# Hypochlorous acid-mediated modification of cholesterol and phospholipid: analysis of reaction products by gas chromatography–mass spectrometry

**Jeroen J. M. van den Berg,[1,*] Christine C. Winterbourn,[†] and Frans A. Kuypers[*]**

Children's Hospital Oakland Research Institute,* 747 Fifty Second Street, Oakland, CA 94609, and Christchurch School of Medicine,† Christchurch, New Zealand

**Abstract** Oxidative modification of membrane lipids by hypochlorous acid could be an important element in the mechanism of membrane disruption by activated neutrophils. We have previously shown that hypochlorous acid reacts with unsaturated fatty acids of membrane phospholipids to give fatty acid chlorohydrins (Winterbourn et al. 1992. *Arch. Biochem. Biophys.* **296:** 547–555). In the present study, we examined the reaction of cholesterol in bilayers with an inert phospholipid carrier. Product separation and identification was performed using gas chromatography–mass spectrometry after trimethylsilyl-derivatization. Unlike the reaction of hypochlorous acid with unsaturated fatty acids, no chlorohydrin derivatives were found with cholesterol. Instead, the main oxidation products were identified as the epimeric cholesterol 5,6-epoxides and 4-hydroxycholesterol, while several other hydroxy- and keto-derivatives were also found in smaller amounts. Analysis of the products obtained after reaction of vesicles composed of a mixture of several unsaturated phospholipid species plus cholesterol revealed that the individual fatty acids and cholesterol all exhibit comparable susceptibilities toward hypochlorous acid. Using myeloperoxidase to generate hypochlorous acid, basically the same products and product distribution were obtained. These studies show that unsaturated phospholipids and cholesterol can be profoundly modified by reaction with hypochlorous acid. This warrants further investigation to define the role of lipid modifications in neutrophil-mediated membrane disruption.—**van den Berg, J. J. M., C. C. Winterbourn, and F. A. Kuypers.** Hypochlorous acid-mediated modification of cholesterol and phospholipid: analysis of reaction products by gas chromatography–mass spectrometry. *J. Lipid Res.* 1993. **34:** 2005–2012.

**Supplementary key words** neutrophils • oxidation • cholesterol • fatty acid • phospholipid • membrane • chlorohydrin • oxysterol

Reactive oxygen species produced by activated phagocytes are essential instruments in the killing of invading pathogens, but are also implied as mediators of host tissue injury (1–3). Activated neutrophils produce superoxide ($O_2^{-\cdot}$) and hydrogen peroxide ($H_2O_2$), which is converted to a large extent into the very reactive hypochlorous acid (HOCl) by the neutrophil's myeloperoxidase (MPO)/$H_2O_2/Cl^-$ system (4). HOCl is known to be strongly microbicidal and can also lyse erythrocytes and damage other cells, but the mechanism by which it disrupts membranes has not been clarified in detail.

Membrane constituents are main targets for HOCl, and its reaction with membrane protein thiols, thioethers, and amines has been described (5, 6). However, little is known about the effect of neutrophil oxidants on the lipid constituents of biological membranes. This is of potential importance, as the drastically altered physical properties of oxidized lipids could have profoundly disturbing effects on membrane structure and function, even at low concentrations. Neutrophils can cause lipid peroxidation in the presence of iron (7, 8), but MPO-derived HOCl inhibits lipid peroxidation (8). We have recently shown that HOCl reacts with unsaturated fatty acid double bonds to give fatty acid chlorohydrins (9). This reaction appears to be very efficient for fatty acid micelles as well as for unsaturated phospholipid vesicles, and the same products were detected when incubating these substrate systems with $MPO/H_2O_2/Cl^-$.

In addition to phospholipids, cholesterol is another important lipid constituent of many cell membranes. It functions to maintain membrane fluidity and flexibility by acting as a 'lubricant', accommodating phospholipid molecular species with very different physical properties into the matrix of the lipid bilayer. Cholesterol is also

Abbreviations: BSTFA, bis(trimethylsilyl)trifluoroacetamide; GC–MS, gas chromatography–mass spectrometry; HOCl, hypochlorous acid; MPO, myeloperoxidase; MSTFA, n-methyl-n-trimethylsilyltrifluoroacetamide; PC, phosphatidylcholine; PBS, phosphate-buffered saline.

[1]To whom correspondence should be addressed.



Downloaded from www.jlr.org by guest, on June 17, 2012

ASBMB JOURNAL OF LIPID RESEARCH

prone to oxidative modification, and the presence of oxidized cholesterol could have deleterious effects on membrane stability. We therefore examined the reactivity of cholesterol towards HOCl in vesicles with a non-oxidizable carrier phospholipid. In addition, we used vesicles containing a mixture of unsaturated phospholipids and cholesterol as a model for a biological membrane in order to compare the reactivity of cholesterol and unsaturated fatty acyl chains. Gas chromatography-mass spectrometry (GC-MS) was used to separate and identify the reaction products.

## MATERIALS AND METHODS

### Materials

1,2-Dipalmitoyl phosphatidylcholine (di16:0-PC), 1-palmitoyl,2-oleoyl phosphatidylcholine (16:0,18:1-PC), 1-palmitoyl,2-linoleoyl phosphatidylcholine (16:0,18:2-PC), 1-stearoyl,2-arachidonoyl phosphatidylcholine (18:0,20:4-PC), 5α-cholesten-3β-ol (cholesterol), and 5α,6α-epoxy-cholestan-3β-ol (cholesterol 5α,6α-epoxide) were obtained from Sigma (St. Louis, MO). 5-Cholestene-3β,4β-diol (4β-hydroxycholesterol), 5α-cholestan-3β-ol-6-one (6-ketocholestanol), 5-cholestene-3β,7α-diol (7α-hydroxycholesterol), 4-cholesten-6β-ol-3-one, and cholestane-3β,5α,6β-triol were purchased from Steraloids (Wilton, NH). Cholestane-3β,5α,6α-triol was a kind gift from Drs. I. Björkhem and E. Lund (Karolinska Institutet, Huddinge, Sweden). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethyl chlorosilane (TMCS) was obtained from Pierce (Rockford, IL). Myeloperoxidase (MPO) was purified as previously described (10). Sodium hypochlorite was purchased from Fischer. Its concentration was determined by reaction with monochlorodimedon ($\epsilon_{290}$ = 19,000 $M^{-1}cm^{-1}$). Chemicals used were of analytical grade, and all solvents used were of HPLC grade purity.

### Reaction of cholesterol-containing phospholipid vesicles with hypochlorous acid

The appropriate lipid mixtures were dried down under nitrogen from a chloroform solution. Phosphate-buffered saline (50 mM phosphate, 110 mM NaCl, pH 7.4; PBS) was added and multilamellar liposomes were formed by vortex mixing. Unilamellar vesicles were made by extrusion of liposomes through filters with 0.1 μm pores (11). The final phospholipid concentration was 0.75 mM. The cholesterol:phospholipid mole ratio in the vesicles was 1:1.

A known amount of NaOCl was added to 1 ml of the vesicle suspension while mixing continuously on a vortex mixer. After incubating at room temperature for 30 min, 100 μl 11 mM $CaCl_2$ was added and the phospholipids were hydrolyzed with 5 IU phospholipase $A_2$ from bee venom in a 30-min incubation at 37°C. Subsequently, the mixture was slightly acidified with HCl and lipids were extracted twice with $CH_2Cl_2$. The level of hydrolysis was tested by TLC on silicagel HR 60 (Merck, Darmstadt, Germany), developed in chloroform-methanol-0.9% NaCl-acetic acid 100:50:5:16 (v/v/v/v). All phospholipid was found to have been converted to lysophospholipid and free fatty acid. Derivatization and GC-MS analysis of the reaction products was performed as described below.

### Reaction of vesicles with myeloperoxidase

To a 1-ml suspension of vesicles containing 0.5 μmol of each of 16:0,18:1-PC, 16:0,18:2-PC, and cholesterol in PBS at 37°C, additions of 20 nmol $H_2O_2$ were made at 10-min intervals. MPO (13 nM) was added at the start and at every second addition of $H_2O_2$. Alternate additions of 2 μM ascorbic acid were also made. This procedure was used to maintain enzyme activity and minimize competition by $H_2O_2$ for the HOCl (9). After 0.3 μmol $H_2O_2$ had been added, the suspension was acidified and extracted as described above for NaOCl-treated samples.

### Derivatization

For GC-MS analysis, hydroxyl groups in cholesterol and cholesterol reaction products were derivatized to the trimethylsilyl (TMS) ether derivatives. HOCl-derived hydroxyl groups in fatty acids were derivatized to the corresponding TMS ethers. Carboxylic hydroxyl groups were derivatized to TMS esters (thus, fatty acids were analyzed as their TMS ester/TMS ether derivatives). For TMS derivatization, samples were dissolved in 100 μl pyridine; 50 μl BSTFA containing 1% TMCS was added and the mixture was heated at 70°C for 1 h. The samples were then used for GC-MS analysis without further extraction.

### Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were carried out using an HP 5790 A Series Gas Chromatograph coupled to an HP 5970 A MSD mass spectrometer (Hewlett-Packard, Palo Alto, CA) equipped with a 15 m DB-1 fused silica column (i.d. = 0.25 mm, film thickness 0.25 μm, J&W Scientific, Folsom, CA). The sample (1 μl) was splitless-injected with helium as carrier gas and the quadrupole mass spectrometer was operated in electron impact mode at 70 eV. The column was kept at 50°C for 3 min and subsequently heated at a rate of 27°C/min to 180°C followed by an increase of 5°C/min to 310°C.

## RESULTS

Incubation of di16:0-PC/cholesterol vesicles with HOCl (HOCl:cholesterol ratio 1:2), followed by extraction and TMS derivatization, typically gave a total ion chromatogram as shown in **Fig. 1.** A peak corresponding to 16:0



Downloaded from www.jlr.org by guest, on June 17, 2012

ASBMB JOURNAL OF LIPID RESEARCH

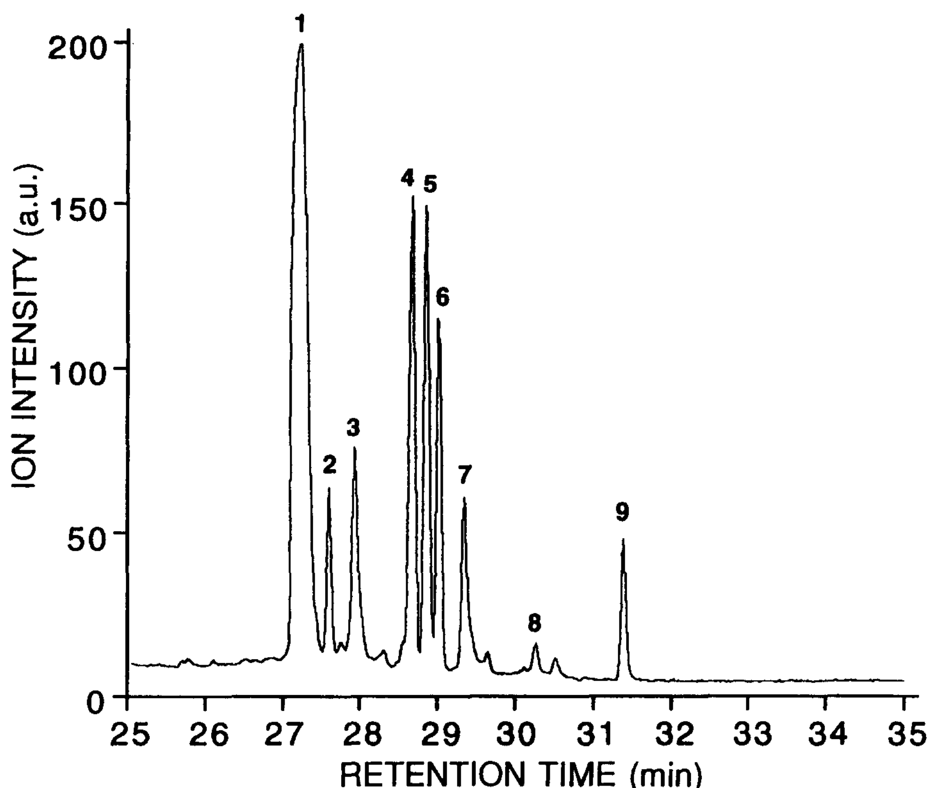


**Fig. 1.** GC–MS analysis of reaction products obtained after reaction of cholesterol with hypochlorous acid. An equimolar mixture of di16:0-PC and cholesterol was used to prepare unilamellar vesicles by extrusion as described in Methods. Cholesterol and PC concentrations were 0.75 mM in a total volume of 1 ml; reagent HOCl was added to a concentration of 0.37 mM giving a HOCl:cholesterol ratio of 1:2. Information on peak identification is given in Table 1; a.u. = arbitrary units.

TMS ester (molecular ion $M^+$ at *m/z* 328, $(M-CH_3)^+$ at *m/z* 313) eluted at 12.6 min (not shown), and the cholesterol starting material was found to elute at 27.25 min (peak **1**). There were several product peaks eluting after cholesterol in the 27.5–31.5 min region. **Table 1** lists retention times, characteristic fragmentation ions, and peak identities for this experiment. By comparison of retention times and fragmentation patterns of the three major product peaks **4, 5,** and **6** with GC–MS data obtained for pure standards, their identity was established as: **4:** cholesterol 5β,6β-epoxide (5β,6β-epoxycholestan-3β-ol) ($M^+$ 474); **5:** cholesterol 5α,6α-epoxide (5α,6α-epoxycholestan-3β-ol) ($M^+$ 474); **6:** 4β-hydroxycholesterol (5-cholestene-3β,4β-diol) ($M^+$ 546). The identity of peak **2** was established as 7α-hydroxycholesterol (5α-cholestene-3β,7α-diol), as GC–MS data for this product were identical to data obtained for a 7α-hydroxycholesterol reference compound. The molecular ion of this compound at *m/z* 546 was usually not observed in the mass spectrum or present at very low intensity; the $(M-90)^+$ fragment at *m/z* 456 was always the base peak. The mass spectrum and retention time of product **3** were virtually identical to those of 4-cholestene-3β,6β-diol ($M^+$ 546), obtained after sodium borohydride reduction of 4-cholesten-6β-ol-3-one, followed by BSTFA derivatization. This identification is consistent with published data (12–14). Comparison with reference compound data established the identity of the small peak **8** at 30.25 min as 6-ketocholestanol (5α-cholestan-3β-ol-6-one) ($M^+$ 474). Peak **7** was identified as 6-hydroxycholesterol (5-cholestene-3β,6-diol), which is the enol form of 6-ketocholestanol. It would normally be expected in minor amounts only, as the keto-enol equilibrium lies far to the keto side. However, the mass spectrum and retention time of peak **7** were virtually identical to those of 6-hydroxycholesterol ($M^+$ 546), which was obtained after TMS derivatization of a 6-ketocholestanol reference compound with n-methyl-n-trimethylsilyltrifluoroacetamide (MSTFA) in the presence of ammonium iodide. It would therefore appear that, under the sample processing conditions used here, 6-ketocholestanol is trapped in its enol form, which could at least partially account for the presence of this compound in the GC–MS chromatogram. The mass spectrum of peak **9** (retention time 31.4 min) indicates that it may be another cholestene-diol, infrequently showing a very faint molecular ion at *m/z* 546, a weak $(M-90)^+$ fragment at 456, and a



Downloaded from www.jlr.org by guest, on June 17, 2012
ASBMB JOURNAL OF LIPID RESEARCH

TABLE 1. Oxidation products of cholesterol with hypochlorous acid

| Peak No.[a] | Retention Time | Characteristic Fragments[b] | Identity[c] | Product Distribution[d] |
|---|---|---|---|---|
| | *min* | *m/z* | | % |
| 1 | 27.25 | 73 (75), 129 (83), 329 (100), 353 (38), 368 (69), 443 (9), 458 (25) ($M^+$) | cholesterol (5α-cholesten-3β-ol) | |
| 2 | 27.6 | 73 (33), 143 (10), 196 (15), 209 (15), 367 (4), 441 (8), 456 (100) ($(M-90)^+$), 546 (2) ($M^+$) | 7α-hydroxycholesterol (5α-cholestene-3β,7α-diol) | 8 |
| 3 | 27.9 | 73 (100), 143 (17), 194 (25), 403 (85), 441 (23), 456 (55), 517 (5), 531 (10), 546 (5) ($M^+$) | 4-cholestene-3β,6β-diol | 15 |
| 4 | 28.65 | 73 (100), 75 (67), 95 (84), 135 (67), 197 (59), 211 (41), 366 (28), 369 (34), 384 (78), 474 (55) ($M^+$) | cholesterol 5β,6β-epoxide (5α,6β-epoxycholestan-3β-ol) | 24 |
| 5 | 28.75 | 73 (100), 75 (81), 95 (71), 135 (49), 197 (34), 211 (32), 366 (46), 369 (43), 384 (89), 474 (48) ($M^+$) | cholesterol 5α,6α-epoxide (5α,6α-epoxycholestan-3β-ol) | 21 |
| 6 | 29.0 | 73 (100), 147 (42), 253 (21), 327 (35), 366 (68), 417 (24), 441 (11), 456 (37), 531 (4), 546 (6) ($M^+$) | 4β-hydroxycholesterol (5-cholestene-3β,4β-diol) | 15 |
| 7 | 29.35 | 73 (65), 147 (9), 233 (7), 417 (9), 441 (100), 456 (6), 531 (66), 546 (13) ($M^+$) | 6-hydroxycholesterol (5-cholestene-3β,6α/β-diol) | 10 |
| 8 | 30.25 | 73 (34), 75 (31), 95 (26), 159 (22), 384 (8), 445 (100), 459 (57), 474 (17) ($M^+$) | 6-ketocholestanol (5-cholestan-3β-ol-6-one) | 2 |
| 9 | 31.4 | 55 (26), 57 (26), 73 (21), 159 (20), 313 (5), 366 (100), 403 (21), 456 (3), 459 (5), 546 (1) | unidentified[e] | 6 |

[a]Peak numbers refer to peaks in Fig. 1.
[b]Mass spectrometric data for TMS-derivatized compounds. Numbers in brackets are relative intensities of fragment peaks relative to the base peak in the spectrum.
[c]Identity of compounds before TMS derivatization. Compounds were identified by comparison of GC-MS data with reference compounds. For details see text.
[d]Product distribution calculated on the basis of peak area. The combined peak areas of the product peaks 2-9 was taken as 100%.
[e]Possibly cholestene-diol or cholestane-triol (see text).

very prominent $(M-90-90)^+$ fragment at *m/z* 366. However, we have not yet been able to establish the positions of the hydroxyl groups. Alternatively, this compound could also be a cholestane-triol, with the molecular ion (*m/z* 636) absent and *m/z* 366 representing a $(M-90-90-90)^+$ fragment. The long retention time points in this direction, but the mass spectrum was clearly distinct from cholestane-3β,5α,6α-triol and cholestane-3β,5α,6β-triol.

When vesicles composed of a mixture of unsaturated phospholipid species (16:0,18:1-PC, 16:0,18:2-PC, 18:0,20:4-PC), and cholesterol were exposed to HOCl, GC-MS analysis (**Fig. 2**) revealed the presence of fatty acid chlorohydrin reaction products as previously described (9), plus the cholesterol reaction products described above. At the HOCl concentration used (HOCl:lipid ratio 1:1) the bulk of the fatty acid reaction products consisted of monochlorohydrin derivatives of 18:1 (peak cluster **15**), 18:2 (peak cluster **14**), and 20:4 (peak cluster **16**), formed by addition of HOCl across one double bond. Small amounts of bischlorohydrin derivatives were also found (peak clusters **17** and **18**). The identity and distribution of cholesterol and fatty acid products formed in the mixture were similar to what was observed when either fatty acid or cholesterol was the only oxidizable substrate. As noted before (9), the monounsaturated 18:1 showed a comparable extent of modification as the polyunsaturated 18:2 and 20:4. A rough comparison on the basis of peak areas suggests that individual unsaturated fatty acyl chains of membrane phospholipids and cholesterol display similar susceptibilities to HOCl-mediated oxidation.

In the reaction of HOCl with cholesterol, no evidence was found for addition of HOCl across the Δ5-6 double bond to form a chlorohydrin derivative. Instead, the major products formed were the isomeric 5,6-epoxides. Fatty acid epoxides can be formed by elimination of HCl from chlorohydrins (9), but only at high pH. To test whether cholesterol epoxide formation was sensitive to pH, the di16:0-PC/cholesterol vesicle experiments were repeated at pH values ranging from 5 to 8. Cholesterol epoxide formation was observed over the entire pH range, with no evidence for chlorohydrin formation. Lowering the pH in the experiments with vesicles consisting of a mixture of unsaturated PC and cholesterol also did not influence cholesterol epoxide formation.

Experiments were also carried out in which vesicles consisting of 16:0,18:1-PC, 16:0,18:2-PC, and cholesterol were treated with the $MPO/H_2O_2/Cl^-$ system instead of reagent NaOCl. The HOCl-generating system was less efficient, with only about 5% modification when $H_2O_2$



Downloaded from www.jlr.org by guest, on June 17, 2012

ASBMB JOURNAL OF LIPID RESEARCH

JOURNAL OF LIPID RESEARCH

ASBMB

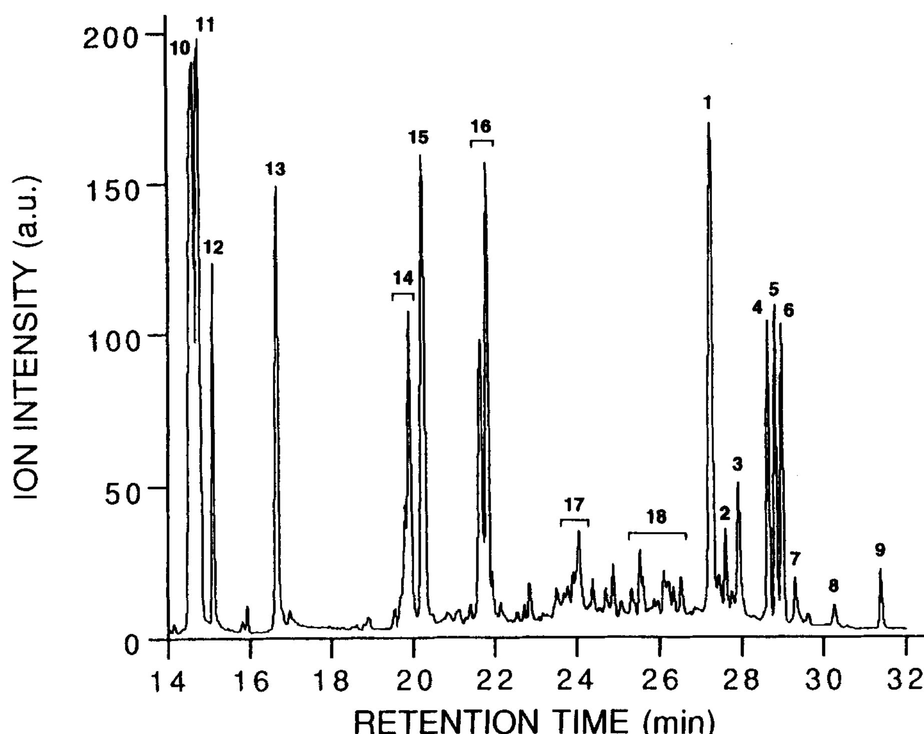


**Fig. 2.** GC-MS total ion chromatogram obtained after reaction of PC/cholesterol vesicles containing equal amounts of 18:1, 18:2, and 20:4 as the *sn*-2 fatty acids with HOCl (HOCl:phospholipid ratio 1:1). After incubation, phospholipids were hydrolyzed with phospholipase $A_2$, and lipids were extracted and derivatized to the corresponding TMS ester/TMS ethers as described in Methods. On the basis of fragment ion structural assignment and comparison of mass spectra and retention times of products with pure standards, the following product identification was obtained: 1 = cholesterol; 2 = 7α-hydroxycholesterol (5-cholestene-3β,7α-diol); 3 = 4-cholestene-3β,6β-diol; 4 = cholesterol 5β,6β-epoxide (5β,6β-epoxycholestan-3β-ol); 5 = cholesterol 5α,6α-epoxide (5α,6α-epoxycholestan-3β-ol); 6 = 4-hydroxycholesterol (5-cholestene-3β,4β-diol); 7 = 6-hydroxycholesterol (5-cholestene-3β,6α/β-diol); 8 = 6-ketocholestanol (5-cholesten-3β-ol-6-one); 9 = unknown (possibly cholestene-diol or cholestane-triol); 10 = 18:2; 11 = 18:1; 12 = 18:0; 13 = 20:4; 14 = 18:1 monochlorohydrin isomers (18:2 reaction products); 15 = 18:0 chlorohydrin isomers (18:1 reaction products); 16 = 20:3 monochlorohydrins (20:4 reaction products); 17 = 18:0 bischlorohydrins (18:2 reaction products); 18 = 20:2 bischlorohydrins (20:4 reaction products).

was added at a mole ratio of 0.2 (relative to total lipid), instead of theoretically 20%. This lower efficiency appeared to be caused to a large extent by reaction of HOCl with MPO (inactivating the enzyme) rather than with the vesicles. This could not be completely avoided despite careful experimentation as described in Materials and Methods. The pattern of product formation, however, was basically the same. No fatty acid bischlorohydrins were formed. The predominant products with their estimated relative amounts (based on peak area calculation) in brackets were: 18:1 monochlorohydrins (33%), 18:0 chlorohydrins (37%), epimeric cholesterol 5,6-epoxides (22%), and 4β-hydroxycholesterol (8%).

## DISCUSSION

Oxidative modification of lipids alters their physical properties to such an extent that they may undermine the structural integrity of the membrane. Most cells have an impressive arsenal of antioxidant compounds and enzymes at their disposal that protects against the generation and accumulation of lipid oxidation products in their membranes. Nevertheless, lipid oxidative damage does occur in vivo and plays a role in many pathological conditions, its suggested role in atherogenesis a prominent example (15-17). Neutrophils and monocytes generate reactive oxygen species including HOCl (2). Reactions of HOCl with membrane constituents resulting in loss of cellular integrity could be involved in bacterial killing as well as cytotoxicity/tissue injury caused by these inflammatory cells. They could also promote atherosclerosis by contributing to oxidative modification of LDL particles either through direct interaction or indirectly after transfer of HOCl-modified lipids from other sites (inflamed tissue, erythrocytes) to LDL.

We have previously shown that HOCl adds across double bonds of unsaturated fatty acid to give fatty acid

Downloaded from www.jlr.org by guest, on June 17, 2012

chlorohydrins (9). This reaction was observed both with fatty acid micelles and with vesicle membranes composed of unsaturated phospholipid species. The present study shows that HOCl also reacts efficiently with cholesterol, a major and important component of most biological membranes, and that in mixed vesicles modification of both cholesterol and fatty acyl groups is observed. **Fig. 3** provides an overview of the reaction products of unsaturated fatty acids and cholesterol with HOCl as they have been identified using GC–MS. Bis- and polychlorohydrins are not given, as formation of such compounds is considered less likely under physiological conditions.

The major products of the reaction of HOCl with cholesterol were the epimeric 5,6-epoxides, with hydroxy-derivatives also formed. No chlorohydrin products were detected. Epoxides can be formed from fatty acid chlorohydrins, but only at a higher pH (8–9) (9). With cholesterol, however, epoxides remained the major oxidation products and no chlorohydrin formation was observed throughout the pH range of 5–8. This indicates a mechanism for the reaction of HOCl with cholesterol that is distinct from the reaction with fatty acids, which is probably governed by the different molecular configurations around their respective susceptible double bonds. A partial identification of reaction products of HOCl with cholesterol supported on microbeads has been provided before and is in general agreement with our present results (18). Hydroxy- and keto-derivatization has also been observed in the reaction of HOCl with other cyclic aromatic compounds (19). It was beyond the scope of this investigation to conduct detailed comparative studies of reaction mechanisms at nonphysiologic pH, and it was therefore not tested whether chlorohydrin formation from cholesterol could be observed at extremely low pH.

The reaction products of unsaturated lipids with HOCl are expected to be relatively stable. Unlike the primary products of peroxidation reactions, lipid hydroperoxides, they are not susceptible to rapid decomposition in the presence of metal ions, generating radicals and propagating oxidative damage. However, HOCl-modified lipids can exert cytotoxic and biophysical effects.

Cholesterol oxidation products have diverse cytotoxic

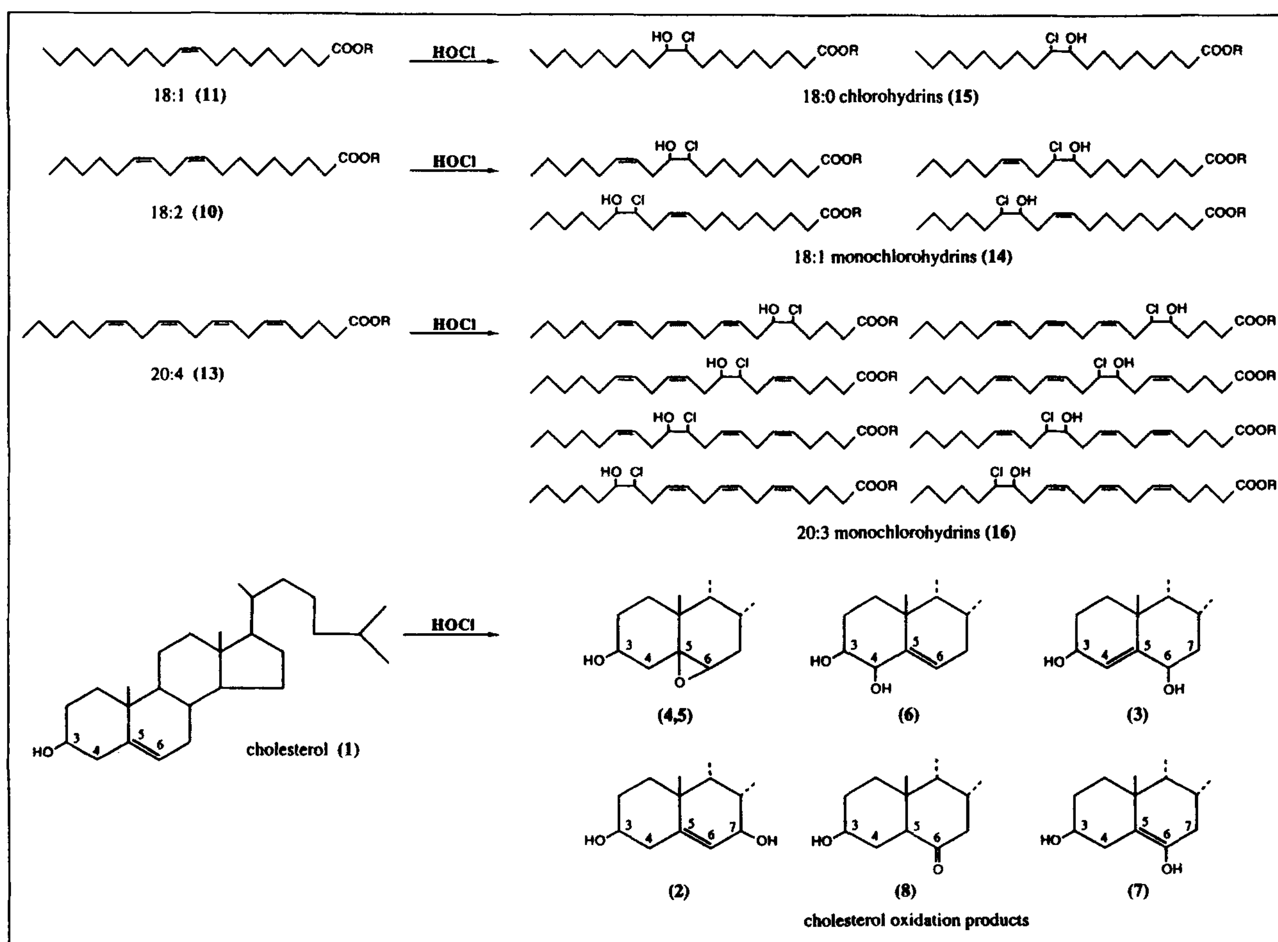


**Fig. 3.** Reaction products of unsaturated fatty acids and cholesterol with hypochlorous acid. Bold numbers in brackets refer to peaks or peak clusters identified in Fig. 2. Only monochlorohydrin derivatives of polyunsaturated fatty acids are given here, as these are the most common reaction products at low HOCl:lipid ratios likely to be important under physiological conditions. Product names are given in the legend of Fig. 2.



Downloaded from www.jlr.org by guest, on June 17, 2012
ASBMB JOURNAL OF LIPID RESEARCH JLR

effects. Epoxy-, keto-, and hydroxy-derivatives of cholesterol affect de novo sterol biosynthesis, DNA synthesis, cellular functions, and cellular growth and proliferation (20). In addition to direct cytotoxicity, the effects that oxysterols exert on plasma membrane structure and function could be an important mechanism by which they mediate their toxic effects. Biophysical data on the effects on membrane structure of all oxidized cholesterol species of interest in the present study are not available, but, in general, oxidation of cholesterol has been shown to affect its function as a lipid modulator in membranes. Thus, lipid monolayer studies have indicated that ketosterols occupy increased molecular surface areas and have lost the condensing effect on unsaturated fatty acyl chains characteristic of cholesterol (21, 22). This is linked to a loss of membrane barrier function, as the glucose permeability of liposomes decreased with increasing cholesterol concentration, but actually increased with increasing ketocholesterol concentration (22). Oxysterols such as 7-hydroxycholesterol have been found to influence erythrocyte and model membrane structure by increasing lipid packing through increased interaction with phospholipid head groups, immobilizing fatty acyl chains, and by increasing protein helical structure (23). In overview, the effects of oxidized cholesterol species are diverse and dependent on the nature of the oxidized species. As several oxidized species are formed from reaction of cholesterol with HOCl, the overall effect is hard to predict and should be considered in combination with phospholipid modifications, as these lipid types interact in a membrane.

The presence of hydroxyl and chlorine moieties makes an HOCl-modified fatty acyl chain much more polar than its parent compound. We have recently shown that the introduction of a hydroperoxyl or a hydroxyl moiety in an unsaturated phospholipid leads to conformational changes in the molecule as expressed in a drastically larger molecular surface area measured in a lipid monolayer system (24). Thus, oxidized phospholipids form membrane packing defects that can result in membrane destabilization, e.g., causing increased permeability and decreased fluidity (25, 26). Although no experimental data are available yet on the physicochemical properties of fatty acid chlorohydrins, similar if not larger disruptive effects would be expected from these compounds. We speculate that the presence of phospholipid chlorohydrins in combination with oxidized cholesterol could be especially detrimental to the membrane architecture.

The present study has shown that unsaturated fatty acyl chains of membrane phospholipids and cholesterol exhibit comparable susceptibilities toward HOCl. The chlorohydrins and epoxides that are the major products have also been observed when composite vesicles are treated with a $MPO/H_2O_2/Cl^-$ system. The consequences of chlorohydrin formation and cholesterol oxidation by HOCl in relation to the bactericidal action and cytotoxicity of neutrophils have hitherto hardly been considered. However, in view of the potentially disrupting effects of their respective reaction products on membrane structure, fatty acid as well as cholesterol modification should be considered as potential contributors to neutrophil-mediated injury. In that regard, we have recently obtained evidence for modification of lipids as well as proteins in membranes of erythrocytes undergoing HOCl-mediated lysis (Vissers, M. C. M., A. Stern, F. A. Kuypers, J. J. M. van den Berg, and C. C. Winterbourn, unpublished results). The identity of the products is being confirmed by GC-MS.

In conclusion, our demonstration of the formation by HOCl and $MPO/H_2O_2/Cl^-$ of fatty acid chlorohydrins and cholesterol oxidation products has identified a number of compounds that have up to now hardly been considered but that could potentially be important mediators of neutrophil bactericidal action and cytotoxicity. These results warrant further investigation to define the role of oxidized lipids, phospholipids as well as cholesterol, relative to oxidation of other substrates in the mechanism of HOCl- and neutrophil-mediated membrane disruption.

We are grateful to Dr. Bertram Lubin for his support of this work and to Drs. Cedric Shackleton, Ingemar Björkhem, and Erik Lund for their expert advice on mass spectrometric analysis and cholesterol oxidations. We also thank Ms. Esther Roitman for GC-MS analysis of samples, and Ms. Maggie Yee for her excellent technical assistance. The present study was supported by National Institute of Health grants HL 27059, DK 32094, and DK 34400, the Health Research Council of New Zealand, and by the State of California, Department of Health and Human Services, through the Tobacco-Related Disease Research Program of the University of California (New Investigator Award 4KT-0265 to J. J. M. van den Berg).

*Manuscript received 14 March 1993 and in revised form 15 June 1993.*

## REFERENCES

1. Fantone, J. C., and P. A. Ward. 1982. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *Am. J. Physiol.* **107:** 397–418.
2. Klebanoff, S. J. 1988. Phagocytic cells: products of oxygen metabolism. *In* Inflammation: Basic Principles and Clinical Correlates. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 391–443.
3. Farber, J. L., M. E. Kyle, and J. B. Coleman. 1990. Biology of disease. Mechanisms of cell injury by activated oxygen species. *Lab. Invest.* **62:** 670–679.
4. Weiss, S. J., R. Klein, A. Slivka, and M. Wei. 1982. Chlorination of taurine by human neutrophils. *J. Clin. Invest.* **70:** 598–607.
5. Albrich, J. M., C. A. McCarthy, and J. K. Hurst. 1981. Biological reactivity of hypochlorous acid: implications for microbicidal mechanisms of leukocyte myeloperoxidase. *Proc. Natl. Acad. Sci. USA.* **78:** 210–214.
6. Schraufstätter, I. U., K. Browne, A. Harris, P. A. Hyslop,



Downloaded from www.jlr.org by guest, on June 17, 2012

ASBMB JOURNAL OF LIPID RESEARCH

J. H. Jackson, O. Quehenberger, and C. G. Cochrane. 1990. Mechanism of hypochlorite injury of target cells. *J. Clin. Invest.* **85:** 554–562.
7. Carlin, G., and R. Djursäter. 1988. Peroxidation of phospholipids promoted by myeloperoxidase. *Free Radical Res. Commun.* **4:** 251–257.
8. Winterbourn, C. C., H. P. Monteiro, and C. F. Galilee. 1990. Ferritin-dependent lipid peroxidation by stimulated neutrophils: inhibition by myeloperoxidase-derived hypochlorous acid but not by endogenous lactoferrin. *Biochim. Biophys. Acta.* **1055:** 179–185.
9. Winterbourn, C. C., J. J. M. van den Berg, E. Roitman, and F. A. Kuypers. 1992. Chlorohydrin formation from unsaturated fatty acids reacted with hypochlorous acid. *Arch. Biochem. Biophys.* **296:** 247–255.
10. Kettle, A. J., and C. C. Winterbourn. 1988. Superoxide modulates the activity of myeloperoxidase and optimizes the production of hypochlorous acid. *Biochem. J.* **252:** 529–536.
11. Mayer, L. D., M. J. Hope, and P. R. Cullis. 1986. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta.* **858:** 161–168.
12. Aringer, L. 1980. Oxidation of 3-oxygenated Δ4- and Δ5-$C^{27}$ steroids by soybean lipoxygenase and rat liver microsomes. *Lipids.* **15:** 563–571.
13. Aringer, L., and L. Nordström. 1981. Chromatographic properties and mass spectrometric fragmentation of dioxygenated $C^{27}$-, $C^{28}$-, $C^{29}$-steroids. *Biomed. Mass Spectrom.* **8:** 183–203.
14. Brooks, C. J. W., W. J. Cole, T. D. Y. Lawrie, J. MacLachlan, J. H. Borthwick, and G. M. Barrett. 1983. Selective reactions in the analytical characterization of steroids by gas chromatography-mass spectrometry. *J. Steroid Biochem.* **19:** 189–201.
15. Quinn, M. T., S. Parthasarathy, L. G. Fong, and D. Steinberg. 1987. Oxidatively modified low density lipoprotein: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc. Natl. Acad. Sci. USA.* **84:** 2995–2998.
16. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
17. Steinbrecher, U. P., H. Zhang, and M. Lougheed. 1990. Role of oxidatively modified LDL in atherosclerosis. *Free Radical Biol. Med.* **9:** 155–168.
18. Foote, C. S., R. B. Abakerli, R. L. Clough, and F. C. Shook. 1980. On the question of singlet oxygen production in leucocytes, macrophages and the dismutation of superoxide anion. *In* Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. W. H. Bannister and J. V. Bannister, editors. Elsevier/North Holland, Amsterdam. 222–230.
19. Ohki, T., K. Maeda, J. Sakakibara, E. Suzuki, and N. Yamanaka. 1993. Structural analysis of oxidation products of urofuran acid by hypochlorous acid. *Lipids.* **28:** 35–41.
20. Smith, L. L., and B. H. Johnson. 1989. Biological activities of oxysterols. *Free Radical Biol. Med.* **7:** 285–332.
21. Demel, R. A., K. R. Bruckdorfer, and L. L. M. van Deenen. 1972. Structural requirements of sterols for the interaction with lecithin at the air-water interface. *Biochim. Biophys. Acta.* **255:** 304–310.
22. Demel, R. A., K. R. Bruckdorfer, and L. L. M. van Deenen. 1972. The effect of sterol structure on the permeability of liposomes to glucose, glycerol and Rb+. *Biochim. Biophys. Acta.* **255:** 321–330.
23. Kucuk, O., L. J. Lis, T. Dey, R. Mata, M. P. Westerman, S. Yachnin, R. Szostek, D. Tracy, J. W. Kauffman, D. A. Gage, and C. C. Sweeley. 1992. The effects of cholesterol oxidation products in sickle and normal red blood cell membranes. *Biochim. Biophys. Acta.* **1103:** 296–302.
24. van den Berg, J. J. M., J. A. F. Op den Kamp, B. H. Lubin, and F. A. Kuypers. 1993. Conformational changes in oxidized phospholipids and their preferential hydrolysis by phospholipase $A_2$: a monolayer study. *Biochemistry.* **32:** 4962–4967.
25. Sugihara, T., W. Rawicz, E. A. Evans, and R. P. Hebbel. 1991. Lipid hydroperoxides permit deformation-dependent leak of monovalent cation from erythrocytes. *Blood.* **77:** 2757–2763.
26. Wratten, M. L., G. van Ginkel, A. Bekker, E. van Faassen, F. Siemssen, A. van't Veld, and A. Sevanian. 1991. Structural and dynamic effects of oxidized lipids in unsaturated lipid membranes in relation to vitamin E activity. *In* Oxidative Damage and Repair. Chemical, Biological and Medical Aspects. K. J. A. Davies, editor. Pergamon Press, New York. 587–593.



Downloaded from www.jlr.org by guest, on June 17, 2012