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On the Metabolism of Thiosulfate Esters

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An enzyme has been demonstrated in rat liver, which converts sulfocysteine to thiosulfate, ammonia and pyruvate. No thiosulfate is formed from aminoethylthiosulfuric acid. Sulfite is formed from both these thiosulfate esters when they are incubated with liver homogenate under anaerobic conditions, and the reaction is augmented by heat denaturation of the homogenate. The reaction is attributed to a nonenzymic reaction with protein bound thiol groups. A possible use of thiosulfate esters as protective substances against ionizing radiation is suggested.

Thiosulfate esters have so far not attracted much interest from a biochemical point of view. One ester, sulfocysteine * [$COOHCH(NH_2)CH_2S_2O_3H$], was demonstrated by Clarke [1] to be a reaction product from cystine and sulfite, but was apparently not considered as a possible normal metabolite. Later work with mould mutants [3,4] has, however, suggested that in these moulds, sulfocysteine may be an intermediate in the metabolic pathway leading from sulfate to cysteine. Recently Cavallini and Stirpe [5] administered sulfocysteine to rats and observed an increased excretion of thiosulfate. They suggested that the compound was deminated to ammonia, pyruvate and thiosulfate and that sulfocysteine was a precursor of the thiosulfate normally excreted in the urine from higher animals.

The present work was undertaken in order to study possible transformations of sulfocysteine and its decarboxylated analogue, β-aminoethylthiosulfuric acid (AETA) in the presence of rat liver and rat kidney. These two compounds were chosen for study as they are more stable than simple aliphatic thiosulfate esters. A preliminary report of the present work has appeared in this journal [6].

* Clarke originally proposed the name cysteine-S-sulfonate, but this could erroneously imply a thiosulfonate structure (RSO_2SH) instead of a thiosulfate ester structure (RS_2O_3H) for the compound. Recently [2] the name S-sulfocysteine has been suggested and it is used in the present paper.

MATERIALS

Attempts were first made to prepare sodium sulfocysteine according to Clarke [1], but in confirmation of the original report it was found that the product obtained was contaminated with copper ions. These could not be removed by cation exchangers; presumeably the copper ions were complexed by sulfocysteine. Attempts to remove the copper by treating the sample with H_2S led to immediate decomposition of the sulfocysteine. The following improved method for the preparation of the compound, giving a copper free product, was then worked out. To 2.4 g cystine (10 mmole) dissolved in 80 ml water and 5 ml 25 % ammonia was added 12.6 g $Na_2SO_3 \cdot 7H_2O$ (50 mmole) and the solution left for 1 h. 30 % H_2O_2 was then added dropwise with efficient stirring until no free cysteine could be detected with phosphotungstic acid [7]. About 3 ml H_2O_2 were required. The solution was then neutralized with 2.4 ml anhydrous acetic acid, and 15 ml 2 M barium acetate solution was added. Barium sulfate was centrifuged off and the supernatant taken to dryness *in vacuo*. The residue was treated successively with 50, 25 and 15 ml hot 95 % ethanol in order to remove sodium acetate, and the ethanol insoluble residue was then dissolved in 6 ml H_2O. By addition of one volume of 95 % ethanol the sodium sulfocysteine was precipitated as crystals which were collected after one night in the refrigerator. 1.74 g of the compound (containing 3 moles of water per 2 moles of salt [1]) was obtained, corresponding to 34 % of the theoretical yield. Recrystallization from ethanol-water (1:1) gave 1.54 g of pure product.

AETA [8] was a gift from Dr. B. Hansen.

METHODS

Thiosulfate was determined colorimetrically as previously described [9]. Sulfate was determined by a turbidimetric method modified from that of Gassner and Friedel [10]. To 3 ml of the sample was added 0.3 ml 2 M trichloroacetic acid and 5.7 ml water, and precipitated proteins centrifuged off. To 3 ml of supernatant was then added 0.4 ml gelatine reagent followed by 0.4 ml of barium chloride reagent [10]. The turbidity was measured in a 1 cm cuvette at 400 mμ against a blank, obtained by adding 0.8 ml gelatine reagent to another 3 ml aliquot of the supernatant. Sulfite was determined by a colorimetric procedure, based on that of West and Gaeke [11]. To the sample was added an equal volume of 0.2 M $HgCl_2$ and the precipitate was centrifuged off. To 0.5 ml of supernatant was then added 2.0 ml of 0.04 % *p*-rosaniline hydrochloride in 0.72 M HCl and 2.0 ml of 0.2 % formaldehyde, and after 3—7 min. the absorbance of 570 mμ was determined. A blank value was obtained by adding $HgCl_2$ to the test system before the thiosulfate ester. Pyruvic acid and ammonia were determined in trichloroacetic acid filtrates, the former according to Friedemann and Haugen [12] and the latter by nesslerization following aeration.

Paper chromatography was carried out with ascending development on Whatman paper No. 1 and either 75 % acetone-water or 80 % phenol-water as solvents. The samples were deproteinized either by trichloroacetic acid or by heating at 100°C for 2 min. The chromatograms were sprayed with ninhydrine or iodoplatinate [13] (the latter reagent demonstrated compounds containing oxidizable sulfur). The presence of disulfides on the chromatograms was in some cases verified by spraying with phosphotungstic acid-sulfite [14]. The identity of the pyruvate formed from sulfocysteine was also established by paper chromatography according to Wieland and Fisher [15].

RESULTS

When sulfocysteine was incubated with rat liver or kidney slices, small amounts of thiosulfate were formed (Table 1). With a rat liver homogenate instead of slices, the yield of thiosulfate was increased. The formation of thiosulfate was not dependent upon the presence of oxygen and the active system was heat labile (Table 1). No thiosulfate was formed from AETA under otherwise similar conditions. Further experiments demonstrated that the formation

Table 1. Thiosulfate formation from sulfocysteine. Sulfocysteine 0.01 M, pH 7.9, slices or homogenate corresponding to 50 mg of rat tissue per ml. Incubated 1 h in Warburg vessels with shaking at 37°. Experiments with slices were made in Ringer phosphate solution, experiments with homogenate in 0.025 M phosphate buffer.

Preparation	Gas phase	S_2O_3 formed μmole/ml
Liver slices	Air	0.18
Kidney »	»	0.13
Liver homogenate	»	0.64
» »	N_2	0.72
heated 2 min. at 100°C	Air	0

of thiosulfate from sulfocysteine was a zero order reaction (under the conditions indicated in Table 1). Active extracts could be prepared from acetone dried rat liver powder and they were not significantly inactivated by dialysis against 0.01 M sodium acetate at 4°C. When such dialyzed extracts were incubated with sulfocysteine the formation of pyruvate and ammonia besides thiosulfate could be easily demonstrated. When the three reaction products were quantitatively determined the results shown in Table 2 were obtained. The somewhat low yields of pyruvate and ammonia can be attributed to a conversion of these compounds to other products by the liver extract [16], and consequently the stoichiometry of the reaction is best represented by

$$-S_2O_3CH_2 \cdot CHNH_2 \cdot COOH + H_2O \rightarrow CH_3 \cdot CO \cdot COO^- + S_2O_3^{2-} + NH_4^+ + H^+$$

However, not only thiosulfate, but also sulfate was formed, when a rat liver homogenate was incubated with sulfocysteine while about the same amount of sulfate was obtained when sulfocysteine was replaced by AETA (Table 3). The responsible system was heat labile. As no sulfate was formed under anaerobic conditions a simple hydrolysis of the thiosulfate ester to sulfate could be excluded and it was then suspected that sulfate was formed through oxidation of another primary reaction product. (The possibility that thiosulfate was this primary product in case of sulfocysteine was excluded by the fact that thiosulfate is not oxidized to sulfate by liver homogenates [17]). A possible sulfate precursor would be sulfite, and this was in fact formed, when sulfocysteine or AETA was incubated with liver homogenates under

Table 2. Products from sulfocysteine. Test system contained 0.01 M sulfocysteine, 0.025 M phosphate and dialyzed acetone powder extract corresponding to 15 mg dry weight/ml. Incubated 1 h at 37° in air.

Experiment No.	S_2O_3 formed μmole/ml	NH_3 formed μmole/ml	Pyruvate formed μmole/ml
1	0.77	0.69	0.57
2	1.36	1.28	1.00
3	1.16	0.98	0.72

Table 3. Sulfate formation from thiosulfate esters. Substrate concentration 0.01 M, phosphate 0.025 M, pH 7.4, homogenate corresponding to 50 mg rat liver per ml. Incubated 1 h at 37°.

Substrate	Homogenate	Gas phase	SO_4 formed μmole/ml
Sulfocysteine	Unheated	Air	0.48
»	»	N_2	0
»	Heated 2 min. at 100°C	Air	0.04
AETA	Unheated	»	0.51
»	Heated 2 min. at 100°C	»	0.04

anaerobic conditions. No sulfite could be detected when the incubation was carried out in air as the sulfite was rapidly oxidized to sulfate, presumably through the action of the sulfite oxidase system [18]. It was somewhat unexpectedly found that heating of the homogenate did not destroy, but on the contrary stimulated sulfite formation (Table 4) and this could only in part be attributed to an inactivation of the heat labile sulfite oxidase, as this "activation" was observed also under anaerobic conditions (Table 4). Further experiments demonstrated that the sulfite formation was completely inhibited by 0.001 M *p*-chloromercuribenzoate, a sulfhydryl group reagent, which suggested that sulfite was formed through a reaction between the thiosulfate ester and a sulfhydryl group. As it is well known that heat denaturation liberates "masked" sulfhydryl groups in proteins, the effect of heat treatment on the homogenate indicated that the active sulfhydryl groups were mainly protein bound. This was also supported by the fact that dialysis of a heat denatured homogenate gave only a 25 % decrease of the sulfite forming activity. (The dialysis was carried out in a nitrogen atmosphere, as otherwise a large decrease of activity was obtained.) Of interest was also that sulfite was formed from sulfocysteine and heat denatured ovalbumin, a protein containing SH-groups, but not from sulfocysteine and the native protein. The

Table 4. Sulfite formation from thiosulfate esters. Conditions as in Table 3 except that the reaction time was only 10 min.

Substrate	Homogenate	Gas phase	SO_3 formed μmole/ml
Sulfocysteine	Unheated	Air	0
»	»	N_2	0.18
»	Heated 2 min. at 100°C	Air	0.38
»	»	N_2	0.55
AETA	Unheated	Air	0
»	»	N_2	0.23
»	Heated 2 min. at 100°C	Air	0.41
»	»	N_2	0.53

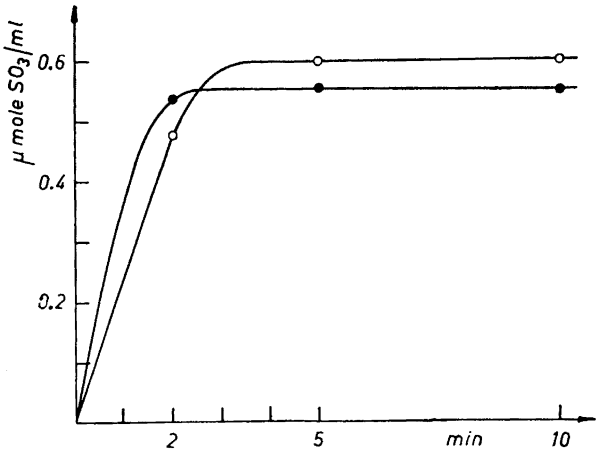

Fig. 1. Relation between glutathione and thiosulfate esters. Thiosulfate ester conc. 0.01 M, glutathione 1 μmole/ml, pH 7.4 and temp. 20°C.
○ ○ ○ Sulfocysteine ● ● ● AETA

reaction between a thiosulfate ester and a sulfhydryl compound gives sulfite and a mixed disulfide according to Ref.[2]

$$R_1SSO_3H + R_2SH \rightarrow R_1SSR_2 + H_2SO_3$$

In the present case the mixed disulfide should be protein bound. Indirect evidence for a formation of a protein bound disulfide from the thiosulfate ester and liver homogenate could in fact be obtained. When the reaction mixtures were deproteinized with trichloroacetic acid, paper chromatography did not reveal any reaction products containing amino groups or sulfur in the supernatant (except thiosulfate in the case of sulfocysteine), but when deproteinization was carried out by heat denaturation, cystine and cystamine were detected in the sulfocysteine and AETA system, respectively. The formation of these symmetrical disulfides could be attributed to a rearrangement of the primary formed mixed disulfide by the heating steps according to 2 PSSR → $(PS)_2 + (RS)_2$ (where P indicates protein).

The formation of sulfite with a simple sulfhydryl compound, glutathione, was also studied for comparative purposes (Fig. 1). It is evident that the reaction stops, when an equilibrium point is reached, as already demonstrated by Stricks and Kolthoff [19] in similar systems by a polarographic technique. The reaction between the thiosulfate esters and inorganic sulfide was also studied, but in this case the reaction was more complicated, as not only sulfide but also thiosulfate was formed. The time course of these reactions in shown in Fig. 2. The results can be explained by a primary formation of sulfite and a persulfide, followed by a slower transsulfuration according to

$$RSSO_3^- + HS^- \rightarrow RSSH + SO_3^{2-} \rightarrow RSH + S_2O_3^{2-}$$

as already suggested by Gutman [20]. Under the reaction conditions used in

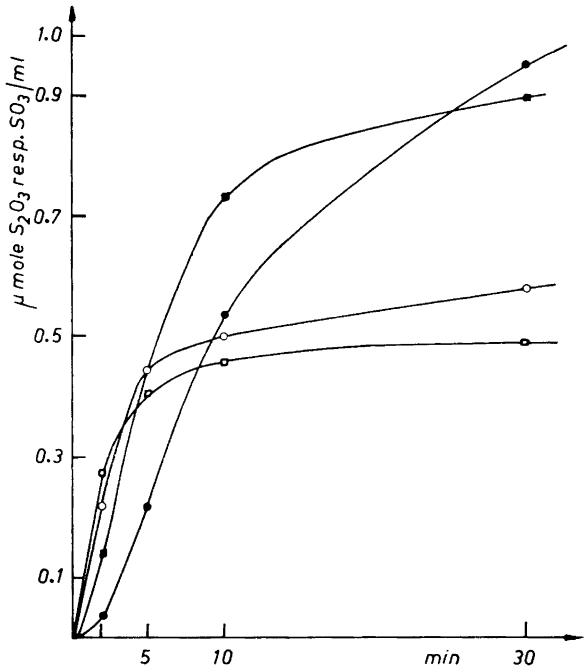

Fig. 2. Relation between inorganic sulfide and thiosulfate esters. Conditions as in Fig. I except that glutathione was replaced by Na_2S, 0.99 μmole/ml.

● ● ● ● Thiosulfate formed from sulfocysteine
■ ■ ■ ■ Thiosulfate from AETA
○ ○ ○ ○ Sulfite from sulfocysteine
□ □ □ □ Sulfite from AETA

this work the thiosulfate ester was present in excess, and consequently the following reaction also occurred

$$RSSO_3^- + RSH \rightarrow (RS)_2 + HSO_3^-$$

as is evident from the fact that the molar yield of sulfite plus thiosulfate was in excess over the sulfide added to the system (*cf.* Fig. 2). The formation of cystine and cystamine, respectively, in the systems was confirmed by paper chromatography, but no persulfide was detected, presumably due to the instability of the latter. The results obtained have a certain resemblance to those obtained in a study of the reaction between a thiosulfonate ester (disulfoxide) and inorganic sulfide [21]. The reaction products were here found to be sulfinate, as expected, but a disulfide and thiosulfonate instead of the expected persulfide.

DISCUSSION

The present work has demonstrated an enzymic formation of thiosulfate from sulfocysteine, with pyruvate and ammonia as the other reaction products, thus confirming the suggestion of Cavallini and Stirpe [5]. This reaction shows a resemblance to the cysteine desulfhydrase reaction [22], where hydrogen sulfide,

pyruvate and ammonia are formed from cysteine, when the latter is incubated with liver. As sulfocysteine is structurally related to cysteine, the same enzyme system may be responsible for both these reactions. It is of interest here that the thiosulfate formation from sulfocysteine in the presence of liver homogenate was strongly inhibited by semicarbazide (87 % inhibition at 0.001 M inhibitor conc.) and thus also in this respect similar to cysteine desulfhydrase [21]. Concerning the possibility that sulfocysteine is a precursor for thiosulfate in higher animals, the available data do not indicate that sulfocysteine is of importance in this respect. It was previously [2] demonstrated that thiosulfate is rapidly formed from β-mercaptopyruvate and sulfite in the presence of liver, and the ability of a liver homogenate to form thiosulfate by this reaction is about 10^4 times higher than its catalytic activity on sulfocysteine.

The ability of thiosulfate esters to form mixed disulfides is of interest in connection with chemical protection against ionizing radiation. Eldjarn and Pihl [23] have attributed the protecting properties of certain sulfhydryl and disulfide compounds (for instance cysteamine) to their ability to form mixed disulfides with proteins. If this theory is valid one should expect that also thiosulfate esters have radiation protective properties and this has, as a matter of fact, been experimentally verified for AETA in connection with the present work [24]. Experiments with other thiosulfate esters are now in progress.

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