3. Mills I. M. *Spectrochim. Acta* **16** (1960) 35; *J. Mol. Spectr.* **5** (1960) 334.
4. Cyvin, S. J. *Acta Chem. Scand.* **13** (1959) 2135; *Spectrochim. Acta* **15** (1959) 828.
5. Kristiansen, L. and Cyvin, S. J. *To be published.*

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On the Mechanism of Rhodanese Inhibition by Sulfite and Cyanide

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The inhibition of rhodanese by cyanide [1] or sulfite [2] has been interpreted as being due to a reaction of the inhibitor with a disulfide bond in the enzyme [3,4]. However, Green and Westley [5] have recently observed that the inhibition of rhodanese by cyanide was more rapid in dilute than in concentrated enzyme solutions, which would not be expected if the inhibition was due to a direct reaction of the inhibitor with the enzyme. Furthermore they found that crystalline rhodanese contained labile sulfur, which could be exchanged with the outer sulfur atom of thiosulfate or removed from the enzyme by incubation with cyanide or sulfite. The sulfur-rhodanese complex was formed when the free enzyme reacted with a sulfur donor, *e.g.* thiosulfate, and thus represents an intermediate in the rhodanese reaction. Since thiosulfate is present in some of the purification steps used for the preparation of crystalline rhodanese [6,7], the latter is obtained in the form of this sulfur complex. Green and Westley suggested that either cyanide or sulfite inactivates rhodanese by removing the labile sulfur from the enzyme; this treatment yields the free enzyme, which was supposed to be very unstable and rapidly denatured. However, one objection against this mechanism is the fact that sulfite is a more potent inhibitor than cyanide [3], although it is a less active sulfur acceptor in rhodanese catalyzed reactions [8].

It has now been observed that the inhibition of rhodanese with sulfite or cyanide required the presence of oxygen (Table 1) and that rhodanese, inhibited by sulfite or cyanide, could be reactivated by treatment with sulfhydryl compounds such as cysteamine, mercaptoethanol, or thioglycolate. Dialysis against phosphate buffer or sodium acetate gave no reactivation. If the enzyme was denatured by incubation with 8 M urea, neither dialysis against buffer nor treatment with sulfhydryl compounds had any reactivating effect. Thus the sulfhydryl compounds could not reactivate urea-denatured rhodanese, and their reactivating effect on sulfite- or cyanide-inhibited rhodanese must apparently be explained by another mechanism. Experiments with ^{35}S-labelled sulfite and crystalline rhodanese (Table 2) furthermore showed that the inhibitor became bound to the enzyme during the inhibition and was liberated again from the inhibited enzyme when the latter was reactivated by cysteamine. The rhodanese preparation used in these experiments was 3 times crystallized (specific activity 252 RU/mg) and contained 1.3 atoms of labile sulfur per molecule enzyme. The labile sulfur was determined by treating the enzyme with an excess of cyanide and colorimetric determination [9] of the thiocyanate formed after the remaining cyanide had been removed by aeration from the acidified sample. The number of labile sulfur atoms per molecule rhodanese found for this preparation agreed with the number of sulfite molecules bound to one molecule of rhodanese during inhibition (Table 2). However, Westley and coworkers [5,10] reported 1.5—1.9 atoms per molecule. When crystalline rhodanese was oxidized with performic acid [11] and cysteic acid determined with an automatic amino

Table 1. Effect of oxygen on sulfite and cyanide inhibition of rhodanese. Rhodanese (partially purified) was incubated with the inhibitor in Warburg flasks containing air or purified nitrogen as indicated. Inhibitor concentration was 0.01 M in all experiments, pH was 7.0 in case of sulfite and 7.4 in case of cyanide, time of incubation was 10 min in case of sulfite and 30 min in case of cyanide. Controls were run in nitrogen with omission of the inhibitor. Rhodanese activity was determined as in Ref. [6].

Inhibitor	*Gas phase*	*Activity* % of control
Sulfite	Air	0.5
,,	Nitrogen	100
Cyanide	Air	4.3
,,	Nitrogen	88.7

Table 2. Inhibition of rhodanese with ^{35}S-labelled sulfite. A sample of 0.1 μmole crystalline rhodanese was incubated with 100 μmoles $Na_2{}^{35}SO_3$ (sp.a. about 0.02 mC/mmole) in air at pH 7.0 and the excess sulfite removed by dialysis against 0.01 M sodium acetate or 0.1 M cysteamine + 0.01 M phosphate buffer pH 7.4. The rhodanese activity was compared with that of the non-treated enzyme.

Treatment	*Activity* %	^{35}S-*incorporated* μmole/μmole enzyme
Sulfite	0.4	1.31
Sulfite + cysteamine	81	0.137

acid analyzer [12] after hydrolysis of the protein, 3.85 moles of cysteic acid per mole of enzyme were obtained. This indicated that rhodanese contained 4 cysteine or half-cystine residues per molecule. The enzyme was then assayed for SH-groups by amperometric titration with silver ions [13] and it was found that the native enzyme reacted with silver ions to a very slight extent without giving an endpoint. However, in the presence of a detergent (5 % dodecyl sulfate) a value of 4.35 SH/mole enzyme was obtained. These results indicated that rhodanese contains 4 cysteine and no cystine residues. When sulfite-inhibited rhodanese was titrated in the presence of detergent only 3.16 SH/mole enzyme was found; thus one SH-group had reacted during the inhibition.

The results now obtained suggest that rhodanese contains an active sulfhydryl group (the free enzyme is represented by ESH in the reactions below) and the enzyme-sulfur complex a persulfide group (ESSH). The otherwise rather unstable persulfide group is probably stabilized by bonding to another site in the enzyme, as the labile sulfur is released when the enzyme-sulfur complex is denatured [5,10]. The inhibition mechanism in case of sulfite would be

$$ESH + O + SO_3^{2-} = ESSO_3^- + OH^-$$

and the reactivation by a sulfhydryl compound (RSH)

$$ESSO_3^- + RSH = ESSR + HSO_3^-$$

followed by

$$ESSR + RSH = ESH + (RS)_2.$$

Similar reactions can be written for the cyanide inhibition except that a thiocyanate ester (ESCN) is formed instead of a thiosulfate ester ($ESSO_3^-$). The possibility that rhodanese contains an active sulfhydryl group has in fact been previously suggested [2,14] from inhibition experiments with sulfhydryl reagents, but it was pointed out [2] that these compounds had a rather weak inhibitory effect on the enzyme. This may be due to the fact that in these experiments the enzyme was present as the rhodanese-sulfur complex which may be less reactive in the presence of inhibitors than the free enzyme.

A full report of the present findings, including the complete amino acid analysis of rhodanese, will be published in this journal.

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1. Lang, K. *Z. Vitamin. Hormon. Fermentforsch.* **2** (1949) 288.
2. Sörbo, B. *Acta Chem. Scand.* **5** (1951) 724.
3. Sörbo, B. *Acta Chem. Scand.* **5** (1951) 1218.
4. Sörbo, B. *Acta Chem. Scand.* **7** (1953) 1137.
5. Green, J. R. and Westley, J. *J. Biol. Chem.* **236** (1961) 3047.
6. Sörbo, B. *Acta Chem. Scand.* **7** (1953) 1128.
7. Westley, J. and Green, J. R. *J. Biol. Chem.* **234** (1959) 2325.
8. Sörbo, B. *Acta Chem. Scand.* **11** (1957) 628.
9. Nyström, C. and Sörbo, B. *Scand. J. Clin. & Lab. Invest.* **9** (1957) 223.
10. Westley, J. and Nakamoto, T. *J. Biol. Chem.* **237** (1962) 547.
11. Pierce, J. G. *J. Am. Chem. Soc.* **77** (1955) 184.
12. Spackman, D. H., Stein, W. H. and Moore, S. *Anal. Chem.* **30** (1958) 1190.
13. Benesch, R. E., Lardy, H. A. and Benesch, R. *J. Biol. Chem.* **216** (1955) 663.
14. Saunders, J. P. and Himwich, W. A. *Am. J. Physiol.* **163** (1950) 404.

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