Glutathione Oxidation by Hypochlorous Acid in Endothelial Cells Produces Glutathione Sulfonamide as a Major Product but Not Glutathione Disulfide*

Juliet M. Pullar‡, Margret C. M. Vissers, and Christine C. Winterbourn

From the Free Radical Research Group, Department of Pathology, Christchurch School of Medicine, Christchurch, New Zealand

Treatment of cells with hypochlorous acid (HOCl) at sublethal doses causes a concentration-dependent loss in reduced glutathione (GSH) levels. We have investigated the products of the reaction of HOCl with GSH in human umbilical vein endothelial cells. Despite a complete loss of GSH, there were only very small increases in intracellular and extracellular glutathione disulfide and glutathione sulfonic acid after exposure to HOCl. 35S labeling of the GSH pool showed only a minimal increase in protein-bound GSH, suggesting that S-thiolation was not a major contributor to HOCl-mediated loss of GSH in endothelial cells. Rather, the products of the reaction were mostly exported from cells and included a peak that co-eluted with the cyclic sulfonamide that is a product of the reaction of GSH with reagent HOCl. Evidence of this species in endothelial cell supernatants after HOCl treatment was also obtained using electrospray mass spectrometry. In conclusion, exposure to HOCl causes the irreversible loss of cellular GSH with the formation of novel products that are rapidly exported from the cell, and resynthesis of GSH will be required to restore levels. The loss of GSH would alter the redox state of the cell and compromise its defenses against further oxidative stress.

Hypochlorous acid is a potent oxidant generated during the neutrophil oxidative burst by the enzyme myeloperoxidase according to the following reaction.

\[ \text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \]

**Reaction 1**

HOCl can react readily with a wide range of functional groups but is extremely reactive with thiol groups (1, 2). A likely target for its reaction in vivo is reduced glutathione (γ-glutamylcysteinylglycine, GSH), the major low molecular weight thiol in most cells (3). GSH plays a fundamental role in defending against injury induced by reactive oxygen species, as well as being an important cellular redox buffer involved in the maintenance of protein thiol groups in a reduced form (3). Previous work has established that exposure to sublethal concentrations of HOCl causes a decrease in the GSH content of several cell types (4–6), including human umbilical vein endothelial cells (HUVEC) (7). However, the products of the reaction in endothelial cells have not been established.

Oxidation of intracellular GSH by oxidants such as hydrogen peroxide and organic peroxides generally leads to the formation of GSSG. This alters the redox state of the cell, but enzymatic reduction means that the effect is transient, and GSH is able to function as a recyclable antioxidant defense. GSH can be further oxidized to the sulfenic, sulfonic, and sulfenic acid derivatives via successive two-electron oxidations of the thiol group. Low molecular weight sulfenic acids are generally not stable (8), and although the initial product of GSH oxidation by hydrogen peroxide is the sulfenic acid, this reacts with more GSH to give GSSG. The reaction of thiols with HOCl yields an initial sulfenyl chloride, which undergoes further reactions (9, 10). Cysteine (when present in excess) gives nearly stoichiometric conversion to the disulfide, but with GSH, two additional stable end products have been identified (1, 11). Based on their mass spectra, these have been provisionally characterized as an internal sulfonamide of GSH (Fig. 1) and an additional oxidation product of GSSG, glutathione thiosulfonate.

In this study, we have investigated the products of the reaction of HOCl with GSH in HUVEC. We have found only minimal formation of GSSG and protein-mixed disulfides. Other oxidation products, including glutathione sulfonamide, were detected. They were present in the extracellular medium, indicating their rapid export from the cell.

**EXPERIMENTAL PROCEDURES**

*Reagents—* Sodium hypochlorite was supplied by Reckitt and Coleman Ltd. (Auckland, New Zealand). The concentration of HOCl was established by reaction with 5-thio-2-nitrobenzoic acid and measurement of \(A_{412} (e = 28200 \text{ M}^{-1} \text{cm}^{-1})\) (12) or alternatively by using the extinction coefficient of hypochlorite \(e_{100} = 350 \text{ M}^{-1} \text{cm}^{-1}\) at pH 10–12. GSSG was supplied by Roche Molecular Biochemicals New Zealand Ltd. [35S]Cysteine was supplied by Amersham Pharmacia Biotech. Cell culture materials including medium 199, fetal bovine serum, and penicillin/streptomycin were from Life Technologies, Inc. CLS type I collagenase was from Worthington, and heparin was from Fisons Pty, Ltd. (Sydney, Australia). Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose (13). All other chemicals were supplied by Sigma or BDH Chemicals New Zealand Ltd. (Palmerton North, New Zealand).

*Cell Culture—* HUVEC were isolated from umbilical cords, obtained with informed consent, by collagenase digestion (14). They were cultured in fibronectin-coated flasks in medium 199 supplemented with fetal calf serum, heparin, endothelial cell growth supplement, penicillin, and streptomycin as in previous studies (7). Cells were grown to confluency in 15- or 32-mm-diameter wells and used after the second or third passage. HUVEC were identified by their typical morphology.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Free Radical Research Group, Dept. of Pathology, Christchurch School of Medicine, P.O. Box 4345, Christchurch, New Zealand. E-mail: juliet.pullar@chmeds.ac.nz.

1 The abbreviations used are: HUVEC, human umbilical vein endothelial cells; DTT, dithiothreitol; HBSS, Hanks’ balanced saline solution; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinopropanesulfonic acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
including the presence of Wieland-Palade bodies and cobblestone pattern on confluence.

Cell Treatment—HOCl was diluted to working concentrations in HBSS (phosphate-buffered saline, pH 7.4, containing 5.5 mM glucose, 0.5 mM magnesium, and 1.0 mM calcium) and used immediately. At this pH, HOCl and OCl− were present in approximately equimolar concentrations (pKa = 7.53), but the solution is subsequently referred to as HOCl. Confluent cells were washed in HBSS, the HOCl was added to the wells, and the cells were incubated at 37 °C for 10 min. The 15-mm wells contained ~120,000 cells and were treated in a 1 ml volume, whereas the 32-mm wells contained about 700,000 cells and were treated in a 2 ml volume.

Glutathione Measurements—Glutathione species (GSH, GSSG, and glutathione sulfonic acid) were analyzed using an adaptation of the Martin and White fluorescent HPLC assay in which thiol groups were blocked with iodoacetic acid, and amine groups were derivatized with dansyl chloride (15). After exposure of cells to HOCl, the supernatants were frozen for later analysis, and adherent cells were extracted with lysing buffer (perchloric acid-diethylenetriaminepentaacetic acid-boric acid-cresol red indicator), and 1 μM of iodoacetic acid. Extracts were transferred to microcentrifuge tubes, the pH was corrected to 8–8.5 with 1.45 M lithium hydroxide, and the samples were left for 1 h in the dark at 25 °C. An equal volume of 4 mg/ml dansyl chloride in acetonitrile was then added, and the samples were incubated in the dark at 25 °C for 30–60 min. Following chloroform extraction, samples were separated by HPLC using a 5-μM NH2-silica LiChrospher column (E Merck, Alltech). Detection was performed with a Jasco FP-920 fluorescent detector with excitation and emission wavelengths of 328 and 542 nm, respectively. Concentrations of GSH, GSSG, and glutathione sulfonic acid in cell samples were calculated by comparing their peak areas to a series of standards of known concentrations. Fresh reagents were made up before each assay was performed.

35S Labeling of Cellular Glutathione—HUVECs grown in 15- or 32-mm wells were preincubated with medium that lacked sulfur-containing amino acids for 2 h and then incubated with 20 μM cycloheximide for 1 h in the same medium. Following this, the cells were loaded with [35S]cysteine (2.5 μCi/ml; specific activity of 1075 Ci/mmol) in the presence of cycloheximide for 2–3 h, again in the deficient medium, as described by Thomas et al. (16). The cells were washed in HBSS with 1% bovine serum albumin to remove any residual radioactivity, followed by HBSS alone. After treatment with HOCl, the supernatant was removed, and the cellular protein was precipitated with ice-cold 5% perchloric acid. An aliquot was removed to represent the total intracellular counts. The protein was pelleted by centrifugation, washed twice in ice-cold ether, and resuspended in 1% SDS for counting. Alternatively, the protein was pelleted by centrifugation, washed twice in 1% SDS, treated with DTT, and resuspended in 1% SDS. Bray’s scintillant was added to each of the fractions, and the radioactivity was determined.

HPLC Separation of 35S-Labeled Intracellular Products—The 35S-labeled components of the extracellular supernatant and intracellular low molecular weight fraction were separated by HPLC on a C18 Nucleosil column with 50 mM formic acid as eluant (11). Samples were spiked with GSH and GSSG, and the fractions were collected every 0.25 min for scintillation counting. The retention times of the labeled peaks were compared with those of the products of the oxidation of GSH by HOCl.

Electrospray Mass Spectrometry—To obtain sufficient material for analysis, HUVECs were grown in 75-cm² flasks and treated with HOCl (290 μM) in a volume of 8 ml. For control and treated cells, supernatants from two flasks were pooled and fractionated to remove salt and enrich them with glutathione sulfonamide, using two consecutive HPLC procedures. After centrifugation to remove debris and concentration by freeze drying, samples were first applied to a Superdex peptide HR 10/30 column (Amersham Pharmacia Biotech) with water as the eluant. Fractions eluting in the position expected for glutathione sulfonamide were concentrated by freeze drying and then applied to a C18 Nucleosil column with 50 mM formic acid as eluant. Fractions eluting in the position expected for glutathione sulfonamide were collected, concentrated by freeze drying, and frozen at −80 °C until analysis.

Electrospray-ionization mass spectrometry was performed in negative ion mode on a VG Platform II mass spectrometer. Samples were in 50% acetonitrile with 0.1% NH4OH. The source temperature was 60 °C, the probe voltage was −3000 V, and the cone voltage was −70 V. Scans from 200 to 700 m/z were acquired every 2 s.

RESULTS

HPLC Analysis of Glutathione Derivatives after HOCl Treatment—We have previously characterized the effects of exposure of HUVECs to HOCl (7). With an oxidant dose of between 5 and 50 nmol of HOCl/1.2 × 10⁶ cells, there is progressive loss of cell viability, oxidation of cell thiols, and inactivation of susceptible thiol enzymes, particularly glyceroldehyde-3-phosphate dehydrogenase. Doses of 25 nmol and less are sublethal, although there is significant oxidation of cell thiols including GSH at these doses. In the current study we have used similar doses of oxidant. Exposure of HUVECs to HOCl for 10 min caused a concentration-dependent loss of intracellular GSH. A small amount of GSH present in the medium was also oxidized (Fig. 2A). When the cells were analyzed for products, small increases in intracellular GSSG were seen with 35 and 50 nmol of HOCl (Fig. 2B). Glutathione sulfonic acid was undetectable in control cells. Levels were increased at 50 nmol of HOCl but only to a small extent (Fig. 2B). Extracellular GSSG and glutathione sulfonic acid were not increased with HOCl treatment (data not shown). Therefore, neither GSSG nor glutathione sulfonic acid accounted for the majority of the observed GSH loss.

Effect of HOCl on 35S-Labeled Endothelial Cell GSH—To investigate the fate of endothelial GSH after exposure to HOCl, intact cells were incubated with [35S]cysteine in the presence of cycloheximide to label the cellular GSH pool. Analysis of control cells by HPLC and scintillation counting indicated that there was almost complete incorporation of the cysteine into GSH (data not shown). Upon fractionation, 81% of the total counts were intracellular, of which 12% were in the protein fraction and 69% were in the low molecular weight fraction. The remaining counts were extracellular. After exposure to increasing concentrations of HOCl, there was a progressive increase in extracellular counts and a corresponding decrease in the intracellular low molecular weight fraction (Fig. 3). The decrease was significant at 25 nmol of HOCl and closely paralleled the loss of GSH shown in Fig. 2. With 50 nmol of HOCl, more than half the counts were extracellular. The increased extracellular counts were not the result of cell lysis, because there was no significant release of 51Cr at this time with these doses of oxidant. The released counts remained relatively constant by guest on July 25, 2018http://www.jbc.org/Downloaded from

FIG. 1. Proposed structure for the novel sulfonamide generated in the reaction of GSH with HOCl. The proposed mechanism for formation is via condensation of the sulfonyl chloride with the amino group of the glutamyl residue of glutathione.
HPLC Separation of $^{35}$S-Labeled Glutathione Oxidation Products—The $^{35}$S-labeled components of the extracellular supernatant and the intracellular low molecular weight fraction were separated by HPLC, and the elution profiles of labeled material compared with that of HOCl-treated GSH. As shown previously (11), the reaction of GSH with HOCl gave two novel products in addition to GSSG (Fig. 5A). The sulfonamide (337 peak) eluted at $5\text{ min}$, immediately prior to GSH at $6.5\text{ min}$. The thiolsulfonate (644 Da) ran as a shoulder on the GSH peak, eluting at about $8\text{ min}$. Analysis of the intracellular fraction for control endothelial cells gave a large peak corresponding to GSH, a small broad peak at the breakthrough position, and no detectable GSSG (Fig. 5B). Treatment with HOCl resulted in an almost complete loss of GSH, a small increase in counts eluting in the position of GSSG, and a small peak at $5.5\text{ min}$ (Fig. 5B).

The extracellular fraction from control cells gave a small peak corresponding to GSH and no other labeled peaks (Fig. 5C). HOCl treatment of cells resulted in several new peaks, one of which migrated in the position of glutathione sulfonamide (Fig. 5C). There were also significant increases in counts in the breakthrough peak at $3–5\text{ min}$ and two additional new peaks, eluting between $8$ and $11\text{ min}$. These peaks were not further characterized.

Experiments were performed with a range of HOCl concentrations, and the percentage distribution of the radiolabeled peaks was calculated as a fraction of the total extracellular soluble counts. For these experiments, it was necessary to grow cells in larger wells to obtain sufficient material to analyze.

Under these conditions, the cells were more sensitive to HOCl, with lower concentrations causing greater toxicity and greater GSH loss. To determine the relative proportions of radiolabeled peaks, HOCl concentrations were used that gave GSH losses of $45–90\%$. After exposure to HOCl, up to $25\%$ of counts are present in the peak that co-elutes with the sulfonamide, in...
creasing from about 5% in control cells (Fig. 6). The early peak increased to about 20% of total counts. The two unknowns that elute between 8 and 11 min were combined for analysis. These peaks account for up to 40% of the total extracellular counts after HOCl exposure.

Electrospray Mass Spectrometry—Electrospray mass spectrometry was used to seek further evidence for the presence of glutathione sulfonamide in endothelial cell supernatants. Pure GSH gave the characteristic signal corresponding to [M – H] at m/z 306 in negative ion mode (Fig. 7a), and glutathione sulfonamide prepared from GSH and HOCl gave a major signal at m/z 336.3 (Fig. 7b). Following a two-step procedure designed to enrich for the sulfonamide, the supernatant from HOCl-treated endothelial cells also gave a signal at m/z 336.3–336.4 (Fig. 7d). Although comparatively weak, this was the strongest signal in the sample. The supernatant from identically treated control cells gave no equivalent peak (Fig. 7c). There was a peak of about one-third the size at m/z 337.1. On repeated injection this signal was consistently 0.7 mass units higher than the major peak in the treated cells and aligned with a shoulder on this peak. On this basis we conclude that only the HOCl-treated endothelial cells gave a signal at the correct m/z for glutathione sulfonamide.
DISCUSSION

We have previously shown that sublethal concentrations of HOCl, either added as a reagent or generated by myeloperoxidase, cause a significant loss of GSH in cultured endothelial cells (7). Analysis of the products of the reaction has given unexpected results. Very little GSSG was measurable either intracellularly or extracellularly, accounting for only a few percentages of the GSH lost. Glutathione sulfonic acid was also a minor product. By labeling the GSH pool with $^{35}$S, we recovered much of the label lost from HOCl-treated cells in the extracellular medium. This was not the result of cell lysis or leakage from the cells. Several major products were detected by HPLC, predominantly in the cell supernatant. One of these would appear to be glutathione sulfonamide. At least two other products were observed but not identified.

The sulfonamide is a unique product that has only been described for GSH oxidation by HOCl. Evidence for its presence in HUVEC comes from two sources. HPLC separation of $^{35}$S-labeled products in the cell supernatant gave a labeled peak eluting at the same time as the sulfonamide synthesized from pure GSH. Also, a species with the predicted molecular mass was detected by electrospray mass spectrometry in the supernatant from HOCl-treated but not control cells. Although the signal was small, in combination with the $^{35}$S data, these findings provide a strong basis for concluding that glutathione sulfonamide is formed in endothelial cells treated with HOCl and represents about 25% of the extracellular counts with HOCl treatment.

Two other HPLC peaks, eluting after GSH and present in the extracellular medium, constituted the other major GSH oxidation products, about 40% of the total extracellular counts with HOCl treatment. They escaped detection by the dansyl chloride assay, which implies that, like the sulfonamide, they lack the amine group required for derivatization. Whether they are related to the sulfonamide or contain chloramine groups remains to be explored. Other labeled products were seen in the breakthrough peak on HPLC. Although glutathione sulfonic acid elutes in this position, it was detected by the dansyl chloride method only intracellularly at the higher HOCl concentrations and is unlikely to be a major contributor to this peak.

Pure thiols are oxidized by HOCl to a mixture of products. The initial product is the sulfenyl chloride that can react with more thiol to give the disulfide or be oxidized further (8–10, 17). GSH forms a mixture of predominantly GSSG and the sulfonamide, which is presumed to form via condensation of the sulfenyl chloride with the $\alpha$-amino group (11). On this basis, if the sulfonamide were formed in cells, we would also expect to see GSSG. The reason only low amounts of GSSG were detected could be that enzymatic reduction prevents its accumulation. Alternatively, the sulfenyl chloride could have a different fate in a cellular environment.

HOCl caused only minimal S-thiolation of cell proteins. Protein-associated counts increased only slightly at the highest dose, and the proportion released with dithiothreitol was similarly increased only at the highest HOCl dose. We have previously reported that up to half of endothelial cell protein thiols are lost under the conditions of HOCl treatment used in the present study (7). Although our results cannot discount a contribution of S-thiolation, perhaps of specific proteins, most of this protein thiol loss must be due to other mechanisms. The limited S-thiolation seen with HOCl may be explained on the basis of the minimal GSSG accumulation and restricted capacity for thiol/disulfide exchange.

These results contrast with the accumulation of substantial amounts of glutathionylated proteins in cells treated with hydroperoxide and organic peroxides (18, 19). S-Thiolation is considered to be one of the mechanisms whereby these oxidants exert a regulatory effect on cell function (20). We and others have shown that at the doses used in the current study, HOCl can activate pathways leading to p53 activation, growth arrest, and apoptosis (21–23). Our results suggest that S-thiolation may not play a major role in the activation of these processes by HOCl.

Our findings with endothelial cells are very similar to those with neutrophils (5). The addition of HOCl or stimulation of the neutrophils to produce endogenous HOCl from myeloperoxidase caused a loss of GSH without GSSG formation and little evidence of S-thiolation. $^{35}$S labeling also showed a peak in the position of the sulfonamide, which was predominantly extracellular and accounted for about 20% of the GSH loss.

It is notable that glutathione sulfonamide and the other major products of the reaction of endothelial cell GSH with HOCl were detected predominantly in the extracellular medium within the 10-min treatment time. It is likely that the majority of the reaction occurred intracellularly. The small amount of GSH present in the medium of resting cells was insufficient to account for the product formed, and the HOCl concentrations used caused an insignificant release of cell constituents in that time (7). Also, the detection of some sulfonamide in cell extracts points to an intracellular origin. It appears, therefore, that the cells have an efficient transport mechanism for exporting these glutathione oxidation products. This may provide protection for the cells, although this possibility remains to be explored. However, the irreversible formation and export of these oxidation products mean that with HOCl, GSH does not function as a recyclable antioxidant. As a consequence the ability of GSH to provide protection against ongoing oxidative stress is diminished and can only be restored by glutathione resynthesis. The altered redox buffering of the cells also has implications for their responses to regulatory signals.

In addition to understanding the impact of reactive oxidants on cell function, it is important to have reliable biomarkers for detecting oxidant formation and its consequences in vivo. Our findings both with endothelial cells and neutrophils suggest that glutathione sulfonamide is a promising candidate for a biomarker of HOCl production by neutrophils in vivo. The reaction with GSH is one of the fastest observed for HOCl (2), making GSH a likely major target for HOCl in vivo. The detection of glutathione sulfonamide as a significant product in both neutrophils and HUVEC and its rapid release from the cells after HOCl exposure suggests it may be present in urine or blood. Further work is needed, however, to establish whether the sulfonamide is indeed unique to HOCl, to determine whether it can be detected with high sensitivity, and to define its biological properties.

Acknowledgment—We thank Dr. S. Brennan for the electrospray mass spectrometry experiments.

REFERENCES

1. Winterbourn, C. C. (1985) Biochim. Biophys. Acta 840, 204–210
2. Folkes, L. K., Candeias, L. P., and Wardman, P. (1995) Arch. Biochem. Biophys. 323, 120–126
3. Meister, A., and Anderson, M. E. (1983) Annu. Rev. Biochem. 52, 711–760
4. Vissers, M. C. M., and Winterbourn, C. C. (1995) Biochem. J. 307, 57–62
5. Carr, A. C., and Winterbourn, C. C. (1997) Biochem. J. 327, 275–281
6. Tatsunami, T., and Fliss, H. (1994) Am. J. Physiol. 267, H1597–H1607
7. Pullar, J. M., Winterbourn, C. C., and Vissers, M. C. M. (1999) Am. J. Physiol. 277, H1505–H1512
8. Claiborne, A., Miller, H., Parsonage, D., and Ross, P. R. (1993) FASEB J. 7, 1483–1490
9. Prutz, W. A. (1996) Arch. Biochem. Biophys. 332, 110–120
10. Armento, W. L., Cane, M., Fernandez, M. J., Garcia, M. V., and Santabella, J. A. (2000) Tetrahedron 56, 1103–1109
11. Winterbourn, C. C., and Brennan, S. O. (1997) Biochem. J. 326, 87–92
12. Vissers, M. C. M., and Fantone, J. C. (1997) Free Radical Biol. Med. 8, 331–337
13. Vissers, M. C. M., and Winterbourn, C. C. (1999) Arch. Biochem. Biophys. 285, 53–59
14. Jaffe, E., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) J. Clin. Invest. 52, 2745–2756
Glutathione Oxidation by Hypochlorous Acid

15. Martin, J., and White, I. N. H. (1991) J. Chromatogr. 568, 219–225
16. Thomas, J. A., Chai, Y. C., and Jung, C. H. (1994) Methods Enzymol. 233, 385–395
17. Silverstein, R. M., and Hager, L. P. (1974) Biochemistry 13, 5069–5073
18. Schuppe, I., Moldeus, P., and Cotgreave, I. A. (1992) Biochem. Pharmacol. 44, 1757–1764
19. Chai, Y.-C., Hendrich, S., and Thomas, J. A. (1994) Arch. Biochem. Biophys. 310, 264–272
20. Cotgreave, I. A., and Gerdes, R. (1998) Biochem. Biophys. Res. Commun. 242, 1–9
21. Vile, G. F., Rothwell, L. A., and Kettle, A. J. (1998) Arch. Biochem. Biophys. 359, 51–66
22. Vile, G. F., Rothwell, L. A., and Kettle, A. J. (2000) Arch. Biochem. Biophys. 377, 122–128
23. Vissers, M. C. M., Pullar, J. M., and Hampton, M. B. (1999) Biochem. J. 344, 443–449
Glutathione Oxidation by Hypochlorous Acid in Endothelial Cells Produces
Glutathione Sulfonamide as a Major Product but Not Glutathione Disulfide
Juliet M. Pullar, Margret C. M. Vissers and Christine C. Winterbourn

J. Biol. Chem. 2001, 276:22120-22125.
doi: 10.1074/jbc.M102088200 originally published online March 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102088200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 23 references, 4 of which can be accessed free at http://www.jbc.org/content/276/25/22120.full.html#ref-list-1