THE INITIAL STEP IN ENZYMATIC SULFITE OXIDATION*

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Previous studies have established the presence in liver of an enzyme system which catalyzes the oxidation of sulfite (1-4). Some of the properties of this system have been described (1, 2), as well as a procedure for its partial purification (4). The system is known to be a complex of several enzymes and involves the reversible participation of hypoxanthine or inosine (4) and a terminal flavoprotein. The present report is concerned with the initial event in this series of reactions.

EXPERIMENTAL

The enzyme preparation used in these studies was identical with that employed previously and designated Fraction F-III A (4). Essentially, it was prepared by extraction of acetone-powdered dog liver with 10 volumes of 0.05 M phosphate buffer, pH 7.8, removal of the protein insoluble at pH 5.5, and that precipitated by brief exposure to 60°, and removal of nucleic acids with protamine. All assays were performed in the presence of 0.01 per cent Versene to prevent sulfite autoxidation. The preparation catalyzes aerobic sulfite oxidation in phosphate or tris(hydroxymethyl)-amino methane buffer and also catalyzes reduction of methylene blue, gallocyanin, and other oxidation-reduction dyes.

The anaerobic reduction of methylene blue proved to be inhibited 50 per cent by 10-4 M arsenite and by 10-4 M p-chloromercuribenzoate (PCMB). This level of sensitivity is below that of the pyruvate and ketoglutarate oxidase systems in which lipoate is employed, but considerably above that of the numerous other enzymes which are sensitive to these agents at concentrations of 0.01 to 0.001 M. In contrast, the aerobic oxidation of sulfite was much less sensitive to arsenite; a concentration of 0.01 M was necessary to achieve 50 per cent inhibition.

It appeared, therefore, that in oxygen the sensitive sulfhydryl groups are maintained in the disulfide form and are less readily available to react with agents such as arsenite. The suggestion thus arose that sulfite oxidation

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may involve reversible reduction and oxidation of a disulfide structure. This was substantiated by the progressive nature of PCMB inhibition. Whereas preincubation with 0.0025 M PCMB was almost without effect, the aerobic oxidation of sulfite was inhibited 5 per cent in the first 10 minutes, 15 per cent in the second 10 minutes, and 40 per cent in the third 10 minutes. When samples of the enzyme preparation were anaerobically incubated with PCMB and examined spectrophotometrically by the Boyer procedure (5), the presence of sulfite (10 μmoles per ml., pH 7.0) elicited

![Fig. 1. Oxidation of sulfite and sulphydryl compounds by partially purified sulfite oxidase. Each flask contained 1.0 ml. of enzyme and one of the following: sulfite, 10 μmoles (●); cysteine, 20 μmoles (○); dimercaptolipoate, 10 μmoles (■); or reduced glutathione, 25 μmoles (▲); in 2.2 ml. of 0.05 M potassium phosphate, pH 7.8, containing 0.01 per cent Versene Fe-III.](http://www.jbc.org/)

the exposure of 0.9 to 2.2 moles of sulphydryl per 100,000 gm. of protein. Because of the crude state of the enzyme preparation, the absolute values here are without significance, but they are compatible with the possibility that sulfite oxidation may involve the reversible participation of disulfide and sulphydryl groups.

This possibility was further supported by the observation that both dimercaptolipoate¹ and cysteine were oxidized by the same preparation,

¹ Dimercaptolipoate was prepared by reduction with sodium borohydride at pH 7; the excess borohydride was discharged with acid and the dimercaptan was extracted into benzene. Almost quantitative yields were obtained when the reaction was followed by observing the absorption of the disulfide form at 330 μμ (7) and
aerobically, at a rate comparable to that of sulfite (Fig. 1). In contrast, the oxidation of reduced glutathione was relatively slow. Further evidence was offered by the surprising observation that the enzyme system respired with borohydride as “substrate.” The addition of 1.0 μmole of oxidized lipoate to a vessel containing 20 μmoles of sulfite accelerated oxygen consumption by 15 to 40 per cent. In contrast, an equivalent amount of cystine effected a 55 per cent decrease in the rate of sulfite oxidation, again suggesting that lipoate, rather than cystine, may be the source of the sulfhydryl group involved in sulfite oxidation (Fig. 2). When

![Graph of Fig. 2](http://www.jbc.org)  
**Fig. 2.** Effect of lipoate and various inhibitors on the oxidation of sulfite (O). Each flask contained 1.0 ml. of enzyme, and 10 μmoles of sulfite in 2.2 ml. of 0.05 M potassium phosphate, pH 7.8, containing 0.01 per cent Versene Fe-III. Appropriate flasks contained 1.0 μmole of lipoate (△), 1.0 μmole of cystine (□), 4 μmoles of cysteine thiosulfonate (■), or 4 μmoles of thiosulfate (○).  

the enzyme preparation was offered a mixture of sulfite (10 μmoles) and dimercaptolipoate (10 μmoles), the rate of oxidation was not significantly greater than that observed when the smaller dimercaptolipoate concentration was employed, thus indicating that the same reaction step is limiting in the enzymatic oxidation of these compounds. However, when cysteine and sulfite were similarly combined, the rate of oxygen consumption, while initially rapid, soon declined markedly, presumably because of the inhibitory effect of the cystine so formed.  

measuring the sulfhydryl produced by the Boyer procedure (5). Per sulfhydryl released, the usual reaction with nitroprusside was about 10 per cent that with cysteine, presumably accounting for the statement that lipoate cannot readily be reduced by borohydride (7).
A series of sulfur-containing acids was tested as possible inhibitors of sulfite oxidation. Benzenesulfonic acid, pyridine-3-sulfonic acid, ethanesulfonic acids, cysteic acid, and cysteinesulfinic acids were found to be without effect. Inorganic thiosulfate, however, proved to be an effective inhibitor of sulfite oxidation both aerobically and in the dye reduction assay (Fig. 2).

The structure of thiosulfate, \( S\text{--SO}_3^- \), suggested a possible mechanism for the initial step in sulfite oxidation. Bisulfite is known to react with disulfides, cleaving them to a sulfhydryl and thiosulfonate (6). Thus, were

\[
\text{RS-S-R + HSO}_3^- \rightarrow \text{R-SH + R-S-SO}_3^-
\]

\( \text{HSO}_3^- \) to react with a disulfide component of the enzyme system, subsequent hydrolysis of the thiosulfonate would yield a second sulfhydryl group and sulfate. Were lipoate the disulfide in question, the mechanism might be depicted as shown in Fig. 3.

![Fig. 3. The initial steps in sulfite oxidation](image)

Since cystine inhibited sulfite oxidation, cysteine thiosulfonate was prepared from cystine (6) and was found to inhibit sulfite oxidation, aerobically and in the dye reduction system, about as effectively as does thiosulfate (Fig. 2). As neither thiosulfate nor cysteine thiosulfonate inhibited the oxidation of dimercaptolipoate or cysteine, it was concluded that these agents may inhibit sulfite oxidation by competing with the active thiosulfonate in the hydrolytic step of the sequence shown in Fig. 3. Accordingly, it seemed desirable to determine whether the enzyme preparation was capable of catalyzing the hydrolysis and subsequent oxidation of lipoate hemithiosulfonate. However, several attempts to prepare this compound, by modifications of the procedure used to prepare cysteine thiosulfonate, failed. Similar results have been reported by Calvin and are in keeping with the unusual nature of lipoate as a disulfide (7). Indeed, since \( E'_0 \) for the lipoate system is 0.2 volt below that of cysteine while \( E'_0 \) for the oxidation of sulfite is of the same order as that of cysteine (8), it is unreasonable to expect significant net synthesis of lipoate hemithiosulfonate. This consideration also accounts for the fact that no significant appearance of \(-\text{SH}\) groups was detected when sulfite and oxidized lipoate in substrate amounts were incubated anaerobically with the enzyme preparation and the reaction followed at 330 m\(\mu\).

The sum of these observations, \textit{viz.} oxidation of cysteine and dimercapto-
lipoate, sensitivity of sulfite oxidation to arsenite and PCMB inhibition, catalysis of sulfite oxidation by lipoate but inhibition by cystine, and inhibition by cysteine thiosulfonate and by thiosulfate, affords strong evidence in support of the reaction sequence shown in Fig. 3 and suggests but does not establish that lipoate is the disulfide compound involved.

Sorbo (9) has postulated a mechanism for the action of rhodanese which involves addition of thiosulfate across an enzyme-bound disulfide group. The similarity of such a reaction with that proposed above, the inhibition of sulfite oxidation by thiosulfate, and the inhibition of rhodanese by sulfite reported by Sorbo led us to investigate the rhodanese activity of our enzyme preparations. Crude preparations of both rat and dog liver sulfite oxidase were rich in rhodanese. However, the final, partially purified preparations were practically devoid of rhodanese activity. Rhodanese and the sulfite-oxidizing system are, therefore, distinct entities.

SUMMARY

The oxidation of sulfite by a partially purified enzyme system from dog liver is inhibited by sulfhydryl reagents, thiosulfate, cysteine thiosulfonate, and cysteine but accelerated by lipoate. The preparation also oxidizes dimercaptolipoate and cysteine. It is concluded that the initial event in sulfite oxidation in this system is reaction with a disulfide to form a thiosulfonate which is hydrolyzed to a sulfhydryl compound and sulfate. It is suggested that lipoate may be the source of the disulfide group.

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