Oxidation of Cholesterol by Dioxygen Species[1]

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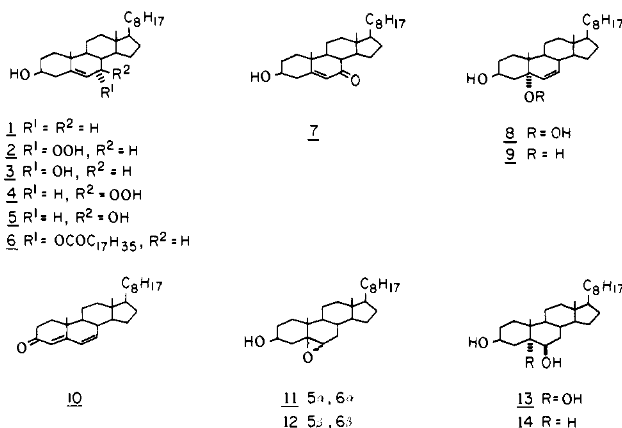
Received March 6, 1978

Abstract: Reaction between cholesterol in aqueous sodium stearate dispersions and H_2O_2 yielded many products of which 12 were isolated and identified. Products included 5,6α-epoxy-5α-cholestan-3β-ol and 5,6β-epoxy-5β-cholestan-3β-ol in 1:8 ratio representing direct reaction between cholesterol and H_2O_2 and 5α-cholestane-3β,5,6β-triol formed by hydration of the 5,6-epoxides; epimeric cholesterol 7-hydroperoxides, epimeric cholest-5-ene-3β,7-diols, and 3β-hydroxycholest-5-en-7-one representing free-radical oxidation of cholesterol by molecular oxygen derived by H_2O_2 disproportionation; 5α-cholest-6-ene-3β,5-diol, cholest-4,6-dien-3-one, and 7α-stearatoxycholest-5-en-3β-ol possibly derived from singlet molecular oxygen released in H_2O_2 disproportionation; and 5α-cholestane-3β,6β-diol. These results complete our studies of reactions between cholesterol and dioxygen species, revealing for the first time the several pathways of oxidation of a biologically important substrate by dioxygen species.

Introduction

The oxidation of biologically important natural products by means dependent on air proceeds by many processes to a variety of product types. Active oxidants may encompass monoatomic, diatomic, or triatomic oxygen species, and in such complexity a satisfactory understanding of the modes of oxidative alteration for a given substrate has not heretofore emerged. Even though controlled means of generation of defined oxygen species be used for reaction, demonstration of the direct participation of dioxygen[2] species depends upon evidence of the retention of the dioxygen oxygen–oxygen covalent bond in peroxide, hydroperoxide, dioxetane, or perepoxide products. Similarly, proof of trioxygen species participation in reactions depends upon demonstration of retention of two successive oxygen–oxygen covalent bonds in trioxide, hydrotrioxide, or molozonide products. Necessarily, work aimed at these demanding proofs is not always attempted, and the participation of dioxygen and trioxygen species is deduced from other arguments or evidence.

In order to provide a complete understanding of the reactions of an important substrate cholesterol (cholest-5-en-3β-ol) (**1**)



with dioxygen species we have examined its reactions with ground-state (triplet) molecular oxygen ($^3O_2$) in a variety of chemical and enzymic conditions,[3] with electronically excited (singlet) molecular oxygen ($^1O_2$),[4] with the dioxygen cation (O_2^+),[5] with the one-electron reduction product superoxide radical anion ($O_2^-\cdot$) or its conjugate hydroperoxyl radical (HOO·),[6] with organic hydroperoxides,[7] and with the two-electron reduction product hydrogen peroxide (H_2O_2), peroxide anion (O_2^{2-}), or hydroperoxide anion (HOO^-). Reaction with $^3O_2$ is best exemplified by the natural air aging of cholesterol where a large number of identified products are formed,[8] but where the major oxidation pathway yields the primary products 3β-hydroxycholest-5-ene 7α-hydroperoxide (**2**) and 3β-hydroxycholest-5-ene 7β-hydroperoxide (**4**),[3a] from which the secondary products cholest-5-ene-3β,7α-diol (**3**), cholest-5-ene-3β,7β-diol (**5**), and 3β-hydroxycholest-5-en-7-one (**7**) derive by thermal degradations.[9] By contrast reaction with $^1O_2$ yields 3β-hydroxy-5α-cholest-6-ene 5-hydroperoxide (**8**) and the epimeric 3β-hydroxycholest-4-ene 6-hydroperoxides.[4] Thermal decomposition of the 5α-hydroperoxide **8** affords the corresponding alcohol 5α-cholest-6-ene-3β,5-diol (**9**) and cholesta-4,6-diene-3-one (**10**).[9b,c] Clearly cholesterol reactions with $^3O_2$ and with $^1O_2$ take different courses.

The oxygen–oxygen bond of other dioxygen species is not retained in products of their reaction with cholesterol, O_2^+ yielding a complex mixture devoid of sterol hydroperoxides,[5] $O_2^-\cdot$ not reacting at all,[6] and H_2O_2 yielding epoxides as described here in detail.

Results

The attack of H_2O_2 on cholesterol in aqueous sodium stearate dispersions gave a complex mixture from which 11 well-known cholesterol oxidation products, **2–5**, **7**, **9**, **10**, isomeric epoxides 5,6α-epoxy-5α-cholestan-3β-ol (**11**) and 5,6β-epoxy-5β-cholestan-3β-ol (**12**), 5α-cholestane-3β,5,6β-triol (**13**), and 5α-cholestane-3β,6β-diol (**14**), were identified. There was additionally recovered a heretofore undescribed derivative, 7α-stearatoxycholest-5-en-3β-ol (**6**), the structure of which is established in a later section.

The time course of the reactions followed chromatographically showed that the 7-hydroperoxides **2** and **4** were formed first and that products **2–6** and **9** were present after 2 h at 70 °C, whereas the epoxides **11** and **12** were detected only after 3 h. Reintroduction of each product into the reaction system established that the 7α-hydroperoxide **2** was partially epimerized to **4** and decomposed to **3** and **7** and the 7β-hydroperoxide **4** decomposed to **5** and **7**. The 3β,7α-diol **3** was epimerized to **5**; the 7-ketone **7** was dehydrated to a minor degree to cholesta-3,5-dien-7-one. The epoxides **11** and **12** were partially hydrated to the 3β,5α,6β-triol **13**. Products **5**, **10**, **13**, and **14** were stable in the system.

The 3β,5α-diol **9** was transformed rapidly into the 3β,7-diols **3** and **5** and into the 7α-stearate ester **6**. Although the 5α-hydroperoxide **8** was not detected as a product, its introduction into the experimental system led to rapid isomerization to the 7α-hydroperoxide **2**, decomposition to the 3β,5α-diol **9** and dienone **10**, and transformation to the 7α-stearate ester **6**. These and subsequent transformations of initial products

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yielded **2–7**, **9**, and **10** as products derived from **8**, with **2–5** being detected after 1 h at 70 °C, **6**, **9**, and **10** after 2 h, and **7** only after 3 h, at which time traces of **8** remained.

The presence of some products and yields of all were sensitive to reaction condition changes. Yields of products **2–7** and **9–14** were only slightly greater at 70 °C than at 50 °C, and all products were detected at 37 °C as well. However, no products were detected at 0 °C or at 25 °C within the times studied. Moreover, adjustment of the pH of the aqueous sodium stearate dispersions of cholesterol within the range pH 5.2–9.6 gave products **2–7** and **9–13** but in diminished amounts in the low pH ranges. Furthermore, products **6**, **9**, and **10** were erratically detected in the pH range 5.2–7.5. Deletion of phosphate (pH adjustment with NaOH) gave products **2–7**, and **9–14**, as did also substitution of methanol for ethanol. Deletion of stearic acid gave poor dispersions in which products **2–5**, **7**, and **11–14** but not **6**, **9**, or **10** were observed, a pattern also obtained using aqueous cholesterol dispersions prepared without any other added components. Dispersions made with *rac*-1,2-dipalmitoyl-*sn*-glycero-3-phosphate, with nonionic surfactants Tween 20 or Tween 80, or with sodium taurocholate or glycocholate over the pH range 7.1–9.2 uniformly gave products **2–5** but only erratically gave products **9** and **10** in diminished yields.

Products **2–7**, **9**, and **10** appeared to derive as a consequence of the disproportionation of H_2O_2 to molecular oxygen. Whereas derivation of **2–5** and **7** by $^3O_2$ action thereby seems the case, formulation of **6**, **9**, and **10** as secondary products of $^1O_2$ action, as discussed later, demanded additional proof. Accordingly, conditions thought to test the means by which products **6**, **9**, and **10** derived were examined. The oxidation of H_2O_2 in aqueous sodium stearate dispersions of cholesterol by NaOCl, possibly yielding $^1O_2$,[10] was attempted, and the same product spate **2–7** and **9–14** in slightly greater yields was isolated. Substitution of ^2H_2O for water in making the cholesterol dispersions in anticipation of extending the lifetime of $^1O_2$ possibly generated[11] led to products **2–5**, **7**, and **11–13**, but **6**, **9**, and **10** were not found despite careful search.

The effects of *rac*-α-tocopherol as both a free-radical scavenger and $^1O_2$ quencher[12] and of 2,6-di-*tert*-butylphenol as an inhibitor of free-radical reactions but not of $^1O_2$ reactions[13] were examined. In both cases the $^1O_2$ products **6**, **9**, and **10** were not found, and products **2–5** were formed only in diminished amounts. The 7-ketone **7** was not detected in either experiment; the 5,6-epoxides **11** and **12** were found only with *rac*-α-tocopherol addition.

The pattern which emerges from these results is that the presence and yields of products **6**, **9**, and **10** are more sensitive to variations of experimental conditions that are the other identified products **2–5**, **7**, and **11–14**.

Structure of 7α-Stearate Ester 6. Recognition of product **6** as a stenediol related to **3**, **5**, and **9** was suggested by the development of a highly diagnostic characteristic blue coloration on thin layer chromatograms of **6** sprayed with sulfuric acid[8a] and by gas chromatographic elution curves of the pyrolysis products of **6** which disclosed cholesta-2,4,6-triene as product.[9b,c] Infrared absorptions at 3400, 1735, and 1660 cm^{-1} indicating hydroxyl, ester carbonyl, and olefin features, respectively, and a low-intensity ion *m/z* 669 recognized as the quasi-molecular ion $(M + H)^+$ recorded in the chemical ionization (CI) mass spectrum of **6**[14] sufficed for assignment of a stenediol monoester structure to **6**. Key fragment ions in the same spectrum included *m/z* 385 recognized as the ion $(M - 284 + H)^+$ derived from the sterol nucleus following ester elimination, *m/z* 367 as principal ion recognized as the ion $(M - 284 - H_2O + H)^+$ derived by double eliminations, and the prominent *m/z* 285 recognized as the quasi-molecular ion of the eliminated fatty acid octadecanoic (stearic) acid.[15-17]

These data confirm a stenediol monostearate structure for **6**. As cholesterol and other 3β-alcohols were not acylated under reaction conditions and **6** differed in melting point from 3β-stearatoxycholest-5-en-7-ol previously described,[18] a 3β,7-diol 7-monostearate structure was suggested. Proton spectra clearly established a 7α-monostearate structure for **6**. A one-proton broad multiplet at 3.55 ppm associated with the axial 3α hydrogen of a 3β-alcohol[19] and a one-proton signal at 5.55 ppm (doublet, J = 5 Hz) associated with a vinyl 6 proton disclosed these aspects of structure, and a poorly resolved one-proton doublet at 4.96 ppm (J = 4 Hz) associated with an equatorial or quasi-equatorial 7β hydrogen of a 7α-acyl ester supports in detail a 5-ene-3β,7α-diol 7α-monoester formulation for **6**.[20,21] The doublet character and magnitude of the coupling constant of the 6-proton signal additionally support the 7α configuration of **6**, as these features are found in proton spectra of 3β,7α-diol **3** and derivatives but not in those of the epimeric 3β,7β-diol **5** and derivatives.[22,23]

Discussion

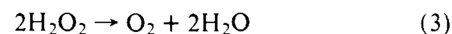
It is possible to associate identified products into four groups by means of qualitative rate and product stability studies. The products **2–5** and **7** are clearly interrelated as are the isomeric 5,6-epoxides **11** and **12** and their common hydration product **13**. Products **6**, **9**, and **10** also compose a related group, the 7α-stearate ester **6** deriving from **9** and all deriving from the 5α-hydroperoxide **8** in control studies. The 3β,6β-diol **14** stands alone apparently unrelated to other products.

The identity of each oxidation product save **14** is such as to allow us to infer the nature of the oxidation process and dioxygen species implicated in formation of that product from cholesterol. The 5,6-epoxides **11** and **12** isolated in low yield in the proportion 1:8 represent attack on cholesterol of H_2O_2 (or HOO^-) in analogy to the previously described attack of organic hydroperoxides on cholesterol where the epoxides were formed in 1:9 to 1:11 ratios.[7] The epoxides are also formed in the same ratio[7] or in the ratio 1:3.5[24] by air oxidation of cholesterol and probably represent thereby the intermediate formation of the 7-hydroperoxides **2** and **4** as previously described.[7] The 3β,5α,6β-triol **13** also represents the epoxidation process as well, for **13** is derived by hydration of both **11** and **12**.

The first-formed and most abundant products **2–5** and **7** by their chemical nature allow us to infer the oxidation of cholesterol by $^3O_2$ in free-radical processes.[3a] As these reactions were conducted under N_2, the only source of $^3O_2$ for these transformations is from the disproportionation of H_2O_2 or by unprecedented processes which will not be postulated at this point. The indicated disproportionation may be by base catalysis of eq 1–3,[25] by catalysis from adventitious traces of transition metal ions, by catalysis promoted by the cholesterol dispersed phase,[26] or by more than one such process.

$$H_2O_2 + HO^- \rightleftharpoons HOO^- + H_2O \quad (1)$$

$$H_2O_2 + HOO^- \rightarrow O_2 + HO^- + H_2O \quad (2)$$



$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad (3)$$

The next most abundant set of products includes the 3β,5α-diol **9**, the dienone **10**, and the 7α-stearate ester **6** derived from the action of H_2O_2 on cholesterol and by the transformation of the 5α-hydroperoxide **8** in the same system. The 3β,5α-diol **9** and dienone **10** are established thermal decomposition products of the 5α-hydroperoxide **8**,[9b,c] no other process for formation of **9** being known. The dienone **10** derived from **8** (but not from **9**) is also recognized as a product of the action of $^1O_2$ on cholest-5-en-3-one.[27]

In view of our demonstrated transformation of **8** to **6**, **9**, and **10** in these dispersions it is tempting to posit that these products indeed derive from **8** not detected but putatively formed as a transient intermediate by oxidation of substrate cholesterol. However, the presence of **8** allows us to infer the action of $^1O_2$

on cholesterol, as no other process is known which yields **8**! The 5α-hydroperoxide **8** is known only as a product of the attack of $^1O_2$ on cholesterol. To avoid this inference of $^1O_2$ action, derived from our best present information, it is necessary to postulate alternative mechanisms for derivation of **8** from cholesterol where experimental evidence is not available[28] or for derivation of **6**, **9**, and **10** by mechanisms not involving **8**.[29] A definitive choice among these possibilities cannot be made now, but the inference of $^1O_2$ action on cholesterol will be discussed further.

The presence of $^1O_2$ in the system can derive from only one process, the disproportionation of H_2O_2, and thermodynamic considerations allow generation of the $^1\Delta_g$ state of $^1O_2$ from H_2O_2.[30] Nevertheless, although chemical and spectral data support the formation of $^1O_2$ from H_2O_2 upon oxidation with hypochlorite or halogen[31] and in certain other reactions,[32] release of $^1O_2$ from H_2O_2 disproportionation has not been previously demonstrated.[33] Moreover, our efforts to provide additional experimental support of the thesis have not been successful. Thus, although we isolated the same product spate **2–7** and **9–14** in slightly improved yield from aqueous cholesterol dispersions containing H_2O_2 and NaOCl in a modification of procedures in which $^1O_2$ is implicated,[10] the 5α-hydroperoxide **8** was not among products. Experiments designed to prolong the lifetime of any $^1O_2$ generated, using ^2H_2O instead of water,[11] also did not lead to **8** as product; indeed, **6**, **9**, and **10** were not detected in this case.[34] Finally, attempted selective suppression of free-radical oxidations by 2,6-di-*tert*-butylphenol and of both $^3O_2$ and $^1O_2$ oxidations using *rac*-α-tocopherol resulted in both cases in the suppression of putative $^1O_2$ products **6**, **9**, and **10** and in diminution of amounts of $^3O_2$ products **2–5**. None of these experimental approaches provided results consistent with anticipation but in each case definitive conclusions for or against the presence of $^1O_2$ in the system cannot be made.

Nonetheless, where $^1O_2$ flux is sufficiently high and prolonged the interception of $^1O_2$ by cholesterol in aqueous systems has been demonstrated. Both human erythrocyte ghosts and synthetic liposomes incorporating an appropriate photosensitizer yield the 5α-hydroperoxide **8** upon photoirradiation.[12a,35] From the weights of isolated products **6**, **9**, and **10** implying $^1O_2$ action and of **2–5** and **7** implying $^3O_2$ action, the ratio of $^1O_2$ to $^3O_2$ released by H_2O_2 disproportionation is 1:3, thus a significant level of $^1O_2$. Moreover, the isolated yields of **6**, **9**, and **10** represent a minimum yield, as **8** and **9** are both rapidly isomerized to **2** and **3**, respectively, in these dispersions. The isomerization of **8** to **2** has been observed in organic solvent solutions,[36] upon pyrolysis,[9b,c] and in the aqueous dispersion of the present study. Furthermore, the quasi-axial **2** and **3** also epimerize to quasi-equatorial **4** and **5**, respectively, under like circumstances.[8e,9c] Therefore, levels of **8** or **9** unique to $^1O_2$ action on cholesterol may be diminished substantially by conversions to **2–5** for which no inference of $^1O_2$ action can be made.

Thus, adequate levels of $^1O_2$ may be released from H_2O_2 disproportionation over several hours to provide a $^1O_2$ flux which allows reaction with cholesterol. The disproportionation of H_2O_2 in aqueous cholesterol dispersions is known to be sensitive to the state of the sterol dispersion, pH, extraneous salts, etc.,[26] and present results likewise suggest that H_2O_2 disproportionation in our cholesterol dispersions is sensitive to pH, solvent, surfactant, and inhibitor effects. Disproportionation to $^1O_2$ is much more sensitive to these effects than is dismutation to $^3O_2$, although both are affected. Our results implying release of $^1O_2$ from H_2O_2 may apply uniquely to the cholesterol dispersions studies, and extension of the generality of H_2O_2 disproportionation to $^1O_2$ to other systems, particularly to biological systems, should be advanced only from additional specific proof.

The twelfth isolated product was the 3β,6β-diol **14** for which no origin can be proposed. Reintroduction of the 5,6-epoxides **11** and **12** individually, indeed of all products, into the aqueous dispersions, with and without added hydrogen peroxide, failed to yield **14** and we conclude that **14** was not formed from any product **2–13** but from cholesterol by other processes. Formation of **14** from cholesterol is a formal hydration, but **14** is not formed from cholesterol by attack of hydroxyl radical[29] or by photosensitized hydration, which yielded instead 5β-cholestane-3β,5-diol and *A*-homo-3a-oxa-3α,5α-cyclocholestane.[37]

Experimental Section

General chromatographic and spectral analysis methods used were those previously described in detail.[3a,4,7,8b] Sterol hydroperoxides were detected on thin layer chromatograms by means of *N*,*N*-dimethyl-*p*-phenylenediamine.[38] Thin layer chromatographic mobilities (R_c) and gas chromatographic relative retention times (t_R) on 3% SE-30 columns are measured in terms of cholesterol as unity. Proton nuclear magnetic resonance spectra were recorded on deuteriochloroform solutions using a Varian XL-100 spectrometer. Chemical shifts are measured downfield from an internal reference of tetramethylsilane. High-resolution mass spectra in both EI and CI (using methane) modes were obtained using a CEC Model 21-110B mass spectrometer. Other mass spectra were obtained in the CI mode using methane, isobutane, or ammonia as reagent gases with Du Pont Model 491 and Finnigan Corp. Model 3200 mass spectrometers, using direct probe for sample introduction.

Identification of the common oxidation products **2–5**, **7**, **9–10**, **13**, and **14** was made by comparison of spectral and chromatographic properties of each with those of an authentic sample, as previously described in detail.[9b] Identity of the 5,6-epoxides **11** and **12** was made by similar comparison of properties of their lithium aluminum hydride reduction products with those of authentic samples.[7]

Aqueous Sodium Stearate Dispersions. Standard aqueous dispersions were prepared by dissolving 250 mg of stearic acid and 300 mg of Na_3PO_4 in 40 mL of ethanol and 60 mL of distilled water, diluting 80 mL of this solution to 500 mL with distilled water (pH 9.5 as prepared), and then adding a hot ethanol solution (25 mg/mL) of highly purified cholesterol to give a final cholesterol concentration of 1 mg/mL.[39] Additions of H_2O_2 (200 mg of 30% H_2O_2, giving a final H_2O_2 concentration of 3.5 mM) and other ingredients, pH adjustment with dilute HCl or NaOH, removal of air under vacuum, and imposition of a N_2 atmosphere afforded the test systems, which were stirred and heated at 70 °C for 6 h. Cooled aliquots taken hourly were neutralized and extracted with three 100-mL portions of diethyl ether, the ether extracts were dried over anhydrous sodium sulfate and concentrated under vacuum, and methanol was added. Crystalline cholesterol was filtered off and the filtrate concentrated under vacuum for analysis or isolations.

In a typical experiment, 504.3 mg of cholesterol oxidized for 6 h at 70 °C gave 285.1 mg of recovered cholesterol, mp 148–150 °C, and chromatography on 0.5-mm thick silica gel HF_{254} chromatoplates with benzene-ethyl acetate (18:7) using four ascending irrigations resolved the major products.

5α-Cholestane-3β,5,6β-triol (13). The least mobile component (R_c 0.10, tan spot with 50% sulfuric acid) was eluted with and crystallized from acetone, yielding 0,8 mg (0.6%) of colorless crystals of 3β,5α,6β-triol **13**, mp 238–243 °C (lit. mp 232–238 °C[40]), t_R 2.70.

5α-Cholestane-3β,6β-diol (14). The sterol resolved at R_c 0.18 giving a red color with 50% sulfuric acid, eluted with and crystallized from acetone, gave 1.7 mg (1.4%) of colorless crystals of 3β,6β-diol **14**, mp 190–193 °C (lit. mp 189–192 °C[40]), t_R 1.81.

Cholest-5-ene-3β,7α-diol (3). From the sterol zone at R_c 0.23 colored intense blue with 50% sulfuric acid there was recovered by elution and crystallization with acetone 8.1 mg (6.8%) of the 3β,7α-diol **3**, mp 183–186 °C (lit. mp 158–161 and 176–187 °C[40]), t_R 2.20.

Cholest-5-ene-3β,7β-diol (5). The sterol zone at R_c 0.30 giving a blue color with 50% sulfuric acid yielded likewise 8.9 mg (7.5%) of crystalline 3β,7β-diol **5**, mp 174–178 °C (lit. mp 172–179 °C[40]), t_R 2.33.

5α-Cholest-6-ene-3β,5-diol (9). The sterol detected at R_c 0.40 upon elution with acetone yielded 1.9 mg (1.6%) of crystalline 3β,5α-diol **9**, mp 147–150 °C (lit. mp 181,[41a] 170–175,[41b] 147–150,[41c] 148–

149,[8e] 147–148 °C[9b]), t_R 0.55 (identified as cholesta-2,4,6-triene derived from **9**[9b,c]).

The next most mobile zone was complex in composition and was characterized by positive sterol hydroperoxide tests, ultraviolet light absorption in situ, and 50% sulfuric acid colors suggesting overlay of tan-colored and blue-colored spots. The total sterol mixture from this zone was eluted and rechromatographed using chloroform–acetone (24:1) and triple ascending irrigation. This treatment resolved the sterol hydroperoxides from other sterols.

Cholesterol 7-Hydroperoxides (2 and 4). The sterol hydroperoxides zone resolved using chloroform–acetone (24:1) eluted with acetone yielded 0.9 mg (0.7%) of a mixture of 7α- and 7β-hydroperoxides **2** and **4** which were not processed as such but which were reduced with sodium borohydride in methanol. The product 3β,7-diols **3** and **5** were then recovered following additional thin layer chromatography and identified by spectral and chromatographic properties with authentic 3β,7α- and 3β,7β-diols **3** and **5**, thus establishing the identities of the epimeric cholesterol 7-hydroperoxides from the reaction.

3β-Hydroxycholest-5-en-7-one (7). The more polar sterol zone exhibiting ultraviolet light absorption and a tan color with 50% sulfuric acid resolved from the epimeric 7-hydroperoxides **2** and **4** by chloroform–acetone (24:1) was eluted with acetone, yielding 16.5 mg of a mixture containing two major components at t_R 1.44 and 2.01 (3% SE-30). Preparative gas chromatography[42] of a small amount of the sample afforded **7** identified by comparison of spectral and chromatographic properties with those an authentic sample of **7**. Reduction of the sample in methanol by excess $NaBH_4$ caused the loss of the t_R 2.01 component (**7**) but not the t_R 1.44 component (**11** and **12**) and the formation of the epimeric 3β,7-diols **3** and **5** whose identities were confirmed by thin layer and gas chromatographic data. Material unaltered by $NaBH_4$ reduction weighed 2.3 mg, thus giving a yield of **7** by difference of 14.2 mg (12.0%).

5,6α-Epoxy-5α-cholestan-3β-ol (11) and 5,6β-Epoxy-5β-cholestan-3β-ol (12). The 2.3 mg of material recovered following borohydride reduction of 16.5 mg of the mixture of **7**, **11**, and **12** contained the isomeric 5,6-epoxides **11** and **12** as evinced by thin layer and gas chromatography. Composition of the mixture was estimated following reduction by lithium aluminum hydride in refluxing dry diethyl ether for 2 h. Products isolated by thin layer chromatography were **14** (derived from the 5β,6β-epoxide **12**[43]), mp 191–192 °C (lit. mp 189–192 °C[40]), t_R 1.83, and 5α-cholestane-3β,5-diol (derived from the 5α,6α-epoxide **11**[43]), mp 224–225.5 °C (lit. mp 223–225 °C[40]), t_R 1.39. Gas chromatographic analysis of the reduced products mixture gave 11% 5α-cholestane-3β,5-diol and 89% **14**,[7] thus providing a ratio of **11** to **12** of 1:8 and calculated yields of 0.25 mg (0.2%) of **11** and 2.05 mg (1.7%) of **12**.

Several unidentified components between the zone occupied by the 7-ketone **7**, the 5,6-epoxides **11** and **12**, and the 7-hydroperoxides **2** and **4** and cholesterol were detected but were of too small an amount to permit isolation. The cholesterol zone was eluted with acetone and recrystallized to yield 105.1 mg of pure cholesterol, identified as such by spectral and chromatographic data, thus for a total recovery of 390.2 mg (77.4%).

7α-Stearatoxycholest-5-en-3β-ol (6). A. From Cholesterol. The most mobile component from the thin layer chromatogram of the original oxidation products mixture was located at R_c 1.09 and contained the 7α-stearate ester **6** and the dienone **10**. The mixture of **6** and **10** was rechromatographed using benzene–ethyl acetate (1:9) in triple ascending irrigations, yielding **10** at R_f 0.66, **6** at R_f 0.37, and a very weak component at R_f 0.41 which gave the same characteristic blue coloration with 50% sulfuric acid as did **6**. Elution of the R_f 0.37 and 0.41 components together, followed by rechromatography using benzene–ethyl acetate (19:1) in five successive ascending irrigations, resolved the sterols. The less mobile zone at R_f 0.27 eluted with acetone yielded 8.1 mg (4.1%) of **6** as a colorless oil which could not be crystallized. The preparation was homogeneous by thin layer chromatography and was characterized: IR (KBr) 3400 (OH), 1735 (carbonyl), and 1660 cm^{-1} (C=CH); NMR ($CDCl_3$) δ 0.67 (s, 3 H, C-18), 0.87 (d, J = 6 Hz, 6 H, C-26/C-27), 0.92 (d, J = 4 Hz, 3 H, C-21), 1.00 (s, 3 H, C-19), 1.26 and 1.56 (-CH_2CH_2), 2.32 (d, J = 7 Hz, 2 H, -$COCH_2$-), 3.55 (m, 1 H, 3α-H), 4.96 (d, J = 4 Hz, 1 H, 7β-H), 5.55 ppm (d, J = 5 Hz, 1 H, 6-H); R_c 1.09 in benzene–ethyl acetate (18:7); R_f 0.37 in benzene–ethyl acetate (1:9); R_f 0.27 in benzene–ethyl acetate (19:1), intense blue color with 50% sulfuric acid; t_R 0.57 (identified as cholesta-2,4,6-triene); MS (rel intensity) by EI m/z 384.3385 (5) (M − $C_{18}H_{36}O_2$), 385.3444 (3) (M − $C_{18}H_{35}O_2$), 366.3276 (5) (M − H_2O − $C_{18}H_{36}O_2$), 351.3064 (1) (M − H_2O − $C_{18}H_{36}O_2$ − CH_3), 284.2715 (13) ($C_{18}H_{36}O_2$), 211.1472 (1) ($C_{16}H_{19}$), 143.0834 (9) ($C_{11}H_{11}$), 129.0908 (24) ($C_7H_{13}O_2$), 128.0609 (6) (C_8H_{10}), 115.0733 (12) ($C_6H_{11}O_2$), 87.0466 (20) ($C_4H_7O_2$), 73.0297 (100) ($C_3H_5O_2$), etc.; MS (rel intensity) by CI (methane) m/z 401.3418 (20) ($C_{27}H_{45}O_2$), 383.3299 (89) (M − $C_{17}H_{35}COOH$ − H), 369.3482 (21) ($C_{27}H_{45}O$), 368.3406 (18) ($C_{27}H_{44}O$), 367.3360 (56) (M − $C_{17}H_{35}COOH$ − OH), 285.2771 (93) ($C_{17}H_{35}COOH$ + H), etc.; MS (rel intensity) by CI (isobutane) m/z 669 (1) (M + H), 423 (10), 405 (2), 385 (10) (M − $C_{17}H_{35}COOH$ + H), 383 (4) (M − $C_{17}H_{35}COOH$ − H), 367 (100) (M − H_2O − $C_{17}H_{35}COOH$ + H), 285 (23) ($C_{17}H_{35}COOH$ + H); MS (rel intensity) by CI (ammonia) m/z 402 (16) (M + NH_3 − $OCOC_{17}H_{35}$), 385 (6), 384 (6) (M − $C_{17}H_{35}COOH$), 383 (6), 367 (100) (M − $C_{17}H_{35}COOH$ − OH), 302 (20) ($C_{17}H_{35}COOH$ + NH_4).[44]

Anal. Calcd for $C_{45}H_{80}O_3$: mol wt, 668.6107. Found: mol wt, 668.6089 (sum of m/z 384.3385 and 284.2704 ions).

The more mobile component at R_f 0.41 recovered with **6** (at R_f 0.27) was eluted with acetone. Evaporation under vacuum gave 3.3 mg (1.7%) of a colorless oil which could not be crystallized but which was characterized: IR (KBr) 3400 (OH), 1720 (CO), and 1660 cm^{-1} (C=CH); R_f 0.41 in benzene–ethyl acetate (19:1); intense blue color with 50% sulfuric acid; t_R 0.57 (cholesta-2,4,6-triene). This component was recognized as probably being the 7β epimer of **6**, 7β-stearatoxycholest-5-en-3β-ol, putatively formed by epimerization of **6** during reaction. A solution of **6** in 5 mL of acetone containing 1 drop of glacial acetic acid held at room temperature was analyzed by thin layer chromatography using benzene–ethyl acetate (19:1). After 30 min the R_f 0.41 component was detected, and after 6 h the apparent ratio of **6** (at R_f 0.37) to the R_f 0.41 component was approximately 7:3. The chemical characterization and identification of R_f 0.41 component was not pursued further.

B. From 5α-Cholest-6-ene-3β,5-diol. A dispersion of 1.005 g of the 3β,5α-diol **9** in 1 L of sodium stearate solution containing 2 mL of 30% H_2O_2 was heated at 70 °C for 6 h under N_2. Chromatographic recovery of products gave 0.512 g of unaltered **9**, 0.124 g of 3β,7α-diol **3**, traces of 3β,7β-diol **5**, and 7α-stearate ester **6**. Rechromatography of the ester **6** using benzene–ethyl acetate (1:9) in four ascending irrigations, elution with acetone, and recrystallization from acetone gave 11.3 mg of **6** as an amorphous solid [mp 55–60 °C; IR (KBr) 3400, 1735, 1660 cm^{-1}] identical in spectral and chromatographic properties with **6** isolated from the reaction of cholesterol described in A.

C. From 3β-Hydroxy-5α-cholest-6-ene 5-Hydroperoxide. A dispersion of 24.3 mg of **8** in 25 mL of sodium stearate solution containing 10 mg of 30% H_2O_2 was heated under N_2 at 70 °C, with aliquots withdrawn for chromatographic analysis at intervals. After 1 h approximately half of the original substrate was isomerized to the epimeric 7-hydroperoxides **2** and **4** and 3β,7-diols **3** and **5**. After 2 h the 3β,5α-diol **9** and 7α-stearate **6** were detected. Recovery of **6** by chromatography afforded a sample identical in chromatographic and spectral properties with **6** isolated under A.

Cholesta-4,6-dien-3-one (10). The component at R_f 0.66 resolved from the 7α-monostearate **6** in the benzene–ethyl acetate (19:1) system, located by its ultraviolet light absorption, was eluted and crystallized from acetone, yielding 1.4 mg (1.4%) of colorless crystals, mp 75–80 °C (lit. mp 79–82 °C[40]), t_R 3.30 (3% SP-2401).[45]

Repetition of these experiments at 0, 25, 37, and 50 °C established that no products formed at 0 or 25 °C, products **2–7** and **9–14** formed at 37 °C in approximately the same amounts as at 70 °C but were not isolated, and products at 50 °C were obtained in the following isolated yields: **2** and **4**, 0.4%; **3**, 6.1%; **5**, 5.7%; **6**, 4.3%; **7**, 9.0%; **10**, 1.0%; **11**, 0.2%; **12**, 1.5%; **13**, 0.4%; **14**, 0.6%.

Special Effects. Standard aqueous sodium stearate dispersions of cholesterol (1 mg/mL) 3.5 mM in H_2O_2 were modified as described and heated at 70 °C under N_2 for 6 h, with hourly chromatographic analysis. Products were isolated in some cases.

A. Deuterium Oxide. A standard dispersion of 26 mg of cholesterol prepared with deuterium oxide (99% deuterium) led to products **2–5**, **7**, **11**, and **12** but **6**, **9**, and **10** were not detected.

B. Hypochlorite. To a standard dispersion of 495 mg of cholesterol heated under N_2 was added dropwise 3 mL of 5% NaOCl (Clorox) over 5 h. Isolated products follow: **3**, 9.1 mg; **5**, 9.6 mg; **6**, 14.3 mg; **7**, 16.1 mg; **9**, 2.5 mg. Products **2**, **4**, and **10–14** were detected but not isolated.

C. Antioxidants. A standard dispersion of 450 mg of cholesterol was

treated with 1.0 g of *rac*-α-tocopherol. Products detected were **2–5**, **7**, **11**, **12**, and **14** but **6**, **9**, and **10** were not found. A standard dispersion of 250 mg of cholesterol was treated with 47 mg (0.25 mmol) of 2,6-di-*tert*-butylphenol. Products found were **2–5**; products **7** and **9–14** were not observed.

D. pH Effects. Portions of standard 1 mg/mL cholesterol dispersions were adjusted with 10% HCl to pH 4.6, 5.2, 7.0, 7.5, 8.0, 8.5, or 9.0 prior to heating. Products **2–5** and **7** were found in all cases; products **6**, **9**, and **10** were present at pH 8.0 and above; products **6** and **9** were found erratically at pH 7.5 and less, being detected in some experiments but not in duplicates.

E. Medium Consumption. Standard cholesterol dispersions were prepared in which methanol was substituted for ethanol. Products **2–7** and **9–14** were found in approximately the same amounts as in the standard system. Deletion of stearic acid in making the dispersions gave a very poor dispersion, and only **2–5** and **7** were detected as products. A standard dispersion of 251 mg of cholesterol prepared using 20 mg of NaOH instead of Na_3PO_4 gave all products **2–7** and **9–14**.

Dispersions free of surfactant were made by adding 200 mg of cholesterol in 100 mL of acetone slowly to 250 mL of distilled water under vacuum in a rotary evaporation. The milky dispersion was rotated under vacuum (ca. 20 Torr) to remove solvent and some water and then filtered through sintered glass, giving 1 mg/mL dispersions of cholesterol stable for days. Heating 250 mL of dispersion containing 100 mg of 30% H_2O_2 at 60 °C for 6 h gave **2–5** and **7** but **6**, **9**, and **10** were not found.

Aqueous cholesterol dispersions (1 mg/mL) were also made by adding hot methanol solutions of cholesterol to distilled water containing 35 mg of Tween 20 (polyoxyethylene (20) sorbitan monolaurate, Pierce Chemical Co., Rockford, Ill.) or Tween 80 (polyoxyethylene (80) sorbitan monooleate); bile salts (180 mg of taurocholic acid or 161 mg of glycocholic acid, each with 15 mg of NaOH); or 150 mg of *rac*-1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. Methanol was removed under vacuum, 100 mg of 30% H_2O_2 added, pH adjusted in some cases, and the dispersions heated at 60 °C under N_2 for 6 h. Products **2–5** were found in all cases. Products **9** and **10** were found in Tween dispersions at pH 7.7 and 9.4 and in glycocholate (but not taurocholate) dispersions at pH 8.9. Only **9** was detected in phosphatidylcholine dispersions at pH 9.2 but not at pH 7.1. Detection of products **9** and **10** in these experiments was overall more erratic than in sodium stearate dispersions.

F. Product Stabilities. Individually prepared standard dispersions were made using 25 mg of pure sterols **2–5** and **7–14** instead of cholesterol and heated under N_2 at 37, 50, and 70 °C for 6 h, with hourly analysis for products. Transformations described in the Results section were observed at all temperatures, the more extensive being at 70 °C. In no case were **6** or **9** formed from any substrate **2–5**, **7**, **10–14**, nor was **10** formed from any substrate **2–7**, **9**, **11–14**. The 3β,6β-diol **14** was not formed from either epoxide **11** or **12** or from any other substrate. The 5α-hydroperoxide **8** was also tested and found to be the least stable of all sterols. At 70 °C over half was isomerized and decomposed to **2–5** within 1 h; **6** and **9** were additionally present at 2 h; **7** and **10** were present after 3 h; **8** could not be detected after 4 h.

Cholesterol Oxidation in Solution. As a control not involving hydrogen peroxide disproportionation 251 mg of cholesterol dissolved in 400 mL of acetonitrile was treated with 25 mL of 30% H_2O_2 and heated under N_2 at 50 °C for 5 h. Chromatographic analysis of reaction products established that only the 5,6-epoxides **11** and **12** had formed and that products **2–7**, **9**, **10**, **13**, and **14** had not formed.

Acknowledgment. The helpful assistance of Dr. D. M. Desiderio, Baylor College of Medicine, Houston, Texas, in providing high-resolution mass spectra, and of Dr. David McAdoo of the Marine Biomedical Institute, Galveston, Texas, for providing some CI mass spectra is gratefully acknowledged. Financial support of these studies was provided by the Robert A. Welch Foundation, Houston, Texas, and the U.S. Public Health Service (NIH Grants HL-10160 and ES-00944).

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(20) Coupling constants for the 7-proton signals for the epimeric 3β,7-diols **3** and **5** are $J_{6,7} = 5.5$, $J_{7,8} = 1.5$, and $J_{6,7} = 1.5$, $J_{7,8} = 5.5$ Hz, respectively; cf. ref 8e; $J_{6,7} = 5.5$ and $J_{6,7} = 1.5$ Hz, respectively, cf. ref 19a. For the 3,7-diacetates of the epimeric 3β,7-diols **3** and **5**: $J_{6,7} \simeq 4.5$ and 2.2 Hz, respectively; cf. ref 19a as corrected; cf. ref 21.
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(29) As **6**, **9**, and **10** do not retain oxygen–oxygen bonds, their formation from cholesterol by reaction with other (not dioxygen) species containing only one oxygen atom might occur. Chief among such other species is the hydroxyl radical (HO·) putatively generated in the thermolysis of H_2O_2. However, oxidation of cholesterol by HO· derived by solvent X-radiolysis or by the Fenton reagent variously yielded **3**, **5**, 7, **11–13**, and cholest-5-ene-

3β,25-diol; cf. (a) J. Weiss and M. Keller, *Experientia*, **6**, 379 (1950); (b) M. Keller and J. Weiss, *J. Chem. Soc.*, 2709 (1950); (c) B. Coleby, M. Keller, and J. Weiss, *ibid.*, 66 (1955); (d) G. R. Clemo, M. Keller, and J. Weiss, *ibid.*, 3470 (1950); (e) M. Kimura, M. Tohma, and T. Tomita, *Chem. Pharm. Bull.*, **20**, 2185 (1972); (f) *ibid.*, **21**, 2521 (1973); (g) A. L. J. Beckwith, *Proc. Chem. Soc., London*, 194 (1958). We have confirmed that HO· generated by ^{60}Co γ-radiolysis of aqueous cholesterol dispersions saturated with N_2O yields **3**, **5**, **7**, and **11–13**, and that neither sterol hydroperoxide **2**, **4**, and **8** nor **6**, **9**, **10**, and **14** were formed.
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(44) Purification of **6** was fraught with unresolved homogeneity problems, as even the best (99% purity) stearic acid used contained demonstrable levels of fatty acid congeners as impurities, leading to octadecenoate, hexadecanoate, and hexadecenoate congeners of **6** as impurities in **6** revealed in CI mass spectral ions (methane or isobutane) *m/z* 283, 257, and 255, respectively.
(45) The component previously suggested as cholesta-4,6-dien-3β-ol found in aqueous sodium stearate dispersions of cholesterol and 5α-hydroperoxide **8**, cf. ref 7, was assigned the dienol identity from its chromatographic mobility, characteristic blue color with sulfuric acid, and ultraviolet light absorption. The component was in fact an unresolved mixture of dienone **10** and 7α-stearate **6** not then recognized as such.

Total Synthesis of *dl*-19-Hydroxyprostaglandin E_1 and *dl*-13-*cis*-15-*epi*-19-Hydroxyprostaglandin E_1

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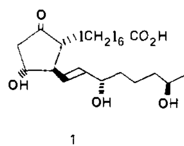
Abstract: A total synthesis of *dl*-19-hydroxyprostaglandin E_1 (**34**) and *dl*-13-*cis*-15-*epi*-19-hydroxyprostaglandin E_1 (**35**) via the conjugate addition of the dioctenyl cuprate reagent **21** derived from *dl*-1-iodo-3-hydroxy-7-*tert*-butyldimethylsilyloxyoct-1-*cis*-ene (**14**) to *dl*-2-(6-carbomethoxyhexyl)-4-tetrahydropyranyloxycyclopent-2-en-1-one (**23**), followed by the stereospecific sulfenate-sulfoxide transformation on the resultant 13-*cis*-prostaglandin analogue, is reported. The preparation of the requisite *cis*-iodooctene **14**, prepared by two synthetic sequences starting from either α-methylcyclopentanone or acrylonitrile, is described as well as the separation of the C-19 α and β isomers.

Recently, two groups[3-6] have demonstrated that the major prostaglandin fraction in human semen consists of 19-hydroxyprostaglandin E_1 and 19-hydroxyprostaglandin E_2, together with lesser amounts of PGEs, PGFs, and 19-OH-PGFs.[7] Previously, in 1966, Hamberg and Samuelsson[8] had identified 19-OH-PGAs and 19-OH-PGBs in human semen, and later Hamberg[9] established the *R* configuration at C-19 for 19-OH-PGB$_1$ (**2**). These 19-OH-PGAs and -PGBs now are con-



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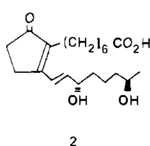

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sidered to have been artifacts resulting from dehydration during isolation and/or storage and analysis, since recent studies have shown that longer storage at a given temperature or storage at a temperature ≥0 °C of fresh seminal fluid results in a decrease in the amounts of 19-OH-PGEs with a concomitant increase in the amounts of 19-OH-PGAs and -PGBs. Although it has not yet been proven, it is highly likely that the naturally occurring 19-OH-PGEs have the 19 (*R*) configuration. The physiological role that these 19-OH-PGEs play in man or in primates[10] has not been yet established. It was therefore of interest to synthesize these prostaglandins for biological studies and evaluation.[11,12]

The total synthesis of *dl*-19-OH-PGE$_1$ reported here follows that which was developed in our laboratory,[13,14] which has as its key step the conjugate addition of the requisite functionalized *cis*-octenyl cuprate to the appropriate hydroxycyclopentenone, in order to take advantage of the high degree of stereochemical control at carbons 8, 11, 12, and 15. The stereospecific sulfenate-sulfoxide rearrangement[14] of the 13-*cis*-15β-hydroxy epimer provides the prostaglandin of natural stereochemistry, except the center at C-19 which is an equal mixture of 19α- and 19β-hydroxy isomers.

The required *cis*-iodovinylcarbinol **14** was prepared first by a six-step sequence as shown in Scheme I. Baeyer–Villiger oxidation of α-methylcyclopentanone with *m*-chloroperbenzoic acid gave lactone **3** in 74% yield after distillation. Linstead and Rydon[15] had previously synthesized this lactone in four steps (15% yield). Condensation of acetylenemagnesium bromide in tetrahydrofuran at 0 °C with lactone **3** gave, in only modest yield (30%), the hydroxy ketone **4** and the unexpected hydroxy ketone **5** in a ratio of ca. 2:1. The NMR spectrum of this mixture also exhibited resonances which were assigned to small

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