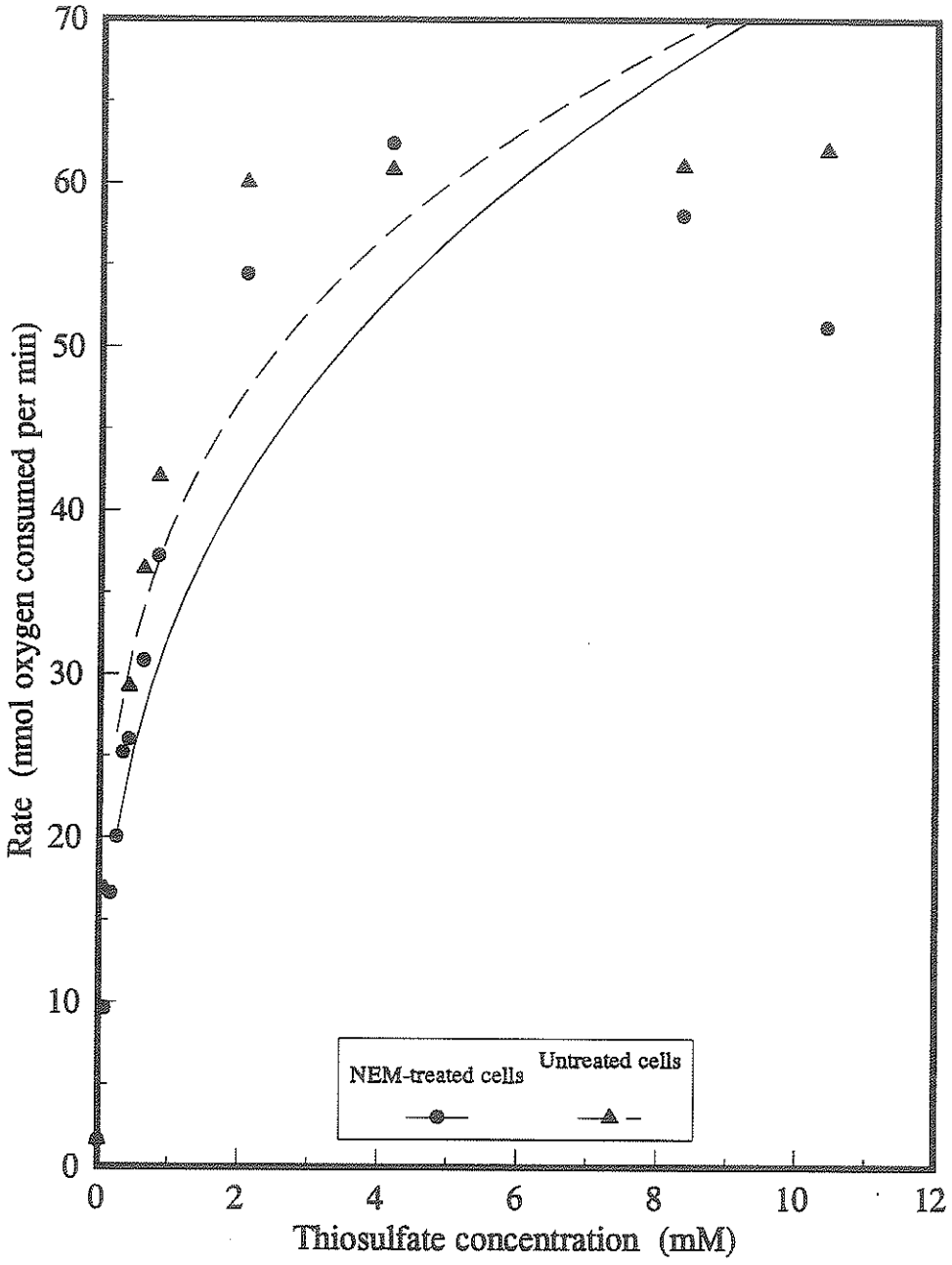

70
60
50
40
30
20
10
0
Rate (nmol oxygen consumed per min)
0
2
4
6
8
10
12
Thiosulfate concentration (mM)
NEM-treated cells
Untreated cells

Figure 7. Velocity plot of thiosulfate oxidation at pH 2.5 by *T. thiooxidans* with and without NEM-treatment.

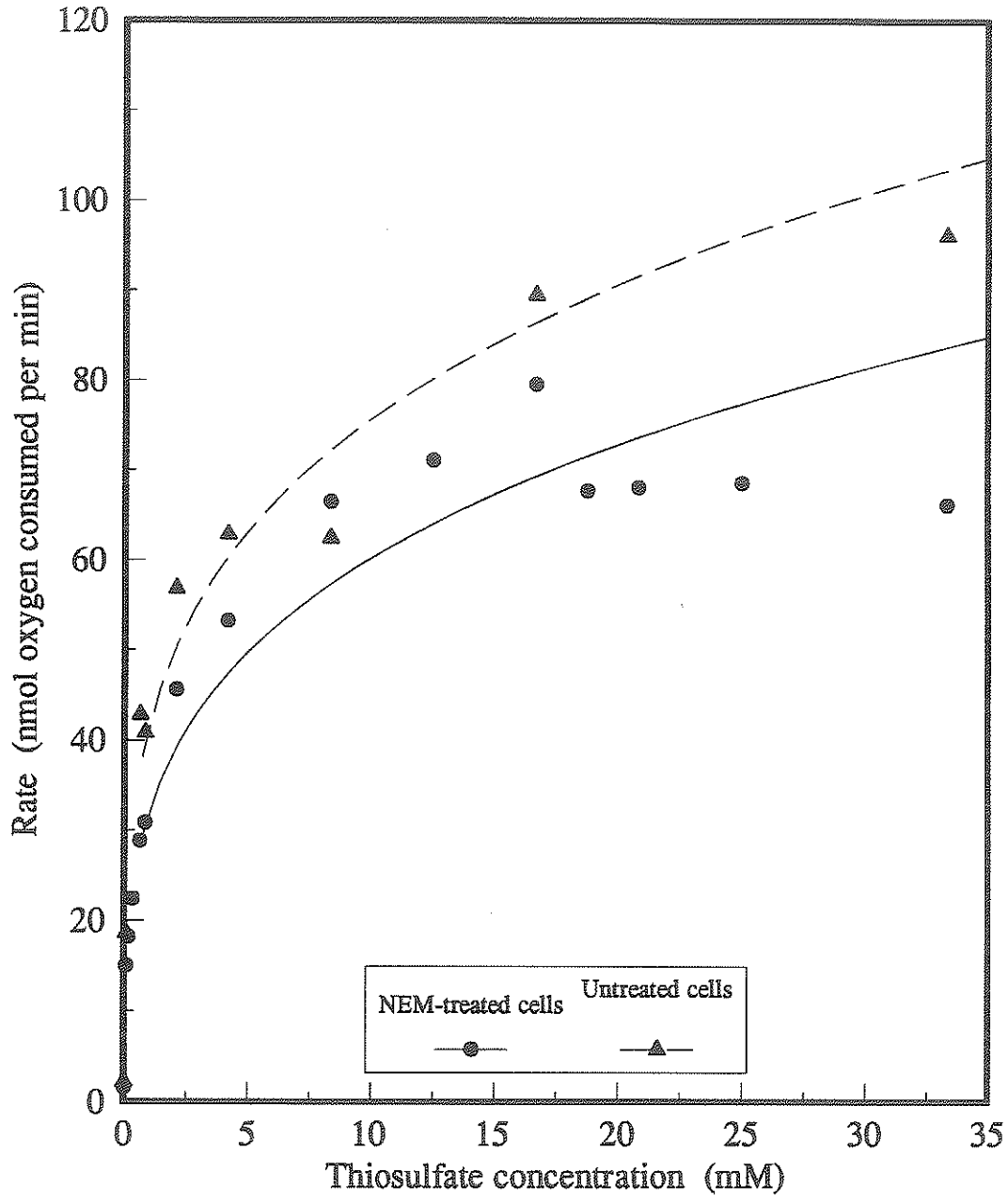

120
100
80
60
40
20
0
Rate (nmol oxygen consumed per min)
0
5
10
15
20
25
30
35
Thiosulfate concentration (mM)
NEM-treated cells
Untreated cells

Figure 8. Velocity plot of thiosulfate oxidation at pH 3.0 by *T. thiooxidans* with and without NEM-treatment.

Figure 9. Velocity plot of thiosulfate oxidation at pH 3.5 by *T. thiooxidans* with and without NEM-treatment.

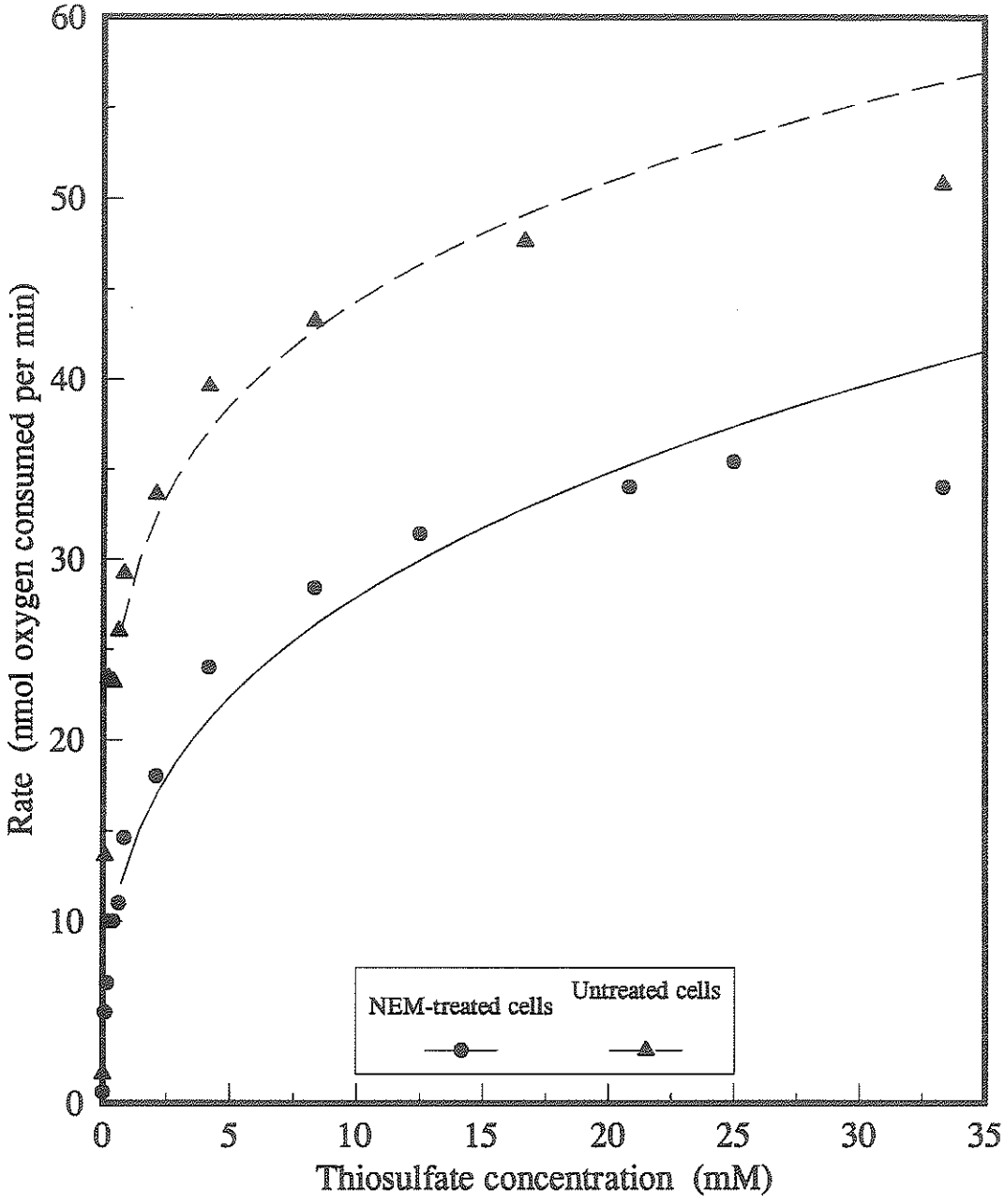

60
50
40
30
20
10
0
Rate (nmol oxygen consumed per min)
0
5
10
15
20
25
30
35
Thiosulfate concentration (mM)
NEM-treated cells
Untreated cells

Figure 10. Velocity plot of thiosulfate oxidation at pH 4.0 by *T. thiooxidans* with and without NEM-treatment.

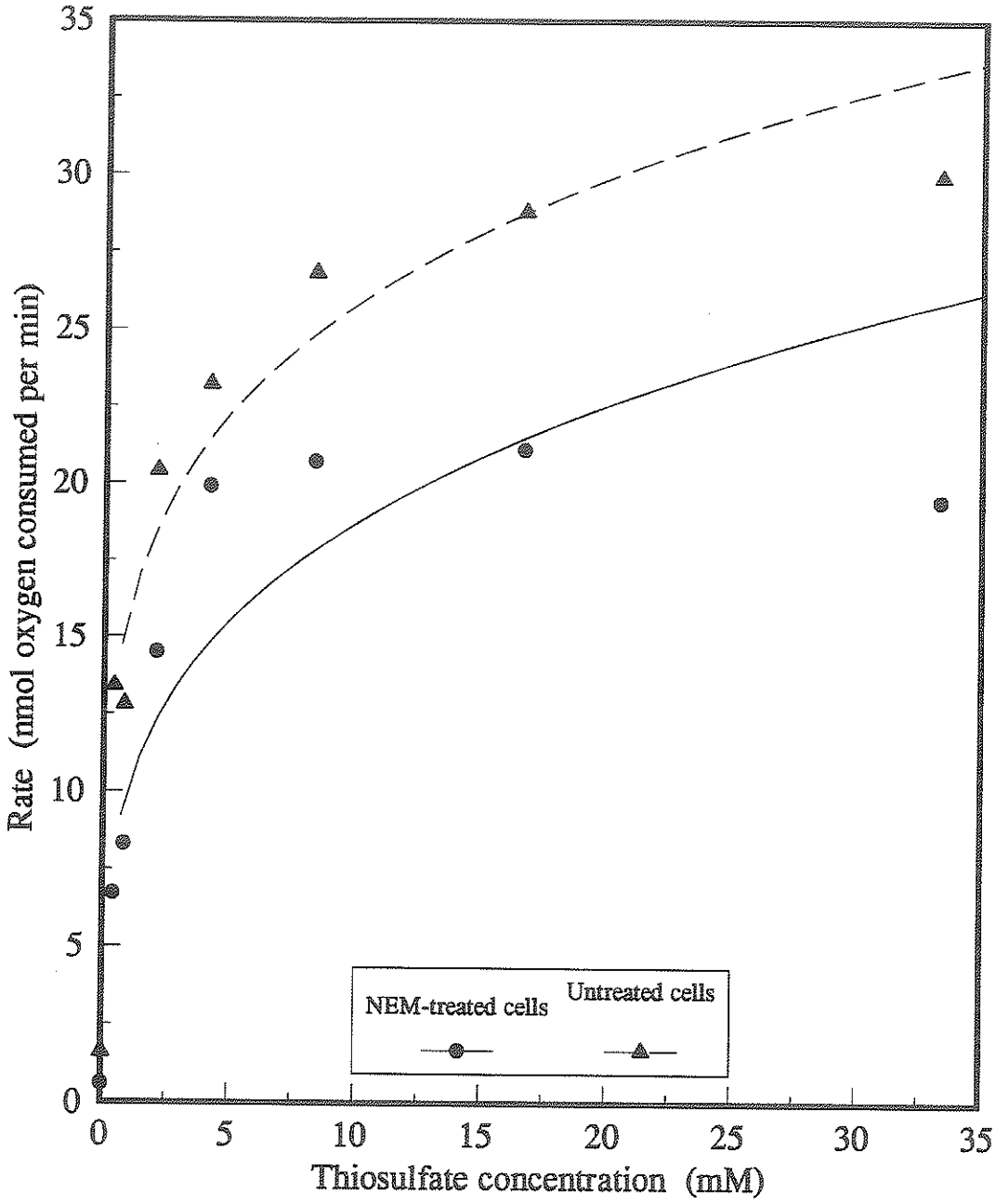

35
30
25
20
15
10
5
0
Rate (nmol oxygen consumed per min)
0
5
10
15
20
25
30
35
Thiosulfate concentration (mM)
NEM-treated cells
Untreated cells

Figure 11. Velocity plot of thiosulfate oxidation at pH 4.5 by *T. thiooxidans* with and without NEM-treatment.

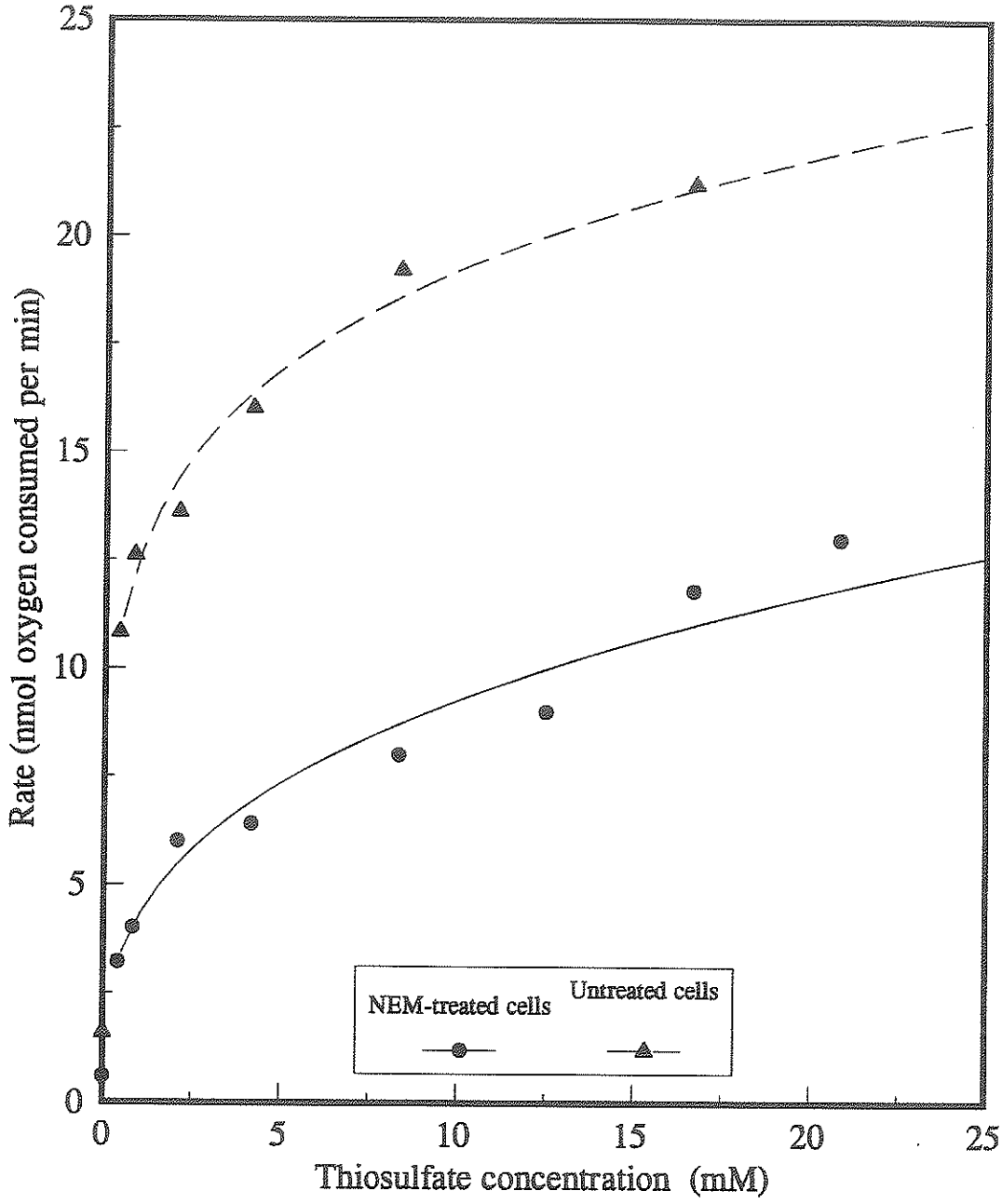

25
20
15
10
5
0
0
5
10
15
20
25
Rate (nmol oxygen consumed per min)
Thiosulfate concentration (mM)
NEM-treated cells
Untreated cells

Figure 12. **Double reciprocal plot of thiosulfate oxidation at pH 1.8 by *T. thiooxidans* with and without NEM-treatment.**

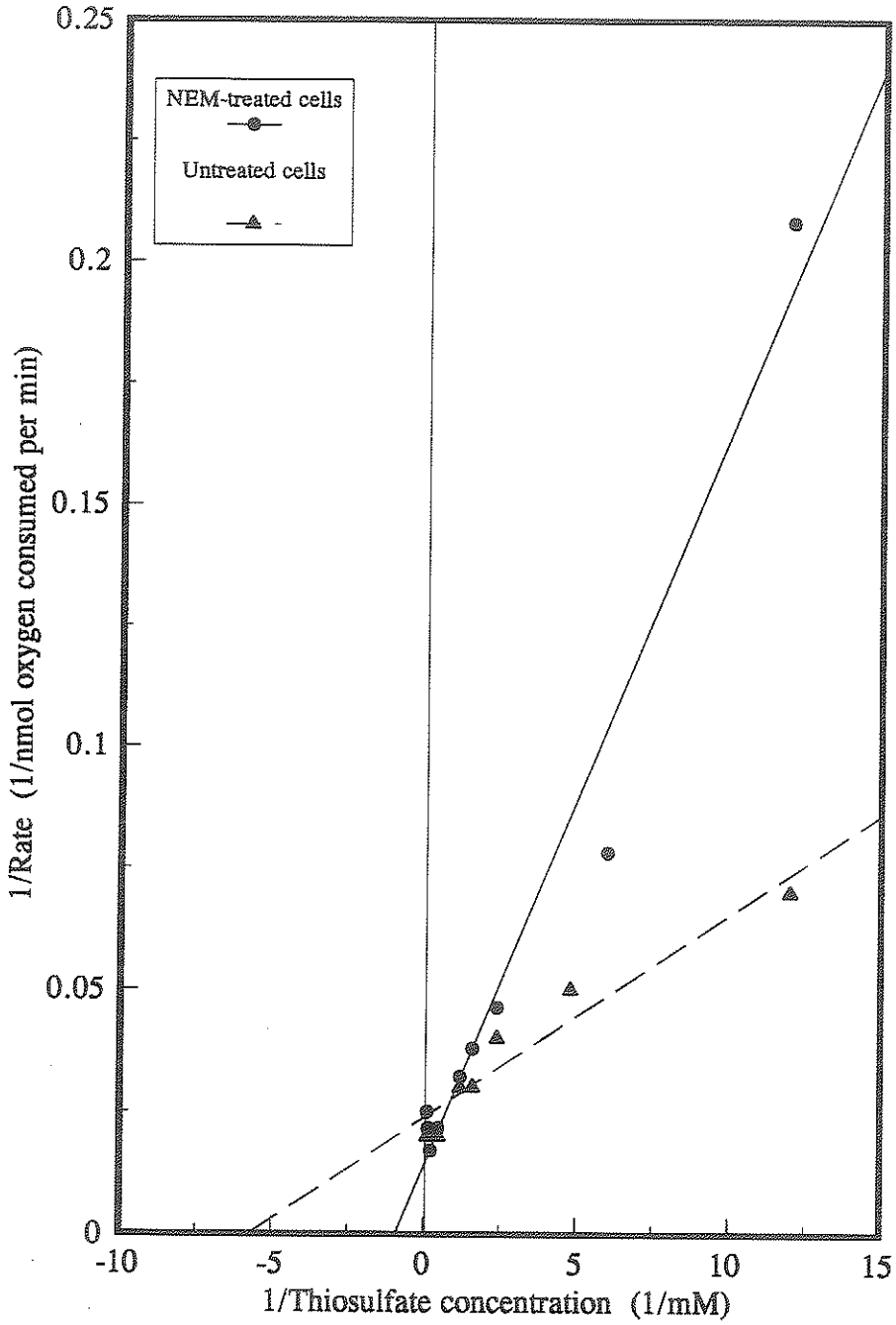

NEM-treated cells
Untreated cells
0.25
0.2
0.15
0.1
0.05
0
-10
-5
0
5
10
15
1/Rate (1/nmol oxygen consumed per min)
1/Thiosulfate concentration (1/mM)

Figure 13. Double reciprocal plot of thiosulfate oxidation at pH 2.0 by *T. thiooxidans* with and without NEM-treatment.

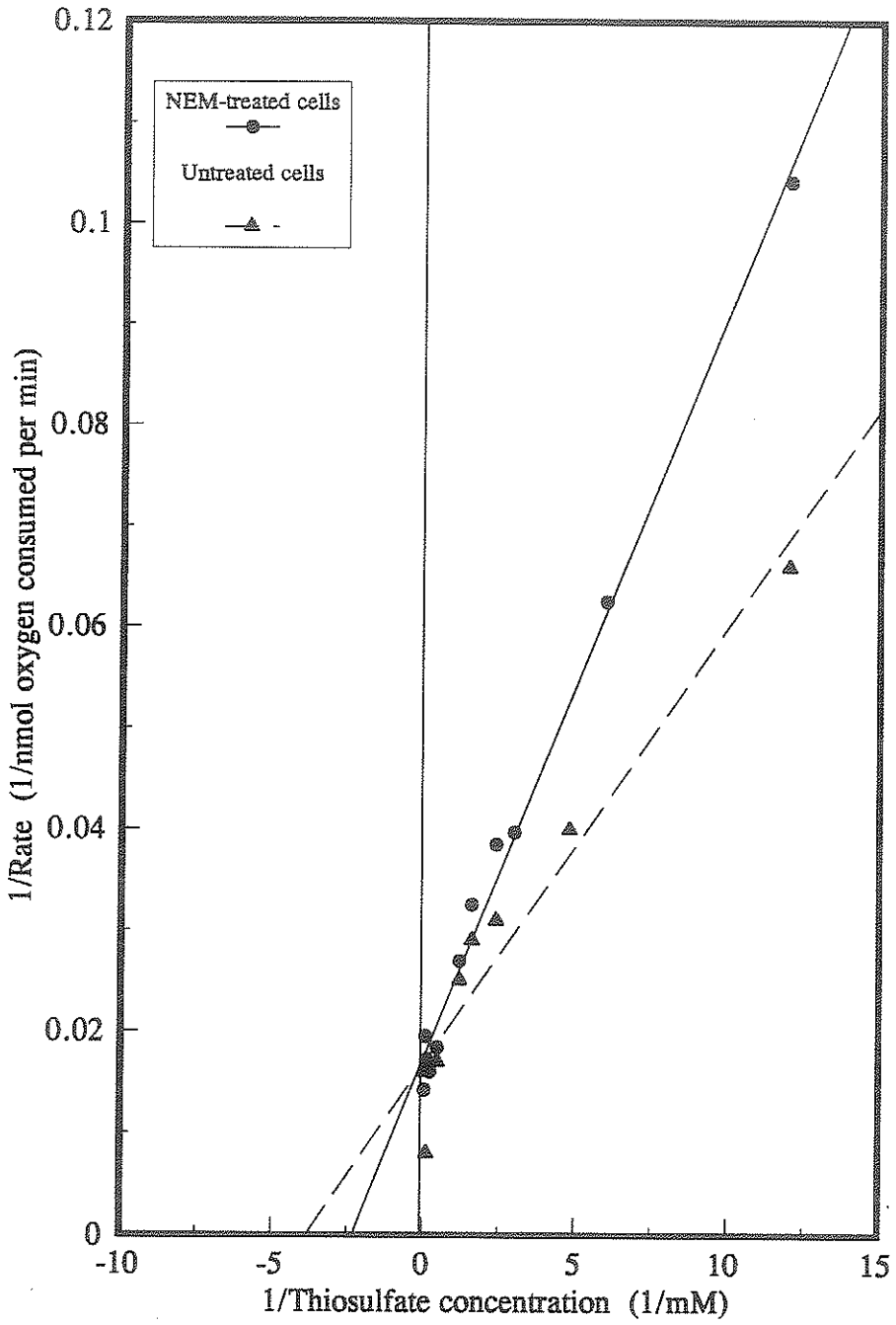

NEM-treated cells
Untreated cells
0.12
0.1
0.08
0.06
0.04
0.02
0
-10
-5
0
5
10
15
1/Rate (1/nmol oxygen consumed per min)
1/Thiosulfate concentration (1/mM)

Figure 14. Double reciprocal plot of thiosulfate oxidation at pH 2.5 by *T. thiooxidans* with and without NEM-treatment.

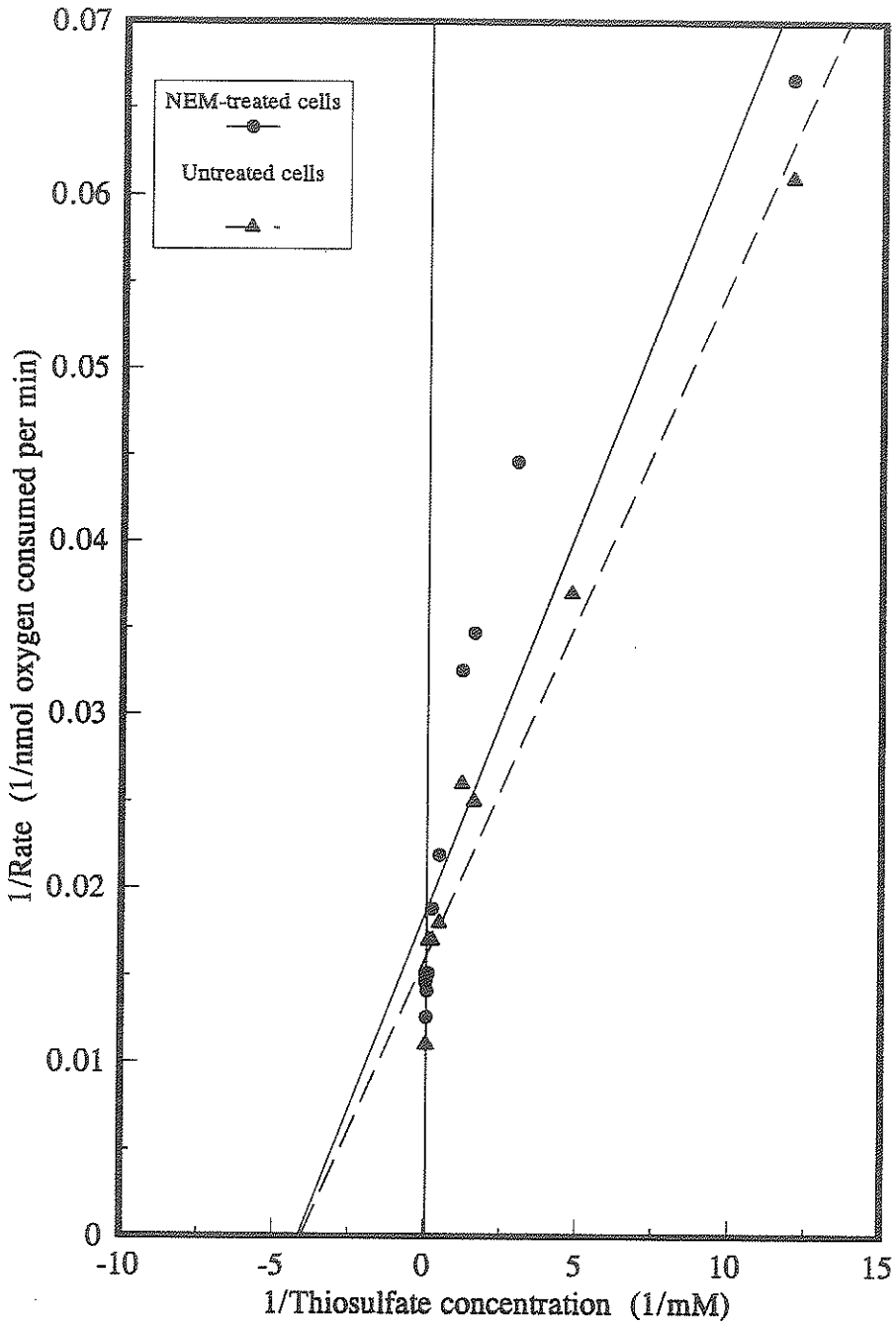

NEM-treated cells
Untreated cells
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1/Rate (1/nmol oxygen consumed per min)
-10
-5
0
5
10
15
1/Thiosulfate concentration (1/mM)

Figure 15. Double reciprocal plot of thiosulfate oxidation at pH 3.0 by *T. thiooxidans* with and without NEM-treatment.

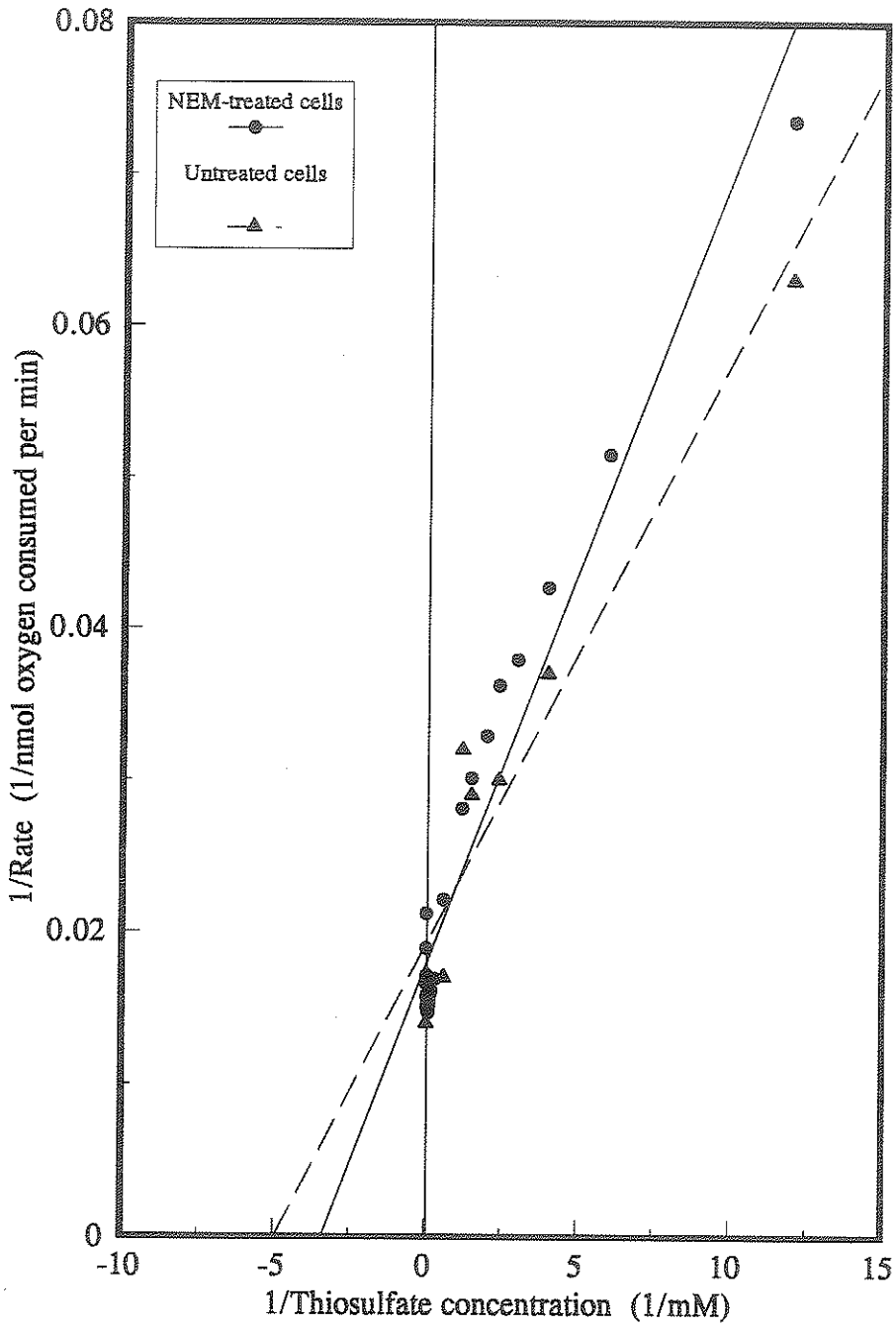

NEM-treated cells
Untreated cells
0.08
0.06
0.04
0.02
0
-10
-5
0
5
10
15
1/Rate (1/nmol oxygen consumed per min)
1/Thiosulfate concentration (1/mM)

Figure 16. Double reciprocal plot of thiosulfate oxidation at pH 3.5 by *T. thiooxidans* with and without NEM-treatment.

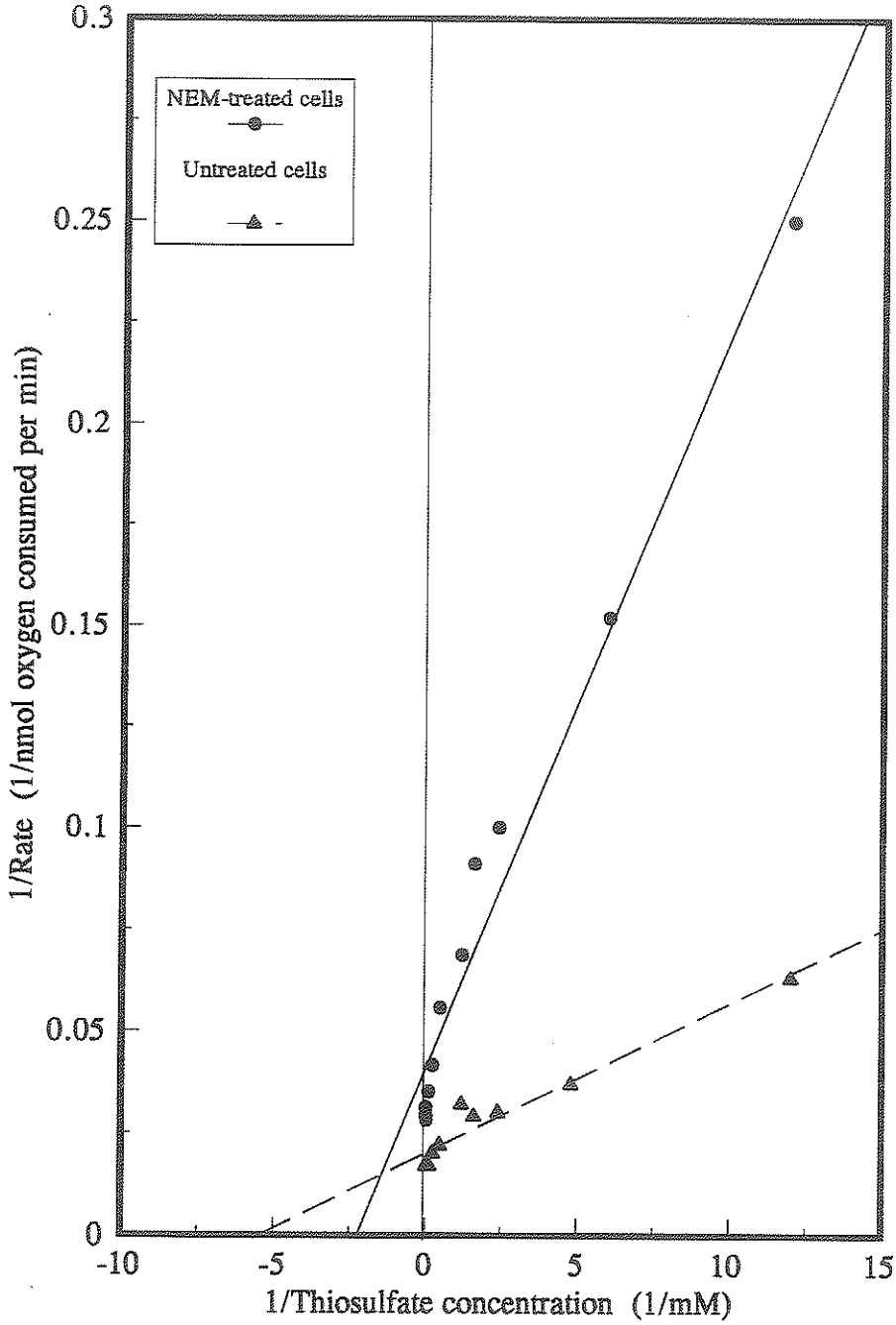

NEM-treated cells
Untreated cells
0.3
0.25
0.2
0.15
0.1
0.05
0
-10
-5
0
5
10
15
1/Rate (1/nmol oxygen consumed per min)
1/Thiosulfate concentration (1/mM)

Figure 17. Double reciprocal plot of thiosulfate oxidation at pH 4.0 by *T. thiooxidans* with and without NEM-treatment.

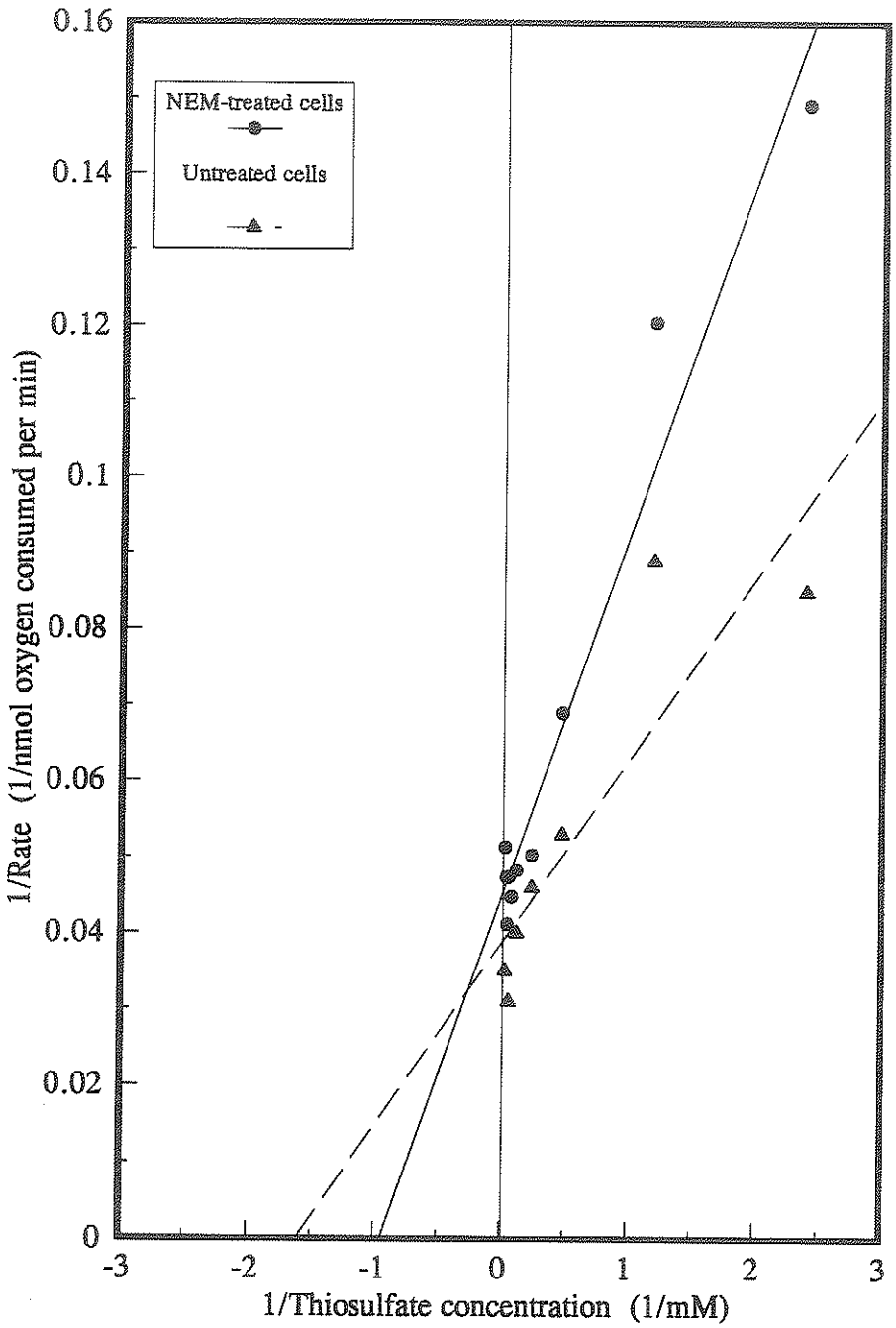

NEM-treated cells
Untreated cells
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-3
-2
-1
0
1
2
3
1/Rate (1/nmol oxygen consumed per min)
1/Thiosulfate concentration (1/mM)

Figure 18. Double reciprocal plot of thiosulfate oxidation at pH 4.5 by *T. thiooxidans* with and without NEM-treatment.

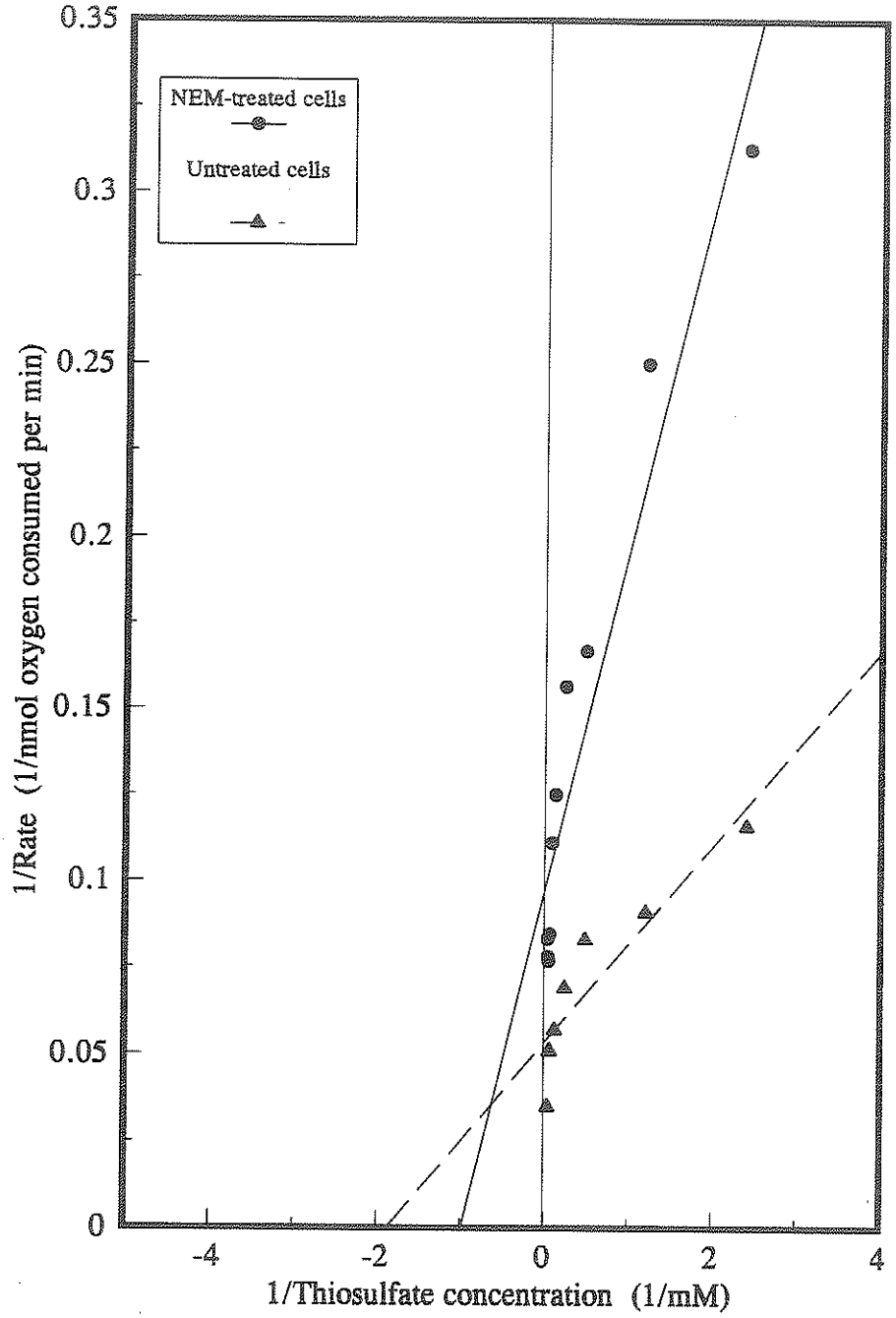

NEM-treated cells
Untreated cells
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-4
-2
0
2
4
1/Rate (1/nmol oxygen consumed per min)
1/Thiosulfate concentration (1/mM)

Figure 19. Double reciprocal plot of thiosulfate oxidation at different pH by cell-free extracts of *T. thiooxidans*.

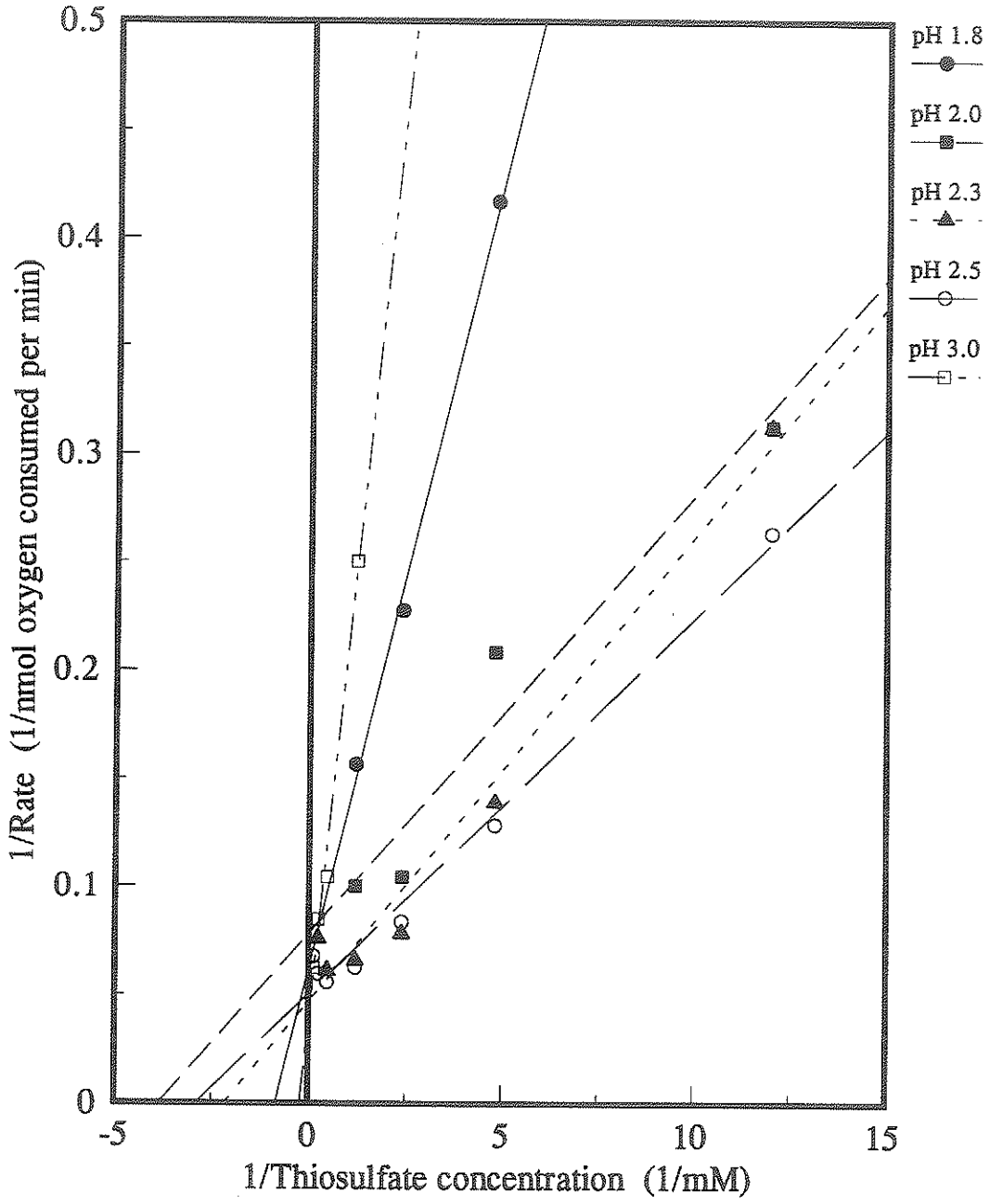
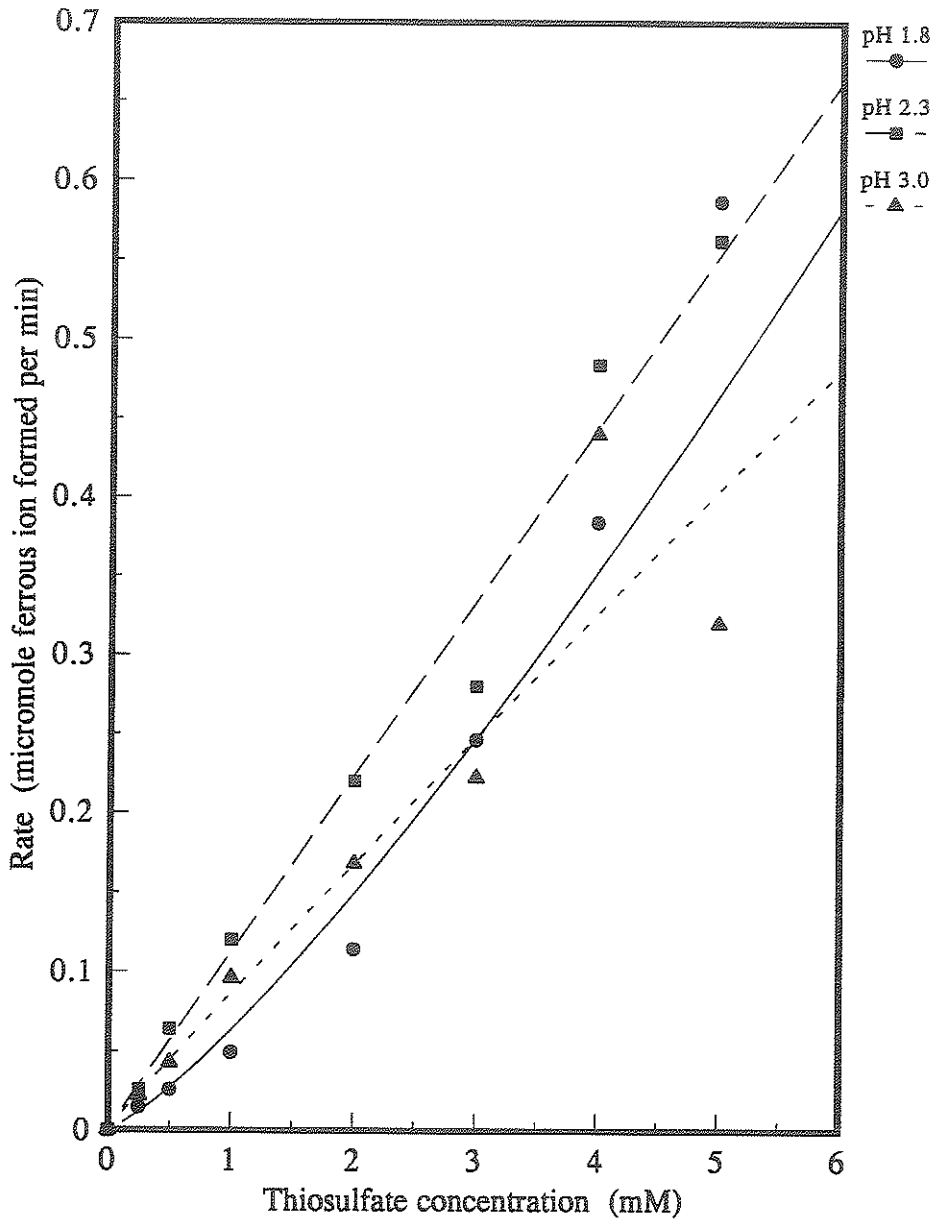
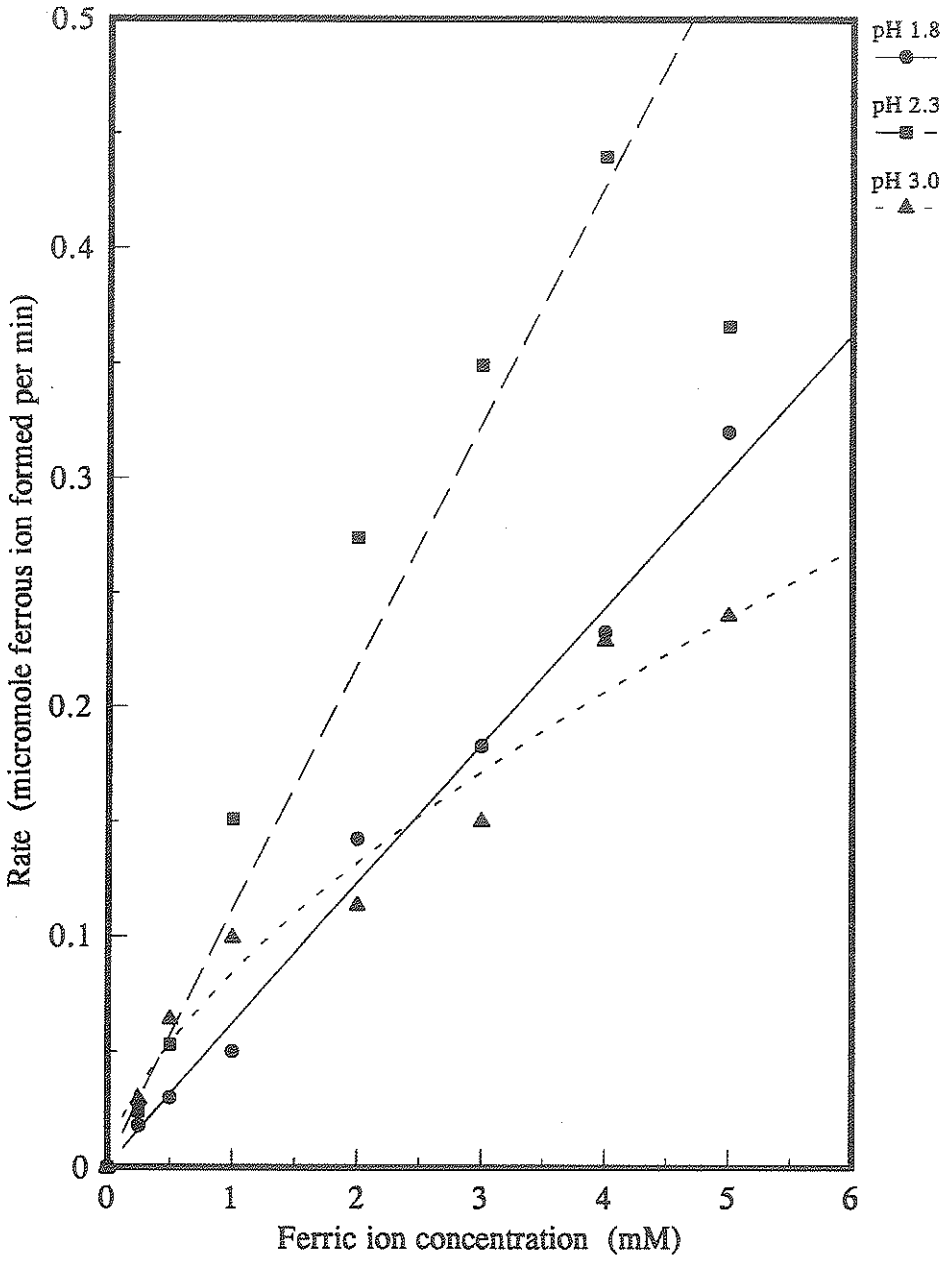

0.5
0.4
0.3
0.2
0.1
0
-5
0
5
10
15
1/Rate (1/nmol oxygen consumed per min)
1/Thiosulfate concentration (1/mM)
pH 1.8
pH 2.0
pH 2.3
pH 2.5
pH 3.0

Figure 20. Velocity plot of different amounts of thiosulfate with a fixed amount of ferric ion (1 mM).




Note: based on ferrous ion formation data

Figure 21. Velocity plot of different amounts of ferric ion with a fixed amount of thiosulfate (1 mM).




Note: based on ferrous ion formation data

Figure 22. Chemical reaction of different amounts of thiosulfate with a fixed amount of ferric ion (1 mM) added at pH 1.8.

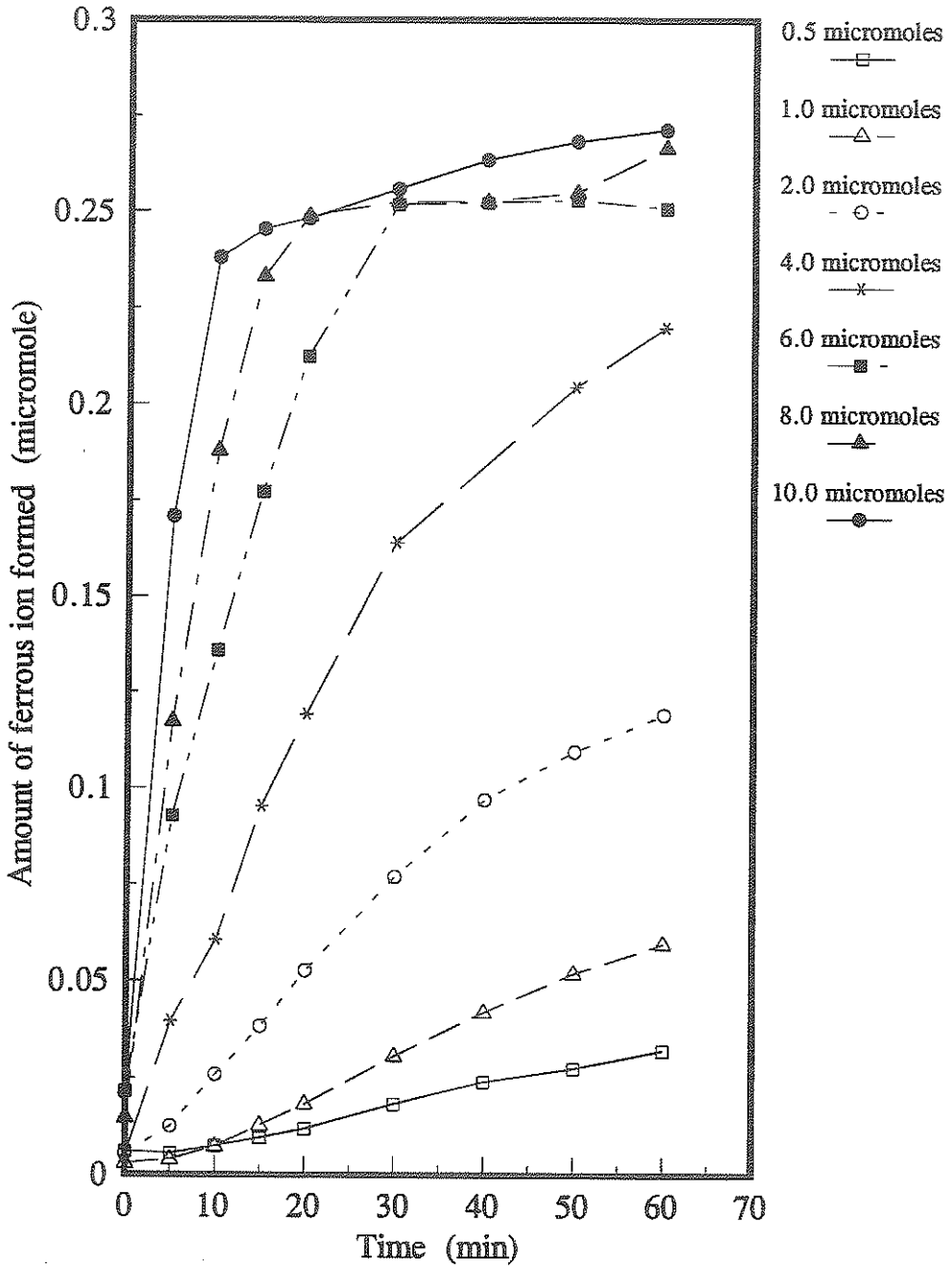

0.3
0.25
0.2
0.15
0.1
0.05
0
Amount of ferrous ion formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 23. Chemical reaction of different amounts of thiosulfate with a fixed amount of ferric ion (1 mM) added at pH 2.3.

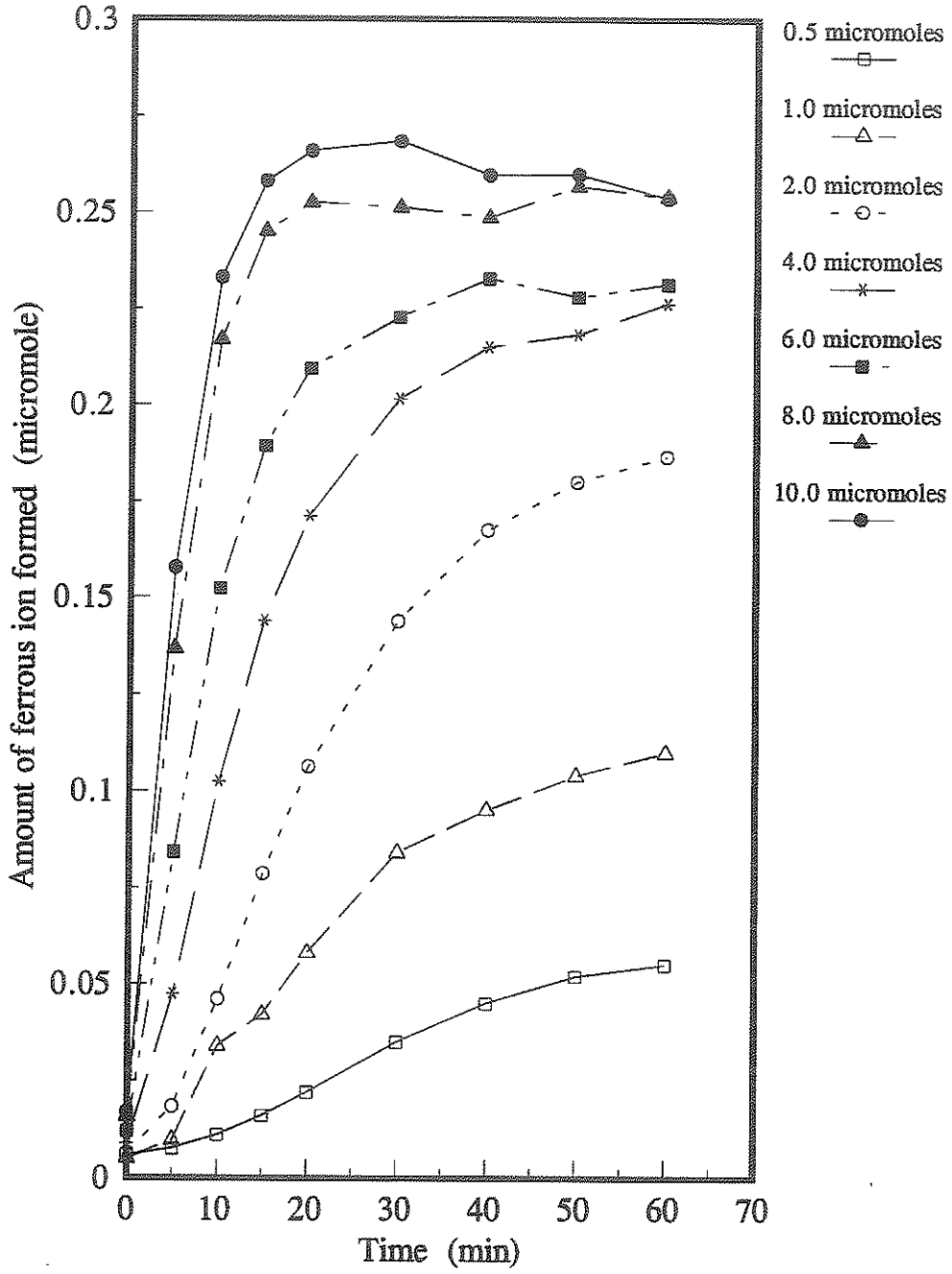

0.3
0.25
0.2
0.15
0.1
0.05
0
Amount of ferrous ion formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 24. Chemical reaction of different amounts of thiosulfate with a fixed amount of ferric ion (1 mM) added at pH 3.0.

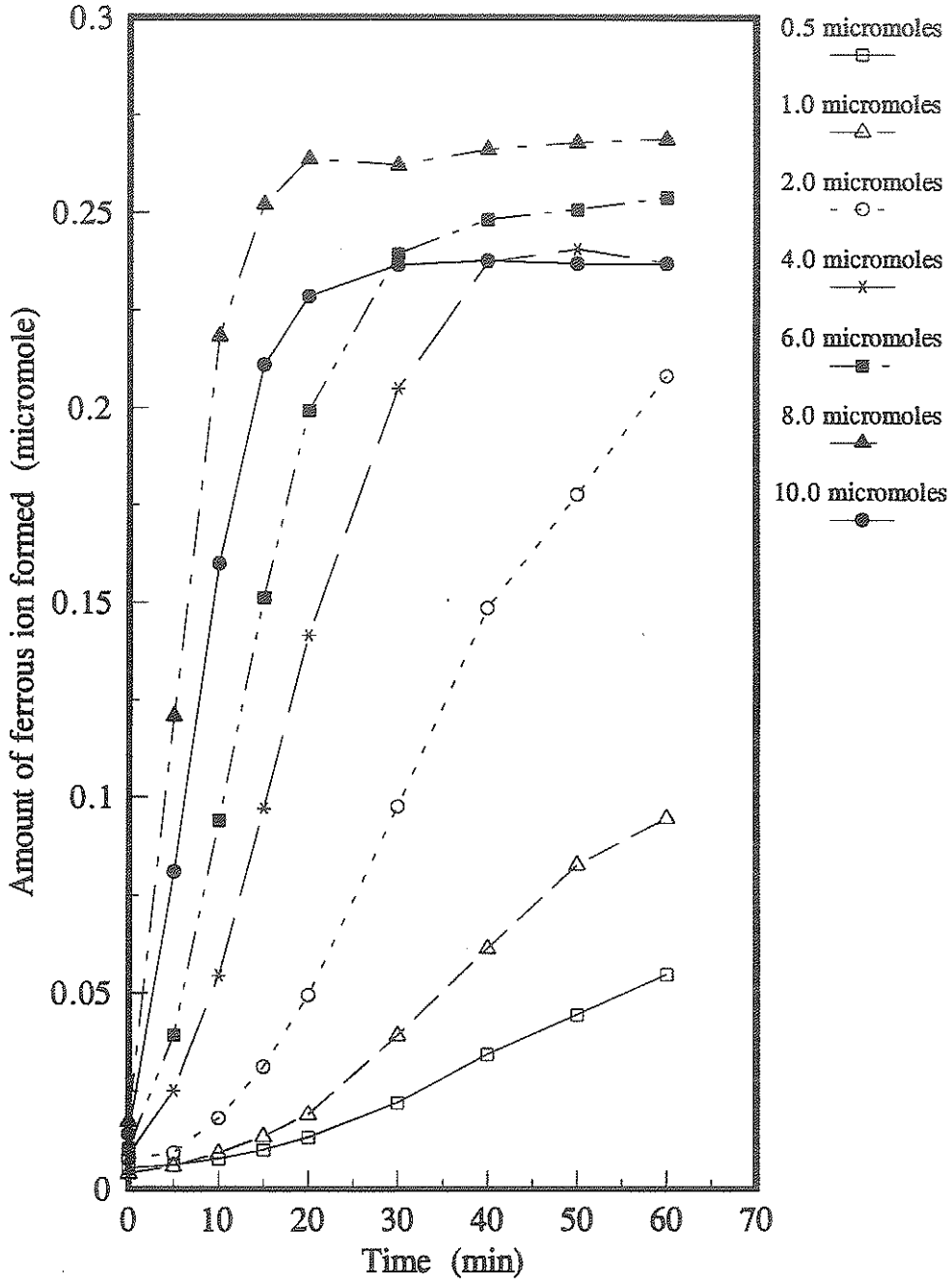

0.3
0.25
0.2
0.15
0.1
0.05
0
Amount of ferrous ion formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 25. Chemical reaction of different amounts of ferric ion with a fixed amount of thiosulfate (1 mM) added at pH 1.8.

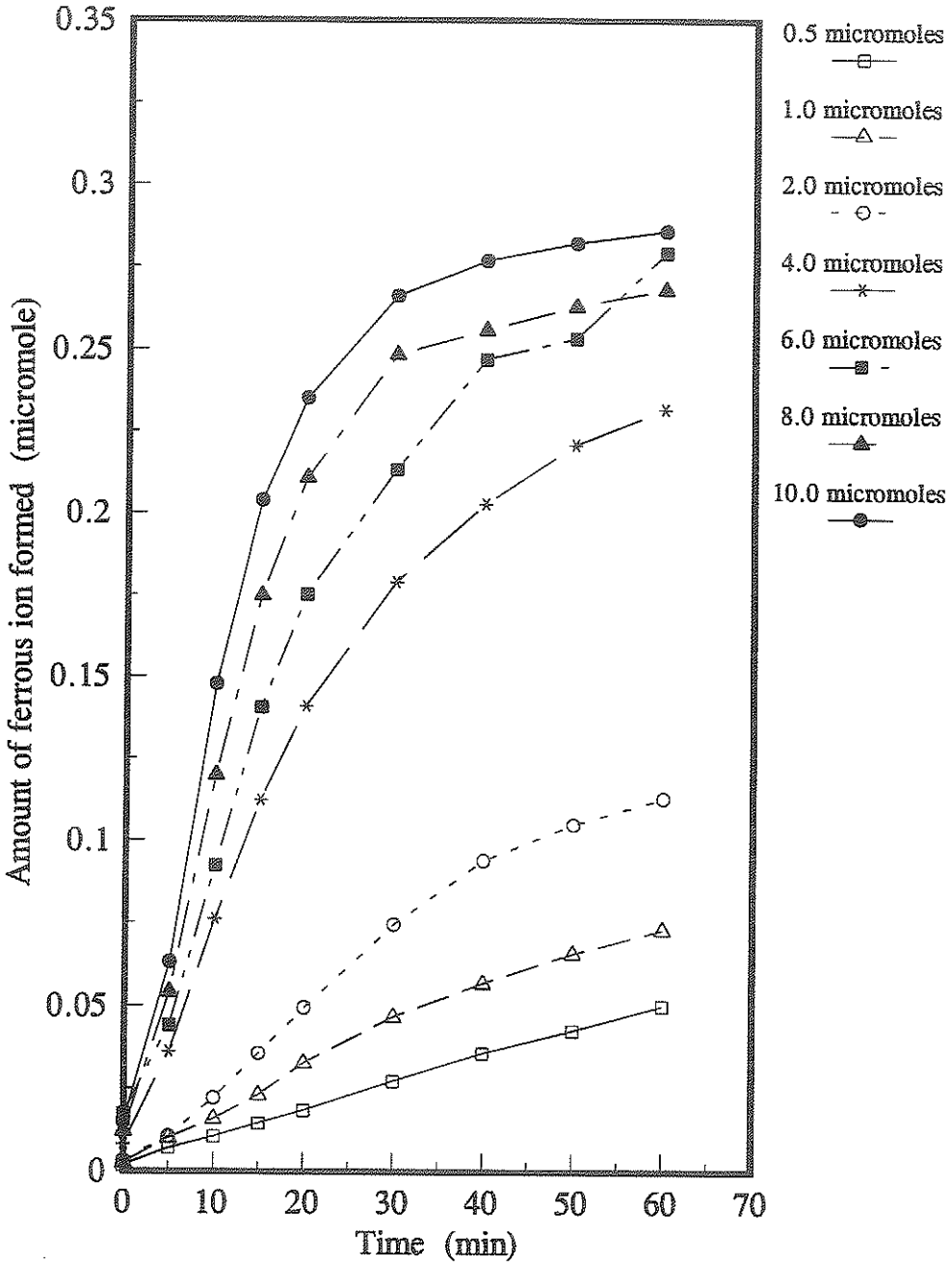

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Amount of ferrous ion formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 26. Chemical reaction of different amounts of ferric ion with a fixed amount of thiosulfate (1 mM) added at pH 2.3.

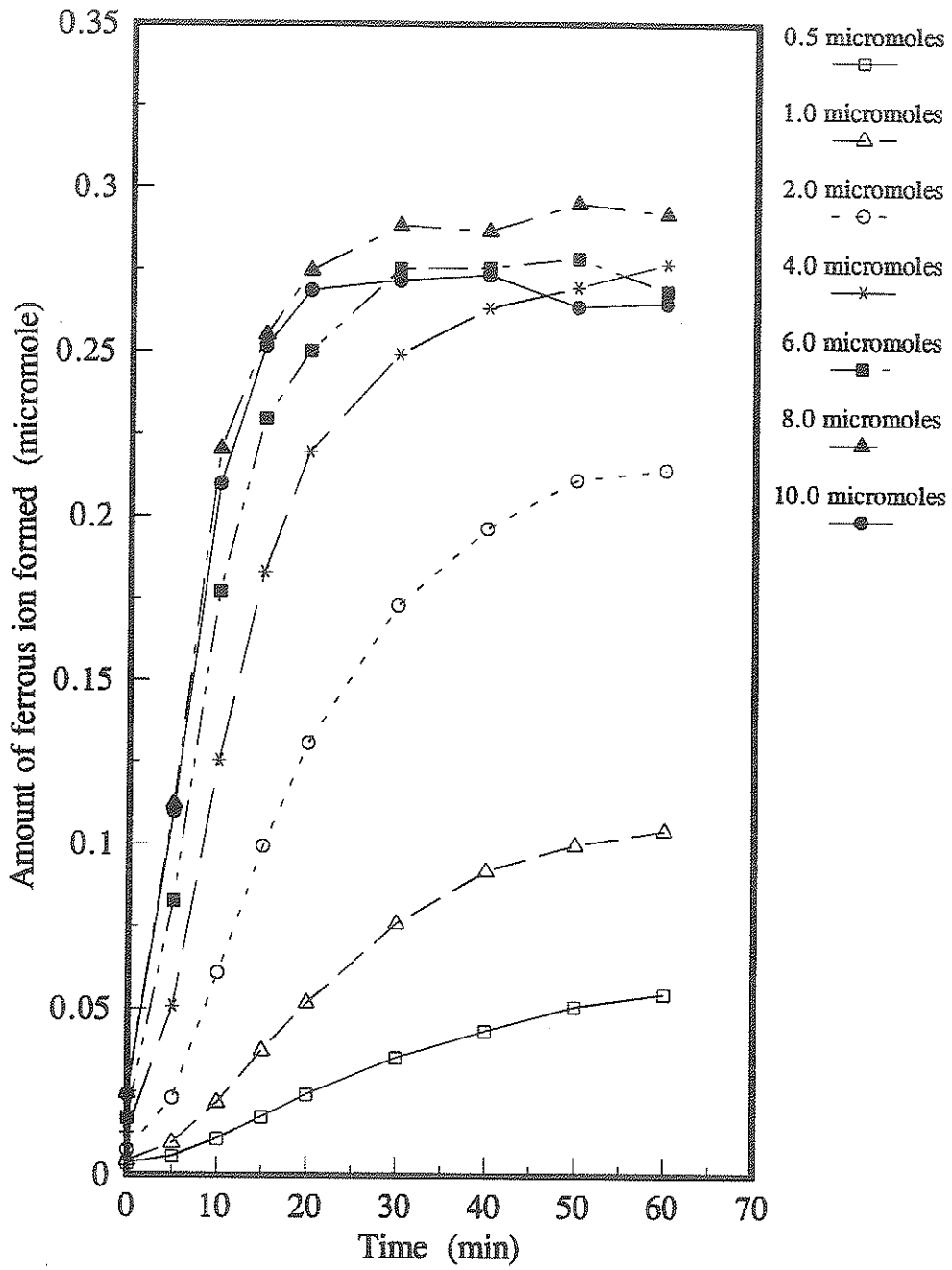

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Amount of ferrous ion formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 27. Chemical reaction of different amounts of ferric ion with a fixed amount of thiosulfate (1 mM) added at pH 3.0.

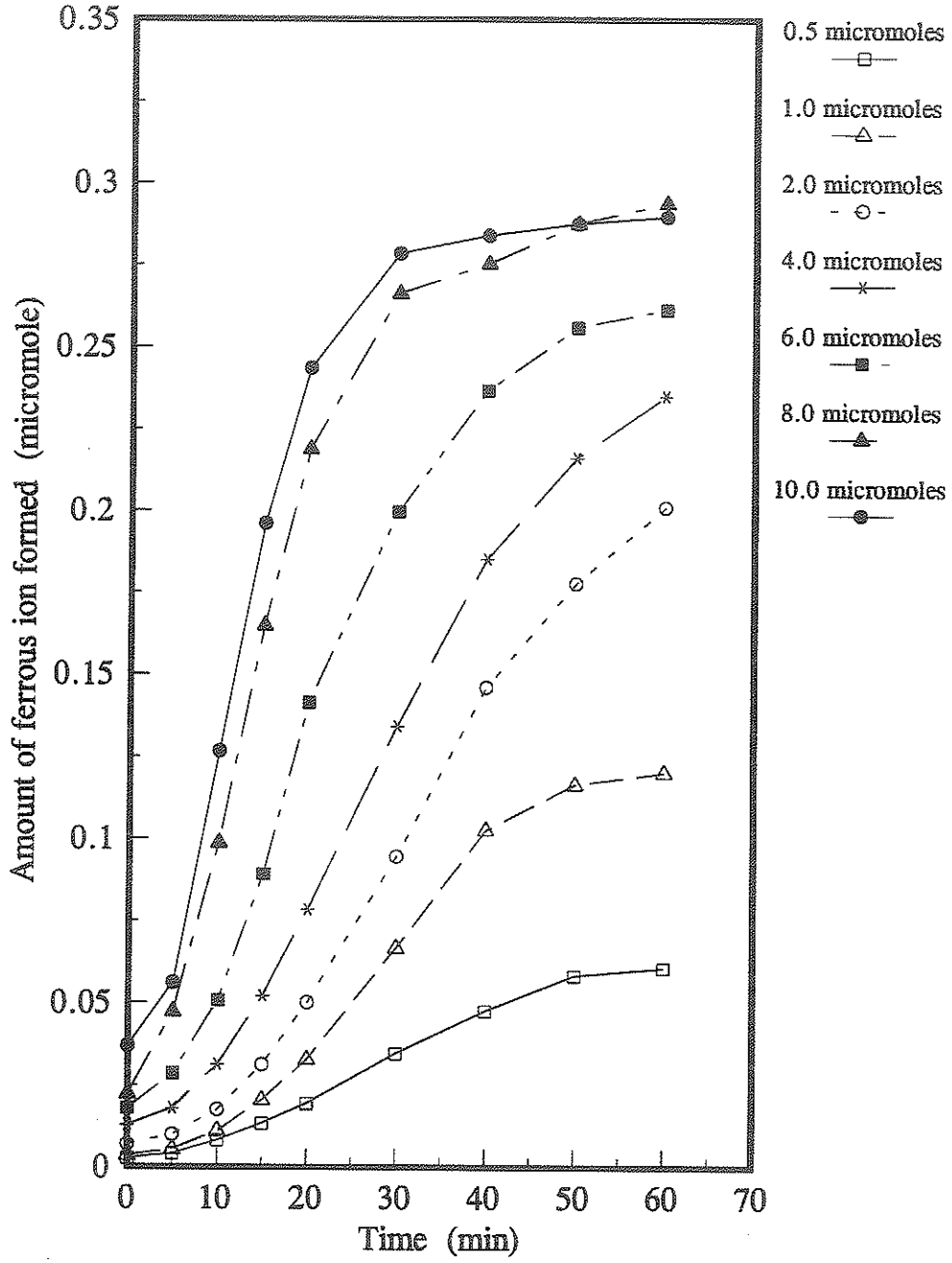
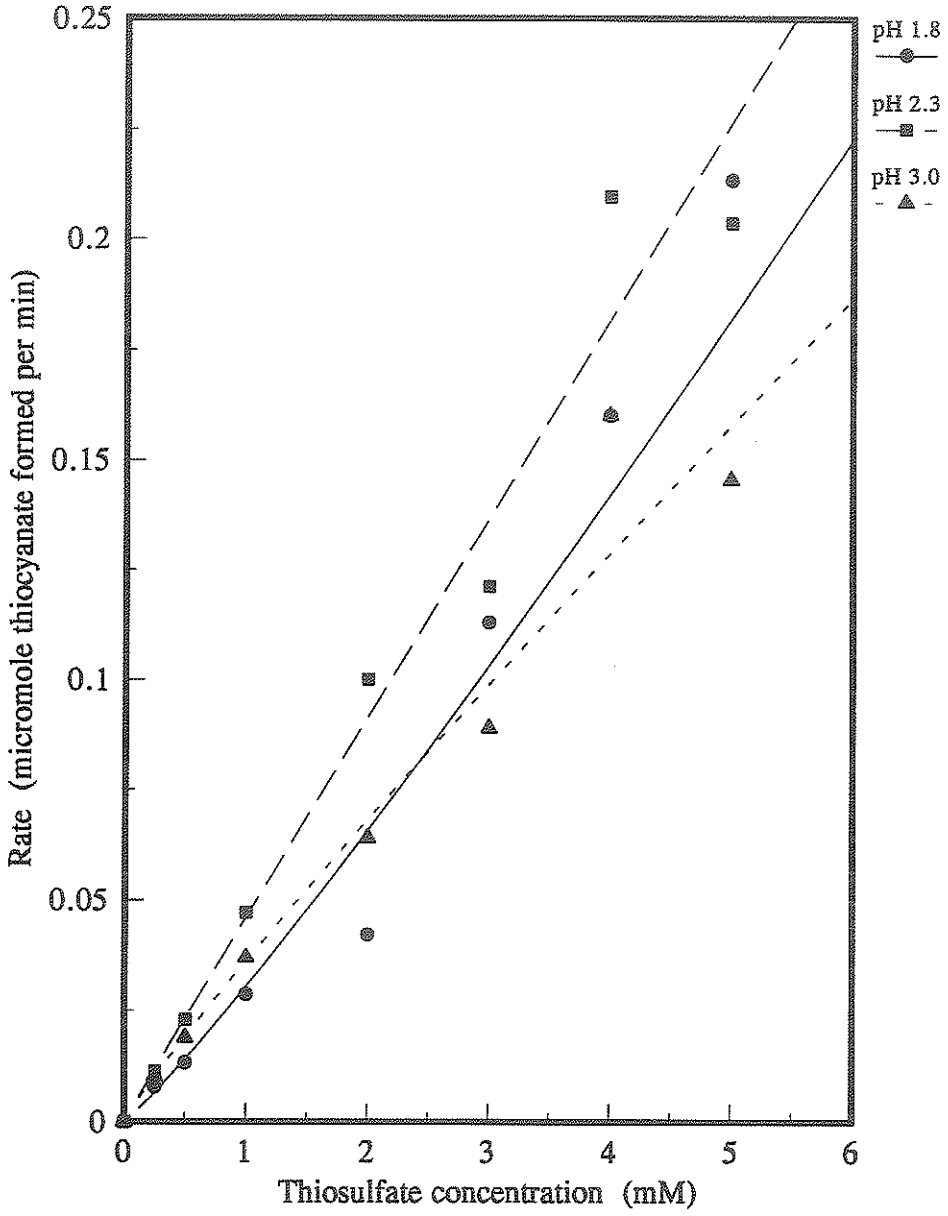
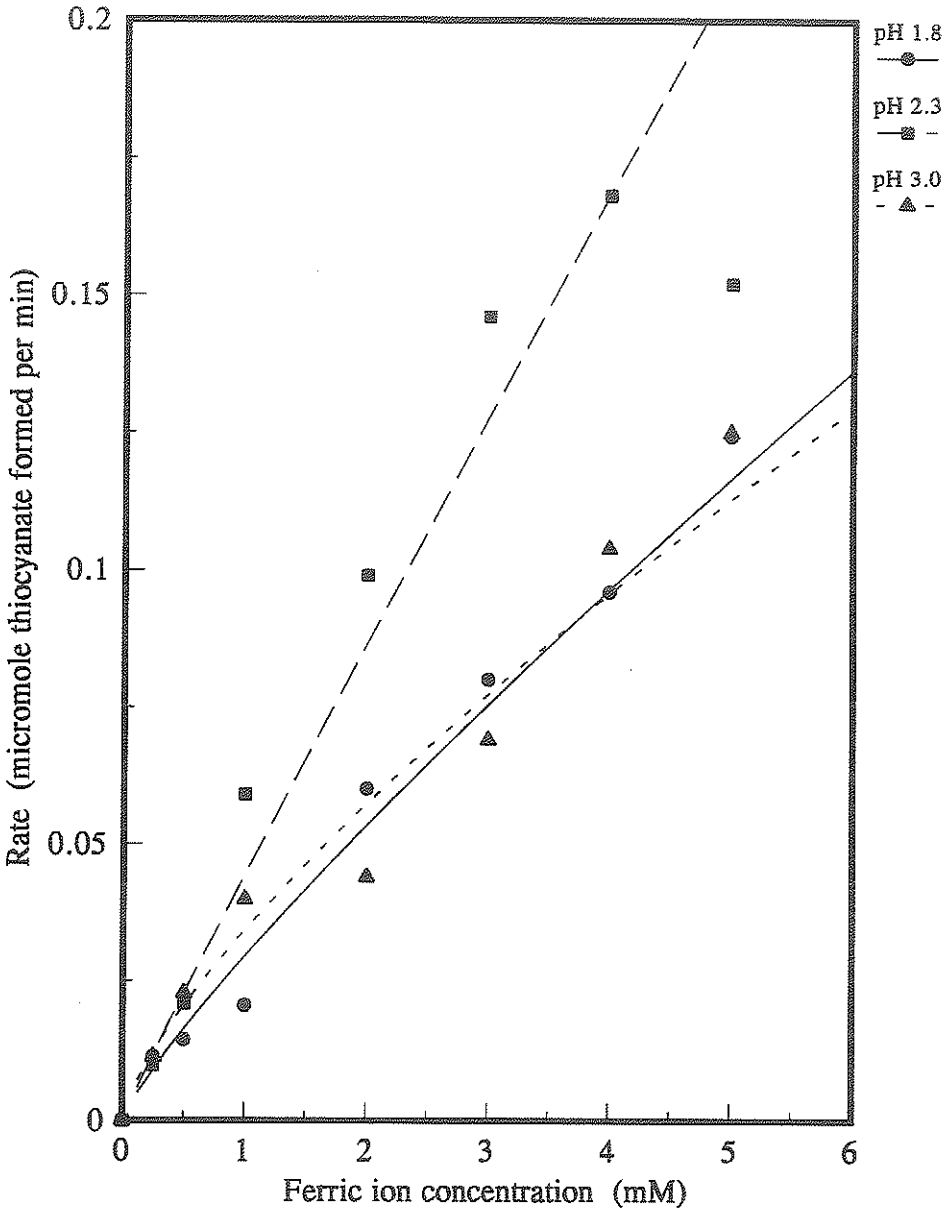

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Amount of ferrous ion formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 28. Velocity plot of different amounts of thiosulfate with a fixed amount of ferric ion (1 mM).




Note: based on thiocyanate formation data

Figure 29. Velocity plot of different amounts of ferric ion with a fixed amount of thiosulfate (1 mM).




Note: based on thiocyanate formation data

Figure 30. Chemical reaction of different amounts of thiosulfate with a fixed amount of ferric ion (1 mM) added at pH 1.8.

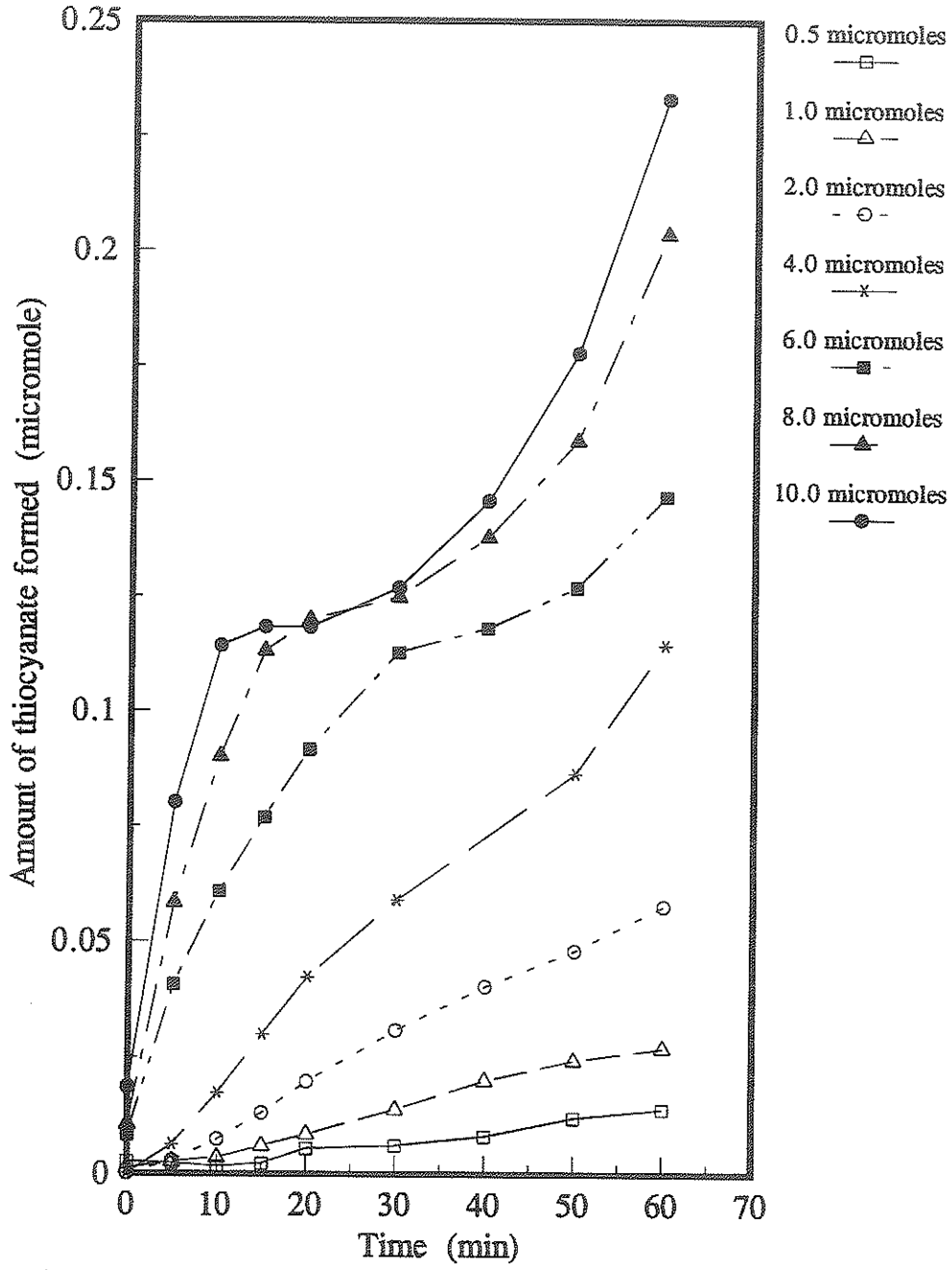

0.25
0.2
0.15
0.1
0.05
0
Amount of thiocyanate formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 31. Chemical reaction of different amounts of thiosulfate with a fixed amount of ferric ion (1 mM) added at pH 2.3.

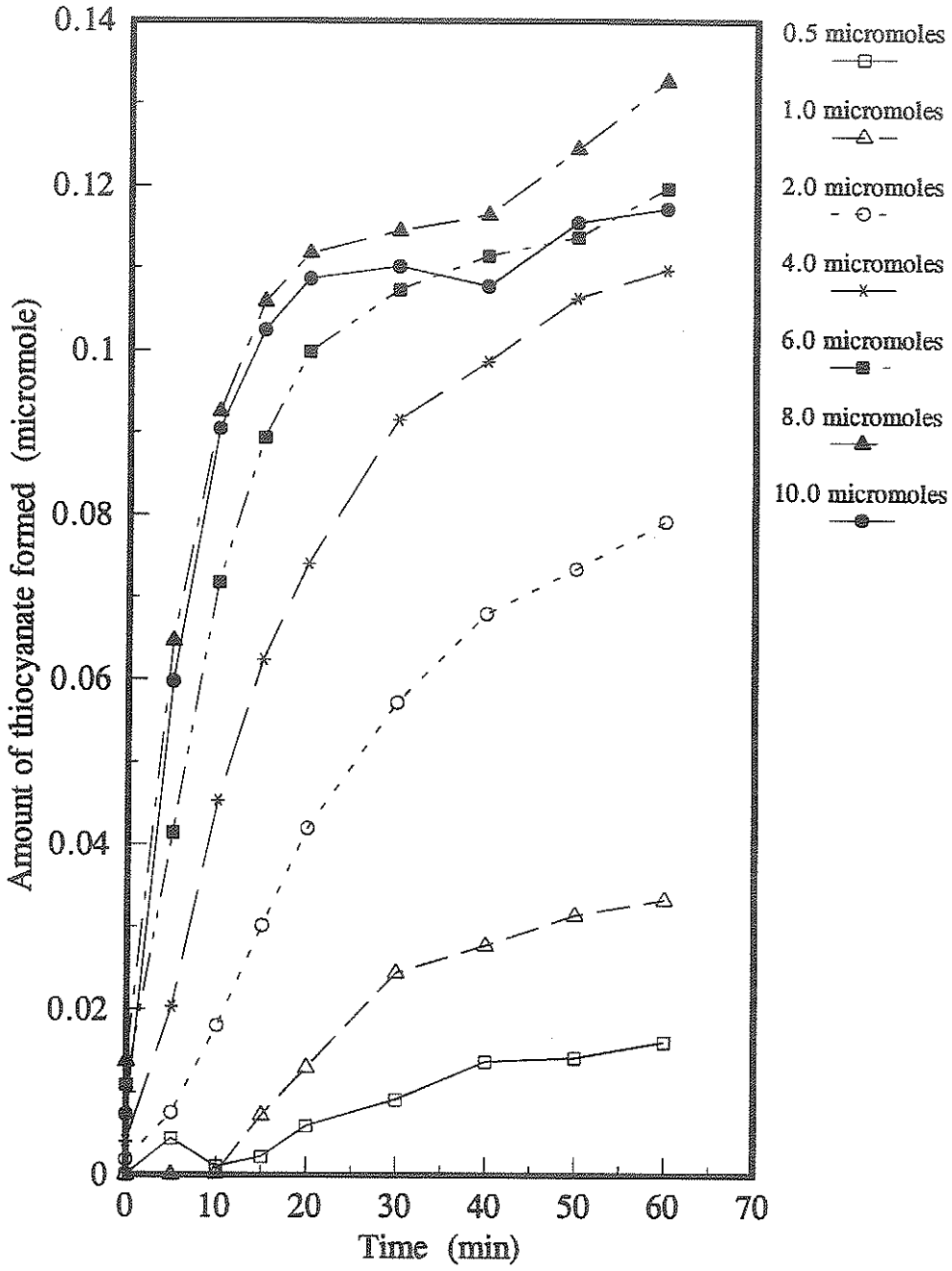

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Amount of thiocyanate formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 32. Chemical reaction of different amounts of thiosulfate with a fixed amount of ferric ion (1 mM) added at pH 3.0.

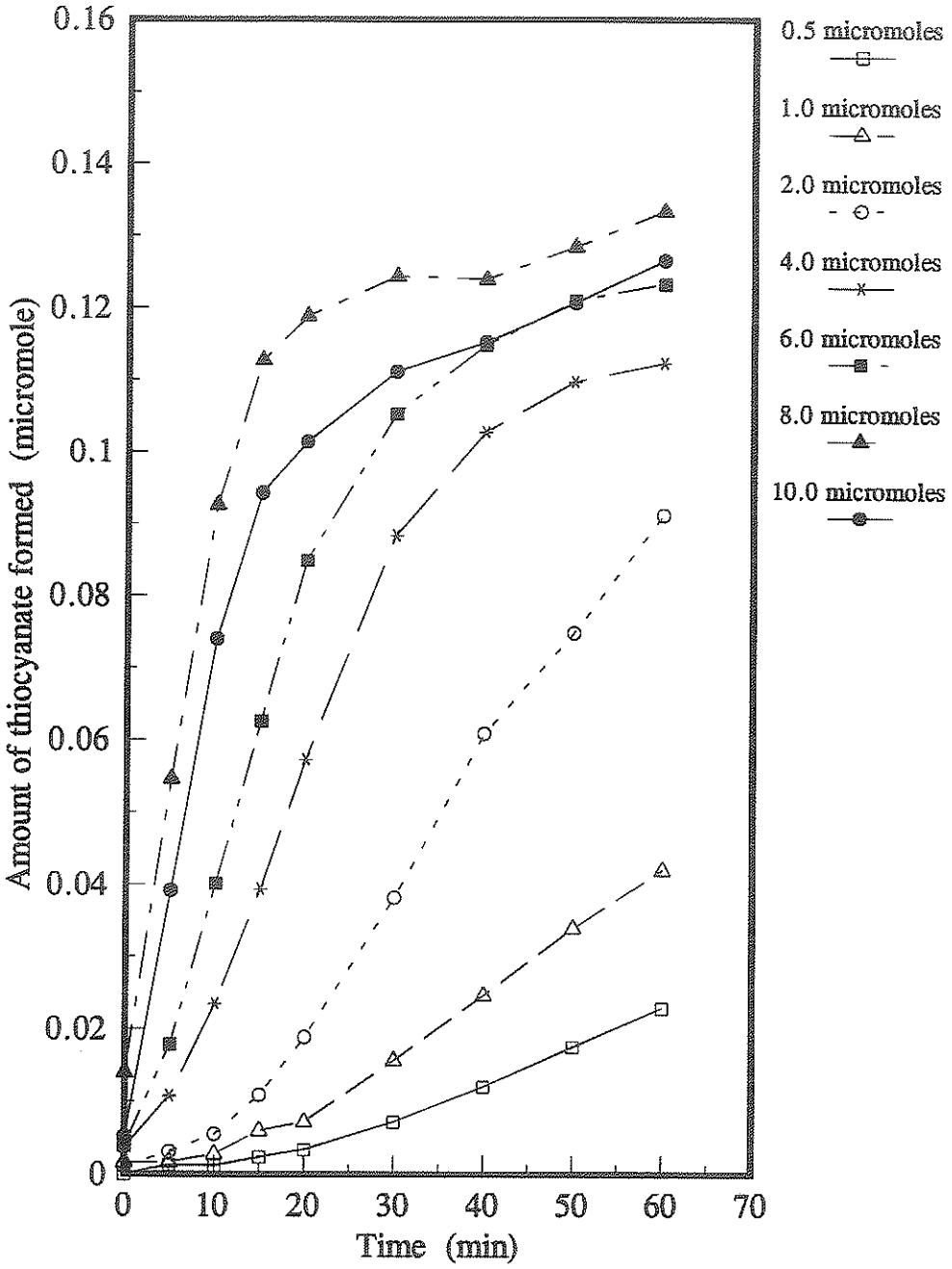

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Amount of thiocyanate formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 33. Chemical reaction of different amounts of ferric ion with a fixed amount of thiosulfate (1 mM) added at pH 1.8.

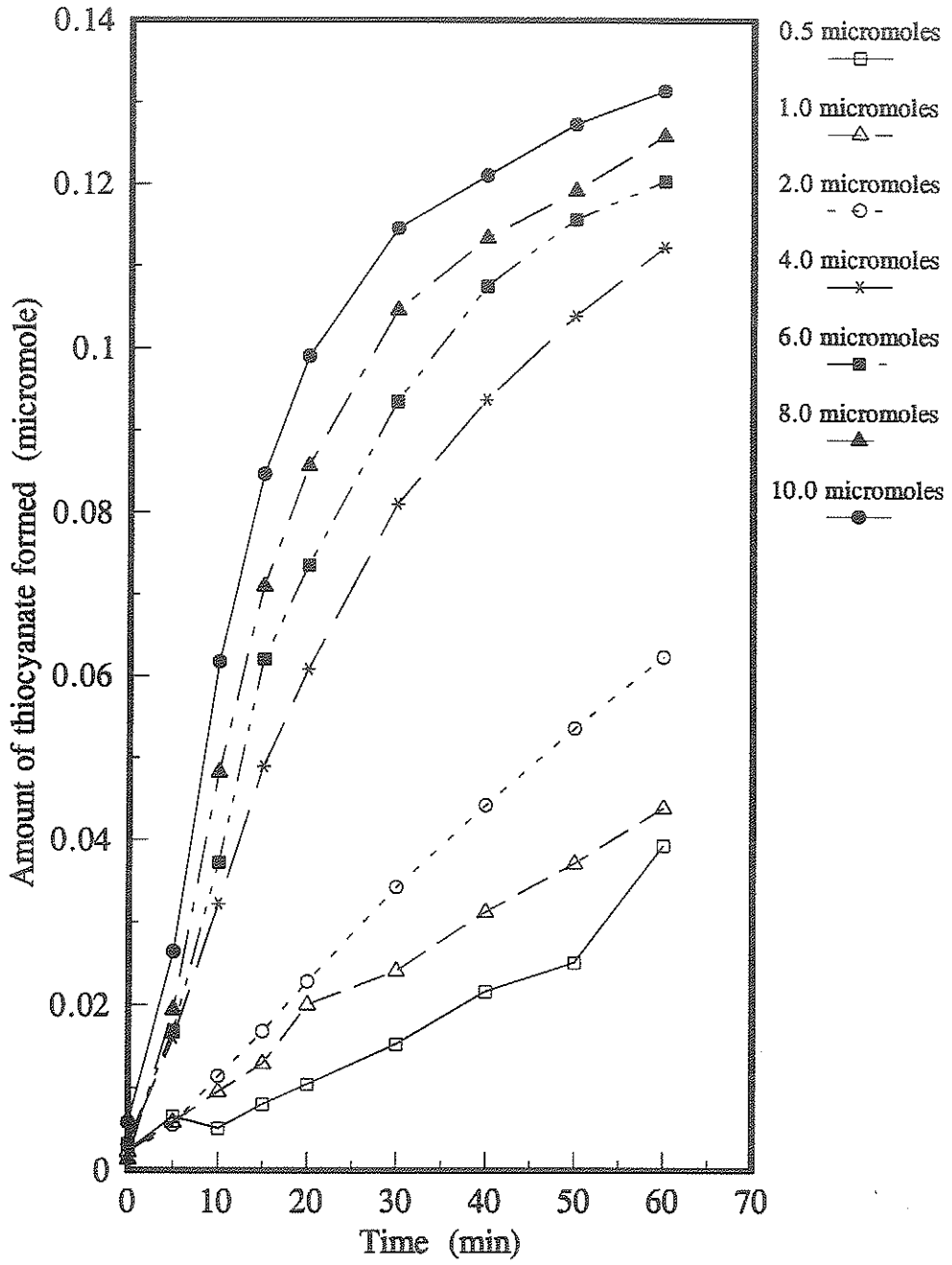

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Amount of thiocyanate formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 34. Chemical reaction of different amounts of ferric ion with a fixed amount of thiosulfate (1 mM) added at pH 2.3.

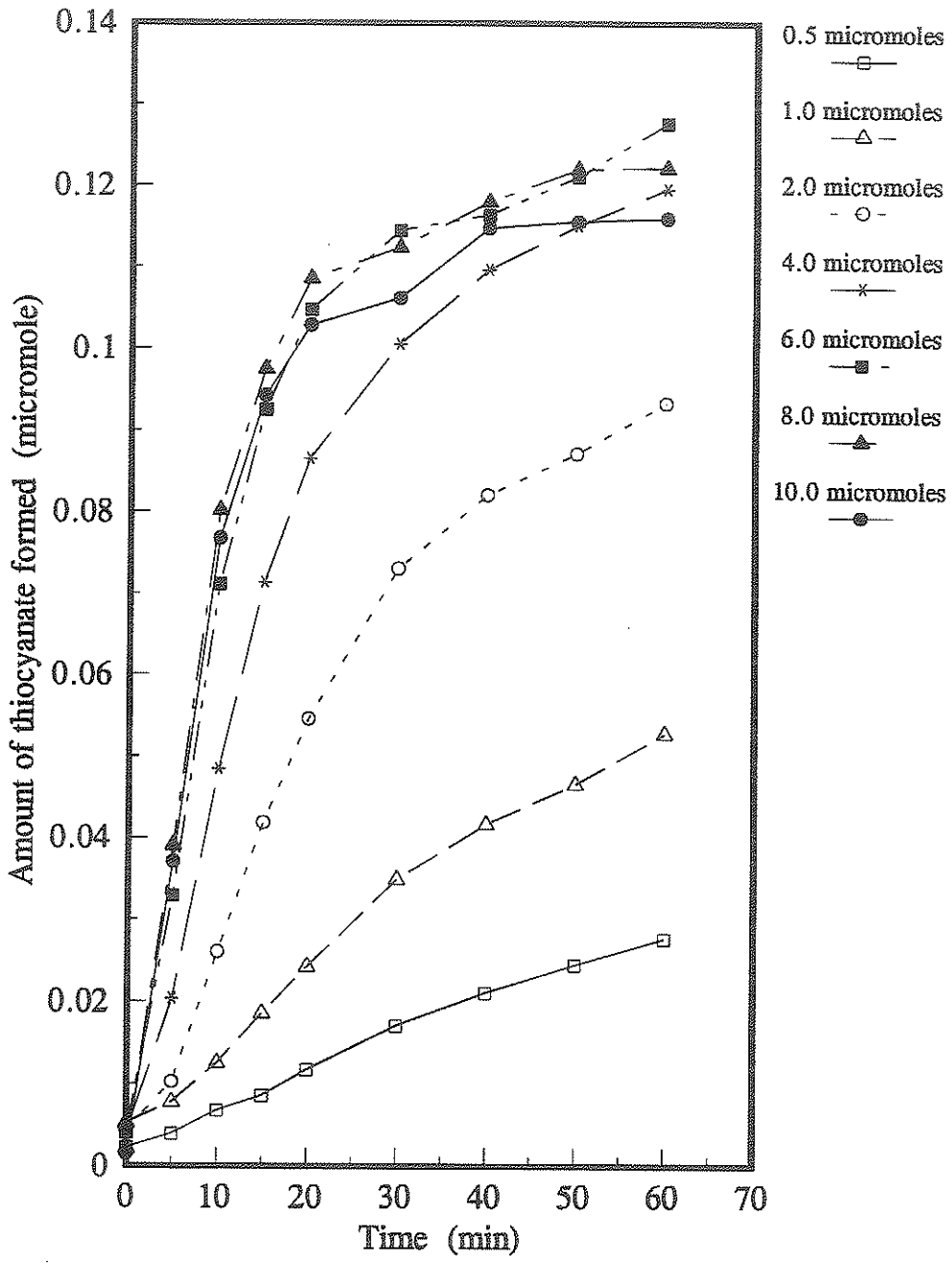

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Amount of thiocyanate formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 35. Chemical reaction of different amounts of ferric ion with a fixed amount of thiosulfate (1 mM) added at pH 3.0.

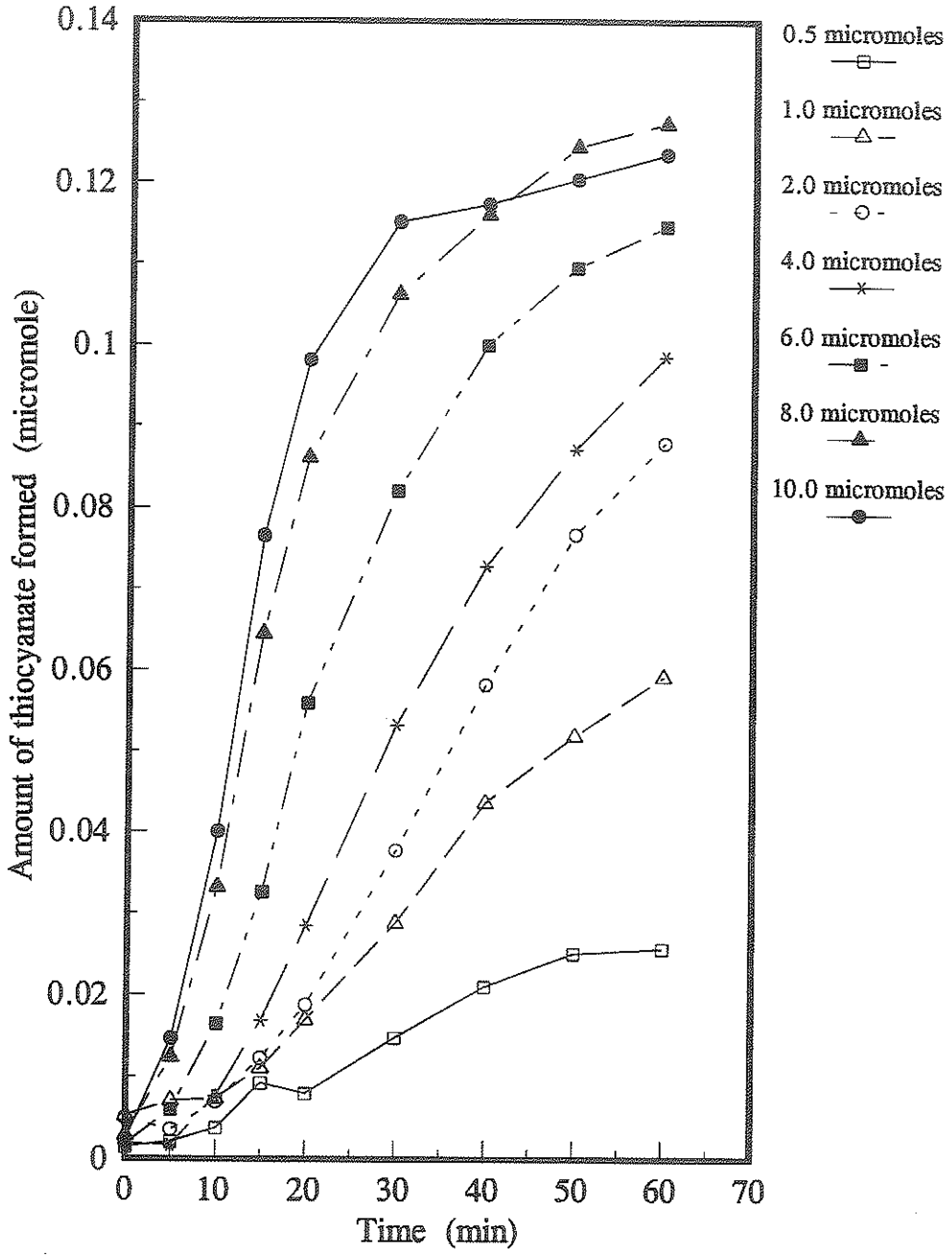

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Amount of thiocyanate formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
1.0 micromoles
2.0 micromoles
4.0 micromoles
6.0 micromoles
8.0 micromoles
10.0 micromoles

Figure 36. Chemical reaction of different amounts of thiosulfate in $\beta$-alanine-$H_2SO_4$ buffer at pH 1.8.

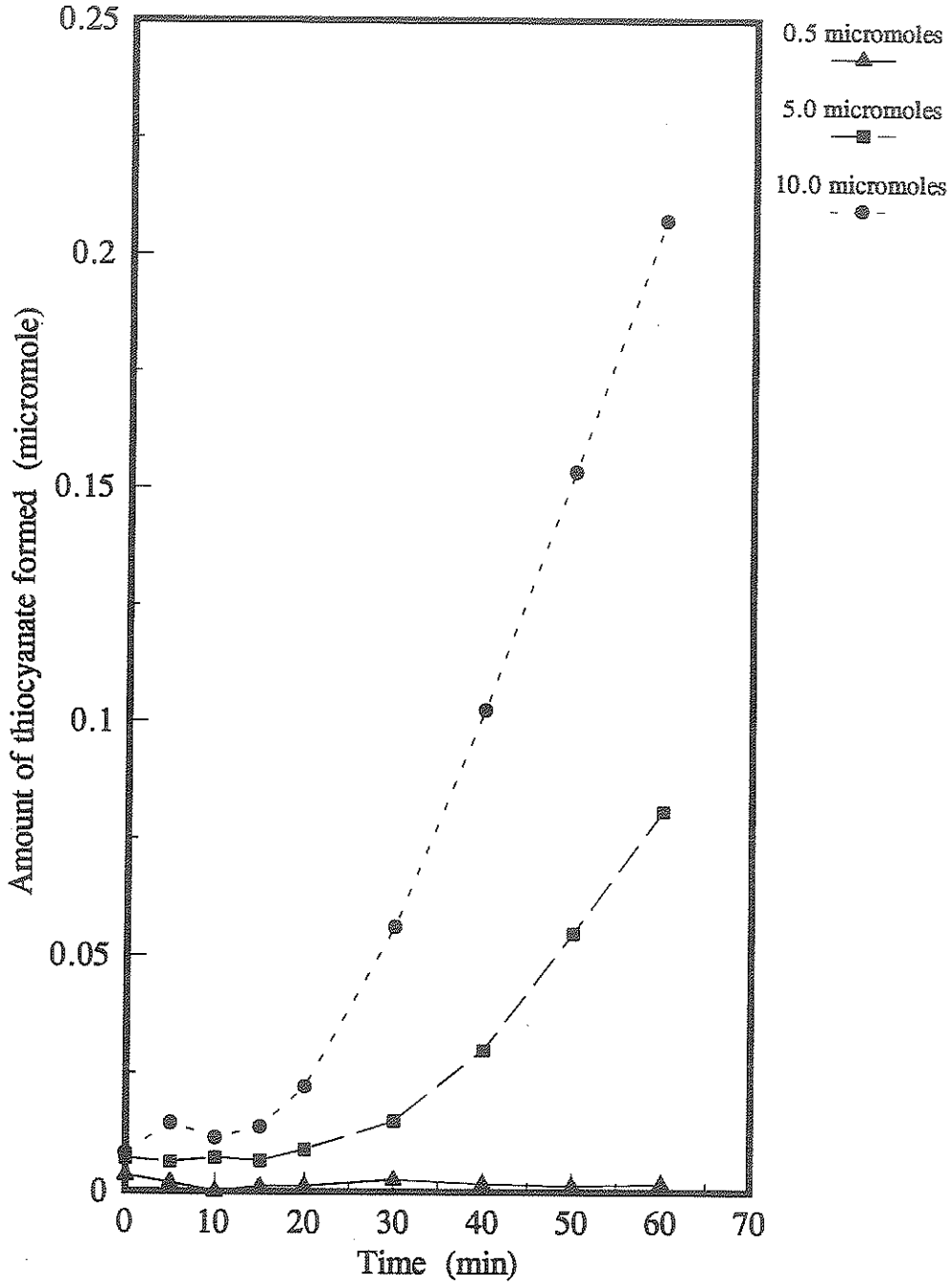

0.25
0.2
0.15
0.1
0.05
0
Amount of thiocyanate formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
5.0 micromoles
10.0 micromoles

Figure 37. Chemical reaction of different amounts of thiosulfate in $\beta$-alanine-$H_2SO_4$ buffer at pH 2.3.

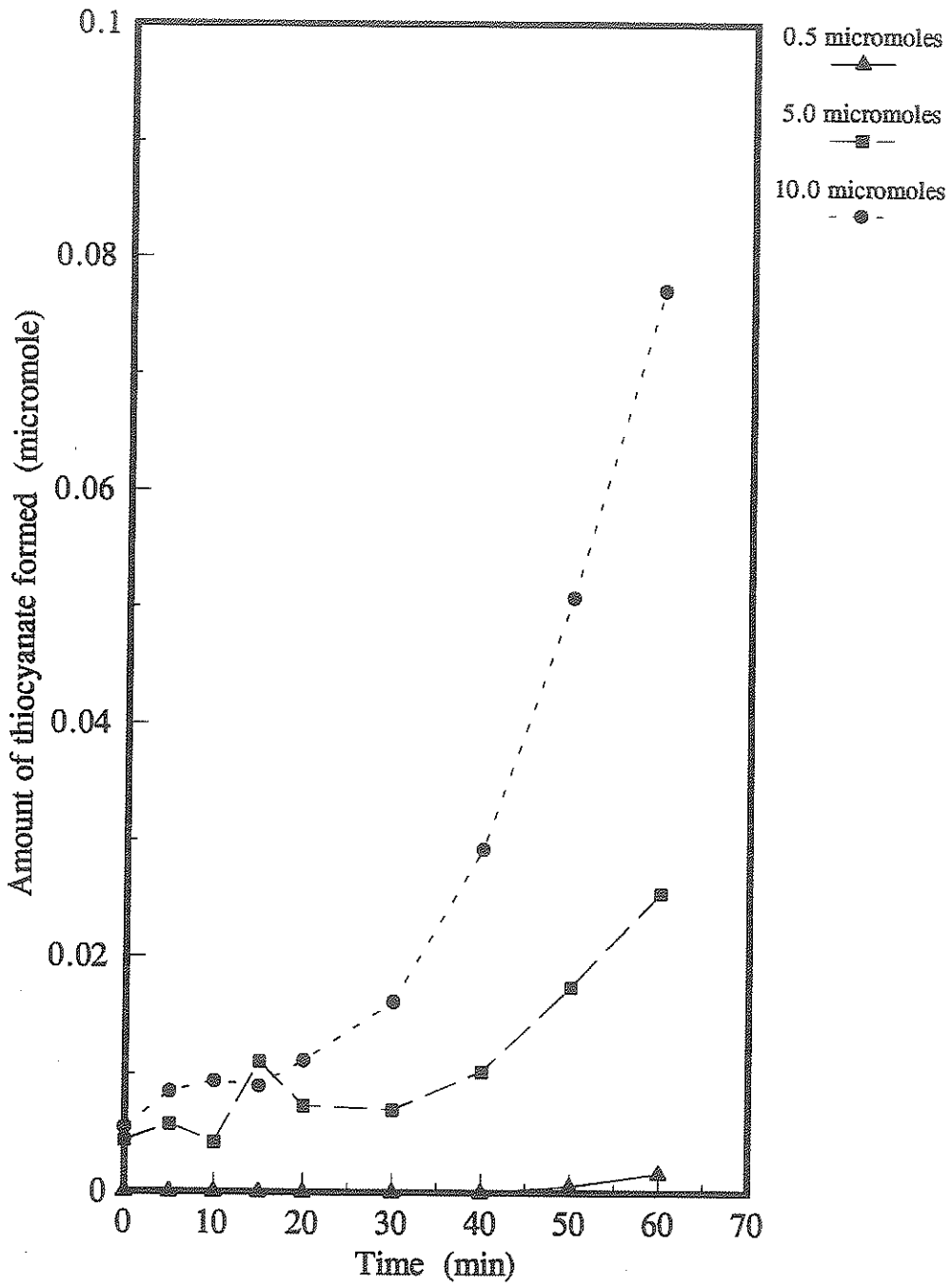

0.1
0.08
0.06
0.04
0.02
0
Amount of thiocyanate formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
5.0 micromoles
10.0 micromoles

Figure 38. Chemical reaction of different amounts of thiosulfate in $\beta$-alanine-$H_2SO_4$ buffer at pH 3.0.

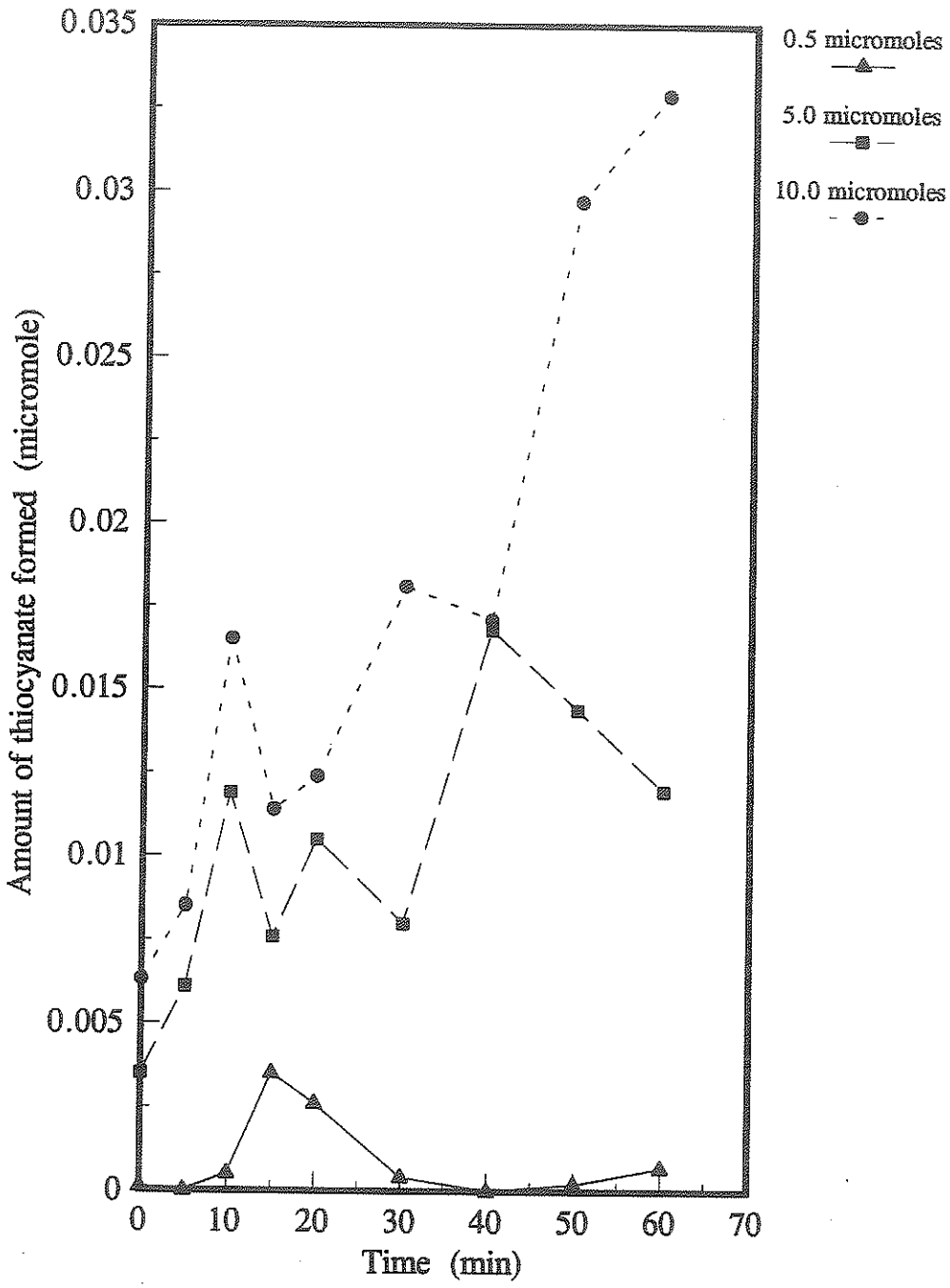
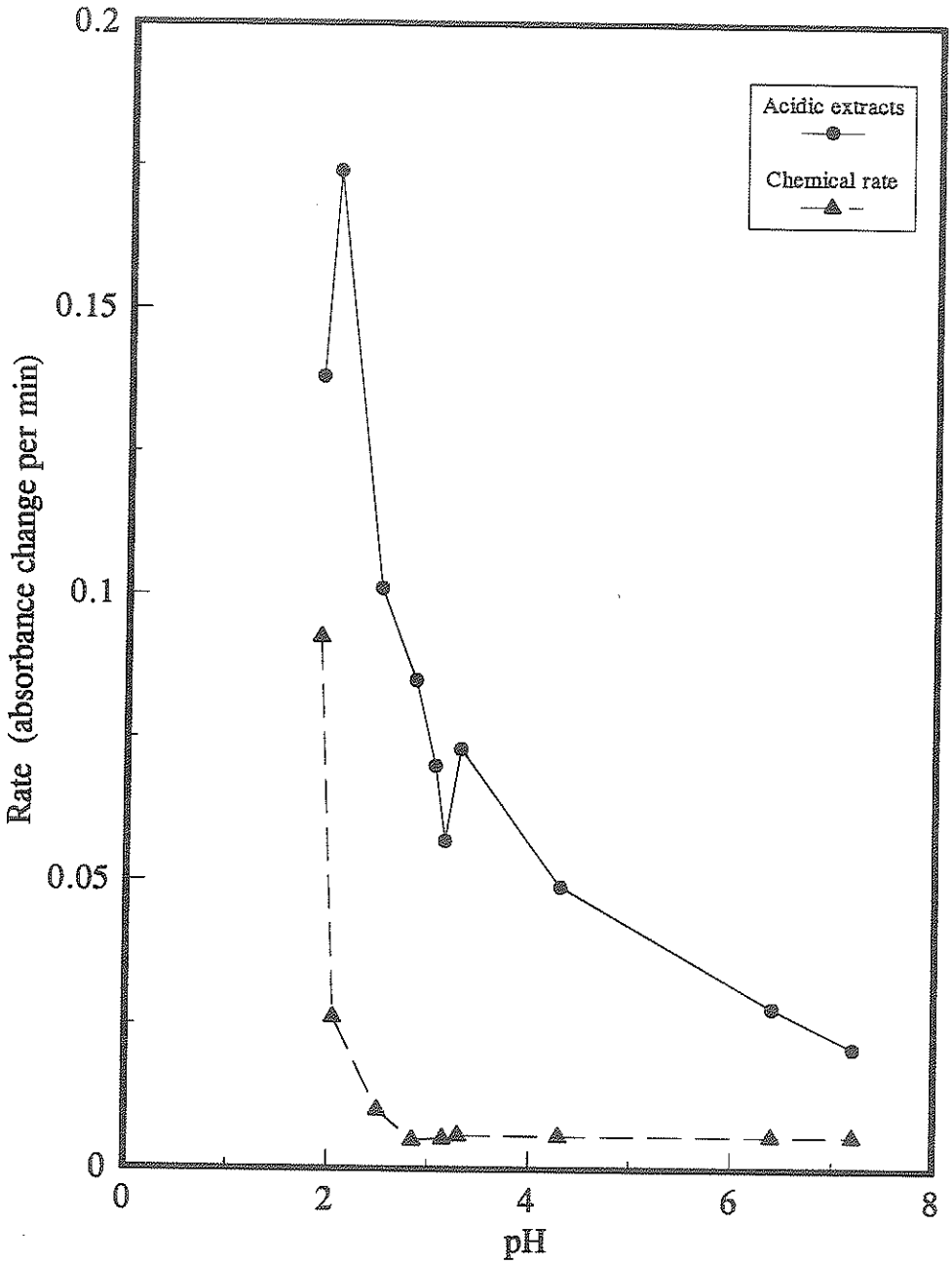

0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Amount of thiocyanate formed (micromole)
0
10
20
30
40
50
60
70
Time (min)
0.5 micromoles
5.0 micromoles
10.0 micromoles

Figure 39. Effect of pH on thiosulfate:ferricyanide oxidoreductase in acidic extracts of *T. thiooxidans*.


0.2
0.15
0.1
0.05
0
Rate (absorbance change per min)
0
2
4
6
8
pH
Acidic extracts
Chemical rate

## *CONCLUSION*

Sulfur oxidation by *T. thiooxidans* was inhibited by a sulfhydryl-binding inhibitor, NEM. Partially purified sulfur-oxidizing enzyme from *T. thiooxidans* was inhibited by NEM, and the inhibition was time-dependent. Cell-free extracts, in the presence of GSH, oxidized sulfur to sulfite, and also formed thiosulfate from sulfur and sulfite.

Sulfide was oxidized to elemental sulfur nearly stoichiometrically with the $O_2$ consumption 0.5 mol $O_2$/mol of sulfide when further oxidation of sulfur was inhibited by NEM. A sensitive and quantitative analytical method was developed for detecting a minute quantity of accumulated elemental sulfur which was formed during the oxidation of sulfide in the presence of NEM. The optimum pH of sulfide oxidation was 5. The lowest $K_m$ was found to be 8 $\mu$M at pH 7.0.

In the presence of different strains of NEM-treated thiobacilli cells (Tt-1 , Tf-2-$S^0$, SM4-Hec-$S^0$ and *T. thioparus*), thiosulfate was oxidized to tetrathionate with the $O_2$ consumption of 0.25 mol $O_2$/mol of thiosulfate. Tetrathionate oxidation was completely inhibited by NEM. The optimum pH of tetrathionate oxidation was 3.0. NEM-treated *T. thiooxidans* oxidized thiosulfate to tetrathionate at an optimum pH 2.3. NEM-treated and -untreated *T. thiooxidans* produced biphasic double reciprocal plots with two $K_m$ values and two $V_{max}$ values. These values increased with increasing pH. Cell-free extracts of *T. thiooxidans* also oxidized thiosulfate to tetrathionate but within a pH range of 1.8 to 3.0.

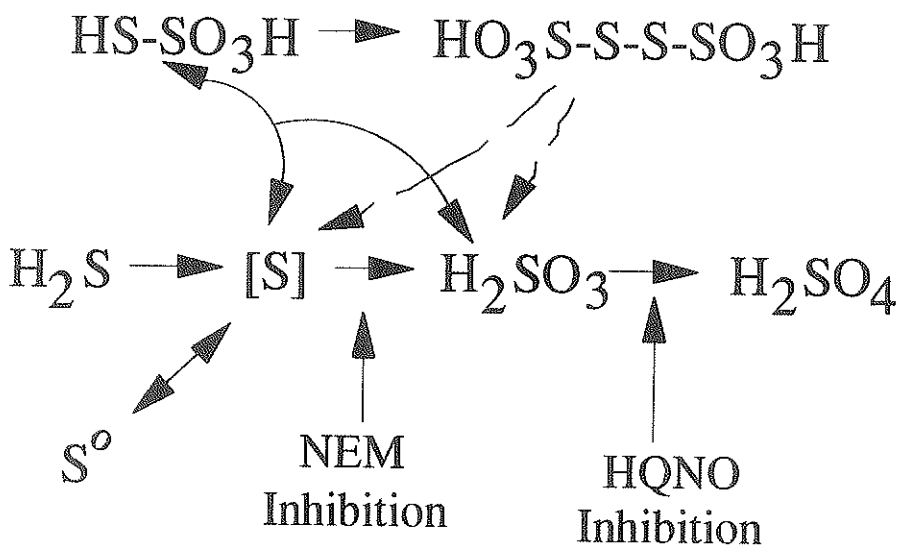
A thiosulfate:ferricyanide oxidoreductase which catalyzed the oxidation of thiosulfate to tetrathionate at an acidic pH range was successfully solubilized from *T.*

*thiooxidans* by an unusual acid treatment. The acidic extracts, thus obtained, contained a native cytochrome c which was reduced by thiosulfate only at an acidic pH. A compound which reduced horse heart cytochrome c at a neutral pH but required sulfite at an acidic pH was also identified in these acidic extracts.

Sulfur, sulfide, thiosulfate and tetrathionate were all oxidized, with stoichiometric $O_2$ consumption, to the level of sulfite when further sulfite oxidation was inhibited by HQNO. Elemental sulfur was stoichiometrically oxidized to sulfite by *T. thiooxidans* in the presence of HQNO at a wide range of pH from 2.5 to 9.0 with equimolar oxygen consumption according to the equation: $S + O_2 + H_2O \rightarrow H_2SO_3$. Sulfide, thiosulfate and tetrathionate were oxidized to sulfite with $O_2$ consumption of 1.5 mol/mol of sulfide, 1 mol/mol of thiosulfate and 1.5 mol/mol of tetrathionate respectively. Sulfur and sulfide were oxidized to sulfite either at pH 2.3 or at pH 7.0 but both thiosulfate and tetrathionate were oxidized to sulfite only at a range of pH from 1.8 to 4.0.

The results presented in this thesis lead to the proposal of a model for the pathway of inorganic sulfur metabolism in *T. thiooxidans* as shown in Scheme 1. In this scheme, sulfur (sulfane sulfur [S]) and sulfite are the key intermediates in the oxidation of elemental sulfur ($S^0$), sulfide, thiosulfate and tetrathionate by *T. thiooxidans*. Sulfur oxidation is inhibited by N-ethylmaleimide (NEM) and sulfite oxidation is inhibited by 2-*n*-hydroxyquinoline-*N*-oxide (HQNO). Sulfane sulfur, when accumulated, is readily converted to elemental sulfur ($S_8$ or $S^0$). Thiosulfate is either oxidized to tetrathionate for further oxidation or split to sulfane sulfur and sulfite for further oxidation via the sulfur- and sulfite-oxidizing systems. When sulfur oxidation is inhibited by NEM,

thiosulfate is oxidized to tetrathionate with stoichiometric $O_2$ consumption. However, both thiosulfate and tetrathionate are oxidized to sulfite when further sulfite oxidation is inhibited by HQNO.




Scheme 1. Oxidation of inorganic sulfur compounds and its inhibition in T. thiooxidans