SULFIDE OXIDATION IN RAT TISSUES*

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INTRODUCTION

The enzymic pathways involved in the catabolism of sulfur-containing amino acids have been reviewed by SINGER AND KEARNEY[1], and more recently by CHATAGNER AND BERGERET[2]. Two distinct routes are recognized by which the sulfur of cysteine is oxidized to inorganic sulfate; one via cysteine sulfinic acid and the other through either the cysteine desulfhydrase or transaminase-desulfurase mechanism to hydrogen sulfide, with subsequent oxidation to sulfate.

The cysteine desulfhydrase mechanism of this latter pathway has been the subject of numerous studies. In mammalian tissues the formation of hydrogen sulfide from cysteine can be demonstrated under anaerobic conditions[1,2,3,4]. Microorganisms of the intestine also produce hydrogen sulfide[5], and in the case of *Escherichia coli* this process is greatly enhanced by aerobic conditions[6]. Relatively little is known about the ability of mammalian tissue to oxidize hydrogen sulfide, or the pathways and mechanisms by which this is accomplished. *In vivo* experiments by DZIEWIATKOWSKI[7] have shown that small doses of ^{35}S-labeled sodium sulfide injected into rats are partially converted to ^{35}S-labeled sulfate. DER-GARABEDIAN AND FROMAGEOT[8] reported that in tissue homogenates, sulfide is oxidized to thiosulfate. In subsequent publications TER-KAREPETIAN[9] *alias* DER-GARABEDIAN reported some of the characteristics of this "enzymic" system which he named sulfide oxidase. Recently SORBO[10] reported the heme-catalyzed oxidation of sulfide to thiosulfate.

The studies reported here represent an extension of some of this earlier *in vitro* work. Our results confirm the formation of thiosulfate by rat-liver extracts, but differ from the earlier reports[8] with respect to some of the characteristics ascribed to the catalytic system involved in this oxidation.

METHODS AND MATERIALS

Rat liver extracts

Young adult male rats of the Wistar strain, ranging from 200 to 300 grams in weight were maintained on pelleted Purina small animal chow. They were starved for 24 hours prior to sacrificing§.

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§ The stimulating effect of starvation on the sulfide oxidase system is described elsewhere[11,19].

References p. 591.

To obtain rat-liver extracts, the animals were lightly anesthetized with ether and decapitated. The livers (and occasionally the kidneys) were immediately exsanguinated by introducing a cannula into the inferior vena cava and perfusing *in situ* with ice cold $M/7.5$ phosphate buffer pH 7.2. The perfused livers were rinsed, blotted, weighed, resuspended in cold phosphate buffer (pH 7.2) and homogenized in a Waring blender for 1 minute. The final homogenates contained 120 mg liver (wet weight) per ml. The rat-liver homogenates were spun at 12000 *g* for 15 min in a refrigerated centrifuge. The precipitate was discarded. Routinely the supernatant extracts were used immediately after preparation for the experiments described below.

The protein content of each liver extract was determined by the method of LOWRY *et al.*[12].

Substrate and test system

Washed sodium sulfide crystals ($Na_2S \cdot 7H_2O$) were dissolved in water to make a $4 \cdot 10^{-2}M$ solution. The test system, consisting of 22 ml of liver extract in buffer and contained in a closed Erlenmeyer flask, was first preincubated for 5 min at 37°C, 3 ml of the sulfide substrate was then added, the mixture shaken and the zero time sample withdrawn immediately. Occasionally larger flasks with proportionally larger volumes were employed. Both the addition of substrate and the withdrawal of aliquots for sulfide and thiosulfate assay, was accomplished through a short length of plastic tubing attached to a glass tube which extended into the reaction mixture. Except for the times when additions were made or samples were withdrawn, the tube was kept tightly closed. This minimized the loss of substrate through hydrogen sulfide escaping to the outer atmosphere during the sampling process. All incubations were conducted (unless indicated to the contrary) in an atmosphere of air in a water bath shaker at 37°C.

Assay for sulfide and thiosulfate

Sulfide oxidation in the system could be followed both by the disappearance of substrate and by the formation of thiosulfate in the reaction mixture. The sulfide remaining in the reaction mixture was measured colorometrically by the methylene blue method as described for inorganic systems by FOGO AND POPOWSKY[13] and used previously for the assay of alkaline phosphatase in biological materials by DOYLE[14]. A 0.3 ml aliquot of the incubation mixture was required for this assay. Thirty minutes after the addition of the N,N-dimethyl-*p*-phenylenediamine sulfate and prior to the assay of the colored solution (in a D. U. Beckman spectrophotometer at 670 mμ), a portion of it was spun to precipitate all cloudiness due to biological material.

The method was standardized both by comparison with the less specific iodine titration technique for hydrogen sulfide in solution[18] and by the theoretical values of sulfide present (based on the original weight of the sodium sulfide crystals). The relationship of the sulfide present and color formation was linear and reproducible in the range tested (Fig. 1).

Thiosulfate formation was determined by chemical and enzymic assay procedures. Routinely KURTENACKER's titration technique[15] for thiosulfate in the presence of sulfide, sulfite and sulfate was employed with the following modifications:

(1) The $10^{-3}M$ iodine solution used for titration was kept in a colored automatic burette under a nitrogen atmosphere, thus no change in strength of the solution occurred over extended periods of time. (2) The titration endpoints were greatly improved by removing residual proteins and phosphates from the aliquot with uranyl acetate.

An aliquot of 10 ml of the reaction mixture was shaken with 5 ml of a neutral $ZnCO_3$ slurry and 2 ml glycerol in a 50 ml volumetric flask. After 10 minutes, 20 ml of an uranyl acetate solution (0.5 g/100 ml) were added and the mixture again shaken. Following another 10 minutes, the mixture was diluted to 50 ml final volume, and filtered. 10 ml of the filtrate were rapidly titrated against $0.001M$ iodine in the presence of 1 ml formol and 3 ml of a 10% acetic acid solution. Potato starch in borate was used as indicator. All samples were run in duplicate.

The method was reproducible and accounted for from 94 to 100% of any thiosulfate added directly to a homogenate before deproteinization. This indicated that adsorption of thiosulfate to the precipitate was negligible.

The method employed for measuring thiosulfate formation using rhodanese, was based on SORBO's assay technique for rhodanese[16]. A system was employed in which the thiocyanate formed by the reaction $CN^- + S_2O_3^= \xrightarrow{\text{rhodanese}} CNS^- + SO_3^=$ was *limited only* by the amount of thiosulfate present in the sample. To 2 ml of the incubation mixture and 0.5 ml of $0.2M$ KH_2PO_4. 8 mg of rhodanese* dissolved in 0.5 ml of $5 \cdot 10^{-4}M$ thiosulfate with a trace of albumin was added, The reaction was started by the addition of 0.5 ml of $0.25M$ KCN, and after five minutes of shaking at room temperature, stopped by the addition of 0.5 ml of formol. Subsequently 5 ml of ferric nitrate reagent was added**. The volume of the mixture was increased to 20 ml with distilled water and immediately filtered. The optical density of the filtrate was determined in a Beckman DU

* 100 g $Fe(NO_3)_3 9H_2O$ plus 200 ml concentrated HNO_3 made up to 1 l.

** Obtained from Mann Biochemical Corp. U.S.A.

References p. 591.

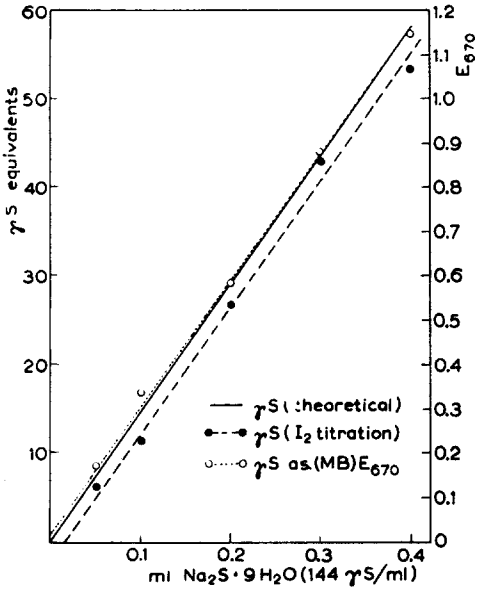

Fig. 1. Iodometric and colorimetric determination of sulfide in tissue extracts.

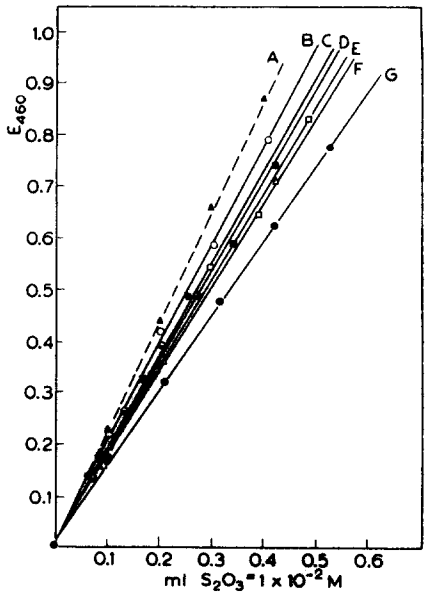

Fig. 2. Standard curves for thiosulfate assay with Rhodanese in the presence of various rat-liver extracts.

spectrophotometer at 460 mμ. Thiosulfate formation was measured as the change in optical density from that of the zero time aliquot. Standard curves were constructed with different rat-liver extracts and known amounts of thiosulfate. The relationship between the thiosulfate added and the optical density at 460 mμ was always linear. However, the slope of standard curves in different liver preparations did not always coincide (Fig. 2), so that to quantitate results, it was necessary to obtain a standard curve for every liver preparation used. It was found experimentally that the intrinsic rhodanese of the crude liver preparation or sulfide substrate was not the modifying factor involved. The presence of sulfide or sulfur in the reaction mixture did not measurably modify the results under the assay conditions employed.

RESULTS AND DISCUSSION

Two parallel systems

When fresh extracts of perfused rat livers in phosphate buffer were incubated at pH 7.3 in a closed system at 37°C with ample sodium sulfide as substrate, thiosulfate was formed at a uniform rate over a 2 h period. This is shown in Fig. 3. For any particular preparation the formation of thiosulfate was proportional to the amount of rat liver extract present. Buffer by itself had no appreciable activity. By exposing the preparation to approximately 100°C in a boiling water bath for 3 min, 50 to 90% of its oxidative ability was destroyed. The residual activity remained despite prolonged boiling. When fresh and boiled homogenate were mixed, the resulting activity represented the mathematical sum of the two. Resuspension and rehomogenization of the heat-denatured precipitate did not reactivate the system. Thus incomplete destruction, heat-activated inhibition, or occlusion of just one system, seemed unlikely.

The presence of two separate systems was supported by several experiments. Figs. 4, 5, and 6 show the effects of pH, incubation temperature and substrate concentration respectively on the heat-labile and heat-stable components of the sulfide-

References p. 591.

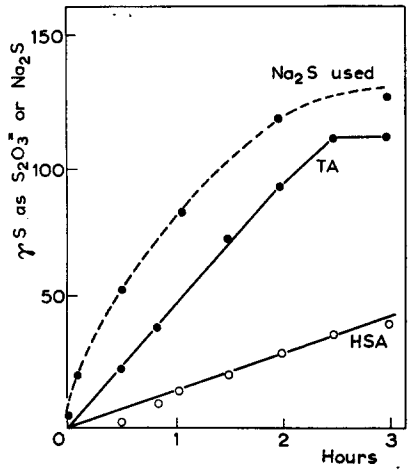

Fig. 3. Thiosulfate formation and sulfide disappearance in the sulfide-oxidizing system of rat liver. TA = total activity (combining heat-stable and heat-labile activity). HSA = heat-stable activity.

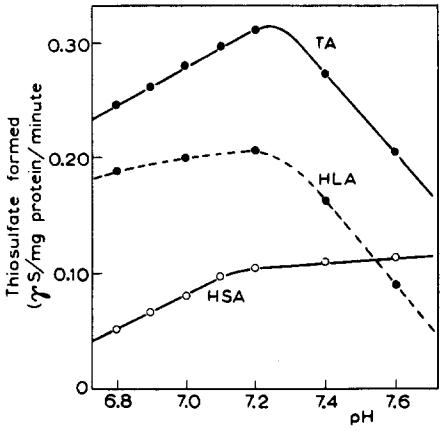

Fig. 4. The effect of pH on the sulfide-oxidizing system. TA = total activity. HSA = heat-stable activity. HLA = heat-labile activity.

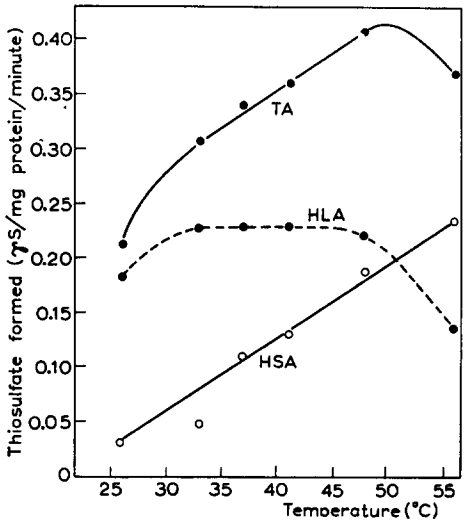

Fig. 5. Effect of temperature on components of sulfide-oxidizing system. TA = total activity. HSA = heat-stable activity. HLA = heat-labile activity.

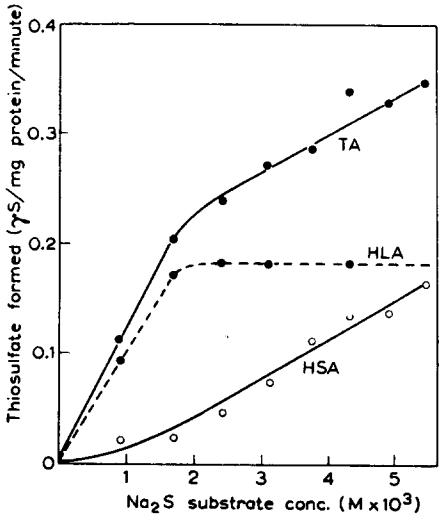

Fig. 6. Effect of substrate concentration on sulfide-oxidizing system. TA = total activity. HSA = heat-stable activity. HLA = heat-labile activity.

oxidizing system. The heat-labile activity in each case, was evaluated as the total activity minus the activity remaining after boiling the preparation for 3 min. The heat-labile component had both a pH optimum around pH 7.2 to 7.3 and a temperature optimum from 35°C to 50°C. The heat-stable component showed neither (Fig. 4 and 5). Furthermore, the heat-labile component of the system became saturated with respect to substrate, yet no substrate saturation could be demonstrated for the heat-stable component within the range of sulfide concentrations compatible with the analytical

References p. 591.

techniques (Fig. 6). Thus it would appear likely that two parallel systems exist in rat liver, both oxidizing sulfide to thiosulfate, one nonenzymic and heat stable and the other with some characteristics of an enzymic system. The two systems may in some way be related to each other. The name "sulfide oxidase" was assigned to the heat-labile system. It corresponds in nomenclature to a heat-stable system described by DER-GARABEDIAN AND FROMAGEOT[8,9].

Masking effect of blood

The heat-labile component of the system was completely masked if the livers were not exsanguinated by perfusion prior to homogenization and extraction. (Table I). If blood was added back to the perfused rat-liver extracts, no apparent decrease in activity was observed upon boiling the preparation as the decreased sulfide-oxidizing ability of the blood-free boiled extract was more than compensated for by the increased activity of the boiled blood; hence the masking effect.

TABLE I

THE MASKING EFFECT OF BLOOD ON THE HEAT-LABILE SULFIDE OXIDASE SYSTEM

Preparation from	*γS of $S_2O_3^=$ formed/ml rx mix/min*			*γS of $S_2O_3^=$ formed/mg protein/min*		
	Fresh T.A.	*Boiled H.S.A.*	*(2)-(3) H.L.A.*	*Fresh T.A.*	*Boiled H.S.A.*	*(5)-(6) H.L.A.*
(1)	*(2)*	*(3)*	*(4)*	*(5)*	*(6)*	*(7)*
Non-perfused liver*	(16.8)	(18.3)	(—1.5)	(0.08)	(0.09)	—(0.01)
Perfused liver	35.1	6.9	28.2	0.31	0.06	0.25
Blood	11.2	40.0	—28.8	0.09	0.31	—0.22
Perfused liver and blood**	46.0	43.5	2.5	0.19	0.18	0.01

* This is a preparation from a different animal to the one used below and thus no direct comparison with reconstituted liver and blood can be made.

** Mixture of 11.3 mg blood protein for every 10 mg of liver protein. Amounts are equivalent to those used for perfused liver and blood, tested separately. T.A. = Total activity. H.S.A. = heat-stable activity. HLA. = heat-labile activity.

Oxygen requirement

Formation of thiosulfate virtually ceased in the absence of oxygen. Very small amounts of thiosulfate formed in a nitrogen atmosphere could be attributed to residual oxygen dissolved in the extract or buffer.

Substrate specificity

No detectable thiosulfate formation could be measured when sulfur or sulfite were used as sole substrates.

End products and stoichiometry

The thiosulfate formed by this system was identified chemically and enzymically as outlined above. Additional qualitative rests were run with solutions free of residual sulfide. The mercury salt techniques, as well as some other tests suggested by KURTENACKER[15] confirmed thiosulfate formation. Sulfide disappearance from the reaction mixture was also measured (Fig. 3). After an initial loss of substrate, presumably to the atmosphere, and equilibration with the vapor phase of the reaction vessel, the rate of sulfide disappearance and thiosulfate appearance corresponded quite closely.

References p. 591.

The stoichiometry of the sulfide-oxidizing system was studied with rat-liver extracts incubated in two-armed Warburg vessels. Into the main compartment 1.5 ml of extract and 1.5 ml phosphate buffer pH 7.3 were introduced. The two sidearms contained 0.4 ml of the sodium sulfide substrate in 0.001 *M* sodium hydroxide and 0.4 ml of 0.001 *M* hydrochloric acid respectively. Oxygen uptake was measured manometrically 5 min after the start of the reaction and every 5 min thereafter for one hour or longer. Due to the escape of some sulfide substrate to the atmosphere of the vessel, the oxygen uptake for the first 5–10 min appeared depressed. It continued at a constant rate thereafter. In calculating oxygen uptake in the usual manner[17], this artifact was eliminated by extrapolating back to zero time from the linear plot representing the time period from 10 to 60 min.

Thiosulfate formation was measured by the rhodanese method. A separate flask was used for each time period. Suitable controls were also run. The correction for endogenous carbon dioxide production was found to be negligible.

TABLE II

STOICHIOMETRY OF SULFIDE-OXIDIZING SYSTEM

Experiment	*Sulfide loss*	*Oxygen uptake*	*Thiosulfate* formed*	*Sulfide S recovered as thiosulfate S*
	μmoles/hour/ml of reaction mixture			
1	4.5	5.0	1.9	86%
Ratio:	(2.3)	: (2.6)	: (1.0)	
2	4.2	4.1	1.6	
Ratio:	(2.7)	(2.6)	(1.0)	76%
Theoretical ratio:	$(2.0)HS^- + (2.0)O_2$		(1.0) $S_2O_3^= + H_2O$	100%

* Rhodanese assay method.

The results of this study are recorded in Table II. The values obtained in two successive experiments were consistent and indicated a comparatively close correspondence to the theoretical ratio of oxygen uptake, sulfide disappearance, and thiosulfate formation. Such discrepancies as do exist, consist of excessive sulfide disappearance and oxygen utilization above that indicated by the thiosulfate formed. This may be due to a variety of reasons, among them (a) a lag period in the system, (b) the formation of by-products other than thiosulfate, or (c) technical difficulties in the assay technique. Thus the escape of sulfide to the outer atmosphere while transferring aliquots would have increased apparent sulfide utilization, and the small return into solution of the hydrogen sulfide of the gaseous phase during the reaction period would have tended to increase the apparent oxygen consumption as measured manometrically while decreasing the apparent sulfide loss.

Distribution of sulfide oxidase activity in rat organs and cell constituents

The heat-labile activity was not confined to the liver, but was also demonstrated in the perfused kidney. Owing to the virtual impossibility of ridding the spleen of blood hemoglobin, no heat-labile system could be demonstrated in this organ. These results are shown in Table III.

References p. 591.

TABLE III

A COMPARISON OF THE SULFIDE-OXIDIZING SYSTEMS IN LIVER, KIDNEY AND SPLEEN

No. of rats pooled	*Organ*	*Perfused*	*γS of $S_2O_3^=$ T.A.*	*formed/mg H.S.A.*	*prot./min H.L.A.*
3	Liver	yes	0.40	0.10	0.30
3	Kidney	yes	0.51	0.17	0.34
3	Spleen	no	0.19	0.38	—
4	Kidney	yes	0.35	0.03	0.32
4	Spleen	no	0.26	0.45	—

T.A. = Total activity (H.S.A. + H.L.A.). H.S.A. = heat-stable activity. H.L.A. = heat-labile activity.

Fractionation of rat-liver cells into their constituent fractions according to a slightly modified Hogeboom and Schneider technique* gave results recorded in Table IV. Although no clear-cut separation was achieved, the sulfide oxidase was found preferentially in the mitochondrial fraction. If rat-liver homogenates prepared in the usual way in phosphate buffer were spun at 100,000 *g* for one h, the greater part of the activity was found in the supernatant fraction indicating that sulfide oxidase was basically a soluble system.

TABLE IV

DISTRIBUTION OF SULFIDE-OXIDIZING ACTIVITY IN RAT LIVER CELLS*

Cell fraction	*Activity in fraction (% of total activity)*	*Heat-labile activity (% of total activity)*
All components	100	47
Nuclei	12	1
Mitochondria	36	16
Microsomes	16	8
Cytoplasmic supernatant	16	7
Recovery (per cent)	81	32

* Cell fractionation by modified Hogeboom method.

Preliminary purification of rat-liver extract

The crude extract in phosphate buffer (pH 7.3) was heated rapidly to 67°C and held at this temperature for 2 min. It was immediately cooled in an ice bucket. The supernatant of the cooled spun preparation contained most of the sulfide oxidase system (Table V). A five- to ten-fold increase in specific activity was consistently noted. Of this, the heat-stable component had the greater share. The sulfide oxidase properties of the crystalline protein ferritin, a component of this system, are being reported separately[18].

Further purification of the system by means of salting-out techniques have, to date, met with only sporadic success. If the preparation was separated into two fractions by salting out with ammonium sulfate (pH 6) to a final concentration of 2*M*, both the precipitated and supernatant fractions, after redissolving and dialysis against distilled water at 4°C, contained approximately the same specific activity. The two fractions differed in that the heat-stable activity (HSA) remained preferentially in the

* B. K. JACOBSEN. Personal communication.

References p. 591.

TABLE V

PURIFICATION OF SULFIDE OXIDIZING SYSTEM

Fraction	*Specific activity**		
	Total activity	*Heat-stable activity*	*Heat-labile activity*
Crude homogenate	1.0	1.0	1.0
Extract	1.1	1.0	1.1
70°C Supernatant	5.5	12.6	3.9
70°C ppt.	0.01	0.4	0

* Activity of components in crude homogenate = 1.0/mg protein. Of the total initial activity, 16% is heat-stable activity and 84% is heat-labile activity.

supernatant portion of the ammonium sulfate-treated preparation, while the reverse was true for the heat-labile fraction (HLA) of the sulfide oxidase system.

All properties described in this communication for the crude rat-liver extract were substantially duplicated in the partially purified system. The only exception was the ratio of heat-labile to heat-stable system, due doubtlessly to some destruction of the former during the purification procedure.

SUMMARY

1. Extracts of rat liver and kidney were shown to catalyze the oxidation of sulfide to thiosulfate. The system responsible for this oxidation contained both a heat-stable and heat-labile component, the former non-enzymic and the latter probably enzymic. Some of the dual characteristics and other properties of the system have been described.

2. The presence of blood masked the heat-labile activity in rat-liver tissue.

3. The greater part of oxygen uptake and sulfide disappearance observable in the system could be accounted for by the thiosulfate formed.

4. Sulfide oxidation to thiosulfate, is most closely associated with the mitochondrial fraction of rat-liver cells. Its relative heat-stability has made possible some purification of the system.

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