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# SULFITE OXIDASE FROM *MERLUCCIUS PRODUCTUS* *

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## Summary

Sulfite oxidase (sulfite:oxygen oxidoreductase, EC 1.8.3.1) was purified 482-fold from liver of the Pacific hake *Merluccius productus*. The molecular weight of the enzyme was found to be 120 000 by gel exclusion chromatography on Sephadex G-100. Electrophoretic analysis on sodium dodecyl sulfate (SDS)-polyacrylamide gel revealed that the enzyme was composed of two subunits whose molecular weight was estimated to be 60 000. The pH optimum of the enzyme was 8.7; $K_s$ for sulfite, $2.5 \cdot 10^{-5}$ M; and that for cytochrome *c*, $3.6 \cdot 10^{-7}$ M. The enzyme elicited an EPR signal at $g = 1.97$ characteristic of pentavalent molybdenum. Colorimetric analysis also disclosed that the enzyme contained 2 mol each of heme and molybdenum per mol of protein.

The fish liver homogenate in isotonic sucrose solution was fractionated by differential centrifugation into nuclei, mitochondria, microsomes and supernatant ($100\,000 \times g$). The major portion of sulfite oxidase activity was found in mitochondria. The sulfite oxidase activity was markedly high in liver and kidney, as compared with that in heart, spleen, muscle, gill and eye.

## Introduction

As reported previously [1], my particular interest in the enzyme sulfite oxidase stems from the fact that waste liquors from paper pulping operations contain sulfite which acts adversely on aquatic ecosystems. Since most of the sulfite ingested by various test animals is metabolized to sulfate by the action

* Taken in part from the doctoral dissertation of Y.O., University of Washington, 1977.

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of sulfite oxidase [2,3], the occurrence of the enzyme in fish would serve as a detoxifying device for them.

I undertook a study designed to demonstrate and characterize sulfite oxidase in fish. Characterization of sulfite oxidase will provide impetus for further studies relating to inducibility and/or variability of the enzyme in fish at an increased sulfite concentration in natural waters, and it will provide a basis for understanding the mechanism by which sulfite may be detoxified by intact fish.

## Materials and Methods

### *Materials*

Fresh or frozen livers from the Pacific hake *Merluccius productus* or the Brown rockfish *Sebastes auriculatus*, which was caught off Puget Sound, WA, were used for sulfite oxidase assay.

### *Purification procedures*

Sulfite oxidase was purified by a modification of the method of Cohen et al. [4].

100 g of frozen fish livers were chopped and homogenized for 1 min in a Waring blender with 500 ml of cold acetone. The homogenate was placed in 2000 ml of cold acetone, stirred for 5 min and then collected on a Buchner funnel. After being washed with cold acetone on the funnel, the residue was dried in vacuo.

50 g of acetone powder was stirred for 30 min at 25°C with 1000 ml of 0.05 M phosphate buffer, pH 8.0. The mixture was centrifuged at 10 000 rev./min and at 4°C for 30 min. Solid $(NH_4)_2SO_4$ was added to the supernatant solution to 45% saturation, while the pH was maintained at about 8.0 with 1 M KOH. The precipitate was collected by centrifugation, dissolved in 0.05 M phosphate buffer, pH 8.0, and dialyzed against this buffer.

The sulfate-free protein solution was adsorbed on a DEAE-cellulose (Whatman DE-52) column (1.5 × 20 cm) equilibrated at 4°C with 3% saturated $(NH_4)_2SO_4$ in 0.05 M Tris-HCl, pH 8.0, and a linear gradient elution was applied with $(NH_4)_2SO_4$ from 3 to 10% saturation in 0.05 M Tris-HCl, pH 8.0.

The enzyme fractions eluted from the DEAE-cellulose column were pooled, precipitated with saturated $(NH_4)_2SO_4$ and dialyzed against 0.05 M Tris-HCl, pH 8.0. The sulfate-free enzyme solution was further purified by gel filtration on a Sephadex G-200 column (1.0 × 80 cm).

### *Assay procedures*

Sulfite oxidase activity was assayed spectrophotometrically by observing ferricyanide reduction at 25°C.

A 0.4 ml portion of enzyme solution was added to 4.6 ml of reaction mixture containing 0.4 mM $K_3Fe(CN)_6$ and 0.1 mM EDTA buffered at pH 8.7 with 0.05 M Tris-HCl. After a recorder was set to zero, 0.02 ml of 0.1 M sodium sulfite was added to the mixture and a decrease in absorbance at 420 nm followed for 10 min. In some cases, 10 $\mu$M ferricytochrome *c* was used instead of ferricyanide and an increase in absorbance at 550 nm followed as described above.

1 unit of enzyme represents the amounts that caused an absorbance change of 0.10/min.

*EPR experiments*

*Purified sulfite oxidase.* An electron paramagnetic (EPR) spectrometer Varian model E-3 was used to characterize pentavalent molybdenum ($Mo^{5+}$) in the enzyme.

0.2 ml of purified sulfite oxidase (2.3 mg/ml) in 0.05 M Tris-HCl, pH 8.0, was mixed with 0.02 ml of 0.1 M sodium sulfite in a quartz tube (3 mm inner diameter, 12 cm long) and frozen in liquid nitrogen. EPR measurement was performed at −100°C by using a modulation amplitude of 12.5 G; the microwave power was 20 mW. Spectra were recorded at a 1-s recorder time constant with a gain of $1 \cdot 10^4$ and with a magnetic field-scan rate of 50 G/min.

*Detection of EPR signal in various fish organs.* EPR signal of molybdenum in various organs taken from Pacific hake or Brown rockfish was examined by the method of Kessler et al. [5].

Liver, heart, spleen, kidney, gill, eye and white muscle were used. Each tissue, after being weighed, was homogenized with an equal volume of 0.25 M sucrose buffered at pH 8.0 with 0.05 M Tris-HCl. A 0.2 ml portion of the homogenate was frozen in an EPR tube and analyzed as mentioned above.

## Results

*Purification of fish liver sulfite oxidase.* The results of each purification step are summarized in Table I. 50 g of acetone powder, corresponding to approx. 500 g fish livers, was extracted with 1000 ml of 0.05 M phosphate buffer, pH 8.0. The buffer extract contained 40.5 mg protein/ml; the specific activity was 3.0 units/mg. Activity of the enzyme in acetone powder was stable at −20°C over 3 months.

The phosphate buffer extract was fractionated with 45% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 0.05 M Tris-HCl buffer, pH 8.0, and dialyzed overnight against this buffer. The resulting solution of 156 ml

TABLE I

PURIFICATION OF FISH-LIVER SULFITE OXIDASE

| Step | Volume (ml) | Protein * (mg) | Activity (units) | Specific activity (units/mg) | Yield (%) | Purification fold |
|---|---|---|---|---|---|---|
| Phosphate buffer extract from acetone powder | 1 000 | 40 500 | 121 400 | 3.0 | 100 | 1.0 |
| 45% $(NH_4)_2SO_4$ precipitate | 156 | 4 320 | 56 600 | 13.1 | 47 | 4.4 |
| Pooled fractions from DEAE-cellulose | 4.0 | 50 | 38 800 | 776 | 32 | 259 |
| Pooled fractions from Sephadex G-200 | 2.0 | 4.7 | 6 800 | 1 447 | 5.6 | 482 |

* Protein determined by the method of Lowry et al. [32].

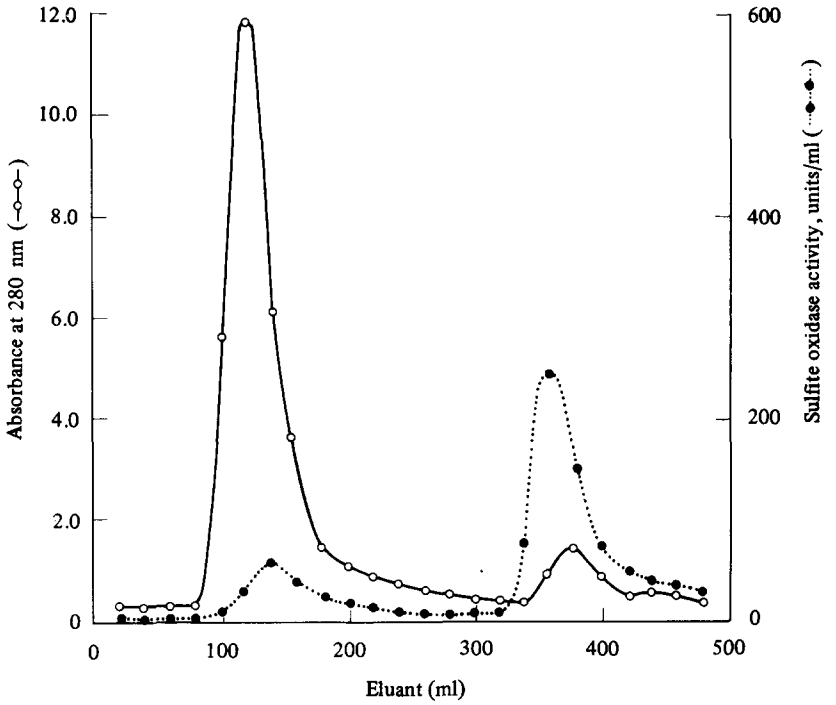


Fig. 1. Elution diagram of fish-liver sulfite oxidase from a DEAE-cellulose column.

contained 27.6 mg protein/ml. The specific activity was 13.1 units/mg; yield, 47% and purification factor, 4.4.

The preparation was placed on a DEAE-cellulose column and a linear gradient elution from 3 to 10% $(NH_4)_2SO_4$ saturation in 0.05 M Tris-HCl, pH 8.0, was applied (Fig. 1). The active fractions were pooled, concentrated with saturated $(NH_4)_2SO_4$ and dialyzed against 0.05 M Tris-HCl, pH 8.0. The dialysate of 4.0 ml contained 12.5 mg protein/ml. The specific activity was 776 units/mg; yield, 32% and purification factor, 260.

The enzyme solution was further passed through a Sephadex G-200 column (Fig. 2). The pooled fractions of the enzyme were concentrated and dialyzed as mentioned above. The final solution of 2.0 ml contained 2.35 mg protein/ml. The specific activity was 1447 units/mg; yield, 5.6% and purification factor, 482.

*Purity.* Purity of the enzyme was tested by polyacrylamide gel electrophoresis [6]. When the gel was stained for protein with 0.2% amido black or with 0.25% Coomassie brilliant blue, it exhibited one major band along with one minor band. Densitometric analysis of the gel indicated that the major band accounts for 85% of the total protein.

*Molecular weight.* Molecular weight of the enzyme was determined by gel-filtration technique on Sephadex G-100 [7]. The molecular weight was estimated as 120 000 when the elution volume was plotted against the logarithm of molecular weights of standard proteins.

Determination of the molecular weight of the enzyme was also made by

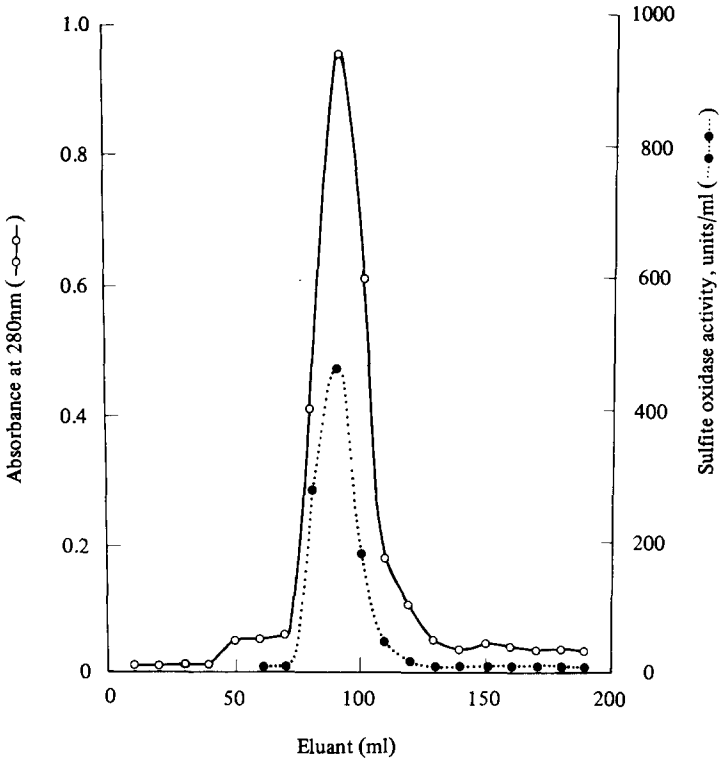


Fig. 2. Elution diagram of fish-liver sulfite oxidase from a Sephadex G-200 column.

electrophoretic technique on SDS-polyacrylamide gel [8]. The molecular weight of the polypeptide chain was estimated as 60 000 from a plot of the electrophoretic mobility against the logarithm of known molecular weights of polypeptide chains. It follows that the enzyme is composed of two subunits.

*Identification and quantitation of heme.* Fish-liver sulfite oxidase exhibited an absorption maximum of protein at 286 nm and a Soret band 410 nm (Fig. 3). When the enzyme solution was reduced with sulfite, the absorption maximum at 410 was shifted to 425 nm. Reduction also caused the appearance of new absorption maxima at 525 and 555 nm. Absorption bands of the reduced pyridine hemochromogen prepared from the enzyme appeared at 525 and 557 nm.

By use of the absorption band at 557 nm for pyridine hemochromogen [9], the heme content was quantitated to be 16.2 nM/mg enzyme, or 1.94 mol/mol enzyme.

*Identification and quantitation of molybdenum.* EPR analysis revealed that fish-liver sulfite oxidase contains pentavalent molybdenum as its component (Fig. 4). The enzyme, when reduced with sulfite, elicited an EPR signal at $g = 1.97$. The height of signal was enriched by an increase of enzyme activity. Response of the signal at pH 8.0 was increased about 1.7-times that at pH 7.0.

The molybdenum content was quantitated by colorimetric method [10] to be 16.6 nM/mg enzyme, or 1.99 mol/mol enzyme.

*Effect of pH.* The effect of variation of pH on enzyme activity was studied

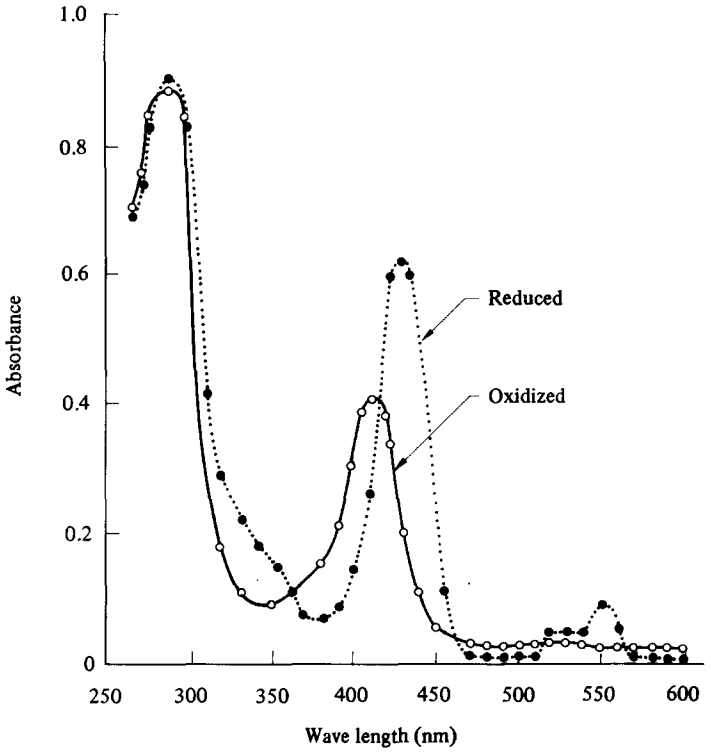


Fig. 3. Absorption spectra of fish-liver sulfite oxidase in its oxidized and reduced forms. The spectrum of the oxidized form (○———○) was obtained from an enzyme solution of 0.26 mg/ml in 0.05 M potassium phosphate buffer, pH 8.0. The spectrum of reduced form (●· · · · · ·●) was derived when sodium sulfite was added to a concentration of 0.5 mM.

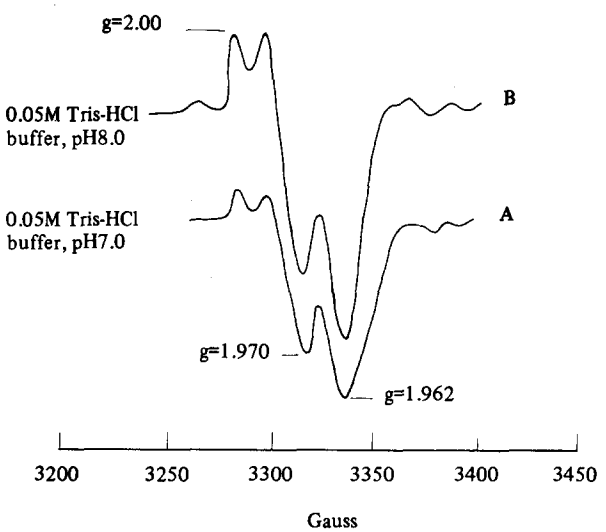


Fig. 4. EPR analysis of purified fish-liver sulfite oxidase. The enzyme (2.3 mg/ml) in 0.05 M Tris-HCl at pH 7.0 (A) and at pH 8.0 (B) was reduced with 0.01 M sodium sulfite.

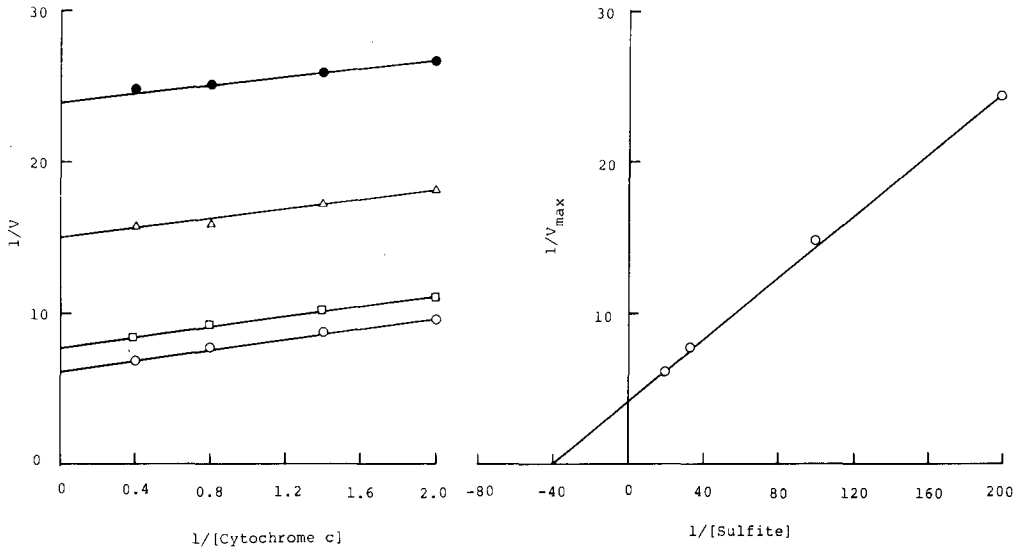


**Fig. 5. Kinetics of fish-liver sulfite oxidase. *V* values were determined from Lineweaver-Burk plots using the indicated concentration of cytochrome *c* (μM) with various sulfite concentrations in 0.05 M Tris-HCl at pH 8.7 and at 25°C (see Fig. 6). Enzyme activity was expressed as mol sulfite oxidized per min per μmol of enzyme active site.**

**Fig. 6. Kinetics of fish-liver sulfite oxidase. The ordinate intercepts of the lines in Fig. 5 were plotted as a function of the reciprocal of mmol concentration of sulfite. $K_S$ for sulfite was found to be $2.5 \cdot 10^{-5}$ M.**

in Tris-HCl buffer and in phosphate buffer. The maximal activity was attained at pH 8.7. At pH 7.0, the activity decreased to less than half of the maximal. The enzyme was inactivated by bringing the pH below 5.0.

*Kinetics.* The rate of cytochrome *c* reduction with varied sulfite concentration was investigated. The ordinate intercepts of the lines in Fig. 5 were plotted as a function of the reciprocal of the sulfite concentration. From the resulting line in Fig. 6, $K_s$ for sulfite was found to be $2.5 \cdot 10^{-5}$ M. In the same manner, $K_s$ for cytochrome *c* was found to be $3.6 \cdot 10^{-7}$ M. A Lineweaver-Burk plot yielded parallel lines of identical slope, regardless of the concentration of the fixed substrate. In accordance with the observed kinetics (ping-pong kinetics), enzymic oxidation of sulfite in the presence of cytochrome *c* (Cyt *c*) can be written as:

$$E \xrightarrow{SO_3^{2-} + 2\,H^+} (EH_2 - SO_3) \xrightarrow[H_2O]{SO_4^{2-} + 2\,H^+} (EH_2) \xrightarrow{2\ Cyt\ c^{3+}} (EH_2 - 2\ Cyt\ c^{3+}) \xrightarrow{2\ Cyt\ c^{2+} + 2\,H^+} E$$

*Heat stability.* There was no significant activity loss of the enzyme at 25°C for 10 min under assay conditions used. However, the activity was strikingly decreased by raising temperature above 50°C. Incubation of the enzyme at 50°C for 5 min caused about 94% activity loss. The enzyme was inactivated almost completely by heating at 56°C for 3 min (Fig. 7).

*Inhibition studies.* Fish-liver sulfite oxidase was inhibited strongly by heavy

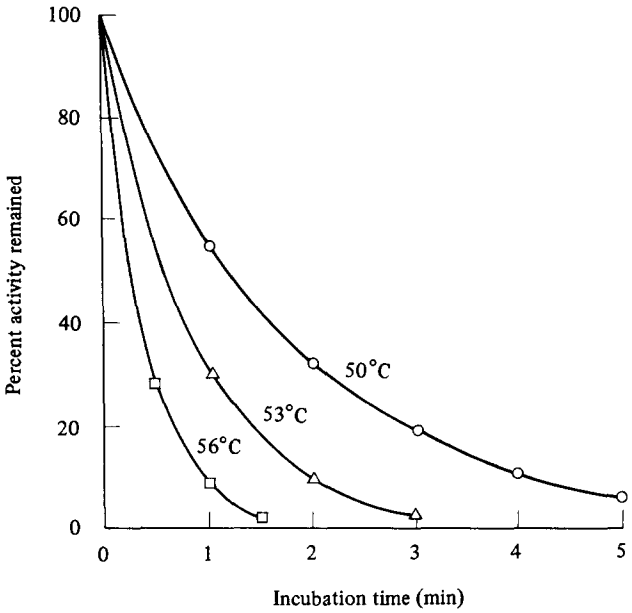


Fig. 7. Effect of temperature on inactivation of fish-liver sulfite oxidase. Reaction was carried out in 5 ml of 0.05 M Tris-HCl containing 0.4 mM ferricyanide and 0.4 mM sodium sulfite at pH 8.7 and the indicated temperatures. At zero time 47 μg of enzyme was added to the mixture and the reduction of ferricyanide was observed for 5 min.

metal cations [1]. Anions such as sulfate also inhibited the enzyme system. With ferricyanide as the electron acceptor, the $K_i$ for sulfate with respect to sulfite was found to be 0.026 M and $K_i'$ to be 0.015 M; the $K_i$ for sulfate with respect to ferricyanide 0.020 M and $K_i'$ 0.020 M. The type of inhibition by sulfate was uncompetitive toward sulfite but noncompetitive toward ferricyanide.

The use of a low concentration of sodium cyanide (0.1 mM) did not affect enzyme activity, although the presence of 10 mM cyanide in assay system caused gradual enzyme inactivation. Approx. 30% of the activity was lost at 25°C for 10 min of incubation.

*Subcellular distribution of sulfite oxidase.* Fish liver was homogenized in isotonic sucrose solution and fractionated by differential centrifugation [11] into nuclei, mitochondria, microsomes and soluble fraction (100 000 × *g*). The acetone powder extract prepared from each fraction was assayed for sulfite oxidase (Table II). The major portion of the activity was observed associated

TABLE II

SUBCELLULAR LOCATION OF SULFITE OXIDASE IN FISH LIVER

| Location | Protein * (mg/ml) | Activity (units/ml) | Specific activity (units/mg) |
|---|---|---|---|
| Nuclei | 3.8 | 1.5 | 0.40 |
| Mitochondria | 6.3 | 16.3 | 2.6 |
| Microsomes | 22.7 | 25.0 | 1.1 |
| Soluble fraction (100 000 × *g*) | 9.2 | 3.5 | 0.38 |

* For protein determination see Table I.

TABLE III

RELATIVE ACTIVITY OF SULFITE OXIDASE IN VARIOUS FISH ORGANS

| Organ | Relative activity * |
|---|---|
| Liver | 1.00 |
| Kidney | 0.80 |
| Gill (lamellae) | 0.41 |
| Muscle | 0.40 |
| Heart | 0.22 |
| Eye (retina and fluids) | 0.21 |
| Spleen | 0.10 |

* The activity was calculated by taking the EPR signal amplitude elicited by fish liver as 1.

with mitochondria; some with microsomes. The activity was very low in nuclear and soluble fractions.

*Sulfite oxidase activity in various fish organs.* Sulfite oxidase activity in various fish organs was tested by EPR spectroscopy (Table III). The activity was remarkably high in liver and kidney, as compared with that in heart, spleen, muscle, gill and eye.

## Discussion

Purified fish-liver sulfite oxidase exhibited absorption of a Soret band characteristic of a hemoprotein (Fig. 3). The molar extinction coefficient ($\epsilon$) of this band was calculated to be 97 600. On reduction of the enzyme with sulfite, new peaks appeared at 425 nm ($\epsilon$ = 145 000), 525 nm ($\epsilon$ = 12 000) and 555 nm ($\epsilon$ = 23 600). These absorption bands are closely related to those of protoporphyrin IX.

The reduced form of the enzyme also elicited EPR signals of molybdenum, viz., doublets at $g$ = 2.00 and $g$ = 1.97 (Fig. 4). Such doublets are believed to be induced by a splitting effect of the dissociable proton on the ligand at the molybdenum center [12].

Fish sulfite oxidase is similar to the mammalian, but is different from the bacterial enzyme in its chemical properties, such as molecular weight, kinetics, pH optimum and inhibition by various substances [1].

High activity of the enzyme in fish liver and kidney (Table III) indicates that both organs are functional in sulfite metabolism. In liver, sulfite seems to be produced from cysteine via cysteine sulfenate, cysteine sulfinate and $\beta$-sulfinylpyruvate through catalysis of transaminase and desulfinase [13]. The resulting sulfite is then oxidized to sulfate by tissue sulfite oxidase. The sulfate is excreted in urine from kidney mostly as inorganic sulfate.

Analysis of fish eyes showed that the lens fluid and retina are low in sulfite oxidase activity (Table III). Lens is recognized to be one of the richest sources of glutathione. Glutathione also exists as *S*-sulfoglutathione. *S*-Sulfoglutathione reacts with reduced glutathione (GSH) to yield sulfite and oxidized glutathione (GSSG) [14]. The increased level of sulfite and GSSG will promote production of *S*-sulfocysteine and cystine from cysteine.

Low sulfite oxidase activity was also observed in fish heart. It appears that

incorporation of sulfite into the blood stream can make a fish highly susceptible to hypoxia by producing sulfhemoglobin in the blood, accompanied by cyanosis. Under this circulatory inefficiency, respiratory function of the fish is likely to be retarded because of oxygen tension loss in the tissue.

In the central nervous system, the presence of exogenous sulfite seems to play an important role in glutamate metabolism. Transaminase catalyzes transaminations from glutamate to oxaloacetate. This enzyme is also involved in transamination from cysteine sulfinate to α-ketoglutarate. β-Sulfinylpyruvate formed from the latter process is a major source of endogenous sulfite. Since transaminase is quite active in central nervous system [15,16], the increased sulfite level in blood stream may pose a significant effect on the transamination process.

Furthermore, sulfite interacts not only with disulfide, sulfhydryl and carbonyl groups, NAD and FAD, forming additional products [17—23], but also with nucleic acids, inducing mutation or modification of their constituent bases [24—31].

The occurrence of sulfite oxidase in fish tissues suggests that fish may have a detoxifying device for conversion of toxic sulfite to innocuous sulfate. The capacity of fish to metabolize sulfite will depend on its detoxification system and adaptability to ever changing environmental conditions. The coexistence of metabolic inhibitors such as heavy metals appears to enhance toxicity with the resulting impairment in growth, metabolism and reproduction of fish.

## Acknowledgements

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