PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Biochemistry of the Chemolithotrophic Oxidation of Inorganic Sulphur

D. P. Kelly

Phil. Trans. R. Soc. Lond. B 1982 **298**, 499-528
doi: 10.1098/rstb.1982.0094

Phil. Trans. R. Soc. Lond. B **298**, 499–528 (1982)
Printed in Great Britain

Biochemistry of the chemolithotrophic oxidation of inorganic sulphur

By D. P. Kelly
Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL, U.K.

A historical review is presented of the elucidation of the mechanisms of oxidation of inorganic sulphur compounds and of electron transport in the thiobacilli. A unitary mechanism, consistent with current knowledge, is proposed. The significance of polythionates is discussed. The relations between oxidation mechanisms, substrate-level and electron transport-dependent phosphorylation, energy-dependent NAD^+ reduction and efficiency of growth are assessed in order to evaluate the efficiency of energy conservation in different species. The unresolved problems are identified for the benefit of those planning further assaults on the last redoubts of the thiobacilli.

Introduction

Es erhebt sich nun die Frage, was unter den natürlichen Lebensbedingungen dieser Organismen die Ausgangs – und Endproduckte ihres stoffwechsel sind. Leider lässt sie sich mit voller Bestimmtheit nicht beantworten.

(The question that now arises is what are the substrates and end products of the metabolism of these organisms in their natural environments. Unfortunately this cannot be answered categorically.) Nathansohn (1902)

It is now the eightieth anniversary of Nathansohn's isolation of the first thiobacillus ever to be obtained and studied in pure culture. We now know that the natural substrates of the bacteria are the following: soluble and metal sulphides; other reduced sulphur compounds such as sulphur or thiosulphate, which arise from chemical oxidation of the sulphide generated by sulphate-reducing bacteria or from other biological processes; polythionates, which arise chemically or microbiologically in terrestrial and aquatic environments; and even organic sulphides of biogenic origin. We also know that sulphate is the ultimate end-product in natural environments, although the extent to which partly oxidized products accumulate cannot be assessed. The mechanisms for inorganic sulphur oxidation among the chemolithotrophs have long been under study and are a rich topic for dispute among microbial biochemists.

The rate and success of progress in elucidating the biochemical pathways is evident from the following quotations.

The oxidation of sulfide, sulfur, or thiosulfate to sulfate provides the energy for the growth of the thiobacilli. The intermediate compounds formed, and the relation of the oxidative steps to energy-storing and energy-utilizing reactions are still largely unknown.
Vishniac & Santer (1957)

Although a wealth of information has accumulated during the past year, neither the path of sulphur during sulphur oxidation by thiobacilli, nor the electron transport, nor the reduction of NAD are completely understood. Schlegel (1975)

The quarter century since Vishniac's words has seen the publication of more than a thousand papers relating to thiobacilli, at least 749 in the years 1969–81, with more than 300 since Schlegel's remarks (Anon. 1982). This mass of information has yielded clarification of all the problems

they identified, but neither statement can yet be said to have been wholly invalidated. I am restricting my survey to work on the thiobacilli, since even less is known about sulphur oxidation in other sulphur-chemolithotrophs, and oxidation by phototrophs is dealt with elsewhere (Trüper, this symposium). It is my purpose to summarize the historical eludication of what we know about the pathway of sulphur compound oxidation and the mechanism and extent of energy conservation from the process.

The early years: the observations of Alexander Nathansohn

> ...*also die Producte der eigentlichen Athmung unserer Bacterien Schwefelsäure und Tetrathionsäure sind.* (...the products of the proper respiration of our bacteria are therefore sulphuric acid and tetrathionic acid.) Nathansohn (1902)

Using an organism that was probably a marine strain of *T. neapolitanus*, Nathansohn (1902) made three observations of fundamental significance. His interpretation of his results has shaped the hypotheses for sulphur oxidation right up to the present day.

First, he demonstrated the production of extracellular elemental sulphur ('*ausgeschiedenen Schwefel*') in cultures growing on thiosulphate. This he attributed to the cleavage of thiosulphate to sulphur and sulphite, the latter subsequently being oxidized to sulphate, giving an equivalence between sulphur precipitation and concomitant sulphuric acid production ('*Die nach der Formel* [equation (1)] *gebildete Schwefelsäure ist aber dem ausgeschiedenen Schwefel Equivalent*'):

$$Na_2S_2O_3 + O \longrightarrow Na_2SO_4 + S. \qquad (1)$$

Secondly, he demonstrated tetrathionate production from thiosulphate. By calculation, he deduced that sulphate and tetrathionate arose in a 2:1 molar ratio (equation (2)):

$$3\begin{vmatrix} SO_3Na \\ \\ SNa \end{vmatrix} + 5\,O \longrightarrow S_2\begin{matrix} SO_3Na \\ \diagup \\ \diagdown \\ SO_3Na \end{matrix} + 2\,SO_4Na_2. \qquad (2)$$

This stoichiometry was certainly suggestive of a specific mechanism for their generation, and led him to the belief that they were both metabolic end-products. Nathansohn recognized, however, that there was potentially an incompatibility between the alternatives of thiosulphate metabolism by a cleaving reaction to give sulphur, or by '*eine Condensation zu Tetrathionsäure*'. He commented that the point to be resolved was whether extracellular sulphur precipitation was a secondary process, with the intracellular oxidation of thiosulphate proceeding by a different mechanism ('... *in ganz andere Weise*').

Thirdly, he showed that adding 5% thiosulphate to the culture filtrates (freed of bacteria) resulted in production of a rich sulphur precipitate, which was not formed if 0.1–0.2% potassium cyanide was added at the same time. These filtrates also contained H_2O_2. These observations are now explicable as H_2O_2 production from terminal respiration and sulphur arising from polythionate decomposition with excess thiosulphate, but at the time suggested to Nathansohn the occurrence of extracellular metabolic reactions.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Fifty years on: the assessment by Vishniac & Santer

Little real advance occurred in the half century after Nathansohn. Vishniac (1949, 1952) and Vishniac & Santer (1957) summarized the likely interrelations of sulphur and the thionic acids (figure 1), retaining the concept of extracellular chemical interactions of polythionates, but intracellular involvement of tetrathionate and trithionate as intermediates of thiosulphate oxidation. Thiosulphate became an intermediate in the metabolism of intracellular sulphur

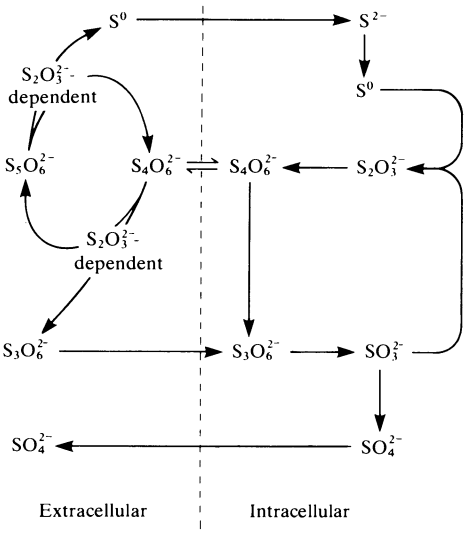


Figure 1. Sulphur oxidation: 1957 model. The scheme proposed by Vishniac & Santer (1957), largely based on the classic paper by Tamiya *et al.* (1941).

(derived from external sulphur by reduction to sulphide and intracellular reoxidation), being produced by condensation of sulphur and sulphite. Their schemes did not, however, explain how sulphur at the reduction level of S^0 or HS^- was converted to sulphite, a six-electron-equivalent oxidation. Looking at their scheme (figure 1) reveals that no oxidation of sulphane-level sulphur occurs until the conversion of trithionate to sulphite, and this step was not explicable in 1957. Vishniac (1952) provided beautiful demonstrations of the apparently sequential production of tetrathionate and trithionate by a thiobacillus, and later demonstrated (Vishniac & Santer 1957) that in the absence of phosphate, thiosulphate and tetrathionate oxidation were incomplete. Oxidation to completion occurred if phosphate or arsenate were added subsequently, indicating that one or more steps of thiosulphate oxidation were phosphate-dependent, possibly involving substrate-level phosphorylation by means of a mixed anhydride of the type $-S-O-PO_3^{2-}$. The production by *T. thioparus* of one or more compounds labelled with both isotopes was shown after supplying [^{35}S]sulphide and [^{32}P]phosphate (Vishniac & Santer 1957).

An enzymological breakthrough: Peck (1960)

The requirement of phosphate for thiosulphate oxidation described above was given a firm enzymological basis by Peck's (1960) demonstration that *T. thioparus* contained enzymes for the conversion of sulphite to sulphate via the $-S-O-PO_3^{2-}$ compound adenylyl sulphate (APS, adenosine phosphosulphate) as was already known for sulphate reduction to sulphite in *Desul-*

fovibrio. *T. thioparus* also contained a thiosulphate reductase and sulphur-producing sulphide oxidase. These, with APS reductase and ADP sulphurylase, could explain the quantitative conversion of thiosulphate to sulphate and sulphur (figure 2). That APS or a similar compound was indeed an intermediate during thiosulphate oxidation by intact organisms was indicated by Santer's (1959) demonstration that ^{18}O from phosphate appeared in the sulphate produced. If all the sulphate formed had passed through a $-S-^{18}O-P^{18}O_3^{2-}$ intermediate, one-quarter of

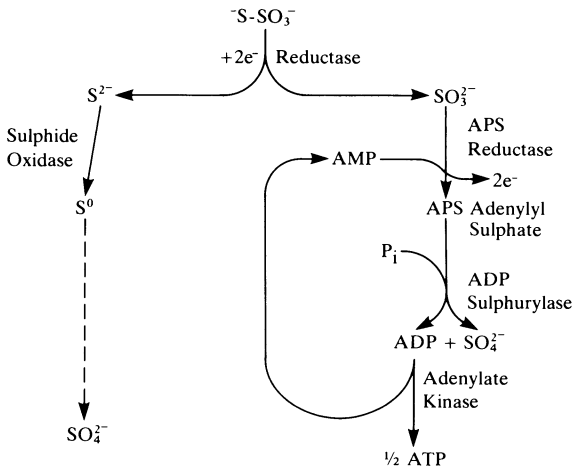


FIGURE 2. Enzymes for a thiosulphate oxidation mechanism involving the adenosine phosphosulphate pathway (Peck 1960).

the oxygen in the product sulphate should be derived from phosphate, compared with Santer's value of 22 %. Since intact cells can convert both atoms of thiosulphate to sulphate, a mechanism for converting sulphide or sulphur to sulphite remained the problem. Peck & Fisher (1962) showed that ^{35}S from sulphane-labelled thiosulphate ($^{35}SSO_3^{2-}$) was converted to sulphate by intact *T. thioparus*, but could be partly trapped by sulphur if an excess of unlabelled sulphur was included as a 'trap' in the reaction mixture. Peck & Fisher (1962) also showed that sulphide could be oxidized to sulphate by extracts of *T. thioparus* (with concomitant phosphate esterification, probably via APS). The ratio of sulphide converted to sulphate (4 μmol) to oxygen consumed (6.6 μatoms) was 1:1.65 compared with the value of 1:4.0 given by

$$H_2S + 2O_2 \longrightarrow H_2SO_4. \quad (3)$$

Thiosulphate was also produced from sulphide, which would give a sulphide consumed:oxygen ratio of 1:2.0 according to

$$2H_2S + 2O_2 \longrightarrow H_2S_2O_3 + H_2O. \quad (4)$$

Peck & Fisher's (1962) data thus give rather low oxygen uptake for the observed sulphate production but do suggest that thiosulphate could conceivably be an intermediate in sulphide oxidation (Peck 1962), thus allowing both the atoms of thiosulphate to pass through sulphite and APS to allow ^{18}O exchange with phosphate. The importance of AMP as an intermediate in the oxidation of sulphite and the sulphane of thiosulphate was proved by the ^{18}O exchange technique by Peck & Stulberg (1962) using *T. thioparus* extracts. The partial reactions studied are shown in figure 3. Their data do not however, enable firm conclusions about the extent to which sulphide conversion to sulphite occurred in their experiments, or the extent to which AMP-independent sulphite oxidase was involved. In one experiment of Peck & Stulberg (1962),

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

a cell-free extract incubated with 40 μmol thiosulphate and ^{18}O-labelled AMP produced 28.6 μmol sulphate, but only 19.4 μmol phosphate were esterified. This indicates that 28.6 μmol sulphite arose from thiosulphate and was then converted to sulphate. All this could, however have come from *sulphone* groups, and the experiments do not give any information about sulphane-sulphur oxidation. If the recovery of esterified phosphate equalled that actually formed (i.e. not decreased by hydrolysis or recycling) the data could indicate that the sulphite was oxidized in a

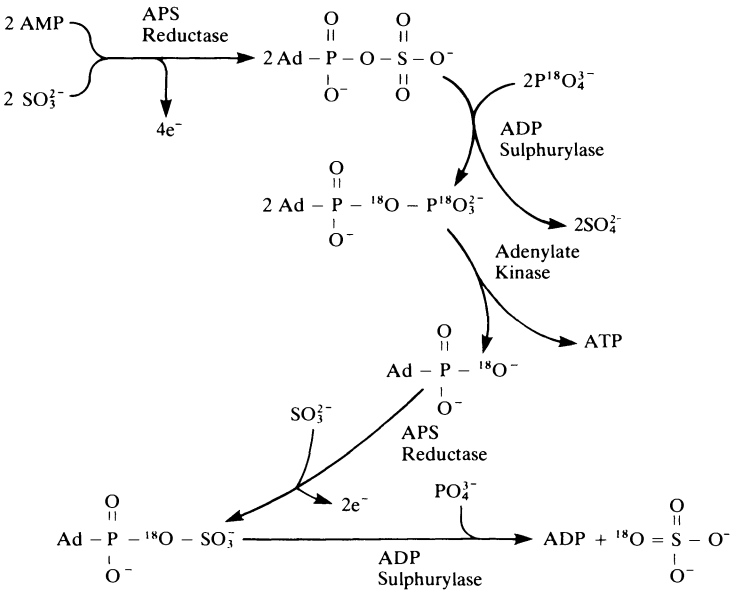


FIGURE 3. ^{18}O incorporation from phosphate into sulphate by the APS pathway (Peck 1962).

2:1 ratio by AMP-requiring and AMP-independent pathways. Enrichment in ^{18}O in the total sulphate produced showed it to be 76% of the value expected if all were derived via APS. The assumption that only 19.4 μmol sulphate arose through APS was, however, wholly consistent with the theoretical ^{18}O enrichment for this amount of sulphate. It is clear that the combination of evidence from the presence of the APS pathway enzymes, ^{18}O exchange and indirect evidence of uncoupler-insensitive phosphorylation in thiobacilli (Kelly & Syrett 1963, 1964, 1966*a*), shows the terminal oxidation of sulphite to involve APS in at least some thiobacilli. There is clearly a case for extending ^{18}O enrichment experiments of the kind employed by Santer (1959) and Peck & Stulberg (1962) to whole cells and extracts capable of *complete* oxidation of thiosulphate to sulphate, using strains that do contain APS reductase and those in which this enzyme has not been detected.

A COMMON PATHWAY FOR OXIDATION OF SULPHUR COMPOUNDS: VIEWPOINTS BY 1970

'Presumably different pathways operate in different organisms' (Schlegel 1975).

Several authors published schemes during the 1960s attempting to synthesize diverse data from studies on various thiobacilli into a similar scheme for sulphur compound oxidation and thereby extend the concept of 'unity in biochemistry' (Kluyver & Donker 1926) to the thio-

bacilli. Unresolved problems that remained included the enzymatic mechanism of the initial scission of thiosulphate and the biochemistry of sulphide conversion to sulphite. APS reductase and cytochrome *c*-linked sulphite oxidase activities had, however, been shown in various thiobacilli (see, for example, Peck *et al.* 1965; Bowen *et al.* 1966; Charles & Suzuki 1965, 1966*a*, *b*; Lyric & Suzuki 1970*a*, *b*; Hempfling *et al.* 1967), and APS reductase remained undetected in *T. novellus* and *Thiobacillus* A2 (Aleem 1965; Charles & Suzuki 1966*a*, *b*; Lyric & Suzuki 1970*c*;

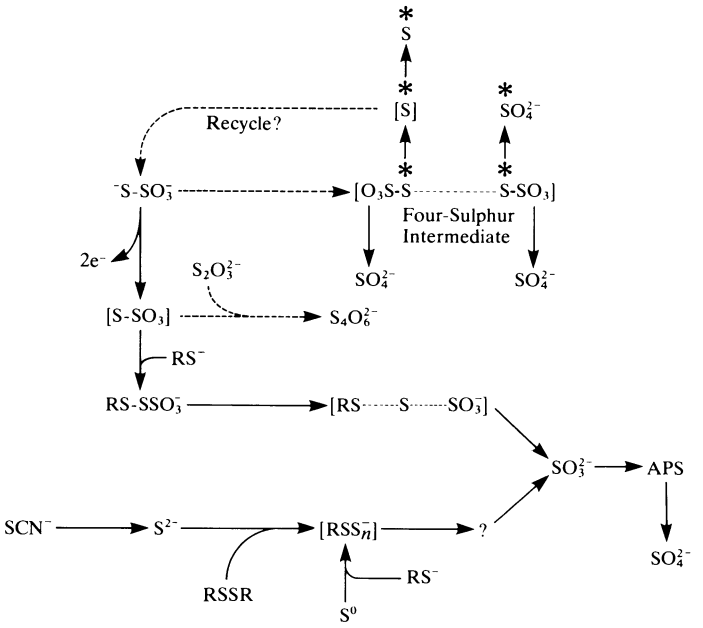


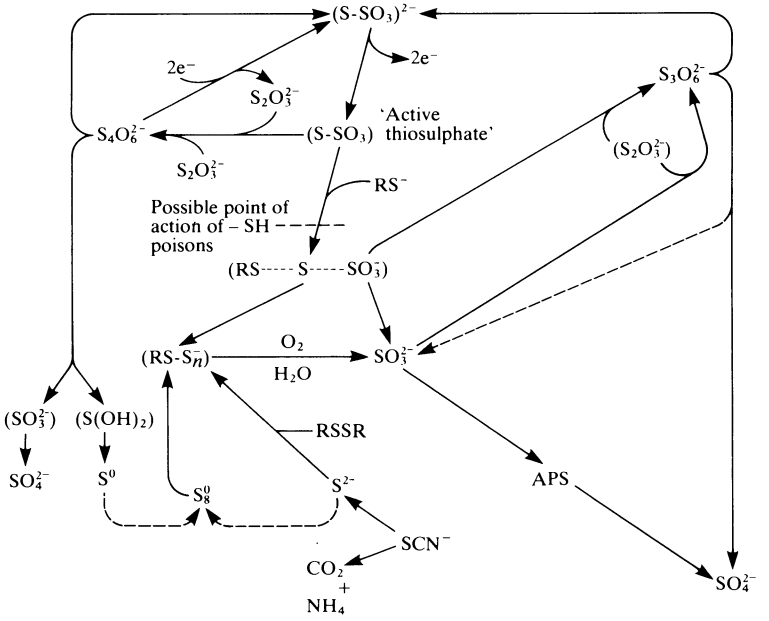
FIGURE 4. The scheme of Trudinger (1964*a*, 1967) to explain the apparently quantitative relation between sulphur and sulphate formation from thiosulphate and his (1967) hypothetical pathway for sulphur in thiobacilli.

Silver & Kelly 1976). A number of studies demonstrated discrimination in rate of oxidation between the two atoms of thiosulphate to sulphate (Skarzynski *et al.* 1957; Peck & Fisher 1962; Kelly & Syrett 1966*b*). Trudinger (1961*b*) demonstrated the existence of the 'thiosulphate-oxidizing enzyme' (whose product was tetrathionate), which was subsequently found in numerous thiobacilli (Kelly 1966; Lyric & Suzuki 1970*d*; Silver & Lundgren 1968*a*) but is extremely low in *Thiobacillus* A2 (Kelly 1973) and *T. denitrificans* (Justin & Kelly 1978*b*). Trudinger (1964*a*) subsequently postulated the occurrence of a four-sulphur intermediate (that was not tetrathionate) in the oxidation of thiosulphate by *T. neapolitanus* X, based on evidence of apparent stoichiometric discrimination between the sulphur atoms in producing several products. These observations led to the generalized scheme given in figure 4 (based on Trudinger (1964*a*, *b*, 1967); Roy & Trudinger (1970)).

The most significant discoveries of the late 1960s were (1) the demonstration of the oxidation (involving a cytochrome *c*) of sulphide by cell-free extracts of *T. concretivorus* (a strain of *T. thiooxidans*) to produce a membrane-bound highly reactive linear polymeric sulphur compound, probably containing sulphur at the level of S^0 rather than being a polysulphide (Moriarty & Nicholas 1969, 1970), and (2) the demonstration in *T. thioparus* and *T. thiooxidans* of a 'sulphur-oxidizing enzyme', producing sulphite from elemental sulphur by using reduced glutathione as a cofactor (Suzuki 1965*a*; Suzuki & Silver 1966). The enzyme was purified 124-fold and was

demonstrated to be an oxygenase containing non-haem iron and possibly labile sulphide. This enzyme activity was subsequently demonstrated in other thiobacilli, including *T. novellus* (Charles & Suzuki 1966*a*, *b*), *T. ferrooxidans* (Silver & Lundgren 1968*b*) and *T. denitrificans* (Justin & Kelly 1978*a*). Cell-free oxidation of sulphur was also reported for *T. thiooxidans*, *T. dentrificans* and *T. neapolitanus* by Adair (1966) and Taylor (1968).

These observations provided a framework within which the enzymatic conversion of sulphide



5. The unitary mechanism for sulphur compound oxidation proposed by Kelly (1968).

via polymeric sulphur to sulphite could be explained. Coupled with the concept of thiosulphate cleavage to sulphite and sulphide or sulphur and of sulphite oxidation by an oxidase or the APS pathway, a scheme for the complete oxidation of thiosulphate could thus be derived (see, for example, Charles & Suzuki 1966*a*, *b*). In 1967 (Kelly 1968) I constructed a hypothetical scheme to draw together all the available observations on sulphur compound metabolism into a unitary mechanism (Figure 5). The relatively simple 'core' of the scheme (due mainly to Suzuki) is summarized in figure 6*a*. This mechanism received further support from the isolation of a membrane-associated thiosulphate-oxidizing complex from *T. novellus* (Oh & Suzuki 1977*a*). This complex contained the constituent enzymes needed for oxidation: rhodanese, sulphur-oxygenase, sulphite oxidase and cytochrome *c* oxidase (Oh & Suzuki 1977*b*). The inability to reconstitute an active complex from the constituent enzymes could indicate that some cofactor was lost during fractionation or that an enzyme other than those identified was involved and that this was lost during the fractionation procedure.

It must not be forgotten that a 'polythionate pathway' still had strong advocates at this time (London & Rittenberg 1964) and had received what seemed to be supportive evidence from cell-free studies. The most likely alternative mechanisms by 1970 were thus the two based on the studies of the groups of Peck and Suzuki on the accumulated experience with polythionates as summarized by London & Rittenberg (1964). These alternatives are shown in figure 6

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

and the background to the thinking of this decade were comprehensively reviewed by Peck (1962, 1968), Vishniac & Trudinger (1962), Burns (1967), Kelly (1968, 1972), Trudinger (1967) and Roy & Trudinger (1970). The nagging problems raised by attributing the conversion of sulphane-level sulphur to sulphite by means of the sulphur-oxidizing enzyme are, however, (*a*) does all energy conservation then accompany sulphite oxidation, because the oxygenase is non-energy conserving, being unlinked to the electron transport chain, (b) the level of the enzyme is very low in all the organisms studied; (*c*) an oxygenase cannot operate in the anaerobe, *T. denitrificans*.

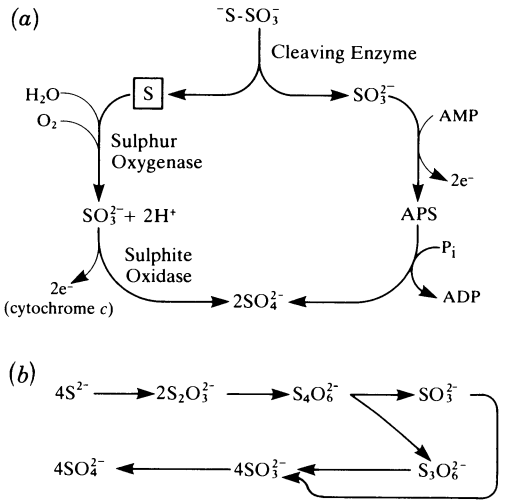


FIGURE 6. Viewpoints by 1970: (*a*) the 'cleavage–oxygenase–APS pathway' scheme due largely to Suzuki and his colleagues; (*b*) a polythionate pathway alternative (London & Rittenberg 1964).

THE ORIGIN AND DEGRADATION OF POLYTHIONATES

I am firmly convinced that polythionates have no central role as free intermediates of inorganic sulphur oxidation in thiobacilli. Their production and metabolism may use the same reversible mechanisms, and their degradation seems always to require reductive or hydrolytic scission to simpler molecules such as thiosulphate and sulphite. In the natural environment it is possible that the extracellular accumulation of polythionates from substrates such as sulphide or thiosulphate may be advantageous to thiobacilli if they thereby make the sulphur less available for competing heterotrophs or for chemical autooxidation or decomposition. It may be that some more significant biochemical role for polythionate may eventually emerge, but the 'central pathway' of inorganic sulphur oxidation seems to be crystallizing in the literature as a system to operate with single-sulphur units, with larger molecules of sulphur being peripheral to the main energy-yielding oxidative process.

No new information on polythionate metabolism has appeared for a decade or more, and the only enzyme ever isolated and characterized is the tetrathionate-producing thiosulphate-oxidizing enzyme of Trudinger (1961 *b*), subsequently shown in various thiobacilli.

In figure 7 I have attempted a schematic summary of the observed and proposed reactions of polythionates in thiobacilli. Thiosulphate may arise by non-enzymic chemical reaction between sulphur and sulphite (Suzuki 1965 *a*; Suzuki & Silver 1966) and possibly by enzymic

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

condensation in *T. thiooxidans* (Imai *et al.* 1962). Further condensation of thiosulphate with sulphite to form trithionate was indicated by ^{35}S-labelling patterns in *T. neapolitanus* (Kelly & Syrett 1966*b*). The oxidative condensation of two thiosulphates produces tetrathionate, as proposed by Nathansohn (1902) and confirmed by Trudinger (1961). Further metabolism of tetrathionate by *T. thiooxidans* was phosphate-dependent (Okuzumi & Kita, 1965), a finding that could reflect the operation of an APS pathway. Okuzumi (1965, 1966*a*) reported anaerobic

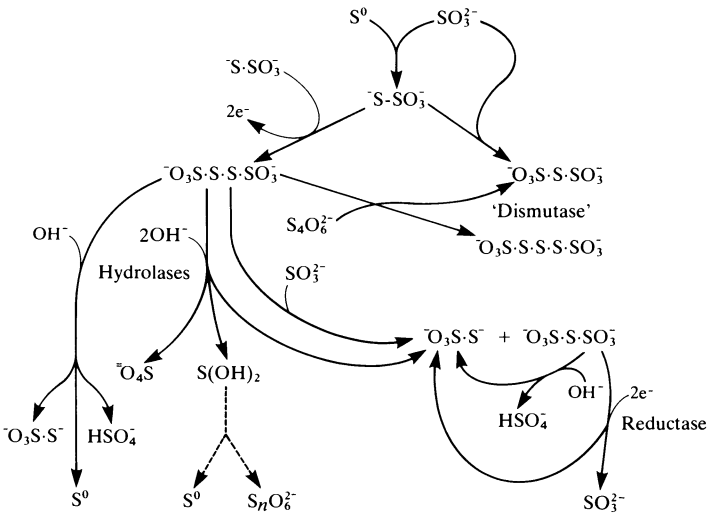


FIGURE 7. Reactions of polythionates.

conversion and enzymic disproportionation of two molecules of tetrathionate into trithionate and pentathionate, apparently catalysed by a 'tetrathionate dismutase' which was activated by iron, nickel or cobalt and inhibited by EDTA, 2,2′-dipyridyl and *o*-phenanthroline. Tetrathionate could also give rise to trithionate (and thiosulphate) by reaction with sulphite, as is known to occur chemically (Trudinger 1964*c*). Trithionate was proposed to be reductively dismuted to thiosulphate and sulphite in *T. thiooxidans* (Okuzumi 1966*b*). Anaerobically, *T. neapolitanus* will decompose both trithionate and tetrathionate (Trudinger 1964*c*, *d*; Kelly & Tuovinen 1975). Trudinger believed that the processes result from hydrolytic scission of either substrate to produce thiosulphate and sulphate, with sulphur or a hydrated sulphur compound of the form $S(OH)_2$ from tetrathionate. The degradative metabolism of polythionates thus seems to involve them in chain-shortening reactions producing thiosulphate or sulphur or sulphite or some combination of all three. None of the reactions shown explains the oxidation of sulphane–sulphur to sulphone–sulphur ($S \longrightarrow SO_3^{2-}$) and any cyclic polythionate pathway involving sequential production of sulphite or sulphate would need to include such a step. An argument against an obligatory role for polythionates in a common oxidation mechanism is the total inability of some strains (e.g. *Thiobacillus* A2 (Kelly 1973; Kelly & Tuovinen 1975)) to produce or metabolize them. Trudinger (1964*d*) proposed a generalized mechanism to explain polythionate degradation with the production of thiosulphate:

$$\text{enzyme} + {}^{-}O_3S.S_n.SO_3^{-} \longrightarrow \text{enzyme–}S_{n-1}.SO_3 + {}^{-}O_3S\text{–}S^{-}.$$

The enzyme complex could break down to produce the other products observed experimentally. It is interesting that, if this process does occur, the enzyme complex formed is analogous to the enzyme-bound polysulphur complexes proposed by Moriarty & Nicholas (1969, 1970).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Thiosulphate oxidation: a scheme for the early 1980s

> Another question of interest is whether the pathway of sulfate reduction is enzymologically the same as the pathway of sulfide oxidation, and even more specifically whether the pathways of sulfate reduction or sulfide oxidation are identical in the various physiological types of microorganisms that carry out these reactions. Peck (1962)

These words of Peck's were proved to be prophetic in the late 1970s by the work of Schedel & Trüper (1979, 1980) using *T. denitrificans*. They demonstrated a sirohaem sulphite reductase,

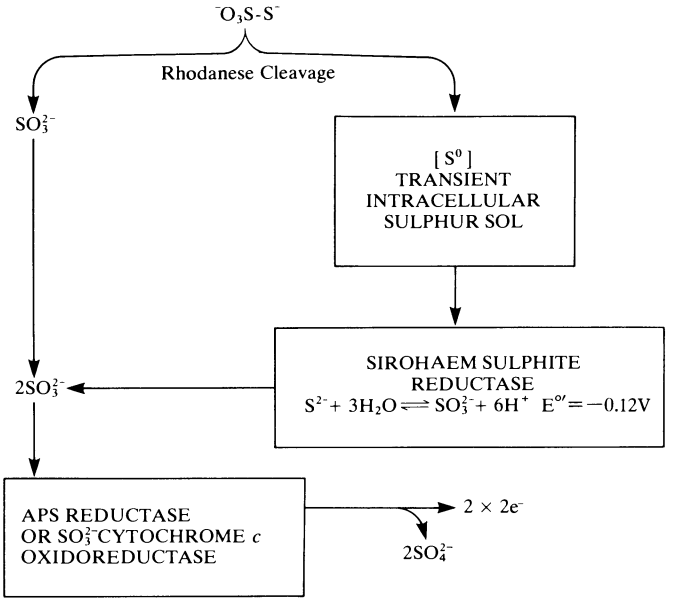


Figure 8. Thiosulphate oxidation: a scheme for the early 1980s.

comprising about 2 % of the total cellular protein, and purified it to homogeneity. *T. denitrificans* effected thiosulphate oxidation after cleaving the ion to sulphite and a highly reactive intracellular hydrophilic sulphur sol by means of a cleaving enzyme that probably carried the functions previously ascribed to separate enzymes: rhodanese and thiosulphate reductase (Schedel & Trüper 1980). If the normal physiological activity of the reductase were actually in the direction of sulphite *formation* from sulphide, its importance is obvious because it provides the missing link in *T. denitrificans* for the conversion of sulphane-sulphur to sulphite. If the enzyme normally uses the sulphur sol generated from thiosulphate as its substrate, the production of sulphite is explained by the operation of only two enzymes. Operation in the oxidative direction is feasible, and was proposed by Schedel & Trüper (1979) to be consistent with a requirement for an electron acceptor capable of receiving electrons from the oxidation at an average redox potential of -0.12 V. The nature of the acceptor has not yet been established and the actual 'reverse' operation of the enzyme not yet achieved. In this context, the observations that non-haem iron and flavin in soluble cofactors may be essential for sulphur oxidation in *T. thiooxidans* could be significant (Takakuwa 1975*a*).

The scheme of figure 8 is a working hypothesis for a mechanism of thiosulphate oxidation in the thiobacilli in general. It assumes that the 'sulphur-oxidizing oxygenase enzyme' is unimportant and that sulphite is produced by a reductase. Subsequently, sulphite is converted to

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

sulphate by the pathways already discussed or in *T. denitrificans* by similar pathways coupled to inorganic nitrogen compound (rather than oxygen) reduction.

The hypothesis in 1982 is thus essentially as proposed 20 years earlier by Peck (1962): that the conversion of sulphur, sulphide and the sulphane-sulphur of thiosulphate and polythionates to sulphate involves sulphite reductase and an APS pathway similar to that which functions in reverse direction in sulphate-reducing bacteria for the generation of sulphide. The appearance of thiosulphate and trithionate as apparent intermediates in many of the studies of sulphide oxidation is also consistent with the operation of sulphite reductase, since these may also be

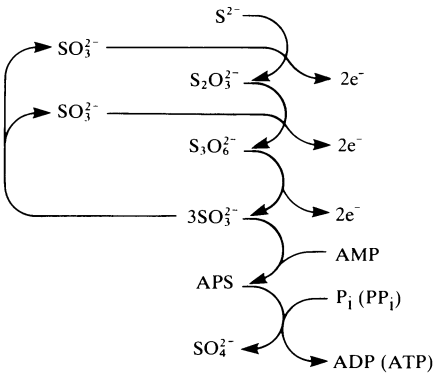


FIGURE 9. Possible products of a sulphite reductase acting as a sulphide oxidase (based on Thauer *et al.* 1977).

formed during sulphite reduction (Lee *et al.* 1973; Kobayashi *et al.* 1974; Schedel & Trüper 1979), though they are quite probably not true intermediates, being derived from normally enzyme-bound partly reduced sulphur species (Chambers & Trudinger 1975). Writing in reverse (figure 9) the model for sulphite reduction given by Thauer *et al.* (1977) one can see that thiosulphate and trithionate are even less plausible as main-line intermediates for the oxidative 'reverse' process because the essential step – sulphide conversion to sulphite – is not related to their formation, unless the sulphane-sulphur of trithionate were the true substrate of the reductase. More likely, sulphite displaces more reduced intermediates from their enzyme complexes (figure 9). A noteworthy observation is that sulphite was aerobically generated from sulphide by cell-free extracts of *T. denitrificans* and required both supernatant and pellet fractions from centrifuging at 144 000 *g* (Aminuddin & Nicholas 1973).

THE YEARS TO 1982: NEW APPROACHES AND NEW DIRECTIONS

The oxidation of inorganic sulphur compounds poses three interlinked problems. One, the main topic of this review, is the actual pathway for sulphur oxidation. The others are, first, the electron transport mechanisms associated with inorganic sulphur oxidation and, secondly, the means by which energy is conserved from sulphur oxidation and expressed as growth.

During the 1970s, there was a relative dearth of new information on sulphur pathways, but a significant advance in studies of electron transport problems and in the study of growth yields and energetic efficiencies of thiobacilli as a possible indirect means of making deductions about energy-yielding processes accompanying the central oxidative pathway for sulphur compounds.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Electron transport in thiobacilli

Both obligate and facultative chemolithotrophic thiobacilli naturally contain all the electron transport components necessary to link NAD(P)$^+$ and oxygen (or oxidized nitrogen compounds in *Thiobacillus* A2 and *T. denitrificans*), since their basic endogenous metabolism is similar to that of any other bacteria. The problem is: at which level do electrons from the oxidation of sulphur compounds enter the chain, how is NAD$^+$ reduced, what are the terminal oxidase systems and how many coupling sites exist for ATP synthesis during electron transport? It has

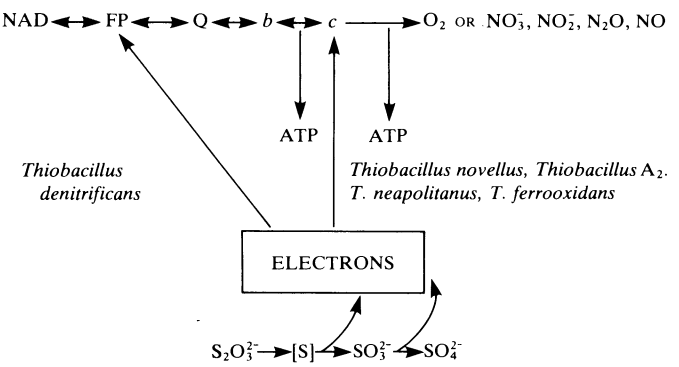


FIGURE 10. Principles of electron transport and phosphorylation in thiobacilli.

been established by considerable work that (*a*) sulphur oxidations do not reduce NAD$^+$ directly, (*b*) electrons enter the chain at the level of cytochrome *c* or in some cases flavoprotein or cytochrome *b*, and (*c*) that NAD$^+$ reduction requires energy-dependent electron flow from cytochromes to NAD$^+$ (Aleem 1969). These problems have been thoroughly discussed by Schlegel (1975) and Kelly (1978). Figure 10 summarizes the principles of electron transport in thiobacilli. A general finding so far is that electrons from sulphur oxidations seem to enter the chain at the cytochrome *c* level in aerobic thiobacilli, but at the flavin or cytochrome *b* level in *T. denitrificans*. This would indicate that two coupling sites for ATP synthesis might be available to *T. denitrificans* for each electron pair transported, but only one for the aerobes. This immediately suggests that the growth of the former should be better: in fact *T. denitrificans* gives much higher growth yields both anaerobically and aerobically than do the acrobic thiobacilli studied to date (Timmer-ten Hoor 1976, 1981; Kelly 1978; Justin & Kelly 1978*a*, *b*; Kuenen 1979). This is discussed in a later section.

The work by Nicholas's group in Adelaide enables the scheme shown in figure 11 to be constructed to explain electron transport from thiosulphate metabolism in *T. denitrificans*. The following notable points emerge from their studies and those of Aleem and his colleagues: (*a*) observations with inhibitors of electron transport and direct measurement of cytochrome reduction indicate nitrate reduction by a sulphite-dependent reductase with electron flow through flavin, quinone and cytochrome *b* to a cytochrome c_{554}, while sulphide oxidation (to the membrane-bound polysulphur) is linked via cytochrome c_{551} to a cytochrome *c*, *d*-nitrite reductase; a mechanism for electron transport during the polysulphur oxidation to sulphite was not established (cf. Schedel & Trüper 1980); (*b*) the nitrite reductase could alternatively transfer electrons to oxygen; (*c*) ATP sulphurylase rather than ADP sulphurylase is proposed as the terminal enzyme for the APS pathway (Aminuddin 1980). This is consistent with Peck's

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

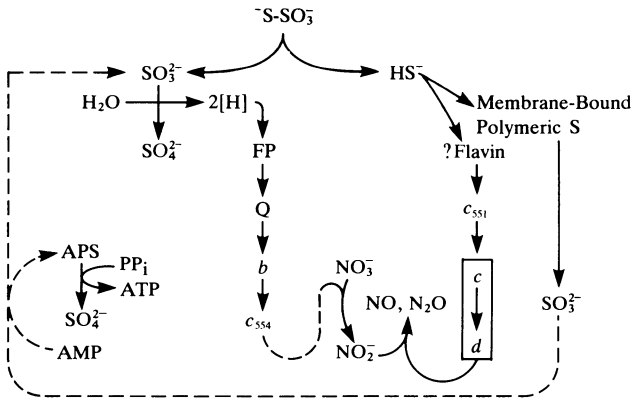


FIGURE 11. Anaerobic oxidation of thiosulphate by *Thiobacillus denitrificans*. (Based on Adams *et al.* (1971), Aminuddin (1980), Aminuddin & Nicholas (1973, 1974*a*, *b*) and Sawhney & Nicholas (1977*a*, *b*).

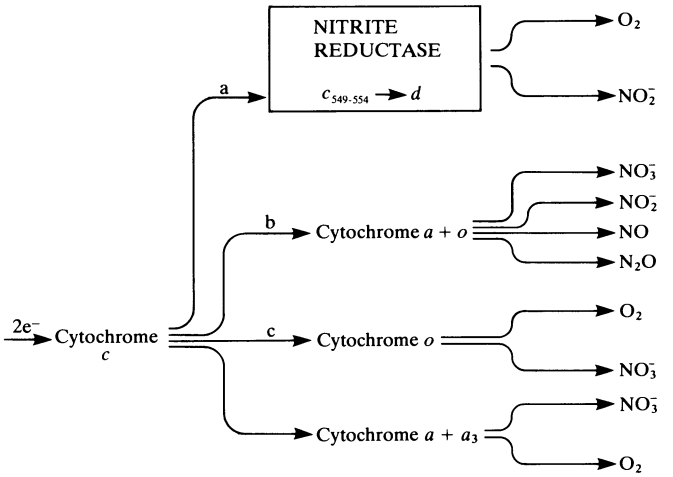


FIGURE 12. The variety of terminal oxidase systems after cytochrome *c* in thiobaclili, deduced from spectrophotometric and inhibitor studies. (*a*) Sawhney & Nicholas (1977); (b) Ishaque & Aleem (1973); (c) Peeters & Aleem (1970).

(1960) observation of a high level of this enzyme in *T. thioparus*. The problem to be resolved if this enzyme is involved is the origin of pyrophosphate for its function.

Possible alternative terminal oxidation reactions are summarized in figure 12.

Clearly the most electronegative point at which electrons from an oxidation can enter the electron transport chain is dependent on the electrode potentials of the electron-donating reaction and of the chain component. Standard electrode potentials ($E^{\circ\prime}$, with the hydrogen couple, $H_2/2H^+ + 2e^-$, as -0.414 V) for many of the reactions important to thiobacilli are given in table 1. It is apparent from these data that there is a physicochemical bar to direct electron transport from any sulphur oxidation to NAD^+, but that the oxidations of sulphide to sulphur or sulphite to sulphate (and trithionate to sulphite) should be able to couple to FAD reduction, and both these and the oxidation of sulphide to sulphite are more electronegative than the cytochrome *b* couple. APS reductase might be able to couple at cytochrome *b*, but APS formation and the overall oxidations of sulphur, thiosulphate or tetrathionate to sulphite (or of

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

thiosulphate to tetrathionate) could only couple to cytochrome *c* reduction. Differences among thiobacilli regarding whether flavin or cytochrome *b* or cytochrome *c* are points of entry for electrons from the oxidations with $E^{\circ\prime}$ below -0.1 V must therefore depend on the specificity of the oxidoreductases that they evolved early in their existence. One could speculate that coupling to the most electronegative acceptor physically possible should have been selectively advantageous, but this does not seem to be so. Drozd (1974) has demonstrated by

Table 1. Electrode potential of some oxidation–reduction reactions of probable importance to thiobacilli

reaction or couple	$E^{\circ\prime}/V$†
NAD/NADH	−0.320
$FAD/FADH_2$	−0.220
cytochrome *b* ox./red.	−0.040
cytochrome *c* (various) ox./red.	+0.200/+0.270
$S_2O_3^{2-}/HS^- + HSO_3^-$	−0.402
S^0/HS^-	−0.270
$S + 3H_2O \longrightarrow H_2SO_3 + 4H^+ + 4e^-$	+0.050
$S_2O_3^{2-} + 3H_2O \longrightarrow 2H_2SO_3 + 2H^+ + 4e^-$	−0.020‡
$H_2SO_3 + H_2O \longrightarrow H_2SO_4 + 2H^+ + 2e^-$	−0.280
$HSO_3^-/S_3O_6^{2-}$	−0.173‡
HSO_3^-/HS^-	−0.116
$S_3O_6^{2-}/S_2O_3^{2-} + HSO_3^-$	+0.225
$AFS/AMP + HSO_3^-$	−0.060
$S_4O_6^{2-}/S_2O_3^{2-}$	+0.024
$S_4O_6^{2-} + 6H_2O \longrightarrow 4H_2SO_3 + 4H^+ + 6e^-$	+0.090‡
$NO_2^- + H_2O \longrightarrow NO_3^- + 2H^+ + 2e^-$	+0.540
$NO + 2H_2O \longrightarrow NO_3^- + 4H^+ + 3e^-$	+0.540
NO_2^-/NO	+0.350
NO/N_2O	+1.175
N_2O/N_2	+1.355
O_2/H_2O	+0.818

† Data taken from Peck (1968), Gundersen (1968) and Thauer *et al.* (1977), or calculated from Latimer (1952).
‡ These data are reasonable estimates of true $E^{\circ\prime}$ values, relative to the $H_2/2H^+ + 2e^-$ couple being −0.414 V. Data published earlier (Kelly 1978) as $E^{\circ\prime}$ values were incorrect as they were in fact E° values relative to the hydrogen gas – hydrogen ion couple taken as an arbitrary zero.

measurement of the outward translocation of protons during thiosulphate oxidation by *T. neapolitanus* that H^+/O values of 2.0 are found, consistent with the presence of only one ATP coupling site, associated with electron transport through cytochrome *c* to oxygen (cf. Ross *et al.* 1968) In table 2 I have summarized some of the published evidence from electron transport inhibitor experiments that demonstrate the use of the cytochrome *b* part of the chain for sulphur compound oxidation in *T. denitrificans*, but apparently not in at least some of the aerobic thiobacilli. The energy dependence of NAD^+ reduction was similarly demonstrated both directly and indirectly in various thiobacilli by using intact cells and cell-free extracts (Aleem *et al.* 1963; Kelly & Syrett 1966*a*; Aleem 1969; Roth *et al.* 1973; Kelly 1978). Energy-dependent reduction of cytochrome *b*, flavin and NAD^+ occurred in *T. neapolitanus* (Aleem 1969) and was confirmed in intact organisms (Roth *et al.* 1973) in which sodium amytal blocked NAD^+ reduction without affecting ATP synthesis during thiosulphate oxidation.

The consequence of electrons entering the chain at cytochrome *c* rather than cytochrome *b*

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

is clearly the likelihood that a P/O ratio of one rather than two will be achieved (figure 10), but it could be argued that only the $b \rightarrow c$ coupling site might be linked to ATP synthesis in anaerobic *T. denitrificans*. The fact that growth yields of *T. denitrificans* are greater than those for the aerobes argues against this, and the highly electropositive $E^{0\prime}$ values for the nitrogen compound couples used by the bacteria (e.g. N_2O/N_2) would at least indicate the possibility that ATP synthesis occurs during electron transport from cytochrome *c* to terminal nitrogen

TABLE 2. EVIDENCE FROM ELECTRON TRANSPORT INHIBITORS FOR THE POINTS OF ENTRY OF ELECTRONS FROM SULPHUR COMPOUND OXIDATION INTO THE ELECTRON TRANSPORT CHAIN IN THIOBACILLI

inhibitor	reaction inhibited	system tested†	A	B	C	D	E	F	G	H	J	K	L	M
rotenone	flavoprotein reduction		++	++	+	+	++	+		0			++	0/+
TTFA			++	+	0	++	+						++	+
amytal			++	++	+	+	++	+	0	+	+	+	++	
atebrine									0			++	+	0
HQNO	cytochrome $b \longrightarrow c$		++					++	0	0	++	+	++	0
dicoumarol			++					++						
antimycin A			++					++	0	0		+	+	0
KCN	terminal oxidase systems		++	++	+	++	++	++	++	++	++	++	+	++
NaN_3			++	++	+	++	+	++		++	++	+		++
CO								++		++		+		
references			1	1	1	1	1	2	3	4	4	3	5	6

Scores: ++, Inhibition greater than 60%; +, inhibition 20–60%; 0, negligible inhibition.

References: (1) Peeters & Aleem (1970), Ishaque & Aleem (1973); (2) Adams *et al.* (1971), Sawhney & Nicholas (1977); (3) Aleem (1965); (4) Aminuddin & Nicholas (1974*a*, *b*); (5) Saxena & Aleem (1965); (6) Oh & Suzuki (1977).

† *Systems*: A, anaerobic NO_3^--dependent $S_2O_3^{2-}$ oxidation by *T. denitrificans*; B, anaerobic SO_3^{2-} oxidation by *T. denitrificans*; C, aerobic SO_3^{2-} oxidation by *T. denitrificans*; D, anaerobic S^{2-} oxidation by *T. denitrificans*; E, aerobic S^{2-} oxidation by *T. denitrificans*; F, SO_3^{2-}-dependent nitrate reductase; G, $S_2O_3^{2-}$-cytochrome *c* oxidoreductase of *T. novellus*; H, NO_2^- reduction or O_2 uptake during sulphide oxidation by the 144000 *g* pellet fraction of *T. denitrificans*; J, SO_3^{2-} oxidation by [H]; K, cell-free 'thiosulphate oxidase' of *T. novellus*; L, ATP-dependent NAD^+ reduction in *T. neapolitanus*; M, thiosulphate oxidation to sulphate by membrane vesicles of *T. novellus*.

oxidants. For example, the nitrate-dependent oxidation of sulphite (figure 10) is considerably energy-yielding in thermodynamic terms (calculated from free energies of formation given by Thauer *et al.* 1977):

$$NO_3^- + SO_3^{2-} \longrightarrow NO_2^- + SO_4^{2-}; \Delta G^0 = -184 \text{ kJ mol}^{-1}.$$

Since the ΔG^0 for ATP synthesis from ADP and orthophosphate is around -31.8 kJ mol^{-1} under standard conditions, and would be unlikely to exceed -50 kJ mol^{-1} under physiological conditions (Thauer *et al.* 1977), there is clearly ample energy available to effect ATP synthesis during this reaction.

Growth yields of thiobacilli on inorganic sulphur compounds

The application of chemostat culture techniques to thiobacilli has opened up a new theoretical approach to elucidating not only the energy efficiency of growth on sulphur compounds but an indirect means of establishing what is likely or possible as routes for inorganic sulphur oxidation, electron transport and coupled ATP and NADH formation (Hempfling & Vishniac 1967; Timmer-ten Hoor 1976, 1981; Kelly *et al.* 1977; Justin & Kelly 1978*a*, *b*; Eccleston & Kelly

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

1978; Kuenen 1979). Pirt's (1965, 1975) graphical procedures may be used to estimate theoretical maximum (or 'true') growth yields, corrected for energy expended in cell 'maintenance' for thiobacilli (see, for example, Hempfling & Vishniac 1967; Eccleston & Kelly 1978; Justin & Kelly 1978*a*; Kuenen 1979) as shown for *T. neapolitanus* in figure 13. Comparing 'true growth yields' reveals the considerable difference in growth efficiency of different species (table 3): the mean of the quoted values for $Y^{max}_{S_2O_3}$ for the aerobic thiobacilli is only 6.7 compared with

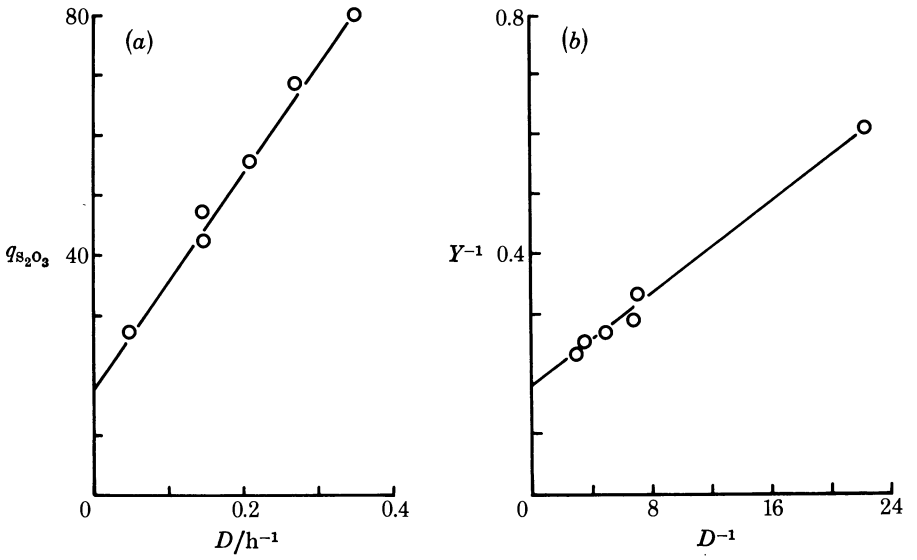


FIGURE 13. Growth yield of *Thiobacillus neapolitanus* strain C in chemostat culture (Kelly, unpublished data). A chemostat culture (420 ml) at 30 °C, pH 6.6, was growth-limited by the input of 40 mM sodium thiosulphate. Biomass (grams dry mass per litre) was determined by direct measurement at six dilution rates. Sulphur precipitation and washout occurred at $D = 0.37$ h^{-1}. Yield (grams dry mass per mole of thiosulphate used) was calculated and plots given of (*a*) specific rate of thiosulphate oxidation ($q_{S_2O_3}$ in millimoles of thiosulphate oxidized per hour per gram of dry mass) against dilution rate; and (*b*) reciprocal of yield against reciprocal of dilution rate. The true or maximum growth yield ($Y^{max}_{S_2O_3}$) is given by the reciprocal of the slope of the q–D plot and as the reciprocal of the Y^{-1} intercept on the Y^{-1}–D^{-1} plot. The value is 5.5 g mol^{-1} by both procedures.

14.7 for aerobic *T. denitrificans*. The data of Timmer-ten Hoor (1976, 1981) are particularly interesting because she shows the equivalence of sulphide and thiosulphate as growth-yielding substrates, consistent with both oxidations being equivalent to the transfer of eight electrons per molecule oxidized, suggesting that each substrate yields the same amount of ATP (by electron-transport and substrate-level phosphorylation). The earlier work of Kelly & Syrett (1963, 1964) supported the occurrence of both substrate-level phosphorylation (presumably by the APS pathway) and electron-transport phosphorylation in *T. neapolitanus* (although Kuenen (1979) could not detect an APS pathway in strain X of *T. neapolitanus*). Kelly & Syrett (1964), however, found sulphide-dependent CO_2 fixation by cell suspensions to be only about 67 % of that with thiosulphate, even though both molecules require two oxygen molecules for oxidation. Re-examining the original 1962 data (Kelly, unpublished) showed that in a total of 13 separate experiments, CO_2 fixation with sulphide was 73 ± 19 % of that with thiosulphate, including three values of 80–101 %. In most experiments the sulphate produced and oxygen consumed were measured and from four experiments in which fixation with sulphide was around 69 % of that with thiosulphate, mean CO_2 fixation was 50.5 and 30.0 nmol fixed per

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

micromole of thiosulphate or sulphide oxidized respectively (based on sulphate recovery) and 30.7 and 20.4 nmol per micromole of oxygen consumed with thiosulphate or sulphide respectively. Based on sulphate formation or oxygen uptake, fixation with sulphide was 59.4 or 66.4 % of that with an equivalent amount of thiosulphate, in agreement with the original published data (Kelly & Syrett 1963, 1964). This 'shortfall' in fixation with sulphide is not easily explicable if indeed the identical chemostat growth yields found by Timmer-ten Hoor (1976, 1981) at one

TABLE 3. OBSERVED YIELDS (Y) AND 'TRUE GROWTH YIELDS' (Y_g^{max}) OF THIOBACILLI AND *THIOMICROSPIRA* GROWING IN CONTINUOUS CHEMOSTAT CULTURE

organism	substrate	Y_g^{max}†	Y† (D = 0.03 h^{-1})	note
T. neapolitanus	$S_2O_3^{2-}$	7.7	—	1
		6.3	—	2
T. neapolitanus X		6.3	—	3
T. neapolitanus C		5.5	—	4
Thiobacillus A2	$S_2O_3^{2-}$	6.4	—	5
		7.0	—	6
T. ferrooxidans	$S_2O_3^{2-}$	7.5	—	7
	$S_4O_6^{2-}$	12.2	—	7
T. denitrificans	$S_2O_3^{2-}$-(NO_3^-)	11.4	6.3	8
	$S_2O_3^{2-}$-(O_2)	14.7	11.7	8
	$S_2O_3^{2-}$-(NO_3^-)	10.0	9.3	9
	S^{2-}-(NO_3^-)	—	9.4	9
	$S_2O_3^{2-}$-(NO_2^-)	—	8.2	9
	$S_2O_3^{2-}$-(O_2)	—	13.2	9
Thiomicrospira denitrificans	$S_2O_3^{2-}$-(NO_3^-)	5.1	5.2	9
	S^{2-}-(NO_3^-)	—	5.9	9
	$S_2O_3^{2-}$-(NO_2^-)	—	5.2	9
	$S_2O_3^{2-}$-(O_2)	—	7.7	9
Thiomicrospira pelophila	$S_2O_3^{2-}$-(O_2)	7.0	—	10

Notes:

(1) This is the lower figure given by Hempfling & Vishniac (1967) from a Y^{-1}–D^{-1} plot, for $D < 0.14\ h^{-1}$.

(2) Calculated from fig. 1 of Hempfling & Vishniac (1967) after estimating steady-state biomass concentration and specific rate of thiosulphate consumption ($q_{S_2O_3}$) from their data and plotting $q_{S_2O_3}$ against dilution rate (D). Y^{max} is given by the reciprocal of the slope of this plot.

(3) Calculated as in (2) from the data of fig. 1 of Kuenen (1979) for D = 0.033–0.33 h^{-1}, assuming input concentration of 40 mM thiosulphate. An identical value was obtained by using these seven data points corrected for dry mass equal to excreted organic carbon (Kuenen 1979; Cohen *et al.* 1979), kindly provided by J. G. Kuenen (personal communication).

(4) Kelly: data of figure 13.

(5) Calculated as in (2) from the data of fig. 2 of Kuenen (1979) for D = 0.015, 0.03 and 0.05 h^{-1} assuming input thiosulphate concentration to be 40 mM.

(6) Previously unpublished data of Wei-Ping Lu & Kelly for D = 0.02–0.08 h^{-1}.

(7) Eccleston & Kelly (1978).

(8) Justin & Kelly (1978).

(9) Timmer-ten Hoor (1981).

(10) J. G. Kuenen, personal communication of an approximate value.

† Expressed as grams dry mass of bacteria produced per mole of sulphur compound oxidized. Data are *not* corrected for electrons used for CO_2 fixation rather than ATP-generating electron transport.

dilution rate are indicative of equivalent Y^{max} values for sulphide and thiosulphate. Possibly in the washed cell suspension experiments the autooxidation of sulphide reduced that available to the bacteria or possibly sulphide exerted an uncoupling effect as a toxic substrate, thereby lowering the efficiency of CO_2 fixation.

The relative contributions of substrate-level and oxidative phosphorylation to ATP synthesis

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

are in any case not yet finally resolved, but estimates of the amount of ATP contributed by the former during thiosulphate oxidation fall in the range 20–40 % (Kelly & Syrett 1963; Hempfling & Vishniac 1967; Timmer-ten Hoor 1976; Kelly 1978; Eccleston & Kelly 1978; Aminuddin 1980). Extensive theoretical use has been made of growth yield data in attempts to assess energy coupling mechanisms during growth dependent on sulphur oxidation. These include evaluation of the energy cost of reduced cytochrome-dependent reduction of NAD^+, the location of coupling sites for ATP synthesis and the actual mechanisms for ATP synthesis (see, for example, Roth *et al.* 1973; Aleem 1975; Timmer-ten Hoor 1976, 1981; Kelly *et al.* 1977; Kelly 1978, 1981; Eccleston & Kelly 1978; Aminuddin 1980). Rather than review this diverse literature, I have presented below new studies on *Thiobacillus* A2 (Wei-Ping Lu, unpublished) that illustrate most of the theoretical and practical assumptions used by recent authors.

Thiosulphate oxidation and ATP synthesis in *Thiobacillus* A2: a growth yield and energy balance assessment

Wenn ich also der Ansicht zuneige, dass in dem Schwefelbacterien und ähnlichen Organismen die Oxidation der anorganischen Substanz wirklich das einzige Glied des abbauenden Stoffwechsels darstellt, so möchte ich damit nicht einer allzu 'einfachen' und groben Verstellung dieses Processes das Wort reden. Die Thatsache der chemosynthetischen Kohlensäure-Assimilation lehrt, wie mir scheint, . . . das die bei der Oxydation frei werdende Energie wenigstens zum Theil nicht als Wärme auftritt, sondern sofort transformirt und dazu verwendet wird, Körper von hohem chemischem Energie potential zu schaffen. Daraus dürfte folgen, dass die Oxydation nicht in der das Plasma durchtränkenden Flussigkeit etwa durch ausgeschiedene Enzyme, erfolgt, sondern im engsten Verbande mit den lebenden, zur Energie-transformation befähigten Theilchen vor sich geht. Sich diese Vorgänge näher ausmalen zu wollen, wäre müssig; . . .

(If I incline to the view that in sulphur bacteria and similar organisms, oxidation of the inorganic substance truly represents the only component of the degradative metabolism, I do not wish to imply an all-to-simple and crude idea of this process. It seems to me that the chemosynthetic assimilation of carbon dioxide shows that little of the energy released from the oxidation escapes as heat, but is immediately transformed and used to produce bodies of high chemical energy potential. It might follow from this that oxidation does not take place in the cytoplasmic solution, perhaps through separate enzymes, but rather in the closest association with living particles, capable of energy transformation. It would be idle to seek to give a more precise picture of these processes;...) Nathansohn (1902)

Given the state of general biochemical knowledge at that time, Nathansohn's assessment of the bioenergetic link of chemolithotrophy to autotrophy and the importance of an integrated intracellular system for energy transformation is remarkably prophetic.

Experiments with cell-free extracts

Recent work in our laboratories has resulted in the preparation from *Thiobacillus* A2 of French pressure cell extracts that effect stoichiometric oxidation of thiosulphate, coupled to ATP synthesis:

$$Na_2S_2O_3 + 2O_2 + H_2O + 4ADP + 4P_i \longrightarrow Na_2SO_4 + H_2SO_4 + 4ATP.$$

Thiosulphate oxidation required factors present in both the supernatant and pellet fractions

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

after centrifuging at 140000 *g* (table 4). Although no oxygen uptake occurred, cytochrome c_{552}, but not cytochrome *b*, was reduced by thiosulphate or sulphite added to the supernatant fraction. This reduction was unaffected by antimycin A or HQNO. Addition of the pellet fraction or of heart cytochrome *c* and bovine heart cytochrome oxidase resulted in cytochrome oxidation and oxygen uptake. In contrast, NADH or succinate did not reduce cytochromes in the 140000 *g* supernatant. Similarly, the pellet fraction did show reduction of cytochromes *c* and *b*

TABLE 4. STOICHIOMETRY OF THIOSULPHATE OXIDATION BY WHOLE CELLS AND EXTRACTS OF *THIOBACILLUS* A2

preparation tested†	thiosulphate disappearance (nmol min^{-1} mg^{-1} protein)	oxygen uptake (nmol min mg^{-1} protein)	$O_2/Na_2S_2O_3$
whole cells	335	621	1.85
S-10000	5.3	11.1	2.09
S-140000	0	0	—
P-140000	4.2	0	—
S + P‡	7.1	14.2	20.0
	thiosulphate added (nmol ml^{-1})	**oxygen uptake (nmol ml^{-1})**	
S-140000§	20	0	—
S-140000§† supplemented with cytochrome *c* (0.1 mM) and cytochrome oxidase (2 units ml^{-1})	20	40.3	2.02
	50	99.5	1.99

† S and P refer to supernatant and pellet fractions from centrifuging French pressure cell extracts at 10000 *g* and centrifuging the S-10000 at 140000 *g*.
‡ 2.8 mg S-140000 protein + 0.3 mg P-140000 protein.
§ 4.6 mg protein ml^{-1}.

when NADH or succinate was added (cytochrome *c* reduction being abolished by antimycin A or HQNO), but not when thiosulphate or sulphite was supplied. These results indicated that although NADH and succinate dehydrogenases transferring electrons to membrane-associated cytochrome C_{552} were present in the pellet fraction, some factor essential to linking sulphite or thiosulphate oxidation to cytochrome reduction was only present in the supernatant fraction. For thiosulphate this could be the primary enzyme in the activation or scission of the molecule as well as the enzyme(s) reducing cytochrome *c*. Rhodanese is among the enzymes present in this fraction, but it will be recalled that these observations are in some contrast to the wholly membrane-associated complex studied by Oh & Suzuki (1977*a*, *b*). The results also show that while limited thiosulphate oxidation can occur in the soluble fraction, oxygen uptake requires membrane-associated cytochrome *c* and cytochrome oxidase.

Both crude extracts (10000 *g*) and a reconstituted mixture of pellet and supernatant from 140000 *g* oxidized thiosulphate completely to sulphate (Table 4). The supernatant fraction from centrifuging at 40000 *g* coupled thiosulphate oxidation to ATP synthesis with a P:O ratio of about unity (table 5). Oxidation and phosphorylation efficiencies were not significantly affected by antimycin A or HQNO, in contrast to NADH-dependent oxidation and phosphorylation, which were severely inhibited (table 5). This demonstrates that qualitative oxidation of thiosulphate to sulphate and coupled ATP synthesis do not involve electron transport through

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 5. PHOSPHORYLATION BY CELL-FREE EXTRACTS OF *THIOBACILLUS* A2

preparation tested†	substrate	inhibitor	oxygen uptake‖ n atom min^{-1}	ATP formed¶ nmol min^{-1}	P/O
S-40 000	$Na_2S_2O_3$	—	39	43	1.12
	$Na_2S_2O_3$	Antimycin A§	32	35	1.11
	$Na_2S_2O_3$	HQNO§	38	40	1.06
	NADH	—	644	334	0.52
	NADH	Antimycin A§	24	0	0
	NADH	HQNO§	20	0	0
S-130 000‡	$Na_2S_2O_3$	—	21	16	0.76
	$Na_2S_2O_3$	2,4-dinitrophenol (0.1 mM)	22	0	0
P-130 000‡	$Na_2S_2O_3$	—	1.4	1.0	0.71

† S and P indicate supernatant and pellet fractions respectively from centrifuging the 10 000 *g* crude extract at 40 000 *g* or centrifuging the 40 000 *g* supernatant at 130 000 *g*.
‡ These fractions contained small amounts of the corresponding P or S fraction.
§ Used at 2 μg mg^{-1} protein. HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.
‖ Reactions were run in Clark oxygen electrode cells and data corrected for endogenous uptake.
¶ Measured by using the Lumac system and corrected for endogenous synthesis.

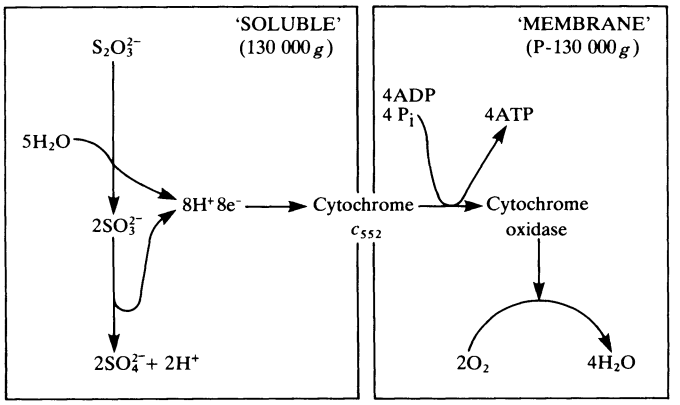


FIGURE 14. Localization of the components of the thiosulphate-oxidizing system of *Thiobacillus* A2 (Wei-Ping Lu & D. P. Kelly, unpublished data).

cytochrome *b* in *Thiobacillus* A2. Thiosulphate oxidation and ATP synthesis were effected by the 130 000 *g* pellet or supernatant fractions, provided that a small amount of the corresponding fraction was also present (table 5). ATP synthesis in the supernatant fraction was abolished by dinitrophenol, which did not inhibit oxidation, indicating that synthesis did not involve substrate-level phosphorylation, in agreement with the apparent absence of APS reductase from the organism. These results are consistent with the scheme shown in figure 14.

Experiments with chemostat culture

In continuous culture on thiosulphate the growth yield (grams dry mass *Thiobacillus* A2 per mole of thiosulphate oxidized) increased with dilution rate from 5.3 at $D = 0.025\ h^{-1}$ to 6.4 at $D = 0.08\ h^{-1}$. The true growth yield ($Y^{max}_{S_2O_3}$) was estimated graphically (cf. figure 13) to be 7.04 g mol^{-1}. Insignificant excretion of organic carbon from the bacteria occurred during growth (as judged from the total organic carbon content of cultures) and their carbon content

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

was 46 % of dry mass. Consequently the $Y^{max}_{S_2O_3}$ represented the fixation of 0.27 mol CO_2. Using calculations similar to those employed for other thiobacilli (Kelly *et al.* 1977; Eccleston & Kelly 1978; Justin & Kelly 1978; Kelly 1978) one can estimate that since CO_2 fixation to the hexose level by the Calvin cycle requires 3 mol ATP and 2 mol NAD(P)H per mole, fixation of 0.27 mol needed 0.81 mol ATP and 0.54 mol NAD(P)H. Reduction of 0.54 mol NAD^+ required 1.08 of the 8 H equivalents liberated per mole of thiosulphate oxidized, leaving 6.92 mol H (from 0.87 mol thiosulphate) for electron transport coupled to ATP synthesis. For biosynthesis and growth commencing at the hexose level of fixed carbon, about 0.88 mol ATP are required per mole of carbon (estimated from Stouthamer 1973). From these figures one can say that the assimilation of 0.27 mol CO_2 required 0.81 mol ATP for fixation and 0.24 mol ATP for biosynthesis, indicating at least 1.05 mol ATP to be available from the oxidation of 0.87 mol thiosulphate (i.e. 1.21 mol ATP *per mole of thiosulphate oxidized for energetic purposes*). In addition, a quantity (x mol) of ATP is required to enable electron flow from reduced cytochrome *c* to produce 0.54 mol NADH. The total ATP produced will thus be $(1.05+x)/0.87$ mol ATP per mole of thiosulphate oxidized for energy generation. The experimentally observed P/O ratio of 1.12 (table 5) indicated that 4.48 mol ATP were formed per mole of thiosulphate. Thus if 3.9 mol ATP arise from 0.87 mol thiosulphate oxidation and 1.05 mol ATP are concurrently consumed for CO_2 fixation and growth, 2.85 mol ATP would remain for other ATP-requiring processes, including the reduction of 0.54 mol NAD^+. If all this were actually expanded in the reduction of the NAD^+, the ATP requirement would be 5.3 mol ATP per mole of NAD^+. (If the P/O ratio were approximated to the nearest whole number, 1.0, ATP production would be 4 mol per mole of thiosulphate, and the requirement for NAD^+ reduction 4.5 mol ATP per mole). This estimation of the energy cost of NAD^+ reduction is higher than those around 2 mol ATP per mole obtained by other calculations based on growth yields (Timmer-ten Hoor 1976, 1981; Kelly *et al.* 1977; Kelly 1978) or direct estimations (Aleem 1975; Saxena & Aleem 1972) and exceeds that expected from the so-called reversal of normal electron flow, which would require ine ATP to be consumed at each of the 'coupling sites' associated with cytochromes $c \longrightarrow b$ and Q/flavin $\longrightarrow NAD^+$. There is some thermodynamic justification for the true ATP requirement to be greater than 2 mol per mole of NAD^+. This may be seen from the following analysis. The standard electrode potential $E^{\circ\prime}$ for cytochrome *c* varies from 0.254 V for heart cytochrome (Lehninger 1975) and 0.27 V for cytochrome c_{522} of *T. denitrificans* (Aubert *et al.* 1958) to lower values reported for other thiobacilli of 0.247 V for cytochrome c_{522} of *T. thiooxidans* (Takakuwa 1975), 0.210 V for cytochrome $c_{553.5}$ and 0.200 V for cytochrome c_{550} of *T. neapolitanus* (Trudinger 1961), 0.140–0.150 V for cytochrome c_{551} of *T. thioparus* (Skarzynski *et al.* 1956) and about 0.200 V for cytochrome c_{552} of *Thiobacillus* A2 (Kula *et al.* 1975). The oxidation of NADH ($E^{\circ\prime} = -0.32$ V) by oxygen ($E^{\circ\prime} = +0.81$ V (Schlegel 1981)) gives a potential difference of 1.13 V and a standard free energy change ($\Delta G^{\circ\prime}$) of 218 kJ mol^{-1} (Schlegel 1981) or 192.9 kJ mol^{-1} per volt. From the standard $\Delta G^{\circ\prime}$ for ATP of 31 kJ mol^{-1}, the efficiency of energy conservation in *producing* 3 ATP from NADH oxidation is only 42.7 %. Taking the ΔG for ATP formation at pH 7.0, under probable physiological conditions, as 43.9 kJ mol^{-1} (as calculated by Thauer *et al.* (1977)) this efficiency of conservation would be 60.4 %. Conversely the potential difference to be bridged in transferring electrons from *Thiobacillus* A2 cytochrome c_{552} to NAD^+ is 0.52 V, equivalent to 100.3 kJ mol^{-1}. For this energy-dependent reduction, the apparent thermodynamic requirement if all this free energy were provided at 100 % efficiency by ATP would thus be 100.3/31.0 or 100.3/43.9 mol ATP (from the two quoted ΔG values for ATP), i.e.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

3.2 or 2.3 mol ATP per mole of NAD^+. Actual requirements of 4.5–5.3 mol ATP would reflect thermodynamic efficiencies in the range 43–51 % (physiological ΔG for ATP) and 61–72 % (standard $\Delta G^{\circ\prime}$ for ATP), or values similar to the efficiency seen in the *formation* of ATP during energy-yielding electron transport. Saxena & Aleem (1972) reported an experimentally determined ATP requirement for 2.55 mol ATP per mole of NAD^+ reduced in cell-free extracts of *T. neapolitanus*. They stated that this reflected an overall energetic efficiency of about 90 %, in agreement with my calculation above. Indeed, if NAD^+ reduction requires a reversal of a chemiosmotic proton-translocating energy-conserving process, reversal should indeed need only 2 mol ATP. It is unclear, however, whether the experiment of Saxena & Aleem (1972) proves this point. Their extracts (15 mg protein per assay) contained adenylate kinase capable of forming ATP at a rate of 10.8 nmol min^{-1}. The time scale for producing 236 nmol NADH during the disappearance of 610 nmol ATP was 20–26 min (from tables 1 and 2 and fig. 2 of Saxena & Aleem 1972). Adenylate kinase could have produced a minimum of 216–281 nmol ATP in that time if sufficient ADP had been available. Since NAD^+ reduction (*ca.* 9 nmol min^{-1}) and adenylate kinase (10.8 nmol min^{-1}) had comparable activity, and the latter's requirements for 21.6 nmol ATP min^{-1} could be met by the production of 23 or 45 nmol ADP min^{-1} from NAD^+ reduction (assuming 2.55 or 5 nmol ATP per mole of NAD^+ reduced), considerable resynthesis of ATP could have occurred. In effect their estimate of '2.5 ATP equivalents per equivalent of NADH' must be the lowest possible estimate and their own data could in fact be consistent with an actual use of 5 ATP per NAD^+.

It is clear that energy calculations based on growth yields are fraught with the danger of numerous errors: no account has been taken of the possible energy cost of ion transport (including that of substrate thiosulphate and nitrate for entry into *T. denitrificans*) or of ammonia assimilation. Similarly, the energy requirement for biosynthesis from hexose could be greater that that calculated from the data of Stouthamer (1973). The estimates are of value in that they indicate upper and lower limits for energy production and consumption and provide a framework for further biochemical investigation.

Some further considerations of energy relations in T. denitrificans and the aerobic thiobacilli

Somewhat different calculation bases from those of Kelly (1978), Kelly *et al.* (1977) and Justin & Kelly (1978) were taken in the studies of Timmer-ten Hoor (1976, 1981). Anje Timmer's approach was essentially to deduce Y_{ATP} values and growth yield from thiosulphate used for energy. This led her (Timmer-ten Hoor 1976, 1981) to deduce that anaerobically or aerobically *T. denitrificans* produced 7.61 or 10.73 mol ATP per mole of thiosulphate respectively, equivalent to 1.68 and 2.68 ATP per $2e^-$ transported to nitrate or oxygen. She further calculated that oxidative phosphorylation could contribute 1.18 ATP per $2e^-$ anaerobically and 2.18 aerobically (Timmer-ten Hoor 1981). The implication of these figures is that anaerobically only one ATP coupling site occurs during electron transport (i.e. one proton translocating loop), whereas two occur aerobically. This would negate the view (e.g. figure 10) that anaerobic electron transport from cytochrome *c* to oxidized nitrogen acceptors could be energy-coupled. This hypothesis creates a further paradox; if the difference between aerobic and anaerobic growth yield of *T. denitrificans* is due largely to there being only one anerobic coupling site, the aerobic thiobacilli might be expected to give growth yields comparable with those of anaerobic *T. denitrificans*. In fact its $Y^{max}_{S_2O_3}$ is 10–11.4 g mol^{-1} (Justin & Kelly 1978; Timmer-ten Hoor 1981)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

in contrast to about 6.7 for the aerobes (table 3). The anerobic $Y^{max}_{S_2O_3}$ for *Thiomicrospira denitrificans* at 5.13 is closer but is low. This organism also appears to give better aerobic than anaerobic yields ($Y^{max}_{S_2O_3}$ = 7.74 compared with 5.15 (Timmer-ten Hoor 1981)).

Several possibilities present themselves to help explain differences among these yield values.

(*a*) P/2e$^-$ values of 1.0 or 2.0 or even both for different electron pairs generated during thiosulphate oxidation in different strains.

(*b*) The presence or absence of substrate-level phosphorylation by the APS pathway.

(*c*) Differences in ATP generation dependent on whether ADP or ATP sulphurylase function in the APS pathway.

(*d*) The possibility that at least some sulphane-sulphur oxidation occurs by the non-energy-conserving sulphur oxygenase in the aerobes.

(*e*) The difference in ATP demand for NAD^+ reduction dependent on whether electrons enter the chain at flavin or cytochrome *c*.

(*f*) Decreased efficiency of CO_2 fixation in the aerobes because of the oxygenase activity of the CO_2-fixing enzyme, ribulose bisphosphate carboxylase. This would not affect anaerobic *T. denitrificans*.

For *T. ferrooxidans* growing on tetrathionate or thiosulphate (Eccleston & Kelly 1978), we assumed the operation of a sulphur oxygenase and an ATP requirement of 2 mol per mole of NAD^+ reduced. This led to estimates of 3 ATP and 5 ATP formed respectively per mole of thiosulphate and tetrathionate oxidized for energetic purposes; indicating P/O ratios of 0.75 and 0.71 respectively. These estimates are probably low but await proof of energy-coupling during sulphane-sulphur oxidation.

Some guidance is given by comparing the amount of thiosulphate used for energy (i.e. ATP production) in the different species. This can be deduced from CO_2 fixed for growth. The elemental composition of all the strains studied is similar. Thus the carbon content values (as a percentage of dry mass) are: *T. denitrificans*, 47.40 ± 1.02 (Justin & Kelly 1978) and 48.3 (Timmer-ten Hoor 1976); *T. ferrooxidans*, 48.72 ± 1.07 (Eccleston & Kelly 1978) and 47.8 (Jones & Kelly, unpublished); *Thiobacillus* A2, 45.2 ± 1.1 (Smith *et al.* 1980; Lu & Kelly, unpublished); *T. neapolitanus*, 47.50 (Tuovinen & Kelly 1973; Smith & Kelly, unpublished); *Thiomicrospira denitrificans*, 44.6 (Timmer-ten Hoor 1981). These give an overall mean carbon content of 47.1 %. Using this figure we can compare growth yields (as CO_2 fixed) for the following: mean $Y^{max}_{S_2O_3}$ for aerobic thiobacilli (table 3), 6.7; anaerobic *T. denitrificans* (mean value of data from nitrate- or thiosulphate-limited cultures (Justin & Kelly 1978; Timmer-ten Hoor 1981)), 10.6; and aerobic *T. denitrificans* (Justin & Kelly 1978), 14.7. The approximate quantities of thiosulphate oxidized via the electron transport chain were calculated on the assumption that 4[H] (i.e. 2 NADH) are required to reduce one CO_2 to the level of cell carbon (table 6). This may be a slight underestimate as Timmer-ten Hoor (1976) estimated a requirement for 4.3 mol of electrons per mole of CO_2, but this does not materially effect the comparison.

Using the $Y^{max}_{S_2O_3\,(energy)}$ values, one can calculate a Y_{ATP} value for the aerobes from Timmer-ten Hoor's estimates of 6.71 and 10.73 ATP per mole of thiosulphate for aerobic and anaerobic *T. denitrificans*, assuming all other factors to be equal. Thus the respective Y_{ATP} values are 1.99 and 1.93 for *T. denitrificans*: a mean of 1.96, which if applied to the aerobes would indicate that 3.93 ATP were produced per thiosulphate oxidized to produce energy: a good agreement with the value of about 4 shown for *Thiobacillus* A2 *in vitro*. This is obviously only a first approximation

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

since the higher growth yields in aerobic and anerobic *T. denitrificans* than for any of the aerobic thiobacilli could be due to the existence of a P/O of 2.0 in the former and of APS-dependent substrate-level phosphorylation in both, but a P/O of only unity and perhaps no substrate-level ATP synthesis in the aerobes. If electrons entered the electron transport chain no lower than the cytochrome *b* level in both anerobic and aerobic *T. denitrificans*, but only a cytochrome *c* in aerobes, the energy demand for NAD^+ reduction would be twice as great in the latter. Comparing aerobic *T. denitrificans* and the other aerobes, 10.73 mol ATP produced by the former from 1 mol thiosulphate supports the growth of 20.68 g or the fixation of 0.812 mol CO_2,

TABLE 6. PROPORTIONS OF HYDROGEN EQUIVALENTS ([H]) FROM THIOSULPHATE OXIDATION USED FOR CO_2 FIXATION AND ELECTRON TRANSPORT IN SEVERAL THIOBACILLI

organism	$Y^{max}_{S_2O_3}$†	CO_2 fixed to give $Y^{max}_{S_2O_3}$ mol	[H] available from $S_2O_3^{2-}$ oxidation	[H] required for observed CO_2 fixation	[H] available for electron transport	thiosulphate oxidized‡ mol	$Y^{max}_{S_2O_3}$ (energy)§
aerobic thiobacilli	6.7‖	0.263	8.0	1.05	6.95	0.869	7.71
anaerobic *T. denitrificans*	10.6¶	0.416	8.0	1.66	6.34	0.793	13.33
aerobic *T. denitrificans*	14.7††	0.577	8.0	2.31	5.69	0.711	20.68

† Calculated as grams per mole of $S_2O_3^{2-}$ consumed for growth.
‡ For energetic purposes, per mole consumed.
§ Calculated as grams per mole of $S_2O_3^{2-}$ oxidized for energetic purposes.
‖ Mean value from table 3.
¶ Mean data of Justin & Kelly (1978) and Timmer-ten Hoor (1981)
†† Justin & Kelly (1978).

requiring the reduction of 1.62 mol NAD^+. If the ATP requirement for this were only 1 mol ATP per mole, or a total of 1.62 ATP, the ATP used for other growth-related processes would become 9.11, or a Y'_{ATP} of 2.27. If this value applied to the aerobes, in which NAD^+ reduction might require 2 mol ATP per mole, the $Y^{max}_{S_2O_3\,(energy)}/Y'_{ATP}$ indicates an ATP production of 3.40 mol plus that required for NAD^+ reduction. This would be 1.22 mol ATP (to reduce 0.61 mol NAD^+ for 0.30 mol CO_2 fixed), giving a total of 4.62 mol ATP per mole of thiosulphate. Such a calculation would be consistent with a P/O of 1.0 plus ATP synthesis from APS via ADP sulphurylase. Table 7 illustrates the maximum ATP production expected from thiosulphate oxidation by using the energy-conserving reactions so far demonstrated or deduced. Timmer-ten Hoor's estimates exceed these theoretical figures for *T. denitrificans*. This can be explained because thiosulphate oxidized to generate [H] for NAD^+ reduction produces sulphite that can still pass through the energy-conserving APS pathway. Thus anaerobically (an additional 0.26 mol $S_2O_3^{2-}$ giving ATP) and aerobically (an additional 0.41 mol ATP) a further 0.52 and 0.82 mol ATP could be available from ATP sulphurylase, giving a total ATP production (compared with Timmer-ten-Hoor's estimates) of 6.52 (6.71) and 10.82 (10.73) mol. The most likely alternative mechanisms of energy generation from thiosulphate among the thiobacilli are summarized in figure 15. This scheme would probably apply equally to sulphide oxidation. A further possibility, not otherwise considered, would be that aerobic thiosulphate (or sulphur) oxidation could be catalysed by a non-energy-linked suphur oxygenase (P/O = 0) to sulphite, the oxidation of which could be coupled at cytochrome *b* or higher (P/O = 2.0).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 7. ATP PRODUCTION FROM THIOSULPHATE IN SEVERAL HYPOTHETICAL MODEL SYSTEMS

(Moles of ATP produced per mole of thiosulphate oxidized via energy-yielding electron transport.)

hypothetical model system	P/O = 1.0	P/O = 2.0	APS pathway using: ADP sulphurylase	APS pathway using: ATP sulphurylase	total ATP produced	most similar experimental observation
A	4.0	0	0	0	4.0	aerobic thiobacilli
B	4.0	0	1.0	—	5.0	
C	4.0	0	0	2.0	6.0	anaerobic *T. denitrificans*
D	—	8.0	1.0	—	9.0	—
E	—	8.0	—	2.0	10.0	aerobic *T. denitrificans*

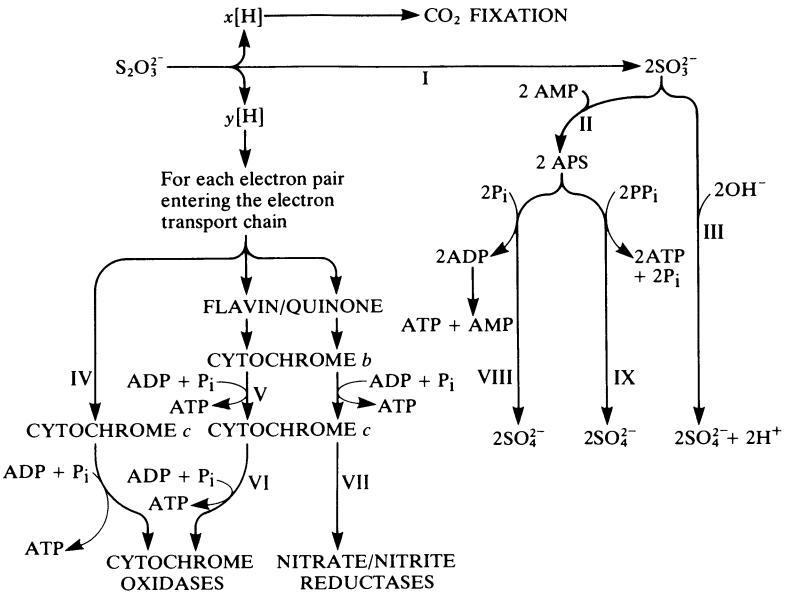


FIGURE 15. The principal energetic alternatives hypothetically available to thiobacilli oxidizing thiosulphate. (I) Oxidation to sulphite by 'reversed sulphite reductase' after scission; (II) APS reductase; (III) sulphite: cytochrome *c* oxidoreductase; (IV) the probable route in *Thiobacillus* A2 and *T. neapolitanus*; (V) 'coupling site II' apparently operating in aerobic and anaerobic *T. denitrificans*; (VI) 'coupling site III' operating in aerobic *T. denitrificans* (and other aerobic thiobacilli); (VII) no energy conservation in electron transport from cytochrome *c* to nitrate–nitrite reductases: this is deduced from growth yields and awaits biochemical testing.

This would give the same ATP synthesis as model A (table 7) but it is an unlikely combination for the organisms studied so far. A $Y^{max}_{S_2O_3}$ value for *T. novellus* does not seem yet to be available, but this might fit the alternative hypothesis if it has a low Y^{max} (i.e. 6–7), since Cole & Aleem (1970, 1973) showed P/O ratios in cell-free extracts of 0.9 for thiosulphate and 1.4 for sulphide, with sulphide-dependent ATP-synthesis being blocked by amytal, rotenone, antimycin A and HQNO, suggesting electron flow and phosphorlation at the level of cytochrome *b*. This is further supported by the apparent need for only 1.3 ATP per NAD^+ reduced by *T. novellus*, consistent with a reversal of electron flow only through one ATP-coupling loop (Aleem 1966)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The use of theoretical calculations like those above, although based on a now large base of biochemical understanding and reasonable experimental data, has probably been pushed to the limit currently possible. The need now is for some more precise biochemical proof.

The future

> The reactions about which least are known are those effecting thiosulphate cleavage, and those converting sulphur to sulphite. (Kelly 1972)

A decade later these words still have truth, though the nature of the processes most likely to operate have been established and are in need of direct experimental verification and demonstration *in vivo*. The problems of inorganic sulphur oxidation and association energy-conservation processes are far from fully solved, as I hope I have illustrated. Three aspects of the sulphur pathway in which more work is required are (*a*) the exact nature of the primary reactions of thiosulphate (or sulphide or sulphur) (*b*) the demonstration of enzymic production of sulphite from sulphur by a system other than the sulphur oxygenase, and the (*c*) elucidation of the enzymology of polythionate degradation. It seems certain that the initial reaction of thiosulphate is scission by an enzyme such as rhodanese to produce sulphite and an enzyme-bound sulphur species. This is essentially a reductive step, since one either postulates a thiosulphate reductase, producing sulphide and sulphite (and hence requiring 2 mol e^- per mole of thiosulphate cleaved), or rhodanese functioning with a reduced acceptor molecule, such as reduced lipoic acid (Silver & Kelly 1976). The $E^{\circ\prime}$ for the thiosulphate/sulphide + sulphite couple is so electronegative (-0.4 V; table 1) that the energy cost of the process to the organism would seem to be potentially very great. Similarly the generation of reduced lipoate for rhodanese (using one dihydrolipoate for each thiosulphate cleaved) is an energy-demanding process in that 1 mol NADH would be required per mole of lipoate reduced by lipoate dehydrogenase. Simply generating reduced lipoate could thus 'cost' a minimum of 1 or 2 mol ATP per mole of thiosulphate used. The observation that growth yields on sulphide and thiosulphate are very similar (Timmer-ten Hoor 1981) argues that there can be no such vast energy burden in the initial metabolism of thiosulphate. Clearly resolution of this problem is of urgent interest.

To conclude, the best working hypothesis on which to hang a mechanism for the chemolithotrophic oxidation of inorganic sulphur, based on the data available in February 1982, should include the following premises.

(*a*) There is a fundamentally common oxidation mechanism operating in all the thiobacilli and metabolically similar chemolithotrophs.

(*b*) Such variations as occur, or appear to occur, in the sulphur oxidation pathway are due to differences in activity or detail of function (e.g. K_m, V_{max}, enzyme concentration) of essentially common enzyme systems.

(*c*) Sulphite may be oxidized by either or both of AMP-dependent or independent routes, the former terminating with ADP- or ATP-sulphurylase.

(*d*) There are major differences in electron transport mechanisms, both in the point at which electrons enter the chain and in the terminal oxidase systems.

(*e*) Polythionates arise from enzyme-bound intermediates such as polysulphur chains, sulphite reductase-oxidase, or from 'activated thiosulphate'. They are mobilized only after scission reactions, and are not necessarily substrates or products of all thiobacilli.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(*f*) Inorganic sulphur oxidation in the intact cells is a highly integrated process, requiring a close association of oxidative enzymes, the membrane-bound electron transport system and associated terminal oxidases or reductases.

There is still much to be done to 'close the book' on the study of sulphur oxidation in thiobacilli. Until that time, let us recall Milton's (1667) words, supposedly describing Hell, that must apply to those laboratories continuing to wrestle with the impact of thiobacilli on the world of organic and inorganic sulphur chemistry:

> Regions of sorrow, doleful shades, where peace
> And rest can never dwell; but torture without end
> Still urges, and a fiery Deluge, fed
> With ever-burning Sulphur unconsumed:
> Such place Eternal Justice had prepar'd...

I am indebted to former and present collaborators and to many other colleagues for discussions extending over 20 years that have helped shape my views in formulating this paper. In particular I thank the following for stimulating ideas, or for letting me see or use experimental results before publication: Harry Peck, Phil Trudinger, Isamu Suzuki, Ron Lyric, Hans Trüper, Gijs Kuenen, Anje Timmer-ten Hoor, John Pirt, Hussain Aleem, David Moriarty Colin Jones, Roger Whittenbury and the late Wolf Vishniac. I am particularly indebted to Wei-Ping Lu from whose unpublished results I have quoted so freely and to Ann Wood for constructive comment on much of the manuscript. Some of the original work reported was supported through research grants or awards by the S.E.R.C., N.E.R.C., the British Council and the U.K. Ministry of Defence.

References

Adams, C. A., Warnes, G. M. & Nicholas, D. J. D. 1971 A sulphite-dependent nitrate reductase from *Thiobacillus denitrificans*. *Biochim. biophys. Acta* **235**, 398–406.

Adair, F. W. 1966 Membrane-associated sulfur oxidation by autotroph *Thiobacillus thiooxidans*. *J. Bact.* **92**, 899–904.

Aleem, M. I. H. 1965 Thiosulfate oxidation and electron transport in *Thiobacillus novellus*. *J. Bact.* **90**, 95–101.

Aleem, M. I. H. 1966 Generation of reducing power in chemosynthesis. III. Energy-linked reduction of pyridine nucleotides in *Thiobacillus novellus*. *J. Bact.* **91**, 729–736.

Aleem, M. I. H. 1969 Generation of reducing power in chemosynthesis. VI. Energy-linked reactions in the chemoautotroph, *Thiobacillus neapolitanus*. *Antonie van Leeuwenhoek* **35**, 379–391.

Aleem, M. I. H. 1975 Biochemical reaction mechanisms in sulfur oxidation by chemosynthetic bacteria. *Pl. Soil* **43**, 587–607.

Aleem, M. I. H., Lees, H. & Nicholas, M. I. H. 1963 Adenosine triphosphate-dependent reduction of nicotinamide adenine dinucleotide by ferro-cytochrome *c* in chemoautotrophic bacteria. *Nature, Lond.* **200**, 759–761.

Aminuddin, M. 1980 Substrate-level versus oxidative phosphorylation in the generation of ATP in *Thiobacillus denitrificans*. *Arch. Microbiol.* **128**, 19–25.

Aminuddin, M. & Nicholas, D. J. D. 1973 Sulphide-linked oxidation linked to the reduction of nitrate and nitrite in *Thiobacillus denitrificans*. *Biochim. biophys. Acta* **325**, 81–93.

Aminuddin, M. & Nicholas, D. J. D. 1974*a* Electron transfer during sulphide and sulphite oxidation in *Thiobacillus denitrificans*. *J. gen. Microbiol.* **82**, 115–123.

Aminuddin, M. & Nicholas, D. J. D. 1974*b* An AMP-dependent sulphite oxidase from *Thiobacillus denitrificans*: purification and properties. *J. gen. Microbiol.* **82**, 103–113.

Anon. 1982 Computer-file search of *Biological Abstracts*, using key word: *Thiobacillus* (University of Warwick Library).

Aubert, P. J., Milhaud, G., Moncel, C. & Millet, J. 1958 Existence, isolement et propriétés physico-chimiques d'un cytochrome *c* de *Thiobacillus denitrificans*. *C.r. hebd. Séanc. Acad. Sci., Paris* **246**, 1616–1619.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Bowen, T. J., Happold, F. C. & Taylor, B. F. 1966 Studies on adenosine 5′-phosphosulfate reductase from *Thiobacillus denitrificans*. *Biochim. biophys. Acta* **118**, 566–576.
Burns, G. R. 1967 *The Sulphur Institute: Technical Bulletin* No. 13. Washington and London.
Charles, A. M. & Suzuki, I. 1965 Sulfite oxidase of a facultative autotroph, *Thiobacillus novellus*. *Biochem. biophys. Res. Commun.* **19**, 686–690.
Charles, A. M. & Suzuki, I. 1966*a* Mechanism of thiosulfate oxidation by *Thiobacillu. novellus*. *Biochim. biophys. Acta* **128**, 510–521.
Charles, A. M. & Suzuki, I. 1966*b* Purification and properties of sulfite: cytochrome *c* oxidoreductase from *Thiobacillus novellus*. *Biochim. biophys. Acta* **128**, 522–534
Chambers, L. A. & Trudinger, P. A. 1975 Are thiosulfate and trithionate intermediates in dissimilatory sulphate reduction? *J. Bact.* **123**, 36–40.
Cohen, Y., de Jonge, I. & Kuenen, J. G. 1979 Excretion of glycolate by *Thiobacillus neapolitanus* grown in continuous culture. *Arch. Microbiol.* **122**, 189–194.
Cole, J. S. & Aleem, M. I. H. 1970 Oxidative phosphorylation in *Thiobacillus novellus*. *Biochem. biophys. Res. Commun.* **38**, 736–743.
Cole, J. S. & Aleem, M. I. H. 1973 Electron-transport-linked compared with proton-induced ATP generation in *Thiobacillus novellus*. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3571–3575.
Drozd, J. W. 1974 Respiration-driven proton translocation in *Thiobacillus neapolitanus* C. *FEBS Lett.* **49**, 103–105.
Eccleston, M. & Kelly, D. P. 1978 Oxidation kinetics and chemostat growth kinetics of *Thiobacillus ferrooxidans* on tetrathionate and thiosulfate. *J. Bact.* **134**, 718–727.
Gundersen, K. 1968 The formation and utilization of reducing power in aerobic chemoautotrophic bacteria. *Z. allg. Mikrobiol.* **8**, 445–457.
Hempfling, W. P. & Vishniac, W. 1967 Yield coefficients of *Thiobacillus neapolitanus* in continuous culture. *J. Bact.* **93**, 874–878.
Hempfling, W. P., Trudinger, P. A. & Vishniac, W. 1967 Purification and some properties of sulfite oxidase from *Thiobacillus neapolitanus*. *Arch. Mikrobiol.* **59**, 149–157.
Imai, K., Okuzumi, M., Katagiri, H. 1962 The pathways of sulfur oxidation on *Thiobacillus thiooxidans*. [In Japanese.] *Koso Kagaku Shimpoziumu* **17**, 132–143.
Ishaque, M. & Aleem, M. I. H. 1973 Intermediates of denitrification in the chemoautotroph *Thiobacillus denitrificans*. *Arch. Mikrobiol.* **94**, 269–282.
Justin, P. & Kelly, D. P. 1978*a* Growth kinetics of *Thiobacillus denitrificans* in anaerobic and aerobic chemostat culture. *J. gen. Microbiol.* **107**, 123–130.
Justin, P. & Kelly, D. P. 1978*b* Metabolic changes in *Thiobacillus denitrificans* accompanying the transition from aerobic to anaerobic growth. *J. gen. Microbiol.* **107**, 131–137.
Kelly, D. P. 1966 Fluoroacetate toxicity in *Thiobacillus neapolitanus* and its relevance to the problem of obligate chemoautotrophy. *Arch. Mikrobiol.* **61**, 59–76.
Kelly, D. P. 1968 Biochemistry of oxidation of inorganic sulphur compounds by microorganisms. *Aust. J. Sci.* **31**, 165–173.
Kelly, D. P. 1972 Transformations of sulphur and its compounds in soils. In *Symposium international sur le Soufre en agriculture 1970* (*Annls agron.*, numéro hors série), pp. 217–232.
Kelly, D. P. 1973 Inorganic sulphur metabolism in *Thiobacillus* A2. In *1st International Congress of Bacteriology, Abstracts II*, p. 71. Jerusalem: International Association of Microbiological Societies.
Kelly, D. P. 1978 Bioenergetics of chemolithotrophic bacteria. In *Companion to microbiology*, (ed. A. T. Bull & P. M. Meadow), pp. 363–386. London: Longman.
Kelly, D. P. 1981 Introduction to the chemolithotrophic bacteria. In *The prokaryotes* (ed. M. P. Starr, H. Stolp, H. G. Trüper, A. Balows & H. G. Schlegel), vol. 1, pp. 997–1004. Berlin: Springer-Verlag.
Kelly, D. P. & Syrett, P. J. 1963 Effect of 2:4-dinitrophenol on carbon dioxide fixation by a *Thiobacillus*. *Nature, Lond.* **197**, 1087–1089.
Kelly, D. P. & Syrett, P. J. 1964 The effect of uncoupling agents on carbon dioxide fixation by a *Thiobacillus*. *J. gen. Microbiol.* **34**, 307–317.
Kelly, D. P. & Syrett, P. J. 1966*a* Energy coupling during sulphur compound oxidation by *Thiobacillus* sp. strain C. *J. gen. Microbiol.* **43**, 109–118.
Kelly, D. P. & Syrett, P. J. 1966*b* ^{35}S Thiosulphate oxidation by *Thiobacillus* strain C. *Biochem. J.* **98**, 537–545.
Kelly, D. P. & Tuovinen, O. H. 1975 Metabolism of inorganic sulphur compounds by Thiobacillus ferrooxidans and some comparative studies on *Thiobacillus* A2 and *T. neapolitanus*. *Pl. Soil* **43**, 77–93.
Kelly, D. P., Eccleston, M. & Jones, C. A. 1977 Evaluation of continuous chemostat cultivation of *Thiobacillus ferrooxidans* on ferrous-iron or tetrathionate. In *Conference – Bacterial Leaching* (ed. W. Schwartz), pp. 1–7. Weinheim: Verlag Chemie.
Kluyver, A. J. & Donker, H. J. L. 1926 Die Einheit in der Biochemie. *Chemie Zelle Gewebe* **13**, 134–190.
Kobayashi, K., Seti, Y. & Ishimoto, M. 1974 Biochemical studies on sulfate-reducing bacteria. XIII. Sulfite reductase from *Desulfovibrio vulgaris* – mechanism of trithionate, thiosulfate and sulfide formation and enzymic properties. *J. Biochem., Tokyo* **75**, 519–529.
Kuenen, J. G. 1979 Growth yields and 'maintenance energy requirement' in *Thiobacillus* species under energy limitation. *Arch. Microbiol.* **122**, 183–188.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Kula, T., Aleem, M. I. H. & Wilson, D. 1975 Oxidation-reduction potentials of the cytochromes of *Thiobacillus* A2. In *Abstracts, Am. Soc. Microbiol. Annual Meeting 1975*, p. 136.

Latimer, W. M. 1952 *The oxidation states of the elements and their potentials in aqueous solutions*. Englewood Cliffs: Prentice-Hall.

Lee, J. P., LeGall, J. & Peck, H. D. 1973 Isolation of assimilatory and dissimilatory sulfite reductases from *Desulfovibrio vulgaris*. *J. Bact.* **151** 529–542.

Lehninger, A. L. 1975 *Biochemistry*. New York: Worth.

London, J. & Rittenberg, S. C. 1964 Path of sulfur in sulfide and thiosulfate oxidation by thiobacilli. *Proc. natn. Acad. Sci. U.S.A.* **52**, 1183–1190.

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

Lyric, R. M. & Suzuki, I. 1970*b* Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. II. Properties of adenosine-5′-phosphosulfate reductase. *Can. J. Biochem.* **48**, 344–354.

Lyric, R. M. & Suzuki, I. 1970*c* Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. III. Properties of thiosulfate oxidation. *Can. J. Biochem.* **48**, 355–363.

Lyric, R. M. & Suzuki, I. 1970*d* Kinetic studies of sulfite:cytochrome *c* oxidoreductase, thiosulfate-oxidizing enzyme, and adenosine-5′-phosphosulfate reductase from *Thiobacillus thioparus*. *Can. J. Biochem.* **48**, 594–603.

Milton, J. 1667 *Paradise Lost*, book I. London: Samuel Symons, printer.

Moriarty, D. J. W. & Nicholas, D. J. D. 1969 Enzymic sulphide oxidation by *Thiobacillus concretivorus*. *Biochim. biophys. Acta* **184**, 114–123.

Moriarty, D. J. W. & Nicholas, D. J. D. 1970 Products of sulphide oxidation in extracts of *Thiobacillus concretivorus*. *Biochim. biophys. Acta* **197**, 143–151.

Nathansohn, A. 1902 Über eine neue Gruppe von Schwefelbacterien und ihren Stoffwechsel. *Mitt. zool. Stn Neapel* **15**, 655–680.

Oh, J. K. & Suzuki, I. 1977*a* Isolation and characterization of a membrane-associated thiosulphate-oxidizing system of *Thiobacillus novellus*. *J. gen. Microbiol.* **99**, 397–412.

Oh, J. K. & Suzuki, I. 1977*b* Resolution of a membrane-associated thiosulphate-oxidizing complex of *Thiobacillus novellus* *J. gen. Microbiol.* **99**, 413–423.

Okuzumi, M. 1965 Studies on biochemistry of the thiobacilli. VII. Metabolism of tetrathionate by *T. thiooxidans*. *Agric. biol. Chem.* **29**, 1069–1073.

Okuzumi, M. 1966*a* Studies on biochemistry of thiobacilli. VIII. Dismutation of tetrathionate by *Thiobacillus thiooxidans*. *Agric. biol. Chem.* **30**, 313–318.

Okuzumi, M. 1966*b* Studies on biochemistry of the thiobacilli. IX. Reduction of trithionate by *Thiobacillus thiooxidans*. *Agric biol. Chem.* **30**, 713–716.

Okuzumi, M. & Kita, Y. 1965 Studies on biochemistry of the thiobacilli. VI. Oxidation of thiosulfate to tetrathionate by *T. thiooxidans*. *Agric. biol. Chem.* **29**, 1063–1068.

Peck, H. D. 1960 Adenosine 5′-phosphosulfate as an intermediate in the oxidation of thiosulfate by *Thiobacillus thioparus*. *Proc. natn. Acad. Sci. U.S.A.* **46**, 1053–1057.

Peck, H. D. 1962 Symposium on the metabolism of inorganic compounds. V. Comparative metabolism of inorganic sulfur compounds in microorganisms. *Bact. Rev.* **26**, 67–94.

Peck, H. D. 1968 Energy-coupling mechanisms in chemolithotrophic bacteria. *A. Rev. Microbiol.* **22**, 489–518.

Peck, H. D. & Fisher, E. 1962 The oxidation of thiosulfate and phosphorylation in extracts of *Thiobacillus thioparus*. *J. biol. Chem.* **237**, 190–197.

Peck, H. D. & Stulberg, M. P. 1962 O^{18} studies on the mechanism of sulfate formation and phosphorylation in extracts of *Thiobacillus thioparus*. *J. biol. Chem.* **237**, 1648–1652.

Peck, H. D., Deacon, T. E. & Davidson, J. T. 1965 Studies on adenosine 5′-phosphosulfate reductase from *Desulfovibrio desulfuricans* and *Thiobacillus thioparus*. *Biochim. biophys. Acta* **96**, 429–446.

Peeters, T. & Aleem, M. I. H. 1970 Oxidation of sulfur compounds and electron transport in *Thiobacillus denitrificans*. *Arch. Mikrobiol.* **71**, 319–330.

Pirt, S. J. 1965 The maintenance energy of bacteria in growing cultures. *Proc. R. Soc. Lond.* **B163**, 224–231.

Pirt, S. J. 1975 *Principles of microbe and cell cultivation*. Oxford: Blackwell.

Ross, A. J., Schoenhoff, R. L. & Aleem, M. I. H. 1968. Electron transport and coupled phosphorylation in the chemoautotroph *Triobacillus neapolitanus*. *Biochem. biophys. Res. Commun.* **32**, 301–306.

Roth, C. W., Hempfling, W. P., Conners, J. N. & Vishniac, W. V. 1973 Thiosulfate- and sulfide-dependent pyridine nucleotide reduction and gluconeogenesis in intact *Thiobacillus neapolitanus*. *J. Bact.* **114**, 592–599.

Roy, A. B. & Trudinger, P. A. 1970 *The biochemistry of inorganic compounds of sulphur*. Cambridge University Press.

Santer, M. 1959 The role of ^{18}O-phosphate in thiosulfate oxidation by *Thiobacillus thioparus*. *Biochem. biophys. Res. Commun.* **1**, 9–12.

Sawhney, V. & Nicholas, D. J. D. 1977*a* Sulphite- and NADH-dependent nitrate reductase from *Thiobacillus denitrificans*. *J. gen. Microbiol.* **100**, 49–58.

Sawhney, V. & Nicholas, D. J. D. 1977*b* Sulphide-linked nitrite reductase from *Thiobacillus denitrificans* with cytochrome oxidase activity: purification and properties. *J. gen. Microbiol.* **106**, 119–128.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Saxena, J. & Aleem, M. I. H. 1972 Generation of reducing power in chemosynthesis. VII. Mechanism of pyridine nucleotide reduction by thiosulfate in the chemoautotroph *Thiobacillus neapolitanus*. *Arch. Microbiol.* **84**, 317–326.

Schedel, M. & Trüper, H. G. 1979 Purification of *Thiobacillus denitrificans* siroheme sulfite reductase and investigation of some molecular and catalytic properties. *Biochim. biophys. Acta* **568**, 454–467.

Schedel, M. & Trüper, H. G. 1980 Anaerobic oxidation of thiosulfate and elemental sulfur in *Thiobacillus denitrificans*. *Arch. Microbiol.* **124**, 205–210.

Schlegel, H. G. 1975 Mechanisms of chemo-autotrophy. In *Marine ecology* (ed. O. Kinne), vol 2, pt 1, pp. 9–60. London: Wiley.

Schlegel, H. G. 1981 *Allgemeine Mikrobiologie*. Stuttgart: Thieme.

Silver, M. & Kelly, D. P. 1976 Rhodanese from *Thiobacillus* A2: catalysis of reactions of thiosulphate with dihydrolipoate and dihydrolipoamide. *J. gen. Microbiol.* **97**, 277–284.

Silver, M. & Lundgren, D. G. 1968*a* The thiosulfate-oxidizing enzyme of *Thiobacillus ferrooxidans*. *Can. J. Biochem.* **46**, 1215–1220.

Silver, M. & Lundgren, D. G. 1968*b* Sulfur-oxidizing enzyme of *Thiobacillus ferrooxidans*. *Can. J. Biochem.* **46**, 457–461.

Skarżyński, B., Klimek, R. Szczepkowski, T. W. 1956 Cytochrome in *Thiobacillus thioparus*. *Bull. Acad. pol. Sci. cl. II Sér. Sci. biol.* **4**, 299–304.

Skarżyński, B., Ostrowski, W. & Krawczyk, A. 1957 *Bull. Acad. pol. Sci. cl. II Ser. Sci. biol.* **5**, 159–164.

Smith, A. L., Kelly, D. P. & Wood, A. P. 1980 Metabolism of *Thiobacillus* A2 grown under autotrophic, mixotrophic and heterotrophic conditions in chemostat culture. *J. gen. Microbiol.* **121**, 127–138.

Stouthamer, A. H. 1973 A theoretical study on the amount of ATP required for synthesis of microbial cell material. *Antonie van Leeuwenhoek* **39**, 545–565.

Suzuki, I. 1965*a* Oxidation of elemental sulfur by an enzyme system of *Thiobacillus thiooxidans*. *Biochim. biophys. Acta* **104**, 359–371.

Suzuki, I. 1975*b* Incorporation of atmospheric oxygen-18 into thiosulfate by the sulfur-oxidizing enzyme of *Thiobacillus thiooxidans*. *Biochim. biophys. Acta* **110**, 97–101.

Suzuki, I. & Silver, M. 1966 The initial product and properties of the sulfur-oxidizing enzyme of thiobacilli. *Biochim. biophys. Acta*. **122**, 22–33.

Takakuwa, S. 1975*a* Studies on the metabolism of a sulfur-oxidizing bacterium. VII. Purification and characterisation of soluble components indispensable for sulfur oxidation by *Thiobacillus thiooxidans*. *Pl. Cell Physiol.* **16**, 1027–1035.

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

Tamiya, H., Haga, K. & Huzisige, H. 1941 Zur Physiologie der chemoautotrophen Schwefelbakterien. I. *Acta phytochim., Tokyo* **12**, 173–225.

Taylor, B. F. 1968 Oxidation of elemental sulfur by an enzyme system from *Thiobacillus neapolitanus*. *Biochim. biophys. Acta* **170**, 112–122.

Thauer, R. K., Jungermann, K. & Decker, K. 1977 Energy conservation in chemotrophic anaerobic bacteria. *Bact. Rev.* **41**, 100–180.

Timmer-ten Hoor, A. 1976 Energetic aspects of the metabolism of reduced sulphur compounds in *Thiobacillus denitrificans*. *Antonie van Leeuwenhoek* **42**, 483–492.

Timmer-ten Hoor, A. 1981 Cell yield and bioenergetics of *Thiomicrospira denitrificans* compared with *Thiobacillus denitrificans*. *Antonie van Leeuwenhoek* **47**, 231–243.

Trudinger, P. A. 1961*a* Thiosulphate oxidation and cytochromes in *Thiobacillus* X. I. Fractionation of bacterial extracts and properties of cytochromes. *Biochem. J.* **78**, 673–680.

Trudinger, P. A. 1961*b* Thiosulphate oxidation and cytochromes in *Thiobacillus* X. II. Thiosulphate oxidizing enzyme. *Biochem. J.* **78**, 680–686.

Trudinger, P. A. 1964*a* Evidence for a four-sulphur intermediate in thiosulphate oxidation by *Thiobacillus* X. *Aust. J. Biol. Sci.* **17**, 577–579.

Trudinger, P. A. 1964*b* Oxidation of thiosulphate by intact cells of *Thiobacillus* X: effects of some experimental conditions. *Aust. J. Biol. Sci.* **17**, 738–751.

Trudinger, P. A. 1964*c* Products of anaerobic metabolism of tetrathionate by *Thiobacillus* X. *Aust. J. Biol. Sci.* **17**, 446–458.

Trudinger, P. A. 1964*d* The metabolism of trithionate by *Thiobacillus* X. *Aust. J. Biol. Sci.* **17**, 459–468.

Trudinger, P. A. 1967 The metabolism of inorganic sulphur compounds by thiobacilli. *Rev. pure appl. Chem.* **17**, 1–24.

Tuovinen, O. H. & Kelly, D. P. 1973 Studies on the growth of *Thiobacillus ferrooxidans*. *Arch. Mikrobiol.* **88**, 285–298.

Vishniac, W. 1949 On the metabolism of the chemolithoautotrophic bacterium *Thiobacillus thioparus*. Ph.D. thesis, Stanford University, California.

Vishniac, W. 1952 The metabolism of *Thiobacillus thioparus*. I. The oxidation of thiosulfate. *J. Bact.* **64**, 363–373.

Vishniac, W. & Santer, M. 1957 The thiobacilli. *Bact. Rev.* **21**, 195–213.

Vishniac, W. & Trudinger, P. A. 1962 Symposium on autotrophy. V. Carbon dioxide fixation and substrate oxidation in the chemosynthetic sulfur and hydrogen bacteria. *Bact. Rev.* **26**, 168–175.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES