On the mechanism of sulfide oxidation in biological systems

BAXTER AND VAN REEN[1] recently observed that when inorganic sulfide was oxidized in the presence of rat-liver preparations, determinations of thiosulfate by two different methods sometimes gave different results. The methods employed were a conventional iodometric titration and an enzymic method[2], based on the conversion of thiosulfate to thiocyanate in the presence of cyanide and the enzyme rhodanese. The rhodanese method always gave higher values than the iodometric titration except when the sulfide had been completely oxidized, in which case essential agreement between the two methods was observed. This suggested that sulfide was oxidized to thiosulfate through an intermediate, which behaved as thiosulfate in the rhodanese assay system but did not reduce iodine. An important difference between the two methods for thiosulfate assay was that the iodometric titrations were carried out on deproteinized samples whereas the enzymic assays were carried out on the untreated reaction system. If, however, deproteinized samples were also used for the enzymic determinations essential agreement between the two methods was again obtained. This was taken as evidence for the intermediate being protein bound, and it was further suggested that this intermediate was formed through sulfitolysis of a protein disulfide bond according to $RSSR + HSO^-_3 \longrightarrow RSH + RSSO^-_3$. (It was assumed that sulfide was oxidized to sulfite in a preceding reaction.) $RSSO^-_3$ was suggested to be the unknown intermediate and was classified as a thiosulfonate. Thiosulfonates are, however, of the formula RSO_2S^-, whereas compounds of the formula $RSSO_3^-$ should be classified as thiosulfate esters[4,5]. The latter are not converted to thiocyanate by cyanide and rhodanese[3], and the reaction mechanism suggested by BAXTER AND VAN REEN is consequently untenable. We have now been able to identify the intermediate described by BAXTER AND VAN REEN as follows.

It was previously reported[6] that certain heme compounds catalyze the oxidation of inorganic sulfide to thiosulfate, and we observed that a compound similar in behaviour to the intermediate of BAXTER AND VAN REEN was formed also in these reactions. Thus when inorganic sulfide (0.005 *M*) and hemin (5.4 μg/ml) in 0.133 *M* phosphate buffer, pH 7.2, was shaken in air, it was found that after 30 min the system contained 0.23 μmole S_2O_3/ml as determined by iodometric titration whereas the rhodanese method gave 1.13 μmoles S_2O_3/ml. Obviously a protein-bound intermediate could not have been formed in this case as no protein was present in the system. It has previously been demonstrated that when sulfide is oxidized in the presence of liver extracts[7] or heme compounds[6] thiosulfate is not the only reaction product as free sulfur is also formed. The possibility was first considered that sulfur was the unknown intermediate, although BAXTER *et al.*[2] stated that sulfur did not interfere in the rhodanese assay for thiosulfate. Sulfur, if present in a colloidal form[3,8], is actually converted to thiocyanate in the presence of cyanide and liver extracts and we observed in fact that colloidal sulfur interfered to a slight but significant extent in the rhodanese assay for thiosulfate, if the latter was carried out on a liver homogenate. But the unknown intermediate could not be identical with colloidal sulfur, as the presence of rhodanese was found to be necessary for the reaction between the intermediate and cyanide, whereas rhodanese does not catalyze the reaction between sulfur and cyanide[3]. However, when sulfide is incompletely oxidized to sulfur, the latter

will react with remaining sulfide to give a polysulfide*. It was now observed that inorganic polysulfide behaved as the unknown intermediate in the two methods for thiosulfate assay. Thus when 3 ml of a polysulfide solution (containing 40 μmoles Na_2S and 10 μmoles S per ml) were added to 27 ml of 0.133 *M* phosphate buffer, pH 7.2, and the thiosulfate content immediately determined, the iodometric method gave 0.22 μmole S_2O_3/ml**, whereas the rhodanese method gave 1.01 μmoles "apparent" S_2O_3/ml. It was also demonstrated that rhodanese was necessary for the conversion of the polysulfide to thiocyanate. From these observations it was concluded that the unknown intermediate in sulfide oxidation was identical with inorganic polysulfide. The observation of BAXTER AND VAN REEN[1] that deproteinization (with $ZnCO_3$) abolished the interference given by the intermediate in the rhodanese assay is explained by the fact that polysulfide is decomposed and removed from the system by this treatment.

It was previously[3] reported that the spontaneous reaction between a polysulfide (with a ratio of sulfide to sulfur corresponding to a disulfide) was so rapid even at a neutral pH that any catalytic effect of rhodanese could not be demonstrated. These experiments were, however, made at a "disulfide" concentration of 0.05 *M*, but if this was lowered to 0.001 *M* the spontaneous reaction became so slow that it was possible to demonstrate that "disulfide" under these conditions was a good sulfur donor for rhodanese (Table I).

TABLE I

INORGANIC "DISULFIDE" AS SUBSTRATE FOR RHODANESE

The test system of pH 7.4 contained 1 μmole "disulfide" (Na_2S_2) or thiosulfate as indicated, 50 μmoles cyanide, 60 μmoles phosphate, 50 μg bovine serum albumin and 7.8 μg crystalline rhodanese (the latter was omitted from the "blank" system) in a final volume of 1.0 ml. Reaction time, 5 min at 20°. The thiocyanate formed was determined colorimetrically[3].

System	*Sulfur donor*	*CNS^- formed μmoles*
Blank	Thiosulfate	0.02
Rhodanese	Thiosulfate	0.44
Blank	"Disulfide"	0.16
Rhodanese	"Disulfide"	0.78

The oxidation of polysulfide ("disulfide") to thiosulfate was also studied and found to be considerably more rapid than that of sulfide in the absence of any added catalyst. Free hemin and hemoglobin had no significant catalytic action on polysulfide (Table II). As these heme compounds catalyze the oxidation of sulfide to thiosulfate[6], their catalytic activity must in this case be due to the fact that they catalyze the oxidation of sulfide to polysulfide, but the oxidation of the latter then proceeds spontaneously. Liver extracts, on the other hand, also catalyzed the oxidation of "disulfide" to thiosulfate (Table II) and consequently the "sulfide oxidase" in liver cannot be identical with hemoglobin, as we previously suggested[6]. The existence of a non-hemoglobin sulfide oxidase in liver has previously been advocated by BAXTER *et al.*[2].

* For a discussion on the chemistry of inorganic polysulfide solutions, see ref.[9]. They actually contain equilibrium mixtures of higher and lower polysulfides.

** Formed by rapid autoxidation of the polysulfide.

TABLE II

THIOSULFATE FORMATION FROM SULFIDE AND "DISULFIDE"

The test system of pH 7.2 contained 15 μmoles Na_2S or 7.5 μmoles Na_2S_2, 400 μmoles phosphate and catalyst as indicated in a final volume of 3 ml. Incubation was carried out with shaking in Warburg vessels for 15 min at 37° and thiosulfate then determined colorimetrically[6]. The values obtained were corrected for corresponding "blanks" (0.10–0.25 μmole $S_2O_3^{2-}$ in case of sulfide and 1.1–1.5 μmoles $S_2O_3^{2-}$ in case of "disulfide").

Catalyst	*Substrate*	$S_2O_3^{2-}$ *formed* μ*moles*
Hemin (32.5 μg)	Sulfide	1.37
Hemoglobin (10 mg)	Sulfide	1.85
Liver extract*	Sulfide	1.50
Hemin (32.5 μg)	"Disulfide"	0.10
Hemoglobin (10 mg)	"Disulfide"	0.13
Liver extract*	"Disulfide"	1.43

* Prepared according to BAXTER *et al.*[2]. Each vessel contained 0.34 g liver (fresh weight).

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Crystalline synthetic porphyrin *c*

Synthetic porphyrin *c* was prepared more than twenty years ago by THEORELL[1] who heated protoporphyrin with L-cysteine in mineral acid solution. ZEILE AND MEYER[2], on the other hand, heated protoporphyrin with HBr and subsequently fused the crude adduct with L-cysteine. Both procedures gave non-crystalline products with the correct analytical data and the expected properties for the structure given in Fig. 1.

In this laboratory, during the course of preparation of porphyrin *c* to be used for metal-ion complexing studies, two modifications in the method of ZEILE AND MEYER[2] were introduced which led ultimately to a crystalline product. Thus in the present instance (a) the porphyrin–HBr adduct was fused with L-cysteine in an oil bath under controlled conditions instead of over an open flame, and (b) the crude