Interception of Discrete Oxygen Species in Aqueous Media by Cholesterol: Formation of Cholesterol Epoxides and Secosterols

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ABSTRACT

The oxidation products of cholesterol oxidized by different dioxygen species, hydroxyl radical, or triatomic species ozone variously implicated in biological oxidations are compared. Some products are unique to the oxygen species involved, whereas others, such as the isomeric cholesterol 5,6-epoxides, are formed broadly by many oxygen species. The resolution of the isomeric cholesterol 5,6-epoxides by capillary column gas chromatography on SE-30 or SE-54 has been achieved.
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INTRODUCTION

Several discrete oxygen species are implicated in specific enzyme oxidations, generalized enzymic lipid peroxidations, endogenous metabolic processes such as phagocytosis, and in nonenzymic peroxidations and autoxidations as well as in the expression of environmental oxygen toxicity. However, demonstration of the unique participation of individual oxygen species is confounded by experimental limitations and by systems dynamics where more than one oxygen species is present.

It has been our interest to devise means of interception of active oxygen species in chemical and biological systems so that from the oxidation products formed one can infer the nature of the oxygen species involved in the oxidations. Of critical importance to applications of these approaches to living systems is full knowledge of the chemistry occurring in aqueous media as well as in conventional organic solvents. Such interceptions of oxidizing species have been probed using the ubiquitous cellular membrane component cholesterol in studies of the monooxygen species hydroxyl radical HO• (1) and the following dioxygen species: ground-state dioxygen $^3O_2$ involved in specific enzymic hydroxylations and in free radical peroxidations (2-5) and autoxidations (6-9), electronically excited (singlet) dioxygen $^1O_2$ implicated in photosensitized oxygenations (10-17) and other cases (17-19), the 1-electron reduced species superoxide radical $O_2^{\overline{\cdot}}$ generated in some enzymic oxidations (20), the 2-electron reduced species peroxide $O_2^{=}$ (18,19) and organic hydroperoxides (21), and the dioxygen cation O_2^+ which is not a likely biological species (22).

We have now extended this approach to the triatomic species ozone O_3. Ozonization of cholesterol and its 3β-acetate in nonaqueous media yields poorly characterized ozonides (23-26); in aqueous media, little chemical work has been conducted (27-29). The present report outlines our recent work on cholesterol ozonization and provides a comparison of all results testing the use of cholesterol for the interception of defined oxygen species.

EXPERIMENTAL

Chromatography

Thin layer chromatography (TLC) was conducted on Kieselgel 60, F-254 chromatoplates (E. Merck GmbH, Darmstadt) and on 10-cm-long Alugram Sil G/UV$_{254}$ aluminum-backed chromatostrips (Machery Nagel, Düren) irrigated with benzene/ethyl acetate mixtures. Sterols were detected by ultraviolet light absorption (254 nm), by N,N-dimethyl-*p*-phenylenediamine spray for peroxides (30), and by 50% sulfuric acid spray followed by heating to full color display and/or to charring. High performance liquid chromatography (HPLC) was conducted on 2 3.9 mm x 30 cm μPorasil microparticulate (10 μm diam) adsorption columns in tandem (Waters Associates, Milford, MA) irrigated with hexane/isopropyl alcohol (24:1, v/v) flowing at 2.0 ml/min. Effluent was monitored at 212 nm with a Perkin-Elmer Corp. Model LC-55 variable wavelength spectrophotometric detector and by differential refractive index using a Waters Associates' Model R4-1 detector (31).

Gas chromatography was conducted with a Hewlett-Packard Model 5880A chromatograph equipped with hydrogen flame ionization detector (nitrogen carrier gas) or with a Finnigan Corp. Model 3300 gas chromatograph-mass

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spectrometer (GC-MS) (helium carrier gas), using fused silica capillary columns, 0.2-mm id, 5-35-m-long wall coated with SE-30 (Applied Science, State College, PA) or SE-54 (Hewlett-Packard, Palo Alto, CA). Splitless injections of 10-500 ng sterols in 1 μl toluene via a Grob injector were made into the capillary columns. Injector and detector temperature was 285 C; oven temperature was programmed from 100 C held for 1 min to 270 C at 20 C/min. When using the quadrupole mass spectrometer for detection, the capillary column was introduced directly into the ionization source, mass spectra being scanned at 40 scans/min over the range 100-500 amu, using 22 eV ionization.

Quantitation of cholesterol 5,6-epoxides was achieved using cholesterol as an internal standard and integration of peak areas on ion chromatograms constructed with ion m/z 386 for cholesterol and m/z 402 for the cholesterol 5,6-epoxides. A plot of (m/z 402)/(m/z 386) ion abundances vs (cholesterol 5,6-epoxide/cholesterol) weight ratio was linear over the range 10-500 ng for both cholesterol 5α,6α-epoxide (*3*) and cholesterol 5β,6β-epoxide (*4*). Useful mass spectra of both 5,6-epoxides were also obtained over the 50-100 ng range.

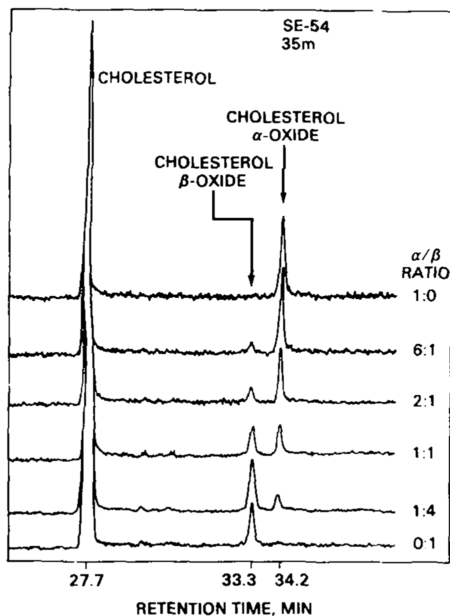


FIG. 1. Capillary column gas chromatography on SE-54 (35 m) of cholesterol and the isomeric cholesterol 5,6-epoxides *3* and *4* in various *3*/*4* ratios.

Ozonization

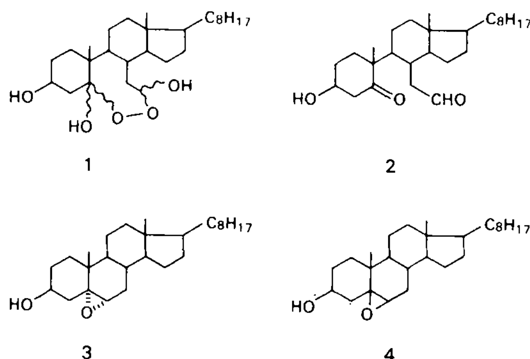
Dispersions of pure cholesterol in water were made by dissolving 100 mg cholesterol in 50 ml acetone, adding the solution under vacuum to 120 ml distilled water, and evaporating the dispersion under vacuum to remove solvent and provide a 1 mg/ml concentration. The dispersion was filtered through sintered glass and used as such. Ozone generated using a Tesla coil leak detector acting on a stream of oxygen flowing at 1 ℓ/min was passed through the cholesterol dispersions at room temperature for 2 hr or until cholesterol was totally destroyed, as evinced by TLC. Sterols were recovered by extraction with equal volumes of benzene and the dried extracts were evaporated under vacuum to yield crude ozonization products. Individual products were isolated by HPLC.

RESULTS

In distinction to cholesterol ozonization in organic solvents where only poorly characterized products are described (23-26), ozonization of cholesterol in water yielded 4 isolable products: a major peroxidic product presumed to be an ozonide hydrate 5ξ,6ξ-epidioxy-5,6-secocholestane-3β,5ξ,6ξ-triol (*1*) and non-peroxidic products 3β-hydroxy-5-oxo-5,6-seco-cholestan-6-al (*2*) and the isomeric cholesterol 5,6-epoxides 5,6α-epoxy-5α-cholestan-3β-ol (*3*) and 5,6β-epoxy-5β-cholestan-3β-ol (*4*) (Fig. 1). Details of identification of these sterols will be reported elsewhere.

The ozonide hydrate *1* appears to be a product of addition of the elements of water to a putative ozonide initially formed and the secoaldehyde *2* appears to be a transformation product of *1* or of an initially formed ozonide. The mechanism by which the cholesterol 5,6-epoxides are formed is uncertain. It is possible that cholesterol is epoxidized by O_3 or other unrecognized oxidant formed early in the reaction. We have been unable to demonstrate the epoxidation of cholesterol by the ozonide hydrate *1* in the manner cholesterol is epoxidized by sterol hydroperoxides (21).

The 4 products *1*-*4* can be resolved by TLC and HPLC. We report here for the first time the resolution of the underivatized cholesterol 5,6-epoxides *3* and *4* by GC with capillary columns (cf. Table 1). The ozonide hydrate *1* is characterized by a positive N,N-dimethyl-*p*-phenylenediamine color test, and both 5,6-secosterols *1* and *2* give a characteristic brown color with 50% sulfuric acid on thin layer chromatograms which is diagnostic of destruction of the sterol Δ^5-double bond. Secosterols *1* and *2* are thus readily distinguished from



SCHEME 1

major products of cholesterol autoxidation by $^3O_2$, where intense blue colors with 50% sulfuric acid characterize the initially formed sterol hydroperoxides and their reduction products, the epimeric cholest-5-ene-3β,7-diols (32).

Even though the ozonization of cholesterol involved molecular oxygen (containing O_3), at no time were autoxidation products detected. Clearly, O_3 reacted with cholesterol more rapidly than $^3O_2$. Moreover, in these simple water dispersions, no HO• oxidations of cholesterol were apparent.

Analysis of ozonization products by HPLC known to resolve the 5,6-epoxides *3* and *4* and by capillary column GC established a *3*/*4* ratio of 1:8. Capillary column gas chromatography of *3* and *4* derived by oxidation of cholesterol in chloroform with *m*-chloroperbenzoic acid and of *3* 3β-acetate and *4* 3β-acetate from cholesterol 3β-acetate similarly oxidized gave other ratios (data incorporated into Table 3).

In the case of the peracid epoxidations, the analysis of products was conducted using a 5-m-long capillary column of SE-54 under the same conditions used with the longer 35-m columns. Using the 5-m column, the retention times were obtained: *3*, 12.28 min; *4*, 12.11 min; *3* 3β-acetate, 13.15 min; *4* 3β-acetate, 12.69 min.

Resolution of the 3β-acetates and 3β-trimethylsilyl ethers of the isomeric 5,6-epoxides *3* and *4* is also readily achieved using capillary columns. Retention data are included in Table 1. Chromatographic resolution of 5,6-epoxides 3β-acetates (33), 3β-benzoates (31,34), and 3β-trimethylsilyl ethers (33) has been previously achieved, and resolution of free sterols *3* and *4* without derivatization by HPLC has been described (31,35). Although small differences in gas chromatographic retention times for *3* and *4* have been noted using packed columns (21,33,36), resolution by that means has not been forthcoming.

Epoxidation of cholesterol by O_3 has not been recognized as occurring, but the 5β,6β-epoxide *4* 3β-acetate was formed in the ozonization of cholesterol 3β-acetate (26). The 5α,6α-epoxide *3* which is interesting as a toxic agent (37,38) has not been recognized as a product of ozonization under any conditions.

DISCUSSION

The successful application of cholesterol or other substrates for interception of active oxygen species depends on the capacity of the interceptor to react with the oxygen species uniquely and at a rate sufficient to compete with other reactions the oxygen species may undergo. The uniqueness of reaction products from cholesterol may now be fairly tested; data in Table 2 summarize the present knowledge.

It is seen that unique products form in several instances. Thus, cholesterol 7-hydroperoxides are uniquely the result of free radical autoxidation or lipid peroxidations. Likewise, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide is found only in systems in which $^1O_2$ is implicated. Furthermore, 5,6-secosterols are unique to O_3 oxidations. The other oxidation products in Table 2 permit no unique inference to be drawn as to the oxidizing species involved.

Table 2 does not include specific enzymic oxidations of cholesterol, but enzymic metabolites may be encountered in systems containing active enzymes. Thus, cholest-5-ene-3β,7α-diol is a hepatic metabolite of cholesterol as well as product of $^3O_2$, O_2^+ and HO• nonenzymic oxidations, and the 5α,6α-epoxide 3 also has enzymic (39,40) and multiple nonenzymic origins.

With exceptions of data in Table 2 for O_2^+ where products listed are for gas-phase oxidations (22) and for $^3O_2$ attack in the sterol side chain and at the 3β-hydroxyl group where products are those of solid-state reactions (7,8), the products listed are those formed in aqueous media. There is no reaction of the defined oxygen species $O_2^{\overline{\cdot}}$ with cholesterol, whether it be generated in chemical, photochemical, electrochemical, or enzyme systems, in aqueous or anhydrous organic solvent media (20).

For the highly reactive HO• and O_3 species, the speed of reaction with cholesterol in water insures product formation, and although $^3O_2$ reactions are sluggish at ambient temperature, products do ultimately form, as $^3O_2$ is a stable

TABLE 1

Chromatographic Properties of Cholesterol Ozonization Products

Sterol	Thin layer chromatography[a] (R_f)			Liquid column chromatography[b] (t_R[min])	Gas chromatography[c] (t_R[min])	
	1	2	3		1	2
Cholesterol	0.70	0.77	0.69	6.0 (3.0)	–	22.7
Cholesterol 3β-acetate	–	–	–	–	–	33.5
Cholesterol 3β-trimethylsilyl ether	–	–	–	–	–	29.5
Cholesterol 5α,6α-epoxide (*3*)	0.40	0.44	0.33	13.0 (6.4)	27.1	34.2
Cholesterol 5α,6α-epoxide 3β-acetate	–	–	–	–	29.4	44.1
Cholesterol 5α,6α-epoxide 3β-trimethylsilyl ether	–	–	–	–	–	36.1
Cholesterol 5β,6β-epoxide (*4*)	0.41	0.45	0.35	14.0 (7.3)	26.6	33.3
Cholesterol 5β,6β-epoxide 3β-acetate	–	–	–	–	28.0	39.8
Cholesterol 5β,6β-epoxide 3β-trimethyl silyl ether	–	–	–	–	–	35.1
Cholesterol ozonide hydrate (*1*)	0.20	0.26	0.14	28.0 (14.2)	dec.	–
3β-Hydroxy-5-oxo-5,6-secocholestan-6-al (*2*)	0.43	0.49	0.37	17.2 (8.2)	24.0	–
3β-Hydroxy-5-oxo-5,6-secocholestan-6-oic acid	0.14	0.19	0.10	–	–	–
5β-5,6-Secocholestane-3β,5α,6-triol	0.00	0.02	0.00	–	–	–

[a]System 1, Kieselgel 60, F-254, benzene/ethyl acetate (3:2, v/v); system 2, Alugram Sil G/UV_{254}, benzene/ethyl acetate (3:2, v/v); system 3, Alugram Sil G/UV_{254}, benzene/ethyl acetate (7:3, v/v).

[b]=μPorasil, hexane/isopropyl alcohol (24:1, v/v), 2.0 ml/min. Data in parentheses are for only one column.

[c]System 1, 25-m SE-30 capillary column; system 2, 35-m SE-54 capillary column.

TABLE 2

Cholesterol Oxidation Products Formed by Defined Oxygen Species in Water

Site of attack	Oxidation products	Oxygen species						
		$^3O_2$	$^1O_2$	$O_2^{\overline{\cdot}}$	$O_2^=$	O_2^+	HO•	O_3
C-7	7-Hydroperoxides	+						
C-7	7-Alcohols[a]	+				+[b]	+	
	7-Ketones[a]	+				+[b]	+	
Side chain[c]	Hydroperoxides	+						
	Alcohols, ketones, etc.	+						
	C_{19}-C_{26} sterols[a]	+						
Δ^5	5,6-Epoxides	+			+	+[b]	+	+
	5,6-Secosterols							+
7α-H/Δ^5	Δ^6-5α-Hydroperoxide		+					
3β-Alcohol[c]	3-Ketones	+						

[a]Secondary products formed from initial hydroperoxides.
[b]Gas-phase reaction products.
[c]Solid-state reaction products.

species not altered by water. For $O_2^=$ and $^1O_2$ interceptions in aqueous media, the stability of the species must be considered. Whether $O_2^=$ is intercepted depends on whether disproportionation dissipates the species before the slower epoxidations occur. In model systems, both disproportionations and epoxidations are observed (18,19). Rapid quenching by water may preclude $^1O_2$ interception by cholesterol, as the reaction is slow. Nonetheless, where an adequate $^1O_2$ flux is maintained, the unique $^1O_2$ oxidation product 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide is formed in aqueous media (10-15).

As the 5,6-epoxides *3* and *4* are formed from cholesterol by attack of several oxygen species (cf. Table 2), their mere presence in a test sample does not imply the oxidizing species involved. The possibility remains that the proportions of *3* and *4* can provide clue to the processes implicated, and data of Table 3 appear to support such a contention. Thus,

TABLE 3

5,6-Epoxides Ratios Obtained in Cholesterol Epoxidations

Epoxidizing conditions	α/β	Ref.
Air (aq dispersions)	1:11	21
Sterol hydroperoxides (aq)	1:10	21
Ozone (aq)	1:8	—[a]
H_2O_2 (aq)	1:8	18,19
Dried egg	ca. 1:5	44
Incubations, liver enzymes	1:3.3 to 1:4.7	5,33,45-47
H_2O_2, Fe (III) acetylacetonate (aq acetonitrile)	1:4	48
Incubations, soybean lipoxygenase	1:3.7 to 1:4	5,33
Air, solid [4-^{14}C]cholesterol	1:3.6	33
Rabbit plasma, liver	1:2.1 to 1:3.4	49
HO^, X-radiolysis (methanol)	ca. 1:2	50
Air-aged USP cholesterol	ca. 1:1	8
MoO_5 (dichloroethane)	1.5:1	51
HO• (aq)	3.5:1	1
Organic peracids	6.8:1	—[a,b]
Methylpyridazine oxide photolysis	8:1	52
NO_2-treated rat lung, in vivo	8:1	53
Human serum	1:0	54
Incubations, bovine adrenal cortex mitochondria	1:0	39,40

[a]Present results.
[b]Conditions: *m*-chloroperbenzoic acid in chloroform; product analysis on 5-m SE-54 capillary column. Cholesterol 3β-acetate gave a 7:3 ratio, the same as previously obtained with perbenzoic acid (55).

β-face attack and a predominance of 5β,6β-epoxide *4* occur in systems involving di- or tri-oxygen species in autoxidations, lipid peroxidations and ozonizations, whereas α-face attack and predominance of 5α,6α-epoxide *3* be the case where one oxygen atom oxidants such as N-oxide photolysis, epoxidases, and peracids are involved. However, the *3/4* ratios may be misleading if preferential loss of the less stable 5β,6β-epoxide *4* occurs. The 1:1 ratio observed in naturally air-aged cholesterol appears to be such a case. The predominance of the 5β,6β-epoxide *4* would have been expected a priori.

In biological systems where only the 5α,6α-epoxide *3* is formed, the intervention of specific cholesterol 5α,6α-epoxidases is indicated. In other instances not listed, where formation of the 5α,6α-epoxide *3* is suggested (41-43), available data are inconclusive, as any combination of the 2 isomers might be present.

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