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Glutathione oxidation in real time by thermospray liquid chromatography–mass spectrometry

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Abstract

The oxidation and reduction of glutathione and oxidized glutathione were studied in real time by liquid chromatography–mass spectrometry during exposure to hydrogen peroxide and mercaptoethanol. By mass spectrometry mixed disulfides and both reversible and irreversible oxidations of sulfur to higher states (sulfinic and sulfonic acids) were directly observed during exposure to hydrogen peroxide. The irreversible oxidation of glutathione to glutathione sulfonic acid could be detected after 30 min exposure of glutathione to 40 m*M* H_2O_2 at 20°C. A peak consistent with glutathione-sulfinic acid was transiently present, suggesting this compound behaved as an oxygen consuming antioxidant. Liquid chromatography–mass spectrometry appears to be an excellent method to study oxidation and reductions of sulfur containing peptides and amino acids.

Keywords: Glutathione; Peptides

1. Introduction

Oxidants and reactive oxygen species can damage a variety of cellular and extracellular components, including membrane lipids and nucleic acids [1,2]. Organisms have a variety of defenses in place to prevent such damage from occurring. One of the major endogenous antioxidants is a tripeptide, glutathione (GSH) [3]. GSH acts as a reducing agent by donating the thiol hydrogen from cysteine and, as GSH is itself oxidized, it forms a disulfide link with either another molecule of GSH forming GSSG, or with a protein thiol to form a mixed disulfide. In addition to GSH, many proteins contain sulfhydryl groups that could have antioxidant properties [4]. More commonly, however, the sulfur-containing amino acids in protein are already oxidized to disulfides, and this disulfide bond is often critical for maintaining tertiary protein structure and function [5,6].

Besides the reversible sulfhydryl to disulfide reactions in which GSH and proteins can participate, there is additional oxidation which sulfur-containing amino acids can undergo to form sulfonates [7–9]. Sulfur in this highly oxidized state can not be easily

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reduced to either disulfides or sulfhydryls [8,10]. Therefore sulfonate formation may lead to irreversible oxidative inactivation of GSH, and is potentially an important consequence of oxidative injury [8,11].

Liquid chromatography–mass spectrometry (LC–MS) is an excellent method to study reactions in aqueous media. Using flow loops one can monitor the loss and simultaneous formation of several species in real time, and transient compounds can sometimes be identified.

We have applied LC–MS to study the oxidation and reduction of glutathione to better characterize reactions involving this important sulfur-containing antioxidant. These studies suggest that oxidation of sulfur in peptides can readily occur under the appropriate conditions.

2. Experimental

GSH, free acid, GSSG, cysteic acid, and mercaptoethanol were obtained from Sigma Chemicals, (St. Louis, MO, USA). 3,3[$^2H_2$] cysteine was purchased from Cambridge Isotopes, Cambridge, MA. 30% Hydrogen peroxide (H_2O_2) was obtained from Aldrich Chemicals, (Milwaukee, WI, USA). Solvents and other reagents, including purified distilled water, were obtained from Fisher Scientific, (Pittsburgh, PA, USA). *N*-methyl-*N* (*tert.*-butyldimethylsilyl) trifluoroacetamide (TBDMS) was obtained from Regis Chemicals, (Morton Grove, IL, USA). [$^2H_2$]cysteic acid was synthesized by reacting [$^2H_2$]cysteine with hydrogen peroxide as previously reported by our laboratory [7].

The pH of solutions was determined using a Fisher Accumet pH meter and adjusted through the careful addition of 0.01–1.0 *M* NaOH.

Reactions were carried out on aliquots in borosilicate glass tubes and in 250 μl glass Hamilton syringes (Hamilton Corp, Reno, Nevada, USA) at 20°C. Real time oxidation was observed using a Finnigan (San Jose, CA, USA) LCQ mass spectrometer with continual monitoring of the mass spectra from m/z 50–700 in water or 50–90% acetonitrile in water (v/v). Oxidation reactions were performed by adding 30% H_2O_2 to solutions for a final concentration of 0.01–2.0% (v/v) [10] and a portion applied to the LCQ mass spectrometer at 3–8 μl/min from a 250 μl glass syringe. In other instances, where indicated, the samples were dried under vacuum, concentrating the H_2O_2. Reduction reactions were performed by adding mercaptoethanol [12] to a final concentration of 400 m*M*, and allowing samples to incubate at 20°C for 1 h, following which the samples were dried under vacuum for 16 h. Data was collected using negative ion electrospray, with a voltage of 3.5 kV, a sheath gas flow of nitrogen at 100 ml/min, capillary voltage at −42 V and a capillary temperature at 180°C.

For gas chromatographic mass spectrometric analyses, samples of GSH and GSSG were made to a final concentration of 0.8 to 3.2 m*M*. GSH was incubated in 15% (2.5 *M*) hydrogen peroxide for 1 h prior to drying under vacuum. For reactions involving GSSG, mercaptoethanol additions were made to a final concentration of 2–4 m*M*. When stated, solutions were carefully brought to a pH of 7.2 ± 0.1 and hydrogen peroxide added to a final concentration of 50–500 m*M*. These solutions were incubated at 20°C for 1 to 18 h. Fifty μl aliquots were dried using a Savant (Farmingdale, NY, USA) vacuum centrifuge system. Approximately 90 mcg of [$^2H_2$] cysteic acid was added to the dried aliquots, and the samples were resuspended in 50 μl 6 N HCl. These samples were incubated at 90°C for 1 h, dried under vacuum at 60°C, and the dried aliquots were derivatized by adding 30 μl of TBDMS and 60 μl of acetonitrile with incubation at 90°C for 1 h. Two μl aliquots were applied to a Hewlett Packard (Avondale, PA USA) 5890 gas chromatograph. Gas chromatography was carried out splitless through a Supelco (Bellfonte, PA USA) 12 m dimethylsiloxane, fused-silica capillary column (I.D. 0.25 mm) using a temperature ramp of 30°C/min from 80 to 300°C with helium as a carrier, and mass spectrometry was performed on a Hewlett Packard 5971A mass spectrometer using a SIM mode for m/z 456 and 454 with the electron multiplier at 1700 V as previously described [7].

Experiments were done over multiple time points, and aliquots measured in triplicate. The mean values and standard deviations are shown where indicated. Significance is defined as $p<0.05$ on a two-tailed, unpaired Students *t*-test.

3. Results

3.1. Spectra of GSH and GSSG at acidic pH during exposure to reductants and oxidants

Fig. 1A and B show the spectra obtained from of 3 m*M* solutions of the free acids of GSH and GSSG (pH 3.9±0.1) by negative ion-electrospray LC–MS at a range of *m*/*z* 50–700. GSH (formula weight 307), Fig. 1A, has a major negative ion at *m*/*z* 306 and a second negative ion from the dimer (formula weight 614) at *m*/*z* 613. GSSG (formula weight 612), Fig. 1B, has a major negative ion at *m*/*z* 611 and a second double charged negative ion at *m*/*z* 305. Fig. 2 show representative spectra obtained when these solutions are reexamined following incubation in a 100 fold molar excess (300 m*M*) of mercaptoethanol for 90 min. followed by concentration through drying (Fig. 2A and B) or a 200 fold molar excess (600 m*M*) of hydrogen peroxide (Fig. 2C and D) for 90 min. at 20°C. Following incubation in mercaptoethanol (formula weight 78) the spectrum of GSH (Fig. 2A) is essentially unchanged, while GSSG (Fig. 2B) consists entirely of negative ions attributed to GSH (*m*/*z* 306, 613) or mixed GSH-mercaptoethanol disulfides (formula weight 383) at *m*/*z* 382, or a disulfide:aggregate (GSSG plus mercaptoethanol, formula weight 690) at *m*/*z* 689. However, after 90 min incubation in hydrogen peroxide, GSH solutions consist of a new species (*m*/*z* 354) (Fig. 2C) while GSSG solutions are unchanged (Fig. 2D). A minor species having *m*/*z* of 338 could be seen if the hydrogen peroxide concentration was reduced. Fig. 2E shows the spectrum *m*/*z* 310–380 of a 500 μ*M* solution of GSH incubated in 35 m*M* H_2O_2 for 60 min.

3.2. Identity of oxidized species

After reaction with H_2O_2 with GSH, the formation of a peak at *m*/*z* 354 (an increase of *m*/*z* 48) (Fig. 2C) is consistent with the addition of three atoms of

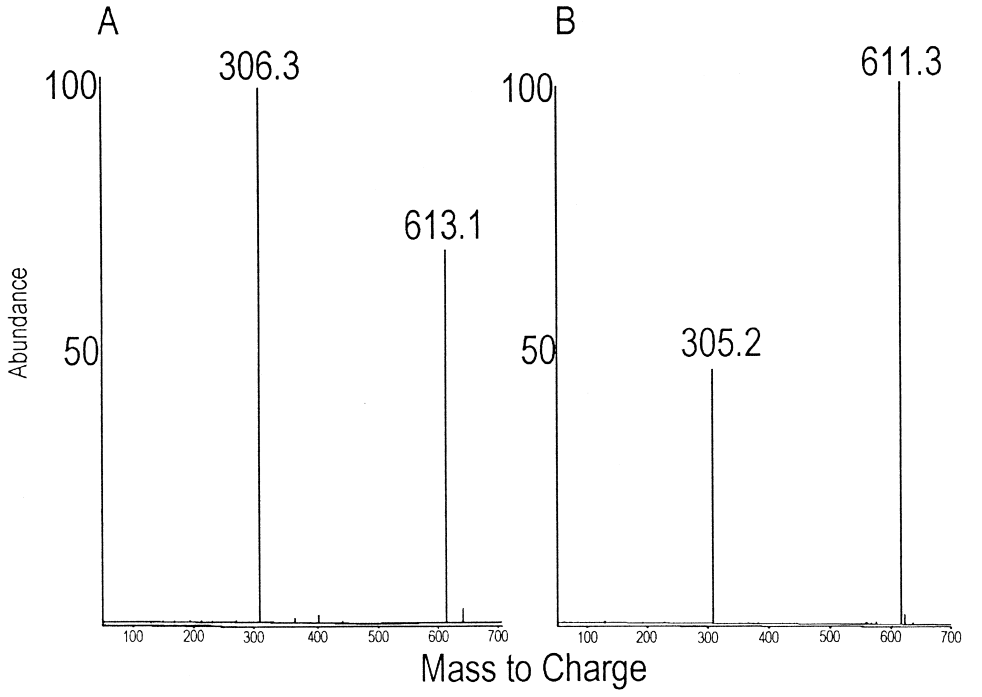

Fig. 1. The electrospray negative ion spectra (*m*/*z* 50–700) generated during infusions into an ion trap liquid chromatography mass spectrometer of: (A) 3 m*M* GSH free acid; (B) 1.5 m*M* GSSG free acid.

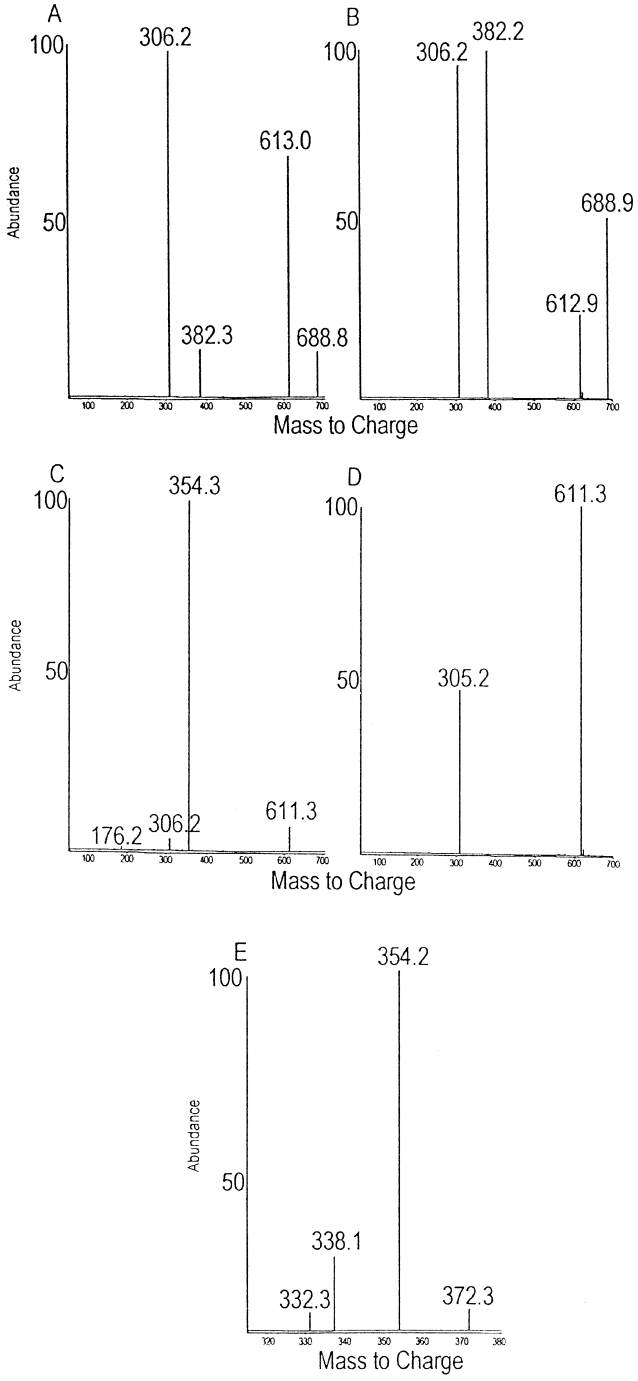

Fig. 2. The electrospray negative ion spectra (m/z 50–700) generated during infusions into an ion trap liquid chromatography mass spectrometer of: (A) resuspended GSH free acid following incubation and drying with 300 m*M* mercaptoethanol; (B) resuspended GSSG free acid following incubation and drying with 300 m*M* mercaptoethanol; (C) GSH free acid following incubation with 600 m*M* hydrogen peroxide for 90 min; (D) GSSG free acid following incubation with 600 m*M* hydrogen peroxide for 90 min.; and, (E) GSH free acid following incubation with 35 m*M* hydrogen peroxide for 60 min.

oxygen per molecule of GSH. The transient species of m/z 338 (an increase of m/z 32) (Fig. 2E) is consistent with the addition of two atoms of oxygen per molecule of GSH. Since this oxidation did not appear to occur with GSSG (Fig. 2D), it suggests the oxidation takes place at the GSH free thiol. To support this, a solution of GSH was split into two aliquots, and one aliquot was incubated with 600 m*M* hydrogen peroxide. The solutions were taken to dryness and hydrolyzed in 6 N HCl for 1 h at 90°C. The component amino acids were derivatized with TBDMS and characterized by electron-impact gas chromatography mass spectrometry. Fig. 3 shows the total ion chromatographs generated from hydrolyzed GSH and hydrolyzed hydrogen peroxide-oxidized GSH at a m/z range of 200–650. In Fig. 3A (hydrolyzed GSH), as expected, the peaks labeled 1 and 2 have spectra and retention times identical with standards containing cysteine and glutamate respectively. Following oxidation, Fig. 3B, the hydrolyzed GSH no longer contains peak 1 (cysteine), peak 2 (glutamate) is unchanged, and a new peak (peak 3) appears having a spectrum and retention time identical to cysteic acid (cysteine sulfonic acid) [7]. The results are consistent with the peak at m/z 338 being glutathione-sulfinic acid.

3.3. Irreversibility of sulfonic acid formation.

The irreversibility of sulfonic acid formation to reduction by thiols has been shown by others [9]. To test this observation, the effect of the addition of concentrated free thiols to sulfonic acids was examined. Separate solutions of cysteic acid (not shown) and GSH-sulfonic acid containing small amounts of GSSG (Fig. 4A) were incubated in 100 fold excess of mercaptoethanol for 1 h at 37°C, and concentrated to dryness at 60°C under vacuum. These solutions were reconstituted in water and examined by negative ion electrospray on LC–MS. There was no change in pure cysteic acid solutions following mercaptoethanol incubation (not shown). Likewise, based on mass spectra there was no apparent reduction of GSH sulfonic acid, while the

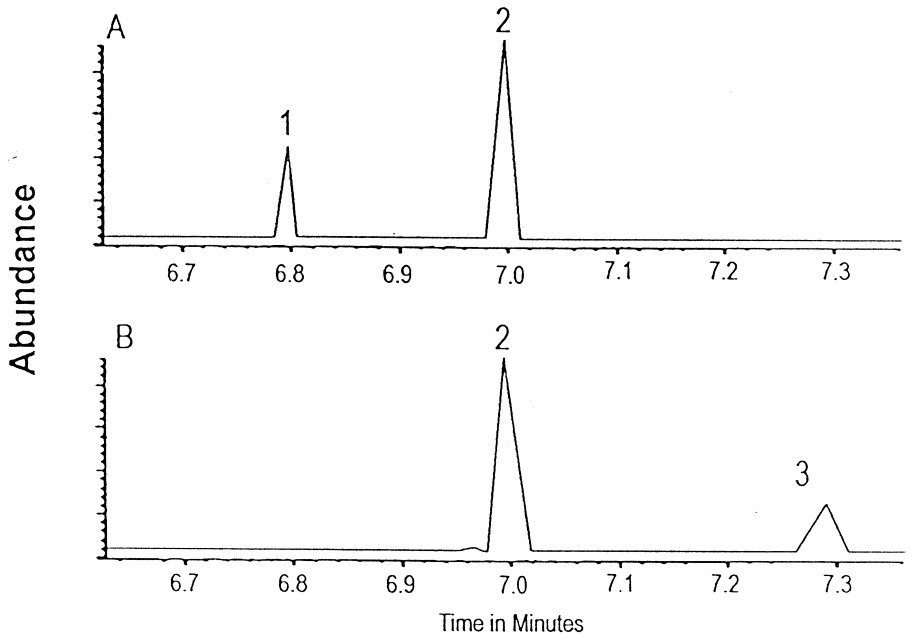

Fig. 3. The total ion chromatograms (m/z 220–650) generated by electron impact gas chromatography mass spectrometry of tert.-butyldimethylsilyltrifluoroacetamide derivatized. (A) GSH,free acid; and (B) oxidized GSH free acid, following hydrolysis into component amino acids. Peak 1 is cysteine, peak 2 is glutamic acid and peak 3 is cysteic acid.

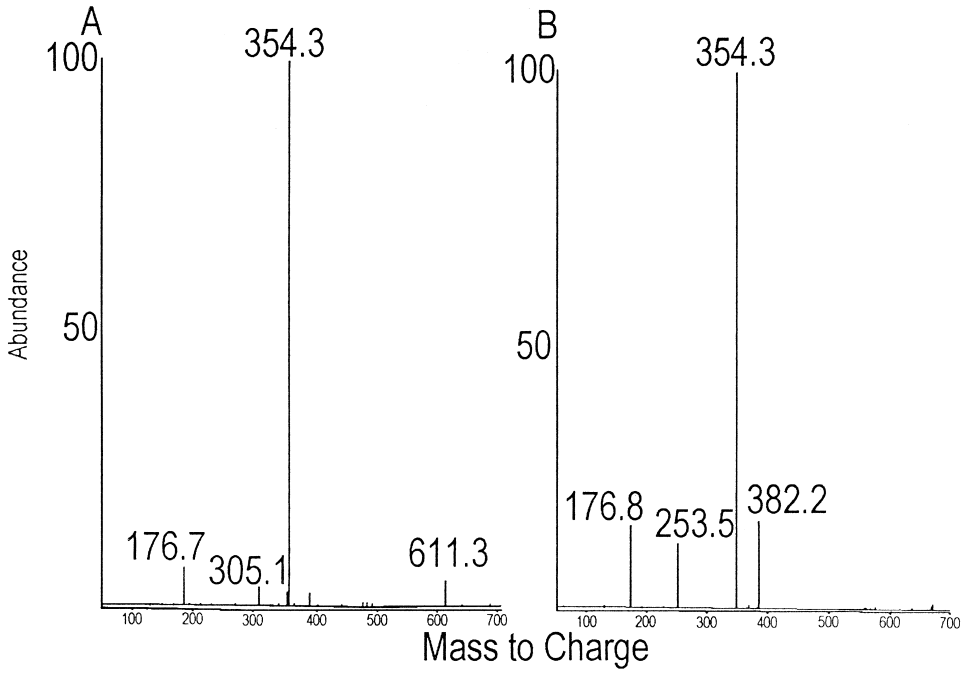

Fig. 4. The electrospray negative ion spectra (m/z 50–700) generated during infusions into an ion trap liquid chromatography mass spectrometer of: (A) GSH-sulfonic acid formed by incubating GSH with hydrogen peroxide; and (B) the same solution in Fig. 4A after incubation at 37°C in a 100-fold excess of BME, followed by concentration of BME by vacuum centrifugation at 60°C.

residual GSSG was converted to mixed GSH:BME disulfides (Fig. 4B).

3.4. Spectra of GSH and GSSG at neutral pH during exposure to reductants and oxidants

To this point, all reactions shown were carried out at the pH of the free acids of GSH and GSSG (pH 3.9±0.1). Reactants were then adjusted to neutral pH (7.2±0.1) through the careful addition of NaOH. Although no differences were apparent in the negative ion spectra of pH adjusted GSH and GSSG (not shown), the pH adjusted GSH behaved quite differently upon exposure to hydrogen peroxide. As shown above (Fig. 2), GSH at pH 3.9 was almost quantitatively converted to GSH-sulfonic acid after a 90 min incubation in a 200 fold excess of hydrogen peroxide. In contrast, at pH 7.2, GSH was rapidly oxidized to GSSG upon the addition of H_2O_2. Fig. 5A shows the negative ion spectrum when GSH, pH 7.2 was incubated in 200 fold molar excess of hydrogen peroxide for 2 min. The GSH ion (m/z 306) was already a minority species, while GSSG ions (m/z 305, 611) predominated. GSSG remained resistant to sulfonate formation at pH 7.2, with no appreciable ion at m/z 354 apparent after 4 h of incubation in a 200 fold excess of hydrogen peroxide (not shown), and only a small amount was apparent after 20 h of incubation (Fig. 5B).

4. Discussion

This report describes direct real time observations of oxidation/reduction reactions involving GSH (Fig. 6). The formation of disulfides, mixed disulfides, sulfinic acids and sulfonic acids of GSH are

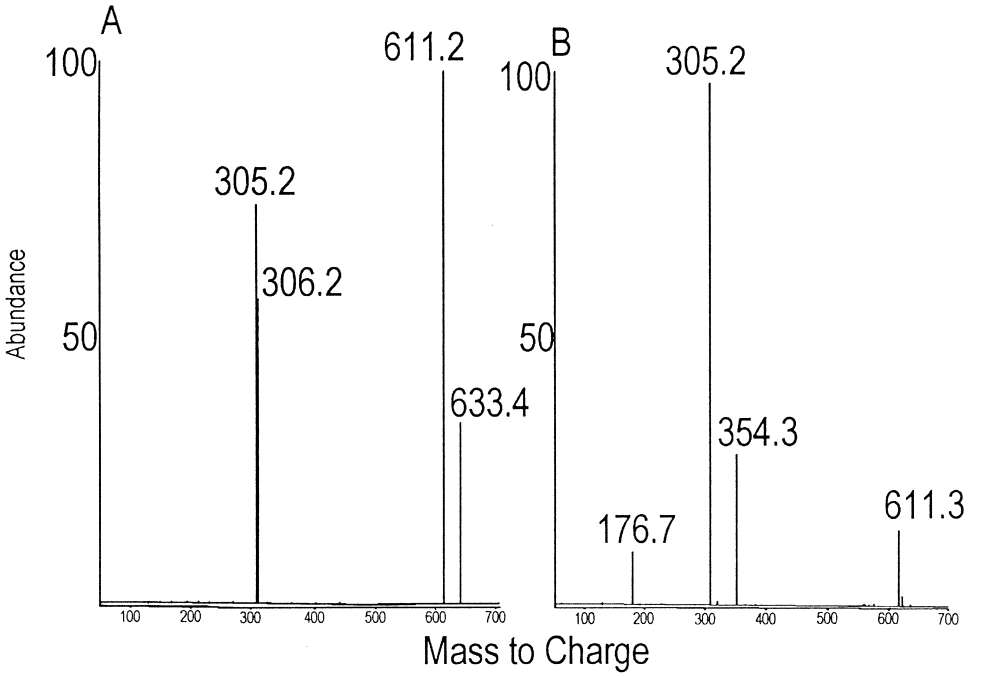

Fig. 5. The electrospray negative ion spectra (m/z 50–700) generated during infusions into an ion trap liquid chromatography mass spectrometer of (A) GSH which had been neutralized to pH 7.2 and incubated with hydrogen peroxide for 2 min; and, (B) GSSG which had been neutralized to pH 7.2 and then incubated with hydrogen peroxide for 20 h.

readily seen. The power of LC–MS includes the ability to observe the simultaneous formation of several species (for example mixed disulfides during reduction or sulfinic and sulfonic acid formation during oxidation).

As conditions are adjusted, one, therefore, has the ability to take relatively complex situations and compare qualitative and quantitative changes in specific reactants. This may help predict reaction outcomes in biological systems.

Our results differ somewhat from those of others [13] in which exposure of GSH to hypochlorous acid resulted in a compound with negative ion electrospray of m/z 336 and m/z 644 consistent with the formation of an internal sulfonamide and a thiol-sulfonate. In contrast, we observed the formation of a transient sulfinic acid, and a more stable sulfonic acid in hydrogen peroxide suggesting different mechanisms of oxidation.

Furthermore, the sulfinic acid was very unstable in hydrogen peroxide, being rapidly converted to the sulfonic acid. This is strong evidence of the antioxidant oxygen consuming properties of the GSH-sulfinic acid. This is an additional antioxidant property of GSH-containing solutions, much in parallel to what has been reported for ascorbic acid [14].

Our specific observations include the ease of irreversible thiol oxidation of GSH at low pH. Although not proven, it suggests other thiol containing peptides may be inactivated through similar mechanisms under similar conditions. LC–MS studies should be able to provide insight into this important and interesting area.

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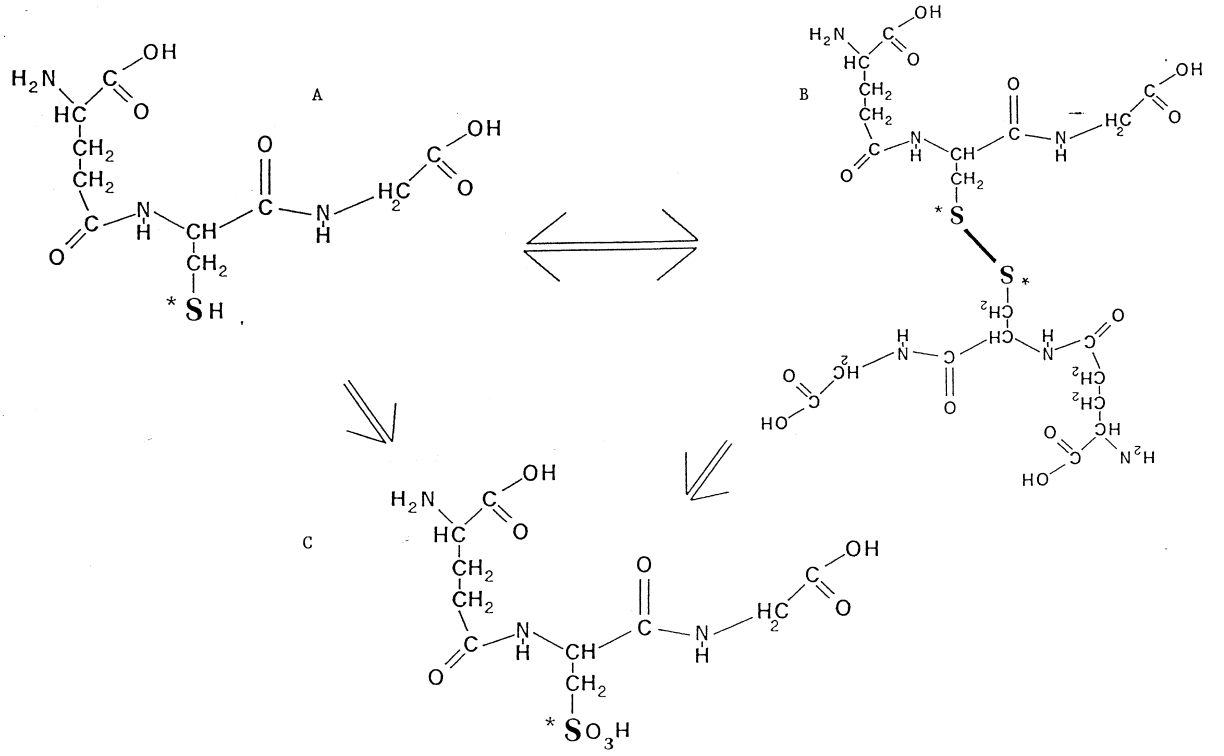

Fig. 6. The structure of (A) GSH, (B) GSSG, and (C) GSH-sulfonate. The arrows represent possible directions in which these compounds can be formed from each other.

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