On the Metabolism of Thiosulfate Esters

BO SÖRBO

Research Institute of National Defense, Medical Dept., and
Nobel Medical Institute, Biochemical Dept., Stockholm, Sweden

An enzyme has been demonstrated in rat liver, which converts
sulfocteine 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, sulfocteine * [COOHCH(NH₂)CH₂S₂O₃H], was
demonstrated by Clarke¹ to be a reaction product from cystine and sulfite,
but was apparently not considered as a possible normal metabolite. Later
work with mould mutants⁵,⁶ has, however, suggested that in these moulds,
sulfocteine may be an intermediate in the metabolic pathway leading from
sulfate to cysteine. Recently Cavallini and Stirpe⁵ administered sulfocteine
to rats and observed an increased excretion of thiosulfate. They suggested
that the compound was dominatated to ammonia, pyruvate and thiosulfate and
that sulfocteine was a precursor of the thiosulfate normally excreted in the
urine from higher animals.

The present work was undertaken in order to study possible transforma-
tions of sulfocteine and its decarboxylated analogue, β-aminoethylthio-
sulfuric 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 journal⁶.

* Clarke originally proposed the name cysteine-S-sulfonate, but this could erroneously imply
a thiosulfonate structure (RSO₃SH) instead of a thiosulfate ester structure (RS₂O₂H) for the
compound. Recently the name S-sulfocteine has been suggested and it is used in the present
paper.

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MATERIALS

Attempts were first made to prepare sodium sulfocysteine according to Clarke, 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; presumably the copper ions were complexed by sulfocysteine. Attempts to remove the copper by treating the sample with H₂S 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 Na₂SO₃·7H₂O (50 mmole) and the solution left for 1 h. 30% H₂O₂ was then added dropwise with efficient stirring until no free cysteine could be detected with phosphotungstic acid. About 3 ml H₂O₂ 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 H₂O. 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) was obtained, corresponding to 34% of the theoretical yield. Recrystallization from ethanol-water (1:1) gave 1.54 g of pure product.

AETA was a gift from Dr. B. Hansen.

METHODS

Thiosulfate was determined colorimetrically as previously described. Sulfate was determined by a turbidimetric method modified from that of Gassner and Friedel. 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 gelatin reagent followed by 0.4 ml of barium chloride reagent. The turbidity was measured in a 1 cm cuvette at 400 μm against a blank, obtained by adding 0.8 ml gelatin reagent to another 3 ml aliquot of the supernatant. Sulfite was determined by a colorimetric procedure, based on that of West and Gaeke. To the sample was added an equal volume of 0.2 M HgCl₂ 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 μm was determined. A blank value was obtained by adding HgCl₂ 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 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 (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. The identity of the pyruvate formed from sulfocysteine was also established by paper chromatography according to Wieland and Fisher.

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

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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.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Gas phase</th>
<th>S₂O₃ formed µmole/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver slices</td>
<td>Air</td>
<td>0.18</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Liver homogenate</td>
<td></td>
<td>0.64</td>
</tr>
<tr>
<td>Heated 2 min. at 100°C</td>
<td>N₂</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>0</td>
</tr>
</tbody>
</table>

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

\[-S₂O₃CH₂·CHNH₂·COOH + H₂O → CH₃·CO·COO⁻ + S₂O₃²⁻ + NH₄⁺ + H⁺\]

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

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.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>S₂O₃ formed µmole/ml</th>
<th>NH₄ formed µmole/ml</th>
<th>Pyruvate formed µmole/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.77</td>
<td>0.69</td>
<td>0.57</td>
</tr>
<tr>
<td>2</td>
<td>1.38</td>
<td>1.28</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>1.16</td>
<td>0.98</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Table 3. Sulfite 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°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Homogenate</th>
<th>Gas phase</th>
<th>SO₄ formed μmole/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfocysteine</td>
<td>Unheated</td>
<td>Air</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>Heated 2 min. at 100°C</td>
<td>N₂</td>
<td>0</td>
</tr>
<tr>
<td>AETA</td>
<td>Unheated</td>
<td>Air</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Heated 2 min. at 100°C</td>
<td>N₂</td>
<td>0.51</td>
</tr>
</tbody>
</table>

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. 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 ovalalbumin, 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.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Homogenate</th>
<th>Gas phase</th>
<th>SO₄ formed μmole/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfocysteine</td>
<td>Unheated</td>
<td>Air</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Heated 2 min. at 100°C</td>
<td>N₂</td>
<td>0.18</td>
</tr>
<tr>
<td>AETA</td>
<td>Unheated</td>
<td>Air</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Heated 2 min. at 100°C</td>
<td>N₂</td>
<td>0.55</td>
</tr>
</tbody>
</table>

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reaction between a thiosulfate ester and a sulphydryl compound gives sulfite and a mixed disulfide according to Ref.\textsuperscript{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 deproteination 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 \( 2 \text{PSSR} \rightarrow (\text{PS})_2 + (\text{RS})_2 \) (where P indicates protein).

The formation of sulfite with a simple sulphydryl 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\textsuperscript{15} 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 is 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

\[ \text{RSSO}_3^- + \text{HS}^- \rightarrow \text{RSSH} + \text{SO}_3^{2-} \rightarrow \text{RSH} + \text{S}_2\text{O}_3^{2-} \]

as already suggested by Gutman\textsuperscript{20}. Under the reaction conditions used in

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

$$\text{RSSO}_3^- + \text{RSH} \rightarrow (\text{RS})_2 + \text{HSO}_3^-$$

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 (disulf oxide) and inorganic sulfide \(^2\). 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 \(^2\), where hydrogen sulfide,
pyruvate and ammonia are formed from cysteine, when the latter is incubated
with liver. As sulfcysteine 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 sulfcysteine 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 desulhydrase. Concerning the possibility that sulfcysteine is a
precursor for thiosulfate in higher animals, the available data do not indicate
that sulfcysteine is of importance in this respect. It was previously demon-
strated that thiosulfate is rapidly formed from β-mercaptopropyruvate and sulfate
in the presence of liver, and the ability of a liver homogenate to form thio-
sulfate by this reaction is about 10^4 times higher than its catalytic activity on
sulfcysteine.

The ability of thiosulfate esters to form mixed disulfides is of interest in
connection with chemical protection against ionizing radiation. Eldjarn and
Pihl have attributed the protecting properties of certain sulphhydryl 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. Experiments with other thiosulfate esters are now in progress.

The valuable technical assistance of Mrs. E. Ekblom is gratefully acknowledged.

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