The pathway of sulfite oxidation in *Thiobacillus thiooxidans* JCM 7814 was investigated. Most of the activities of sulfite oxidation were in the membrane fractions of the cells. The addition of AMP (adenosine 5'-monophosphate) did not affect the activity. Two other reference strains of *T. thiooxidans* also gave the same results. Sulfite oxidation by *T. denitrificans* with an APS (adenosine 5'-phosphosulfate) pathway was evidently stimulated by the addition of AMP, while none of the effects occurred upon the addition to *T. novellus*, which has an AMP-independent pathway. Although *T. denitrificans* produced APS from sulfite and AMP, *T. thiooxidans* JCM 7814 neither consumed AMP nor produced APS. *T. thiooxidans* JCM 7814 had sulfite dehydrogenase activity. These results showed that *T. thiooxidans* JCM 7814 has the only AMP-independent pathway. The presence of cytochromes a, b, and c was also shown from the difference spectrum of the membrane fraction prepared from *T. thiooxidans* JCM 7814 cells.

*Thiobacillus thiooxidans* is an obligate chemoautotrophic bacterium that can use inorganic sulfur compounds as energy sources. These compounds are biologically oxidized and then finally produce sulfate. It has been known that sulfite is an intermediate compound positioned just before sulfate when sulfur compounds are oxidized. The two pathways are already known for the oxidation from sulfite to sulfate in Thiobacilli. One of them is an APS pathway depending on AMP; this pathway was found in *T. denitrificans* and *T. thioparus*. Another is an AMP-independent pathway appeared in *T. novellus*, *T. thioparus*, and *T. ferrooxidans*. Although the presence of an APS reductase system in *T. thiooxidans* has been reported by Peck, the sulfite oxidation by this organism has not been identified as the APS pathway or the AMP-independent pathway.

In this paper, sulfite oxidation in *T. thiooxidans* JCM 7814 was investigated to identify the pathway and to discover its relationship with cytochromes.

**Materials and Methods**

**Microorganism.** The strain used in this study was *Thiobacillus thiooxidans* JCM 7814, which was kindly supplied by Dr. H. Shiota of Iwate University. Five standard strains, *T. thiooxidans* IFO 13724, *T. thiooxidans* IFO 13701, *T. denitrificans* JCM 3689, *T. thioparus* JCM3859, and *T. novellus* IFO 12443, were used to compare with *T. thiooxidans* JCM 7814.

**Cultivation methods.** The strains of *T. thiooxidans* were cultivated in Silverman 9K medium containing 1% sodium thiosulfate instead of sulfur. Two ml of seed culture was inoculated into each of five 500 ml shaking flasks containing 100 ml of medium. The flasks were incubated at 30°C for 4 days on a reciprocal shaker. The culture medium (500 ml) was transferred into a jar fermentor containing 10 liters of the medium described above. The cultivation was done at 30°C for 4 days. The aeration rate was 0.5 vvm and agitation speed was 300 rpm. Culture pH was kept at 5.0 with titration of 5 NaOH to neutralize the sulfurous acid produced. Cells were harvested by centrifuging the culture medium at 9,000 rpm. The harvested cells were washed twice with chilled water and then resuspended in 0.05 M Na citrate-NaOH buffer (pH 6.0). The cells suspension was stored at 4°C until use.

*T. denitrificans* JCM 3689 was cultivated in S-8 Thiobacillus medium. The cultivation was done anaerobically in a jar fermentor containing 5 liters of the medium at 30°C for 3 days. The pH was kept at 7.0. The agitation speed was 100 rpm.

*T. thioparus* JCM 3859 was cultivated in Thiobacillus No. 1 medium without agar. The cultivation was done in a jar fermentor containing 5 liters of the medium at 30°C for 3 days. The pH was kept at 6.8. The aeration rate was 0.5 vvm and agitation speed was 300 rpm.

*T. novellus* IFO 12443 was cultivated in a heterotrophic medium consisting of 10 g of Na2S2O3·5H2O, 1.0 g of KH2PO4, 1.0 g of NH4Cl, and 10 g of thioglycollate medium (Difco Laboratories, Detroit, U.S.A.) without agar in one liter of tap water. The pH of this medium was adjusted to 7.2. The cultivation was done in a jar fermentor containing 4 liters of the medium at 30°C for 2 days. The aeration rate was 0.5 vvm and agitation speed was 300 rpm.

**Method of cell fractionation.** A modification of the preparation method described by Tano et al. was used. The washed cells (300 mg dry wt./20 ml) were suspended in 0.05 M Tris-HCl buffer (pH 7.0) containing 0.2 mM EDTA Na2·2H2O. The cell suspension was disrupted by sonication (20 KHz) at 0°C for 60 min. The sonicate was centrifuged to remove residual cells and debris at 15,000 × g for 30 min at 4°C. The supernatant was further centrifuged at 144,000 × g for 60 min at 4°C to obtain the supernatant fraction (soluble fraction) and the precipitate (membrane fraction). The membrane fraction was resuspended in 10 ml of 0.05 M Tris-HCl buffer (pH 7.0) containing 0.2 mM EDTA Na2·2H2O. The soluble fraction and the membrane fraction were dialyzed against 0.05 M potassium phosphate buffer (pH 8.0) containing 0.2 mM EDTA Na2·2H2O. The dialyzed fractions were used to assay enzyme activities.

**Assay of sulfite oxidation.**

**Oxygen uptake method.** The change of dissolved oxygen concentration in a reaction cell was measured with a biological oxygen monitor (Rank Brothers Ltd., Cambridge, UK) at 30°C. The reaction mixture consisted of 1.5 ml of distilled water, 0.5 ml of 0.05 M potassium phosphate buffer (pH 8.0) containing 0.2 mM EDTA Na2·2H2O, 0.5 ml of 0.05 M Na2SO4 containing 5 mM EDTA Na2 in the reaction cell. After the concentration of oxygen in the reaction cell reached saturation by aeration for 1 min, the reaction was started by adding Na2SO4. Activity is expressed as μmol-O2/hr/mg-protein.

**Colorimetric method:** A reduction of ferricyanide was measured with a change of absorbance at 420 nm. The reaction mixture contained 1.0 ml of distilled water, 1.1 ml of potassium phosphate buffer (pH 8.0), 0.5 ml of 0.01 M K3[Fe(CN)6], and 0.2 ml of enzyme in a cuvette (1 cm path) equilibrated at 30°C. The reaction was started by adding 0.2 ml of 0.05 M Na2SO4 containing 10 mM EDTA Na2 to the reaction mixture. The change of absorbance at 420 nm was measured. The solution was kept at 30°C for 10 min. The solution was mixed with 0.5 ml of 3.0 mM Na2HPO4 containing 1.0 ml of distilled water instead of 0.05 M Na2SO4. The optical density at 420 nm was measured after 10 min. Activity was expressed as μmol-ferricyanide reduced/hr/mg-protein.

**Protein measurement.** Protein was measured by Lowry's method. The standard protein used was bovine serum albumin.

**Thin-layer chromatography.** A reaction mixture to detect AMP, APS,
ADP, and ATP contained: 0.05 M Tris–HCl buffer (pH 8.0), 1.1 ml; 0.01 M K_{2}Fe(CN)_{6}, 0.5 ml; 5 mM Na-AMP, 1.0 ml; enzyme, 0.2 ml; and 0.05 M Na_{2}SO_{4} in 10 mM EDTA Na_{4}, 0.2 ml. The reaction mixture was incubated for 60 min at 30°C. Methanol containing 1 N NH_{4}OH was added to the mixture to stop the reaction and then it was centrifuged to remove denaturated protein. The supernatant was applied and developed on TLC to detect AMP, APS, ADP, and ATP. The samples (1–5 μl) were spotted onto a cellulose thin-layer plate (Merck No. 5787). The developing solvent was n-butanol, acetone, acetic acid, 5% NH_{4}OH, and distilled water (4:5:1.5:1:1:2). After the development, the plate was dried and then nucleotides were detected by irradiation with ultraviolet light.

Spectrometry of membrane fraction. Difference spectra were recorded with a refractometer method using a 1-cm cuvette in a Shimadzu model MPS-2000 spectrophotometer. The reaction mixture contained 15 mg of protein from the membrane fraction, 50 μmol of Tris–HCl buffer (pH 8.0), 50 μmol of sodium sulfite, 10 μmol of EDTA in a total volume of 3.0 ml. The reference cell (oxidized preparation) had hydrogen peroxide in the reaction mixture instead of sodium sulfite.

Chemicals. APS-Na_{2} was purchased from Sigma Chemical Co., St. Louis, U.S.A. AMP-Na, ADP-K, ATP-Na_{3} were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Thin-layer cellulose plate (No. 5787) was supplied by Merck & Co., Rahway, U.S.A.

Results and Discussion

Distribution of sulfite oxidation in T. thiooxidans JCM 7814 cells

Two methods for assay of the oxygen uptake rate and colorimetry were used to identify both the distribution of sulfite oxidation activity in cells and the effects of addition of AMP on the oxidation activity. The strains of IFO 13724 and IFO 13701 were also used for reference. The results showed that the membrane fraction had 10 to 20 times higher activity, depending on the assay methods, than the soluble fraction regardless of whether AMP was added or not (Table I). Low but evident activity was detected in the soluble fractions of the three strains; this differed from the previous results, which showed the activities could not be detected at all in soluble fractions of T. thiooxidans. The results shown in Table I indicated that T. thiooxidans JCM 7814 could oxidize sulfite independently of AMP, and that most of the sulfite oxidation was in the membrane fraction.

Effects of AMP addition on sulfite oxidation activity in other Thiobacillus species

Two pathways of sulfite oxidation by Thiobacillus are known. One is the adenosine 5'-phosphosulfate pathway found in T. denitrificans, the other is an AMP-independent pathway in T. novellus. T. thioparus has both the pathways. This was confirmed by using type cultures of the three strains. The sulfite-oxidizing activity was measured by the ferricyanide reduction method. When AMP added to soluble fractions of T. denitrificans and T. thioparus, the sulfite-oxidizing activities increased to 40-fold and 7-fold, respectively (Table II). These observations indicated the presence of APS reductase (AMP-dependent reduction of ferricyanide by sulfite) in T. denitrificans and T. thioparus. On the other hand, neither fraction of T. novellus was activated by AMP addition. From these results, it was ascertained that the fractions having the AMP pathway in sulfite oxidation were activated by AMP while the fractions containing the AMP-independent pathway were not activated by AMP.

These results shown in Tables I and II indicated that T. thiooxidans JCM 7814 used the AMP-independent pathway to oxidize sulfite.

<table>
<thead>
<tr>
<th>Table I. Distribution of Sulfite-oxidizing Enzyme in Membrane and Soluble Fractions from Three Strains of T. thiooxidans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane fraction</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>JCM 7814 + AMP</td>
</tr>
<tr>
<td>IFO 13724 + AMP</td>
</tr>
<tr>
<td>IFO 13701 + AMP</td>
</tr>
<tr>
<td>AMP</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table II. Effects of AMP Addition of Ferricyanide Reduction in Membrane and Soluble Fractions from T. denitrificans, T. thioparus, and T. novellus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane fraction</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>T. denitrificans + AMP</td>
</tr>
<tr>
<td>JCM 3869 − AMP</td>
</tr>
<tr>
<td>T. thioparus + AMP</td>
</tr>
<tr>
<td>JCM 3859 − AMP</td>
</tr>
<tr>
<td>T. novellus + AMP</td>
</tr>
<tr>
<td>IFO 12443 − AMP</td>
</tr>
</tbody>
</table>

![Fig. 1. Densitometric Patterns of Production of Adenosine 5'-Phosphosulfate during Sulfite Oxidation by the Membrane Fraction from T. thiooxidans JCM 7814 (A) and by the Soluble Fraction from T. denitrificans (B) on Thin-layer Chromatography.](image-url)
Detection of adenosine 5'-phosphosulfate on thin-layer chromatography

To confirm the sulfite oxidation pathway in *T. thiooxidans* JCM 7814, the association of APS production and AMP consumption was investigated. *T. denitrificans* has the APS pathway, thus it produces APS by APS reductase and consequently follows to ADP by ADP sulfurylase. The soluble fraction of *T. denitrificans* JCM 3869 was incubated with the reaction mixture described in Materials and Methods. The results are shown in Fig. 1. From this figure, it can be seen that the APS production started immediately after the incubation and the product was consecutively transferred to ADP, while AMP did not change at all when it was incubated with the membrane fraction of *T. thiooxidans* JCM 7814. None of the products were detected from the reaction mixture even after 1 hr of incubation. A similar result was observed when AMP was added to the soluble fraction of *T. thiooxidans* JCM 7814 (data not shown). These results indicated that the sulfite oxidation of *T. thiooxidans* JCM 7814 was AMP-independent.

Sulfite dehydrogenase activity and cytochromes of membrane fraction from *T. thiooxidans* JCM 7814

From the results obtained above, it was suggested that *T. thiooxidans* JCM 7814 should have a sulfite dehydrogenase, which shows sulfite oxidation coupling with cytochrome c oxidase. The sulfite dehydrogenase activity was measured by a modification of the method of Charles and Suzuki. The results indicated that the membrane fraction of *T. thiooxidans* JCM 7814 had the activity with cytochrome c reduction (data not shown). AMP-independent oxidation of sulfite in Thiobacillus is tightly coupled with electron transport by cytochromes, so the presence of cytochromes in the membrane fraction was investigated by measuring a difference spectrum.

As shown in Fig. 2, sulfite oxidation of *T. thiooxidans* JCM 7814 was followed by the reduction of cytochromes. The difference spectrum of the cytochromes in the membrane fraction had peaks at 523, 552, and 607 nm, with shoulders at 531 and 565 nm. When the membrane fraction was reduced with sulfite or dithionite, peaks and shoulders of both spectra appeared at the same wavelength. The peak at 607 nm corresponded with a type cytochrome and the peaks at 523 and 552 nm corresponded with the c type cytochrome. The shoulders at 530 and 565 nm corresponded with b type cytochrome. Therefore the spectrum suggested that an electron from sulfite dehydrogenase was transferred to cytochrome c as an electron acceptor during sulfite oxidation.

The results presented here and the considerations mentioned in each section indicated that sulfite oxidation in *T. thiooxidans* JCM 7814 is conducted by sulfite dehydrogenase. This conclusion is consistent with the following observations: (1) the addition of AMP did not affect the activity, (2) the strain neither consumed AMP nor produced APS, and (3) the reduction of cytochromes was associated with sulfite oxidation.

References