ENZYMATIC BASIS FOR ASSIMILATORY AND DISSIMILATORY SULFATE REDUCTION

H. D. PECK, JR.

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

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ABSTRACT

Peck, H. D., Jr. (Oak Ridge National Laboratory, Oak Ridge, Tenn.). Enzymatic basis for assimilatory and dissimilatory sulfate reduction. J. Bacteriol. 82: 933-939. 1961.—Two pathways for the reduction of sulfate to sulfite in bacteria have been previously described. The substrate for sulfate reduction by extracts of yeast is 3′-phosphoadenosine-5′-phosphosulfate (PAPS) and, in contrast, the substrate for sulfate reduction in extracts of Desulfovibrio desulfuricans is adenosine-5′-phosphosulfate (APS). The enzymes catalyzing these reductions have been termed PAPS-reductase and APS-reductase, respectively. Since yeasts are “assimilatory sulfate reducers”, i.e., reduce only enough sulfate to satisfy nutritional requirements for sulfur, and D. desulfuricans is a “dissimilatory sulfate reducer”, i.e., utilizes sulfate as its terminal electron acceptor in anaerobic respiration, the pathway of sulfate reduction was determined in 25 microorganisms to ascertain whether there is a correlation between the pathway of sulfate reduction and the physiological role of sulfate in the metabolism of bacteria. Assimilatory sulfate reducers reduced sulfate in the form of PAPS, and, with one exception, APS-reductase was found only in dissimilatory sulfate reducers. APS-reductase was also found in the Thiobacilli in high specific activity and is involved in the oxidation of reduced sulfur compounds to sulfite.

A large and diverse group of microorganisms reduces sulfate in the small amounts required for the synthesis of cellular material, as evidenced by the ability of these organisms to grow on sulfate as their sole source of sulfur. This small-scale reduction of sulfate has been termed assimilatory sulfate reduction (Postgate, 1959), by analogy with the classification of the types of bacterial nitrate reductions proposed by Kluyver (1953). In assimilatory sulfate reduction, H₂S is not usually produced in detectable amounts from sulfate, except as a transient intermediate. However, H₂S may be produced by these organisms in fairly large amounts from sulfur-containing amino acids, inorganic compounds more reduced than sulfate (Tanner, 1917), and from the organic material released upon death and autolysis of the organisms.

A much smaller group of microorganisms reduces sulfate in great excess of nutritional requirements and produces massive amounts of sulfide. Sulfate functions in the anaerobic respiration of these organisms as the terminal electron acceptor, and the amount of sulfide produced is proportional to the amount of H₂ or organic material dissimilated (Starkey, 1960). This large-scale reduction of sulfate to sulfide has been termed dissimilatory sulfate reduction (Postgate, 1959), also by analogy with bacterial nitrate reduction. The major organisms known to be dissimilatory sulfate reducers are species of Desulfovibrio and certain Clostridia.

Furthermore, recent studies on the mechanism of sulfate reduction in microorganisms indicate the existence of at least two different metabolic pathways for the reduction of sulfate (Peck, 1959; Wilson and Bandurski, 1958a; Hilz, Kittler, and Knappe, 1959). Yeasts carry out assimilatory sulfate reduction, as evidenced by their ability to grow on sulfate as their sole source of sulfur (Schultz and McManus, 1949). Cell-free extracts of yeast have been prepared that will reduce sulfate to sulfite and also sulfide (Wilson and Bandurski, 1958a; Hilz et al., 1959). This reduction requires, in addition to enzyme and sulfate, adenosine triphosphate (ATP) and reduced triphosphohydride nucleotide (TPNH) or reduced lipoic acid as electron donor (Hilz and Kittler, 1960). The reduction first involves the formation of the sulfur-containing nucleotide, 3′-phosphoadenosine 5′-phosphosulfate (PAPS),
by the combined action of two enzymes. ATP-sulfurylase catalyzes the formation of adenosine-5'-phosphosulfate (APS) and inorganic pyrophosphate from ATP and sulfite (equation 1). PAPS is then formed by the phosphorylation of APS in the 3' position in the presence of the enzyme, APS-kinase (equation 2; see Lipmann, 1958).

\[
\text{ATP} + \text{SO}_4^{2-} \xrightarrow{\text{ATP-Sulfurylase}} \text{APS} + \text{PP} \quad (1)
\]

\[
\text{APS} + \text{ATP} \xrightarrow{\text{APS-kinase}} \text{PAPS} + \text{ADP} \quad (2)
\]

Finally, PAPS is reduced to sulfite and 3',5'-diphosphoadenosine (PAP), as shown in equation 3.

\[
\text{TPNH} + \text{PAPS} \rightarrow \text{TPN}^+ + \text{PAP} + \text{HSO}_3^- \quad (3)
\]

This enzymatic activity will be referred to as PAPS-reductase.

*<i>D. desulfuricans</i>* exhibits dissimilatory sulfate reduction, and cell-free extracts of this organism have been prepared that reduce sulfate to sulfite and sulfide (Peck, 1959; Ishimoto, 1959). The reduction requires, in addition to sulfate and extract, ATP and an unknown electron donor that can be replaced by reduced methyl viologen. Extracts synthesize only APS from ATP and sulfite; and APS, rather than PAPS, is the form in which sulfate is reduced to sulfite. The reduction of APS to adenosine monophosphate (AMP) and sulfite is shown by equation 4.

\[
\text{APS} + 2e = \text{AMP} + \text{SO}_4^{2-} \quad (4)
\]

The enzyme catalyzing the reduction of APS has been termed APS-reductase.

The difference in the pathway of sulfate reduction in these two different physiological types of microorganisms that reduce sulfate suggested that the PAPS pathway might be characteristic of assimilatory sulfate reduction, and the APS pathway characteristic of dissimilatory sulfate reduction. Therefore, a survey of various microorganisms was conducted to ascertain whether the pathway of sulfate reduction could be correlated with the role of sulfate in the metabolism of the organism.

**Materials and Methods**

The following organisms were grown in Difco Antibiotic Medium 3 (Difco Laboratories, Detroit, Mich.) at 37 C on a rotary shaker: *Escherichia coli* Crooks (ATCC 8739), *Aerobacter aerogenes* (ATCC 8724), *Proteus mirabilis* (ATCC 9921), *P. vulgaris*, *Aeromonas punctata* (ATCC 11163), *Bacillus subtilis* (ATCC 6633), *B. polymyxa* (ATCC 10401), *B. megaterium*, and *Pseudomonas hydrophila*. *E. coli* was also grown in stationary culture on the same medium at 37 C. *Clostridium pasteurianum* (ATCC 6013), *Streptococcus faecalis* strain 10-C-1, *Staphylococcus aureus* (ATCC 10537), and *Leuconostoc mesenteroides* strain H77 were grown in Difco Antibiotic Medium 3 in stationary culture at 30 C. Baker's yeast was purchased from the National Yeast Corp. (Belleville, N. J.) and cell-free extracts prepared as described by Wilson and Bandurski (1958b). *Rhodopseudomonas palustris* (ATCC 11168) was grown photosynthetically on the medium of Kohlmiller and Gest (1951). *D. desulfuricans* Hildenborough was cultured at 30 C as described by Peck (1959); *Thiobacillus thioparus* (ATCC 8158) at 30 C as described by Starkey (1934); *T. thiooxidans* (ATCC 8065) at 30 C with sodium thiosulfate or elemental sulfur as energy source, as described by Starkey (1937); *T. denitrificans* (isolated by the enrichment culture technique by Earl Fisher, Jr.) at 30 C, as described by Baalsrud and Baalsrud (1954); *C. nigricans* (ATCC 7946) at 55 C, as described by Campbell, Frank, and Hall (1957); and *Vibrio choliniticus* at 37 C, as described by Hayward and Stadtman (1959). Other preparations were obtained from members of this laboratory.

Cells were washed once with distilled water or 0.025 M phosphate, pH 7, and cell-free extracts prepared by passing cell suspensions, in 0.05 M tris(hydroxymethyl)aminomethane (tris), pH 8, through a French pressure cell (American Instrument Co., Silver Spring, Md.). Extracts were centrifuged at 20,000 × g for 30 min and stored at -20 C. In the case of the thiobacilli, the elemental sulfur formed during growth was removed from suspension by low-speed centrifugation.

The activities of APS-reductase and PAPS-reductase were determined by measuring the acid-volatile S\(^{35}\) produced in reaction mixtures from S\(^{35}\)-labeled APS or PAPS during incubation with enzyme. Reaction mixtures were incubated in Warburg vessels with double side arms for 10 min under an atmosphere of helium with 0.05 ml of 2 N NaOH in the center well. The reaction mixture used for the assay of APS-reductase contained, in \(\mu\)moles: tris, pH 8, 100; ethyl-
enediaminetetraacetate, disodium salt (EDTA), 5; Na₂SO₄, 10; Na₂SO₃, 10; and APS³⁻, 0.05 (of various specific activities). The total volume of the reaction mixture was 0.5 ml and contained the different extracts diluted in such a manner that the concentration of substrate was not limiting. The electron donor for this reduction was methyl viologen (4 µmoles), reduced under helium in a side arm of the Warburg vessel by freshly prepared sodium hydrosulfite (5 µmoles). The reaction was initiated by the addition of reduced methyl viologen from one side arm and stopped by the addition of 0.1 ml of 10 N H₂SO₄ from the other side arm. After 60 min the 2 N NaOH in the center well was removed and the radioactivity determined, as described by Peck (1960). The reaction mixture used for the assay of PAPS-reductase contained, in µmoles: tris, pH 8, 100; EDTA, 5; Na₂SO₄, 10; glucose-6-phosphate, 5; and TPN, 0.3. The reaction mixture also contained, in a volume of 0.5 ml; enzyme in appropriate amounts; PAPS³⁻, 0.01 µmoles (of various specific activities); and Zwischenferment (Sigma Chemical Co., St. Louis, Mo.), 0.2 mg. Other conditions and additions were the same as described for the assay of APS-reductase. Protein was determined by the method of Lowry et al. (1951).

APS³⁻ was prepared by incubating ATP and SO₄²⁻ with a cell-free extract of *D. desulfuricans*, and isolated by adsorption on charcoal, elution with ammonical ethanol, and high-voltage paper electrophoresis, as previously described (Peck, 1960). APS was chemically synthesized by the method of Baddiley, Buchanan, and Letters (1957), and assayed after hydrolysis in 0.1 N HCl for 30 min at 37 C by measuring the appearance of AMP with adenylc deaminase, prepared by the method of Nikiforuk and Colowick (1955). PAPS was prepared by incubating ATP and SO₄²⁻ with partially purified sulfate-activating enzymes from yeast (Fraction III; Robbins and Lipmann, 1958), and isolated by adsorption on charcoal, elution, and paper electrophoresis. PAPS concentration was determined by its adsorption at 260 nm and by its radioactivity.

**RESULTS AND DISCUSSION**

*Assimilatory sulfate reduction.* The production of sulfide from sulfate is not commonly observed in microorganisms, even when they are reducing and incorporating sulfate into cellular material. The failure to detect sulfide under such conditions has been cited as evidence tending to rule out sulfide as an intermediate in the reduction of sulfate by these organisms. In the absence of sulfide production, other criteria can be employed to demonstrate that a given microorganism can reduce sulfide to the level of sulfide.

For example, the ability to grow in a medium with sulfate as the sole source of sulfur indicates that an organism can reduce sulfate to the level of sulfide and incorporate reduced sulfur compounds into cellular material. Inability to grow on sulfate as the sole source of sulfur may indicate that the organism is either unable to reduce sulfate to the level of sulfide or is unable to incorporate reduced sulfur compounds into cellular material. Another criterion, the incorporation of SO₄²⁻ 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 cells grown on complex media, the enzymes responsible for the reduction of sulfate may be repressed (Bourgeois, Wiane, and Lelouchier-Dagnelie, 1960; Mager, 1960) and negative results obtained. However, under other cultural conditions, it might be possible to demonstrate the presence of sulfate-reducing enzymes. A third criterion for assimilatory sulfate reduction seems to be the presence of the enzyme PAPS-reductase. The presence of this enzyme is shown in this paper to be correlated with the ability of a given organism to carry out assimilatory sulfate reduction.

*E. coli* can grow on a minimal medium, with sulfate as its sole source of sulfur, and also will incorporate SO₄²⁻ into sulfur-containing amino acids (Cowie, Bolton, and Sands, 1951). Mager (1960) reported the presence of PAPS-reductase in extracts of this organism, and indicated that the enzyme in this case is specific for reduced diphosphopyridine nucleotide (DPNH) rather than TPNH. As indicated in Table 1, PAPS-reductase is present in both aerobically and anaerobically grown cells of *E. coli*. Reduction of PAPS occurs when TPNH, reduced methyl viologen, or DPNH (not shown in Table 1) is employed as electron donor. The small amount of APS-reductase activity detected in extracts of aerobically grown cells probably represents conversion of APS to PAPS by APS-kinase (equation 2). In other experiments with aerobi-
### Table 1. Pathway of sulfate reduction in various physiological types of microorganisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>APS-reductase*</th>
<th>PAPS-reductase*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methyl viologen</td>
<td>Methyl viologen</td>
</tr>
<tr>
<td>1. Assimilatory sulfate reducers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli (grown aerobically)</td>
<td>0.4</td>
<td>2.4</td>
</tr>
<tr>
<td>E. coli (grown anaerobically)</td>
<td>0.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Yeast</td>
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<td>0</td>
</tr>
<tr>
<td>Aerobacter aerogenes</td>
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<td>5.6</td>
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<td>Proteus mirabilis</td>
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<td>0.1</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>Pseudomonas hydrophila</td>
<td>0</td>
<td>4.0</td>
</tr>
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<td>Aeromonas punctata</td>
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<td>Clostridium kluyveri</td>
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<td>C. pasteurianum</td>
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</tr>
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<td>Rhodopseudomonas spheroides</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>R. palustris</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Dissimilatory sulfate reducers</td>
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<td>0</td>
</tr>
<tr>
<td>Desulfovibrio desulfuricans</td>
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<td></td>
</tr>
<tr>
<td>Clostridium nigrificans</td>
<td>310</td>
<td>0.4</td>
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<tr>
<td>Vibrio cholnicus</td>
<td>907</td>
<td>0</td>
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<tr>
<td>3. Sulfur oxidizers</td>
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<tr>
<td>Thiobacillus thioparus</td>
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<td>T. thioides</td>
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<td>0</td>
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<td>T. thiooxidans</td>
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<td>0</td>
</tr>
<tr>
<td>Chromatium sp.</td>
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<td>0</td>
</tr>
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</table>

* Specific activity expressed as mmol of acid-volatile sulfur formed/hr/mg protein.

† No activity was observed with TPNH.

R. spheroides will grow photosynthetically when supplied with small amounts of organic sulfur in the form of biotin and thiamine. R. palustris does not require any preformed organic sulfur for photosynthetic growth (Hutner, 1946). Both organisms, therefore, seem to be capable of reducing sulfate. R. spheroides reduced PAPS to a limited extent only with reduced methyl viologen as electron donor, and R. palustris showed no reducing activity toward PAPS or APS. Ibanez and Lindstrom (1959) demonstrated the photoreduction of sulfate by chromatophores of *Rhodospirillum rubrum*. The failure to observe good reduction of these sulfur-containing nucleotides with these photosynthetic organisms may be due to the fact that no special precautions were taken to insure the integrity of chromatophores or the presence of light during the incubation period. However, the only sulfur-containing nucleotide reduced was PAPS.

Extracts of *L. mesenteroides*, *S. faecalis*, *S. aureus*, *Propionibacterium* spp., and *M. lacticiticus* did not reduce either APS or PAPS to form acid-volatile sulfur. The first three organisms have been shown to require preformed sulfur-amino acids for growth (Dunn et al., 1944; Rabinowitz and Snell, 1947; Fildes and Richardson, 1937) and are therefore assumed to be unable to reduce sulfate to the level of sulfate. It was not possible to obtain comparable data for *Propionibacterium* spp. and *M. lacticiticus*; however, because their nutritional requirements seem to be complex, it is quite probable that they are unable to reduce sulfate.

Extracts of *B. subtilis*, *B. polymyza*, and *B. megaterium* did not reduce either PAPS or APS when grown on a complex or minimal medium. These organisms will grow on defined media with sulfate as the sole source of sulfur (Knight and Proom, 1950). In addition, the formation of small amounts of radioactive sulfide from $^{35}S^{2-}$ has been observed with whole cells of *B. megaterium* (Bromfield, 1953). In these respects the bacilli 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 a difference in sulfate metabolism is at present unknown.

These limited observations indicate that the PAPS pathway of sulfate reduction is charac-
teristic of microorganisms that reduce sulfate solely for assimilatory purposes.

Dissimilatory sulfate reduction. Peck (1959) showed that *D. desulfuricans*, a dissimilatory sulfate reducer, reduces sulfate in the form of APS. The reaction, catalyzed by APS-reductase (equation 4), is reversible, does not require lipoic acid for activity, and is not inhibited by arsenite, as in PAPS-reductase. APS-reductase is further differentiated from PAPS-reductase by its inability to use TPNH or DPNH as electron donor and to utilize 3',5'-diphosphoadenosine as substrate in the reverse reaction, i.e., the formation of APS (Peck, 1961).

As shown in Table 1, extracts of *D. sulfuricans* do not reduce PAPS, and APS is reduced only with reduced methyl viologen as electron donor. The specific activity of APS-reductase is 10 to 20 times that observed for PAPS-reductase in assimilatory sulfate reducers. Other strains of *D. desulfuricans* seem also to utilize the APS-pathway of sulfate reduction (Peck, unpublished results; Ishimoto, 1959).

*C. nigrificans* is a dissimilatory sulfate reducer and a spore-forming thermophilic organism (Campbell et al., 1957). When cell-free extracts of this organism were supplemented with ATP and $^{35}$O$^{-}$ under an atmosphere of molecular hydrogen, they reduced sulfate to acid-volatile sulfur. Since the reduction was completely inhibited by MoO$_4^{2-}$, the first step in the reduction of sulfate by this organism seems to be the formation of APS by the enzyme ATP-sulfurylase. Both ATP-sulfurylase and ADP-sulfurylase are present in these extracts (Peck, unpublished results). As shown in Table 1, 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. A very small activity was observed with PAPS and may be due to the hydrolysis of PAPS to APS by a 3'-nucleotidase. In all respects, the mechanism of sulfate reduction in *C. nigrificans* seems identical to that in extracts of *D. desulfuricans*.

Another organism, *V. cholinicus*, has been isolated by Hayward and Stadtman (1959) and appears 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$^{-}$ during the oxidation of choline by extracts of this organism (Hayward and Stadtman, 1960). Extracts of *V. cholinicus* exhibit only APS-reductase with reduced methyl viologen and show no PAPS-reductase activity (Table 1). These results indicate that *V. cholinicus* is a dissimilatory sulfate reducer. Recently it has been claimed that *V. cholinicus* was incorrectly named and that the organism is actually *D. desulfuricans* (Senez, 1960).

Microorganisms that can utilize sulfate as their terminal electron acceptor in respiration (dissimilatory sulfate reducer) all utilize the APS-pathway of sulfate reduction; in contrast, organisms that reduce sulfate solely for nutritional purposes utilize the PAPS pathway of sulfate reduction. Thus the role of sulfate in the physiology of an organism manifests itself on the biochemical level in the different pathways of sulfate reduction. Exceptions to this scheme may eventually be found; however, from the evidence available, this idea seems to be justified.

Organisms that oxidize thiosulfate. Since inorganic sulfur compounds not only function as electron acceptors in anaerobic respiration but also function as electron donors for energy-yielding reactions, the pathway of sulfate metabolism was investigated in organisms that oxidize sulfur compounds. Some heterotrophic organisms oxidize reduced sulfur compounds; however, the oxidation does not seem to be intimately associated with the energy-yielding reactions of these microorganisms (van Niel, 1953). In contrast, the thiobacilli obtain all the energy required for growth from the oxidation of reduced sulfur compounds. The presence of APS-reductase in high specific activity and the absence of PAPS-reductase in cell-free extracts of *T. thioparus* has been reported (Peck, 1960). As shown in Table 1, this observation has been expanded to include *T. thiooxidans* and *T. dendrificans*. APS-reductase is also present in high specific activity, when determined by the AMP-dependent reduction of Fe(CN)$_6^{3-}$ by sulfite (Peck, 1961). The complete oxidation of thiosulfate to sulfate by whole cells of *T. thioparus* requires phosphate or arsenate, and, in the course of the reaction, O$^+$ is transferred from orthophosphate to the sulfate formed during the oxidation. A reaction sequence has been proposed for the oxidation of thiosulfate that presents an explanation of these observations and involves APS-reductase (Peck, 1960).

The *Thiorhodaceae* and *Chlorobacteriaceae*
constitute other groups of microorganisms that utilize sulfur compounds in the major energy-yielding reactions. In these organisms, however, the oxidation of sulfur compounds is closely coupled to photosynthesis, and little is known about their sulfur metabolism. Hendley (1955) has observed the reduction of sulfate to sulfide in whole cells of *Chromatium*, and the presence of APS-reductase in extracts of a *Chromatium* sp. has been observed (Table 1). The activity of this enzyme, however, was very low compared to that observed in the thiobacilli and dissimilatory sulfate reducers. Maximum activity may depend upon the presence of light and an intact photosynthetic apparatus (Ibanez and Lindstrom, 1959). Since extracts of *E. coli* and *P. vulgaris* did not contain APS-reductase when grown heterotrophically in the presence of thiosulfate, it appears that the formation of APS-reductase is not a general response of microorganisms to growth in the presence of thiosulfate. Rather, APS-reductase is thus far found only in microorganisms whose major energy-yielding reactions involve either the oxidation or reduction of inorganic sulfur compounds. The presence of APS-reductase in the thiobacilli and the demonstration of its reversibility (Peck, 1961) indicate that these organisms can produce a high-energy sulfate from the oxidation of reduced sulfur compounds; the sulfate then can be exchanged for phosphate to form ADP. The contribution of this reaction to the total energy production of these autotrophic organisms is at present unknown.

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