Characterization of the oxidation products of the reaction between reduced glutathione and hypochlorous acid

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Reduced glutathione (GSH) is one of the most preferred biological substrates of myeloperoxidase-derived hypochlorous acid and is a likely target for neutrophil oxidants. We have used HPLC to show that the oxidation of GSH by hypochlorous acid gives two major, stable products in addition to glutathione disulphide (GSSG). The most prevalent product lacks free amine and thiol groups, and was shown by electrospray MS to have a molecular mass of 337 Da. This corresponds to GSH with a gain of two oxygen atoms and a loss of two hydrogen atoms, and is consistent with the product being an internal sulphonamide. The other novel product has a molecular mass of 644 Da, and has amine groups but no free thiols. These properties are consistent with it being glutathione thiolsulphonate. Whereas GSSG in the cell is recycled enzymically, formation of these higher oxidation products is likely to be irreversible. Hypochlorous acid, therefore, could compromise the cell by depleting GSH. The putative sulphonamide may be unique for oxidation by hypochlorous acid and thus provide a useful marker of neutrophil oxidant activity.

INTRODUCTION

Neutrophils and monocytes are a major physiological source of reactive oxygen species. These cells generate substantial amounts of the strong oxidant, hypochlorous acid (HOCl), via the myeloperoxidase-catalysed oxidation of chloride by hydrogen peroxide [1,2]. One of the fastest known reactions of HOCl is with thiols [3,4]. For reduced glutathione (GSH), this reaction has a rate constant of $\geqslant 10^7$ $M^{-1}\cdot s^{-1}$ [5], which is about two orders of magnitude greater than that for the reaction of HOCl with amines and comparable only with its reaction with thioethers such as methionine [3]. HOCl is able to penetrate cells and oxidize intracellular thiols [6–8]. As GSH is the major low-molecular-mass intracellular thiol, it will be a prime target for any HOCl generated in the body. It is important, therefore, to characterize the products of this reaction.

It was recognized a number of years ago that, when excess GSH is oxidized by HOCl, not all the HOCl consumed is accounted for by the formation of glutathione disulphide (GSSG) [3,9]. This contrasts with other thiols, such as cysteine, where there is almost stoichiometric conversion to the disulphide. These results suggest that further oxidation of GSH to products such as the sulphonic acid occurs. Prutz [10] showed recently that excess HOCl reacts with GSH with a 4:1 stoichiometry, and proposed the formation of the sulphonyl chloride and chloramine as final products. However, the oxidation products have not been identified directly, and the product distribution under the more physiological conditions of excess GSH over HOCl has not been established.

We have characterized the products of the reaction between GSH and HOCl using a combination of HPLC and electrospray MS. We show that two major products in addition to GSSG are formed, in amounts that vary depending on reaction conditions.

EXPERIMENTAL

HOCl (in equilibrium with hypochlorite; pK 7.5) was added with rapid mixing to GSH either in 50 mM phosphate buffer, pH 7.4 or 6.0, or in borate buffer, pH 8.5. Concentrations were varied as described for individual experiments. GSH concentrations were measured using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) [11], and hypochlorite solutions were standardized by diluting in pH 10 buffer and measuring A_{292} (ϵ 350 $M^{-1}\cdot cm^{-1}$). GSSG was measured by adding NADPH and glutathione reductase [11]. Amine concentrations were measured fluorimetrically with *o*-phthalaldehyde (excitation 340 nm; emission 455 nm) [12]. Calibration against glycine showed that the thiol groups of GSH did not interfere in the assay. In some experiments, amine groups were also measured with ninhydrin. Both methods gave the same results.

HPLC was performed on a 250 mm Nucleosil C18 5 μm column (Alltech Associates, Deerfield, IL, U.S.A.) using a Philips HPLC instrument with diode array detection. The solvent was 50 mM aqueous formic acid (2.4 ml/l) and the flow rate 0.8 ml/min. In some instances, fractions were collected for MS or for thiol or amine analysis.

Electrospray-ionization MS was performed in both the positive- and the negative-ion modes on a VG Platform II mass spectrometer. Samples were delivered at a flow rate of 10 μl/min in 50 % (v/v) acetonitrile with either 0.1 % ammonium hydroxide (negative ion) or 0.1 % formic acid (positive ion). The source temperature was 60 °C, and for negative ion analysis the probe voltage was −3000 V and the cone voltage was −70 V. Scans from 100 to 800 m/z were acquired every 2 s.

Sodium hypochlorite was obtained from Reckit and Colman (Auckland, New Zealand). Biochemicals were from Sigma (St. Louis, MO, U.S.A.).

RESULTS

Treatment of GSH with HOCl at pH 7.4 resulted in the loss of thiol groups and the formation of GSSG. The amount of GSSG formed did not account for all the GSH lost, and the ratio of thiol loss to HOCl added was less than the value of 2 expected for

Abbreviation used: DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.
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Table 1 Stoichiometry of product formation from HOCl and GSH

GSH was measured using DTNB, GSSG using NADPH and glutathione reductase, and amine groups using *o*-phthalaldehyde. GSSG concentrations are expressed as GSH equivalents. Results are means ± S.D. from 3–6 analyses.

		Changes in concn. (mol/mol of HOCl added)			
[GSH] (mM)	[HOCl] (mM)	GSH lost	GSSG formed	Amines lost	Amines lost/ GSSG formed (mole ratio)
pH 7.4					
6.0	1.0	1.36	0.96 ± 0.07	0.05 ± 0.19	0.06
6.0	2.0	1.19	0.87 ± 0.05	0.15 ± 0.08	0.18
6.0	3.0	1.18	0.80 ± 0.02	0.16 ± 0.08	0.21
6.0	4.0	1.10	0.77 ± 0.02	0.20 ± 0.05	0.26
6.0	5.0	1.07	0.75 ± 0.01	0.23 ± 0.04	0.31
0.6	0.3	1.14	0.85 ± 0.03	0.27 ± 0.08	0.32
1.2	0.6	1.20	0.91 ± 0.05	0.22 ± 0.11	0.24
6.0	3.0	1.18	0.80 ± 0.02	0.16 ± 0.08	0.21
12.0	6.0	1.13	0.77 ± 0.05	0.21 ± 0.08	0.27
pH 6.0					
6.0	3.0	0.87	0.49 ± 0.03	0.19 ± 0.08	0.39
pH 8.5					
6.0	3.0	1.54	1.25 ± 0.05	0.31 ± 0.08	0.25

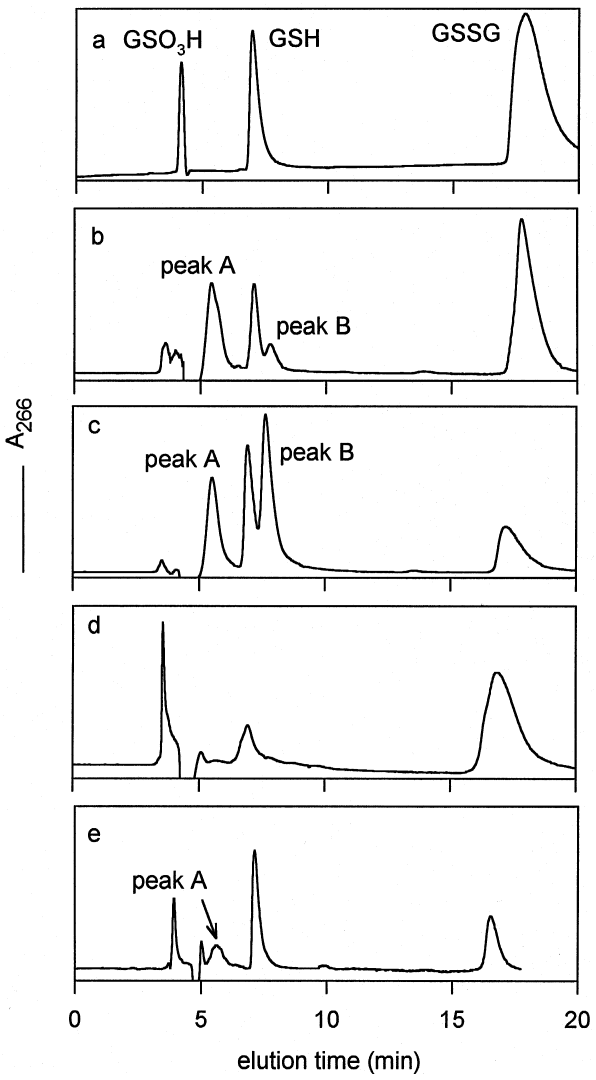


Figure 1 HPLC separation of the products of GSH oxidation by HOCl

(**a**) GSH, GSSG and glutathione sulphonic acid (GSO_3H) standards; (**b**) 10 mM GSH plus 6 mM HOCl at pH 7.4; (**c**) 10 mM GSH plus 6 mM HOCl at pH 6.0; (**d**) 10 mM GSH plus 6 mM HOCl at pH 8.5; (**e**) 0.5 mM GSH plus 0.1 mM HOCl at pH 7.4. HPLC traces were recorded at 226 nm under the conditions described in the Experimental section. The sample volume was 30 μl for (**b**)–(**d**) and 100 μl for (**e**). For (**e**), 0.5 μl of formic acid was added to minimize the trough at 4.5 min. The absorbance reference line corresponds to 0.05 unit for (**a**)–(**d**) and 0.02 unit for trace (**e**).

stoichiometric conversion into the disulphide (Table 1). The reaction was accompanied by the loss of amine groups, which accounted for most of the GSH that was not converted into GSSG. As shown in Table 1, increasing the HOCl concentration at constant GSH concentration gave a progressive decrease in the GSH lost per mol of HOCl added, and an increase in amine loss relative to GSSG formation. Varying the GSH concentration while keeping the HOCl/GSH mol ratio constant had little effect on the product distribution. At pH 6, less GSSG was formed than under the same conditions at pH 7.4, whereas at pH 8.5 more of the GSH was converted into GSSG.

The products were separated by HPLC and their mobilities compared with those of standards (Figure 1a). At pH 7.4 (Figure 1b), there was one product peak at 17 min, corresponding to GSSG, and two other new peaks at 5 and 8 min (A and B respectively). Only a small peak was evident at the position of the sulphonic acid standard. Under these conditions, λ_{max} for GSH, GSSG and peak A was 226 nm, whereas for peak B it was 232 nm. There was no difference between HPLC traces obtained immediately after mixing the reagents and after 24 h at room temperature, indicating that the products were formed rapidly and were stable. There was no oxygen requirement, as the same amounts of each product were produced from well aerated and nitrogen-bubbled solutions. Addition of 0.5 mol of HOCl to GSSG did not generate peak A or peak B. The yields of these peaks did not alter when GSSG was present at a 2-fold excess over GSH (results not shown). Treatment of 20 mM GSH with 8 mM hydrogen peroxide at pH 7.4 produced only GSSG, with no peak A or peak B being evident.

At pH 6.0 there was relatively more of peak B produced and less of GSSG (Figure 1c). At pH 8.5 GSSG was by far the major product, peaks A and B were scarcely detectable, and a minor product was eluted in the position of the sulphonic acid standard (Figure 1d). These traces agree with the data in Table 1 showing that the amount of GSH oxidized per mol of HOCl increases with increasing pH.

HPLC fractions from the reaction carried out at pH 7.4 were collected and analysed. As shown in Figure 2 (upper panel), only the GSH peak contained free thiol groups. No amine groups were detected in peak A, but a shoulder on the GSH peak suggested that amines were present in peak B. To confirm this, HPLC fractions were collected from the reaction mixture at pH 6.0, where peak B is more prevalent. As shown in the lower panel of Figure 2, the shoulder on the fluorescence trace that was not seen with the GSH standard indicates that amine groups are present in peak B. The lower ratio of amine fluorescence to A_{226} for this peak suggests that it has a higher molar absorption coefficient than GSH.

The peaks were collected and subjected to electrospray MS. GSH and GSSG gave the expected signals corresponding to $[M-H]$ at m/z 306 and 611 respectively in negative-ion mode,

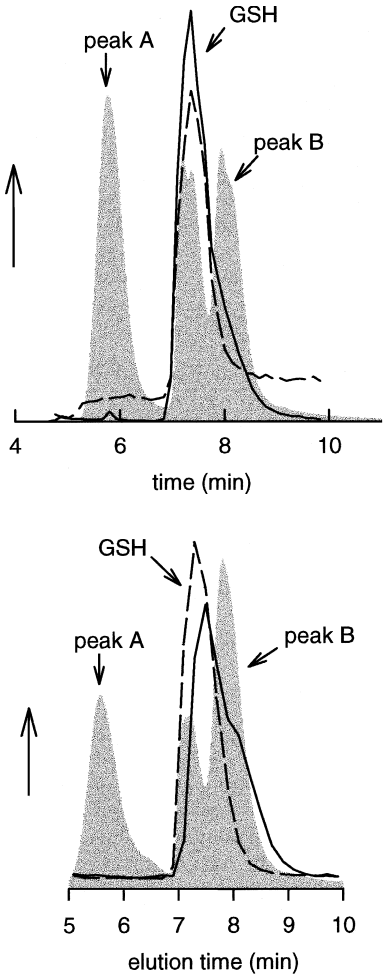


Figure 2 Amine and thiol content of 0.2 ml HPLC fractions collected after the reaction of GSH with HOCl

Upper panel: GSH (20 mM) plus 12 mM HOCl at pH 7.4 (25 μl injected). Shaded trace, A_{226}; broken line, thiol absorbance at 412 nm after dilution to 1 ml and reaction with DTNB; solid line, amine fluorescence after dilution to 1 ml and reaction with *o*-phthalaldehyde. Lower panel: GSH (20 mM) plus HOCl (16 mM) at pH 6.0 showing amine fluorescence (solid line) and A_{226} (shaded trace); the broken line shows the amine fluorescence for 12 mM GSH standard. The vertical arrow corresponds to an A_{226} of 0.04 unit, an A_{412} (thiols) of 0.2 unit or a relative fluorescence (amines) of 2000.

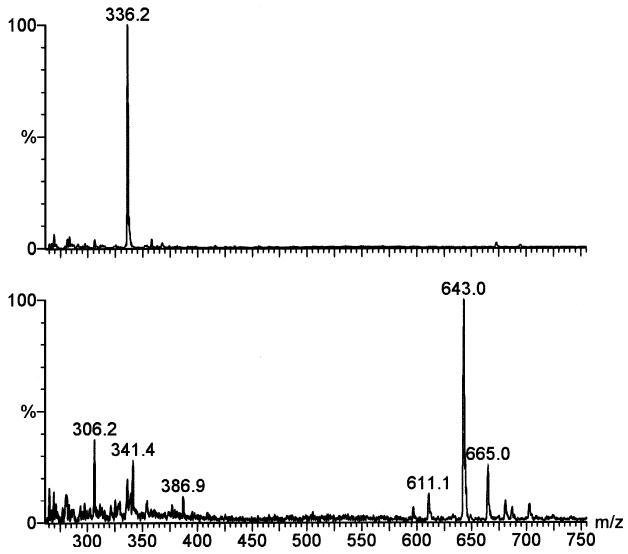


Figure 3 Negative-ion electrospray mass spectra of products of the reaction between GSH and HOCl

GSH (20 mM) was allowed to react with HOCl (16 mM) and samples were collected from the top of peak A (upper spectrum) and peak B (lower spectrum). Electrospray MS was carried out as described in the Experimental section.

Table 2 Variation in product distribution from the reaction of GSH with HOCl

Reactions were carried out at pH 7.4 and were analysed by HPLC with UV monitoring at 226 nm. Results are from one of several experiments, all of which gave similar concentration dependencies.

		Relative peak area		
[GSH] (mM)	[HOCl] (mM)	GSSG/GSH	Peak A/GSSG	Peak B/GSSG
10	1	0.24	0.15	–*
10	2	1.0	0.20	–*
10	4	2.2	0.23	0.036
10	6	5.2	0.29	0.048
10	8	58	0.33	0.056
1	0.6	3.6	0.3	–*
5	3	5.9	0.27	0.024
10	6	5.2	0.29	0.048
20	12	4.8	0.30	0.076

* No distinct peak B.

and at 2 units higher in positive-ion mode. Peak A gave a major signal at m/z 336 in negative-ion mode (Figure 3) and at m/z 338 in positive-ion mode (results not shown), corresponding to GSH with a gain of two oxygen atoms and a loss of two hydrogen atoms. Peak B in negative-ion mode gave a major $[M-H]$ ion at m/z 643 and a smaller signal at m/z 306, corresponding to the $[M-H]$ ion for GSH (Figure 3). The presence of some contaminating GSH is not surprising considering the incomplete resolution of these two peaks (Figure 2, lower panel). Equivalent peaks at 2 mass units higher plus their corresponding Na adducts were seen in the positive-ion mode (results not shown). The mass of 644 units corresponds to GSSG plus two oxygen atoms. Although the breakthrough peak at pH 8.5 co-eluted with the sulphonic acid standard, no definitive mass spectrum for it or any other compound could be identified in this fraction.

Variation in the relative proportions of the products as a function of GSH concentration and of the HOCl/GSH mole ratio was investigated. The relative peak areas (Table 2) reflect changes in product distribution, but because standards were not available for peaks A and B, these cannot be equated directly with concentrations. There was progressively greater GSH loss and gain in GSSG with increasing concentrations of HOCl added to 10 mM GSH, as shown by an increase in the GSSG/GSH ratio. The amount of peak A formed relative to GSSG increased. This is consistent with the greater amine loss at higher HOCl concentrations shown in Table 1. Increasing the GSH concentration while keeping the HOCl/GSH mole ratio constant at 0.6 increased the proportion of GSH converted into peak B relative to GSSG, with little effect on peak A. This investigation was mostly carried out with greater than millimolar GSH and HOCl concentrations to overcome the relative insensitivity of

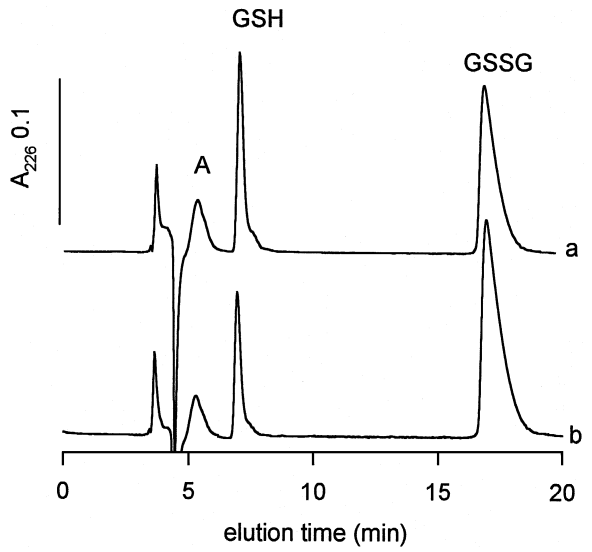


Figure 4 HPLC separation after reaction of 10 mM GSH with 4 mM HOCl in the absence (a) or presence (b) of 20 mM glycine, at pH 7.4

HPLC conditions were as for Figure 1. No distinct peak B is evident with these reactant concentrations.

Table 3 Effects of taurine, lysine and glycine on the oxidation products from GSH and HOCl

GSH (5 mM) was allowed to react with HOCl (2 mM) at pH 7.4 and the products were separated by HPLC. Peak areas were measured at 226 nm. The amino compounds were present at 20 mM. Results are means of duplicate experiments, for which the values for relative areas varied by less than 2. Note that, under these conditions, peak B was only a small shoulder on the GSH peak (see Figure 4).

	Relative peak area		
Amino compound added	Peak A	GSSG	GSH
None	18	33	48
Taurine	8.4	76	15.6
Lysine	10.5	79	10.6
Glycine	9.0	86	7.8

UV detection of underivatized glutathione. However, peak A was also detectable at the more physiologically relevant HOCl concentration of 100 μM (Figure 1e).

Our evidence suggests that one of the products of the reaction of HOCl with GSH is formed through an intramolecular reaction between the amine group and the oxidized thiol. Therefore we investigated whether equivalent intermolecular products are formed with other amines. However, no new product peaks were evident when taurine, glycine or lysine was present during the reaction. Instead, each gave concentration-dependent enhancement of GSSG formation at the expense of peaks A and B, and a greater loss of GSH for an equivalent amount of HOCl added (as shown in Figure 4 for a 2-fold excess of glycine). The three amino compounds had comparable reactivity in causing these changes (Table 3), although glutamic acid (not shown) was considerably less effective, and at 20 mM caused only a small change in product distribution.

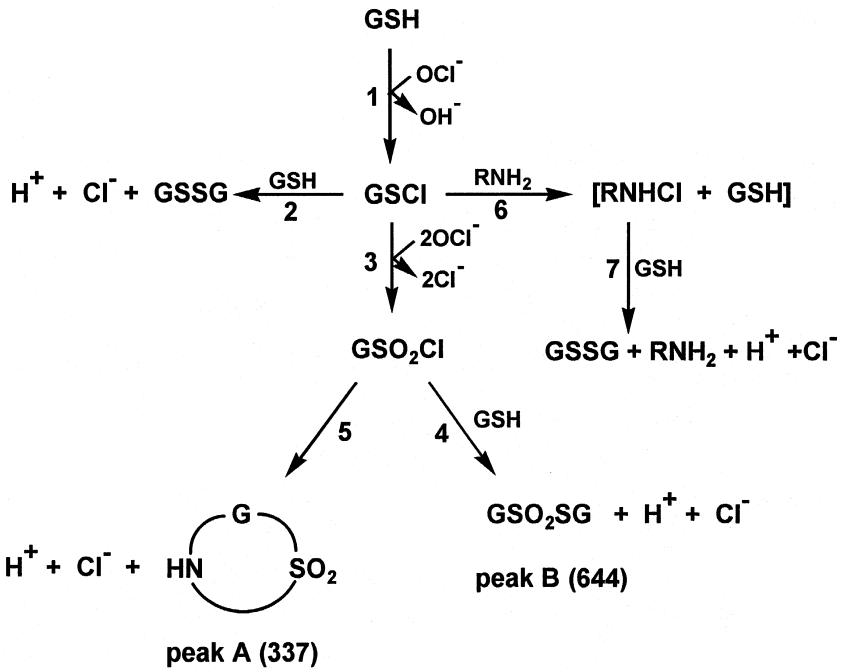
DISCUSSION

GSH is an important biological antioxidant that is likely to be a prime target for HOCl generated by myeloperoxidase. We have shown that the reaction of GSH with HOCl generates higher oxidation products of GSH, in addition to GSSG. Formation of such products is implied from the results of previous studies [3,9], and formation of the sulphonic acid and sulphonyl chloride has been proposed [5,10]. However, these were found not to be significant end-products. Instead there were two major products, with masses of 337 and 644 Da. The reaction was studied mainly at high GSH and HOCl concentrations because of ease of detection, but our results indicate that they are likely to be relevant at the HOCl concentrations generated physiologically. The 337 Da product was observed with 100 μM HOCl and, from the concentration trends shown in Table 2, we expect that detection at lower concentrations is limited only by the insensitivity of the analytical method.

The 337 Da product lacked free amino and thiol groups. These properties, plus the gain of 30 mass units over GSH, are all consistent with it being an internal sulphonamide. Formation of sulphonamides by elimination of HCl from sulphonyl chlorides and amines is a well known reaction [13] (as in the dansylation of proteins), and would produce a compound of the requisite molecular mass. Such products tend to be unstable and to be hydrolysed, whereas ours was unchanged over days, raising the possibility that it rearranges rapidly to a more stable structure. Straight HCl elimination would give a nine-membered ring, also suggesting that an initial sulphonamide may not be the stable end-product. The product with a mass of 644 Da, which contained free amine but not thiol groups, is likely to be the thiolsulphonate of glutathione (GSO_2SG). This compound is produced when GSSG is oxidized by performic acid [14,15].

The formation of these products, plus the observed concentration-dependences of the product distribution, can be explained by the mechanism shown in Scheme 1. The first step (reaction 1) produces the sulphenyl chloride, as proposed by others [16]. It is likely to be the fast reaction for which Folkes et al. [5] measured the rate constant. The sulphenyl chloride will react readily with more GSH to give GSSG (reaction 2). To explain the presence of higher oxidation products, this must occur in competition with further oxidation by HOCl (reaction 3) to give the sulphonyl chloride. Reaction 4 represents formation of the thiolsulphonate (peak B), and reaction 5 an internal HCl elimination to give the putative sulphonamide (peak A). Reaction 4, being bimolecular in GSH, will be favoured over reaction 5 at high GSH concentrations, and this can explain why such conditions gave more of peak B. The observed increase in peak A relative to GSSG with increasing HOCl/GSH ratios is predicted by this mechanism as, at the higher ratios, reaction 3 would compete better with reaction 2. Although peak B could theoretically arise via oxidation of GSSG [14,15], this was not observed at the HOCl concentrations we used, probably because there was preferential oxidation of the amine groups to give chloramines. The different product profiles at pH 6 and 8.5 can largely be explained by an increase in the rate of reaction 2 with increasing pH, and an alternative breakdown pathway for the sulphonyl chloride at the higher pH. Hydrolysis to the sulphonic acid would be likely [13], although we could not confirm this by MS. We found no evidence of sulphonic acid formation via hydrolysis of the sulphenyl chloride, as proposed by Silverstein and Hager [16], as this requires oxygen and there was no oxygen-dependence in our system.

Formation of peak A appears to involve an intramolecular reaction of the amine and thiol groups of GSH. No equivalent



Scheme 1 Proposed scheme for the reaction of GSH with HOCl

Abbreviations: GSCl, glutathione sulphenyl chloride; GSO_2Cl, glutathione sulphonyl chloride; GSO_2OH, glutathione sulphonic acid; GSO_2SG, glutathione thiolsulphonate; RNH_2 and RNHCl, exogenous amine and its corresponding chloramine. As the products of reaction 7 react rapidly with each other, the chloramine is likely to be formed only as a transient species.

reaction with external amines was observed, suggesting that the structure of GSH facilitates the internal reaction. In the presence of amines, the proportion of GSH converted into peak A was decreased. This might be explained by the HOCl reacting directly with the amine in preference to GSH, to give a chloramine that then oxidizes the thiol exclusively to GSSG. However, amines react about 100 times more slowly than GSH with HOCl [3,5], so, with only a 2-fold excess, chloramine formation will be minor. It is much more likely that the amine competes for the sulphenyl chloride (Scheme 1, reaction 6), with the chloramine formed then oxidizing GSH to the disulphide (reaction 7).

The ability of HOCl to generate higher oxidation products of GSH has implications for how well GSH could protect cells against oxidative injury caused by neutrophils or monocytes. GSH will function best as an antioxidant when it is oxidized to GSSG and recycled by glutathione reductase. The formation of higher oxidation products is likely to be irreversible, leading to depletion of GSH that can only be replaced by new synthesis. HOCl, therefore, could compromise the cell far more than oxidants such as hydrogen peroxide that yield only GSSG. Another interesting possibility is that the higher oxidation products have biological activity. Glutathione thiolsulphonate, for example, can cause irreversible inhibition of placental $NADP^+$-linked prostaglandin reductase/dehydrogenase [17].

The 337 Da derivative of GSH has not been described previously. Its formation with HOCl, but not hydrogen peroxide, plus the proposed reaction mechanism involving a sulphonyl chloride, suggest that it may be unique for HOCl. Since GSH is likely to be a prime target for HOCl, this compound may provide a specific biological marker for HOCl production and a valuable probe for assessing the contribution of neutrophil oxidants to inflammatory injury.

The physiological or diagnostic relevance of these novel glutathione oxidation products depends on their being formed in cells or biological media exposed to HOCl. At the millimolar GSH concentrations that exist in most cells, the 337 Da compound is the more likely of the two to be formed in appreciable amounts. However, it is possible that the reactions that we observed with amino compounds could suppress its formation in more complex biological systems. Direct identification in cell systems is not possible using our current detection method because of insensitivity, and the 337 Da product would not be detected by standard assays for glutathione derivatives that rely on free thiol or amine groups. However, we have preliminary evidence, obtained with neutrophils in which GSH was prelabelled with ^{35}S, that the reactions that we have observed are physiological [18]. Treatment of the cells with reagent hypochlorite, or stimulation with PMA, generated a labelled product that migrated on HPLC in the same position as peak A. We are currently developing a more sensitive HPLC assay to confirm the identity of this product directly.

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