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VISUALIZATION OF HEPATIC SULFITE OXIDASE IN CRUDE TISSUE PREPARATIONS BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

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SUMMARY

Sulfite oxidase (sulfite:O_2 oxidoreductase, EC 1.8.3.1) has been purified to homogeneity from rat liver and has been found to be similar to those previously isolated from other sources. The purified enzyme is a molybdohemoprotein and on addition of sulfite displays the $g = 1.97$ electron paramagnetic resonance signal of pentavalent molybdenum. The shape of the signal undergoes uniquely characteristic changes with alteration of pH and of anionic composition. Rat liver homogenates display a well-defined EPR signal at $g = 1.97$. The effects of pH and of anions on the "native" signal in liver homogenates are identical to those produced on the EPR signal of purified enzyme. This is in contrast to the insensitivity of the molybdenum EPR signal of purified rat liver xanthine oxidase to these conditions. The subcellular distribution of the native signal corresponds to the distribution of sulfite oxidase activity. In addition, the amplitudes of native EPR signals at $g = 1.97$ in various tissues of the rat and in livers from several other species paralleled the sulfite oxidase activity.

INTRODUCTION

Peisach et al. [1] observed an EPR signal of molybdenum near $g = 1.96$ in rat liver and rat liver mitochondria. By comparison of mitochondria from rat liver and beef heart Peisach et al. [1] concluded that the EPR signal was not related to proteins involved in mitochondrial electron transport, but could arise from one of the soluble molybdoproteins xanthine oxidase, aldehyde oxidase and sulfite oxidase.

Xanthine oxidase, aldehyde oxidase and sulfite oxidase are the only molybdenum-containing enzymes known to be present in animal tissues. The molybdenum centers of these enzymes have been singularly amenable to examination by EPR spectroscopy, as is evident from the studies on xanthine oxidizing enzymes from milk [2, 3] and chicken liver [4], aldehyde oxidase from rabbit liver [5, 6] and

sulfite oxidase from bovine [7] and chicken [8] livers. Indeed, EPR was instrumental in the recognition of sulfite oxidase as a molybdoprotein [7].

Recently the intermembrane space of mitochondria has been identified as the intracellular habitat of sulfite oxidase (sulfite:O_2 oxidoreductase, EC 1.8.3.1) of rat liver [9–11]. In the course of those studies it was observed that slices and crude homogenates of rat liver, when examined by EPR spectroscopy, displayed a well-defined signal at $g = 1.97$ characteristic of pentavalent molybdenum. The unusual clarity of the signal even in unfractionated liver samples suggested the possibility of direct visualization of a single protein in such tissue samples. Sulfite oxidase, xanthine oxidase, or a hitherto unrecognized molybdoprotein were the possible alternatives as the source of this EPR signal. Aldehyde oxidase activity has not been detected in rat liver [9].

We have examined the qualitative and quantitative aspects of this "native" $g = 1.97$ signal in comparison to those exhibited by the molybdenum centers of rat liver sulfite oxidase and xanthine oxidase. The characteristic alterations in the line shape of the signal under varying conditions have conclusively identified the native signal as arising from sulfite oxidase in its natural milieu. The experimental basis for this identification is presented in this article.

EXPERIMENTAL PROCEDURE

Spectrophotometric assays were performed with a Gilford model 2400 recording spectrophotometer thermostatted at 25 °C. EPR spectroscopy was performed with a Varian E-9 HF spectrometer with a 9.5 GHz microwave bridge assembly and capability for field modulation at 100 kHz. Samples were maintained at −100 °C for all EPR spectroscopy. Other parameters were variable and are specified as required. Preparation of samples to be analyzed was as previously described [7].

Homogenization of tissues was performed in 1 vol. of 0.25 M sucrose buffered with 0.01 M Tris–HCl, pH 7.0, using four strokes with the teflon pestle of a Potter–Elvehjem type homogenizer driven at 1150 rev./min by a 3/8 inch drill. Subcellular fractions were prepared by the method of Schneider and Hogeboom [12] using 10 vol. of 0.25 M sucrose containing 0.01 M Tris–HCl, pH 7.0. Protein was determined by the method of Lowry et al. [13] with bovine serum albumin as a standard.

Sulfite oxidase activity in liver homogenates or at various stages of purification was assayed as previously described [9]. Rat liver xanthine oxidase was prepared by the method of Rowe and Wyngaarden [14].

The molybdenum and heme content of purified sulfite oxidase were determined as described previously [8].

Acrylamide gel electrophoresis was performed by the method of Davis [15]. Previously described procedures were used to stain the gels for heme [16] and sulfite oxidase activity [17]. Protein bands were stained with amido black. Sodium dodecyl-sulfate gel electrophoresis was performed by the method of Weber and Osborn [18].

EDTA at a concentration of 0.1 mM was present in all phosphate buffers used in enzyme purification procedures for sulfite oxidase and xanthine oxidase. All of these buffers were at pH 7.8.

RESULTS

Purification of rat liver sulfite oxidase

Rat liver sulfite oxidase was purified by a procedure adapted from those previously described for bovine [7] and avian [8] sulfite oxidase. Fresh or frozen livers were homogenized in a Waring blendor with 5 vol. of 0.01 M potassium phosphate. Sodium deoxycholate (10%, w/v) was added to the homogenate to a final concentration of 0.1%. This insured complete removal of sulfite oxidase from any residual intact mitochondria. The homogenate was then brought to 20% saturation with solid $(NH_4)_2SO_4$, heated to 58 °C and clarified by centrifugation. The supernatant solution was brought to 50% saturation with solid $(NH_4)_2SO_4$, and after centrifugation the precipitate was redissolved in 5 vol. of 0.01 M potassium phosphate. Acetone fractionation was performed and the precipitate from the 40–60% acetone fraction was immediately resuspended in 0.01 M potassium phosphate. The suspension was clarified by centrifugation and the supernatant solution mixed with an equal volume of saturated $(NH_4)_2SO_4$ in 0.05 M Tris. The resulting pellet was dissolved in 0.05 M potassium phosphate and the solution dialyzed against the same buffer and applied to a 2 cm × 15 cm column of DEAE-cellulose previously equilibrated with the buffer. The column was then washed with 0.05 M phosphate until the unadsorbed proteins were eluted. A linear gradient from 0.05 to 0.20 M potassium phosphate was then applied to the column. Active fractions were pooled, concentrated and dialyzed against 0.05 M potassium phosphate. Gel filtration chromatography of this material on a column of Sephadex G-200 (2.5 cm × 90 cm) yielded pure sulfite oxidase.

As can be seen in Fig. 1, after a small amount of material was eluted at and near the void volume of the column, the enzyme was eluted with congruent protein, activity, and 413-nm absorption patterns. Enzyme prepared in this manner has a 413 nm/280 nm ratio of 0.8 and gives a single band on acrylamide gels when stained for protein, heme or activity. A typical purification procedure is summarized in Table I.

Fig. 2 shows that the purification procedure does not affect the electrophoretic mobility of sulfite oxidase, indicating that no gross change in the protein has occurred during purification. In addition, the single activity band on acrylamide gels of the initial homogenate argues for a single protein species responsible for sulfite oxidation in rat liver. Analysis of this enzyme yielded a heme to molybdenum ratio of one, similar to that for bovine and avian sulfite oxidases. Sodium dodecylsulfate gel electrophoresis on purified enzyme gave a subunit molecular weight of 58 000, as compared to 56 000 for the enzyme from chicken liver determined in the same experiment. Sodium dodecylsulfate gels to which both purified chicken and rat sulfite oxidases were applied confirmed this difference in subunit size.

EPR signal of the purified enzyme

Rat liver sulfite oxidase, like the enzyme purified from bovine, human and chicken livers, does not evince a molybdenum EPR signal when examined in the pH range 7–9 in the absence of substrate. However, when 0.01 M sodium sulfite is added, the characteristic molybdenum signal of the enzyme appears. As can be seen in Fig. 3A, the shape and *g*-value of this signal are strongly influenced by the pH of the solution. Bovine, chicken and human liver sulfite oxidase display similar shifts in signal

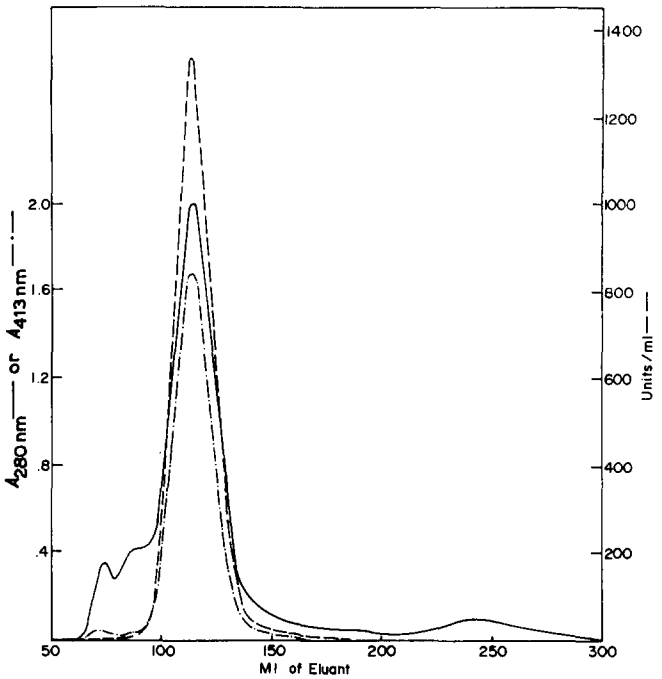

Fig. 1. Elution pattern of rat liver sulfite oxidase from Sephadex G-200. Conditions are described in the text.

shape with changes in pH [7, 8]. It has been observed, in the case of the chicken liver enzyme [8], that phosphate and other anions cause characteristic changes in the shape of the molybdenum EPR signal at pH 7.0. Fig. 3B shows the effect of 0.1 M potassium phosphate on the EPR signal manifest in 0.1 M Tris–HCl at pH 7.0. The

TABLE I

PURIFICATION PROCEDURE FOR RAT LIVER SULFITE OXIDASE

Procedure	Volume (ml)	Protein (mg)	Units	Specific activity (units/mg)	Yield (%)	Purification
1. 0.01 M Phosphate Homogenization	3710	124 285	105 735	0.85	100	1.0
2. Heating to 58 °C	2870	49 651	107 625	2.17	100	2.6
3. 50% $(NH_4)_2SO_4$ Precipitation	540	26 676	107 980	4.05	100	4.8
4. 40–60% Acetone Fraction + 50% $(NH_4)_2SO_4$ precipitation	35.3	2 944	77 600	26.4	73.4	31.0
5. Pooled fractions from DEAE-cellulose	500	79.5	44 594	561	42.2	660
6. Sephadex G-200 peak fractions	40	38.8	37 332	963	35.3	1130

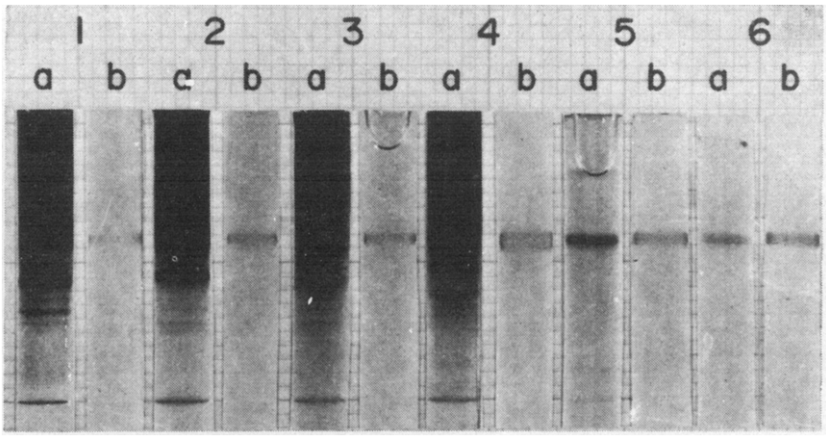

Fig. 2. Acrylamide gels of sulfite oxidase at various steps during the purification procedure. The numbers above the gels correspond to purification stages outlined in Table I. Gels labelled (a) have been stained for protein; those labelled (b) have been stained for sulfite oxidase activity, as described previously [15] .Amounts of protein in μg applied to the gels were (1) 837, (2) 433, (3) 1258, (4) 834, (5) 19.5 and (6) 0.24.

resulting signal is identical in shape to that of chicken liver sulfite oxidase which has undergone the same treatment.

Thus, the EPR signal of the molybdenum center of rat liver sulfite oxidase is particularly sensitive to changes in both the pH and anion composition of the medium. In contrast, the EPR signal of molybdenum in xanthine oxidase generated on the addition of xanthine is insensitive to the above mentioned alterations in the composi-

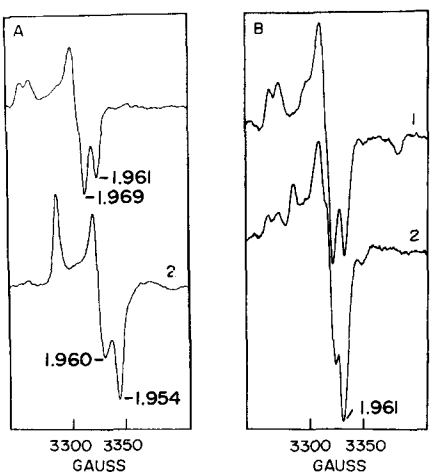

Fig. 3. Effect of pH and anions on the molybdenum EPR signal of purified rat liver sulfite oxidase (7 mg/ml). (A) Enzyme in 0.1 M Tris–HCl at pH 7.0 (1) and pH 9.0 (2) reduced with 0.01 M sodium sulfite. (B) Enzyme at pH 7.0 in 0.1 M Tris–HCl (1) and after addition of 0.1 M potassium phosphate (2) reduced with 0.01 M sulfite. EPR conditions were as follows: microwave frequency, 9.12 GHz; microwave power, 5 mW: modulation amplitude, 4 G; time constant, 1.0 s; gain, 5000; and scan rate, 50 G/min.

tion of the medium as shown in Fig. 4. Consequently, the effects of both pH and anions served to distinguish the paramagnetic molybdenum centers of sulfite oxidase and xanthine oxidase in cruder tissue preparations.

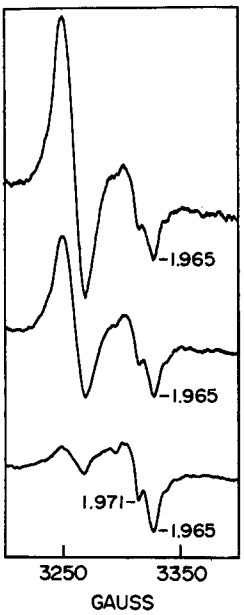

Fig. 4. Effect of pH and anions on the molybdenum EPR signal of rat liver xanthine oxidase. Enzyme at a concentration of 5 mg/ml in (1) 0.05 M potassium phosphate, pH 7.0, (2) 0.1 M Tris–HCl, pH 7.0, and (3) 0.1 M Tris–HCl, pH 9.0, was reduced with an equal volume of deoxygenated 1.5 mM xanthine. Spectra were recorded under the following conditions: microwave frequency, 9.12 GHz; microwave power, 5 mW; modulation amplitude, 8 G; time constant, 3.0 s; gain (1) 10 000, (2) 5000, (3) 2500; and scan rate of 50 G/min.

EPR signal of rat liver homogenates

Fresh rat liver was homogenized in 1 vol. of 0.25 M sucrose buffered with either 0.01 M Tris–HCl, pH 7.0 or 0.1 M Tris–HCl, pH 9.0. The homogenates with no exogenous reductant were examined by EPR spectroscopy. As can be seen in Fig. 5A, EPR signals at $g_m = 1.97$ and $g_m = 1.96$ were present at pH 7.0 and pH 9.0, respectively. When 0.1 M potassium phosphate, pH 7.0, was added to a liver homogenate prepared in 0.25 M sucrose containing 0.01 M Tris–HCl, pH 7.0, the characteristic change in shape which occurs in the EPR signal of the purified enzyme was observed, as shown in Fig. 5B. Thus the effects of pH and of anions on the native signal in liver homogenates and on the EPR signal of purified enzyme are identical.

Dialysis of the liver homogenate for 16 h resulted in the disappearance of the native EPR signal. As shown in Fig. 6, however, addition of 0.01 M sodium sulfite to the dialyzed homogenate regenerated a molybdenum signal similar to the native signal and to the specific EPR signal of sulfite oxidase. Addition of xanthine, on the other hand, failed to produce the EPR signal.

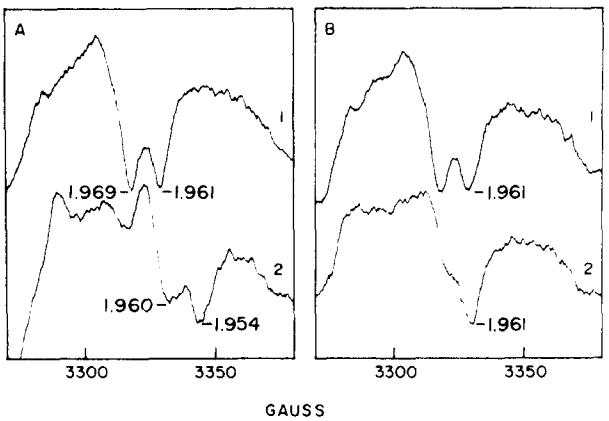

Fig. 5. Effect of pH and anions on the molybdenum EPR signal of rat liver homogenates. (A) Isotonic liver homogenates in Tris-HCl at pH 7.0 (1) and pH 9.0 (2) prepared as described in the text. (B) Homogenate in 0.01 M Tris-HCl, pH 7.0, (1) and after addition of 0.1 M potassium phosphate, pH 7.0, (2). EPR conditions were: microwave frequency, 9.12 GHz; microwave power, 5 mW; modulation amplitude, 8 G; time constant, 3.0 s; gain, 20 000; and scan rate of 50 G/min.

EPR signal of liver slices

Immediately after decapitation of the animals, livers were perfused in situ with 0.25 M sucrose containing 0.1 M Tris-HCl at either pH 7.0 or 9.0. The livers were then excised, cut into small slices and packed into a quartz EPR tube. Spectra,

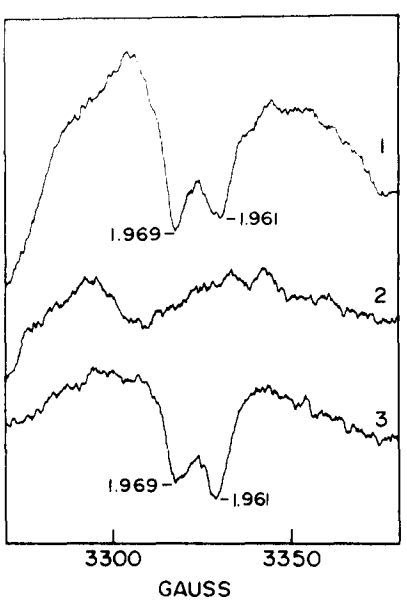

Fig. 6. Effect of dialysis on the molybdenum EPR signal of rat liver homogenates. (1) undialyzed homogenate (2) homogenate in 0.25 M sucrose buffered with 0.01 M Tris-HCl, pH 7.0, dialyzed for 16 h against the same medium (3) dialyzed homogenate after addition of 0.01 M sulfite. EPR conditions were as in Fig. 5.

recorded as usual at −100 °C, are shown in Fig. 7. As can be seen, EPR signals at $g_m = 1.97 =$ at pH 7.0 and at g_m 1.96 at pH 9.0 characteristic of those in the homogenate and purified enzyme were present in the perfused liver samples.

Correspondence of activity and EPR signal in subcellular fractions

It has been shown that sulfite oxidase is localized in mitochondria in rat liver and that an EPR signal attributable to pentavalent molybdenum is also present in

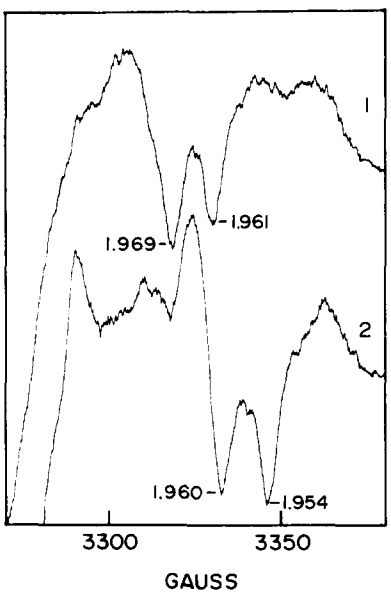

Fig. 7. Effect of pH on the molybdenum EPR signal of rat liver slices. Rat livers were perfused in situ with 250 ml of a solution containing 0.25 M sucrose, 0.4 mM KF and 0.1 M Tris–HCl, pH 7.0, (1) or pH 9.0 (2). KF was added to inhibit acid production by glycolysis. EPR conditions were as in Fig. 5 except that a scan rate of 25 G/min was used.

intact mitochondria [9]. Xanthine oxidase, the other molybdenum-containing enzyme of rat liver, exists in the cytosol [19]. The data present in Fig. 8 show that the subcellular distribution of the $g_m = 1.97$ EPR signal of liver homogenate is identical to that of sulfite oxidase activity, 75% of which is recovered in the mitochondrial fraction. Analysis of the fractions for xanthine oxidase activity confirmed that this enzyme is entirely confined to the cytosol.

Sulfite oxidase activity and EPR signal in various organs of the rat

Sulfite oxidase has been found in organs other than liver, albeit at lower levels. When 1:1 homogenates of liver, kidney, heart, lung and adrenal in 0.25 M sucrose containing 0.01 M Tris–HCl, pH 7.0 were examined, similar signals at $g_m =$ 1.97 were observed, the magnitudes of which were commensurate with the enzyme activity in each organ. Brain, muscle, blood, adipose tissue and thymus displayed neither activity nor the characteristic EPR signal of sulfite oxidase.

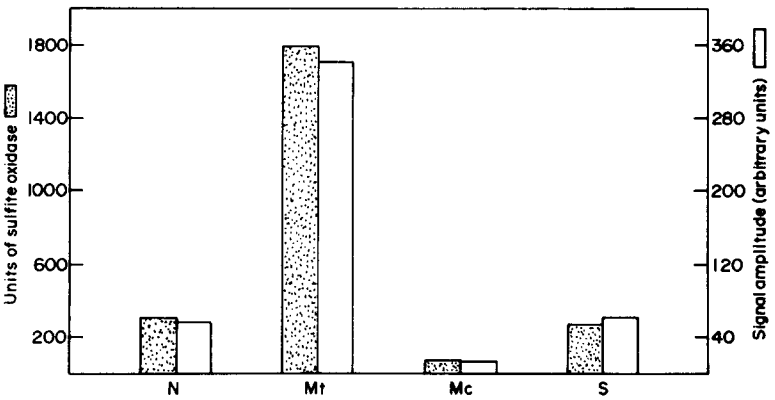

Fig. 8. Subcellular distribution of sulfite oxidase activity and the molybdenum EPR signal of rat liver. N, nuclei (600 × *g* pellet); Mt, mitochondria (5000 × *g* pellet); Mc, microsomes (100 000 × *g* pellet); and S, soluble fraction (100 000 × *g* supernatant solution). The bars represent recovery of sulfite oxidase activity or amplitude of molybdenum EPR signal in the subcellular fractions derived from 7.5 g of liver.

EPR signals of livers from other species

Homogenates of chicken, pig, human and rabbit livers were prepared in 0.25 M sucrose containing 0.01 M Tris–HCl at pH 7.0 and examined by EPR spectroscopy. In each case, the $g_m = 1.97$ signal was observed, corresponding in magnitude to the amount of sulfite oxidase activity.

DISCUSSION

Sulfite oxidase has now been purified and characterized from three animal sources. The isolation procedure and physicochemical properties of the rat liver enzyme are quite similar to those previously described for the bovine [7] and avian [8] enzymes.

The molybdenum centers of the enzymes from all three sources are strikingly identical in their sensitivity to variations in the pH and anion composition of the medium, as reflected in their EPR spectra which are the only observable attributes of the molybdenum moieties of enzymes containing that metal. This remarkable constancy of the EPR properites of sulfite oxidase is in contrast to the dissimilarity of the molybdenum EPR spectra of xanthine oxidizing enzymes from milk [2, 3], chicken liver [4] and *Micrococcus lactilyticus* [20].

The effect of pH on the line shape and *g*-value of sulfite oxidase has been ascribed to the dissociation of a proton proximal to the molybdenum center whose ligand field is thereby altered from an axially symmetric to an asymmetric structure [7]. The effect of anions on the EPR signal may stem from the same interaction which underlies the inhibition by anions of the egress of electrons from sulfite oxidase to the one electron acceptors ferricytochrome *c* and ferricyanide [8].

These conveniently observable criteria have been applied towards the identification of the native $g_m = 1.97$ signal observed in liver slices and homogenates. In homogenates, the native signal mimics the molybdenum signal of purified sulfite

oxidase in responding to changes in pH and anion composition. In addition, perfusion of liver with isotonic solutions at pH 7.0 or at pH 9.0 is sufficient to produce the anticipated change in the characteristics of the EPR signal. The effects of environmental alterations on the *g*-values of the EPR signal of purified sulfite oxidase and those of the native signal were always identical. It is thus conclusively established that the native EPR signal at $g_m = 1.97$ in liver preparations directly reflects the presence of sulfite oxidase.

The reason for terming the $g_m = 1.97$ EPR signal as "native" is that it is observed in tissue slices or homogenates even without added sulfite. In fact, addition of sulfite fails to augment the signal, indicating that the native signal is an index of the sulfite oxidase activity present in the homogenate. This conclusion is further corroborated by the correspondence between the observed signal amplitude and the sulfite oxidase activity of homogenates of a variety of rat tissues as well as of livers of diverse origin.

In 1969, there was a report of a fatal instance of sulfite oxidase deficiency in a human [21, 22]. In 1970, the enzyme was recognized as a molybdohemoprotein [7]. It is obvious that in the case of a complex enzyme dependent on multiple cofactors for activity, an apparent genetic deficiency of the enzyme could result from lesions in the primary structure of the protein or from aberrations connected with the cofactors of the enzyme. The results presented above should enable the monitoring of the molybdenum EPR signal of sulfite oxidase, in addition to its activity, in liver biopsy samples if future instances of deficiency of the enzyme come to light.

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