SOME ASPECTS OF SULFIDE OXIDATION BY RAT-LIVER PREPARATIONS*

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INTRODUCTION

Hydrogen sulfide may be formed in the mammalian body through the action of bacteria in the GI tract[2] or via the cysteine desulfhydrase mechanism of tissues[2]. The existence and some properties of a system in rat liver and kidney which oxidizes sulfide to thiosulfate has been described[3,4,5]. A heat-labile component from beef liver performing a similar function has been characterized[6]. Recently SORBO has described a heme-catalysed oxidation of sulfide to thiosulfate[7].

Despite this widespread ability of tissues to oxidize sulfide to thiosulfate, the mechanism and intermediates of this reaction are unknown. The formation of one molecule of thiosulfate from sulfide involves the transfer of 8 electrons and therefore presumably at least 4 reaction steps.

The following communication reports the results of experiments which further characterize the system from the rat liver, some of its reaction mechanism and possible intermediates.

METHODS AND MATERIALS

Adult male rats of the Wistar strain ranging in weight from 200 to 300 g were used for all experiments. In order to increase the capacity of their livers to oxidize sulfide, they were starved for 24 h prior to decapitation[4]. The perfusion of the rat liver and the preparation of the partially purified extracts have been previously described[5]. The preparation and measurement of the hydrogen sulfide substrate, the conditions of incubation and both the iodometric and enzymic methods for the assay of thiosulfate were conducted as reported earlier[5]. The measurement of sulfite in the presence of sulfide and thiosulfate was carried out iodometrically by the method of KURTENACKER[8].

RESULTS AND DISCUSSION

Reaction mechanisms and intermediates in sulfide oxidation to thiosulfate

a. The potential function of peroxide as an oxidizing agent in the rat liver system. It has been reported that catalase is inhibited by sulfide[9]. Thus any peroxide formed in the partially purified rat-liver extract could presumably act as an oxidizing agent

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of sulfur compounds. The possible participation of this type of mechanism in the biological oxidation of sulfide was tested experimentally by generating peroxide from perborate. The reaction mixture was equilibrated with perborate for 5 minutes at 37°, prior to the addition of the sulfide substrate. In the system containing the rat-liver extract an increase of up to 40 % in the titratable thiosulfate was noted (Table I). This might be attributable to the enrichment of the reaction vessel's atmosphere with oxygen during the equilibration period (when the catalase was still active). On the other hand, the results with "buffer only" suggest that in the biological system, peroxide might further oxidize thiosulfate to tetrathionate thus making the 40 % increase too low a figure.

TABLE I

PARTICIPATION OF PEROXIDE IN THE OXIDATION OF SULFIDE TO THIOSULFATE

*System and Additions**	*$S_2O_3^=$ formation***
Complete system	100
Complete system + perborate (H_2O_2)	140 (Max)
Complete system + pyruvate $1 \cdot 10^{-2}$ *M* (final concn.)	99
Complete system + pyruvate $5 \cdot 10^{-2}$ *M* (final concn.)	113
Buffer + substrate only	6
Buffer + substrate + perborate (H_2O_2)	—35***
Buffer + substrate + pyruvate $1 \cdot 10^{-2}$ *M*	11

* 25-ml system consisting of 11 ml rat-liver extract in buffer, 11 ml 0.15 *M* phosphate buffer pH 7.3 with additions where indicated and 3 ml sulfide substrate. Initial concentration of sulfide (Na_2S) in system $5 \cdot 10^{-3}$ *M*.

** As measured by I_2 titration after 30 min of incubation with substrate. Complete active system = 100.

*** This value is negative because of a decrease from the zero blank reading. In another experiment in which thiosulfate was added to a buffer solution, the addition of the above amount of perborate at room temperature removed within 5 min almost one-half of the titratable thiosulfate.

To clarify this point, a more definitive experiment was designed. Pyruvate traps hydrogen peroxide by reacting with it non-enzymically[10]. Table I shows that the inclusion of smaller or larger doses of sodium pyruvate in the complete reaction mixture in no way inhibited the formation of thiosulfate. Thus the involvement of free peroxide as the primary oxidizing agent of the sulfide-oxidizing system in rat liver appears unlikely.

b. Formation of a protein-bound intermediate. When the stoicheiometry of the system was studied[5] it was noted that thiosulfate formation as measured iodometrically appeared to be substantially less than when the enzymic assay technique with rhodanese was employed. The two methods only gave identical results when the reaction was permitted to go to completion.

Fig. 1 shows the relationship of the two assay systems in an experiment in which product formation was studied as a function of incubation time.

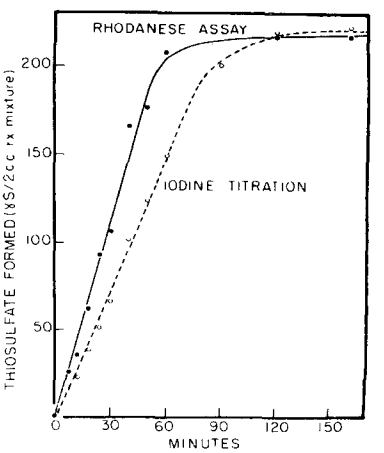

Fig. 1. Apparent thiosulfate formation by sulfide-oxidizing system.

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Samples were incubated for differing periods of time but were otherwise identical. After 30 or 60 min of incubation, the rhodanese assay registered almost twice as much "thiosulfate" formed as did the iodine titration. It was noted that sulfide disappearance corresponded quite closely to the rhodanese assay for thiosulfate formation.

Iodometric thiosulfate measurements were conducted on an aliquot of the deproteinized sample while the rhodanese assays were performed using aliquots which were not deproteinized. Conditions were maintained, however, which precluded the possibility of further oxidation of the sulfide in these samples. When the samples for analysis by rhodanese were deproteinized and lyophilized prior to reacting with cyanide in the presence of the enzyme, identical values were obtained both for iodine titration and rhodanese assay of thiosulfate (Table II). The zinc carbonate deproteinization does not coprecipitate thiosulfate[8,5]. This is confirmed by the data recorded in Table II. Furthermore, these results demonstrate that lyophilization did not destroy any of the thiosulfate of the sample. The conclusion was, therefore, reached that thiosulfate or some similar compound which could act as substrate for rhodanese, adhered to the precipitated proteins.

TABLE II

A COMPARISON OF THIOSULFATE FORMATION AS MEASURED IODOMETRICALLY AND ENZYMICALLY BOTH WITH AND WITHOUT PRIOR DEPROTEINIZATION OF THE SAMPLE

Incubation time (min)	*Addition* $S_2O_3^=$/ml extract*	*Method of thiosulfate assay***			
		I_2 titration on deprot. sample	*Rhodanese on non-deprot. sample*	*I_2 titration on deprot. lyoph. sample*	*Rhodanese on deprot. lyoph. sample*
0	0	0	0	0	0
0	1.85	1.63	1.65	—	—
30	0	1.33	3.13	1.37	1.27
60	0	2.79	5.59	2.77	2.71

* Value represents ml of $1 \cdot 10^{-3}$ M $S_2O_3^=$.

** Values represent ml of $1 \cdot 10^{-3}$ M $S_2O_3^=$ formed per ml of rat-liver-homogenate extract of the reaction mixture. Each reaction vessel contained 10 ml of rat-liver extract, phosphate buffer pH 7.3 and addition to make a final volume of 25 ml.

Substrates other than thiosulfate for impure preparations of rhodanese have been reported. Both sulfide and sulfur were checked for their effects on the assay procedure and were found to be inactive. β-Mercaptopyruvate has been reported to react with cyanide in the presence of crude rhodanese to form thiocyanate[22]. β-Mercaptopyruvate will also titrate with iodine before deproteinization of a sample, but it is removed from solution by the zinc carbonate-deproteinization procedure*. Theoretically, therefore, it could be the compound acting as the supplementary substrate for rhodanese. This seems unlikely, however, in view of the high oxygen uptake[5] and the failure of high concentrations of pyruvate (which might be expected to stimulate β-mercaptopyruvate production) to inhibit thiosulfate formation as measured iodometrically. MEISTER[12] has shown, furthermore, that in the absence of cyanide, *in vitro* rat-liver preparations decompose β-mercaptopyruvate to hydrogen sulfide and pyruvate quite rapidly while the reverse reaction did not appear to occur.

* Unpublished data: the β-mercaptopyruvate was kindly supplied by Dr. A. MEISTER.

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SORBO recently reported that thiosulfonates may act as substrates for rhodanese[13]. A compound of this nature has also been postulated by FRIDOVICH AND HANDLER[14] as an intermediate in the conversion of sulfite to sulfate. Such a compound if attached to protein would be co-precipitated with the protein fraction in the zinc carbonate deproteinization procedure. Thus, on the basis of known facts, the following scheme is postulated.

$$HS^- \longrightarrow 1\,\tfrac{1}{2}O_2 \longrightarrow HSO_3^- \quad (A)$$

$$R\text{–}S\text{–}S\text{–}R + HSO_3^- \longrightarrow RSH + R\text{–}S\text{–}SO_3^- \quad (B)$$

$$RS\text{–}SO_3^- + HS^- \longrightarrow S_2O_3 + RSH \quad (C)$$

$$2RSH + \tfrac{1}{2}O_2 \longrightarrow R\text{–}S\text{–}S\text{–}R + H_2O \quad (D)$$

$$\text{Sum: } 2HS^- + 2O_2 \longrightarrow S_2O_3 + H_2O \quad (E)$$

Assuming reaction (C) to be the rate-limiting step, a temporary accumulation of $R\text{–}S\text{–}SO_3^-$ would be expected, as reflected by the difference of the iodine and rhodanese assay for thiosulfate (Fig. 1).

Thiosulfate inhibits the sulfite oxidase system of FRIDOVICH AND HANDLER[15], presumably through the hydrolytic reaction for the conversion of the sulfur moiety of thiosulfonate to sulfate; *i.e.*: $R\text{–}S\text{–}SO_3^- + H_2O \rightarrow SO_4^= + RSH + H^+$. It might also be inhibited by the action of sulfide substrate on the (metallo?) flavoprotein involved in the reaction sequence[14]. The virtually complete suppression of this final step in the sulfite oxidase mechanism would afford the possibility of an accumulation of the intermediate $R\text{–}S\text{–}SO_3^-$ which in the presence of sulfide is converted to thiosulfate. Such a mechanism could well explain the reason for thiosulfate accumulation in *in vitro* systems of tissue extracts.

c. Sulfite as an intermediate. Whether free sulfite actually occurs as an intermediate in the reaction sequence (A), is not yet known. Acetaldehyde, a known sulfite-trapping agent, inhibited thiosulfate formation in the system, but the mode of this inhibition remains obscure. Only very limited reactivation or apparent reactivation could be achieved by the addition of substantial doses of sulfite after acetaldehyde inhibition. On the other hand, thiosulfate formation was greatly enhanced both in the

TABLE III

THE EFFECT OF SULFITE ADDITION ON THE FORMATION OF THIOSULFATE FROM SULFIDE

Incubation mixture	*Thiosulfate formed*	*Sulfide disappeared*	*Sulfite disappeared*
	γS/ml reaction mixture		
Homogenate extract in buffer*			
+ HS^-	68	94	—
+ HSO_3^-	0	—	160
+ HS^- + HSO_3^-	92	73	69
Buffer only			
+ HS^-	13	75	—
+ HSO_3^-	0	—	180
+ HS^- + HSO_3^-	56	68	65

* The extract contained 3.7 mg rat liver protein/ml. Approximately 150γS of sulfide and 200γS of Na_2SO_3 were added as indicated per ml of reaction mixture. The incubation was run in phosphate buffer at pH 7.25 and 37° for 2 h.

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uninhibited extract and in the buffer system when sulfite was added (Table III). In addition, the results recorded in Table III demonstrate that after prolonged incubation more thiosulfate was formed in the liver extract than could be accounted for by the sulfide disappearance. Under these conditions, therefore, sulfite sulfur appears to participate in the synthesis of thiosulfate. SORBO has recently described a similar enzymic condensation in which sulfite reacted with β-mercaptopyruvate to form thiosulfate[16]. (His results do not exclude the possibility that the β-mercaptopyruvate was first degraded to sulfide and pyruvate.) The ability of sulfide to inhibit the oxidation of sulfite is common to both the extract and the buffer system. In the former, this is doubtlessly due to the inhibition of the sulfite-oxidase system[15] while in the latter it can be attributed to the removal by sulfide of metal contaminants of the buffer solution which are known to catalyse sulfite oxidation non-enzymically[17].

d. Inhibition of the sulfide-oxidizing system. The effect of inhibitors on the purified heat-labile sulfide-oxidizing system is reported in detail elsewhere[6]. The crude extract and the 67° supernatant of rat liver were inhibited in their ability to oxidize sulfide to thiosulfate by $4 \cdot 10^{-3}$ *M* KCN (50–100 %), $4 \cdot 10^{-4}$ *M* iodoacetate (30–50 %) even when the excess iodoacetate was first dialized away; $4 \cdot 10^{-2}$ *M* acetaldehyde (100 %) $4 \cdot 10^{-3}$ *M* acetaldehyde (40 %), $4 \cdot 10^{-4}$ *M* α,α'-dipyridyl (0–30 %) N_2 atmosphere (92–98 %) carbon monoxide gassing (50 %), but this may have been due to a lack of oxygen in the reaction vessel.

Sodium azide and sodium fluoride did not inactivate the system.

e. Effect of chelators. The effect of Na_4 ethylenediaminetetraacetate (EDTA) and 8-hydroxyquinoline are presented in Table IV. At a concentration of 10^{-4} *M*, EDTA enhanced the rate of thiosulfate formation in the complete rat-liver system from 70 to 180 %. While the degree to which the total activity was stimulated varied with different preparations[18], the same concentration of EDTA always elicited a similar quantitative response from the heat-stable component of the system.

At concentrations of $2 \cdot 10^{-4}$ *M*, 8-hydroxyquinoline stimulated the complete rat liver system by 60 to 100 %. In contrast to EDTA, however, it had no effect on the

TABLE IV

EFFECT OF EDTA AND 8-HYDROXYQUINOLINE ON THE SULFIDE-OXIDIZING SYSTEM OF RAT LIVER

Chelator *Molar concn. in system*	*Thiosulfate formation* *γS of $S_2O_3^=$/mg protein/min*		
	Total activity (TA)	*Heat-stable activity* (HSA)*	*Heat-labile activity (TA)−(HSA)=(HLA)*
None	0.22	0.08	0.14
EDTA $4 \cdot 10^{-7}$	0.24	0.08	0.16
$4 \cdot 10^{-6}$	0.28	0.12	0.16
$4 \cdot 10^{-5}$	0.41	0.28	0.13
$4 \cdot 10^{-4}$	0.42	0.31	0.11
None**	0.26	0.02	0.24
8-Hydroxyquinoline $4 \cdot 10^{-7}$	0.27	0.02	0.25
$4 \cdot 10^{-6}$	0.28	0.03	0.25
$4 \cdot 10^{-5}$	0.34	0.04	0.30
$2 \cdot 10^{-4}$	0.42	0.04	0.38

* Sample was heated to 100° for 3 min. Coagulated protein was rehomogenized.

** This represents a different rat-liver preparation from the one used for the EDTA experiment.

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heat-stable component of the system. Thus, while EDTA stimulated the heat-stable fraction of the sulfide-oxidizing system, the effect of 8-hydroxyquinoline was primarily on the heat-labile fraction. The response to chelators, therefore, provides evidence in addition to the kinetic studies[5,9] differentiating the heat-labile (HLA) and the heat-stable (HSA) fractions of the sulfide-oxidizing system in rat liver. Chelators by themselves had no sulfide-oxidizing ability.

Dialysis, ashing and the effect of EDTA

Dialysis did not inactivate either the heat-labile or the heat-stable fraction of the system. After the addition of EDTA, however, a sizable amount of the increased sulfide-oxidizing capacity could be dialized away. This is shown in Table V. The first change of dializing buffer indicated that at least a portion of the catalytic factor had migrated through the dializing sack. This factor was non-proteinous and in all likelihood a metal–versene complex.

TABLE V

DIALYSIS OF RAT-LIVER PREPARATIONS STIMULATED WITH EDTA

	Thiosulfate formation γS of $S_2O_3^=$/mg protein/min		
	Non-dialized	*Dialized***	*Dialysis Δ*
Extract (TA)	0.25	0.26	+0.01
Extract + EDTA*	0.67	0.40	—0.27
Boiled extract (HSA)	0.07	0.07	0
Boiled extract (HSA) + EDTA*	0.34	0.19	—0.15

* Concentration of EDTA in system $2 \cdot 10^{-4}$ *M*.
** Dialized 1/200,000 with 6 changes of phosphate buffer *M*/15, pH 7.3 at 3° over 24-h period.

When samples of the rat-liver extract in phosphate buffer were ashed at 500°, the resulting salts were usually inactive as catalysts in the sulfide oxidation. The addition of low concentrations of EDTA to the ash invariably resulted in a very active catalyst. These results lent further support to the theory that an EDTA-metal chelate could act as a catalyst in sulfide oxidation. 8-Hydroxyquinoline did not activate the ash.

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SUMMARY

1. The sulfide-oxidizing system in rat liver does not require peroxide for its operation.

2. The existence of a protein-bound intermediate, possibly a thiosulfonate, has been demonstrated.

3. Sulfite may be involved as an intermediate in the *in vitro* formation of thiosulfate from sulfide. A reaction mechanism has been proposed which accounts for the accumulation of thiosulfate in *in vitro* systems.

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4. Some effects of inhibitors and chelators on the rat-liver system have been described. While 8-hydroxyquinoline stimulated the heat-labile fraction, EDTA stimulated the complete system through its action on the heat-stable fraction.

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THE OXIDATION OF SULFIDE TO THIOSULFATE BY METALLO-PROTEIN COMPLEXES AND BY FERRITIN*

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INTRODUCTION

In previous papers[1,2] some aspects of sulfide oxidation to thiosulfate by rat liver preparations were reported. It was noted in particular that chelating agents such as 8OH quinoline and EDTA§, at specific molar concentrations, differentially stimulated the heat-labile and heat-stable components of the rat-liver system[2,3]. Ashed samples of this system were "reactivated" by EDTA in their ability to oxidize sulfide to thiosulfate. It was postulated that an EDTA-metal chelate acted as a non-enzymic catalyst for these oxidations.

As dialysis did not change the sulfide-oxidizing capacity or the response to

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§ The following abbreviations have been used: EDTA – The tetra sodium salt of ethylene diamine tetraacetic acid. 8OH quinoline – 8 hydroxyquinoline.

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