ON THE FORMATION OF THIOSULFATE FROM INORGANIC SULFIDE BY LIVER TISSUE AND HEME COMPOUNDS

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Hydrogen sulfide is a highly toxic compound; its toxicity is in the same range as that of hydrogen cyanide. Since inorganic sulfide can be formed in the bodies of higher animals[1], any detoxification mechanisms for this compound may be of physiological importance. (At the pH existing in tissues, inorganic sulfide is mainly present as hydrosulfide ion and hydrogen sulfide, but, for the sake of convenience, the name "sulfide" is used in this paper to cover both these forms, neglecting to denote which is the one actually participating in a reaction.) Previous investigations on the fate of inorganic sulfide added to tissues *in vitro* have given different results with respect to the reaction products formed. SMYTHE[2] found that sulfide was rapidly oxidized in the presence of liver tissue, and the reaction products were identified as free sulfur and polythionates together with small amounts of sulfate. GARABÉDIAN, on the other hand, in a series of papers[3–9] described an enzyme, "sulfide oxidase", from liver, which oxidized sulfide to thiosulfate. The aim of the present investigation was first to determine whether thiosulfate or a polythionate is formed, when sulfide was oxidized in the presence of liver. Preliminary experiments soon demonstrated that thiosulfate, but no polythionate, was formed, and the catalyst that was responsible for the reaction was then studied. A preliminary report of the present work[10] has already appeared in this journal.

MATERIALS

Rat hemoglobin was prepared by hemolyzing rat blood with distilled water, whereupon the hemoglobin spontaneously crystallized on refrigeration. It was centrifuged off, dissolved in 0.005*M* ammonia, and recrystallized by dialysis against distilled water. The hemoglobin from an aliquot of the rat blood hemolysate was converted to hemin by addition of 10 vol. of acetone, containing 1% HCl, to 1 vol. of sample. The protein precipitate was centrifuged off and washed with 5 vol. of acetone–HCl. The acetone fractions were combined and evaporated *in vacuo*, and the hemin was finally dissolved in 0.0125 *M* ammonia. Recrystallized hemin was also prepared from horse blood according to FISCHER[11]. Catalase was prepared from beef liver and twice crystallized from ammonium sulfate[12]. Myoglobin was a gift from Ing. Å. ÅKESON and beef-heart cytochrome *c*, purified on ion-exchange chromatography, was a gift from Dr. S. KUBY. Other compounds were commercial products of analytical purity. Distilled water, deionized on ion-exchange columns was used throughout this work. Rat-liver homogenates were prepared from 1 part of rat liver with 3 vol. of water in a Bühler blendor.

METHODS

The oxidation of sulfide was carried out at 37°C in Warburg vessels of 15–20 ml volume. The final volume of the test system was 3 ml and the pH 7.4. The main compartment contained the sample and phosphate buffer (in most experiments also pyrophosphate buffer, *vide infra*), and the reaction

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was started by adding the desired amount of 0.1 *M* sodium sulfide from the side arm. This gave an immediate increase in pressure, due to the hydrogen sulfide liberated at pH 7.4. The usual calculation of oxygen consumption from the manometer readings could not be carried out, since gaseous hydrogen sulfide was also consumed at the same time. For any valid calculations of the oxygen consumption under these conditions, it is necessary to know the quantitative composition of the reaction products formed. However, when all the sulfide has been consumed in the reaction, the calculation can be made as usual, since the change in pressure due to consumed oxygen is evidently the difference between the final reading and the manometer reading, before addition of sodium sulfide to the main compartment. Such calculations were made in some experiments with hemin and the absence of hydrogen sulfide in the vessel at the end of the experiment was then verified with lead acetate paper.

Analyses for thiosulfate and polythionates were made according to a recently described colorimetric method[13]. The oxidation of sulfide was stopped by addition to each vessel of 0.5 ml 1 *M* cadmium acetate and the precipitate, consisting of cadmium phosphate and cadmium sulfide, was centrifuged off. Thiosulfate was determined in the supernatant by addition to 1 ml of the latter of 2 ml 0.2 *N* ammonia, 1.2 ml water, 0.5 ml of 0.1 *M* KCN, 0.30 ml of 0.1 *M* $CuCl_2$, and 0.5 ml of ferric nitrate reagent (200 g $Fe(NO_3)_3 \cdot 9\ H_2O$, 200 ml 65% nitric acid, and water to 1000 ml) in the order indicated. The absorbancy in a 1 cm cuvette was then determined at 460 mμ and corrected for that of a blank, obtained by adding the ferric nitrate before cyanide and cupric chloride. Recovery experiments demonstrated that the yield of thiosulfate in the cadmium precipitation steps was 95%. The presence of polythionates was tested for by adding water and cyanide, to 1 ml of supernatant ammonia, as before, and incubating the samples 30 min at room temp. 0.5 ml ferric nitrate reagent, followed by 0.3 ml $CuCl_2$, was then added, and the absorbancy at 460 mμ determined against the same blank.

RESULTS

When sulfide was shaken with a liver homogenate in an air or oxygen atmosphere, thiosulfate, but no polythionate, could be demonstrated as a reaction product. A quantitative study of the thiosulfate formation was first complicated by certain technical difficulties, since sulfide was oxidized to thiosulfate with a considerable velocity in the presence of the phosphate buffer alone. Attempts to depress this "blank oxidation" by careful cleaning of the vessels and replacing the chemicals used with those from other manufacturers were unsuccessful. From the work of KREBS[14] it is known that this blank oxidation is caused by traces of heavy metal impurities. We now observed that the blank oxidation was to a large extent reduced by the presence of 0.005 *M* pyrophosphate and that the presence of this compound had no effect on the formation of thiosulfate in the liver homogenate (Table I). The catalyst, active in liver, was in part heat-stable (Table I) since 60% remained after 2 min at 100° C. Experiments were then carried out with rat blood. When a fresh blood hemolysate was shaken with sulfide only an insignificant oxygen consumption and thiosulfate formation was observed. The red colour of the blood was unchanged. However, with a corresponding

TABLE I

THIOSULFATE FORMATION WITH RAT LIVER

Reaction conditions: Sulfite 0.005 *M*, phosphate 0.05 *M*, 0.005 *M* pyrophosphate if indicated, and 1 ml of liver homogenate in a final vol. of 3.0 ml. 30 min reaction time at 37°. The blank values (buffer alone) have been subtracted from the corresponding values obtained with liver.

Catalyst	*Pyrophosphate*	*Thiosulfate formed (μmoles)*
Buffer alone	—	0.72
Buffer alone	+	0.13
Liver	—	1.61
Liver	+	1.57
Liver, heated to 100°C for 2 min	+	0.96

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TABLE II

THIOSULFATE FORMATION WITH RAT BLOOD

The samples contained 0.5 ml of a 12.5% rat blood hemolysate or an equivalent amount of hemoglobin or hemin. The test system contained 0.005 *M* pyrophosphate. Other conditions as in Table I.

Catalyst	*Thiosulfate formed (μmoles)*
Blood	0.20
Blood, heated to 100°C for 2 min	1.90
Hemoglobin	1.58
Hemin	3.46

amount of crystalline rat hemoglobin instead of the blood hemolysate, the results were different. A rapid oxygen consumption ensued and thiosulfate was formed (Table II). Concomittantly, the colour of the system changed from red to green, demonstrating that the hemoglobin was now converted to sulfhemoglobin. The different results obtained with blood and with pure hemoglobin indicate that there is a factor present in rat blood that protects the hemoglobin against conversion to sulfhemoglobin. Also, after heating a blood hemolysate to 100°C for 2 min a rapid oxidation of the sulfide and formation of thiosulfate occurred (Table II). Blood, in contrast to liver, was thus activated by heat denaturation. It is known[15,16] that hemin catalyses the oxidation of sulfide to free sulfur, and the possibility was now considered that thiosulfate is also formed in this reaction, although not demonstrated as a reaction product by previous investigators. When hemoglobin in a blood hemolysate was converted to free hemin and its action on sulfide investigated, a rapid oxidation with formation of sulfur was observed in confirmation of earlier reports, but also the formation of thiosulfate in good yields could be demonstrated (Table II). This is in disagreement with the results of HAUROWITZ[16], who claimed that the oxygen consumption in the hemin-catalyzed oxidation of sulfide only corresponded to an oxidation to the free sulfur stage, but KREBS[15], on the other hand, found that oxygen was consumed in excess of that necessary for the conversion of sulfide to free sulfur. The formation of thiosulfate in the hemin-catalyzed oxidation of sulfide was then further studied. The effect of hemin concentration is shown in Fig. 1, and, very interestingly, a pronounced maximum of thiosulfate formation was observed. This maximum yield corresponds to about 50% conversion of sulfide to thiosulfate. The lower yields obtained with higher concentrations of hemin cannot yet be explained, but may indicate that other hemin-catalyzed reactions, competing with the thiosulfate-producing reaction(s) occur in the system. (Control experiments showed that neither was thiosulfate destroyed when incubated with hemin under the experimental conditions, nor had hemin any effect on the thiosulfate analysis.) Increasing the sulfide concentration gave an increase of thiosulfate formed, both at the maximum and with an excess of hemin as shown in Fig. 2. Replacing air with oxygen in the system had no significant effect on the thiosulfate yield at the optimum concentration of hemin, but gave an increased formation of thiosulfate when an excess of hemin was present (Table III). The reaction was found to occur with the same velocity in the absence and presence of light.

The oxygen consumption was determined in some experiments and found to agree with that calculated from the amount of thiosulfate formed, under the assumption that free sulfur was the other reaction product. Thus, when 15.0 μmoles of sulfide were

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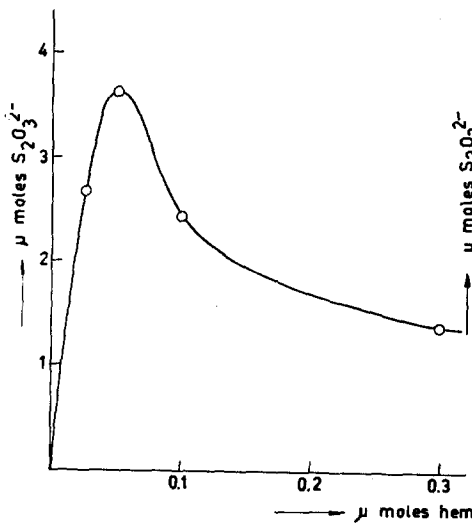

Fig. 1. Effect of hemin concentration on thiosulfate formation. The samples contained indicated amount of recrystallized hemin from horse blood. Other conditions as in Table II.

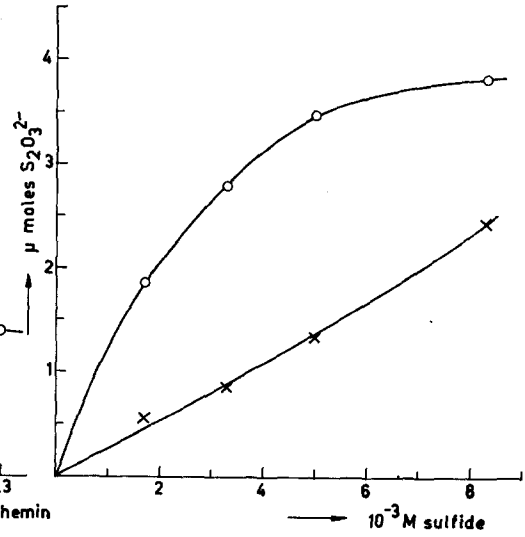

Fig. 2. Effect of sulfide concentration on thiosulfate formation. Conditions as in Table II, except that the sulfide concentration was varied. O O O, 0.05 μmoles hemin; × × ×, 0.30 μmoles hemin.

TABLE III

EFFECT OF OXYGEN ON THE HEME-CATALYZED REACTION. Reaction conditions as in Table II.

Amount of hemin (μmoles)	*Gas phase*	*Thiosulfate formed (μmoles)*
0.05	Air	2.69
0.05	Oxygen	2.68
0.30	Air	1.20
0.30	Oxygen	2.39

oxidized in the presence of 0.3 μmoles hemin, all sulfide was consumed in about 20 min. The reaction was stopped after 30 min when 1.25 μmoles of thiosulfate had been formed, corresponding to an oxygen consumption of 56 μl, according to the reaction $2\ H_2S + 2\ O_2 \longrightarrow H_2S_2O_3 + H_2O$. The rest of the sulfide, 12.5 μmoles, corresponds to 140 μl O_2, according to $2\ H_2S + O_2 \longrightarrow 2\ S + 2\ H_2O$. The calculated oxygen consumption was then 196 μl and the consumption actually observed was 195 μl.

Thiosulfate is spontaneously formed from a mixture of free sulfur and sulfite, but when a mixture of sulfide and sulfite was incubated with hemin, the yield of thiosulfate was even less than that obtained with sulfide alone. Consequently, sulfite seems not to be a precursor to thiosulfate in these systems and the reaction mechanism for thiosulfate formation in these systems is completely unknown.

The catalytic activity of some heme proteins was also investigated. Myoglobin behaved as hemoglobin, sulfide being oxidized concomitantly with the transformation of myoglobin into sulfmyoglobin. Catalase was inactive, but an active catalyst was obtained after heat denaturation of this enzyme. Pure cytochrome *c* was also inactive. Cytochrome *c*, prepared according to KEILIN AND HARTREE[17], was in preliminary

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experiments found to be active; recent work[18] has shown, however, that such preparations are contaminated with hemin-containing impurities. When the experiments were repeated with more purified cytochrome *c*, the latter was found to be inactive.

Finally, vitamin B_{12} was investigated and found to be without catalytic activity.

The question now arises whether the catalytic effect of liver homogenates can be attributed to the presence of free hemin or for instance sulfhemoglobin. The presence of free hemin in normal tissues is disputed[19], and if present it occurs in too small amounts to explain the catalytic activity of liver. With respect to hemoglobin, a comparison between the data in Tables I and II shows that hemoglobin from about 0.25 ml blood has the same catalytic activity as 1 g of liver tissue. The blood content of unperfused liver was of this order of magnitude (as verified by hemoglobin determinations[20] in the liver homogenates). But the fact that hemoglobin in a blood hemolysate was catalytically inactive, not being converted to sulfhemoglobin, appears to refote the assumption that hemoglobin was the active catalyst in liver. However, the colour of the liver homogenates in the test systems always changed from brownish-red to green, demonstrating that here a conversion of hemogloinb to sulfhemoglobin had occurred. A possible explanation of the different behaviour of hemoglobin in liver and in blood is that the protecting factor present in blood is destroyed in a liver homogenate. Another fact, which at first seems difficult to reconcile with the view that hemoglobin is the active catalyst in liver, is the partial heat lability of the liver activity in contrast to that of hemoglobin. However, the following experiment elucidates this point further. Rat hemoglobin was mixed with an inactive protein, bovine serum albumin, to give a solution containing 0.4% hemoglobin and 5.3% albumin. When this mixture was heated to 100°C, the catalytic activity decreased to 40% of the original value, which was attributed to an inclusion of the corresponding amount of hemoglobin in the precipitate of denatured protein. A similar phenomenon could explain the heat lability of "sulfide oxidase" in liver homogenates. It also illustrates the fact, that the heat lability of a catalyst in a biological system is not necessarily a proof of the catalyst being an enzyme. Of interest is also the finding of GARABÉDIAN[9] that his "sulfide oxidase" was in part heat-stable but completely destroyed by ashing. This is also compatible with the identity of hemoglobin and "sulfide oxidase".

DISCUSSION

The fact that free hemin is an active catalyst for the oxidation of sulfide, in contrast to the hemo proteins catalase, cytochrome *c*, hemoglobin and myoglobin, suggests that binding sites on the hemin, necessary for its catalytic activity on sulfide, are in the hemo proteins bound to the protein part of the molecule. Active catalysts are obtained from the hemo proteins by heat denaturation (except from cytochrome *c*, which is heat-stable) indicating that the active sites on the hemin are released by this treatment. As hemoglobin and myoglobin are activated also when converted to sulfhemoglobin and sulfmyoglobin, respectively, it is suggested that a similar detachment of the hemin occurs in this process.

Has the "sulfide oxidase" any physiological importance? As thiosulfate is a normal constituent in urine[21,22] and sulfide may be formed in the body, this appears at first probable. However, the "sulfide oxidase" activity can be demonstrated only

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under rather unphysiological conditions, involving the formation of sulfhemoglobin, which is never formed in the body under normal conditions. Recently[10,23] another metabolic pathway for the formation of thiosulfate has been detected. It consists of a transsulfuration between β-mercaptopyruvate and sulfite, and thiosulfate is more probably formed by this route in the normal animal.

SUMMARY

Thiosulfate, but no polythionate, is formed when inorganic sulfide is oxidized in the presence of liver. Free hemin is a very active catalyst for the oxidation of sulfide to thiosulfate. Of heme proteins investigated, hemoglobin and myoglobin were inactive catalysts per se, but became active after conversion to sulfhemoglobin and sulfmyoglobin. Rat blood contains a factor, which protects hemoglobin against conversion to sulfhemoglobin. Catalase and cytochrome *c* do not catalyze the oxidation of sulfide. The "sulfide oxidase" activity in liver homogenates is attributed to the presence of hemoglobin in these preparations.

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