SYMPOSIUM ON METABOLISM OF INORGANIC COMPOUNDS

V. COMPARATIVE METABOLISM OF INORGANIC SULFUR COMPOUNDS IN MICROORGANISMS

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I. Introduction

Although a voluminous literature exists concerning the biology of microorganisms that oxidize and reduce inorganic sulfur compounds, until recently little was known about the enzymatic mechanisms involved in the metabolism of these compounds. This is surprising when one considers the role that these microorganisms have played at one time or another in the development of our concepts concerning the coupling of energy-producing mechanisms with biosynthetic reactions, comparative biochemistry, and the evolution of life. In addition, these microorganisms and the transformations of sulfur compounds effected by them are important in certain basic biological and geological phenomena that are in some cases of great economic importance. The extent of the metabolism of sulfur compounds by microorganisms in nature is evident to the most casual of observers by the widespread production of hydrogen sulfide and occurrence of black iron sulfides. The biological aspect of inorganic sulfur metabolism has recently been the subject of an excellent review by Postgate (95) and is one of the most interesting and fascinating features of these organisms.

The transformations of inorganic sulfur compounds in nature have been formalized in the so-called sulfur cycle (see reference (132)) that is comparable in many respects to the better known nitrogen cycle. The microorganisms that participate in the sulfur cycle are physiologically diverse and comprise both heterotrophic and autotrophic organisms, the latter exhibiting unique modes of existence. The metabolism of inorganic sulfur compounds has been the subject of reviews by Butlin and Postgate (13) and Fromageot and Senez (30). In this discussion, the enzymes involved in the metabolism of inorganic sulfur compounds will be emphasized.
and allusion made to the more biological aspects of these reactions only where their interpretation is influenced by a knowledge of the enzymatic mechanisms.

**A. General Aspects of Inorganic Sulfur Metabolism**

Although mammals can oxidize reduced sulfur compounds, i.e., sulfide (7), thiosulfate (114), and sulfite (38), and incorporate sulfate into various organic molecules (67), they are unable to reduce sulfate to the level of sulfide (23) and must depend upon plants and bacteria to provide them with their reduced sulfur compounds. In Fig. 1 the basic essentials of the sulfur cycle are shown with most of the ancillary reactions omitted. The sulfur compounds included in the cycle are those that accumulate or are readily detectable in nature. Elemental sulfur has not been included as an intermediate in the sulfur cycle since it does not seem to be an intermediate in either the reduction of sulfate (93) or oxidation of sulfide (9).

Although many heterotrophic microorganisms reduce sulfate, the sulfur does not usually appear as sulfide but rather is incorporated into cellular material and returned to the sulfur cycle by other reactions. The main group of organisms producing large amounts of sulfide directly from sulfate are the so-called “sulfate-reducing bacteria” (see Fig. 1, right).

*Thiobacillus* species are chemoautotrophic organisms that derive the energy required for growth from the oxidation of reduced sulfur compounds (Fig. 1, left) and are the most well known of the nonphotosynthetic sulfur bacteria (130). *Beggiaota* species and allied organisms also carry out the oxidation of reduced sulfur compounds; however, these organisms will not be discussed since almost nothing is known about their nutrition or metabolism (24).

The second large physiological group of microorganisms that oxidize reduced compounds of sulfur are photosynthetic organisms belonging to the families *Thiorhodaceae* and *Chlorobacteriaceae* and typified by the genera *Chromatium* and *Chlorobium*. The enzymatic reactions concerned with the oxidation of reduced sulfur compounds are essentially unknown in these organisms; however, recent observations suggest that the enzymes of sulfur oxidation are intimately related to the photosynthetic apparatus (44).

Some heterotrophs are also capable of oxidizing reduced inorganic sulfur compounds and it has been suggested that these organisms are the major group of organisms responsible for the oxidation of sulfur compounds in soils. These heterotrophic organisms are incompletely described; however, the isolation of a facultative autotroph, *Thiobacillus novellus*, may indicate that there is a gradation in the ability of these organisms to oxidize reduced sulfur compounds and to derive energy from these oxidations, a capability reaching its greatest development in the autotrophic *thiobacilli* (130).

Until recently the intermediates in the oxidation and reduction of inorganic sulfur compounds have been unknown. In the center of the sulfur cycle shown in Fig. 1 are listed some of the more stable sulfur compounds that have at one time or another been implicated or suggested as intermediates in inorganic sulfur metabolism. A very simplified scheme for the oxidation of sulfide or reduction of sulfate (59) is shown in Fig. 2 with the valence states of the sulfur atoms indicated. This scheme, which is an initial formulation of the pathway of sulfate reduction, indicates that there may be four steps in the oxidation or reduction of these sulfur compounds. However, simple inorganic sulfur compounds with an oxidation level of +2 and 0 (with the exception of elemental sulfur) are extremely unstable and probably could not exist under physiological conditions. Alternatively, it might be postulated that

\[
\text{SO}_4^{(-2)} \rightarrow \text{SO}_3^{(-2)} \rightarrow \text{SO}_2^{(-2)} \rightarrow \text{S}^{(-2)}
\]

**Fig. 2.** Postulated inorganic intermediates in the reduction of sulfate. Valence states of the sulfur atoms are shown in parentheses.
METABOLISM OF INORGANIC SULFUR COMPOUNDS

at some oxidation level, the inorganic sulfur is incorporated into an organic molecule and then oxidized or reduced. A third group of compounds that might function as intermediates in the oxidation and reduction of sulfur compounds are the complex inorganic compounds of sulfur, such as the polysulfides or polythionates (S3O7−, S2O8−, S4O6−). The polythionates, in particular, are formed by many microorganisms, and schemes for the oxidation of reduced sulfur compounds have been proposed that utilize known chemical reactions of the polythionates (65, 130).

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. On a physiological basis, organisms that are capable of reducing sulfate can be classified into two distinct types (93). Most organisms can reduce sulfate, as evidenced by their ability to grow on sulfate as a sole source of sulfur; however, they do not form detectable amounts of sulfide directly from sulfate. This small-scale reduction of sulfate has been termed “assimilatory sulfate reduction” by analogy to Kluver’s classification of the various types of nitrate reduction (58). Another, and much smaller, group of microorganisms utilizes sulfate as the terminal electron acceptor in anaerobic respiration and produces massive amounts of hydrogen sulfide. This large-scale reduction of sulfate has been termed “dissimilatory sulfate reduction,” again by analogy to Kluver’s classification. Organisms that might be classified as “incidental dissimilatory sulfate reducers” have not been described; however, many organisms that are not “dissimilatory sulfate reducers” can reduce inorganic sulfur compounds other than sulfate (31, 80). Organisms that oxidize reduced sulfur compounds can be categorized into three groups on the basis of the role that sulfur plays in their physiology; “heterotrophic sulfur oxidizers,” “autotrophic sulfur oxidizers” that include the thiobacilli, and the “photosynthetic sulfur oxidizers.”

The transformation of inorganic sulfur compounds is intimately connected with the energy metabolism of the dissimilatory sulfate reducers and the autotrophic and photosynthetic sulfur oxidizers. This aspect has been the subject of considerable speculation and interest in the past, and recent observations on the metabolism of sulfate in higher animals suggest the possibility that there is a closer relationship between energy and sulfur metabolism than has been previously suspected.

B. The Activation of Sulfate

Although mammals are unable to reduce sulfate to the level of sulfide, they are capable of utilizing sulfate for the synthesis of various sulfated carbohydrates, lipids, and phenols. The reactions and enzymes involved in the esterification of sulfate have been recently reviewed by Lipmann (67) and Gregory and Robbins (33). The reaction requires adenosine triphosphate (ATP) for the initial activation of the sulfate, and two sulfur-containing nucleotides have been shown to be intermediates. The structures of these nucleotides, adenosine 5'-phosphosulfate (APS) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) are shown in Fig. 3. The first step in the metabolism of sulfate is the formation of APS and pyrophosphate (PPi) by the reaction of ATP and sulfate in the presence of the enzyme, ATP-sulfurylase (equation 1). The enzyme requires Mg2+ for activity and has been characterized and purified from baker’s yeast by Robbins and Lipmann (98). The equilibrium of this reaction lies far toward ATP and sulfate (K = 10−8, pH 8.0, 37 C) and to observe a net formation of APS it is necessary to “pull” the

\[
\text{ATP} + \text{SO}_4^{2−} \rightleftharpoons \text{APS} + \text{PPi},
\]

Fig. 3. Structures of APS and PAPS
reaction by removing the other product, PPi, with inorganic pyrophosphatase. It is interesting that the group potential of the sulfate (18 to 19 kcal) is much higher than that of phosphate. Under certain conditions, ATP-sulfurylase catalyzes another reaction, in which orthophosphate (P) is produced from ATP in the presence of pyrophosphatase and group VI anions (MoO₄²⁻, SeO₄²⁻, WO₄²⁻, CrO₄²⁻, and SO₄²⁻). The mechanism of these reactions seems to be similar to the well-known arsenolysis reactions (135).

The next step in the metabolism of sulfate is the phosphorylation of APS in the 3' position by ATP to yield PAPS and adenosine diphosphate (ADP) (99), as shown by equation 2. The enzyme responsible for the phosphorylation has been termed APS-kinase. PAPS is the so-called "active sulfate" of mammalian tissue and, in the presence of the appropriate enzymes (termed sulfokinases), can transfer sulfate to phenols, lipids, and carbohydrates. The other product of this transfer is 3',5'-diphosphoadenosine (PAP).

The formation of PAPS has been observed in extracts of Escherichia coli (42) and many fungi (22, 41, 117). The formation of a sulfur-containing nucleotide having the same electrophoretic mobility as PAPS has been observed in extracts of many bacteria (Peck, unpublished data). Recently, it has been suggested that specialized bacteria are the agents responsible for the reduction of sulfate in many insects (34). There is a paucity of data concerning the occurrence of sulfated compounds in bacteria, and the transfer of sulfate from PAPS (or possibly APS) to form a sulfated compound has not been described in bacteria. In addition, the transfer of sulfate from PAPS to phenols has not been observed, and the extracts examined seem to be lacking the specific sulfokinase (117). Recently a sulfate-containing polysaccharide has been isolated and characterized from bacteria and this compound may be useful in studying the mechanism of sulfate transfer in bacteria (125).

The occurrence of the choline ester of sulfuric acid in fungi has been known for a number of years (138). Kaji and Gregory (56) have shown that the sulfate donor for the synthesis of sulfocholine is PAPS and it appears that the transfer is catalyzed by a specific sulfokinase (117). Although some fungi accumulate fairly large amounts of sulfocholine, the exact role of this compound in the metabolism of fungi is unknown. It is of interest that the occurrence of sulfocholine in certain groups of fungi does seem to have some taxonomic significance (5, 35, 52).

One other enzyme that utilizes APS as substrate has been described from yeast. This enzyme, ADP-sulfurylase, catalyzes the phosphorylation of APS to form ADP and sulfate (99), as shown in equation 3.

\[
\text{APS + P} → \text{ADP + SO}_4^{2-} \tag{3}
\]

This enzymatic activity has been separated from ATP-sulfurylase and therefore appears to be catalyzed by a specific enzyme. The formation of APS by this enzyme has not been observed and, consequently, an equilibrium constant has not been calculated. However, the equilibrium of this reaction is probably quite similar to the equilibrium of the reaction catalyzed by ATP-sulfurylase, since both reactions involve the hydrolysis of a single phosphate bond. In contrast to ATP-sulfurylase, a requirement for Mg²⁺ has not been reported. The enzyme catalyzes an arsenolysis of APS (99) but does not catalyze the liberation of P from ATP in the presence of group VI anions (Peck, unpublished data). From a consideration of these observations, it does not seem that ADP-sulfurylase is involved in the formation of APS, and its role in the metabolism of APS in yeast is at present unknown.

II. ASSIMILATORY SULFATE REDUCTION

A. The Reduction of Sulfate

Although the production of hydrogen sulfide from some inorganic and organic compounds is frequently reported (2, 12, 17, 31, 80, 97, 137), the production of sulfide directly from sulfate is not commonly observed in microorganisms even when they are apparently reducing and incorporating sulfur into cellular materials. In the absence of sulfide production, other criteria can be employed to demonstrate that a given microorganism can reduce sulfate to the level of sulfide and is an assimilatory sulfate reducer.

The incorporation of S⁰O₄⁻ into the reduced sulfur compounds of cellular material constitutes a direct demonstration of the ability of a given organism to reduce sulfate to the level of sulfide. When observations of this type are made with organisms grown in complex media, the enzymes responsible for the reduction of sulfate may be
repessed (10), and negative results obtained; however, if these organisms are grown under other cultural conditions, it might be possible to demonstrate the presence of sulfate-reducing enzymes.

Many yeasts grow well on a defined medium in which the main source of sulfur is sulfate (106) and also incorporate sulfur from $\text{S}_2\text{O}_3^{2-}$ into cysteine and methionine (136). These organisms therefore seem to be in all respects assimilatory sulfate reducers. A cell-free system from yeast that reduces sulfate to sulfite and sulfide in the presence of $\text{Mg}^{2+}$, ATP, and reduced triphosphopyridine nucleotide (TPNH) has recently been described independently by Wilson and Bandurski (134) and by Hilz, Kittler, and Knappe (42). These investigators have further shown that the actual intermediate for the reduction of sulfate to sulfite is PAPS, formed as indicated previously (equations 1 and 2). Recent evidence obtained with partially purified yeast sulfate reductase indicates that this enzyme may be capable of reducing APS; however, the activity observed with APS is much less than that observed with PAPS (133). Hilz et al. (42) have shown that $\text{MoO}_4^{2-}$, which interferes with ATP-sulfurylase, the first enzyme involved in the activation of sulfate, inhibits the formation of sulfite from ATP and sulfate. To demonstrate that the effect of $\text{MoO}_4^{2-}$ was on the formation and not on the reduction of PAPS, this substrate was formed from labeled $p$-nitrophenylsulfate and PAP by a purified sulfokinase, as shown in equation 4. The fact that $\text{MoO}_4^{2-}$ did not inhibit

$$\text{NO}_2 \text{OSO}_3^- + \text{PAP} \rightleftharpoons \text{PAPS} + \text{NO}_2 \text{OH}$$

the reduction of PAPS under the latter condition indicated the effect of $\text{MoO}_4^{2-}$ was solely on ATP-sulfurylase, and that PAPS was formed by the previously described reaction sequence for the activation of sulfate. Employing the $p$-nitrophenylsulfate system, it was also shown that thiosulfate competitively inhibits the activation of sulfate but has no effect on the production of sulfite from PAPS. This is of interest in connection with the data of Cowie, Bolton, and Sands (21), who indicated that thiosulfate as well as other sulfur compounds inhibited the utilization of $\text{S}_2\text{O}_3^{2-}$. Sulfite itself may inhibit the formation of PAPS by its effect on ATP-sulfurylase (135).

The PAPS-reducing enzyme (hereafter called PAPS-reductase) is inhibited completely by arsenite ($3 \times 10^{-4} \text{ M}$) and seems therefore to require an intact vicinal dithiol for activity (42). The enzyme has been purified by both Hilz et al. (42) and Bandurski, Wilson, and Asahi (6) from yeast; however, there is some disagreement as to the nature of the sulphydryl involvement. Hilz et al. (42) reported that lipoic acid will restore activity of PAPS-reductase when added to inactive preparations and recently showed that reduced lipoic acid or its amide will replace TPNH as electron donor (40).

On the other hand, Bandurski et al. (6) separated PAPS-reductase from yeast into two heat-labile components (fractions A and B) and a heat-stable component (PrSS) that seems to be a protein. All three components are required for the reduction of PAPS with TPNH. However, a partial reaction, the oxidation of TPNH and appearance of sulphydryl groups in PrSS, can be observed in the presence of fraction A. Since Hilz et al. (42) postulated that lipoic acid is involved in this reductase, it is surprising that PrSS contains almost no lipoic acid (6). Fraction A will act as a diaphorase, coupling TPNH oxidation with ferriyanide reduction. From these considerations, the enzyme described by Bandurski et al. (6) resembles in many respects dihydrothioctyldehydrogenase (70, 107). The reaction mechanism has been postulated essentially as follows by Hilz et al. (42):

$$\text{RSS} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{R(SH)S} \rightleftharpoons \text{PAP} - \text{OSO}_3^- + \text{R(SH)}_2 \rightleftharpoons \text{PAP} - \text{OH} + \text{R} \text{SSO}_3^-$$

$$\text{R} \text{SSO}_3^- \rightleftharpoons \text{RSS} + \text{HSO}_3^-$$

where R may be either lipoic acid or the heat-stable protein of Bandurski et al. (6). Although the reactions are indicated as being reversible, evidence pertaining to this aspect has not yet been reported. PAPS-reductase has been reported.
to be present in extracts of *E. coli* (42, 69); however, the electron donor in this case for the reduction of PAPS is claimed to be reduced diposphopyridine nucleotide (DIPNH) rather than TPNH (69).

It has previously been postulated that sulfite was an intermediate in the reduction of sulfate from results obtained with isotopes (21, 62) and biochemical mutants (18, 43, 111). Recent enzymatic studies completely support this view since sulfite is one of the products of the reaction catalyzed by PAPS-reductase. These enzymatic observations also confirm results, obtained with biochemical mutants, which indicate that reduction of sulfate to sulfite is a complex reaction requiring at least three factors (18).

**B. The Reduction of Sulfite**

Interpretation of results obtained with biochemical mutants leads one to believe that at the oxidation level of sulfite, further reduction of sulfite may depend upon its incorporation into an organic molecule (111). Enzymological evidence pertinent to this aspect is scanty; however, data have been presented that indicate sulfite can be incorporated into cysteine to form cysteic acid and hydrogen sulfide (16). For this reaction to be of importance in the reduction, presumably the cysteic acid would be reduced to cysteine by reversing the cysteinesulfinic acid pathway of cysteine oxidation (112). However, this pathway does not seem to be involved in the reduction of sulfite since the over-all reaction is not reversible (112). In addition, Cobey and Handler (19) have shown that extracts of *E. coli* do not incorporate sulfite labeled with sulfur-35 into cysteinesulfinic acid. Although these particular compounds of cysteine do not seem to be intermediates in the reduction of sulfite, similar types of compounds may be involved. Regarding the interpretation of nutritional studies, it should be cautioned that, in addition to the fact that many of these putative intermediates of sulfur metabolism are unstable, the organic sulfur may be converted to inorganic sulfur before oxidation or reduction (29). On an enzymatic level there is little evidence to suggest that sulfite is incorporated into a small organic molecule before reduction (69).

An inorganic pathway of sulfite reduction has been indicated in studies with sulfur-35 (21) and by some of the results obtained with biochemical mutants (18). An inorganic pathway involving the formation of thiosulfate as an intermediate in the reduction of sulfite has been postulated since mutants can be obtained that utilize neither sulfate nor sulfite but can utilize thiosulfate as their sole source of sulfur. Similar conclusions were made from studies on the incorporation of $S_{35}O_3^-$ into cellular materials in the presence of thiosulfate. Since recent studies (described in Section IIIC) indicate that thiosulfate can be metabolized by a reductive cleavage to sulfite and sulfide (the presumptive product of sulfate reduction), it is not necessary to place thiosulfate directly on the pathway of sulfate reduction.

A TPN-specific sulfite reductase, which reduces sulfite to sulfide, was found in extracts of *E. coli* (69). It is described by the over-all reaction shown in equation 8. This enzymatic activity

$$\text{SO}_3^- + 3\text{TPNH} + 5\text{H}^+ \rightarrow \text{H}_2\text{S} + 3\text{TPN}^+ + 3\text{H}_2\text{O}$$

from *E. coli* has been enriched about 150-fold, requires flavin adenine dinucleotide for activity, and is inhibited by KCN. Several interesting aspects of this enzyme were reported by Mager (69). He noted that preparations of hydroxylamine reductase also exhibited sulfite-reductase activity and that the ratio of these two activities was constant throughout purification and under various adverse treatments. Since sulfite in low concentrations inhibits hydroxylamine reduction and the $K_m$ for sulfite is 100-fold lower than that for hydroxylamine, Mager postulated that sulfite reduction is the "true physiological function" of the enzyme and that the reduction of hydroxylamine is an "incidental" capacity of the same enzyme. Furthermore, the formation of both sulfite reductase and hydroxylamine reductase by proliferating cells of *E. coli* is inhibited specifically by the presence of cysteine or methionine in the growth medium. It is postulated that this inhibition reflects a feedback repression by cysteine and methionine of sulfite reductase. This result agrees with the data of Cowie et al. (21) that were obtained from studies on the biosynthesis of cysteine from sulfite with whole cells of *E. coli*. The repression of sulfite reductase, rather than PAPS-reductase or the enzymes required for the activation of sulfate, by cysteine and methionine (69), may indicate that sulfite itself is required for the formation of some cellular components.
The fact that sulfite reductase has been purified 150-fold, with apparently no evidence forthcoming to indicate that more than one enzyme is involved in this reduction, suggests that a single enzyme catalyzes the reduction of sulfite to sulfide. In addition, no factors except flavin adenine dinucleotide (FAD) are required for the reduction. The enzymological data indicate that sulfide is an intermediate in the reduction of sulfite and therefore sulfide is probably the inorganic form of sulfur that is incorporated into organic cellular materials.

C. The Reduction of Thiosulfate

Thiosulfate can serve as the sole source of sulfur for the growth of many organisms, and the production of hydrogen sulfide from thiosulfate has been frequently observed (17). Although the production of sulfite and sulfide from thiosulfate has been less frequently observed (78), the reductive cleavage of thiosulfate to sulfite and sulfide seems to be the first step in the metabolism of thiosulfate. The reduction of tetrathionate, a proposed intermediate in the oxidation of inorganic sulfur compounds, involves its initial conversion to thiosulfate by tetrathionase (87).

An enzyme, isolated from yeast and characterized by Kaji and McElroy (55), catalyzes the reductive cleavage of thiosulfate to sulfite and sulfide. The electron donor for this reduction may be either glutathione (GSH), cysteine, or homocysteine. The reaction for the reductive cleavage of thiosulfate with glutathione is given by equation 9. This enzyme is remarkably stable

$$2 \text{GSH} + \text{S}_2\text{O}_3^{2-} \rightarrow \text{GSSG} + \text{SO}_4^{2-} + \text{H}_2\text{S}$$

(9)

to heat and is inhibited by sulfite. In view of this latter observation, a mechanism for the reaction has been proposed that is similar to one suggested for the oxidation of sulfite (28). Thiosulfate reacts with a disulfide group, presumably on the enzyme, to yield

$$\begin{array}{c}
\text{Enzyme} \\
S\text{SO}_3^{2-} \\
\end{array}$$

which is reductively cleaved to yield sulfite and sulfide.

A second pathway of thiosulfate reduction may be present in some organisms (18) since mutants, unable to grow on low concentrations of thiosulfate, will grow in the presence of high concentrations of thiosulfate. This growth does not seem to result from the nonenzymatic cleavage of thiosulfate to sulfite and sulfide or elemental sulfur because these mutants cannot grow on any of these three compounds. Studies with mutants (77) suggest that thiosulfate and serine can react to form cysteine-S-sulfonate, which can then be cleaved to yield cysteine as one of the products. However, there is no enzymological evidence to support the presence of this second pathway of thiosulfate reduction.

D. The Metabolism of Elemental Sulfur and Sulfide

The evidence available, and particularly that obtained with cell-free systems, indicates that sulfur compounds are reduced to sulfide and then incorporated into cellular materials. An enzyme, which has been isolated from yeast, forms cysteine from serine and hydrogen sulfide (105), as shown in equation 10.

$$\text{H}_2\text{COH} + \text{H}_2\text{S} \rightarrow \text{H}_2\text{CSH} + \text{H}_2\text{O}$$

(10)

This enzyme, serine-sulphydrase, requires pyridoxal phosphate for activity. Thus, enzymes have been described for the reduction of sulfate to sulfide and the incorporation of the sulfur into organic materials.

Elemental sulfur does not seem to be an intermediate in this reaction sequence but is probably nonenzymatically reduced to sulfide and then metabolized. Thus, GSH is capable of reducing elemental sulfur to sulfide in the absence of an enzyme (118). Nevertheless, in some instances (the thiobacilli and certain photosynthetic organisms), this reduction may be catalyzed by a specific enzyme.

III. DISSIMILATORY SULFATE REDUCTION

A. General Aspects

The general physiology and taxonomy of the dissimilatory sulfate-reducing bacteria have recently been reviewed in detail by several authors (93, 119, 139). Therefore, the more enzymological aspects of sulfate reduction are emphasized in this section. Surprisingly few bona fide dissimilatory sulfate reducers have been de-
scribed, and a large portion of the physiological and enzymological observations concerning the reduction of sulfate have been made with the classic sulfate reducer, Desulfovibrio desulfuricans. Thermophilic sporeforming sulfate-reducing bacteria have been identified as Clostridium nigricans (15). Most of the other dissimilatory sulfate reducers seem to be species of Desulfovibrio. Many of the older species of Desulfovibrio are now regarded as D. desulfuricans; however, it has not been possible to reisolate all of the older species for examination (108). The application of newer techniques in the cultivation of these organisms and the use of biochemical properties in taxonomy, such as the presence or absence of various pigments (94), should greatly aid in classifying these organisms. Recently, employing both taxonomic and biochemical criteria for identification, two apparently new species of sulfate-reducing bacteria were described (1, 20).

The sulfate-reducing bacteria can no longer be regarded as organisms that can grow heterotrophically with sulfate and an organic electron donor or autotrophically with sulfate, hydrogen, and carbon dioxide. Sulfate cannot be considered an obligatory electron acceptor for the growth of D. desulfuricans under all conditions, since Postgate has shown that it is possible to grow this organism on pyruvate in the absence of sulfate (90). The presence of a clostridial “phosphoroclastic” reaction has been demonstrated in cell-free extracts. Thus, pyruvate is metabolized to hydrogen, carbon dioxide, and acetyl phosphate (74) and high-energy phosphate from this latter compound can be utilized to produce ATP (84). Independently, both Postgate (96) and Mechals and Rittenberg (71) showed that in contrast to earlier views (14), D. desulfuricans is not a true autotrophic organism since the carbon for the synthesis of cellular materials is not derived in large measure from carbon dioxide. They presented evidence that the carbon molecules of the cells are derived mainly from either impurities in the media or added yeast extract. These organisms are also unique among heterotrophic organisms since carbon from certain organic substrates is not assimilated into cellular material during growth, i.e., the organic electron donor can function solely as an energy source. A similar situation has been described in the photosynthetic bacteria (25). Nevertheless, D. desulfuricans can obtain the energy required for growth from the reduction of sulfate with hydrogen (109).

Although, from these considerations, one might expect to find eventually a graded series of sulfate-reducing organisms that vary from “incidental sulfate reducers” to “autotrophic sulfate reducers,” enzymological and physiological studies suggest the possibility that growth with sulfate as the sole electron acceptor may constitute a unique type of metabolism. Evidence supporting this idea has been forthcoming from the mechanism of sulfate reduction and from the studies on the energy metabolism of these organisms.

B. The Reduction of Sulfate

Whole cells of D. desulfuricans rapidly reduce sulfate, sulfite, and thiosulfate to sulfide in the presence of molecular hydrogen (89). The overall reaction for the reduction of sulfate is shown by equation 11. Most strains of this organism contain a very active hydrogenase that is present even when an organic electron donor is utilized for growth. The hydrogenase has been purified and studied by Sadana and Jagannathan (100) and more recently by Krasna, Riklis, and Rittenberg (61). The purified enzyme requires Fe²⁺ for maximal activity (101), a fact suggested by the low hydrogenase content of cells grown in iron-deficient media (91). The observation that sulfate-reducing organisms that do not contain hydrogenase grow satisfactorily in the presence of organic electron donors and sulfate, demonstrates that the presence of hydrogenase is not essential for the reduction of sulfate.

D. desulfuricans is the first nonphotosynthetic anaerobic organism in which the presence of cytochrome was demonstrated (47, 92). This cytochrome, identified as cytochrome c₄, has a low potential (Eₒ' = -0.205) (92) and was shown to function as an electron carrier for the production of hydrogen and carbon dioxide from formate and for the reduction of sulfite and thiosulfate with hydrogen in extracts (48). It has also been implicated as an electron carrier for the reduction of sulfate with hydrogen, since reduced intracellular cytochrome c₄ is oxidized in the presence of sulfate (92).

Although cell-free extracts of D. desulfuricans contain enzymatic systems that reduce thiosul-
fate, sulfite, and tetrathionate to sulfide with molecular hydrogen (48), initial attempts to obtain the reduction of sulfate in extracts were unsuccessful. The reduction of sulfate with hydrogen by whole cells is competitively inhibited by selenate; however, the reduction of sulfite or thiosulfate is not affected by this anion (88). Moreover, molybdate ion similarly inhibits sulfate reduction but not the reduction of sulfite (46). Since Wilson and Bandurski (135) observed that the enzyme ATP-sulfurylase catalyzes a rapid liberation of P1 from ATP in the presence of inorganic pyrophosphatase and group VI anions, the inhibition of sulfate reduction, but not sulfite reduction, by MoO42- and SeO42- suggested that ATP-sulfurylase was involved in the reduction of sulfate by D. desulfuricans. Cell-free extracts of this “sulfate reducer” contain ATP-sulfurylase, as indicated by the release of P1 from ATP in the presence of MoO42-, WO42-, and CrO42- (82). When extracts were supplemented with ATP and sulfate and placed under an atmosphere of hydrogen, hydrogen was utilized, as shown in Table 1, and, in the presence of S35O42-, radioactive acid-volatile sulfur (sulfide or sulfite) was produced (50, 82). If either ATP or sulfate was omitted, there was no utilization of hydrogen. ATP was not required by whole cells for the reduction of sulfate and actually seemed to inhibit the reduction. The fact that MoO42- inhibited sulfate reduction in whole cells as well as in extracts indicates that the reduction of sulfate observed in extracts is identical with that observed in whole cells.

When cell-free extracts were passed over a column of Amberlite resin to remove cytochrome c3 (48), the ability to reduce sulfate with hydrogen was lost as well as the abilities to reduce thiosulfate and decompose formate to hydrogen and carbon dioxide. The addition of purified cytochrome c3 restored all three activities. This constitutes evidence that cytochrome c3 functions either directly or indirectly in the reduction of sulfate. The activity of Amberlite-treated extracts could not be restored by the addition of DPN, TPN, or flavin; however, the addition of methyl viologen stimulated hydrogen utilization with sulfate and ATP about 8-fold over that observed in crude extracts. Methyl viologen also facilitates the reduction of sulfite and thiosulfate with molecular hydrogen, and this one-electron dye presumably functions as an efficient electron carrier between hydrogenase and these reductases (48). These observations indicate that the reaction sequence for electron transport between hydrogenase and each of these reductases is probably identical.

The stoichiometry for the reduction of sulfate in the presence of ATP and hydrogen indicated that APS was the form in which sulfate was reduced by D. desulfuricans (82), rather than PAPS (the form in which sulfate is reduced in assimilatory sulfate reducers). The formation of a single labeled nucleotide when cell-free extracts of D. desulfuricans are incubated with ATP and S35O42- (82) has been reported. This nucleotide has the same electrophoretic mobility as APS, moving slightly faster than ADP, and is hydrolyzed by 0.1 M HCl at 37 °C for 30 min to AMP and sulfate. In Fig. 4 the effects of various treatments on the formation of this labeled nucleotide by the extract are shown. Heating the extract for 3 min in a boiling water bath completely destroys the ability of extracts to form the nucleotide. ATP is required, and the presence of MoO42- inhibits formation of the nucleotide. The observed inhibition by MoO42- also indicates that the la-

### Table 1. Effect of ATP and molybdate on sulfate reduction with hydrogen in whole cells and cell-free extracts

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<thead>
<tr>
<th>Reaction mixture</th>
<th>Activity, μl of H2/15 min</th>
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<tr>
<td></td>
<td>Cell-free extract*</td>
</tr>
<tr>
<td>Complete</td>
<td>86</td>
</tr>
<tr>
<td>Minus Na2SO4</td>
<td>0</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>0</td>
</tr>
<tr>
<td>Plus 10 μmoles of molybdate</td>
<td>0</td>
</tr>
</tbody>
</table>

* Complete system: 125 μmoles of phosphate buffer (pH 7.5), 10 μmoles of MgCl2, 10 μmoles of ATP, 40 μmoles of Na2SO4, 0.8 μmole of methyl viologen, and 15 mg of protein. Total volume, 2.0 ml; 0.2 ml of 20% KOH in center well; temperature, 30 °C; gas phase, hydrogen.
† Complete system: 125 μmoles of phosphate buffer (pH 7.15), 10 μmoles of MgCl2, 40 μmoles of Na2SO4, 10 μmoles of ATP, 0.5 ml of a 20% suspension of cells. Total volume, 2.0 ml; 0.2 ml of 20% KOH in center well; temperature, 30 °C; gas phase, hydrogen.
‡ ATP omitted.
beled nucleotide, APS, is formed by the enzyme, ATP sulfurylase.

The absence of a labeled nucleotide corresponding to PAPS suggests that the extracts are incapable of forming this compound. However, the failure to observe the formation of PAPS could be caused by the rapid hydrolysis or metabolism of this nucleotide. PAPS, labeled with sulfur-35, was prepared and shown to be reduced to acid-volatile sulfur in extracts of yeast supplemented with TPNH. As shown in Fig. 4, after incubation of the labeled nucleotide with D. desulfuricans extract, it was still possible to demonstrate the presence of labeled PAPS. This result indicated that the failure to detect PAPS was a consequence of the absence of APS-kinase rather than the rapid destruction of the nucleotide. The ability of extracts to form acid-volatile sulfur from APS35, PAPS35, and ATP plus S34O4− was examined and the results are shown in Table 2. The electron donor for these reductions was methyl viologen reduced by the enzyme, hydrogenase, that is present in crude extracts of D. desulfuricans (100). No acid-volatile sulfur is produced from APS hydrolyzed in 0.1 M HCl for 30 min at 37 C; however, APS itself does give rise to the production of acid-volatile sulfur. The amount of acid-volatile sulfur produced represents complete reduction of the added APS to acid-volatile sulfur. The low activity exhibited by ATP and S34O4− indicates that APS is neither phosphorolized to ADP and S34O4− nor pyrophosphorolized to ATP and S34O4− and the S34O4− then reduced by another pathway. This conclusion is also substantiated by the observation that the label of APS35 is not diluted during reduction by a large pool of unlabeled S34O4−. These results conclusively demonstrate that APS rather than PAPS is the substrate for sulfate reduction by D. desulfuricans. The enzyme catalyzing the reduction of APS to adenosine monophosphate (AMP) and sulfate has been termed APS-reductase (equation 14).

The reduction of sulfate in these extracts has been shown to require at least two enzymatic components (Peck, unpublished data). When crude extracts of D. desulfuricans are heated for 2 min at 64 C, the abilities to reduce sulfate with hydrogen in the presence of ATP and to liberate P1 from ATP in the presence of MoO4− are destroyed. Inorganic pyrophosphatase, ATP-sulfurylase, and APS-reductase were not apprecia-

![Fig. 4. Formation of APS and stability of PAPS in extracts of Desulfovibrio desulfuricans.](image)

Each reaction mixture contained: tris buffer (pH 8.0), 300 μmoles; MgCl2, 10 μmoles; and, where indicated, ATP, 20 μmoles; Na2SO4, 2.5 μmoles (3.5 × 104 counts per minute per μmole); Na2MoO4, 2 μmoles; PAPS35, 0.018 μmole (1.0 × 104 counts per minute per μmole); and 5.0 mg of crude extract in a volume of 1 ml. After 15 min, the reaction was stopped by placing in a boiling water bath for 3 min. The nucleotides were adsorbed on charcoal, eluted, and separated by electrophoresis. The outlined areas represent ultraviolet quenching, and the shaded areas represent radioactivity as determined by radi autography.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Acid-volatile sulfur produced (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolyzed APS35</td>
<td>2</td>
</tr>
<tr>
<td>APS35</td>
<td>100</td>
</tr>
<tr>
<td>S34O4− + ATP</td>
<td>16</td>
</tr>
<tr>
<td>PAPS35</td>
<td>0</td>
</tr>
</tbody>
</table>

Methods: Each flask contained: tris(hydroxymethyl)aminomethane buffer (tris), pH 8.0, 300 μmoles; MgCl2, 10 μmoles; methyl viologen, 0.8 μmole; EDTA, 10 μmoles; and, where indicated, APS35, 0.1 μmole (3,015 counts per min); hydrolyzed APS35, 0.107 μmole (3,230 counts per min); Na2SO4, 2.5 μmoles (63,700 counts per min); ATP, 5 μmoles; PAPS35, 0.018 μmole (8,330 counts per min). Each reaction mixture contained 4.56 mg of crude extract heated at 60 C for 3 min. After incubation of reaction mixtures at 30 C for 20 min under H2, 0.3 ml of 12 N H2SO4, was tipped from a side arm and acid-volatile S35 absorbed in 0.2 ml of n KOH.
bly affected by this exposure to heat. When ATP-sulfurylase, prepared from yeast (98), was added to these heated extracts, the ability to reduce sulfate in the presence of ATP and hydrogen was restored. These data therefore indicate that ATP-sulfurylase is the enzyme responsible for the formation of APS in the reduction of sulfate. These two activities, ATP-sulfurylase and APS-reductase, also have been separated by electrophoresis with Geon (98). Since heated extracts do not reduce sulfate but do have ADP-sulfurylase, it appears that this enzyme cannot form APS to any extent under the conditions of the experiment. The fact that group VI anions inhibit the reduction of sulfate in whole cells indicates that this conclusion is also valid for whole cells.

Although evidence has not been presented, a third enzyme, inorganic pyrophosphatase, is probably required for maximal activity since the equilibrium of the reaction catalyzed by ATP-sulfurylase lies far toward ATP and sulfate. Removal of PPi would serve to “pull” the reaction toward APS formation. The pathway of sulfate reduction in *D. desulfuricans* can be summarized by the following equations:

\[
\text{ATP} + \text{SO}_4^{2-} \rightleftharpoons \text{APS} + \text{PPi}, \tag{12}
\]

\[
\text{PPi} + \text{H}_2\text{O} \rightarrow 2 \text{P}_{\text{i}}, \tag{13}
\]

\[
\text{APS} + 2e^- \rightleftharpoons \text{AMP} + \text{SO}_4^{2-}, \tag{14}
\]

Although the substrates for the reduction of sulfate by yeast and *D. desulfuricans* are analogous, the enzymes catalyzing these reactions seem to be entirely different. APS-reductase does not produce acid-volatile sulfur from PAPS, although PAPS-reductase does show slight activity toward APS (133). In addition, TPNH will not function as electron donor for the reduction of APS; neither will arsenite inhibit nor lipoic acid stimulate the reduction of APS in partially inactivated preparations. Another interesting property of APS-reductase is that the reaction is reversible (85). In the presence of extract, sulfate, AMP, and ferriyanide, ferriyanide is reduced and APS formed. The stoichiometry of the reaction indicates that the over-all reaction can be represented by equation 15. The reaction is unique in that the biologically utilizable energy is produced in the form of a high-energy sulfate rather than high-energy phosphate. The reaction is not specific for AMP and, as shown in Table 3, other sulfate-containing nucleotides can be formed.

The formation of the sulfate derivatives of guanosine 5'-phosphate (GMP), inosine 5'-phosphate (IMP), and 2'-deoxyribosyl adenine 5'-phosphate (dAMP) have also been demonstrated by the use of S35-labeled sulfite. The importance of these sulfur-containing nucleotides in the reduction of sulfate by *D. desulfuricans* is unknown.

It is of interest that neither TPN, DPN, flavin, or cytochrome c4 will function as electron acceptor in this oxidation. Reduced cytochrome c4 does not supply electrons for the reduction of APS by dilute or purified preparations of APS-reductase and therefore does not seem to be the immediate electron donor for APS-reductase.

Thus, the pathway of sulfate reduction in *D. desulfuricans* is quite different from that described in yeast, and the data indicate that at least two pathways of sulfate reduction exist in bacteria.

### C. The Reduction of Other Sulfur Compounds

The role of sulfite as an intermediate in the dissimilatory reduction of sulfate has been well es-
established by data obtained with both intact cells and extracts of \textit{D. desulfuricans}. The formation of sulfite during the reduction of sulfate by whole cells was reported by Millet (75). In extracts, the formation of sulfite from ATP and sulfate has been observed (82). Cell-free extracts (95) reduce sulfite to sulfide with molecular hydrogen and the reaction requires cytochrome \( c_3 \), hydrogenase, sulfite reductase and possibly other components (51, 76) for the over-all reduction shown by equation 16. Postgate (89) presented evidence

\[
\text{SO}_3^{2-} + 3\text{H}_2 \rightarrow 3\text{H}_2\text{O} \]  \hspace{1cm} (16)

which indicates that thiosulfate, although reduced to sulfide by intact cells, is not an intermediate in the reduction of sulfite. As in the case of sulfate reduction, methyl viologen will couple sulfite reductase with hydrogenase in the absence of cytochrome \( c_3 \) (48). Recently, an assay method, which employs the oxidation of reduced methyl viologen as an index of activity, was devised for this sulfite reductase (51). This method seems to be a direct assay of sulfite reductase and should eliminate many of the difficulties encountered when hydrogen utilization is used for assay. When assayed in partially purified preparations, cytochrome \( c_3 \) will not couple sulfite reductase with hydrogenase and, as in the case of APS-reductase, such inactivity indicates that other components are required for the transfer of electrons from reduced cytochrome \( c_3 \) to the sulfite reductase. Similarly TPN, DPN, and flavin do not seem to be active in this system.

The sulfite reductase of \textit{D. desulfuricans} has not been characterized well enough to decide conclusively whether it is similar to the sulfite reductase of \textit{E. coli}, and the nature of the electron donor should be investigated much more thoroughly in this system. The one major difference is the greater sensitivity of sulfite reductase of \textit{E. coli} to KCN. The unique property of the sulfite reductase from \textit{E. coli} is its ability to reduce hydroxylamine as well as sulfite. Although cell-free extracts of \textit{D. desulfuricans} reduce hydroxylamine with hydrogen and cytochrome \( c_3 \) or methyl viologen as electron carrier (110), this reduction seems to be a nonenzymatic process. The observations of Mager (69) do indicate that the question of the presence of hydroxylamine-reducing activity should be re-examined in extracts of \textit{D. desulfuricans}. Whole cells and extracts reduce both tetrathionate and thiosulfate to sulfide (95). The reduction of tetrathionate seems to involve preliminary cleavage to thiosulfate by the enzyme tetrathionase, and then reduction of the thiosulfate. Thiosulfate is reductively cleaved to sulfite and sulfide, as in yeast, and its reduction with hydrogen requires cytochrome \( c_3 \). The latter cannot be replaced by DPN, TPN, or flavin. Methyl viologen will also function as electron carrier between hydrogenase and thiosulfate in this reaction; however, the role of GSH and other sulfhydryl reagents in this system have not been investigated. In contrast to sulfite reductase and APS-reductase, cytochrome \( c_3 \) seems to donate its electrons directly to thiosulfate reductase (48).

In Fig. 5, the sulfur cycle is again shown, and the intermediates that have been demonstrated to function in the reduction of sulfate to sulfite are inserted. Although two pathways for the reduction of sulfate to sulfite have been reported (the PAPS-pathway in yeast and the APS-pathway in \textit{D. desulfuricans}) the reduction of sulfite and other oxidized sulfur compounds to sulfide seems to be catalyzed by similar enzymes.

\textbf{D. The Energy Metabolism of Desulfovibrio desulfuricans}

In spite of the fact that \textit{D. desulfuricans} is not an autotrophic organism, it can obtain the energy required for growth from the oxidation of hydrogen with sulfate (109). The increased incorporation of \(^{32}\)P-labeled P by during this oxidation of hydrogen with sulfate suggests that the organism...
can transform some of the chemical energy liberated into high-energy phosphate (49, 72, 116). These observations are particularly interesting since the organism requires energy to utilize sulfate as terminal electron acceptor. The fact that high-energy phosphate is required for the reduction of sulfate but not the reduction of sulfite or thiosulfate, makes this system (the reduction of sulfate, thiosulfate, or sulfite with hydrogen) ideally suited for inhibitor studies on the mechanism of phosphorylation that has been postulated to occur concomitantly with the oxidation of hydrogen.

In all probability, the inhibition of sulfate reduction in whole cells by group VI anions indicates that only the enzyme ATP-sulfurylase is involved in the formation of APS. This implies that two high-energy phosphates are required for each sulfate reduced to sulfide, or each four hydrogen molecules oxidized, if it is assumed that PP₁ cannot give rise directly to high-energy phosphate. This implication is probably correct, because the formation of APS depends upon the removal of PP₁.

It has been shown that 2,4-dinitrophenol (DNP) has no effect on the reduction of thiosulfate or sulfate with hydrogen in cell-free extracts (84). Therefore, it can be concluded that DNP does not have an inhibitory effect on the enzymes or electron transport sequence involved in these reductions. Since in whole cells, DNP completely inhibits the reduction of sulfate but does not affect the reduction of thiosulfate, it was apparent that DNP prevented the cells from producing the high-energy phosphate required for the reduction of sulfate but not thiosulfate. Thus, it was postulated that an oxidative phosphorylation was occurring during the oxidation of hydrogen with sulfate (84), and uncoupling of the oxidative phosphorylation with DNP manifested itself by failure to reduce sulfate but not thiosulfate.

Another interesting inhibitor of sulfate reduction in whole cells is methyl viologen. As noted previously, methyl viologen efficiently couples hydrogenase with sulfite, thiosulfate, and APS-reductase in cell-free extracts. The fact that with whole cells methyl viologen inhibits sulfate reduction but stimulates thiosulfate reduction with hydrogen is ascribed to the ability of methyl viologen to shunt electrons around the natural electron carriers participating in the oxidative phosphorylation. Thus the generation of high-energy phosphate, required for the activation of sulfate before its reduction, is prevented.

Whole cells of D. desulfuricans can reduce DNP to aminophenols that are much less inhibitory for sulfate reduction. When the DNP is reduced, the restoration of sulfate reduction does not occur. However, if, after the reduction of DNP, a catalytic amount of pyruvate, thiosulfate, or sulfite is added, sulfate reduction is almost immediately restored to the rate observed in the controls. Since pyruvate can supply ATP by the formation of acetyl phosphate, and since sulfite and thiosulfate, by virtue of their ability to act as electron acceptors, participate in the postulated oxidative phosphorylation, it seems likely that these three compounds can supply enough ATP to initiate sulfate reduction and thereby cause this “sparking” phenomenon (84). All these observations are consistent with the occurrence of oxidative phosphorylation during the oxidation of hydrogen in the presence of various sulfur compounds.

Nevertheless, it was a disturbing fact that cells, grown heterotrophically on lactate and sulfate, were capable of oxidative phosphorylation during the oxidation of hydrogen. Inspection of the equations of the reactions involved in the lactate-sulfate fermentation (equations 17 to 21) reveals

\[
2\text{CH}_3\text{CHOHCOOH} \rightarrow 2\text{CH}_3\text{COCOOH} + 4\text{H}^+. \tag{17}
\]

\[
2\text{CH}_3\text{COCOOH} + 2\text{HOP}_2\text{O}^\text{+} \rightarrow 2\text{CH}_3\text{COOPO}_4\text{O}^\text{2-} + 2\text{CO}_2 + 4\text{H}^+. \tag{18}
\]

\[
2\text{CH}_3\text{COOPO}_4\text{O}^\text{2-} + \text{AMP}^\text{+} + 2\text{H}^+ \rightarrow 2\text{CH}_3\text{COOH} + \text{ATP}^\text{4-}. \tag{19}
\]

\[
\text{SO}_4^\text{2-} + \text{ATP}^\text{4-} + 8\text{H}^+ \rightarrow 8\text{H}_2\text{O} + \text{AMP}^\text{+} + 2\text{HPO}_4\text{O}^\text{2-} + 2\text{H}^+. \tag{20}
\]

\[
2\text{CH}_3\text{CHOHCOOH} + \text{SO}_4^\text{2-} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 8\text{H}^+ + 2\text{H}_2\text{O}. \tag{21}
\]

that there is no net production of high-energy phosphate from substrate-level phosphorylation during this fermentation. Therefore, even when grown on organic electron donors, oxidative phosphorylation is probably necessary to obtain the energy required for growth (84).

Not only is the pathway of sulfate reduction unique in the sulfate-reducing bacteria, but the energy requirement for sulfate reduction necessitates that these organisms obtain energy by
some means other than substrate-level phosphorylation during growth on many electron donors and sulfate. Thus many electron donors can probably be viewed as the equivalent of molecular hydrogen in the metabolism of these organisms, since their carbon is not incorporated into cellular material (6). Although cytochrome \( c_2 \) presumably functions in some manner in the oxidative phosphorylation, other sulfate-reducing bacteria do not possess this cytochrome. It will be of great interest both to investigate the electron transport carriers and energy metabolism in these organisms and to determine whether or not there is an oxidative phosphorylation in each of the four reduction steps postulated in the reduction of sulfate. Thus anaerobic growth with sulfate as sole electron acceptor is a unique metabolism with respect to the role of organic electron donors, energy metabolism, and the pathway of sulfate reduction.

IV. The Distribution of APS-reductase and PAPS-reductase in Microorganisms

Since yeasts, assimilatory sulfate reducers, reduce sulfate in the form of PAPS, and \( D. \) desulfuricans, a dissimilatory sulfate reducer, reduces sulfate in the form of APS, it seemed that the PAPS-pathway of sulfate reduction might be characteristic of assimilatory sulfate reduction and the APS-pathway of sulfate reduction characteristic of dissimilatory sulfate reduction. Table 4 lists the results of a survey designed to ascertain whether the pathway of sulfate reduction in a given organism could be correlated with the role of sulfate in the metabolism of that organism. The reduction of both APS and PAPS was examined in the presence of TPNH (generated from glucose 6-phosphate by Zwischenferment) or reduced methyl viologen as electron donor. Activity was indicated by the formation of acid-volatile sulfur and varied in the various preparations from 0.1 to 30 \( \text{mmoles} \) of acid-volatile sulfur per hr per mg of protein.

The electron donor for the reduction of PAPS varies considerably in different organisms. Yeast utilizes only TPNH as electron donor for this reduction, whereas \( E. \) coli will utilize reduced methyl viologen, TPNH, and DPNH (69) for the reduction. In extracts of \( C. \) pasteurianum and \( R. \) spheroides, only methyl viologen will function as electron donor. Thus, there seems to be a variability in the specificity of PAPS-reductase for the electron donor from exclusively pyridine nucleotide (yeast) to exclusively some unknown electron donor that is replaceable by methyl viologen (C. pasteurianum). \( R. \) spheroides reduced PAPS to a limited extent only with reduced methyl viologen as electron donor, and \( R. \) pseudomonas palustris showed no reducing activity toward PAPS or APS. Ibanez and Lindstrom (44) demonstrated the photoreduction of sulfate by chromatophores of \( R. \) rubrum. The failure to observe good reduction of these sulfur-containing nucleotides with photosynthetic organisms may be due to the fact that no special precautions were taken with respect to the competence of chromatophores or to the presence of light during the incubation period. However, the only nucleotide that was reduced was PAPS.

Although very slight PAPS-reductase activity was found in extracts of \( B. \) terminalis, extracts of \( B. \) subtilis, \( B. \) polymyza, and \( B. \) megaterium did not reduce either PAPS or APS when grown on a complex or

<table>
<thead>
<tr>
<th>Organism</th>
<th>APS-reductase</th>
<th>PAPS-reductase</th>
<th>Assimilatory sulfate reduction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Baker's yeast</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aerobacter aerogenes</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas hydrophila</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aeromonas punctata</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium kluyveri</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium pasteurianum</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rhodopseudomonas spheroides</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rhodopseudomonas palustris</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus polymyza</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus terminalis</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Plus signs in this column indicate that an organism can either incorporate \( \text{S}^{32}\text{O}_4^- \) into cellular material containing reduced sulfur compounds or can grow on sulfate as the sole source of sulfur.
minimal medium. These organisms will grow on a defined medium with sulfate as the sole source of sulfur (60) and, in addition, the formation of small amounts of radioactive sulfide from $^{35}$O$_{2}^{-}$ has been observed with whole cells of *B. megaterium* (11). In these respects, the *Bacillus* species seem to be assimilatory sulfate reducers. Whether the failure to observe the reduction of PAPS or APS is due to technical difficulties, such as rapid hydrolysis of added sulfur-containing nucleotides, or to differences in the sulfate metabolism of these organisms is at present unknown.

From these limited observations it therefore seems likely that the PAPS pathway of sulfate reduction is characteristic of microorganisms that reduce sulfate solely for assimilatory purposes. However, it must be cautioned that exceptions to this idea may be found in the future.

Organisms that reduce APS but not PAPS are listed in Table 5. APS is reduced only with reduced methyl viologen as electron donor and the specific activity of APS-reductase is usually 20 to 100 times that observed for PAPS-reductase in assimilatory sulfate reducers. Other strains of *D. desulfuricans* seem to also utilize the APS-pathway of sulfate reduction (Peck, unpublished data).

*C. nigrificans* is a dissimilatory sulfate reducer (15) and extracts of this organism, when supplemented with ATP and $^{35}$O$_{2}^{-}$ under an atmosphere of molecular hydrogen, reduce sulfate to acid-volatile sulfur. Since this reduction was completely inhibited by MoO$_{4}^{2-}$, the first step in the reduction of sulfate by this organism seemed to be the formation of APS by the enzyme ATP-sulfurylase. Both ATP-sulfurylase and ADP-sulfurylase have been shown to be present in these extracts (Peck, unpublished data). As shown in Table 5, these extracts formed acid-volatile sulfur from APS and the specific activity of APS-reductase is high compared to the specific activity of PAPS-reductase in assimilatory sulfate reducers. In all respects, the mechanism of sulfate reduction in *C. nigrificans* appears identical to that in extracts of *D. desulfuricans*.

Another organism, *Vibrio cholinicus*, isolated by Hayward and Stadtman (36), seems to be a dissimilatory sulfate reducer. Growth on choline is stimulated when sulfate is added to the medium, and in addition radioactive sulfide is produced from $^{35}$O$_{2}^{-}$ during the oxidation of choline by extracts of this organism (37). As shown in Table 5, extracts of *V. cholinicus* exhibit only APS-reductase with reduced methyl viologen in high specific activity and show no PAPS-reductase activity. These results indicate that *V. cholinicus* is a dissimilatory sulfate reducer and recently it has been claimed that *V. cholinicus* was incorrectly named and the organism is actually *D. desulfuricans* (4). Thus, all dissimilatory sulfate reducers that were examined reduced sulfate in the form of APS and exhibited no activity for PAPS.

In addition to microorganisms that reduce sulfur compounds, extracts of organisms that oxidize compounds of sulfur more reduced than sulfate were examined for the presence of APS-reductase and PAPS-reductase. Three species of *Thiobacillus* grown on thiosulfate as energy source showed APS-reductase, in some instances equal in specific activity to that observed in the dissimilatory sulfate-reducing bacteria, and no PAPS-reductase. Since cell-free extracts of *E. coli* and *Proteus vulgaris* did not contain APS-reductase when grown heterotrophically with added thiosulfate (Peck, unpublished data), it did not seem that the presence of APS-reductase is a general response of microorganisms to growth in the presence of thiosulfate. These are extremely interesting observations in view of the fact that thiobacilli obtain the energy required for growth by the oxidation of reduced sulfur compounds. The role of APS-reductase in the sulfur metabolism of these organisms is discussed subsequently.

### Table 5. Pathway of sulfate reduction in dissimilatory sulfate reducers and organisms that oxidize reduced sulfur compounds

<table>
<thead>
<tr>
<th>Organism</th>
<th>APS-reductase</th>
<th>PAPS-reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desulfurobrio desulfuricans</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium nigrificans</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio cholinicus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Thiobacillus thioparatus</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Thiobacillus thiooxidans</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Thiobacillus denitrificans</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

### V. The Oxidation of Reduced Sulfur Compounds

#### A. General Aspects

The formation of sulfate and other sulfur compounds from more reduced sulfur compounds,
both organic and inorganic, is commonly observed in cultures of single microorganisms (30) and in mixed cultures (32). In addition, sulfate may be formed from the hydrolysis of organic sulfates by enzymes termed sulfatases (33). The pathway for the oxidation of cysteine to sulfate has been described by Singer and Kearney (112) in extracts of *P. vulgaris*. The reaction sequence involves the formation of a series of oxidized derivatives of cysteine that terminate in the production of sulfate; the sulfate thus formed is oxidized to sulfate. Many organisms can apparently oxidize cysteine to sulfate; however, there may be some differences in the intermediates involved (27, 79). Generally speaking, it is quite possible that inorganic sulfur compounds, such as sulfide, can react with the proper organic acceptor to form an organic intermediate and be oxidized to sulfate. The pathway, described by Singer and Kearney (112) for the oxidation of cysteine, does not seem to be of major importance for the oxidation of inorganic sulfur compounds since some organisms that have been shown to oxidize cysteine to sulfate do not oxidize reduced inorganic sulfur compounds to sulfate. Therefore, the enzymatic reactions discussed in this section will mainly concern the formation of sulfate and other inorganic sulfur compounds from sulfide by an inorganic pathway.

The stable sulfur compounds reportedly formed during the oxidation of reduced sulfur compounds are sulfite, thiosulfate, and elemental sulfur. In addition, various polythionates, in particular tetrathionate, are commonly formed from the oxidation of reduced sulfur compounds (130). The formation of polythionates has not been observed during the reduction of sulfate although some organisms may reduce polythionates to sulfide.

Many heterotrophic organisms are capable of oxidizing reduced sulfur compounds, and the products are usually sulfate or polythionates. However, neither the organisms nor the enzymatic reactions involved have been characterized to any extent. The thiobacilli and the photosynthetic bacteria are the best studied with regard to the over-all oxidation of reduced sulfur compounds. Until recently, little information has been available concerning the enzymatic mechanisms involved in these oxidations.

**B. The Oxidation of Reduced Sulfur Compounds by Heterotrophic Microorganisms**

It has been known for many years that certain heterotrophic organisms can oxidize reduced sulfur compounds (30, 130). These organisms can be separated into two general physiological groups. The first group can oxidize elemental sulfur and thiosulfate to polythionates, and the second group can convert the polythionates to sulfate. In mixed cultures these organisms can convert reduced sulfur compounds to sulfate. The only enzyme studied that is possibly involved in this transformation is tetrathionase, which forms tetrathionate from thiosulfate (87, 129). Evidence has been presented that indicates the oxidation of thiosulfate to tetrathionate probably does not supply energy for the growth of cells capable of catalyzing this oxidation (7, 129). The mechanism for the formation of other polythionates is at present unknown; however, it may be possible that tetrathionase can also form other polythionates by a mechanism similar to that suggested by Lees (65) for the oxidation of thiosulfate.

Very little is known concerning the oxidation of elemental sulfur, sulfide, or sulfite in these microbial systems. Elemental sulfur is probably nonenzymatically reduced to sulfide (118), and the sulfide is either reoxidized to elemental sulfur or oxidized to thiosulfate. The formation of thiosulfate from sulfide has been observed in extracts of various mammalian tissues (7, 45, 115) and *Thiobacillus thiocyanidans* (121) and seems to be a unique reaction. Protein fractions, ferritin, and various metalloprotein complexes (88, 115) can catalyze this oxidation of sulfide and a mechanism for this oxidation has been proposed. Although intermediates, if any, have not been isolated, it seems that free polythionates are not involved (9, 121). It is interesting that in mammals, thiosulfate is either excreted as such or metabolized in such a manner that the two sulfur atoms are not equivalent (114).

The oxidation of sulfite to sulfate is nonenzymatically catalyzed by various metal ions (38). In the presence of various chelating agents, it is possible to observe the enzymatic formation of sulfate from sulfite and the oxidation has been most extensively studied in mammalian tissue (28). This enzyme, sulfite oxidase, requires hypoxanthine as a cofactor and probably in-
volves a disulfide group, possibly lipoic acid, since the reaction is inhibited by arsenite. At present, the reactions involved in the oxidation of these inorganic compounds by heterotrophic microorganisms can only be inferred from the nature of these reactions in other organisms.

C. The Oxidation of Reduced Sulfur Compounds by Photosynthetic Microorganisms

The chlorobacteriaceae and the thiorniaceae grow photosynthetically under anaerobic conditions and require reduced sulfur compounds (or certain other compounds) that are oxidized to sulfur or sulfate. Although little is known about the actual pathway of the metabolism of inorganic sulfur compounds, these organisms appear not to derive energy from the oxidation of such reduced sulfur compounds (63). Grossly, the pathway of oxidation of reduced sulfur compounds seems to be similar to that in other microorganisms. Elemental sulfur is formed as a transitory intermediate in the oxidation of the thiosulfate (68), and polythionates are utilized (63) and may be formed during the oxidation of sulfide (57). Hendley (39) has observed some reduction of $^{35}$S-labeled sulfate to sulfide in whole cells of *Chromatium* sp. and APS-reductase has been found in extracts of this organism; however, the activity was low compared to that observed in the thiobacilli and the dissimilatory sulfate reducers (Peck, unpublished data). These observations suggest that the oxidation of reduced sulfur compounds proceeds by way of a non-phosphorylative pathway in these organisms. However, the oxidation of reduced sulfur compounds is closely coupled to photosynthesis (68) and maximal activity may depend upon the presence of light and an intact photosynthetic apparatus (49).

D. The Oxidation of Reduced Sulfur Compounds by the Thiobacilli

The thiobacilli are a small group of microorganisms whose energy metabolism is uniquely adapted to obtain all the energy required for growth from oxidation of inorganic sulfur compounds to sulfate, and which utilize carbon dioxide as the source of carbon for the synthesis of cellular materials. In addition to being autotrophic, the thiobacilli also exhibit other interesting biological phenomena. These organisms are obligately autotrophic with the exception of *T. novellus*, which will grow on organic substrates and is therefore a facultative autotrophic organism (130). One species, *T. thiooxidans*, is tolerant of extremely acid environments, and is capable of growing at pH values less than 1; however, other strains vary in their tolerance toward acid. *T. denitrificans* can utilize nitrate anaerobically in place of oxygen as electron acceptor and produces molecular nitrogen (3). Some of the thiobacilli can oxidize thiocyanate or ferrous ion and thus obtain the energy required for growth (130). In view of these differences, it is interesting that recently Johnstone, Townshend, and White (54) claimed that various strains of thiobacilli can be derived from a single species of these organisms. The general physiology of the thiobacilli has been reviewed by Vishniac and Santer (130), Larsen (64), and Umbricht (128). Lees (65) has recently reviewed the energy metabolism of these organisms.

Most of the thiobacilli are capable of oxidizing sulfide, elemental sulfur, thiosulfate, tetra-thionate, and, in some cases, sulfate to sulfite (81). The usual substrates for growth, however, are either elemental sulfur or thiosulfate, thiosulfate being the preferred substrate for technical reasons. When grown on thiosulfate, thiobacilli form elemental sulfur either as a transitory intermediate or in quantities approaching the amount of sulfite produced (*T. thioparus*); however, the amount of sulfite produced seems to depend not only upon the species but also upon the conditions employed to culture the organism (130). During the oxidation of thiosulfate by whole cells of *T. thioparus*, polythionates consisting mainly of tetra-thionate are rapidly formed (130), and schemes for the oxidation of reduced sulfur compounds involving polythionates as intermediates have been proposed by Tamiya, Haga, and Huzisige (124), Vishniac and Santer (130), and more recently by Lees (65). Lees proposed that the polythionates are enzyme-bound intermediates and that the reactions involving polythionates take place at the surface of the cell. Only at the level of sulfite does the sulfur atom enter the cell, at which time it is oxidized to sulfate. Thus the problem of the oxidation of different reduced sulfur compounds can be viewed as the problem of the oxidation of sulfide. Enzymological evidence to support this scheme is
scanty, consisting mainly of the observation that cell-free extracts will oxidize thiosulfate to tetrathionate (126). This reaction seems similar to the reaction catalyzed by the enzyme tetrathionase (87). Most of the evidence supporting the polythionate pathway is derived from observations made with whole cells. Although not a universal observation (81), many strains of thiobacilli form and oxidize tetrathionate and other polythionates. In addition, the rapid formation of polythionate from radioactive thiosulfate has been observed (127) and Vishniac and Santer (130) have reported the formation of thiosulfate and polythionate during the oxidation of S\textsuperscript{35}\textsubscript{a}-labeled sulfide. Other observations have suggested a role of phosphate in the oxidation of thiosulfate (104).

The presence of P\textsubscript{i} is required for the complete oxidation of thiosulfate and tetrathionate to sulfate, and the phosphate can be replaced by arsenate. This result indicates that phosphate may be involved in the oxidation of reduced sulfur compounds at the substrate level. In addition, the phosphate requirement for both tetrathionate and thiosulfate oxidation indicates that at some point these compounds are oxidized by the same pathway. Santer (103) recently presented evidence that further substantiates the direct role of P\textsubscript{i} in the oxidation of thiosulfate. When whole cells oxidized thiosulfate in the presence of O\textsubscript{2}-labeled P\textsubscript{i}, O\textsubscript{2} was transferred from the P\textsubscript{i} to the sulfate formed during the oxidation. The transfer is of sufficient magnitude to indicate that one of the oxygen atoms of each of the sulfate molecules formed is derived from the phosphate. These results suggested the possibility that APS or PAPS might be intermediates in the oxidation of reduced sulfur compounds by these organisms.

Since extracts of the thiobacilli had only been observed to oxidize thiosulfate to tetrathionate, it was possible that the failure to observe the complete oxidation of thiosulfate to sulfate was due to the fact that the initial step in the oxidation of thiosulfate was a reduction, i.e., the reductive cleavage of thiosulfate to sulfide and sulfite, as previously described in other organisms. Several observations indicated that the reductive cleavage of thiosulfate was involved in thiosulfate metabolism of these organisms. Skarzynski and Ostrowski (113) have reported that intact cells of T. thioparus incorporate the ligand or outer sulfur atom of thiosulfate into cellular materials and elemental sulfur. However, Santer et al. (104) were unable to confirm this observation and indicated that both sulfur atoms of thiosulfate are metabolized in the same manner. Other evidence indicates that, in the presence of an unlabeled pool of elemental sulfur, the preferential incorporation of the outer sulfur atom of thiosulfate into elemental sulfur can be observed (Peck and E. Fisher, unpublished data). The presence of thiosulfate-reductase in extracts of T. thioparus is suggested by the observation that sulfide is produced by reaction mixtures containing GSH and thiosulfate (121) and the presence of thiosulfate reductase, as indicated by the formation of both sulfide and sulfate from GSH and thiosulfate, has been demonstrated in extracts of T. thioparus. The products of this reduction could be the precursors of the sulfate and elemental sulfur which are formed during the growth of this organism. Thus sulfide is oxidized to elemental sulfur by extracts of T. thiooxidans (121) and T. thioparus (82), and sulfate could be produced by the oxidation of sulfite, possibly by a sulfite oxidase similar to that described in mammalian tissues (28) and T. denitrificans (73). However, such a reaction sequence does not offer an explanation for the role of P\textsubscript{i} in the oxidation of thiosulfate.

Extracts of T. thioparus have been shown to contain other enzymatic activities that may be concerned in the oxidation of reduced sulfur compounds (83). Extracts of this organism reduce APS to sulfite and AMP in the presence of reduced methyl viologen, and the specific activity of APS-reductase in these extracts is comparable to that observed in the dissimilatory sulfate-reducing bacteria. In contrast, PAPS is not reduced with either reduced methyl viologen or TPNH as electron donor. ATP-sulfurylase, ADP-sulfurylase, and adenylate kinase are also present in high specific activity in these extracts. From a consideration of results obtained with both whole cells and extracts, the reaction sequence (equations 22 to 27) for the oxidation of thiosulfate to sulfate has been proposed (83).

\[
4H^+ + 4e^- + 2S_2O_3^{2-} \xrightarrow{\text{thiosulfate reductase}} 2SO_4^{2-} + 2H_2S \tag{22}
\]

\[
2H_2S + O_2 \xrightarrow{\text{sulfite oxidase}} 2S_2^0 + 2H_2O \tag{23}
\]

\[
2SO_4^{2-} + 2AMP \xrightarrow{\text{APS-reductase}} 2APS + 4e^- \tag{24}
\]
Thiosulfate is postulated as being reductively cleaved to sulfide and sulfate by thiosulfate reductase and the sulfide oxidized to elemental sulfur by sulfide oxidase. Sulfite is oxidized in the presence of AMP to yield APS and the high-energy sulfate exchanged for phosphate to yield ADP by the action of ADP-sulfurylase. ATP can then be formed from ADP by adenylic kinase. The sum of equations 22 to 26 (equation 27) indicates that a net production of high-energy phosphate should occur during the oxidation of thiosulfate. ADP- and ATP-sulfurylase can exchange the high-energy sulfate for phosphate or pyrophosphate, respectively; however, the extent of participation of each enzyme in this exchange probably depends upon the availability of P<sub>1</sub> and PP<sub>1</sub>. Since P<sub>1</sub> is required for the complete oxidation of thiosulfate, ADP-sulfurylase has been proposed as the enzyme most likely involved in the formation of high-energy phosphate from APS.

The reaction sequence explains the effects of P<sub>1</sub> and arsenate observed with whole cells. A deficiency of P<sub>1</sub> will result in an accumulation of APS and consequent depletion of the AMP present in the cells and, thus, inhibition of the oxidation of sulfite or thiosulfate. Arsenate may function to replace P<sub>1</sub> in two ways. First, arsenate will replace P<sub>1</sub> by catalyzing the arsenolysis of APS (99), thereby regenerating AMP for the oxidation of sulfite. A second possible action of arsenate may be to liberate phosphate from phosphate-containing organic compounds in the cells, thereby replenishing the P<sub>1</sub> pool in these cells.

A cell-free system that oxidizes thiosulfate in the presence of GSH has been described (83). Further observations with this system (86) demonstrate that in the presence of GSH, thiosulfate is reductively cleaved to sulfite and sulfide, and the sulfite oxidized to sulfate with the production of one high-energy phosphate per sulfate formed. To observe phosphorylation, it is necessary to supplement reaction mixtures with NaF and ethylenediaminetetraacetate (EDTA) to inhibit hydrolytic enzymes and to add AMP and P<sub>1</sub> as substrates for the phosphorylation. As shown in Table 6, the phosphorylation was not inhibited by DNP and therefore is probably not the result of oxidative phosphorylation. This is further supported by the observation that, in the presence of P<sub>32</sub>-labeled P<sub>1</sub>, almost all of the esterified phosphate was found to be present in ADP. This result is to be expected from a consideration of the postulated reaction sequence (equations 22 to 26) since NaF is known to inhibit adenylic kinase. Furthermore, it was observed that even in the presence of a pool of unlabelled ATP, almost all of the esterified phosphate was found in the ADP. From these results, it was concluded that ADP was the initial product of this phosphorylation and could not have been formed in some manner from ATP. It was also possible to demonstrate a dependency on AMP for phosphorylation and oxygen utilization, and AMP is the only nucleotide with which significant phosphorylation has been observed. Arsenate will stimulate the formation of sulfate when conditions are such that there is an absence of P<sub>1</sub> and only a catalytic amount of AMP. Thus it seems that arsenate can stimulate the oxidation of thiosulfate by the arsenolysis of APS. The nature of the phosphorylation and the products of the oxidation demonstrated that sulfate is produced and ADP formed in a 1:1 ratio in accord with the postulated reaction sequence. Oxygen utilization and the effect of arsenate

<table>
<thead>
<tr>
<th>Additions</th>
<th>P&lt;sub&gt;1&lt;/sub&gt; utilized</th>
<th>O&lt;sub&gt;2&lt;/sub&gt; utilized</th>
<th>Esterified phosphate</th>
<th>Esterified phosphate (P&lt;sub&gt;32&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>2.53</td>
<td>3.47</td>
<td>2.69</td>
<td>2.26</td>
</tr>
<tr>
<td>Complete plus 2,4-dinitrophenol (2 X 10&lt;sup&gt;-4&lt;/sup&gt; M)</td>
<td>3.06</td>
<td>4.66</td>
<td>3.72</td>
<td>3.22</td>
</tr>
</tbody>
</table>

**Materials:** The complete system contained: tris (pH 8.0), 300 μmoles; EDTA, 10 μmoles; NaF, 100 μmoles; AMP, 10 μmoles; Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 40 μmoles; GSH, 40 μmoles; TPN, 0.67 μ mole; P<sub>32</sub>, 5 μmoles (containing 2.28 X 10<sup>4</sup> counts; min μ mole). Thiobacillus thioparus extract, 26 μg in a total volume of 2.0 ml. Time, 30 min.
can also be explained by this scheme. Similar results were obtained using sulfite rather than thiosulfate as substrate for this oxidation (Table 7).

Extracts of *T. thioparus* will form APS in the presence of enzyme, AMP, SO$_4^{2-}$, and Fe(CN)$_6^{3-}$, as was observed with extracts of *D. desulfuricans* (85). IMP, GMP, and dAMP were the only nucleotides tested that substitute for AMP in this reaction and form the corresponding sulfur-containing nucleotides. The facts that PAP will not function in this system, that arsenite does not inhibit the production of APS, and that extracts do not reduce PAPS, indicate that APS-reductase is quite different from PAPS-reductase (85). Studies on the stability, inactivation, and fractionation of APS-reductase activity, which was measured with reduced methyl viologen as electron donor and ferriyanide as electron acceptor, indicate that these activities are catalyzed by a single enzyme. However, some differences have been observed between the APS-reductase of *D. desulfuricans* and that of *T. thioparus*. The enzyme is much more labile in extracts of *T. thioparas* than in extracts of *D. desulfuricans*. In addition, the APS-reductase of *T. thioparus* but not that of *D. desulfuricans* catalyzes a rapid exchange between sulfite and the sulfate of APS (Peck and W. J. Payne, unpublished data). This difference may be ascribed to the unknown electron carriers, which may differ with the physiological function of the enzyme, in the oxidation or reduction of APS. The demonstration of the reversibility of APS-reductase, the critical and novel reaction in the oxidation of thiosulfate, indicates that the thiobacilli can produce a high-energy sulfate at the substrate level from the oxidation of reduced sulfur compounds. All the evidence available indicates that sulfite is an intermediate in the oxidation of thiosulfate (and other reduced sulfur compounds) and is oxidized to sulfate by a phosphorylative pathway rather than by the sulfate oxidase described in mammalian tissue. An effect of AMP on the oxidation of sulfite was observed previously in plants, but neither phosphorylation or APS formation was reported (123).

Although sulfite can be produced from the reductive cleavage of thiosulfate, it is possible that sulfite can be formed by other mechanisms, namely, the postulated polythionate cycle (65). The main evidence for the involvement of thiosulfate reductase in the oxidation of thiosulfate, aside from the presence of the enzyme in extracts, is the absolute requirement for substrate amounts of GSH for oxygen utilization, sulfate production, and ADP formation. Extracts are capable of oxidizing thiosulfate to tetrathionate; however, added tetrathionate will not function as substrate for sulfate production or ADP formation unless GSH is added (Table 8). GSH rapidly and non-enzymatically reduces tetrathionate to thiosulfate, which is then oxidized as previously described. Therefore, it appears that, in these extracts, sulfite is produced solely from thiosulfate by thiosulfate reductase.

The possible involvement of sulfide in the oxidation of elemental sulfur has been suggested

---

**Table 7. Oxidation of SO$_4^{2-}$, SO$_2^{−}$, and S$^−$**

<table>
<thead>
<tr>
<th>System</th>
<th>O$_3$ utilized</th>
<th>P$_i$ esterified</th>
<th>SO$_4^{2−}$ produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>Extract</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ SO$_4^{2−}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ GSH</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ SO$_4^{2−}$ + GSH</td>
<td>2.7</td>
<td>2.0</td>
<td>4.2</td>
</tr>
<tr>
<td>+ SO$_2^{−}$</td>
<td>2.5</td>
<td>4.5</td>
<td>7.8</td>
</tr>
<tr>
<td>+ S$^−$</td>
<td>3.3</td>
<td>1.8</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*Materials:* Each reaction mixture contained: tris (pH 8.0), 300 µmoles; AMP, 10 µmoles; EDTA, 10 µmoles; NaF, 100 µmoles; P$_i$, 5 µmoles; and, where indicated, Na$_2$SO$_4$, 10 µmoles; GSH, 40 µmoles; Na$_2$S, 10 µmoles; and Thiobacillus *thioparus* extract, 29.5 mg, in a total volume of 2.0 ml. Time, 30 min.

**Table 8. Oxidation of thiosulfate and tetrathionate by extracts**

<table>
<thead>
<tr>
<th>Additions</th>
<th>P$_i$ esterified</th>
<th>SO$_4^{2−}$ produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>S$_2$O$_3^{−}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GSH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S$_2$O$_3^{−}$ + GSH</td>
<td>1.7</td>
<td>2.0</td>
</tr>
<tr>
<td>S$_2$O$_3^{−}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S$_2$O$_3^{−}$ + GSH</td>
<td>1.9</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*Materials:* Each reaction mixture contained: tris (pH 8.0), 300 µmoles; AMP, 10 µmoles; EDTA, 10 µmoles; NaF, 100 µmoles; P$_i$, 5 µmoles; and, where indicated, Na$_2$SO$_4$, 10 µmoles; K$_2$S$_2$O$_4$, 10 µmoles; GSH, 40 µmoles. Thiobacillus *thioparus* extract, 40 mg in a total volume of 2.0 ml. Time, 40 min.
by Starkey (120), who observed the formation of small amounts of hydrogen sulfide from elemental sulfur. When sulfide was added to reaction mixtures designed to permit phosphorylation and sulfate formation with an extract of *T. thioparatus*, the formation of sulfate and ADP was observed. In addition, it was also possible to detect the formation of thiosulfate but not polythionate. Since the oxidation of sulfide to thiosulfate in mammalian tissues and *T. thiooxidans* does not seem to involve the formation of polythionates, a reaction sequence for the oxidation of elemental sulfur and sulfide to sulfate can be proposed. The scheme utilizes known reactions and does not involve the polythionates as intermediates, as indicated by equations 28 and 29. The thiosulfate

\[
S^\ell + 2GSH \rightarrow H_2S + GSSG \quad (28)
\]

\[
2SH^\ell + 2O_2 \rightarrow S_2O_3^{2-} + H_2O \quad (29)
\]

is then oxidized to sulfate, as described in equations 22, 24, and 25. The second reaction has been discussed previously. Tetraithionate and other polythionates do not function as intermediates in this scheme, and their occurrence during the oxidation of reduced sulfur compounds by the thiobacilli as well as by heterotrophic organisms is regarded as incidental. With regard to the elemental sulfur formed by many of these organisms during the oxidation of thiosulfate, sulfide originating from the reductive cleavage of thiosulfate may be oxidized to elemental sulfur to supply electrons for the regeneration of GSH that was oxidized during the reductive cleavage of thiosulfate.

The reactions postulated for the oxidation of thiosulfate (equations 22 to 26) also offer an explanation for the O\(^{18}\) data obtained by Santer (103), if the transfer of O\(^{18}\) is assumed to occur by established mechanisms. Since the pool of intracellular AMP is small compared to the amount of substrate oxidized, AMP would rapidly become labeled with O\(^{18}\) from P\(_1\) as shown in equations 30 and 31.

\[
2\text{Ado-}P-\text{O}^{18}-\text{PO}_4^{3-} \rightleftharpoons 2\text{Ado-}P-\text{O}^{18}_2 + 2\text{Pi} \quad (30)
\]

\[
\text{Ado-}P-\text{O}^{18}_2 + \text{ATP} \rightarrow \text{Ado-}P-\text{O}^{18} + \text{ADP} + \text{Pi} \quad (31)
\]

During subsequent cycles of oxidation, O\(^{18}\) will be transferred from the AMP to sulfate, as shown by equations 32 and 33.

\[
\text{SO}_4^{2-} + \text{Ado-}P-\text{O}^{18}_2 \rightleftharpoons \text{Ado-}P-\text{O}^{18} + \text{Pi} \quad (32)
\]

\[
\text{Ado-}P-\text{O}^{18} + \text{PO}_4^{3-} \rightarrow \text{Ado-}P-\text{O}^{18}_2 + \text{Pi} \quad (33)
\]

Santer (103) observed that 22 or 23% of the O\(^{18}\) present in the P\(_1\) was transferred to sulfate during the oxidation of thiosulfate. Considering that these results were obtained with whole cells, his data are in excellent agreement with the 25% value expected if sulfite were oxidized by the reaction sequence just described. Furthermore, the results of Santer can now be interpreted to indicate that the APS pathway or phosphorylative pathway is the major, if not sole, pathway for the oxidation of sulfite to sulfate in these organisms, since each sulfate formed must have been at some time covalently bonded through an oxygen atom to phosphate.

The mechanism of the transfer of O\(^{18}\) has been studied in cell-free extracts of *T. thioparatus* (Peck and M. P. Stulberg, unpublished data). In the presence of O\(^{18}\)-labeled P\(_1\), O\(^{18}\) is transferred to sulfate during the oxidation of thiosulfate (Table 9). However, owing to a dilution of the labeled P\(_1\) during the course of the reaction, it was not possible to calculate the amount of O\(^{18}\) that should have been observed in the sulfate produced. Consequently, each of the reaction cycles
required for the transfer of O\textsuperscript{18} from P\textsubscript{i} to sulfate was studied separately. For O\textsuperscript{18} to be transferred to sulfate, O\textsuperscript{18} must first be incorporated into AMP, as shown in equations 30 and 31. Sulfite, AMP, ferricyanide, and O\textsuperscript{18}-labeled P\textsubscript{i} were incubated with a high concentration of sodium fluoride and the ADP formed was isolated and degraded to AMP by myokinase. As shown in Table 9, the observed and calculated values for the O\textsuperscript{18} content of the AMP are in good agreement. The transfer of O\textsuperscript{18} from AMP to sulfate (indicated by equations 32 and 33) was demonstrated by preparing AMP labeled with O\textsuperscript{18} and observing the transfer of the O\textsuperscript{18} to the sulfate that was produced during the oxidation of thiosulfate in the presence of GSH. Again, good agreement was obtained between the calculated and observed value for the O\textsuperscript{18} content of the sulfate produced during the oxidation (Table 9). These results are in agreement with those obtained by Santer with whole cells of T. thioparus. They demonstrate conclusively that sulfite is oxidized to sulfate by the APS pathway in extracts as well as in whole cells. Further work is necessary to define more clearly the pathway(s) and intermediates involved in the oxidation of sulfide to sulfate.

Figure 5 indicates the most probable intermediates in the oxidation of sulfide and other reduced sulfur compounds to sulfate. A number of features of this scheme should be emphasized. First, elemental sulfur and polythionates (indicated as tetrathionate) are not indicated as being intermediates in this oxidation. Secondly, thiosulfate is an intermediate in the oxidation of sulfide, and the reaction is catalyzed by a sulfide oxidase. Thirdly, in the oxidation of thiosulfate to sulfate, the initial attack on the thiosulfate is the reductive cleavage of the thiosulfate to sulfide and sulfate. Lastly, sulfite can be oxidized to sulfate by a phosphorylative pathway, involving APS, and by a nonphosphorylative pathway, involving sulfite oxidase.

**E. The Energy Metabolism of the Thiobacilli**

The uniqueness of chemosynthesis as a mode of existence has prompted a search for the biochemical phenomena uniquely characteristic of chemosynthetic organisms. However, as data became available concerning the biochemistry of these organisms, particularly of the thiobacilli, the similarity of these forms of life to other forms, rather than their uniqueness, has been emphasized. Thus, the thiobacilli contain the usual amino acids (26) and phosphorylated intermediates (66) found in most tissues, and they fix carbon dioxide by a reaction sequence apparently common to all autotrophic organisms (65). The production of a high-energy sulfate at the substrate level from the oxidation of sulfate is a reaction that at present is physiologically unique to the thiobacilli. Although it is doubtful that this reaction plays an important role in the pattern of chemosynthesis, as exhibited by the thiobacilli, its role in the energy economy of these organisms should be investigated in detail. The inhibition of oxidation by carbon monoxide, cyanide, and azide (53), the stimulation of oxidation by DNP (131), and the presence of cytochromes (126) suggest that these organisms can generate biological energy by means of oxidative phosphorylation. On the other hand, the observation that T. thiopiridans does not contain cytochromes (122), the failure of Santer to observe an effect of DNP on the transfer of O\textsuperscript{18} from P\textsubscript{i} to sulfate (103), and the mere presence of this substrate sulfurylation suggest that oxidative phosphorylation may not be as important in the energy metabolism of these organisms as previously suspected. Considering the apparent complexity of the oxidation of sulfide to thiosulfate, it may be possible that the electrons produced

---

**Table 9. O\textsuperscript{18} transfer during the oxidation of thiosulfate in extracts of Thiobacillus thioparus**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>AMP</th>
<th>O\textsuperscript{18} compound added</th>
<th>Product isolated</th>
<th>Atoms % excess O\textsuperscript{18}</th>
</tr>
</thead>
<tbody>
<tr>
<td>S\textsubscript{2}O\textsubscript{3}\textsuperscript{-} + GSH</td>
<td>4</td>
<td>PO\textsubscript{4}\textsuperscript{3-}</td>
<td>SO\textsubscript{4}\textsuperscript{-}</td>
<td>Calculated 0.03 Observed 0.08</td>
</tr>
<tr>
<td>SO\textsubscript{2}\textsuperscript{+} + Fe(CN)\textsubscript{6}\textsuperscript{3-}</td>
<td>40</td>
<td>PO\textsubscript{4}\textsuperscript{3-}</td>
<td>ADP 1.66</td>
<td></td>
</tr>
<tr>
<td>S\textsubscript{2}O\textsubscript{3}\textsuperscript{-} + GSH</td>
<td>20</td>
<td>AMP</td>
<td>SO\textsubscript{4}\textsuperscript{-}</td>
<td>0.11 0.08</td>
</tr>
</tbody>
</table>

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during this oxidation are at too high a potential to participate in oxidative phosphorylation. This problem might be approached by studying the molecular growth yields of these organisms when grown on different substrates and determining if enough energy could be produced from this substrate sulfurylation to account for the observed growth of these cells. It would also be of interest to observe the changes in the enzymes concerned with the metabolism of thiosulfate when heterotrophically grown cells of *T. novellus* (102) are adapted to the chemosynthetic mode of life.

It is possible that the thiobacilli may be found to have other unique reactions involving sulfur-containing nucleotides. Sulfur-containing nucleotides other than APS can be formed during the oxidation of sulfite to sulfate and these nucleotides apparently cannot be phosphorylated by ADP-sulfurylase. It is tempting to speculate that such sulfur-containing nucleotides may be directly utilized by these cells in reactions involving nucleotide activation (e.g., amino acid activation). In this respect it is interesting that high-energy sulfate has about twice the energy of high-energy phosphate, and utilization of a high-energy sulfate rather than PPi for biosynthetic reactions could result in the conservation of most of the energy in the sulfate bond.

The postulations of Umbreit (128) concerning the separation of the generation of biological energy in the form of high-energy phosphate and its utilization for the fixation of carbon dioxide seem to be essentially correct, although perhaps not warranted by the data existing at the time (64). The stimulation of carbon dioxide fixation by the addition of ATP to cell-free extracts and the independent production of high-energy phosphate during the oxidation of thiosulfate has been demonstrated (65). Thus, it should now be possible to reproduce these experiments in a cell-free system. Reducing power, if needed, can probably be obtained from the oxidation of reduced sulfur compounds.

Although the thiobacilli may be considered as unique with regard to the specific mechanism involved in the production of biological energy, and possibly in other respects, they conform to the general pattern of autotrophic metabolism in that they have independent reaction sequences for producing high-energy phosphate and reducing power, and for utilizing these products for the fixation of carbon dioxide.

**VI. Literature Cited**

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