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Persulfide Properties of Thiocystine and Related Trisulfides

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Thiocystine (bis[2-amino-2-carboxyethyl]trisulfide) functions as a persulfide in transferring its sulfane sulfur to thiophilic acceptors. This occurs by formation of a reactive intermediate, thiocysteine (alanine hydrogen disulfide). In the absence of an acceptor sulfur is released in elemental form. Thiocystine is relatively stable in the pH range of 2–9. However, its conversion to unstable thiocysteine is accelerated by sulfhydryl compounds, rhodanese, or reagents that cleave sulfur–sulfur bonds to yield sulfhydryl groups. Since thiocystine has been detected in biological systems, it is proposed that it provides a storage form of sulfane sulfur. Trisulfides related to thiocystine show qualitatively similar properties.

INTRODUCTION

Thiocystine (bis[2-amino-2-carboxyethyl]trisulfide) was first isolated by Fletcher and Robson (*1*) from the hydrochloric acid hydrolysates of proteins that are rich in cystine. The trisulfide and a small amount of accompanying tetrasulfide were artifacts produced by acid-catalyzed exchange reactions between cystine and its decomposition products. Sandy *et al.* (*2*) isolated thiocystine and the corresponding mixed trisulfide of glutathione from *Rhodopseudomonas spheroides* by a mild extraction procedure involving ion exchange. The products were evidently naturally occurring in this organism. Massey *et al.* (*3*) proposed the probable presence of the corresponding trisulfide of glutathione in commercial samples. Szczepkowski and Wood (*4*) showed thiocystine was produced by the action of cystathionase on cystine and further that it served as a substrate for rhodanese. Thiocystine was formed when cystine was incubated with cystathionase prepared from *R. spheroides* (Wider de Xifera *et al.* (*5*)).

When it was incubated in the rhodanese assay system, thiocystine was observed by Abdolrasulnia and Wood (*6*) to be more unstable than had been previously reported. The compound readily transferred persulfide sulfur to the enzyme but also decomposed rapidly to free sulfur and cystine.

The cause of the instability has been investigated. This report demonstrates thiocystine to be relatively stable in the pH range from 2 to 9. However, rapid decomposition to free sulfur and cystine occurs in the presence of sulfhydryl compounds, sulfite, or cyanide. This effect is due to the intermediary formation of

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the unstable persulide, thiocysteine (CySSH). Similar effects were noted in trisulfides related to thiocystine.

EXPERIMENTAL

Thiocystine, trithiodipropionic acid, and homothiocystine (bis[2-amino-2-carboxypropyl]trisulfide) were prepared according to the method of Savige *et al.* (*7*) and *N,N'*-diacetylthiocystamine (bis[2-acetamidoethyl]trisufide) as described by Buckman and Field (*8*). The trisulfides were characterized by cold cyanolysis according to the directions of Fletcher and Robson (*1*). Carboxymethylthiocysteine was prepared by exchanging dithiodiglycolic acid with cysteine and separating the products by electrophoresis according to the method of Flavin (*9*). The compound was also prepared by reacting cystine monosulfoxide (*7*) with thioglycolic acid essentially according to the procedure of Schöberl and Gräfjé (*10*). Mercaptosuccinic acid was recrystallized from a preparation obtained from National Aniline Company. Oxidized glutathione and lipoic acid were obtained from Sigma Chemical Company. Other chemicals were the best obtainable from commercial sources.

Crystalline rhodanese was prepared from beef liver essentially as described by Horowitz and de Toma (*11*).

Methods

Spectral measurements were done with a Cary 14 recording spectrophotometer and turbidimetric measurements were performed at 460 or 620 nm with a Beckman DK2A ratio recording spectrophotometer equipped with a constant-temperature cell compartment adjusted to 25°C.

Thiocyanate formation was measured by adding 1 ml of Goldstein's reagent (*12*) to a suitable aliquot of a reaction mixture and comparing the ferric thiocyanate color in a Bausch & Lomb Spectronic 20 spectrophotometer with a thiocyanate standard treated in the same manner.

Disulfide spots on paper were detected with the reagent of Toennies and Kolb (*13*).

RESULTS AND DISCUSSION

When thiocystine was dissolved in a glycine–phosphate buffer, pH 7.4, the solution was stable for at least 6 hr. However, when a trace of an electrophilic substance such as cyanide was added, the solution quickly became turbid and deposited free sulfur. The other product was cystine (*1*).

$$\mathrm{CySSSCy} \rightarrow \mathrm{CySSCy} + \mathrm{S^0} \qquad [1]$$

$$\mathrm{Cy} = -\mathrm{CH_2}-\underset{\displaystyle \mathrm{NH_3^+}}{\underset{|}{\mathrm{CH}}}-\mathrm{COO^-}$$

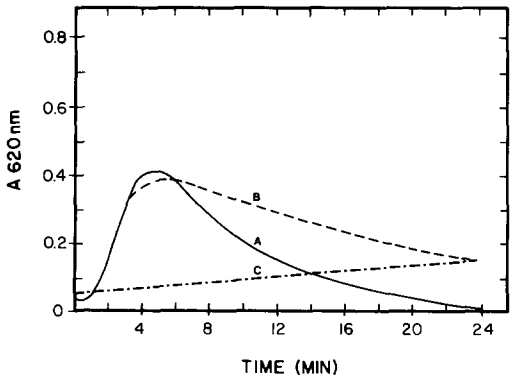

FIG. 1. Decomposition of thiocystine catalyzed by cyanide or sulfite ion. The rate of sulfur formation at 25°C was measured by absorbance at 620 nm. (A) The system contained in 4.5 ml: 20 μmole of thiocystine, 20 μmole of sodium cyanide, and 200 μmole of bis(1-hydroxymethly)aminomethane buffer, pH 9.0. (B) The system contained in 4.5 ml: 20 μmole of thiocystine in 0.2 *N* HCl, 80 μmole of sodium cyanide, 900 μmole of glycine, 1.75 μmole of EDTA, 0.2 *N* sodium hydroxide equivalent to the HCl used, and 350 μmole of phosphate buffer, pH 7.4. (C) The same system as (B) except that 20 μmole of sodium sulfite was substituted for sodium cyanide.

Figure 1 shows the time course of the reaction at pH 7.4 and 9.0. The turbidity reached a maximum at 4–5 min at pH 9 and then declined as settling of the elemental sulfur and slow formation of thiocyanate proceeded. There was no thiocyanate formation at pH 7.4, indicating the absence of cyanolysis. Addition of mercaptoethanol, which facilitates the reaction between cyanide and free sulfur (*14*), did not permit thiocyanate formation but reduced the amount of turbidity due to persulfide formation. Nor was any hydrogen sulfide detected from reaction between the free sulfur and a mercapto group.

$$S^0 + 2HSCH_2CH_2OH \rightarrow H_2S + (-SCH_2CH_2OH)_2 \qquad [2]$$

At pH 9.0 thiocyanate formation reached a value of 5.6 μmole in 20 min and increased to 7.5 μmole in 90 min. When the experiment was done in the presence of 0.02 *M* mercaptoethanol, the thiocyanate formed was 7 μmole at 20 min and increased to 14 μmole in 35 min. Sulfide ion was detected qualitatively (reaction [2]).

The rate of free sulfur formation when sulfite was subsituted for cyanide was much lower. This was probably due to partial conversion of free sulfur to thiosulfate. As reported by Szczepkowski and Wood (*4*) cysteine catalyzed the decomposition of thiocystine. In Fig. 2 mercaptosuccinate, utilized as a model mercaptan, accelerated the decomposition of thiocystine over approximately the same course as cyanide:

$$CySSSCy + RS^- \rightarrow CySSCy + RS^- + S^0 \qquad [3]$$

In the absence of the thiol, virtually no turbidity occurred during a period of 24 min.

The mechanism of the decomposition described by reaction [3] may be

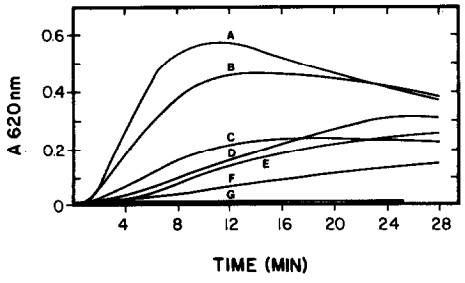

FIG. 2. Effect of oxidized glutathione or lipoic acid on the decomposition of thiocystine catlayzed by mercaptosuccinate. Turbidity was due to separation of free sulfur. (A) The system contained in 4.5 ml: 20 μmole of thiocystine, 0.5 μmole of mercaptosuccinate, 900 μmole of glycine, 1.75 μmole of EDTA, and 350 μmole of phosphate buffer, pH 7.4. (B) The system of (A) plus 20 μmole of lipoic acid, pH 7.4. (D) The system contained in 4.5 ml: 20 μmole of thiocystine, 200 μmole of bis(1-hydroxymethly)aminomethane buffer, pH 8.6. (E) The system of (D) pluse 20 μmole of oxidized glutathione, pH 8.6. (F) The system of (D) plus 20 μmole of lipoic acid, pH 8.6. (G) The system of (A) without mercaptosuccinate.

formulated as intermediate production of unstable thiocysteine by S–S bond scission catalyzed by SH groups.

$$CySSSCy + RS^- \rightleftharpoons CySS^- + RSSCy \quad [4]$$

$$CySS^- \rightarrow CyS^- + S^0 \quad [5]$$

$$CyS^- + RSSCy \rightleftharpoons CySSCy + RS^- \quad [6]$$

Cyanide ion produces sulfhydryl groups initially by attack on an S–S bond (*15*). The sulfhydryl groups function in a catalytic role and cause extensive decompostion of thiocystine.

Due to its intrinsic instability, attempts to isolate thiocysteine have been unsuccessful. Flavin (*9*) detected the formation of a derivative, the mixed disulfide of thioglycolic acid and cysteine (carboxymethylthiocysteine), by adding iodoacetate to an incubation mixture in which the cystathionase of a *Neurospora* mutant acted on cystine. Carboxymethylthiocysteine was isolated by electrophoresis and compared to an authentic sample. In our study, when 20 μmole of iodoacetic acid was added to 10 μmole of thiocystine and 1 μmole of sodium cyanide in the pH range of 8–9, a ninhydrin-positive, disulfide-positive compound was formed.

$$CySSSCy + ICH_2COOH \rightarrow CySSCH_2COOH + CySCH_2COOH + HI \quad [7]$$

It migrated on paper at pH 4.0 in an electric field at the same rate as an authentic sample of carboxymethylthiocysteine. The same results was obtained when the cyanide catalyst was omitted. Thus iodoactate attacks the trisulfide with scission of an S–S bond. Carboxymethylcysteine was not detected as a product of the reaction because it has the same electrophoretic mobility as a carboxymethylthiocysteine. It can be distinguished from the latter by lack of a disulfide reaction with cyanide and nitropruside reagent.

Reaction [4] is reversible (*4*). Therefore, addition of disulfide reduces thiocy-

steine formation and hence the amount of the turbidity observed. This was demonstrated as shown in Fig. 2 by adding oxidized glutathione or lipoic acid to the medium. Both compounds were effective in diminishing the rate of thiocystine decomposition, but lipoic acid, which is a good acceptor of persulfide sulfur (*16*), was more efficient at both pH 7.4 and 8.6. In addition, mercaptosuccinate acted to decrease the turbidity by forming persulfide and hydrogen sulfide. With the small amount of sulfhydryl compound used, this effect was more marked at pH 8.6 than 7.4 due to the greater ionization of the sulfhydryl group.

Rao and Gorin (*17*) showed the persulfide group absorbs weakly at 330–350 nm. Figure 3 shows the development of the characteristic absorption of the persulfide group when thiocystine was incubated with cysteine. The low absorption was due in part to the reversal of the reaction by the cystine formed as a product (reaction [4], where R is Cy).

The decrease in the absorption peak with time (curve B) reflects the instability of the persulfide species. When cyanide was added to the alkaline medium, there was an immediate loss of the characteristic absorbance of the persulfide group due to thiocyanate formation and the absorption spectrum was that of thiocystine (curve C).

The instability of thiocystine in the presence of sulfhydryl groups prompted investigation of closely related trisulfides. Table 1 shows the time required to produce visible clouding when the compounds were incubated at room temperature with either NaCN or mercaptosuccinate at pH 7.4 or 9.0. The effect of removal of the amino group is to stabilize the trisulfide group against attack by cyanide or sulfhydryl compounds.

It is apparent that thiocystine is most unstable to sulfhydryl groups at pH 7.4 followed by homothiocystine, *N,N'*-diacetylthiocystamine, and trithiodipropionic acid (desaminothiocystine).

Cyanide exerts its effect by scission of the disulfide bond.

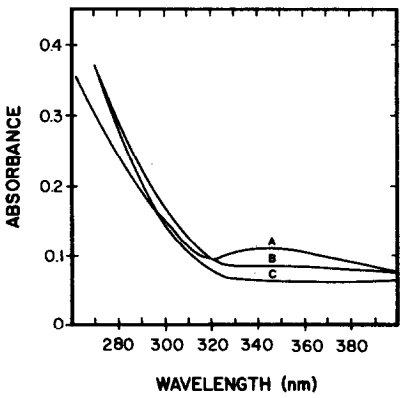

FIG. 3. Formation of a persulfide by the reaction of thiocystine and cysteine. All the reactions were run in 44 m*M* bis(1-hydroxymethyl)aminomethane buffer, pH 8.6. (A) 0.5 m*M* cysteine and 0.28 m*M* thiocystine 15 min after preparation. When the solution was made 0.28 m*M* in cyanide, a tracing identical to curve (C) was obtained. (B) curve (A) after 2.5 hr. (C) 0.28 m*M* thiocystine without cyanide.

TABLE 1

Decomposition of Trisulfides[a]

Trisulfide	Agent (μmole)	Time for clouding to occur (min) pH 7.4	pH 9.0
Trithiodipropionic acid	Mercaptosuccinate		
	0.25	none	none[a]
	0.5	none	—
	NaCN		
	20	none	none
	80	150[c,d]	none[b,d]
Homothiocystine	Mercaptosuccinate		
	0.05	8	15
	0.25	5	5
	NaCN		
	20	none	—
	80	8.5	none[b]
N,N'-Diacetylthiocystamine	Mercaptosuccinate		
	0.05	14	6
	0.2	1.5	—
	NaCN		
	20	none	none[b]
	50	1	—
Thiocystine	Mercaptosuccinate		
	0.05	1	2
	NaCN		
	20	4	2[b]

[a] Incubations were done at room temperature (25–27°C) in a volume of 4.5 ml with 20 μmole of trisulfide and mercaptosuccinic acid or NaCN in the amounts indicated. The solutions were observed for 10–20 min or until a clearly discernible haze had developed. At pH 7.4 the solution also contained 350 μmole phosphate, 900 μmole glycine, and 1.75 μmole EDTA; at pH 9.0 the solution contained 200 μmole of bis(1-hydroxymethyl)aminomethane. Homothiocystine and thiocystine were dissolved in 0.2 *N* HCl before addition to the solutions. The acid was neutralized by NaOH.

[b] Strong SCN^- test.

[c] Weak SCN^- test.

[d] Sulfur precipitated in 15 hr.

$$RSSR + NaCN \rightleftharpoons RSNa + RSCN \quad [8]$$

Concentrations of cyanide necessary to cleave the disulfide are much higher than mercaptosuccinate principally because of the reversibility of the reaction (*18*).

However, both thiocystine and homothiocystine react irreversibly with cyanide (*19*). At pH 9.0 the higher concentration of cyanide ion produces thiocyanate ion; the intermediary persulfide reacts faster with cyanide and less decomposes to free

sulfur. Thus the clouding that developed from the cyanide treatment was of lower density than occurred in the presence of mercaptosuccinate.

Rhodanese is a sulfhydryl enzyme that exists as a dipersulfide in the active state. Although the enzyme is crystallized with persulfide groups, measurements of the ratio of persulfide to enzyme range from 1.0 to 1.7 (*20, 21*). In storage the enzyme characteristically loses some persulfide as free sulfur (*22*). Thus, unless it is stored in the presence of thiosulfate, the enzyme will have some active sulfhydryl groups available. When thiocystine was incubated with thiosulfate-free rhodanese at pH 7.4, a small amount of turbidity developed after 7 min due to separation of free sulfur (Fig. 4).

$$\text{EnzS}^- + \text{CySSSCy} \rightleftharpoons \text{EnzSS}^- + \text{CySSCy} \qquad [9]$$

$$\text{EnzSS}^- \rightleftharpoons \text{EnzS}^- + \text{S}^0 \qquad [10]$$

Reaction [11], the summation of reaction [9] and [10] (compare [4]–[6]), shows the catalytic effect of rhodanese in decomposing thiocystine to sulfur in the absence of an acceptor.

$$\text{EnzS}^- + \text{CySSSCy} \rightarrow \text{CySSCy} + \text{S}^0 + \text{EnzS}^- \qquad [11]$$

Studies on the reactions of rhodanese indicate that a persulfide form (thiocysteine moiety) is the most stable form of the enzyme (*6, 24*). Hence, the consequences of the reaction of thiocystine with rhodanese are probably given by reaction [9] where the more stable enzyme persulfide forms rather than thiocysteine (*6*). Volini and Wang (*22*) have noted that the enzyme persulfide decomposes slowly to yield free sulfur. This is formulated in reaction [10]. At the same time any cysteine that would form according to the competing reaction [3] (where RS^- is $EnzS^-$) would also catalytically accelerate the decomposition of thiocystine. The 7-min time lag before the appearance to sulfur signified decomposition of thiocystine in the presence of rhodanese is also seen in Figs. 1 and 2 where it was in the range of 1 to 3 min. With no evidence of intermediates such as higher polysulfides on hand, the simplest explanation for the time lag is an intermediate retention of the first free sulfur in colloidal suspension that did not record turbidimetrically under the conditions of the experiment until precipitation began.

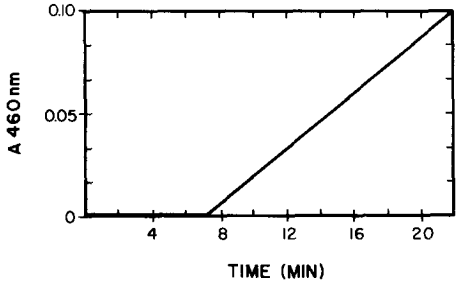

FIG. 4. Effect of rhodanese on the decomposition of thiocystine. Turbidity due to separation of free sulfur. The system contained 13 μmole of thiocystine, 0.16 μmole (5.9 mg) of rhodanese, 300 μmole of phosphate buffer, 1.75 μmole of EDTA, and 900 μmole of glycine in a total volume of 3 ml, final pH 7.4.

The relationships expressed above indicate that trisulfides, in the presence of sulfhydryl groups, cyanide, sulfite, and probably other electrophilic groups, generate persulfides. Persulfides are generally unstable and rapidly decompose to free sulfur and mercaptans if thiophilic acceptors are not present. However, in functioning as substrates for rhodanese in biological systems, persulfides transfer their sulfane sulfur to form thiosulfate, thiocyanate, and at least some iron–sulfur proteins (*24*). Persulfides may have only a transient existence, but the trisulfides provide stable precursors that may well represent a storage form of sulfane sulfur in biological systems.

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