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.

References p. 578.

chelators of the rat-liver system, it followed that the formation of an active EDTA-metal chelate in the biological system was due to metal ions which, prior to the EDTA addition, were attached to the protein.

The present paper reports a study concerning the properties of a number of metallo-protein complexes as sulfide-oxidizing catalysts and the effect of chelators on such systems. In these experiments iron–protein combinations both artificial (Fe-bovine plasma albumin) and naturally occurring (ferritin) showed pronounced potential for oxidizing sulfide to thiosulfate. Ferritin was by far the most active catalytic agent in this respect, but did not correspond, in all of its properties to the partially purified sulfide-oxidizing system of rat liver[1,2].

METHODS AND MATERIALS

The test system and all methods for measuring the sulfide substrate and thiosulfate product have been previously described[1,2].

Crystalline bovine-plasma albumin was obtained from Armour Laboratories (Chicago, Ill.). All metal salts were of the highest purity obtainable commercially. In the initial experiments the metal concentrations for the metal–protein complexes were chosen to approximate the biological rat-liver system.

Ferritin and apoferritin were crystallized from horse spleen by the method of GRANNICK[4]. Prior to use, solutions of the cadmium–ferritin salt were extensively dialized against triple distilled water.

RESULTS AND DISCUSSION

Model sulfide-oxidizing systems

Work by KREBS[5] and SCHOBERL[6] has indicated that the inclusion of metals in a non-enzymic reaction mixture containing sodium sulfide, increased the rate of oxygen uptake as measured manometrically. KREBS' order of effectiveness for the metals to stimulate oxygen uptake corresponds quite well with the order of the metals to stimulate thiosulfate formation as listed in Table 1. This ability is not a function of the metal-sulfide solubility product.

TABLE I

THE OXIDATION OF SULFIDE TO THIOSULFATE IN THE PRESENCE OF METAL IONS AND METAL–PROTEIN COMPLEXES

Metal ion $4 \cdot 10^{-6}$ *M*	*Protein* mg/ml	$S_2O_3^=$ *formed** μmoles/ml/h	*Solubility product MeS 18°*
None	None	0.0–0.1	
None	8	0.0–0.1	
Cu^{++}	—	0.3	$8.5 \cdot 10^{-45}$
Cu^{++}	8	0.5–1.1	
Co^{++}	—	0.2	$3 \cdot 10^{-26}$
Co^{++}	8	1.1–1.4	
Ni^{++}	—	1.6	$1.4 \cdot 10^{-24}$
Ni^{++}	8	1.1–1.6	
Fe^{++}	—	0.2	$37 \cdot 10^{-19}$
Fe^{++}	8	0.9–1.2	
Mn^{++}	—	0.5	$1.4 \cdot 10^{-15}$
Mn^{++}	8	0.8–0.9	

Incubation in phosphate buffer pH 7.3 at 37°. All systems turned brown or black upon addition of substrate. Sulfide substrate concentration $5 \cdot 10^{-3}$ *M*.

* Variability caused by method of mixing protein and metal salt, time lapse before phosphate buffer addition, etc.

References p. 578.

Metal chelates, especially those of iron and cobalt can react reversibly with oxygen and thus act as oxygen carriers[7]. When EDTA was added to the various cations in phosphate buffer, (both metal ion and EDTA at a final concentration of $1 \cdot 10^{-6}$ or $1 \cdot 10^{-4}$ *M*) nickel, iron (both ferrous and ferric), cobalt and magnesium showed increased sulfide-oxidizing properties as the metal chelate. The sulfide-oxidizing properties of copper, manganese, molybdenum, and zinc ions were not enhanced but occasionally depressed by the addition of EDTA. The optimal ratio of molar concentrations of metal to chelator was 1:1. High metal concentration in the system suppressed the rate of sulfide oxidation through combination with a substantial portion of the substrate. The addition of EDTA reversed this trend. The details of these experiments will be reported elsewhere.

Metal–protein combinations of Ni^{++}, Mn^{++}, Cu^{++}, Fe^{++}, (Fe^{+++}) and Co^{++} had considerably higher catalytic activity in oxidizing sulfide to thiosulfate than did the corresponding metal alone. This is shown in Table I. Of the crystalline proteins tested, including lysozyme, gelatin and ovalbumin, crystalline bovine-plasma albumin gave the greatest degree of stimulation.

The optimal ratio of protein to metal ion was tested with iron. Assuming a molecular weight of 70,000 for crystalline bovine-plasma albumin, a molecular ratio 0.5 to 1.0 of Fe^{+++} to 1.0 of protein appeared optimal at the concentrations indicated in Fig. 1. For as yet unknown reasons, there was considerable variability in these experiments.

EDTA and 8-hydroxyquinoline were tested on systems in which the metal concentration was adjusted relative to the protein level so as to approximate those found in the rat-liver system. By this criterion the iron concentration in liver was calculated to be at least ten times higher than that of any other metal (*i.e.*, 5 g of liver contain approximately 1 g of protein and 0.6 mg of iron[8]). The results in Table II show that,

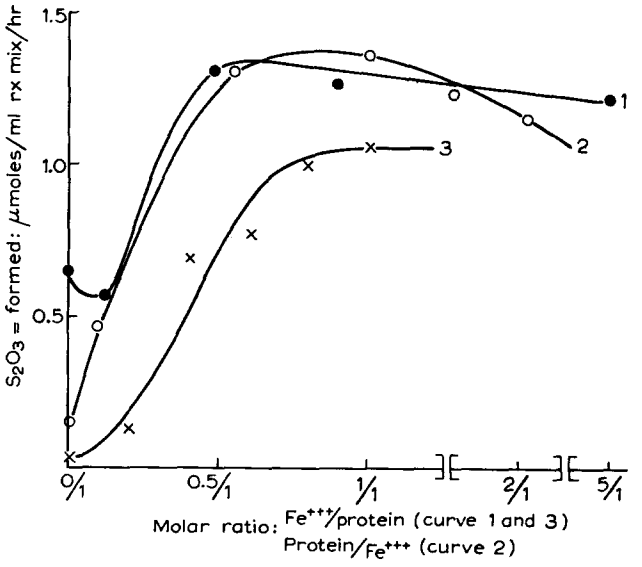

Fig. 1. The effect of metal/protein ratio on thiosulfate formation. Protein concentration for experiments represented by curve 1 is $4.4 \cdot 10^{-5}$ *M* and for curve 3 is $2 \cdot 10^{-5}$ *M*. Ferric (or ferrous) ion concentration for experiment represented by curve 2 is $4 \cdot 10^{-5}$ *M*.

References p. 578.

under the above stipulated conditions, only the Fe-protein complex (possibly by virtue of its higher molar metal concentration) was stimulated by versene and 8-hydroxyquinoline. The Fe-protein complex was also "inactivated" by heating to 100°. The "inactive" system was stimulated by EDTA and to a small extent by 8-hydroxyquinoline. The Fe-protein model system differed primarily from the biological system[2] in that it was almost completely inhibited by $\alpha\alpha'$ dipyridyl.

TABLE II

THE EFFECT OF CHELATORS ON METAL–PROTEIN COMPLEXES

*Metal–protein complex**		*Fresh (F) or heat-denatured** (HD)*	*Additions*			
Metal ion	*Molar concn.*		*None*	*EDTA* $4\cdot10^{-5}$ *M*	*8-hydroxy-quinoline* $2\cdot10^{-4}$ *M*	*α α' dipyridyl* $4\cdot10^{-4}$ *M*
			Thiosulfate formation μmoles/ml rx mix/h			
Fe^{+++}	$4\cdot10^{-5}$	F	1.2	1.9	3.8	0.2
		HD	0.1	1.4	0.6	0.1
Co^{++}	$4\cdot10^{-6}$	F	1.4	0.1	0.5	—
		HD	0.3	0.1	0.2	—
Cu^{++}	$4\cdot10^{-6}$	F	0.6	0.4	0.4	0.3
		HD	0.1	0.1	0.2	—
Mn^{++}	$4\cdot10^{-6}$	F	0.9	0.1	1.0	0.2
		HD	0.1	—	0.4	—
Ni^{++}	$4\cdot10^{-6}$	F	1.1	0.2	0.3	0.6
		HD	0.2	—	0.2	—

* Protein concentration for all experiments was $4\cdot10^{-5}$ *M*.

** Heat denatured by heating complex in boiling water bath for 3 min. The substrate concentration of Na_2S was $5\cdot10^{-3}$ *M*; all runs were made in phosphate buffer pH 7.3 at 37°.

Ferritin as a sulfide-oxidizing catalyst

The catalytic oxidation of sulfides by haem or haemoglobin have been studied by KREBS[9], HAUROWITZ[10] and most recently by SORBO[11]. Some results of a study with the naturally occurring Fe-protein, ferritin, as a sulfide-oxidizing catalyst are presented below. Ferritin was obtained from horse spleen. The initial specific activity of horse-spleen extract was higher than that found in perfused rat-liver extract[2], *i.e.*, 1.1 *vs.* 0.4 γS of $S_2O_3^=$ formed per mg protein per min. Ferritin crystals had a specific activity at least 45 times greater than that found in extracts of rat liver from starved rats[12] (18.0 *vs.* 0.4). The increase in activity with purification of the horse-spleen ferritin is shown in Table III.

It is not possible at this time to decide whether ferritin simulates the heat-labile or the heat-stable activity component of the sulfide-oxidizing system of rat liver. Upon heat denaturation at 100°, the solution became brown as iron was released from the protein. As the metal by itself had activity (see Table I) only partial inactivation by boiling was to be expected. The addition of crystalline bovine plasma albumin (20-fold excess over ferritin w/w) enhanced rather than inhibited the rate of thiosulfate formation. However, when *this* mixture was heat denatured at 100°, less than 20 % of the original activity was retained (Table IV).

ICHIHARA AND MCELROY[13] have shown that hypoxanthine is a cofactor for the heat-labile sulfide oxidase of beef liver. GREEN AND MAZUR[14] found that xanthine

References p. 578.

TABLE III

THE CATALYTIC SULFIDE-OXIDIZING PROPERTIES OF HORSE-SPLEEN FRACTIONS OBTAINED DURING FERRITIN ISOLATION

Fraction	*Sulfide oxidized to thiosulfate* γS of $S_2O_3^=$ formed/mg protein/minute*
Spleen extract**	1.1
80° supernatant	2.0
25% $(NH_4)_2$ SO_4 ppt.***	2.9
Crude ferritin (Cadmium salt)***	18.0
Non-crystallizable ferritin	4.8

* Conditions of assay as described previously for the rat-liver system.
** Supernatant of horse-spleen homogenate in $M/15$ phosphate buffer pH 7.3, spun at 3000 g for 15 min.
*** Redissolved and dialized against buffer prior to use.

TABLE IV

EFFECT OF CHELATORS AND PROTEIN ON SULFIDE OXIDATION BY FERRITIN

Addition to system	*Concentration*	*Thiosulfate formed**	
		Ferritin	*Denatured ferritin***
Standard system		100	97
Dialized		85	110
+ Protein***	2.2 mg/ml	124	27
+ EDTA	$4 \cdot 10^{-4}$ M	139	230
+ EDTA and dialized		32	58
+ 8 Hydroxyquinoline	$2 \cdot 10^{-4}$ M	29	45
+ $\alpha\alpha'$ Dipyridyl	$4 \cdot 10^{-4}$ M	10	12

The system was incubated at 37° pH 7.3 for 15 or 30 min. Ferritin concentration was 0.088 mg/ml. Initial sulfide concentration was $5 \cdot 10^{-3}$ M.
* Ferritin has been assigned a value of 100 = 18γS of $S_2O_3^=$ formed per mg protein per min. (± 15% for experiments conducted at different times).
** Heat denatured in boiling water bath for 3 min at pH 7.3. The protein was denatured prior to the addition of chelators.
*** Crystalline bovine plasma albumin added to ferritin before heat denaturation.

oxidase catalyzed the reduction of the ferric ion of ferritin. The addition of hypoxanthine and xanthine oxidase to ferritin stimulated thiosulfate formation by 35% over the rate of ferritin alone*.

Under the experimental conditions, the rate of thiosulfate formation was directly proportional to the concentration of ferritin in the system. Like the heat-stable fraction of rat liver[2], ferritin showed no pH optimum in the phosphate buffer range and no absolute substrate saturation.

Studies with chelators are summarized in Table IV. The results are similar to those reported for the model iron-protein system and also correspond in part to some of the data obtained with the heat-stable system of rat liver[2]. The enhancement of oxidizing activity by EDTA corresponds to a similar effect observed by MAZUR *et al.*[15] for the oxidation of adrenaline with ferritin as the catalyst. Sulfide oxidation by ferritin is inhibited by KCN, $\alpha\alpha'$ dipyridyl, iodoacetate and a nitrogen atmosphere.

* Personal communication of unpublished data from Dr. AKIRA ICHIHARA.

References p. 578.

Dialysis in the presence of EDTA reduced ferritin activity due, no doubt, to the removal of iron (see Table IV).

Apoferritin had little or no activity, but was restored to almost full activity by the addition of ferric ion. Cupric ion could not be substituted*.

In mammals, the oxidation of sulfide by ferritin may well have considerable physiological significance. Ferritin is strategically located throughout the mucosa of the gastrointestinal tract[16] so that it could act as catalyst for the oxidation of sulfide produced by intestinal microorganisms[17,18]. Thus, sulfide originating from microbial metabolism in the gastrointestinal tract may be prevented from ever reaching the bloodstream.

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SUMMARY

1. Metals, metal chelates and metal–protein complexes have been tested as sulfide-oxidizing agents. The conditions for maximal catalytic activity by these systems have been described.

2. At physiological concentrations iron (Fe^{++} or Fe^{+++}) added to inert protein (crystalline bovine plasma albumin) most closely mimicked the biological sulfide-oxidizing system in rat liver.

3. Ferritin oxidized sulfide to thiosulfate at a rate 45 times greater than did active rat-liver extracts.

4. Some of the catalytic properties of ferritin in sulfide-oxidation have been described and a new physiological function for ferritin has been suggested.

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