Some Derivatives of Glutathione

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Although glutathione has been much studied, its oxidation products have not been adequately characterized. The first product of oxidation, the disulphide, GSSG, is a familiar compound, although it is doubtful whether it has been obtained pure. The kinetics of the reaction of GSSG with silver salts have been investigated (Cecil, 1950; Cecil & McPhee, 1957), but the product, the sulphinic acid, GSO₂H, was not characterized. The final product of oxidation, the sulphonic acid, GSO₃H, has also not been characterized. The thiosulphonate, GSO₂·SG, was thought to be a product of the reaction of GSSG with naphthalenesulphonyl chloride (Saunders 1933), but no convincing evidence of its existence has been put forward. The thiosulphate, GS·SO₂H, which is found in lens extracts, was described by Waley (1959). Apart from their relevance to the chemistry of glutathione, compounds such as GSO₂H represent bound forms of derivatives of cysteine which are intermediates in sulphur metabolism, and it is conceivable that the peptides also play a part in sulphur metabolism.

This paper describes the preparation and properties of GSO₂H, GSO₃H and (probably) GSO₂·SG, and also gives some further properties of ophthalmic acid (γ-glutamyl-α-amino-α-butyrylglycine), an analogue of glutathione in the lens (Waley, 1956, 1958).

The sulphinic acid, GSO₂H, was prepared by the reaction of silver nitrate with GSSG:

\[ 2\text{GSSG} + 3\text{Ag}^+ + 2\text{H}_2\text{O} \rightarrow \text{GSO}_2\text{H} + 3\text{GSAg} + 3\text{H}^+ \]

The conditions were similar to those described by Cecil & McPhee (1957), except that more concentrated solutions were used. This, and the other reactions described in this paper, were conveniently followed by paper electrophoresis (Table 1). The sulphinic acid was obtained crystalline, in good yield, after purification by ion-exchange chromatography. This step removes both unchanged GSSG and inorganic salts; pyridine-formate buffers proved convenient, as they were both volatile and ninhydrin-negative.

The sulphinic acid (GSO₂H). This proved to be a comparatively stable compound; it thus resembles cysteinesulphinic acid (CYSO₂H), but differs from simple aliphatic sulphinic acids, which are readily oxidized. The stability of GSO₂H and of CYSO₂H towards oxidation may be due to their existing as dipolar ions in which the sulphinic acid (the most strongly acidic group present) is ionized.

Reduction of GSO₂H by hydroiodic acid proceeded quantitatively, according to the equation:

\[ 2\text{GSO}_2\text{H} + 6\text{HI} \rightarrow \text{GSSG} + 3\text{I}_2 + 4\text{H}_2\text{O} \]

The corresponding reduction of CYSO₂H was carried out by Lavine (1936).

Hydrolysis of GSO₂H by n-hydrochloric acid at 100°C was rapid, and about 25% of the sulphinic acid had been hydrolysed to the constituent amino acids after 4 min. Vigorous acid hydrolysis (6N-hydrochloric acid at 110°C for 16 hr.) gave glycine and glutamic acid as the main ninhydrin-positive products; cysteinesulphinic acid evidently decomposed under these conditions.

The infrared spectrum of the compound (as a Nujol mull) showed a strong band at 967 μ, which is probably a S=O stretching mode, and which is also found in potassium benzenesulphonate (Detoni & Hadzi, 1955).

On electrometric titration, the value of pK₁ due to the sulphinic acid group could not be measured as it was too low; the values of pK₂, pK₃ and pK₄ were 296, 484 and 1052 respectively. These may be ascribed to the two carboxyl groups and the amino group.

The sulphonic acid (GSO₃H). This was prepared by the oxidation of GSH with hydrogen peroxide in formic acid, and was obtained as crystals. The infrared spectrum of GSO₃H showed strong bands at 965 and 862 μ, which fall within the ranges

<table>
<thead>
<tr>
<th>Distance moved (cm.)</th>
<th>Compound</th>
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<tbody>
<tr>
<td></td>
<td>At pH 4</td>
</tr>
<tr>
<td>GSH</td>
<td>9-5  10-3</td>
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<tr>
<td>GSSG</td>
<td>10-3 10-6</td>
</tr>
<tr>
<td>GSO₂H</td>
<td>13-5  4-1</td>
</tr>
<tr>
<td>GSO₃H</td>
<td>14-2  2-8</td>
</tr>
<tr>
<td>CySO₂H</td>
<td>16-0  1-1</td>
</tr>
<tr>
<td>CySO₃H</td>
<td>17-1  0</td>
</tr>
<tr>
<td>CySO₂·SCy</td>
<td>Decomposes 8-9</td>
</tr>
<tr>
<td>Ophthalmic acid</td>
<td>9-2  10-4</td>
</tr>
</tbody>
</table>

Table 1. Mobilities in paper electrophoresis at pH 4 and at pH 2-3

Electrophoresis on no. 52 paper was carried out for 4 hr. at 10V/cm. in the apparatus of Cliffe & Waley (1958). The buffers used were pyridine-acetate, pH 4 (Grassmann, Hannig & Plocki, 1955), and 10% (v/v) acetic acid, pH 2-3.
commonly observed (Bellamy, 1958) for S=O stretching vibrations of sulphonic acids. As with GSO₂H, pK₅ could not be measured; the values of pK₂, pK₃ and pK₄ were 2-82, 4-29 and 10-12 respectively.

**Preparation of the thiosulphonate (GSO₂·SG).** This proved difficult. The procedure of Saunders (1933) gave a complex mixture containing at least five nihydros-peracids or compounds. Fractionation by ion-exchange chromatography did not lead to any pure product, but some GSO₂H was probably formed. If GSO₂·SG is indeed formed (and the mechanism of its formation under these conditions is obscure) it would not be expected to be stable in alkaline solution: the corresponding derivative (CySO₂·SCy) from cysteine decomposed even at pH 8. Attempts were made to oxidize GSSG with peracetic acid, or with hydrogen peroxide in acetic acid, but the main product detected by paper electrophoresis was GSO₂H. The cleavage of GS·SO₄H by GSO₂H was also studied, but no new products were formed. Some reaction occurred between GSO₂H and GSH in the presence of dicyclohexyl carbodi-imide (in aqueous pyridine), but the extent of reaction was small. In the oxidation of cystine to the corresponding thiosulphonate, CySO₂·SCy, Toennies & Lavine (1936) found that anhydrous conditions were necessary; they oxidized cystine perchlorate with perbenzoic acid in acetonitrile solution. We successfully repeated this preparation, but with monoperphthalic acid as the oxidizing agent. This thiosulphonate decomposed during paper electrophoresis at pH 4, but was more stable at a lower pH, in 10 % (v/v) acetic acid. The method of Toennies & Lavine (1936) was not directly applicable to the preparation of GSO₂·SG, since the perchlorate of GSSG was insoluble in acetonitrile. However, the toluene-p-sulphonate of GSSG was readily soluble in dimethylformamide, and, after oxidation with monoperphthalic acid, the solution gave a spot on paper electrophoresis in 10 % (v/v) acetic acid attributed to GSO₂·SG, as well as spots due to GSSG and to GSO₂H.

Confirmation that GSO₂·SG was present in the reaction mixture was obtained from the reaction with cysteine (CySH). Thiosulphonates are readily cleaved by nucleophilic reagents, especially mercaptans (Parker & Kharasch, 1959), and in this case the reaction takes the course:

\[
\text{CySH} + \text{GSO}_2 \cdot \text{SCy} \rightarrow \text{GS} \cdot \text{SCy} + \text{GSO}_2 \text{H}
\]

The reaction, studied by paper electrophoresis, was carried out by adding a drop of a solution of CySH to the reaction mixture on the starting line of the electrophoresis paper (before running). The spot due to GSO₂·SG disappeared, and a new spot, assigned to the mixed disulphide, GS·SCy, appeared. The mixed disulphide was similarly formed from the reaction of CySO₂·SCy with GSH:

\[
\text{GSH} + \text{CySO}_2 \cdot \text{SCy} \rightarrow \text{GS} \cdot \text{SCy} + \text{CySO}_2 \text{H}
\]

Further evidence that GSO₂·SG had been formed was obtained from the infrared spectrum of the impure material, which gave a band at 8-94 μ. This corresponds to a band at 8-01 μ in CySO₂·SCy, and has been used as evidence that the structure is CySO₂·SCy rather than CySO₂·SOCy (Sweetman, 1959). Although the instability of the GSO₂·SG precluded its purification, the evidence given above suggests that it was, in fact, obtained in an impure state.

**Properties of ophthalamic acid.** The infrared spectrum of ophthalamic acid showed the following features. The band at 5-93 μ is assigned to the unionized carboxyl group of glycine, the bands at 6-08 and 6-65 μ are probably the amide I and amide II bands, and the band at 8-14 μ is probably due to a carboxyl group.

On titration, ophthalamic acid gave pK₁, pK₂ and pK₅ values of 2-39, 3-95 and 9-38 respectively. These are assigned to the carboxyl groups of glutamic acid (pK₁) and of glycine (pK₅), and to the amino group (pK₂). The value of pK₅ is close to that (9-2) recorded by Martin & Edsall (1958) for the amino group of S-methylglutathione, and confirms their general conclusions about the ionization of glutathione itself. A small difference, however, is made to the microconstants of glutathione [calculated by the method of Martin & Edsall (1958)] if ophthalamic acid, rather than S-methylglutathione, is taken as the model compound.

**EXPERIMENTAL**

Melting points are uncorrected. The analyses were carried out by Weller and Strauss, Oxford. The pK values were obtained from titration curves carried out at 20° and at an ionic strength of 0-18 x 10⁻¹⁸.

The sulphonic acid (GSO₂H) from glutathione. Oxygen was bubbled through GSH (307 mg.) in 0-2 x NaOH (5 ml.) until a portion of the solution gave a negative nitroprusside test (about 2-5 hr.). Paper electrophoresis at pH 4 (Cliffe & Waley, 1958) then showed only one ninhydrin-positive spot, in the position of GSSG. This solution of GSSG was then treated with 76 ml. of acetate buffer, pH 6-1 (prepared from 47-5 ml. of 0-2 x sodium acetate and 2-5 ml. of 0-2 x acetic acid, diluted to 100 ml.), and 19 ml. of 0-1 x AgNO₃. The silver mercaptide started to separate at once, and, after the mixture had been kept for 25 hr. (in the dark), the solid was centrifuged down and the supernatant stored overnight at 10°. Further solid was centrifuged down, and the supernatant was diluted to 200 ml.; the sulphonic acid was isolated by ion-exchange chromatography on a column (2-5 cm. diam. x 11-4 cm. long) of Dowex 1 (X4; acetate form; 200–400 mesh). The column, which was covered to prevent decomposition of the silver compounds by light, was eluted at 4 ml./min. with pyridine–formate, pH 5-2.
[4-3 ml of 90% (w/v) formic acid, 16-4 ml of pyridine and water to 1 l.]; fractions of 18 ml were collected. After ten fractions, the eluting agent was changed to pyridine-formate, pH 3 [275 ml of 90% (w/v) formic acid, 124 ml of pyridine and water to 1 l.] The ninhydrin-positive fractions (12 and 13) were stored overnight at −10°C, centrifuged, and the supernatant was dried in vacuo. The residual gum was dissolved in the minimum volume of water and diluted with acetone. The sulphonic acid separated as a colourless solid, m.p. 187° (decomp.) (yield 64-1 mg., 76%) (Found: C, 35-6; H, 5-1; N, 12-2; S, 9-2. C₃H₃₂N₉O₆S requires C, 35-4; H, 5-05; N, 12-4; S, 9-4%). Reduction of the sulphonic acid by HI was carried out by adding 0-1 ml of a solution of GSO₂H (1-22 mg.) in water (0-5 ml.) to 2M-KI (0-25 ml.) and 2N-HCl (0-25 ml.). A blank solution without any sulphinic acid was treated similarly. After being kept in the dark for several hours, the iodine liberated was titrated with 25-7 mm-Na₂S₂O₃ with sodium starch glycollate as indicator. The titre after subtraction of the blank (mean of four titrations) was 0-0833 ml., the calculated value being 0-0838 ml.

The sulphonic acid (GSO₂H) from glutathione. Hydrogen peroxide (2 ml of 30%, w/v) was added to 98-100% (w/v) formic acid (30 ml.), and, after 5 min., GSH (1-5 g.) was added. Further portions of hydrogen peroxide (1 ml.) were added after 15 min. and after 30 min. After 70 min. the solution was diluted with water (70 ml.), frozen and dried in vacuo. Oxidation was incomplete (as judged by paper electrophoresis), and so the material, in 10 ml. of formic acid, was added to 20 ml. of formic acid to which 2 ml of 30% (w/v) hydrogen peroxide had been added 60 min. earlier. After oxidation for 60 min. the solution was diluted with water (40 ml.) and dried in the frozen state. The residue (1-5 g.) was purified for analysis by precipitation from water by acetone: the first material to separate was oily; the supernatant was decanted, and from it the sulphonic acid crystallized: [α]D²⁰ = −15-2° (c 1-3 in water) (Found: C, 31-9; H, 5-4; N, 11-1. C₃H₃₂N₉O₆S.H₂O requires C, 32-2; H, 5-1; N, 11-2%).

The thiosulphinic acid (GSO₂-SCy) from cysteine. This was prepared by the oxidation of cystine perchlorate with monoperphthalic acid (cf. Toennies & Lavine, 1936), and was isolated as a crystalline solid (Found: C, 26-7; H, 4-2; N, 10-5; S, 23-2. Calc. for C₄H₁₇N₃O₄S: C, 26-5; H, 4-4; N, 10-3; S, 23-5%).

Summary

1. The sulphinic acid (GSO₂H) from glutathione, prepared by fission of GSSG with silver salts, has been isolated.
2. The sulphonic acid (GSO₂H) from glutathione, prepared by oxidation of GSH with performic acid, has also been isolated.
3. The preparation of the thiosulphinic acid, GSO₂-SCy, has been attempted by oxidation of GSSG with monoperphthalic acid; the unstable product, which could not be purified, showed the reactions expected of GSO₂-SCy.
4. The infrared spectrum and titration constants of opthalmic acid are discussed.

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References