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# Hypochlorite-Induced Oxidation of Thiols: Formation of Thiyl Radicals and the Role of Sulfenyl Chlorides as Intermediates

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Activated phagocytic cells generate hypochlorite (HOCl) via release of hydrogen peroxide and the enzyme myeloperoxidase. HOCl plays an important role in bacterial cell killing, but excessive or misplaced production of HOCl is also known to cause tissue damage. Studies have shown that low-molecular-weight thiols such as reduced glutathione (GSH), and sulfur-containing amino acids in proteins, are major targets for HOCl. Radicals have not generally been implicated as intermediates in thiol oxidation by HOCl, though there is considerable literature evidence for the involvement of radicals in the metal ion-, thermal- or UV light-catalysed decomposition of sulfenyl or sulfonyl chlorides which are postulated intermediates in thiol oxidation. In this study we show that thiyl radicals are generated on reaction of a number of low-molecular-weight thiols with HOCl. With sub-stoichiometric amounts of HOCl, relative to the thiol, thiyl radicals are the major species detected by EPR spin trapping. When the HOCl is present in excess over the thiol, additional radicals are detected with compounds which contain amine functions; these additional radicals are assigned to nitrogen-centered species. Evidence is presented for the involvement of sulfenyl chlorides (RSCl) in the formation of these radicals, and studies with an authentic sulfenyl chloride have demonstrated that this compound readily decomposes in thermal-, metal-ion- or light-catalysed reactions to give thiyl radicals. The formation of thiyl radicals on oxidation of thiols with HOCl appears to compete with non-radical reactions. The circumstances under which radical formation may be important are discussed.

*Keywords:* Hypochlorite; EPR; thiols; spin trapping; radicals; myeloperoxidase

## INTRODUCTION

The respiratory burst of activated phagocytic cells both *in vitro* and *in vivo* results in the generation of $O_2^{-}\cdot$ and $H_2O_2$ and the release of the enzyme myeloperoxidase.[1] This enzyme catalyses the reaction of $H_2O_2$ with $Cl^-$ to give the potent oxidant HOCl.[2] This species is in equilibrium with its anion $^-OCl$ at physiological pH values as a result of its pKa of 7.5;[3] HOCl is used below to indicate the physiological mixture of these two species. HOCl plays an important role in bacterial cell killing,[4] but excessive or

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misplaced production of HOCl is also known to cause tissue damage. This is believed to be important in a number of diseases including atherosclerosis, inflammatory conditions and some cancers,[5] with, for example, elevated levels of HOCl-modified proteins being found in human atherosclerotic plaques and sites of acute inflammation (e.g. [6–9]; reviewed in [10,11]).

*In vitro* studies have shown that low-molecular-weight thiols, such as reduced glutathione (GSH), can be major targets for HOCl.[12–15] Thus reaction of HOCl with GSH has been shown to give rise to the disulfide (GSSG) together with a number of other products at higher oxidation states; these include the sulfinic acid ($GSO_2H$),[16] sulfonic acid ($GSO_3H$),[16] glutathione thiolsulfonate ($GSO_2SG$),[17] and an internal sulfonamide.[17] There are, at present, conflicting reports in the literature as to the quantitative importance of each of these materials (see, e.g.,[16, 17]). The overall stoichiometry of GSH oxidation is however, well established with 4 molecules of HOCl consumed per molecule of GSH fully oxidised.[4,17–19] This figure has been suggested to arise via reaction of 3 molecules of HOCl with the thiol function, whilst the last oxidises the α-amino group possibly to a chloramine (RNHCl).[17, 19, 20] Oxidation of free Cys has been reported to yield the disulfide, cystine, in almost stoichiometric amounts,[12] though evidence has also been presented for the formation of significant yields of cysteic acid ($RSO_3H$).[21, 22]

Sulfur-containing amino acids in proteins are readily modified by HOCl.[23–25] Thus Cys residues react rapidly with HOCl to give oxyacids and cystine,[16, 21] whereas Met residues are oxidised to the sulfoxide.[26] These reactions can result in enzymatic inactivation (e.g. of dihydrolipoamide dehydrogenase[27] and $\alpha_1$-proteinase inhibitor[28, 29]). Other amino acid residues are also targets for HOCl, with Tyr residues undergoing ring chlorination to give 3-chlorotyrosine and 3,5-dichlorotyrosine in relatively low yields (see, e.g.[9, 30]) and Lys residues being readily converted to semi-stable chloramine (RNHCl) intermediates.[31] Trp, Arg and His residues also undergo oxidation, though the products of these reactions are less well characterised (reviewed in[32]). The extent of reaction of HOCl with these different species depends on the relative molar ratios of HOCl to protein, and the abundance of the different residues within the protein, though it well established that reaction is fastest with the sulfur-containing amino acids.[12, 22]

The mechanisms by which the products generated from GSH, Cys and protein thiols are formed are incompletely understood. In particular, there is uncertainty as to the role, and significance, of sulfenyl and sulfonyl chlorides as intermediates in these processes[17, 19, 22, 33, 34] and which pathways operate under different reaction conditions. This is due, at least in part, to the known instability of these materials in aqueous solution.[22, 33] One possible reason for the different product profiles obtained in various studies could be the availability of different reaction channels for intermediate species, such as sulfenyl or sulfonyl chlorides, depending on their accessibility (e.g. free solution vs within a protein matrix), and presence of other reactive agents such as excess thiol, water molecules and metal ions. Thus it has been suggested that sulfenyl or sulfonyl chlorides can undergo further reaction with excess thiol, with amine functions, water molecules, and metal ions (e.g.[17, 19, 33–36]). Radicals have not generally been implicated as intermediates in thiol oxidation by HOCl, though there is considerable evidence in the chemical literature for the involvement of radicals in the metal ion-, thermal- or UV light-catalysed decomposition of sulfenyl or sulfonyl chlorides due to the relatively weak nature of the S-Cl bond (see, e.g.,[37] and references therein). We have therefore examined the potential generation of thiyl ($RS^{\cdot}$) and other radicals during the reaction of HOCl with a range of thiols by use of EPR spectroscopy with spin trapping, and provide evidence for the generation of such species under certain circumstances.

## MATERIALS AND METHODS

### Materials

The water used was filtered through a four-stage Milli Q system (Millipore-Waters, Lane Cove, NSW, Australia) equipped with a 0.2 μm pore-size final filter. pH control was achieved by the use of Chelex 100-treated 100 mM phosphate buffer, pH 7.4. All compounds were commercial materials of analytical reagent grade obtained from Aldrich or Sigma (Castle Hill, NSW, Australia) and used as supplied, with the exception of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) which was purified before use by treatment with activated charcoal. HOCl solutions were prepared immediately prior to use by dilution of a concentrated stock solution [ca. 1 M in 0.1 M NaOH (BDH, Poole, Dorset, U.K.)] into phosphate buffer (as above). HOCl concentrations were determined from the absorbance (at 292 nm) of $^{-}$OCl at pH 11.[3]

### Oxidation Conditions

Oxidations were carried out by addition of HOCl to fresh solutions of the substrate at either 21 or 4 °C, with the samples subsequently incubated at 4, 21, or 37 °C for varying periods. The decomposition of $CCl_3SCl$ was investigated by the addition of freshly-prepared saturated aqueous solutions of this compound, or neat substrate, to solutions of the spin trap DMPO unless stated otherwise in the text. Radical formation was subsequently investigated by incubation of the samples for various periods as described in the text.

### EPR Spectroscopy

EPR spectra were recorded at room temperature using a Bruker EMX X-band spectrometer with 100 kHz modulation and a cylindrical ER 4103TM cavity. Samples were contained in a flattened, aqueous-sample cell (WG-813-SQ, Wilmad, Buena, NJ, U.S.A.) and recording of the spectra was initiated within 90 s of the start of the reaction, except where specified otherwise. Hyperfine couplings were measured directly from the field scan and confirmed by spectral simulation using the program WINSIM.[38] The correlation coefficients between simulated and experimental data were > 0.90. Typical EPR spectrometer settings were: gain $1 \times 10^6$, modulation amplitude 0.1 mT, time constant 0.16 s, scan time 84 s, resolution 1024 points, centre field 348 mT, field scan 10 mT, power 25 mW, frequency 9.76 GHz, with 4 scans averaged. UV photolysis experiments were carried out by exposure of samples contained in a standard (suprasil quartz) aqueous EPR solution cell to light from a 100 W mercury/xenon source for fixed periods before examination by EPR spectroscopy. Visible light photolysis experiments employed a standard tungsten filament light source.

## RESULTS

Electron paramagnetic resonance (EPR) spectroscopy with spin trapping was employed in these studies to detect thiyl radical formation, as direct detection of thiyl radicals in aqueous solution by EPR is difficult as a result of the large g anisotropy of these species,[39] and their rapid reaction with a variety of other molecules including excess thiol anion and $O_2$.[40] DMPO was employed as the spin trap as it has been previously shown that this trap reacts rapidly with thiyl radicals to give detectable adducts (see, e.g.,[41]). The reaction of thiols with HOCl was studied under three different condition regimes: i) sub-stoichiometric, ii) stoichiometric and iii) with the HOCl in excess over the thiol concentration. Incubations were carried out either by the addition of HOCl to buffered solutions which contained both the thiol and the spin trap DMPO, or by adding the spin trap immediately, or at defined time periods, after the addition of the HOCl to the thiol.

TABLE I EPR parameters of thiyl- ($RS^{\cdot}$), nitrogen- and carbon-centred radical adducts observed on reaction of HOCl with thiol compounds using DMPO as a spin trap

| *Substrate* | *Radical assignment* | *Hyperfine coupling constants / mT*[a] | | |
|---|---|---|---|---|
| | | *a(N)* | *a(H)* | *a(other)* |
| Cysteine | Thiyl | 1.54 | 1.75 | |
| | Nitrogen-centred | 1.57 | 1.93 | 0.17 (1N) |
| | Carbon-centred[b] | 1.60 | 2.35 | |
| Cystine | Nitrogen-centred | 1.57 | 1.94 | 0.16 (1N) |
| Homocysteine | Thiyl | 1.53 | 1.68 | |
| | Carbon-centred | 1.57 | 2.30 | |
| N-acetyl-cysteine | Thiyl | 1.52 | 1.67 | |
| | Nitrogen-centred | 1.57 | 1.94 | 0.17 (1N) |
| | Carbon-centred[b] | 1.59 | 2.28 | |
| Cysteamine | Thiyl | 1.49 | 1.74 | |
| | Nitrogen-centred | 1.46 | 1.78 | 0.28 (1N) |
| Glutathione | Thiyl | 1.58 | 1.62 | |
| | Nitrogen-centred | 1.57 | 1.93 | 0.16 (1N) |
| | Carbon-centred[b] | 1.57 | 2.38 | |
| 3-Mercaptopropionic acid | Thiyl | 1.53 | 1.70 | |
| 2-Mercaptoethane sulfonic acid | Hydroxyl | 1.49 | 1.49 | |
| $Cl_3CSCl$ | Thiyl | 1.45 | 1.62 | |
| | Carbon-centred[b] | 1.49 | 2.10 | |

a. ± 0.01 mT.
b. Minor species.

In all cases where HOCl was added to pre-mixed solutions of the thiol and spin trap, EPR signals were detected (Figure 1). When the thiol compound was present in excess, the major initial signal detected with GSH, Cys, N-Ac-Cysteine, homo-Cysteine ($^{+}NH_3CH(CH_2\ CH_2SH)COO^{-}$), cysteamine ($HSCH_2CH_2{}^{+}NH_3$), and 3-mercapto-propionic acid ($HSCH_2CH_2COO^{-}$), has been assigned in each case to the corresponding thiyl radical adduct to DMPO on the basis of the hyperfine coupling constants of the signals and comparison with previously reported values;[41] these data are collected in Table I. These thiyl radical adducts are, in general, short lived (half-lives of the order of a few minutes) and rapid scanning of the magnetic field was required for the detection of some of these species. In the case of 2-mercaptoethane sulfonic acid ($HSCH_2CH_2SO_3^{-}$) the major signal present has been assigned to DMPO-OH (see Table I), and is assigned to this spacies. The authentic thiyl radical adduct from this substrate has (a(N) ≃ a (H) 1.53 mT). Weak signals assigned to carbon-centred adducts were also detected with a number of the thiols (see Table I); these species may arise from subsequent (hydrogen abstraction) reactions of the initial thiyl radicals. When longer time scans were employed, or where multiple accumulations were carried out, the thiyl radical adduct signal diminished in intensity

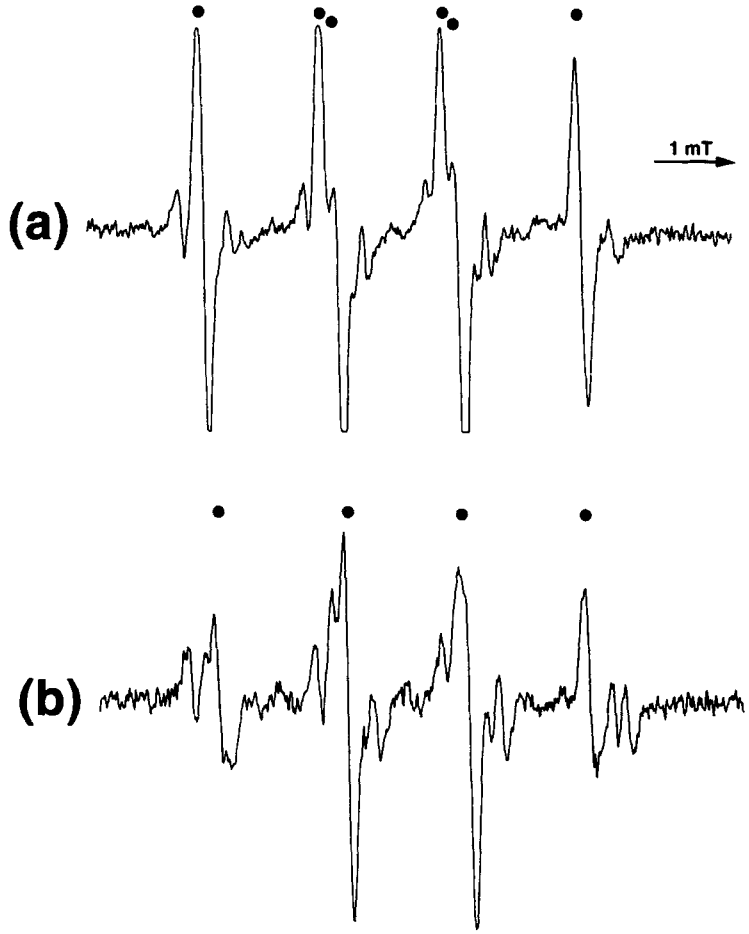


FIGURE 1 EPR spectra observed 2 min after reaction of (a) N-acetyl-cysteine (6 mM) and (b) GSH (3.75 mM) with HOCl (3 mM) in the presence of DMPO (125 mM) at pH 7.4. The features marked [•] are assigned to thiol-derived radical adducts. The other features in the spectra are attributed to the formation of nitrogen-centred radical adducts (see Fig. 2)

and a further signal assigned to DMPO-OH was detected; the mechanism of origin of the latter species was not investigated further, though it may be generated as a result of the decay of the thiyl species. Replacement of some of the thiol compounds with their corresponding disulfide analogous (e.g. cystine) resulted in the loss of the thiyl radical signals.

The detection of all of these signals was dependent on the presence of the spin trap, and the time at which this was added compared to the HOCl. In the complete absence of the trap, no signals were detected. When the spin trap was added 30 s after the HOCl was mixed with the thiol, weak, but distinct, adduct signals were still detected in most cases. With increasing time periods between the addition of HOCl and DMPO the intensity of the thiyl adduct signals decreased. With time delays of > ca. 90 seconds only control level of adducts were observed. In the absence of the thiol, strong signals were detected from a direct reaction of HOCl with the trap as reported previously;[43] the parameters of this chlorimine species are very different to all

the other adducts detected. In contrast, omission of the HOCl resulted either in a complete lack of EPR-active species, or the detection of very weak signals assigned to thiyl radical adducts arising from auto-oxidation of the thiol. The intensity of these signals, and hence extent of thiol auto-oxidation, was found in preliminary experiments to be dependent on the presence of trace transition metal ions in the reaction solutions. Use of buffers which had been treated extensively with Chelex-100 resin minimised the occurrence of such processes, and such treatment was employed in all the studies reported here. Addition of low concentrations of $Fe^{2+}$ ions (250 μM) or exposure to UV light (120 s) enhanced the intensity of the thiyl radical signals obtained on treatment of GSH with HOCl. Though such treatments also increased the intensity of the signals observed in the absence of added HOCl, this enhancement was much more marked in the presence of HOCl, suggesting the formation of an intermediate species whose degradation is catalysed by these treatments.

When high ratios of HOCl to thiol were employed (i.e. with HOCl in excess) somewhat different behaviour was observed. Though the thiyl radical adduct was still detected with all the substrates, further radical adduct signals were also detected with GSH, Cys, N-Ac-Cys, and cysteamine. These additional adducts were found to build-up with increasing time after the addition of HOCl, but the formation of these species does not appear to be linked to the decay of the thiyl radical adduct signal. Furthermore, the concentration of these species was found to increase with higher excesses of HOCl, suggesting that the radicals that give rise to these adducts are formed as a result of HOCl reaction with the substrate at additional sites to those that react with sub-stoichiometric concentrations of HOCl. Thus with GSH, Cys, N-Ac-Cys, and cysteamine signals which have been assigned to nitrogen-centred radical adducts were detected (Figure 2). The signals observed with Cys were also detected with cystine confirming that these signals arise from reaction at sites other than the thiol function (Figure 2). The identity of these additional species and their hyperfine coupling constants are given in Table I; the assignment of these species has been made by comparison with data from other systems.[44, 45]

In order to investigate the role of pre-formed sulfenyl chlorides (RSCl) in the formation of the thiyl radicals detected in the above experiments, the decomposition of a model compound $CCl_3SCl$ was investigated under identical conditions. Incubation of this compound with DMPO (125 mM) at either room temperature or 37 °C resulted in the detection of radical adduct signals which have been assigned to the corresponding thiyl radical adduct (i.e. the DMPO adduct of $CCl_3S^{\cdot}$) on the basis of the short life-time and hyperfine coupling constants of this signal (see Table I) and their similarity to other thiyl radical adducts (Figure 3).[41] The decay of this radical adduct species was rapid ($t_{1/2}$ < ca. 10 minutes) when low concentrations of the substrate were employed; this presumably arises from a rapid rate of decay of the radical adduct, compared to its rate of formation, coupled with depletion of the substrate. At longer incubation times further weak signals from DMPO-OH and a species assigned to a carbon-centred radical were also detected (see Table I). Omission of either the spin trap or $CCl_3SCl$ from these incubations resulted in the loss of these signals. In contrast when similar experiments were carried out at 4 °C, only weak signals were detected. Pre-incubation of $CCl_3SCl$ in aqueous buffer for 25 mins prior to the addition of the spin trap resulted in a marked decrease in the intensity of the thiyl radical adduct and an increase on the intensity of the carbon-centred species. Exposure of mixtures of freshly-prepared solutions of $CCl_3SCl$ and DMPO to UV or visible light for short periods (< 30 s) at 21°C resulted in dramatic increases in the intensity of the signal from the thiyl radical adduct, but no further species were observed. These results are consistent with the rapid ther-

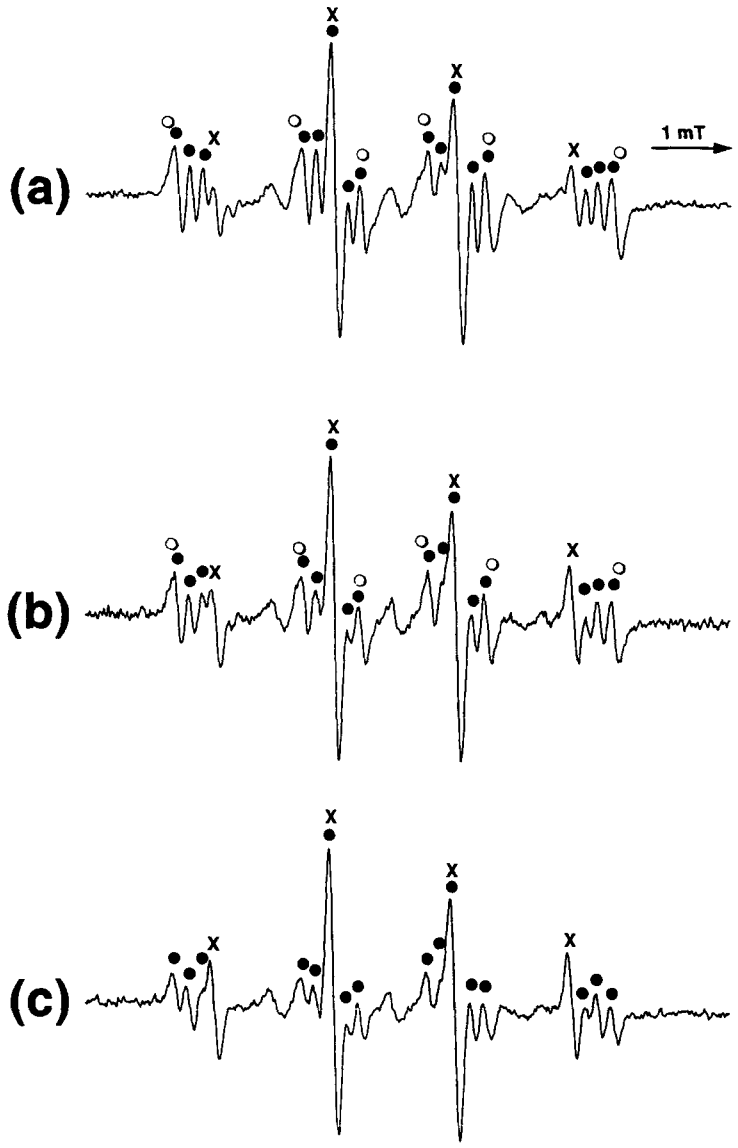


FIGURE 2 EPR spectra observed on reaction of (a) N-acetyl-cysteine (6 mM), (b) GSH (3.75 mM) and (c) cystine (saturated solution) with HOCl [3 mM in (a) and (b); 0.6 mM in (c)] in the presence of DMPO (125 mM) at pH 7.4 after incubation at 20 °C for (a) 30 min, (b) 10 min and (c) 20 min. The signals marked [•] are attributed to the formation of nitrogen-centred radical adducts. The signals marked [○] are assigned to a carbon-centred radical. Features marked [x] are assigned to the well-characterised DMPO-OH adduct

mal and photolytic decomposition of the sulfenyl chloride to thiyl radicals via homolysis of the (weak) S-Cl bond. The formation of these thiyl radicals appears to compete with other reactions which deplete the substrate via non-radical generating pathways; the latter are probably hydrolysis reactions, though this has not been investigated further.

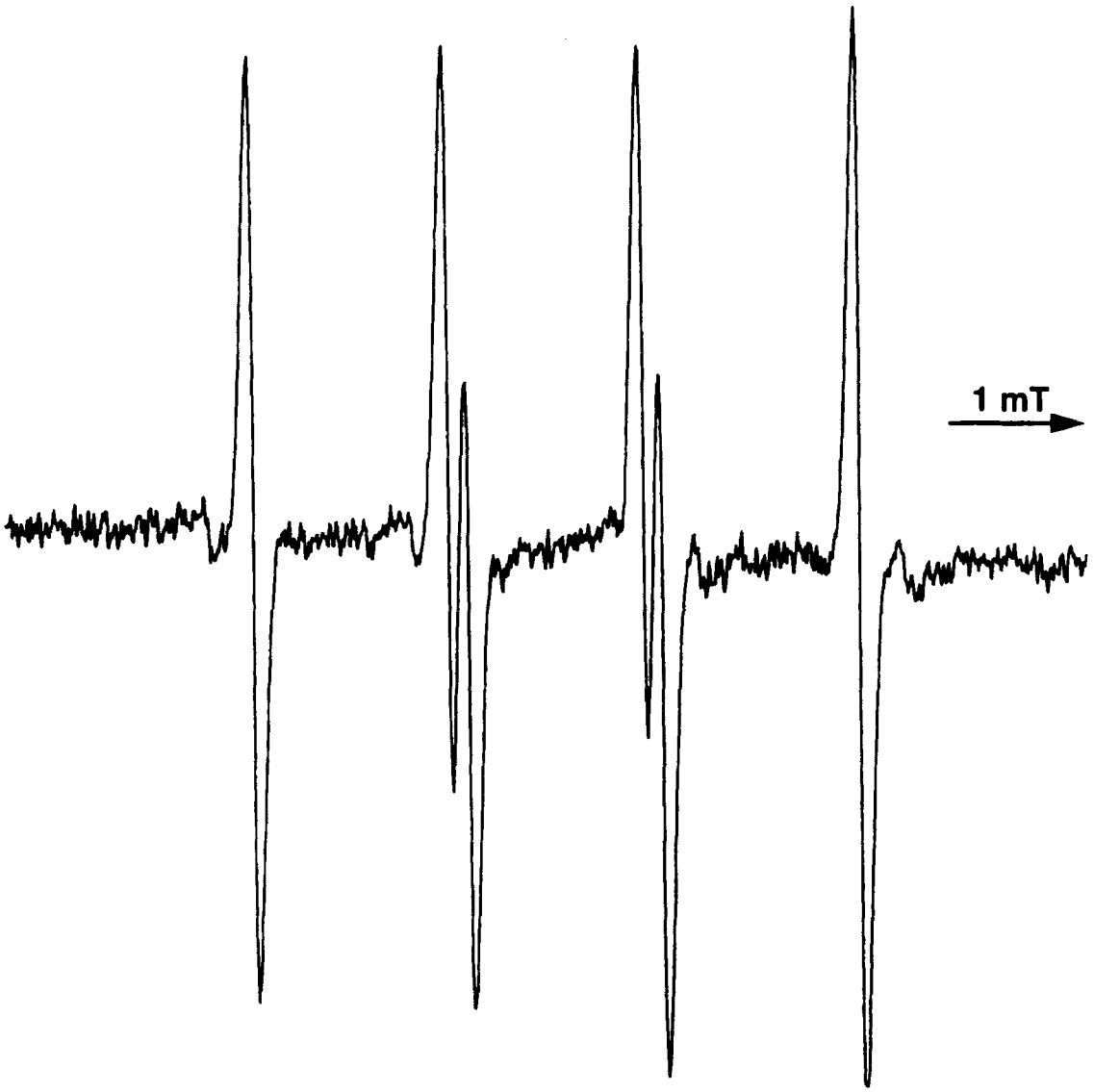


FIGURE 3 EPR spectrum observed on decomposition of $Cl_3CSCl$ (ca. 45 mM) in the presence of DMPO (125 mM) at pH 7.4 and 20 °C. The major signal observed is attributed to the generation of a thiyl-derived radical adduct. The other weak features in the spectra are attributed to low concentrations of a carbon-centred radical adduct (see Table I for parameters)

## DISCUSSION

The results obtained in this study are consistent with the formation of thiyl radicals from a wide range of thiol containing compounds on reaction with HOCl. The detection of these radicals required only the presence of added HOCl and does not appear to be a metal-ion mediated process as extensive treatment of the reactant solutions with Chelex-100 resin had no effect on the observed signals. Furthermore, in the absence of added HOCl only weak, or no, signals were

detected from the auto-oxidation of these thiols. These radicals are detected with both sub-stoichiometric as well as excess HOCl, suggesting that these species can be major intermediates under some circumstances. The observation that the formation of these intermediates does not depend on the presence of metal ions, suggests that these radicals are not formed as a result of reactions such as (1) or (2) followed by (3) which have been previously postulated to occur.[34, 46] Additional evidence against the occurrence of such reactions arises from the observation that only weak, or no, signals from the $HO^{\cdot}$ adduct to DMPO (DMPO-OH) were detected at early stages in the reaction, even though it is known that such radicals react at diffusion-controlled rates with this trap[47] which was present in the reaction mixtures at an excess over the thiol concentration.

$$HOCl + Fe^{2+} \rightarrow HO^{\bullet} + Cl^{-} + Fe^{3+} \quad (1)$$

$$HOCl + Fe^{2+} \rightarrow HO^{-} + Cl^{\bullet} + Fe^{3+} \quad (2)$$

$$X^{\bullet} + RSH \rightarrow XH + RS^{\bullet} \qquad (X^{\bullet} = HO^{\bullet}, Cl^{\bullet}) \quad (3)$$

The role of other functional groups in the formation of the thiyl radicals also appears to have been excluded, as thiyl radicals have been detected even in the absence of groups such as amine or carboxyl groups with which HOCl is known to react. Thus, though HOCl reacts readily with amine functions to yield chloramines which can subsequently decompose to give nitrogen-centred radicals,[44, 45] which might then react with free thiol groups to give the observed thiyl radicals, reaction (4), the detection of thiyl radical signals with 3-mercaptopropionic acid which does not contain this type of function appears to eliminate this possibility. Similarly the detection of thiyl radicals with substrates which lack carboxyl groups eliminates the potential involvement of species such as RC(O)OCl which have been suggested to be formed on reaction of carboxyl groups with HOCl, and which might decompose to give radicals.

$$RNHCl \rightarrow\rightarrow RNH^{\bullet} + RSH \rightarrow RNH_2 + RS^{\bullet} \quad (4)$$

The observation that thiyl radicals are readily detected with the model sulfenyl chloride $Cl_3CSCl$ suggests a possible mechanism for the formation of the observed species. This compound has been shown to readily undergo thermal decomposition to give thiyl radicals. This process can be readily accelerated in the presence of UV or visible light, and is also known to be catalysed by metal ions (e.g.[36, 37] and references therein). Thus it is possible that the thiyl radicals detected in this study are generated by a reaction mechanism which involves the initial formation of a sulfenyl chloride from bi-molecular reaction of HOCl with a free thiol group (reaction (5)). This species once formed can then undergo a number of competing reactions including thermal decomposition to thiyl radicals, reaction with excess thiol anion (reaction (6)[33]), reaction with $H_2O$ to give oxygenated sulfonic acid species (cf. reaction (7)[22, 33]), and reaction with excess HOCl to give sulfonyl chlorides and further down-stream products (cf. reactions (8) – (10)[17]). The intermediacy of a sulfenyl chloride in these reactions is consistent with both the observation of thiyl radicals even when the spin trap is added some time after the initial mixing of thiol with HOCl, and also with the observed enhancement of thiyl radical formation when the reaction mixture is treated with either $Fe^{2+}$ or UV light; both of these treatments are known to enhance the degradation of sulfenyl chlorides to thiyl radicals.[37]

$$RSH + HOCl \rightarrow RSCl + H_2O \quad (5)$$

$$RSCl + RS^{-} \rightarrow RSSR + Cl^{-} \quad (6)$$

$$RSCl + xH_2O \rightarrow\rightarrow RSO_2H/RSO_3H \quad (7)$$

$$RSCl + 2HOCl \rightarrow RSO_2Cl + 2Cl^{-} + 2H^{+} \quad (8)$$

$$RSO_2Cl + H2O \rightarrow RSO_3H + Cl^{-} + H^{+} \quad (9)$$

$$RSO_2Cl \rightarrow\rightarrow \text{glutathione thiosulfonate/ glutathione sulfonamide} \quad (10)$$

The likely fate of the thiyl radicals observed in the current study are reaction with either a further thiyl radical to give the disulfide (a known reaction product of reaction of HOCl with thiols), reaction with a thiol anion to give initially the disulfide radical anion (and hence the disulfide and superoxide radicals in the presence of $O_2$[40]) or reaction with $O_2$ to give a thiyl peroxyl radical.[40] The last of these reactions is reversible, but can result in the formation of oxyacids, which are known products of some thiol oxidations by HOCl (e.g.[16, 21, 22, 33]). Thus most of the products detected in previous studies are consistent with, but do not prove or require, the involvement of thiyl radicals in the reaction mechanism, and cannot readily distinguish between the radical and non-radical pathways. One possible method of examining the significance of radical processes in thiol oxidation by HOCl is via quantification of the extent of radical adduct formation. However this is difficult to achieve with short-lived adducts such as these thiyl species, even though the rate constant for addition of a number of thiyl radicals to DMPO have been determined.[41]

It is likely that the (uni-molecular) radical-mediated pathway is a minor process with low molecular weight thiols under most circumstances given that (bi-molecular) reactions of the sulfenyl chloride with other substrates such as excess thiol (reaction (6)) are likely to be fast in free solution. However this may not always be the case, especially when the sulfenyl chloride is formed in some situations, such as on proteins, where bi-molecular reactions are slowed down by steric or electronic factors, or in situations where the sulfenyl chloride is generated in the presence of metal ions or UV light; under such circumstances thiyl radical formation may be a significant fate of the initially generated sulfenyl chloride.

### *Acknowledgements*

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