On the Mechanism of Oxidation of Cholesterol at C-7 in a Lipoxygenase System*

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Incubation of [7-2H2]cholesterol with soybean lipoxygenase and linoleic acid in the presence of oxygen gave a mixture of 5-cholestone-3β,7α-diol, 5-cholestone-3β, 7β-diol, 3β-hydroxy-5-cholestone-7-one, 5α, 6α-epoxycholestan-3β-ol, and 5β, 6β-epoxycholestan-3β-ol. The conversion into the 7-oxygenated products was associated with a very high internalmolecular isotope effect (Kp/KD = 15-17), suggesting that the rate-limiting step in the overall conversion is likely to be the abstraction of hydrogen at C-7 in a radical reaction. Evidence that linoleic acid is to some extent directly involved was obtained with the use of [7-3H]cholesterol. Incubation of [7-3H]cholesterol resulted in a significant incorporation of 3H in the reisolated linoleic acid fraction.

The isotope effect associated with conversion of [7α-3H]cholesterol into 7-oxygenated products in the lipoxygenase system was 2-3, indicating that the extraction of hydrogen is nonstereospecific. Incubation of [7-2H2]cholesterol with 13-hydroperoxy-9,11-octadecadienoic acid gave the above 7-oxygenated products with relatively small isotope effects (Kp/KD = 3-4).

It is concluded that the most important mechanism for oxidation of cholesterol at C-7 in the lipoxygenase system involves participation of radicals and that a carbon-centered linoleic acid radical can extract hydrogen directly from cholesterol. Fatty acid hydroperoxides and their secondary products seem to be less important as initiators in connection with oxidation of cholesterol.

It is well established that peroxidation of membranes and other structures containing cholesterol and unsaturated fatty acids yields a number of oxygenated cholesterol products in addition to fatty acid hydroperoxides and their degradation products (1, 2). It has been shown that NADPH-dependent lipid peroxidation in liver microsomes gives a mixture of 5-cholestone-3β,7α-diol, 5-cholestone-3β,7β-diol, 3β-hydroxy-5-cholestone-7-one and that the formation of these products parallels the formation of TBA-reacting compounds (3, 4). Incubation of cholesterol with linoleate and lipoxygenase is also known to give the above monooxygenated products of cholesterol in addition to hydroperoxides of cholesterol and fatty acids (4, 5).

Detailed and careful studies by Teng and Smith (4, 5) have shown that oxidation of cholesterol in the above two systems yields the 7α- and 7β-hydroperoxides of cholesterol as primary products. These hydroperoxides are then reduced to the corresponding diols or dehydrated to give 3β-hydroxy-5-cholestone-7-one. In the lipoxygenase system there was no evidence of a direct interaction between cholesterol and the enzyme, indicating that intermediates or products of the lipoxygenase reaction on the unsaturated fatty acids may be responsible for the oxidation of cholesterol. Since peroxidation of polyunsaturated fatty acids by lipoxygenase is known to involve participation of free radicals, Teng and Smith suggested that such species may be of importance for oxidation of cholesterol. They also pointed out that the transformations were similar to radiation-induced free radical oxidation of cholesterol by molecular oxygen. The exact nature of the species responsible for oxidation of cholesterol in the two systems could, however, not be defined.

In the present work we have studied the mechanism of oxidation of cholesterol in a system containing lipoxygenase + linoleate. To obtain information about the rate-limiting step in the reaction and a possible transfer of hydrogen from cholesterol to linoleate, we have used cholesterol specifically labeled with 2H or 3H in the 7α- and the 7β-positions.

MATERIALS AND METHODS

Reagents and Unlabeled Compounds

Unlabeled cholesterol (5-cholestone-3β-ol) was purchased from E. Merck AG, Darmstadt, Federal Republic of Germany (F. R. G.). Linoleic acid, soybean lipoxygenase (lyophilized, Type I), hemtoporphyrin, 3β-hydroxy-5α-cholestan-6-one, and NADPH were purchased from Sigma. Deuterium-labeled reagents with an isotopic purity of more than 98% were purchased from Aldrich-Caemic KG, Steinheim, F. R. G. All other chemicals and solvents were standard commercial high purity materials. Tetrahydrofuran was distilled from lithium aluminum hydride prior to use.

Labeled Compounds

[26,26,26,27,27,27-2H6]Cholesterol was obtained from Stohler (chemical purity > 92% by GC, isotopic purity > 98% by GC-MS). [7-2H2]Cholesterol with a specific radioactivity of 881 GBq/mmol was purchased from Du Pont-New England Nuclear (radiochemical purity 98%, according to manufacturer).

Synthesis of (f)7,7,2H2]Cholesterol—500 mg of 3β-hydroxy-5α-cholestan-6-one and 250 mg of sodium methoxide were suspended in a mixture of 4.5 ml of [D2]water, 3 ml of tetrahydrofuran, and 3 ml of CH2O·[2H]. The suspension was kept at 40°C with continuous stirring for 16 h. After slight acidification with deuter-

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ochloric acid, followed by dilution with water, the material was extracted with diethyl ether, washed with water, and dried with MgSO4. The solvent was then removed under reduced pressure. The product, [5,7,7-2H4]-5β-hydroxy-5α-cholestan-6-one, was acetylated with acetic anhydride (2:1, room temperature, stirring overnight). The suspension was then converted into cholesterol as described by Corey and Gregoriou (6), except that the dehydrated with phosphorous oxychloride was followed by dilution with water, the material was >98% when calculated according to Biemann (7).

The synthesis of 5β-cholestan-3β,5α-diol was carried out for 20 min and that sodium borodeuteride was added as described in Organic Synthesis 57. (8). Sodium borodeuteride was added to sodium borohydride when [6,7,7-2H]-cholesterol was the desired end product. Typical overall yield was >85%. The isotopic purity was >95% when calculated according to Biemann (7).

The reaction was terminated with the addition of 200 ml of methanol and the product was purified with analytical reversed-phase HPLC prior to use, yielding a >99.5% (GC) pure compound. The distribution of the different species was determined by GC-MS (cf. below). Synthesis was performed as described by Hamborg (8). Linoleic acid, 100 mg in 0.5 ml of ethanol, was dissolved in 100 ml of 0.1 M sodium borate buffer, pH 9.0, containing 0.745 g of KCl. Three portions of 100,000 units each of soybean lipoxigenase were added at 15-min intervals. The suspension was kept at 0°C with continuous stirring. The reaction was terminated with the addition of 200 ml of methanol after 30 min, followed by acidification with hydrochloric acid and extraction with 600 ml of diethyl ether. The ether phase was washed twice with water, dried with MgSO4, and evaporated in vacuo. The residue was purified on a 5-g silica gel column (Silica Gel 60, Fisons) with ethyl acetate as the eluent. The fraction containing the desired product was evaporated and the residue was dissolved in 1 ml of acetonitrile.

The reaction was performed in the same way as described by Corey and Gregoriou (6), except that the dehydration with phosphorous oxychloride was followed by dilution with water, the material was >98% when calculated according to Biemann (7).

**Synthesis of 5β,7α-DH Cholesterol—**This compound was synthesized from 5β-acetoxy-7α-bromo-5α-cholestan-6-one as described by Corey and Gregoriou (6). Sodium borodeuteride replaced sodium borohydride in the reduction. The isotopic purity was >95% when calculated according to Biemann (7).

Synthesis of 13-Hydroperoxy-9,11-octadecadienoic Acid—The synthesis was performed as described by Hamborg (8). Linoleic acid, 100 mg in 0.5 ml of ethanol, was dissolved in 100 ml of 0.1 M sodium borate buffer, pH 9.0, containing 0.745 g of KCl. Three portions of 100,000 units each of soybean lipoxigenase were added at 15-min intervals. The suspension was kept at 0°C with continuous stirring. The reaction was terminated with the addition of 200 ml of methanol after 30 min, followed by acidification with hydrochloric acid and extraction with 600 ml of diethyl ether. The ether phase was washed twice with water, dried with MgSO4, and evaporated in vacuo. The residue was purified on a 5-g silica gel column (Silica Gel 60, Fisons) with ethyl acetate as the eluent. The fraction containing the desired product was evaporated and the residue was dissolved in 1 ml of acetonitrile.

**Cholesterol Used**—The cholesterol used was a mixture of [26,26,26,7,7a,7-2H]-cholesterol and cholesterol as described in Organic Synthesis 57. Cholesterol, 300 mg in 300 μl of acetonitrile, was added immediately before the first addition of lipoxigenase. The extraction was performed without prior acidification, and the ether extract was, after evaporation in vacuo, purified with solid-phase cartridges and trimethylsilylated. Typical yield was about 15%.

**Chromatography**

**HPLC—**Reversed-phase analytical HPLC was performed using a Nova-Pak C18 cartridge (8 x 100 mm, 4-μm particle size, from Waters, Inc.) with a methanol-water gradient (0-1 min: 90/10, v/v); 1-30 min: linear gradient from 90/10 to 100/0, 30-32 min, isocratic); 1-30 min: linear gradient 96:4:0.1-96:4:0.1; 15-30 min: linear gradient 96:4:0.1-80:20:0.1; 30-35 min: isocratic; 1-30 min: linear gradient 100:0-96:4:0.1; 15-30 min: linear gradient 96:4:0.1-80:20:0.1; 30-35 min: isocratic 80:20:0.1. Typical retention times were (min): cholesterol, 35.5; acetoxycholesterol, 35.5a; cholesterol, 27.3. Radioactivity in an LKB-Wallac Rackbeta 1217 scintillation counter. The initial incubation was acidic with 6 m hydrochloric acid to pH 4 and extracted with diethyl ether twice more, and the recombined ether phases (alkaline phase) were washed with water. The alkaline phase was evaporated, dissolved in a minimal amount of methanol, and subjected to semipreparative reversed-phase HPLC. A fraction corresponding to the alkali cholesterol was collected from 10.7 to 15.7 min of retention time. This fraction could also be expected to contain some 5β-cholestan-3β,5α-diol and more polar cholesterol oxygenation products such as cholesterol-3β,5α,6β-triol. This partially purified fraction was rechromatographed with radio-HPLC using the normal-phase system described below.

The acid-phase which contained most of the material, was chromato-graphed in 15 portions using the straight-phase reversed-phase system described below. Fractions corresponding to the alkali cholesterol were collected from 12.7 to 15.7 min. The combined phases were subjected to reversed-phase analytical HPLC using the fatty acid system. The eluent was collected between 19 and 22 min and assayed for radioactivity in an LKB-Wallac Rackbeta 1217 scintillation counter. The oxidized sample was hydrolyzed for 12 h in 1 M NaOH in 80% EtOH at room temperature under argon atmosphere, extracted, and subjected to semipreparative reversed-phase HPLC as described.
fraction corresponding to linoleic acid was rechromatographed with straight-phase radio HPLC (Fig. 3).

**Incubation of Cholesterol with 13-Hydroperoxy-9,11-octadecadienoic Acid**—Typically, 300 µg of a cholesterol mixture was dissolved in 1 ml of ethanol together with 1 mg of 13-hydroperoxy-9,11-octadecadienoic acid. The solution was kept in a closed container at 100 °C for 4 h, or at 37 °C overnight.

The solvent was evaporated, and the steroids were purified with solid-phase cartridges (cf. below). The incubation was also performed at 100 °C in borate buffer containing linoleic acid as in the lipoxygenase system with and without 50 mM of ferrous sulfate. Unless otherwise stated, data refer to the conditions first mentioned.

**Incubation of Cholesterol with Acetone Powder of Rat Liver Microsomes**—Acetone powder of liver microsomes from male Sprague-Dawley rats (200-250 g) was prepared as described previously (10), except that no EDTA was used in the homogenizing buffer. The incubations with cholesterol were performed as described previously (11).

**Treatment of Cholesterol with Singlet Oxygen**—800 µg of cholesterol was dissolved in 2 ml of pyridine containing 0.6 mg of hematoporphyrin. The solution was irradiated with a 254-nm UV source with continuous oxygen bubbling for 2 h. The temperature was kept at 5-10 °C. After evaporation of the solvent under reduced pressure, the residue was purified on thin layer chromatography. The 5α-hydroperoxy-6-cholesten-3β-ol thus obtained was allowed to stand overnight in chloroform in order to get 7α-hydroperoxy-5-cholesten-3β-ol (12). Samples were drawn at different times and analyzed with GC-MS, in most cases upon reduction with lithium aluminium hydride.

**Purification of Steroids with Solid-Phase Cartridges**—The steroids were purified using Bond-Elut NH₄ cartridges (Analytichem Corp.) as described by Kaluczny et al. (13). Only the neutral lipid fraction was collected.

**Common Analysis Procedure and Calculation of Isotope Effects**—The purified steroid extracts were subjected to reversed-phase analytical HPLC, and the samples were collected at the retention times corresponding to 5-cholestone-3β,7α-diol, 3β-hydroxy-5-cholesten-7-one, 5α,6α-epoxycholestan-3β-ol, 5β,6β-epoxycholestan-3β-ol, and cholesterol. The fractions were analyzed as trimethylsilyl ether with GC-MS in the selected ion monitoring mode in order to determine the isotope distribution of the species. Ions detected were: 5-cholestene-3β,7α-diol and 5α,6α-epoxycholestan-3β-ol, 3β-hydroxy-5-cholesten-7-one, molecular ion (m/z 458 for the unlabeled compound). Additional full-spectrum analyses were performed in order to ensure the identity of the recovered compounds.

In order to distinguish between the 3β-hydroxy-5-cholesten-7-one formed from [7,7-3H₂]cholesterol and that formed from unlabeled cholesterol, [26,26,26,27,27,27-3H₆]cholesterol was used as a 7-unlabeled substrate instead of unlabeled cholesterol. Since the incubation mixture was contaminated to a very small degree with unlabeled cholesterol (in incubations with acetone powder, to a high degree, about 10%), it seemed advisable to have an additional deuterium label in the 7,7-3H₂-labeled species in order to facilitate the mass spectrometric analysis. A 6-3H-label was found to be stable under the conditions employed. Thus, in general, incubations were performed with about equal parts of [8,7α,7β-3H₂]cholesterol and [26,26,26,27,27,27-3H₆]cholesterol.

As for [6,7-3H₂]5-cholestone-3β,7α and β-diol, part of the 6- as well as the 7-label is lost in the mass spectrometric fragmentation of the molecular ion to the M-TMSOH ion used for measurement. The reason for using this ion was its very high abundance compared to the molecular ion. By using synthetic unlabeled 6,7α-3H₂-labeled 5-cholestone-3β,7β-diol and 6,7β-3H₂-labeled 5-cholestone-3β,7α-diol as standards, it was possible to define the exact isotope pattern of each species of interest. Using [6,7α,7β-3H₂]cholesterol alone or in combination with an equal amount of [3H₂]cholesterol, it was thus possible to calculate both the loss of 3H from the 7-positions and the intermolecular isotope effect in the conversion (relative rate of formation of product from [3H₂]cholesterol and [6,7α,7β-3H₂]cholesterol).

In the calculation of isotope effects, the mass spectrometric intensities from [3H₂]-labeled 5-cholestone-3β,7β-diol was divided by the mass spectrometric intensities recorded from [3H₂]-labeled 5-cholestone-3β,7α-diol (after correction for presence of very small amounts of unlabeled compound in some experiments). This gives a crude measure of the Kᵢ/K₀ value. In case there was a small difference between the amount of [7-3H₂]- and [7-3H₆]cholesterol used as substrate, the Kᵢ/K₀ value was corrected for this. In the calculation of isotope effect, it must be assumed that the amount of product formed is small in relation to the amount of substrate. In all the experiments performed in which the isotope effect was calculated, the degree of conversion was always less than 10%. The isotope effect in the conversion into 3β-hydroxy-5-cholesten-7-one was calculated according with the difference that the mass spectrometric intensities corresponding to 3H₂-labeled product was recorded.

**RESULTS**

When [4-14C]cholesterol was incubated with linoleic acid and soybean lipoxygenase under the conditions described under "Materials and Methods," there was an efficient conversion into 5-cholestone-3β,7α- and β-diol, 3β-hydroxy-5-cholesten-7-one, and 5α,6α- and 5β,6β-epoxycholestan-3β-ol (Fig. 1a). The yield of these products is given in Table I.

The above experiment was repeated with a mixture of [26,26,26,27,27,27-3H₆]cholesterol and [6,7α,7β-3H₂]cholesterol. It was shown that the conversion of [6,7α,7β-3H₂]cholesterol into the two diols led to the loss of one atom of 3H. The conversion into 3β-hydroxy-5-cholesten-7-one led to loss of two atoms of 3H whereas all the 3H was retained in 5α,6α- and 5β,6β-epoxycholestan-3β-ol. The relative rate of formation of both the two diols and the ketone is considerably slower with [6,7α,7β-3H₂]cholesterol than with [3H₂]cholesterol as precursor (Fig. 2). As shown in Table II, the intermolecular isotope effect (Kᵢ/K₀) was calculated to
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**Table I**

*Extent of conversion (%) of [4-14C]cholesterol into monooxygenated products under the different conditions*

For experimental details, see "Materials and Methods." The extent of conversion was calculated after radio HPLC analysis as shown in Fig. 1, A-C. The results shown are those obtained from one typical experiment. Several experiments with each incubation condition gave essentially the same results.

| Incubation condition                                  | 5-Cholestene-3β,7α-diol + 5-cholestene-3β,7β-diol | 3β-Hydroxy-5-cholesten-7-one | 5α,6α-Epoxycholestan-3β-ol | 5β,6β-Epoxycholestan-3β-ol | Cholestane-3β,5α,6α,6β-tetrol |
|-------------------------------------------------------|---------------------------------------------------|-----------------------------|----------------------------|-----------------------------|--------------------------------|
| [4-14C]Cholesterol + linoleic acid + soybean lipoxygenase | 6.3                                               | 0.8                         | 0.5                        | 1.8                         | 0.6                            |
| [4-14C]Cholesterol + 13-hydroperoxyoctadec-11,11-dienoic acid | 2.4                                               | 1.1                         | 0.8                        | 1.6                         | <0.1                           |
| [4-14C]Cholesterol + delipidated rat liver microsomes + NADPH | 3.9                                               | 2.5                         | <0.1                       | 1.5                         | 1.2                            |

*This compound is formed by hydrolysis of the two epoxides.*

**Fig. 2.** Ion chromatogram obtained in GC-MS analysis of the 5-cholestone-3β,7α-diol fraction obtained after incubation of equimolar amounts of [26,26,26,27,27,27-2H6]cholesterol and [6,7,7-3H3]-cholesterol with lipoxygenase + linoleate.

be 15-17 in the conversion into the three 7-oxygenated products.

When the above experiment was repeated in the absence of oxygen, there was no significant conversion into oxygenated products. Addition of Fe2+ to 50 μM did not further increase the degree of conversion (results not shown). Addition of EDTA to 50 μM did also not affect the conversion.

[4-14C]Cholesterol as well as the above mixture with deuterated cholesterol species was also incubated with isolated 13-hydroperoxy-9,11-octadecadienoic acid under conditions described under "Materials and Methods" (Fig. 1B). There was only a low yield of different oxysterols (Table I). The intermolecular isotope effect measured in the conversion of [6,7,7-3H3]cholesterol into the 7-oxygenated products was much lower than those obtained in the lipoxygenase system as above, there was no significant intermolecular isotope effect in the conversion of [6,7,7-3H3]cholesterol into the two epoxides (Table II).

For reasons of comparison, [4-14C]cholesterol and the mixture of deuterated cholesterol species were also incubated with delipidated rat liver microsomes + NADPH (Table I, Fig. 1C). The intermolecular isotope effects obtained here in the conversion into the 7-oxygenated products were lower than those obtained in the lipoxygenase system but higher than those obtained with the isolated 13-hydroperoxy-9,11-octadecadienoic acid (Table II). When the above cholesterol species were treated with singlet oxygen as described under "Materials and Methods," there was a small conversion into 5-cholestone-3β,7α-diol and 3β-hydroxy-5-cholesten-7-one with little or no isotope effect.

In order to study stereospecificity aspects, a mixture of [1H2]cholesterol and [6,7α-2H3]cholesterol was incubated with the lipoxygenase system and linoleic acid (Table II). The isotope effect obtained in two different experiments varied between 2 and 3.

In order to study the mechanism of extraction of hydrogen from cholesterol in the lipoxygenase system, [7-2H3]cholesterol was incubated with lipoxygenase and linoleic acid. Linoleic acid and linoleic acid products were separated from cholesterol and its product as described under "Materials and Methods" (reversed-phase semipreparative HPLC followed by straight-phase HPLC or vice versa). 3H-Labeled material was eluted with the same retention volume as linoleic acid (Fig. 3). It was uncertain, however, whether all the 3H-containing fatty acid was linoleic acid or its conjugated diene, i.e. octadec-9,11-dienoic acid. Under the chromatographic conditions employed, these two fatty acids did not separate, and a compound with strong absorption at 234 nm eluted at a retention close to that of linoleic acid. The total recovery of 3H in the linoleic acid fraction was only about 10,000 cpm in an incubation with 250 Mcpm of [7-2H3]cholesterol. Since the total conversion of the labeled cholesterol into 7-oxygenated products was about 5% and since only one of the two H atoms would be extracted in the conversion, about 5 Mcpm could be expected to be lost from [7-3H]cholesterol if there were no isotope effect at all in the conversion.

Since the tritium isotope effects could be expected to be at least 25-50 in the loss of hydrogen from cholesterol (14) this would correspond to an expected loss of H of about 100,000 cpm. There may, however, well be an isotope effect also in the addition of hydrogen from cholesterol to a linoleic acid radical, and thus it was not possible to calculate the exact degree of transfer of hydrogen from cholesterol to linoleic acid radicals.

**DISCUSSION**

Teng and Smith (5) showed that oxidation of cholesterol by soybean lipoxygenase in incubations including ethyl linoleate as prime substrate gave the epimeric α- and β-hydroperoxides in the proportion 1:3 to 2:3. The 7-hydroperoxides were apparently the first formed oxidation products...
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**TABLE II**

| Incubation condition | 5-Cholestene-3\(^\alpha\)-7α-diol | 5-Cholestene-3\(^\beta\)-7β-diol | 3\(^\beta\)-Hydroxy-5\(^\beta\)-cholesten-7-one | 5α,6α-Epoxycholestan-3β-ol | 5δ,6δ-Epoxycholestan-3β-ol |
|----------------------|----------------------------------|---------------------------------|-------------------------------------|-----------------------------|-----------------------------|
| [7-\(^2\)H\(^2\)]- + [7-\(^2\)H\(^2\)]Cholesterol + linoleic acid + soybean lipoygenase | 15.4 | 16.0 | 17.2 | 1.0 | 1.0 |
| [7-\(^2\)H\(^2\)]- + [7-\(^2\)H\(^2\)]Cholesterol + 13-hydroperoxyoctadec-9,11-dienoic acid | 4.0 | 3.5 | 6.5 | 1.1 | 1.0 |
| [7α-\(^2\)H\(^2\)] + [7-\(^2\)H\(^2\)]Cholesterol + linoleic acid + soybean lipoygenase | 2.6 | 2.9 | 2.3 | | |
| [7-\(^2\)H\(^2\)]- + [7-\(^2\)H\(^2\)]Cholesterol + delipided rat liver microsomes + NADPH | 8.3 | 9.3 | 14.9 | | |
| [7-\(^2\)H\(^2\)]- + [7-\(^2\)H\(^2\)]Cholesterol + singlet oxygen (hematoporphyrin sensitizer + hv) | 1.4 | 1.1 | 1.0 | 1.0 | |

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Fig. 3. Radio-HPLC of \(^2\)H-labeled products obtained after incubation of [7-\(^2\)H\(^2\)]cholesterol together with linoleic acid and lipoygenase under the conditions described under "Materials and Methods."

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of cholesterol with the epimeric 3\(^\beta\),7-diols and the 7-ketone as secondary products.

We also found 5α,6\(^\alpha\)- and 5\(^\beta\),6\(^\beta\)-epoxycholestan-3\(^\beta\)-ol as products of cholesterol in the lipoygenase reaction. The 5,6-epoxides formed from [7-\(^2\)H\(^2\)]cholesterol had retained all the \(^2\)H. This as well as the absence of an isotope effect (cf. below) clearly excludes the possibility that the above 5,6-epoxides are intermediates in the reaction. On the basis of previous work, Smith (15) has suggested that cholesterol 5,6-epoxides may be formed directly by attack of hydroperoxide on cholesterol.

The aim of the present work was to elucidate the initiation step in connection with the oxidative attack at C-7 in cholesterol in the lipoygenase system. Since the 5α-saturated analogue of cholesterol, cholestanol, is not oxidized in the lipoygenase system\(^2\) it is evident that this attack requires allylic hydrogens at C-7. Since the overall oxidation of cholesterol by the lipoygenase system is dependent upon addition of linoleate and since the enzyme is not directly involved (4), it appeared possible that formation of 13-hydroperoxy-9,11-octadecadienoic acid is a prerequisite for the conversion. In accordance with this possibility, incubation of cholesterol with the above hydroperoxide gave all three 7-oxygenated products. The yield was, however, very low in relation to that obtained with linoleate and lipoygenase. In addition the isotope effect in the hydroperoxide-dependent oxidation of [7-\(^2\)H\(^2\)]cholesterol was much lower than that obtained with linoleate and lipoygenase. It can thus be concluded that presence of 13-hydroperoxy-3,11-octadecadienoic acid is not of major importance for the oxidation at C-7 of cholesterol in the lipoxynogenase system.

A more likely mechanism is that carbon-centered or oxygen-centered linoleic acid radicals may initiate the reaction by extracting one of the allylic hydrogens in cholesterol. A significant transfer of \(^2\)H from [7-\(^2\)H\(^2\)]cholesterol to linoleic acid (or its conjugated isomer) was found, suggesting at least some participation of a carbon-centered linoleic acid radical. It should be pointed out that transfer of \(^2\)H to a peroxyl radical or to an oxy1 radical yielding alcohol or hydroperoxide would lead to loss of \(^2\)H and go undetected.

Extraction of hydrogen from an unsaturated fatty acid in connection with a lipoxygenase reaction is known to be associated with a marked isotope effect, and \(K_{D}/K_{0}\) values of 8-9 have been reported (16, 17). The intermolecular isotope effect observed here in connection with conversion of [7-\(^2\)H\(^2\)]cholesterol into the 7-oxygenated products was of the same magnitude, as could be expected if the extraction of hydrogen from cholesterol involves a radical reaction. When extraction of hydrogen is the rate-limiting step in a reaction, a marked isotope effect can be expected if this hydrogen is replaced with deuterium or tritium (14, 18). If the substituted hydrogen is lost in a step which is not rate limiting, only small isotope effects can be expected. It has been stated that the lower limit of primary isotope effects of deuterium which may be interpreted as identifying the slowest or rate-limiting step in a multistep reaction is about 8 (19). The \(K_{D}/K_{0}\) values obtained in the lipoxygenase reaction were, however, well above 8 in all experiments performed. It is thus tempting to suggest that extraction of one of the allylic hydrogens from C-7 in cholesterol is the rate-limiting step in the oxidation of cholesterol under the conditions studied here. It may be mentioned in this connection that monoxygenation of [7α-\(^2\)H\(^2\)]cholesterol catalyzed by the liver microsomal cholesterol 7α-hydroxylase is not associated with an isotope effect, indicating a different type of reaction (20).

The very high degree of discrimination between unlabeled and 7-\(^2\)H\(^2\)-labeled cholesterol in the abstraction of one of the allylic hydrogens in the lipoygenase system is probably dependent upon the specific properties of the attacking radical and the degree of hydrophobicity of the surroundings. We have failed to demonstrate such a high isotope effect when oxidizing [7-\(^2\)H\(^2\)]cholesterol in nonenzymatic model systems containing a synthetic radical initiator. In preliminary experiments we used 4,4'-azoisobutyronitrile as a radical generator and obtained isotope effects in the oxidation of [7-\(^2\)H\(^2\)]cholesterol varying between 3 and 6, depend-

\(^2\) E. Lund, U. Diczfalusy, and I. Bjorkhem, unpublished results.
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ing on the solvent used and whether the free acid or the ethyl ester was used. The highest isotope effects were obtained under the most hydrophobic conditions with a lipophilic solvent and with an esterified reagent. Additionally, in the lipoxygenase system the interaction between cholesterol and the linoleic acid radical must occur in a lipophilic milieu since the cholesterol is solubilized in linoleate micelles. This situation is similar to that occurring in vivo where cholesterol is invariably associated with unsaturated fatty acids in membranes and lipoproteins and where a substantial part of the cholesterol may also be esterified to an unsaturated fatty acid.

Both in the lipoxygenase system and in the incubations with 13-hydroperoxy-9,11-octadecadienoic acid, 5-cholestene-3β,7α-diol, and 5-cholestene-3β,7β-diol were formed in about equal amounts. In principle this may be due to a nonstereospecificity in the abstraction of hydrogen, or to a nonstereospecificity in connection with the addition of oxygen, or to an epimerization of the initially formed 7-hydroperoxides. The fact that the isotope effect in the conversion of stereospecifically labeled [7α-2H]cholesterol into the two 7-diols was reduced to 2-3 shows that the initial extraction of one of the two allylic hydrogens must be nonstereospecific.

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REFERENCES
1. Girotti, A. W. (1985) in Free Radicals in Biology (Pryor, W. A., ed) Vol. 5, pp. 1–28, Academic Press, New York
2. Halliwell, B., and Gutteridge, J. M. C. (1989) Free Radicals in Biology and Medicine, Second ed., Clarendon Press, Oxford
3. Johansson, G. (1971) Eur. J. Biochem. 21, 68–79
4. Teng, J. I., and Smith, L. L. (1976) Bioorg. Chem. 5, 99–119
5. Teng, J. I., and Smith, L. L. (1973) J. Am. Chem. Soc. 95, 4060–4061
6. Corey, E. J., and Gregoriou, G. A. (1958) J. Am. Chem. Soc. 81, 3127–3133
7. Biemann, K. (1966) Mass Spectrometry: Organic Chemical Applications, p. 204, McGraw-Hill Book Company, New York
8. Hamberg, M., and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329–5335
9. Makita, A., and Wells, W. W. (1973) Anal. Biochem. 5, 523–530
10. Björkhem, I., and Åkerlund, J.-E. (1988) J. Lipid Res. 29, 136–143
11. Aringer, L. (1980) Lipids 15, 563–571
12. Schenk, G. O., Gollnick, K., and Neumüller, O. A. (1957) Liebig’s Ann. Chem. 603, 46–59
13. Kaluczny, M. A., Duncan, L. A., Merritt, M. V., and Epps, D. E. (1965) J. Lipid Res. 26, 135–140
14. Melander, L. (1960) Isotope Effects on Reaction Rates, p. 65, Ronald Press, New York
15. Smith, L. L. (1981) Cholesterol Autoxidation, pp. 214–217, Plenum Press, New York
16. Egmond, M. R., Veldink, G. A., Vliegenthart, J. F. G. and Boldingh, J. (1973) Biochem. Biophys. Res. Commun. 54, 1178–1184
17. Hamberg, M. (1984) Biochem. Biophys. Acta 793, 129–132
18. Northrop, D. B. (1981) Annu. Rev. Biochem. 50, 103–131
19. Northrop, D. B. (1975) Biochemistry 14, 2644–2651
20. Björkhem, I. (1971) Eur. J. Biochem. 18, 299–304