Hepatic Sulfite Oxidase

CONGRUENCY IN MITOCHONDRIA OF PROSTHETIC GROUPS AND ACTIVITY*

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SUMMARY

Rat liver sulfite oxidase (EC 1.8.3.1) has been localized, both by activity measurement and by prosthetic group analyses, to the intermembrane space of mitochondria.

The apparent activation of sulfite-dependent cytochrome c reduction by liver homogenates in the presence of detergents was not duplicated with either ferricyanide or oxygen as electron acceptor. This is presumably due to the inaccessibility of sulfite oxidase in its mitochondrial compartment to exogenous cytochrome c.

Sulfite-cytochrome c reductase of rat liver mitochondria was found to have an identity different from either the rotenone-insensitive DPNH-cytochrome c reductase or succinate-cytochrome c reductase.

Examination of rat liver mitochondria at -100° by electron paramagnetic resonance spectroscopy revealed the EPR signal at g = 1.97, deriving from pentavalent molybdenum. The characteristic effect of pH on the shape of this signal identified it as that of the molybdenum center of sulfite oxidase. When rat liver mitochondria were treated with digitonin or dialyzed in hypotonic media the EPR signal was no longer observed. The signal was, however, restored by the addition of either sulfite or DPNH to the treated mitochondria. The enzyme was released into the nonparticulate aqueous phase by each of these treatments. The above observations have also been made with sulfite oxidase of chicken liver.

Under anaerobiosis the cytochromes of rat liver mitochondria were reduced by sulfite to the same extent as by succinate. Addition of sulfite to digitonin solubilized aqueous extracts of mitochondria generated a cytochrome b₅-like spectrum with features similar to that generated by sulfite on purified sulfite oxidase. Such a spectrum was not elicited by sulfite from particulate preparations corresponding to the inner and outer membranes of mitochondria.

Aerobically, the oxidation of sulfite by hepatic mitochondria resulted in the esterification of inorganic phosphate with the ATP-sulfite ratio approaching unity and sulfite-O₂ ratio of 2.0.

Recently, an enzyme capable of oxidizing sulfite to sulfate has been purified (1) and characterized (2, 3) from bovine liver. It has been shown that an animal exposed to sulfur dioxide (4) or given parenteral bisulfite (5) excretes 80 to 90% of the sulfur as sulfate in the urine. This enzyme is felt to be responsible for detoxification of sulfur dioxide and sulfite to sulfate. Further evidence for the essentiality of this enzyme is the report of a child completely deficient in sulfite oxidase, who excreted no sulfate in his urine and who suffered from severe neurologic defects (6, 7).

Bovine sulfite oxidase was found to contain both molybdenum (3) and a b₅-like cytochrome (2). These two electron transport carriers have now been identified as part of the enzyme in avian and human liver while molybdenum has also been found in the enzyme from bacteria and plants (8). There has been some controversy as to the intracellular localization of this enzyme in animal liver. The enzyme was first reported to be localized in the 100,000 × g pellet (9), while a later report demonstrated that most of the activity was in the 600 × g pellet (10). Recently, however, it has been convincingly demonstrated that in the rat liver, sulfite cytochrome c reductase activity is associated with the mitochondrial fraction and is easily solubilized by hypotonic or digitonin treatment, indicating that the enzyme is probably within the mitochondrial intermembranous space (11).

Utilizing the spectral properties of both the heme (optical) and the molybdenum (EPR), in addition to the activities of the enzyme with one and two electron acceptors, it has been possible to confirm these findings. The enzyme itself is in the mitochondrial intermembranous space and can transfer electrons by a cyanide insensitive pathway directly to oxygen or, in the absence of cyanide, to the terminal mitochondrial electron transport carries with concomitant dinitrophenol-sensitive ATP production and a P:2e ratio approaching 1.0.

The abbreviation used is: EPR, electron paramagnetic resonance.

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EXPERIMENTAL PROCEDURES

Cytochrome c (type III) and DPNH were obtained from Sigma, and sodium sulfite from the Allied Chemical Corporation. Rotenone (97 to 98%) was kindly provided by Dr. L. Fishbein, and antimony c was a generous gift from Ayerst Laboratories. Digitonin, obtained from Fisher Chemicals, was recrystallized from hot ethanol. Other compounds were obtained from commercial sources at the highest available states of purity.

Spectrophotometric assays were performed with a Gilford model 2400 absorbance indicator equipped with a thermostated cell compartment. All kinetic experiments were performed at 25°C. Visible spectra of clear solutions were recorded with a Cary model 14 spectrophotometer and spectra of mitochondria were obtained using an Aminco-Chance dual wave length split-beam spectrophotometer.

EPR spectroscopy was performed with a Varian E-9HF spectrometer, with a 9.5 GHz microwave bridge assembly. A Nicolet model 1072 signal averaging system and integrator was used for signal enhancement. All recordings were performed at −100°C with a modulation frequency of 100 KHz. Relatively high temperature and low power were employed to see the methylcobalamin signal which saturates easily with decreasing temperature. All other parameters were variable and are specified in each figure. Preparation of samples to be analyzed was as previously described (3).

Centrifugation was performed in a Sorvall model RC-2B and ultacentrifugation in a Beckman model L3-50.

Mitochondria were isolated by the following procedure. Male Charles River rats, weighing between 200 and 300 g, were killed by decapitation and their livers were removed, washed in cold 0.25 M sucrose, blotted dry, weighed, and then minced in 4 volumes of 0.25 M sucrose. Homogenization was performed using a Teflon-pestled Potter-Elvehjem-type homogenizer attached to a 1/4-inch drill spun at 1150 rpm. Four strokes with the homogenizer led to incomplete cell disruption but insured that integrity of organelles was only minimally disturbed. Subcellular fractions were prepared by the method of Schneider and Hogeboom (12) with all fractions being washed twice with sucrose. Subcellular markers were assayed as follows: DNA by the diphenylamine method of Schneider (13); 8-nucleotidase by the method of Mitchell and Hawthorne (14); glucose 6-phosphatase by the method of Mitchell and Hawthorne (14); glutathione reductase by the method of Dodgson et al. (16). Inorganic phosphate was assayed by the method of Lowry and Lopez (17). Protein was determined by the method of Lowry et al. (18) with bovine serum albumin as a standard.

Sulfite oxidase was assayed in purified samples as previously described (1). However, in homogenates or subcellular fractions, the conditions utilized were as follows.

Reduction of Cytochrome c—Ten microliters of 0.1 M sulfite were added to cuvettes containing 0.04 mM ferricytochrome c, 0.5 mM sodium cyanide, 0.1 mM EDTA, and the protein in a 2.5-ml solution buffered at pH 8.5 with 0.1 M Tris-HCl in 0.25 M sucrose. The increase in absorbance at 550 nm was followed. These assays were performed in the presence or absence of 0.04% sodium deoxycholate. One unit of activity is defined as the amount of protein causing an absorbance change of 1.0 per min under these conditions.

Ferricyanide Reduction—Assay conditions were similar to the above except for 0.4 mM potassium ferricyanide replacing the cytochrome c. The reduction of ferricyanide was followed at 420 nm.

Colorimetric Assay for Sulfite Oxidation—Conditions were similar to the above except for the absence of both potassium ferricyanide and cytochrome c. At various intervals, 0.10-ml aliquots were removed and added to 5 ml of West and Gaeke reagent (19) made from 0.1 M disodium chloromercurate, 0.2% formaldehyde, and 0.04% hydrochloric acid-bleached p-rosaniline hydrochloride in a mixture of 10:1:1. The assay mixture was centrifuged after 15 min at room temperature, and the precipitated protein removed. The optical density of the resulting clear solution was read at 560 nm after a period of at least 30 min and compared with standards containing 0.01 to 0.15 μmole of sulfite. The use of this disulfomercurate assay has the advantage over the previously employed assay (1) using basic fuchsin (20) in that the color developed is quite stable over a period of at least 2 hours.

Rotenone-insensitive DPNH-cytochrome c Reductase—Five hundredths milliliters of 0.01 M DPNH was added to 2.45 ml of solution containing 0.04 mM ferricytochrome c, 0.5 mM sodium cyanide, 4 μM rotenone, 0.1 mM EDTA, and protein, in either 0.05 M potassium phosphate (pH 7.5) or 0.1 M Tris-HCl (pH 8.5) in 0.25 M sucrose. Optical density changes were recorded after 550 nm.

Digitonin Solubilization—Mitochondria in either 0.25 M sucrose or in 0.22 M mannitol, 0.07 M sucrose, 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 7.4 with 0.5 mg % bovine serum albumin were mixed in the cold for 10 min with various aliquots of a 20-μg per ml solution of digitonin in the same buffer, and centrifuged using the method of Schnaitman and Greenawalt (21). Both pellets and the final supernate were then dialyzed against 100 volumes of 0.05 M potassium phosphate pH 7.5 containing 0.1 mM EDTA with two changes of buffer before analysis. For EPR spectroscopy the final supernate was then concentrated by dialysis in vacuo in collodion bags.

RESULTS

Effect of Sodium Deoxycholate on Enzymatic Activity—It has previously been reported that sulfite oxidase is activated by sodium deoxycholate (10) and Triton X-100 (11). Fig. 1 shows the effect of sodium deoxycholate on the rate of reduction of sulfite oxidase activity with increasing concentrations of sodium deoxycholate.
cytochrome c by sulfite in the presence of rat liver homogenate. These assays were performed in 0.25 M sucrose to maintain the structural habitat of the enzyme in the absence of detergents. Maximum activity was observed with 0.04% sodium deoxycholate and was unaffected by the presence or absence of sucrose. This amount of deoxycholate was then used when assaying for enzymatic activity in homogenates or subcellular fractions. Since this apparent activation was reported using cytochrome c as an oxidant, it was felt necessary to determine whether this increase in activity was due to an activation of the enzyme or to a change in its structural localization making it more accessible to cytochrome c. It has previously been shown that the same enzyme that reduced cytochrome c was able to reduce ferricyanide and oxygen (1). When the effect of 0.04% deoxycholate was studied with all three electron acceptors, it became apparent (Table I) that there was no activation of either ferricyanide or oxygen reduction. This was also true at deoxycholate concentrations of 0.004 and 0.08%.

**TABLE I**

**Effect of sodium deoxycholate on oxidation of sulfite with various electron acceptors**

Aliquots of a rat liver homogenate were assayed for sulfite oxidation with indicated electron acceptors with and without 0.04% sodium deoxycholate in the assay mixture. Activity is expressed as millimicroequivalents of electrons per min per mg of protein.

| Electron acceptor | Activity without deoxycholate | Activity with deoxycholate |
|-------------------|-------------------------------|-----------------------------|
| Cytochrome c       | 3.9                           | 23.9                        |
| Ferricyanide       | 56.7                          | 56.1                        |
| Oxygen            | 3.9                           | 3.8                         |

![Fig. 2](https://via.placeholder.com/150)

**Fig. 2.** Subcellular distribution of sulfite oxidase and marker enzymes. Sulfite oxidase was assayed with respect to reduction of cytochrome c (1), ferricyanide (2), and oxygen (3), as described in the text. Cytochrome c reduction was measured in the presence of 0.04% sodium deoxycholate. Markers were succinate-cytochrome c reductase (4), cytochrome c oxidase (5), adenosine triphosphatase (6), glucose 6-phosphatase (7), and DNA (8). N represents 600 

Intracellular Localization of Sulfite Oxidase—The results of the above experiments suggested an intracellular locale for sulfite oxidase that was not accessible to a macromolecule such as cytochrome c. In order to clarify this, the subcellular localization of the enzyme was determined. In agreement with recent reports (11, 22) and in contradistinction from previous observations (9, 10), rat liver sulfite oxidase was found to be localized predominantly in the 5000 x g pellet. As can be seen in Fig. 2, sulfite oxidase fractionates together with two known mitochondrial enzymes, succinate-cytochrome c reductase and cytochrome oxidase. In other experiments, using arylsulfatase as a lysosomal marker, and involving a 5000 x g and a 9000 x g centrifugation, sulfite oxidase sedimented predominantly in the heavier fraction and arylsulfatase predominantly in the lighter one. Up to 15% of sulfite oxidase was present in the supernate and this could be accounted for by the sulfite-cytochrome c reductase activity present in the homogenate in the absence of deoxycholate. Using more vigorous homogenization procedures, the fraction of activity in the supernate could be greatly increased. The use of mild homogenization techniques explains the fact that 30% of the total activity was found in the 600 x g pellet. As can be seen in Fig. 2, similar fractional amounts of both of the mitochondrial marker enzymes sedimented at 600 x g. It can also be seen that the reduction by sulfite of either ferricyanide or oxygen is localized to the same fraction as is the reduction of cytochrome c. When the activities of the various fractions were compared in the presence and absence of deoxycholate, as expected, ferricyanide and oxygen reductase activities were unaffected. Cytochrome c reductase activity, however, was negligible in all three pellets without deoxycholate and unaffected by deoxycholate in the 100,000 x g supernate. Sulfite oxidase has also been localized to the mitochondrial fraction of chicken and rabbit liver and displays a similar deoxycholate de-
ependence for cytochrome c reduction. In addition, lung mito-
chondria isolated from rabbits oxidized sulfite, albeit at a much lower rate.

Intramitochondrial Localization of Sulfite Oxidase—In addition to sulfite oxidase, there are two mitochondrial electron transport systems that can transfer electrons to cytochrome c: the inner membrane electron transport particle and the outer membrane rotenone-insensitive DPNH-cytochrome c reductase. In order to determine if sulfite oxidase was related to either of these enzyme systems, inner and outer membrane particles were prepared by the digitonin procedure of Schmittman and Greenawalt (21). The results of incubating mitochondria with 0.2 mg of digitonin per mg of mitochondrial protein can be seen in Table II. The 9500 × g pellet, representing the mitochondrial inner membrane and matrix, contained succinate-cytochrome c reductase. The 100,000 × g pellet, representing the mitochondrial outer membrane, contained the rotenone-insensitive DPNH-cytochrome c reductase, whereas sulfite-cytochrome c reductase was solubilized and was found in the 100,000 × g supernate representing the space between the inner and outer mitochondrial membranes. This is in agreement with previous observations (11) and explains why deoxycholate was required for cytochrome c reduction by sulfite. When all three cytochrome c reductases in isolated mitochondria were assayed in 0.25 M sucrose, as shown in Table III, deoxycholate stimulated the reduction by succinate and sulfite, but inhibited the reduction by DPNH. Inhibition of rotenone-insensitive DPNH-cytochrome c reductase by deter-
gents has previously been observed (21).

In addition, when rotenone-insensitive DPNH-cytochrome c reductase and sulfite-cytochrome c reductase were assayed together in the presence of deoxycholate, their rates were com-
pletely additive. Ferricyanide reduction by sulfite and DPNH in the presence of rotenone but in the absence of deoxycholate was also additive thus precluding an effect of the detergent. The results of these experiments are shown in Table IV. Fur-
thermore, sulfite reduction of ferricyanide in intact mitochon-
dria, and of cytochrome c in deoxycholate-treated mitochondria was inhibited by sulfite, as was reported for purified bovine en-
zyme (1). In contrast to this, sulfite stimulated ferricyanide and cytochrome c reduction by DPNH.

Structural Localization of Sulfite Oxidase—It was possible to purify rat liver sulfite oxidase by a modification of the procedure applied to bovine liver (1). The purified preparation was quite similar to the bovine (1) and avian (8) enzymes in its molyb-
denum and heme contents. Further, the rat liver enzyme also displayed a pH-dependent alteration in the shape of its sulfite-
induced molybdenum EPR signal, as seen in Fig. 3. These spectral properties of the enzyme were utilized to adduce physical

| Table II |

Fractionation of sulfite-, DPNH (rotenone-insensitive), and succinate-cytochrome c reductase activities of digitonin-treated mitochondria

Mitochondria were treated with 0.2 mg of digitonin per mg of protein and fractionated according to the procedure of Schmittman and Greenawalt (21). All activities are related to the total mitochondrial sample used for fractionation. Assay methods are described in the text.

| Fraction        | Sulfte → cytochrome c | DPNH → cytochrome c | Succinate → cytochrome c |
|-----------------|-----------------------|----------------------|-------------------------|
|                 | Total units           | Per cent             | Total units             | Per cent             | Total units | Per cent |
| Mitochondria    | 168.6                 | 100                  | 624                     | 100                  | 424.8       | 100      |
| 9,500 × g pellet | 9.5                   | 5.6                  | 187.2                   | 30.0                 | 295.1       | 69.5     |
| 100,000 × g pellet | 5.2                  | 3.1                  | 331.5                   | 53.1                 | 30.1        | 7.1      |
| 100,000 × g supernate | 150.0               | 92.9                 | 105.7                   | 10.9                 | 21.1        | 5.0      |
| Total recovery  | 101.6                 | 100.0                | 81.6                    |                      |             |

| Table III |

Effect of 0.04% deoxycholate on mitochondrial sulfite-, rotenone-insensitive DPNH-, and succinate-cytochrome c reductase

All assays were performed in 0.1 M Tris-Cl, pH 7.8, in 0.25 M sucrose.

| Without deoxycholate | With deoxycholate |
|----------------------|-------------------|
| Sulfite              | 0.5               | 3.4               |
| DPNH                 | 0.1               | 3.0               |
| Succinate            | 1.1               | 3.8               |

* Activity expressed as ΔA 550 nm per min per mg of protein.
Mitochondrial pellets were washed three times with 0.25 M sucrose. As shown in Fig. 4, the EPR signal of molybdenum was present in each sample, and the alteration of the g\textsubscript{meta} value of the signal with pH was identical to that seen with purified rat liver sulfite oxidase, proving that the observed signal was in fact derived from mitochondrial sulfite oxidase. The symmetrical signal observed at about 3250 gauss in Fig. 4 and in other figures is not related to sulfite oxidase, since it is not present in the EPR spectrum of purified enzyme. The origin of this signal is being investigated further.

The EPR signal characteristic of the molybdenum component of sulfite oxidase was observed in intact mitochondria even without added sulfite and represents the molybdenum signal observed by Peisach et al. (23) in mitochondria. Indeed, addition of sulfite had no effect on the amplitude of the signal. Dialysis of mitochondria for several hours against 0.25 M sucrose solutions of pH 7 to 9 did not abolish the signal but did engender the expected alterations in the shape of the signal.

When mitochondria were dialyzed against a hypotonic solution (0.01 M Tris-HCl at pH 9.1) the native molybdenum signal was no longer observed; but on the addition of sulfite, the typical alkaline spectrum was generated (Fig. 5). Interestingly, a signal similar in shape and in amplitude was also produced by DPNH, as shown in Fig. 5. The magnitude of the signal was not significantly increased when sulfite and DPNH were added together to the mitochondria.

When mitochondria subjected to hypotonic dialysis were centrifuged at 100,000 \texttimes g, all of the sulfite-induced molybdenum signal was seen to be present in the aqueous supernatant solution, while the pellet exhibited no molybdenum signal with or without added sulfite. The sharp signal at 3250 gauss was not seen in the EPR spectra of the aqueous supernatant solution with or without sulfite. Further, DPNH was incapable of eliciting the molybdenum signal in the supernatant extract obtained by hypotonic treatment. It was thus apparent that hypotonic treatment of mitochondria had released sulfite oxidase from its intramitochondrial location and that concomitant with the process the enzyme had acquired a dependence on the addition of sulfite (and DPNH or sulfite in the case of uncentrifuged preparations) to exhibit the molybdenum signal.

As mentioned earlier, sulfite oxidase activity was solubilized by the addition of digitonin (Table II). In order to characterize the various mitochondrial subfractions with regard to the prosthetic groups of the enzyme, preparations corresponding to those in Table II were examined in 0.25 M sucrose at pH 8.9 for enzymic molybdenum by EPR and in 0.05 M phosphate buffer, pH 7.8, for the heme prosthetic group by absorption spectroscopy. The results depicted in Fig. 6 show that both the EPR signal of molybdenum and the optical difference spectrum of the b\textsubscript{5} like cytochrome, dependent on the addition of sulfite, were entirely present in the 100,000 \texttimes g supernate corresponding to the intermembranous space of mitochondria. The same signal was generated by the addition of DPNH to the extract. The pH dependence of the shape of the EPR signal generated by sulfite in a similar 100,000 \texttimes g extract is illustrated in Fig. 7.

As indicated in Figs. 6 and 7, the magnitude of the sharp signal at 3250 gauss, relative to the molybdenum signal, in digitonin-solubilized supernatant extracts was variable. In the ex-

**Fig. 4 (left)**. Effect of pH on native mitochondrial EPR signal. Mitochondrial pellets were washed three times with 0.25 M sucrose. The final pellet was mixed with an equal volume of 0.1 M Tris-HCl in 0.25 M sucrose at pH 7.0 (A), pH 8.0 (B), and pH 9.0 (C). Protein concentration was 29.5 mg per ml. EPR conditions were identical to those in Fig. 3 except that the time constant was 1.0 s and the gain was 10,000. The g\textsubscript{meta} values were 1.973 (A) and 1.961 (C). Addition of sulfite or DPNH did not augment the signal.

**Fig. 5 (center)**. EPR signals of mitochondria at pH 9.1. Mitochondria were dialyzed against 0.01 M Tris-HCl pH 9.1, 0.1 mM EDTA. EPR signals were obtained on dialyzed mitochondria (A); mitochondria + 0.01 mM sulfite (B); mitochondria + 0.2 mM DPNH (C). EPR conditions were identical with those in Fig. 4.

**Fig. 6 (right)**. Left, EPR signals from whole mitochondria and fractions obtained after treatment with digitonin as described in Table II. A, intact mitochondria at 100 mg of protein per ml. B, 9000 \texttimes g pellet resuspended to 46 mg of protein per ml. C, 100,000 \texttimes g pellet resuspended to 18 mg of protein per ml. D, 100,000 \texttimes g supernate at 64 mg of protein per ml. Each spectrum is an average of 4 scans, and all other EPR conditions are identical to those of Fig. 4, with the exception that the microwave frequency = 9.120 GHz, microwave power = 5 milliwatts and gain = 20,000. Right, reduced versus oxidized difference spectra of mitochondria and digitonin fractions. A, whole mitochondria; B, 9000 \texttimes g pellet; C, 100,000 \texttimes g pellet; D, 100,000 \texttimes g supernate. Each fraction was diluted with buffer to 4 mg of protein per ml. A portion of each sample was then reduced with 1 mM SO\textsubscript{4}\textsuperscript{2-}, and the difference spectra were recorded after 2 min.

**Fig. 7. Effect of pH on the sulfite-dependent EPR signal of the 100,000 \texttimes g supernatant obtained after digitonin treatment of mitochondria.** Experimental conditions were as described in Table II. Spectra were recorded in 0.1 M Tris-HCl at pH 8.9 (A) and pH 7.0 (B). Conditions for recording spectra were identical with those in Fig. 6 except that the frequency was 9.132 GHz.
experiment shown in Fig. 7, this signal was not observed. Significantly, the molybdenum signal was not generated by the addition of DPNH to the latter extract. The relationship of the material responsible for the signal at 3250 gauss to the interaction between DPNH and sulfite oxidase is under investigation. In summary, the experiments in Figs. 6 and 7 clearly established the congruency of activity and prosthetic groups of sulfite oxidase in a specific compartment of hepatic mitochondria.

With the use of digitonin to solubilize sulfite oxidase from rat liver mitochondria, the \( b \)-like cytochrome of the enzyme was clearly observable in difference spectra using sulfite, DPNH or dithionite, with an \( \alpha \)-peak at 554 nm and a \( \beta \)-peak at 525 nm and a Soret difference peak at 424 nm (Fig. 8). Sulfite oxidase, purified from rat liver, shows identical difference maxima. Addition of DPNH to the sulfite-reduced digitonin-solubilized fraction produced about a 20% increase in absorbance at the maxima, whereas addition of sulfite to a DPNH-reduced fraction yielded no increase in absorbance. Sulfite was able to reduce 67% and DPNH 87% of the dithionite-reducible heme. These results were obtained using initially aerobic samples and, whereas the reduction by sulfite was complete within 2 min, reduction by DPNH required 10 to 15 min. It should also be noted that the digitonin concentration in this experiment was sufficient to liberate more than 50% of the rotenone-insensitive DPNH-cytochrome \( c \) reductase activity into the supernate. DPNH was found not to reduce purified rat liver sulfite oxidase nor to reduce cytochrome \( c \) in the presence of the enzyme; and sulfite had previously been shown to be unable to reduce purified microsomal cytochrome \( b \) with or without the flavoprotein reductase (2). The data presented here suggest some interaction between sulfite oxidase and DPNH, presumably mediated by a flavoprotein. That the cytochrome of sulfite oxidase, though similar to microsomal cytochrome \( b \), is spectrally distinct, can be seen by examining the spectra at liquid nitrogen temperatures. Fig. 9 shows that even at concentrations of heme twice that of cytochrome \( b \), there is complete symmetry of the \( \alpha \)-peak with a maximum at 554 nm while purified \( b \) shows a double \( \alpha \)-peak and a triple \( \beta \)-peak as previously described (24).

**Oxidation of Sulfite by Intact Mitochondria**—When mitochondria were reduced by sulfite and allowed to become anaerobic and examined spectrophotometrically, a typical mitochondrial difference spectrum was obtained, as seen in Fig. 6, which was indistinguishable from that obtained with succinate. However, unlike succinate reduction, reduction by sulfite was unaffected by antimycin A at 25 \( \mu \)g per ml. It was also unaffected by seco-barbital and rotenone. An attempt was made to specifically observe the reduction of the \( b \)-like cytochrome with sulfite as was previously done with external DPNH (25). However, there was no reproducible difference which could be attributed to a heme in a sulfite plus succinate versus succinate difference spectrum.

**Metabolism of Sulfite by Mitochondria**—Because of the observation that sulfite can reduce mitochondrial cytochromes, experiments were performed to determine whether the oxidation of sulfite could be coupled to ATP formation. Results of two independent experiments, shown in Table V, indicate that 1 molecule of ATP is formed for every molecule of sulfite oxidized, in agreement with the results of Hunter and Ford (26). It was also determined, using an oxygen electrode, that 1 molecule of \( O_2 \) was consumed for 2 molecules of sulfite oxidized by mitochondria. Although both cyanide and azide prevented ATP formation, they had no effect on sulfite oxidation indicating that, in the presence of these cytochrome oxidase inhibitors, oxygen reduction occurred by another mechanism. It was also found that rotenone could not block ATP formation when sulfite was being oxidized.

**DISCUSSION**

The use of detergents on homogenates and subcellular fractions has been found to produce several fold increases in the activities of mitochondrial enzymes (21). This has been attributed to enzyme activation (21). However, as is clear from the results obtained using sodium deoxycholate, sulfite oxidase itself is maximally active in cell homogenates when assayed with respect to its ability to reduce small molecules such as ferricyanide and oxygen, and the apparent activation of its cytochrome \( c \) reductase activity is therefore a result of the inaccessibility of cytochrome \( c \) to the enzyme in its subcellular compartment. Sucinate cytochrome \( c \) reductase activity was also apparently stimulated by the addition of deoxycholate and this might also indicate a permeability problem for cytochrome \( c \). Rotenone-insensitive DPNH-cytochrome \( c \) reductase activity, however, was maximally active.

![Fig 8. Reduced versus oxidized difference spectra of digitonin-treated rat liver mitochondria. Dialyzed digitonin-treated supernate (4 mg of protein per ml) as described in Fig. 6 was placed in sample and reference cuvettes. Sample cuvettes were then treated with either 1 mM sulfite (C), 0.5 mM DPNH (B) or solid digitonize (C) and the absorption spectra recorded at room temperature.](image-url)

![Fig 9. Low temperature spectra of reduced sulfite oxidase and microsomal cytochrome \( b \). 1, 2.5 ml of sulfite oxidase (0.35 mg per ml) in 0.05 M phosphate pH 7.5 was reduced with 1 mM sulfite and after 2 min of incubation frozen in liquid nitrogen. The spectrum was recorded in a Cary 14 using similarly frozen buffer as reference. 2, 2.5 ml of purified microsomal cytochrome \( b \) (0.04 mg per ml) was reduced with 1 mM DPNH plus catalytic amounts of DPNH-cytochrome \( b \) reductase and treated as 1. Pyrex tubes, 12 mm X 75 mm, were used as sample cuvettes. All spectra were recorded twice without any change in shape.](image-url)
Although the molybdenum signal was present after only 1 to 2 min of incubation, similar to that obtained with sulfite, the complete reduction of the heme by DPNH required 10 to 15 min, while reduction by sulfite was again complete within 1 to 2 min. Whether this is due to more than the concentration differences involved remains to be determined.

The release of sulfite oxidase and the sulfite-reducible heme and molybdenum by digitonin confirms a previous report that hypotonic treatment of mitochondria also released the same protein (22). It has also been reported that similar treatment of mitochondria releases two heme pigments with molecular weights of 120,000 and 12,000, respectively (28). Since DPNH was found to reduce about 20% more of this pigment than did sulfite, perhaps DPNH was also reducing the 12,000 molecular weight cytochrome in addition to reducing sulfite oxidase.

Purified sulfite oxidase, unlike either microsomal $b_2$ or mitochondrial $b_2$ (24), when examined spectrally at liquid nitrogen temperature, produces a symmetrical $a$ peak at 551 nm on reduction by sulfite.

The presence of a native molybdenum signal in intact mitochondria and isotonically dialyzed mitochondria suggests that perhaps the signal observed when sulfite is added to the enzyme is not due to a reduction of the molybdenum but due to a change in its environment. Among other molybdenum-containing enzymes, purified aldehyde oxidase has a native EPR signal attributed to molybdenum (29) which can be augmented by addition of substrate, while xanthine dehydrogenase of Micrococcus lactilyticus has a native molybdenum EPR signal unaffected by substrate (30). Using purified chicken liver sulfite oxidase and xanthine dehydrogenase, it has been observed that, at acid pH, some EPR detectable molybdenum can be seen without the addition of substrate.3 Purified bovine (3) and chicken (8) sulfite oxidase contain 2 moles of molybdenum per mole of enzyme. In addition, at low concentrations of chicken liver sulfite oxidase, the observed weight average molecular weight decreases, approaching that of the subunit (8). Perhaps in intact mitochondria sulfite oxidase exists in its monomeric form with molybdenum in its pentavalent state. Upon release of the enzyme the molybdenum signal might be eliminated by dimerization of the enzyme resulting in spin-coupling interactions between the two molybdenum centers. Reduction by sulfite of dimeric enzyme would serve to change the conformation of the protein to minimize this interaction and allow the unpaired electron to be spectrally observed.

The observation of a molybdenum EPR signal in liver slices and mitochondria by Peisach et al. (23) can be attributed to sulfite oxidase both on the basis of the observed effect of D$_2$O and our observation of the pH-dependence of the signal. The characteristic effect of pH on the shape of the signal in liver mitochondria shows that all of the native molybdenum EPR signal in these preparations is ascribable to sulfite oxidase. In addition, on analysis for the other two known mammalian molybdenum-containing enzymes, we were unable to find any aldehyde oxidase in rat liver using the $N^2$-methylnicotinamide assay as described for rabbit liver (31), while xanthine oxidase activity was present in the 100,000 g supernate as previously described (32).

Sulfite oxidase by coupled mitochondria in the absence of inhibitors of electron transport resulted in one ATP formed per sulfite oxidized. Although sulfite reduces all of the mitochondrial cytochromes under anaerobic conditions, it is likely that the electrons from sulfite feed from sulfite oxidase to intramitochondri
drial cytochrome c whence they are transferred to cytochrome oxidase and then to oxygen. Using an ultrastructural stain, Seligman et al. (33) demonstrated that cytochrome c is localized to the outer surface of the inner membrane. Such a location makes it readily accessible to the intermembranous enzymes such as sulfite oxidase. In the presence of uncouplers or inhibitors, sulfite is oxidized as well but without generating any ATP. Since the b$_5$-like cytochrome of sulfite oxidase is auto-oxidizable and unaffected by cyanide or azide (2), the enzyme can act directly to reduce oxygen.

One further point needs to be emphasized. The present study has shown that cytochrome c is indeed a natural, and seemingly physiological electron acceptor for sulfite oxidase and that the cytochrome c reductase activity is not an artifact of preparation. It has been shown that this activity of the enzyme is extremely susceptible to inhibition by anions (1, 8). Whether anions might poise the interaction of the sulfite oxidase-containing electron transport system with the energy generative system is being investigated.

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