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, GSO_2H , was not characterized. The final product of oxidation, the sulphonic acid, GSO_3H , has also not been characterized. The thiolsulphonate, $\text{GSO}_2\cdot\text{SG}$, 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, $\text{GS}\cdot\text{SO}_3\text{H}$, which is found in lens extracts, was described by Waley (1959). Apart from their relevance to the chemistry of glutathione, compounds such as GSO_2H 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 GSO_2H , GSO_3H and (probably) $\text{GSO}_2\cdot\text{SG}$, and also gives some further properties of ophthalmic acid (γ -glutamyl- α -amino-*n*-butyrylglycine), an analogue of glutathione in the lens (Waley, 1956, 1958).

The sulphinic acid, GSO_2H , was prepared by the reaction of silver nitrate with GSSG:

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 (GSO_2H). This proved to be a comparatively stable compound; it thus resembles cysteinesulphinic acid (CySO_2H), but differs from simple aliphatic sulphinic acids, which are readily oxidized. The stability of GSO_2H and of CySO_2H 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 GSO_2H by hydroiodic acid proceeded quantitatively, according to the equation:

The corresponding reduction of CySO_2H was carried out by Lavine (1936).

Hydrolysis of GSO_2H by *N*-hydrochloric acid at 100° was rapid, and about 25% of the sulphinic acid had been hydrolysed to the constituent amino acids after 4 min. Vigorous acid hydrolysis (6*N*-hydrochloric acid at 110° 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 9.67μ , which is probably a $\text{S}=\text{O}$ stretching mode, and which is also found in potassium benzenesulphinic acid (Detoni & Hadzi, 1955).

On electrometric titration, the value of pK_1 due to the sulphinic acid group could not be measured as it was too low; the values of pK_2 , pK_3 and pK_4 were 2.96, 4.84 and 10.52 respectively. These may be ascribed to the two carboxyl groups and the amino group.

The sulphonic acid (GSO_3H). This was prepared by the oxidation of GSH with hydrogen peroxide in formic acid, and was obtained as crystals. The infrared spectrum of GSO_3H showed strong bands at 9.65 and 8.62μ , which fall within the ranges

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 & Plöckl, 1955), and 10% (v/v) acetic acid, pH 2.3.

Compound	Distance moved (cm.)	
	At pH 4	At pH 2.3
GSH	9.5	10.3
GSSG	10.3	10.6
GSO_2H	13.5	4.1
GSO_3H	14.2	2.8
CySO_2H	16.0	1.1
CySO_3H	17.1	0
$\text{CySO}_2\cdot\text{SCy}$	Decomposes	8.9
Ophthalmic acid	9.2	10.4

commonly observed (Bellamy, 1958) for S=O stretching vibrations of sulphonic acids. As with GSO_2H , pK_1 could not be measured; the values of pK_2 , pK_3 and pK_4 were 2.82, 4.29 and 10.12 respectively.

Preparation of the thiosulphonate ($\text{GSO}_2\cdot\text{SG}$). This proved difficult. The procedure of Saunders (1933) gave a complex mixture containing at least five ninhydrin-positive compounds. Fractionation by ion-exchange chromatography did not lead to any pure product, but some GSO_2H was probably formed. If $\text{GSO}_2\cdot\text{SG}$ 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 ($\text{CySO}_2\cdot\text{SCy}$) 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 GSO_2H . The cleavage of $\text{GS}\cdot\text{SO}_3\text{H}$ by GSO_2H was also studied, but no new products were formed. Some reaction occurred between GSO_2H 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, $\text{CySO}_2\cdot\text{SCy}$, 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 $\text{GSO}_2\cdot\text{SG}$, 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 $\text{GSO}_2\cdot\text{SG}$, as well as spots due to GSSG and to GSO_2H .

Confirmation that $\text{GSO}_2\cdot\text{SG}$ 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:

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 $\text{GSO}_2\cdot\text{SG}$ disappeared, and a new spot, assigned to the mixed disulphide, $\text{GS}\cdot\text{SCy}$,

appeared. The mixed disulphide was similarly formed from the reaction of $\text{CySO}_2\cdot\text{SCy}$ with GSH:

Further evidence that $\text{GSO}_2\cdot\text{SG}$ had been formed was obtained from the infrared spectrum of the impure material, which gave a band at $8.94\ \mu$. This corresponds to a band at $9.01\ \mu$ in $\text{CySO}_2\cdot\text{SCy}$, and has been used as evidence that the structure is $\text{CySO}_2\cdot\text{SCy}$ rather than $\text{CySO}\cdot\text{SOCy}$ (Sweetman, 1959). Although the instability of the $\text{GSO}_2\cdot\text{SG}$ precluded its purification, the evidence given above suggests that it was, in fact, obtained in an impure state.

Properties of ophthalmic acid. The infrared spectrum of ophthalmic acid showed the following features. The band at $5.93\ \mu$ is assigned to the unionized carboxyl group of glycine, the bands at 6.08 and $6.65\ \mu$ are probably the amide I and amide II bands, and the band at $8.14\ \mu$ is probably due to a carboxyl group.

On titration, ophthalmic acid gave pK_1 , pK_2 and pK_3 values of 2.39, 3.95 and 9.38 respectively. These are assigned to the carboxyl groups of glutamic acid (pK_1) and of glycine (pK_2), and to the amino group (pK_3). The value of pK_3 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 ophthalmic acid, rather than *S*-methylglutathione, is taken as the model compound.

EXPERIMENTAL

Melting points are uncorrected. The analyses were carried out by Weiler and Strauss, Oxford. The pK values were obtained from titration curves carried out at 20° and at an ionic strength of $0.16M$.

The sulphinic acid (GSO_2H) *from glutathione.* Oxygen was bubbled through GSH (307 mg.) in $0.2N$ -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 $0.2M$ -sodium acetate and 2.5 ml. of $0.2N$ -acetic acid, diluted to 100 ml.), and 19 ml. of $0.1M$ - AgNO_3 . 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 sulphinic acid was isolated by ion-exchange chromatography on a column (2.5 cm. diam. $\times$ 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 [278 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° , centrifuged, and the supernatant was dried *in vacuo*. The residual gum was dissolved in the minimum volume of water and diluted with acetone. The *sulphinic 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. $C_{10}H_{17}N_3O_8S$ requires C, 35.4; H, 5.05; N, 12.4; S, 9.4%). Reduction of the *sulphinic acid* by HI was carried out by adding 0.1 ml. of a solution of GSO_2H (1.22 mg.) in water (0.5 ml.) to 2 M-KI (0.25 ml.) and 2 N-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- $Na_2S_2O_3$ 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 sulphinic acid (GSO_2H) 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 *sulphinic acid* crystallized: $[\alpha]_D^{25} -15.2^{\circ}$ (c 1.3 in water) (Found: C, 31.9; H, 5.4; N, 11.1. $C_{10}H_{17}N_3O_8S_2H_2O$ requires C, 32.2; H, 5.1; N, 11.2%).

The thiosulphonate ($GSO_2 \cdot SG$) from glutathione. Solid GSSG was prepared by the oxidation of the barium salt of GSH, followed by precipitation of $BaSO_4$ and evaporation of the supernatant.

Monoperphthalic acid (Bachmann & Cooper, 1944; Böhme, 1940) (0.2 ml. of an ethereal solution containing 2 mol.prop. of the per-acid, as determined by iodometric titration) was added to GSSG (61.2 mg., 1 mol.prop.) and toluene-*p*-sulphonic acid (38 mg., 2 mol.prop.) in dimethyl-formamide (1 ml.). The solution was stored in the dark, and $CHCl_3$ (2 ml., 2 ml. and 0.5 ml.) was added on successive days. Finally, light petroleum (b.p. $60-80^{\circ}$) (1 ml.) was added, and the solid centrifuged down and triturated under acetone (yield, 78.9 mg.).

The thiosulphonate ($CySO_2 \cdot SCy$) 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 $C_6H_{12}N_2O_8S$: C, 26.5; H, 4.4; N, 10.3; S, 23.5%).

SUMMARY

1. The *sulphinic acid* (GSO_2H) from glutathione, prepared by fission of GSSG with silver salts, has been isolated.

2. The *sulphonic acid* (GSO_3H) from glutathione, prepared by oxidation of GSH with performic acid, has also been isolated.

3. The preparation of the thiosulphonate, $GSO_2 \cdot SG$, has been attempted by oxidation of GSSG with monoperphthalic acid; the unstable product, which could not be purified, showed the reactions expected of $GSO_2 \cdot SG$.

4. The infrared spectrum and titration constants of ophthalmic acid are discussed.

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