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Oxysterols: Formation and Biological Function

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18.1. Introduction

The association between plasma cholesterol (CHOL) and atherosclerosis has been a topic of research for many decades and continues to be a major field of investigation (McNamara, 2000; Kromhout, 2001). A considerable number of studies, both in animals and humans, have provided evidence that prolonged high levels of plasma CHOL increase the risk of developing atherosclerosis. However, the mechanisms by which CHOL contributes to the initiation and progression of atherosclerosis are still the subject of intense research. The observation that oxidized low-density lipoprotein (LDL) triggers early steps in atherogenesis (Steinberg *et al*., 1989; Ross, 1993) has focused attention on the role(s) of oxidizable components of LDL [namely the polyunsaturated fatty acid (PUFA) content], and the involvement of antioxidant vitamins, such as vitamins C and E and the carotenoids (especially β-carotene) in the prevention of cardiovascular diseases (Gey *et al*., 1991, 1993; Kushi *et al*., 1996). A number of compounds have been identified in oxidatively-modified LDL that elicit the development of atherosclerosis (Ross, 1999) and fatty streak formation (Lusis, 2000). In several cases, the compounds arise from oxidized lipids, including CHOL oxidation products or oxysterols (OS). When LDL is oxidized, the associated PUFA content is reduced, and the levels of lipid peroxides, aldehydes and OS increase (Patel *et al*., 1996; Chang *et al*., 1997). Several OS are of interest as possible reactive mediators of the structural and functional changes of the vascular system that are characteristic of the atherosclerotic

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Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.
Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

process (Sevanian *et al.*, 1995; Smith, 1996; Guardiola *et al.*, 1996; Brown and Jessup, 1999; Russell, 2000; Schroepfer, 2000; Leonarduzzi *et al.*, 2002). OS may also be linked to a wide range of other biological effects in humans and animals, including cytotoxicity, mutagenesis, carcinogenesis, necrosis, apoptosis, immunosuppression and the development of gallstones (Brown and Jessup, 1999; Lyons and Brown, 1999; Lizard *et al.*, 2000; Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002). On the other hand, OS may regulate the expression of genes that participate in the metabolism of both sterols and fats, are intermediates in the transfer of sterols from the periphery to the liver and serve as substrates for the synthesis of bile acids (Russell, 2000). However, the mechanistic details and the identity of some of the native OS species have not been established with certainty.

18.2. Formation of Oxysterols

Oxysterols are defined as oxygenated derivatives of cholest-5-en-3β-ol (cholesterol) (Figure 18.1) or precursors of CHOL that may be formed directly by autoxidation or by the action of a specific monooxygenase, or that may be secondary to enzymatic or nonenzymatic lipid peroxidation (Guardiola *et al.*, 1996; Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002). These OS may be formed in the human body by endogenous free-radical attack on CHOL or by enzymatic processes, mainly in the biosynthesis of bile acids and steroid hormones. In addition, OS may be formed exogenously by autoxidation of CHOL in foods. The nomenclature and abbreviations of OS are presented in Table 18.1. It should be emphasized at this point that the OS that occur in

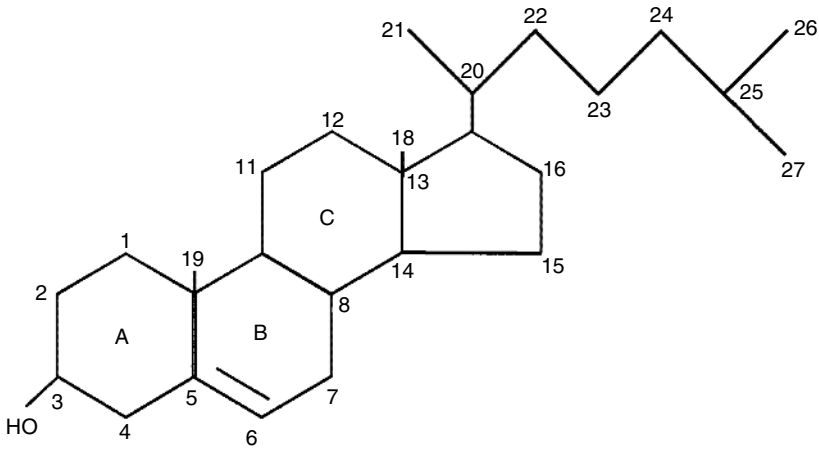

Figure 18.1. The cholest-5-en-3β-ol (cholesterol) molecule, showing ring labelling and carbon numbering.

Table 18.1. Nomenclature and Abbreviations of Cholesterol Oxidation Products

Systematic name	Trivial name	Abbreviation
Cholest-5-en-3β-ol	Cholesterol	CHOL
3β-Hydroxycholest-5-en-7-yl	Cholesterol 7-radical	7-CHOL$^{\bullet}$
3β-Hydroxycholest-5-en-7-peroxyl radical	Cholesterol 7-peroxyl radical	CHOO$^{\bullet}$
3β-Hydroxycholest-5-en-7-radical	Cholesterol 7-alkoxyl radical	CHO$^{\bullet}$
3β-Hydroxycholest-5-ene-7α-hydroperoxide	7α-Hydroperoxycholesterol	7α-OOH
3β-Hydroxycholest-5-ene-7β-hydroperoxide	7β-Hydroperoxycholesterol	7β-OOH
3β-Hydroxycholest-6-ene-5α-hydroperoxide	5α-Hydroperoxycholesterol	5α-OOH
3β-Hydroxycholest-4-ene-6α-hydroperoxide	6α-Hydroperoxycholesterol	6α-OOH
3β-Hydroxycholest-4-ene-6β-hydroperoxide	6β-Hydroperoxycholesterol	6β-OOH
3β-Hydroxycholest-5-ene-25-hydroperoxide	25-Hydroperoxycholesterol	25-OOH
3β-Hydroxycholesterol-5-ene-20α-hydroperoxide	20α-Hydroperoxycholesterol	20α-OOH
Cholest-5-ene-3β,7α-diol	7α-Hydroxycholesterol	7α-OH
Cholest-5-ene-3β,7β-diol	7β-Hydroxycholesterol	7β-OH
Cholest-6-ene-3β,5α-diol	5α-Hydroxycholesterol	5α-OH
Cholest-4-ene-3β,6α-diol	6α-Hydroxycholesterol	6α-OH
Cholest-4-ene-3β,6β-diol	6β-Hydroxycholesterol	6β-OH
Cholest-5-ene-3β,4α-diol	4α-Hydroxycholesterol	4α-OH
Cholest-5-ene-3β,4β-diol	4β-Hydroxycholesterol	4β-OH
Cholest-5-ene-3β,25-diol	25-Hydroxycholesterol	25-OH
Cholest-5-ene-3β,20α-diol	20α-Hydroxycholesterol	20α-OH
3β-Hydroxycholest-5-en-7-one	7-Ketocholesterol	7-keto
3β-Hydroxycholest-4-en-6-one	6-Ketocholesterol	6-keto
5α-Cholestane-3β,5,6β-triol	Cholestanetriol	Triol
5,6α-Epoxy-5α-cholestan-3β-ol	Cholesterol 5α,6α-epoxide	α-epoxide
5,6β-Epoxy-5β-cholestan-3β-ol	Cholesterol 5β,6β-epoxide	β-epoxide
Cholesta-3,5-diene		3,5-diene
Cholesta-3,5-dien-7-one		7-keto-3,5-dien
Cholesta-3,5,7-triene		3,5,7-triene
Cholest-5-en-3-one	5-Cholesten-3-one	3-keto-5-en
Cholest-4-en-3-one	4-Cholesten-3-one	3-keto-4-en
Cholesta-5,7-dien-3β-ol		5,7-dien-3-ol

biological membranes are normally present in trace amounts and always with a great excess (10^3- to 10^6-fold) of CHOL.

18.2.1. Cholesterol Autoxidation

Early research on the oxidation of CHOL has been reviewed extensively and evaluated by Smith (1987, 1992, 1996), Schoepfer (2000) and Lercker and Rodriguez-Estrada (2002). CHOL, with its double bond between the C-5 and C-6 of the B-ring (Figure 18.1), readily undergoes oxidation *via* a free radical mechanism to form ~75 OS products (Smith, 1996). Among the potential initiators of CHOL oxidation are preformed lipid

hydroperoxides, transition metal ions, activated oxygen species and radiation (Smith, 1992). The hydroperoxides of PUFAs, formed during lipid oxidation, may be necessary to initiate the oxidation of CHOL. Homolytic scission of hydroperoxides formed in the propagation reaction of lipid peroxidation by the action of transition metal ions (Fe^{2+} and Cu^{+}) could result in the formation of fatty acid peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals capable of abstracting the reactive allylic 7-hydrogen atom from CHOL, yielding the carbon-centered radical, 3β-hydroxycholest-5-en-7-yl (7-$CHOL^{\bullet}$) (Figure 18.2), thereby initiating the oxidation process. The 7-$CHOL^{\bullet}$ radical reacts with O_2 to yield the 3 β-hydroxycholest-5-en-7-peroxyl radical ($CHOO^{\bullet}$), stabilized in turn by hydrogen abstraction from an unsaturated fatty acid (LH) or another CHOL molecule to yield epimeric 7-hydroperoxycholesterol (7-OOH), 3β-hydroxycholest-5-ene-7α-hydroperoxide, 7α-OOH, and 3β-hydroxycholest-5-ene-7β-hydroperoxide, 7β-OOH (Smith, 1992). The 7-OOH are unstable under conditions involving heat and/or storage and reducing agents. Reducing agents transform the 7-OOH to the corresponding 7α- and 7β-hydroxycholesterol. In the absence of reducing agents, the reduction of the 7-OOH to the 7-alcohols (7-OH) may be regarded as involving thermal or transition metal ion-catalyzed homolysis of the peroxide bond, yielding cholesterol 7-alkoxyl radicals ($CHO^{\bullet}$) (Figure 18.2). The $CHO^{\bullet}$ radical, in turn, abstracts hydrogens from other molecules to form the major autoxidation OS products, cholest-5-ene-3β,7α-diol (7α-OH) and cholest-5-ene-3β,7β-diol (7β-OH) (Figure 18.2) (Smith, 1987). Dehydration of the intermediate $CHO^{\bullet}$ radical results in the formation of 3β-hydroxycholest-5-en-7-one (7-keto). Alternatively, dehydrogenation of 7α- and 7β-OH leads to the formation of 7-keto derivative, decomposition of which by abstraction of the OH group at position 3, leads to the formation of a conjugated triene, cholesta-3,5-dien-7-one (7-keto- 3,5-dien).

Formation of isomeric 5, 6-epoxides is mediated by addition of peroxyl radicals (arising either from fatty acid hydroperoxides or 7α- and 7β-OOH) to the 5,6 double bond of CHOL, with subsequent heterolytic cleavage to produce 5,6α-epoxy-5α-cholestan-3β-ol (α-epoxide), and 5,6β-epoxy-5β-cholestan-3β-ol (β-epoxide) (Figure 18.3) (Bortolomeazzi *et al.*, 1994; Chang *et al.*, 1997). Both epoxides are usually detected in oxidized CHOL (Lercker and Rodriguez-Estrade, 2002). Generally, epoxidation of the B-ring with peroxyl radicals favors the formation of the β-epoxide rather than the α-isomer. Hydration of either epoxide, in an acidic environment, generates 5α-cholestane-3β-5,6 β-triol (triol).

CHOL is very stable during heating at 100°C for 24 h, but is unstable at temperatures above 120°C (Osada *et al.*, 1993a). When CHOL is heated at 120°C, 7-keto is the predominant OS formed (Osada *et al.*, 1993a). Heating at 150°C results in the production of OS 7-keto $>$ α-epoxide

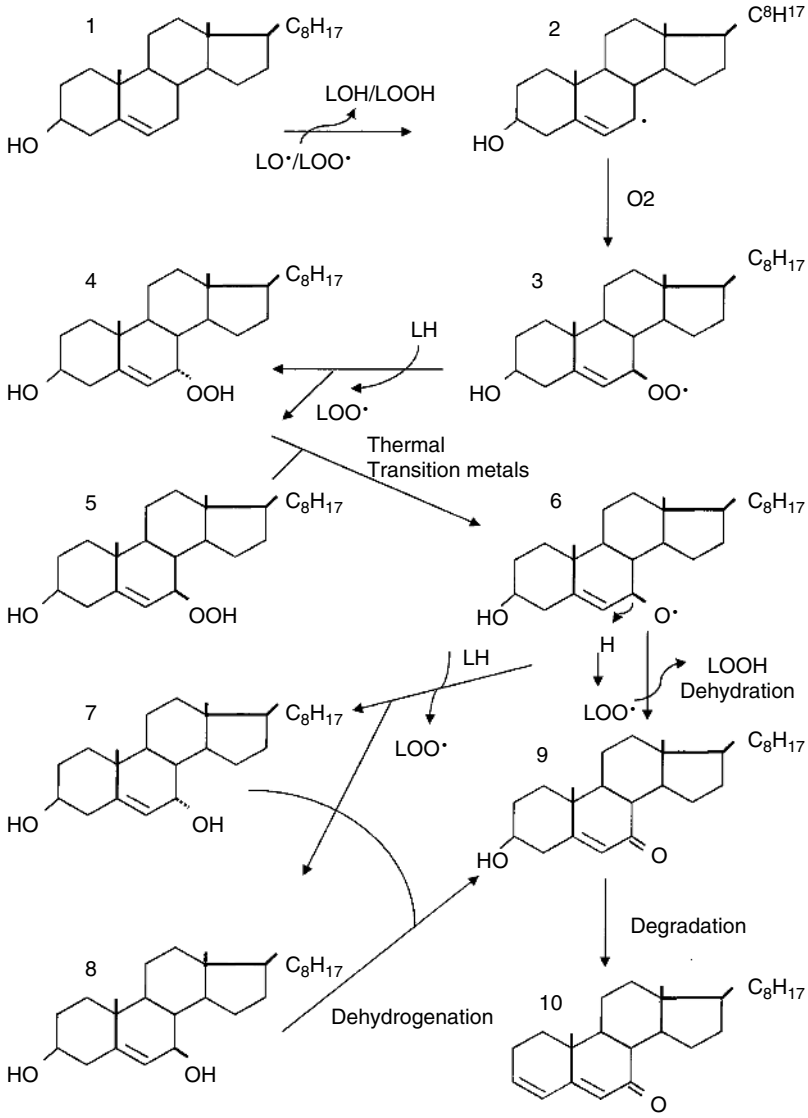

Figure 18.2. Cholesterol autoxidation initiated by peroxy ($LOO^{\bullet}$) or alkoxyl ($LO^{\bullet}$) radicals arising from peroxidation of polyunsaturated fatty acids (LH). Compounds are as follows: (1) CHOL; (2) 7-$CHOL^{\bullet}$; (3) $CHOO^{\bullet}$; (4) 7α-OOH; (5) 7β-OOH; (6) $CHO^{\bullet}$; (7) 7α-OH; (8) 7β-OH; (9) 7-keto; (10) 7-keto-3,5-dien. For abbreviations, see Table 18.1.

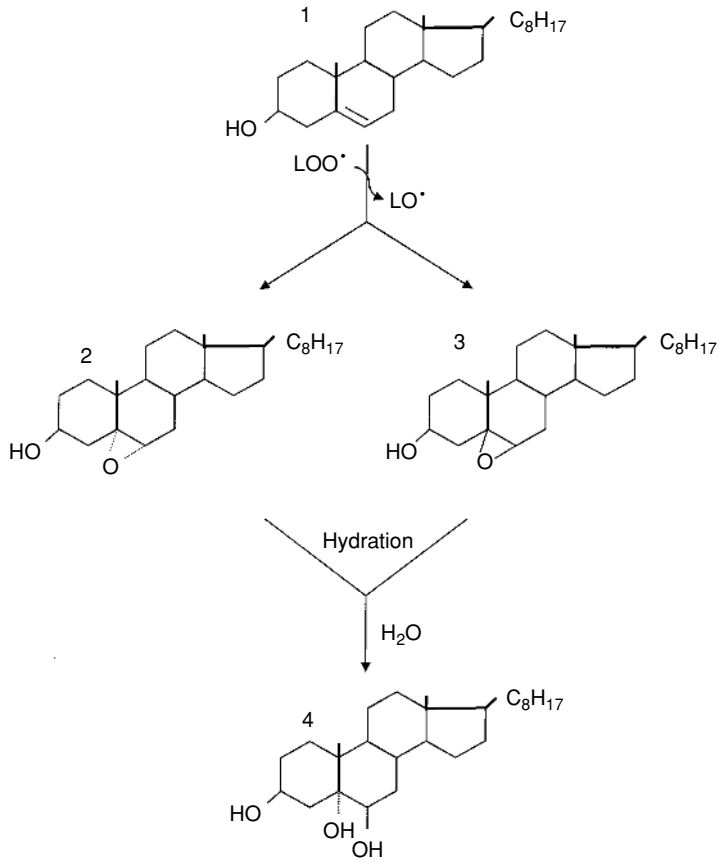

Figure 18.3. Scheme for the formation of cholesterol epoxides. Compounds are as follows: (1) CHOL; (2) α-epoxide; (3) β-epoxide; (4) triol. For abbreviations, see Table 18.1.

> β-epoxide > 7α-OH > 7β-OH. The production of OS at 200°C is low due to the decomposition of CHOL (Osoda *et al.*, 1993a).

When oxidation products such as 7α-OH, 7β-OH and 7-keto are heated they can generate other oxygenated compounds (Lercker and Rodriguez-Estrada, 2002). Dehydration of 7-keto, with subsequent abstraction of the OH group at position 3, leads to the formation of a conjugated triene with a keto group, 7-keto-3,5 dien (Figure 18.4). Abstraction of the OH from position 3 is favored by the presence of a double bond between C-5 and C-6 (Lecker and Rodriguez-Estrada, 2002). Elimination of a molecule of water from the OH group at position 7 of 7α-OH and 7β-OH, gives rise to

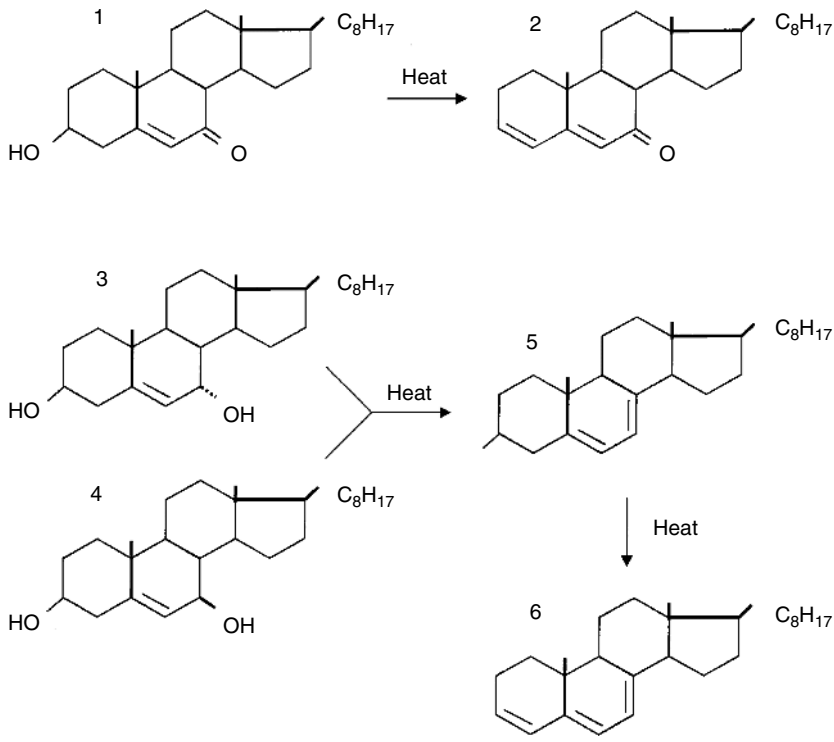

Figure 18.4. Effect of heat on some cholesterol oxidation products. Compounds are as follows: (1) 7-keto; (2) 7-keto-3,5-dien; (3); 7α-OH; (4) 7β-OH; (5) 5,7-dien-3-ol; (6) 3,5,7-triene. For abbreviations, see Table 18.1.

conjugated cholesta-5,7-dien-3β-ol (5,7-dien-3-ol) and subsequently to conjugated cholestra-3,5,7-triene (3,5,7-triene).

Oxidative processes also occur at a very slow rate; they involve oxidation of the 3β-hydroxyl of CHOL and lead to the formation of cholest-5-en-3-one (3-keto-5-en), which rearranges rapidly to cholest-4-en-3-one (3-keto-4-en) with a conjugated double bond structure (Figure 18.5) (Smith, 1987). The 3-keto-5-en can also be oxygenated to the epimeric 6-hydroperoxycholesterols,3-β-hydroxycholest-4-ene-6α-hydroperoxide (6α-OOH) and 3β-hydroxycholest-4-ene-6β-hydroperoxide (6β-OOH).

Singlet oxygen, in the excited state ($^1O_2$), reacts relatively slowly with CHOL *via* a non-radical mechanism; the rate constant for $^1O_2$ addition to CHOL is $\sim 6.7 \times 10^{-4}\,mol^{-1}\,s^{-1}$, which is about 25% that of methyl arachidonate (Girotti, 2001). Only three hydroperoxides are produced in $^1O_2$-mediated reactions:3β-hydroxycholest-6-ene-5α-hydroperoxide

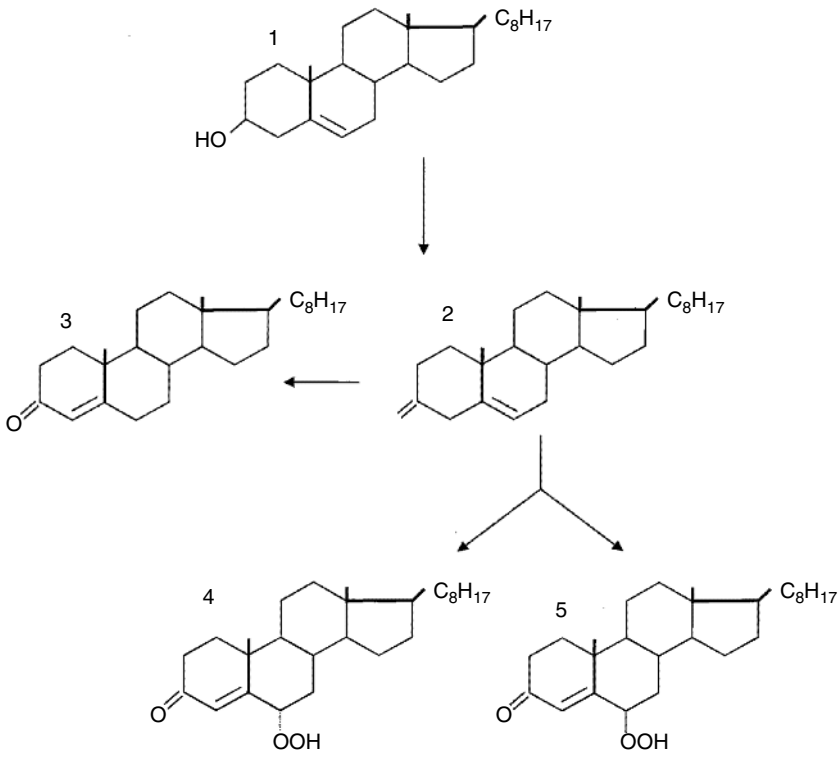

Figure 18.5. Formation of some minor cholesterol products. Compounds are as follows: (1) CHOL; (2) 3-keto-5-en; (3) 3-keto-4-en; (4) 6α-OOH; (5) 6β-OOH. For abbreviations, see Table 18.1.

(5α-OOH), 6α- and 6β-OOH (Figure 18.6). The rate of accumulation of 5α-OOH in photoperoxidizing membranes exceeds that of 6α- or 6β-OOH by at least five-fold (Smith, 1996; Girotti, 2001). Although $^1O_2$ does not produce 7α-, and 7β-OOH directly, these peroxides could be derived from 5α-OOH *via* allylic rearrangement and isomerization and, in turn, form 7-α, 7β-OH and 7-keto (Girotti, 2001). Small amounts of decomposition products of 5α-OOH, such as cholest-6-ene-3β,5α-diol (5α-OH), are also formed *via* the photoxidative mechanism (Smith, 1996).

The effects of riboflavin or fatty acid methyl esters on the photooxidation of CHOL have been investigated (Hu and Chen, 2002; Chien *et al.*, 2003). The data show that the presence of riboflavin or fatty acids methyl esters facilitates the degradation of CHOL and the production of OS. Riboflavin had a more pronounced effect than fatty acid methyl esters. 7-Keto

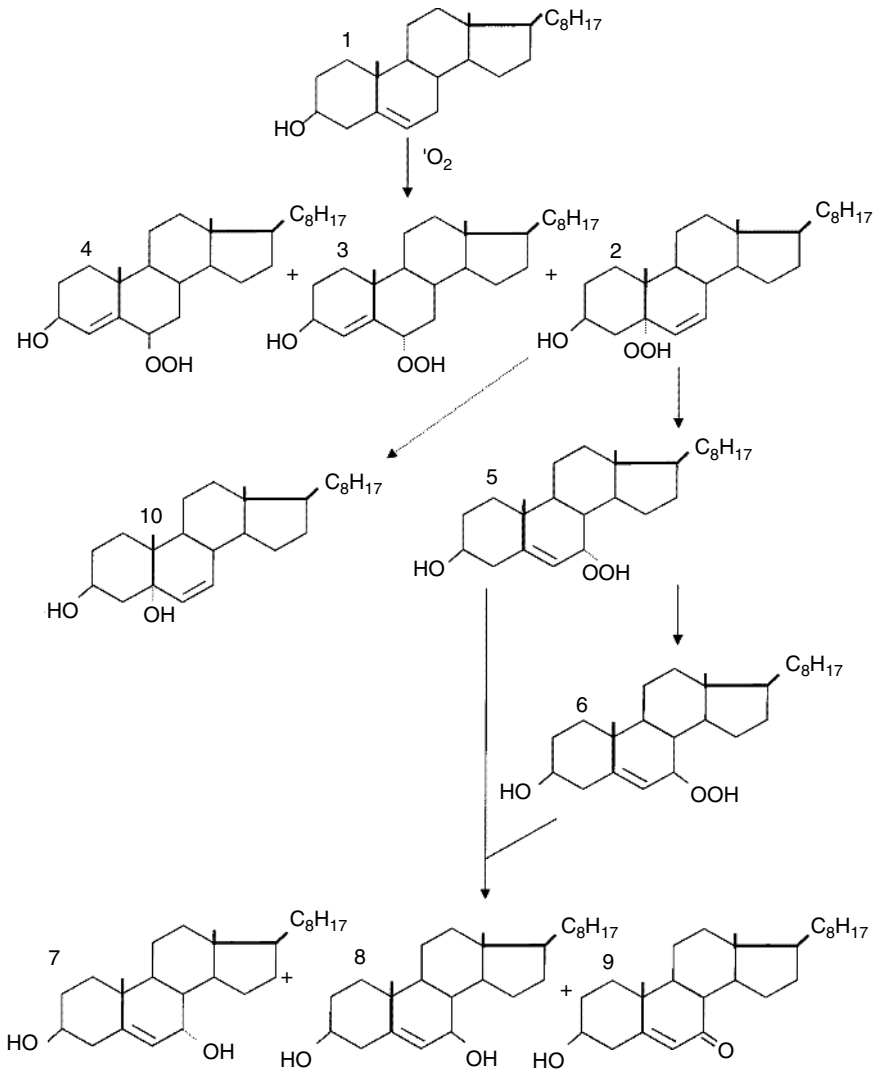

Figure 18.6. Main cholesterol oxidation products formed by singlet oxygen. Compounds are as follows: (1) CHOL; (2) 5α-OOH; (3) 6α-OOH; (4) 6β-OOH; (5) 7α-OOH; (6) 7β-OOH; (7) 7α-OH; (8) 7β-OH; (9) 7-keto; (10) 5α-OH. For abbreviations, see Table 18.1.

was generated in largest amounts, followed by β-epoxide, 7-keto-3,5 dien, α-epoxide, 7α- and 7β-OH. The formation of 7-keto-3,5-dien was probably due to dehydration of 7-keto by energy liberated during illumination (Chien

et al., 2003). The presence of docosahexaenoic ($C_{22:6}$) acid methyl esters favoured the formation of α- and β-epoxides (Hu and Chen, 2002).

Initiation of oxidation by a water-soluble initiator, 2,2[1]-azobis (2-amidinopropan) dihydrochloride (AAPH), leads to the accumulation of 7-keto as the dominant OS (Nielsen *et al.*, 1996a). The isomeric 7α- and 7β-OH were also formed in small amounts, and were found to dehydrogenate to 7-keto through a two-step radical reaction. 7α-OOH, which was formed in higher concentrations than 7β-OOH, was dehydrogenated in the oxidizing system to 7-keto at a rate approximately half of that for 7β-OOH. Nielsen *et al.* (1996a) concluded that 7-keto was not only a product of dehydration of the isomeric forms of 7-OOH, but may also be a product of dehydrogenation of the isomeric forms of 7-OH when exposed to a carbon-centered radical. On the basis of these findings, lipid peroxy radicals formed in foods or in biological systems would appear to be responsible, at least in part, for the generation of 7-keto from the isomeric 7-OOH.

The oxidative mechanisms and pathways for CHOL oxidation are reasonably well documented and are considered to involve a series of free radical chain reactions similar to that for fatty acid oxidation. However, the kinetics of CHOL oxidation has received little attention until recently. Chien *et al.* (1998) defined the major pathways (Figure 18.7) and calculated the rate constants for these reactions (Table 18.2). The reaction can be divided into

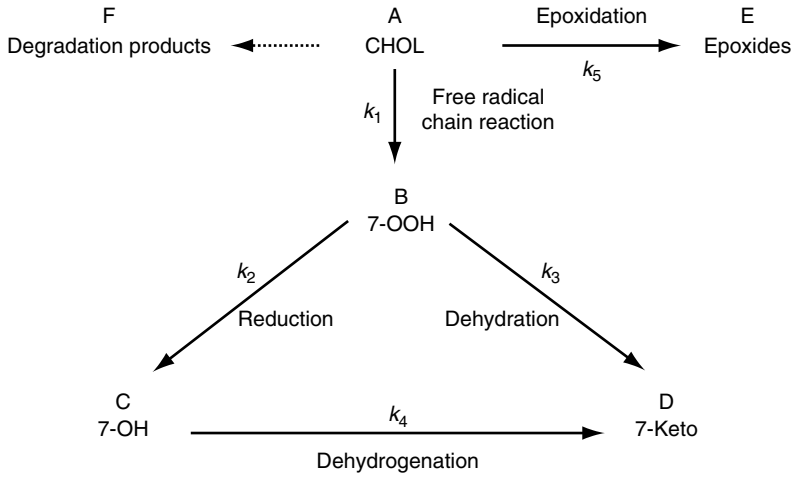

Figure 18.7. Major pathways of cholesterol oxidation: A, CHOL; B, 7-OOH (7α-and 7β-OOH); C, 7-OH (7α-and 7β-OH); D, 7-keto; E, epoxides (α-and β-epoxides). k_1-k_5, rate constants of the reactions of cholesterol oxidation. For abbreviations, see Table 18.1.

Table 18.2. Rate Constants of the Major Pathways of Cholesterol Oxidation[a]

Equation	Reaction	k/h[b]
CHOL $\xrightarrow{k_1}$ 7-OOH (7α-and 7β-OOH)	free radical chain reaction	1587 ± 1
7-OOH $\xrightarrow{k_2}$ 7-OH (7α-and 7β-OH)	Reduction	781 ± 107
7-OOH $\xrightarrow{k_3}$ 7-keto	Dehydration	805 ± 2
7-OH $\xrightarrow{k_4}$ 7-keto	Dehydrogenation	3 ± 2
CHOL $\xrightarrow{k_5}$ epoxides (α-and β-)	Epoxidation	1357 ± 358

[a] Adapted from Chien *et al.* (1998)
[b] Mean ± standard deviation
For abbreviations, see Table 18.1.

two major routes: free radical formation at C-7 of CHOL and epoxidation, and one minor route: degradation. Oxidation *via* the C-7 route includes the formation of 7-OOH (7α- and 7β-OOH) (B) from CHOL (A) through free radical reaction, formation of 7-OH (7α- and 7β-OH) (C) from 7-OOH; formation of 7-keto (D) from 7-OOH through dehydration and formation of 7-keto (D) through dehydrogenation of 7-OH. The epoxidation route includes the formation of α- and β-epoxides (E) from cholesterol through 7-OOH, and probably involves the interaction of a hydroperoxyl radical and CHOL (Bortolomeazzi *et al.*, 1994). In addition, CHOL may be degraded to form other products (F). The reactions for the formation of 7-OOH (7α- and 7β-) and epoxides (α- and β-epoxides) followed a second-order reaction model, while the formation of 7-OH, 7-keto and the dehydrogenation of 7-OH conformed to a first order reaction model. The data in Table 18.2 show that the value of k_1 is very large, indicating that the formation of 7-OOH increases very rapidly during the initial phase of oxidation, followed by epoxidation, dehydration, reduction and dehydrogenation. The sum of k_2 and k_3 is only slightly lower than k_1, indicating that 7-OOH is rapidly and quantitatively degraded to form 7-OH and 7-keto. The data also show that the dehydration step (B→D) proceeds more rapidly than the reduction step (B→C), and that 7-keto is formed, albeit at a slow rate ($k_3 = 3 \pm 2$/h), from 7-OH. This model may help to explain the relatively high concentration of 7-keto compared to other OS products present in many food products. Chien *et al.* (1998) also observed that the epoxides were formed in the greatest amount and increased linearly over the heating period of the kinetic study. They concluded that differences in the heat treatment of cholesterol may greatly affect the formation and degradation rates of 7-OOH.

18.2.2. Initiation of Cholesterol Oxidation

Among the potential initiators of CHOL oxidation are pre-formed hydroperoxides, transition metals, peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals, hydroxyl radicals ($HO^{\bullet}$), HOCl, H_2O_2 (Smith, 1996) and nitrogen oxides (Lai *et al.*, 1995). In model systems, cholesterol fatty acyl esters are oxidized by peroxynitrite $ONOO^{-}$ (Van Der Vliet *et al.*, 1994) and copper (Malavasi *et al.*, 1992). The pattern of OS formed generally includes the 7α-OH and 7β-OH (predominating), 7-keto, 7-keto-3,5-dien, α- and β-epoxide (predominating), and 7α- and 7β-OOH (Smith, 1996). The same pattern, without the 7α- and 7β-OOH, but with the two epoxides (α-epoxide predominant) arise by $HO^{\bullet}$ attack. Oxidation by HOCl produces the two epoxides and cholest-5-ene-3β, 4β-diol (4β-OH), while several other hydroxyl and keto derivatives were present in smaller amounts (Van den Berg *et al.*, 1993). Zarev *et al.* (1999) observed a markedly lower production of OS (7-keto, 7β-OH and β-epoxide) induced by $O_2^{\bullet}/HO^{\bullet}$ free radicals than by copper.

Sterol 3β-fatty acyl esters are also susceptible to oxidation, with the formation of three types of oxidized esters: esterified OS, sterol esters of oxidized fatty acyl moieties and OS esters of oxidized fatty acids (Smith, 1996). According to Lercker and Rodriguez-Estrada (2002), cholesteryl esters oxidize more rapidly than CHOL when heated. Oxidation of 3β-fatty acyl esters proceeds from initial radical generation at the ester carbonyl group to give esterified OS (Sevilla *et al.*, 1986). Interaction between CHOL and other lipid components, such as triacylglycerol, fatty acid methyl esters and fatty acids, also affect CHOL oxidation (Lercker and Rodriguez-Estrada, 2002). Differences in the susceptibility of CHOL to oxidation in model systems are related to differences in the degree of unsaturation of existing triacylglycerols (Ohshima *et al.*, 1993; Osada *et al.*, 1993b; Li *et al.*, 1994), and a significant linear relationship between the level of OS and peroxide value was also observed (Li *et al.*, 1994). It is interesting to note that oxygen uptake (an index of PUFA oxidation) was not observed when a mixture of triolein and CHOL was incubated (Ohshima *et al.*, 1993). No detectable levels of sterol oxidation products were formed, and the levels of oleic acid also remained unchanged after storage at 25°C for up to 100 d. However, for mixture of fish liver triacylglycerols and CHOL, a very significant increase in oxygen uptake was observed after 38 d of storage, as well as a continuous increase in OS content with a concurrent decrease in PUFA residues. Lowering the degree of unsaturation of fish oil triacylglycerols was effective in extending the induction phase prior to the propagation phase of CHOL oxidation (Li *et al.*, 1994).

Increasing the degree of unsaturation of PUFA increases the concentration of labile *bis* allylic hydrogen atoms, making it more likely that one of

these hydrogens may be abstracted by $LOO^{\bullet}$ or other radicals (Pryor, 1994; Morrissey *et al*., 2000). The rate of oxidation of PUFA is proportional to the number of doubly allylic hydrogen atoms in a given PUFA molecule (Pryor, 1994). On a scale where oleate ($C_{18:1}$) undergoes autoxidation too slowly to measure, linoleate ($C_{18:2}$), with two allylic hydrogens undergoes oxidation half as fast as does linolenate ($C_{18:3}$) with four allylic hydrogens. The rate of oxidation of an olefin is expressed as the "Oxidizability Factor" (Pryor, 1994). The oxidizability factor of oleic acid ($C_{18:1}$) is practically zero and the value for $C_{18:2}$, $C_{18:3}$, $C_{20:4}$ and $C_{22:6}$ is 20, 41, 55 and 102, respectively. Thus, the oxidizability of $C_{22:6}$ (10 doubly allylic hydrogen atoms) is five-times greater than that of $C_{18:2}$ (two doubly allylic hydrogen atoms). The data of Ohshima *et al*. (1993) and Li *et al*. (1994) clearly indicate that where the oxidizability factor is very low (as for oleic acid), CHOL oxidation is unlikely to occur. On the other hand, where the oxidizability factor is high (as for fish oil, rich in $C_{22:5}$, $C_{22:6}$), CHOL oxidation occurs readily. The results strongly suggest that $LOO^{\bullet}$, $LO^{\bullet}$ and other radicals from PUFA peroxidation initiate the CHOL oxidation process and promote the propagation stage, probably by abstracting reactive allylic 7-hydrogens from CHOL molecules. The presence of chain-breaking antioxidants, such as vitamin E, inhibits fatty acid and CHOL oxidation in LDL (Halliwell and Gutteridge, 1999), and in muscle-based foods (Monahan *et al*., 1992; Galvin *et al*., 1998a).

The oxidation of CHOL induced by ionizing radiation generates a large number of products originating primarily from the reactive allylic 7-hydrogen in CHOL (Sevilla *et al*., 1986). Both the 25-peroxy and 7-peroxy cholesterol radicals are formed initially at low temperatures, but at higher temperatures, the 25-peroxy radical reacts rapidly, leaving only the 7-peroxy species. Ionizing radiation yields the usual OS, including 7-keto, α- and β-epoxides and 3-keto-4-en (which originates from the A-ring) and the levels of the isomeric epoxides generally exceed that of 7-keto. Radiation-induced oxidations are also accompanied by rearrangement of the isomeric 5,6-epoxides to 6-keto and of the epimeric diols (7α- and 7β-OH) to 7-keto (Smith, 1996).

18.3. Oxysterols in Food Products

During the processing and storage of foods of animal origin, events that result in the formation of fatty acid radicals and hydroperoxides are also likely to lead to the formation of OS. The variables that are important in relation to fatty acid oxidation and OS formation in foods include composition of the food matrix, PUFA content and oxidizability, CHOL level,

processing methods, processing times and temperatures, pH, packaging conditions, composition of the atmosphere, pro- and antioxidants and water activity. Food products that are highly susceptible to CHOL oxidation include milk powders, meat and meat products (including fish), cheese and egg and egg products.

Methodologies employed for the isolation, characterization and quantitation of OS in foods and other biological systems vary considerably in sophistication, with separation and quantitation based on approaches ranging from simple TLC to sophisticated GC, GC-MS, HPLC and NMR methods (Schroepfer, 2000). Extensive reviews have been published on procedures used for sample extraction and purification (Ulberth and Buchgraber, 2002) and determination of OS by GC (Guardiola *et al.*, 2002), HPLC (Rodriguez-Estrada and Caboni, 2002) and by TLC (Lebovics, 2002). Recently, Shan *et al.* (2003) characterized the chromatographic behaviour of a large number of OS on a variety of stationary and mobile phases, and discussed their applications in the isolation and determination of OS in biological systems. Details of methods used for purification and enrichment of OS by various saponification methods and transesterification of lipids were published by Ubhayasekera *et al.* (2004).

Paniangvait *et al.* (1995), in a comprehensive review, noted that research on the occurrence of OS in foods had been based on reliable methods only since the mid 1980s and proposed that there was an urgent need for repeated analyses of OS in foods using standard methods of analysis. Until this is done, many values for OS in foods must be considered approximations. A major deficiency in almost all of the early studies was a lack of serious attention to the problems of artifactual generation of OS from CHOL during processing and analysis of various samples (Rose-Sallin *et al.*, 1995; Schroepfer, 2000). Rose-Sallin *et al.* (1995) presented data on the artifactual generation of OS from CHOL during repeated ($n = 20$) analysis of one sample of milk powder containing 1 mg CHOL per gram. The results indicated that, under the conditions studied, at least 2% of the CHOL in the sample underwent autoxidation during analysis. Specifically, OS levels (ng/g milk powder), formed artifactually, were as follows: 7α-OH, <10; 7β-OH, 80 ± 30; 7-keto, 1490 ± 380; triol, 10 ± 5; and 25-OH, 340 ± 140 (total OS, 1920).

In an attempt to standardize the analysis of OS, a round-robin test on whole milk powder and skim-milk powder was organised by Appelqvist (1996). Analyses of OS were carried out in 17 laboratories and the differences in the level found led Appelqvist (1996) to conclude that it is still premature to establish "true" values for the level of OS in certain foods. The results of a second round-robin study on egg and milk powders showed that several OS in the samples were not determined or were below the determin-

ation limit of some of the laboratories (Dutta *et al*., 1999; Dutta and Savage, 2002). The critical control points in the analysis were identified by Dutta and Savage (2002) and include extraction, saponification, enrichment, use of an internal standard, recovery of the OS during work-up steps, response factors, linearity range, limits of detection and quantification. Overall, analysis of OS in foods is a multi-step method and is rather difficult because OS are a minor fraction of unoxidized CHOL (present 10^3- to 10^6- fold in excess) in foods. In addition, CHOL itself is a minor component because it is found associated with bulk lipids such as triacylglycerols, polar lipids and other lipid components. According to Dutta and Savage (2002), small amounts of OS present in foods and the multistep analytical procedures, the lack of authentic standards of defined structure, purity and different approaches taken by different research groups contribute to concerns about the reliability of different published values for the level of OS in the same foods.

A number of review papers have been published recently on the formation and content of OS in eggs and egg products (Galobart and Guardiola, 2002), milk and milk products (Stanton and Devery, 2002), meat and meat products (Kerry *et al*., 2002), sea-foods and sea-food products (Ohshima, 2002) and in other foods (Evangelisti and Zunin, 2002). Readers are encouraged to explore these reviews for information on the origin and content of OS in food systems.

18.3.1. Oxysterols in Dehydrated Systems

Spray-dried, CHOL-containing foods are susceptible to oxidation of the fatty acids and sterols during subsequent storage at ambient temperature for prolonged periods. In general, commercial milk powders contain very low levels of OS (Rose-Sallin *et al*., 1993; Angulo *et al*., 1997; McCluskey *et al*., 1997). The major OS found in whole- and skim-milk powders were the oxidized C-7 OS derivatives, 7α-OH, 7β-OH and 7-keto and the total OS level found ranged from 0.26 to 1.9 mg/kg (Rose-Sallin *et al*., 1995) and from 0.1 to 1.1 mg/kg (Angulo *et al*., 1997). The latter group observed that storage time at 20°C increased levels of both 7-keto and 7β-OH and that the rate of formation of 7β-OH was significantly greater than that of β-epoxide (Chan *et al*., 1993). However, the secondary OS compounds, α- and β-epoxides and triol, were formed in much higher quantities when the powders were stored at 40°C (Chan *et al*., 1993) or at 55°C (Angulo *et al*., 1997). Exclusion of light and oxygen significantly reduced the development of OS in whole milk powders during storage (Chan *et al*., 1993; McCluskey *et al*., 1997).

The drying technology used exerts a major influence on triacylglycerol oxidation and on the levels of OS in milk and other powder products, both immediately after drying and upon storage. Nitrogen oxides (NO_x), which

include nitric oxide and nitrous oxide, are produced from air as a result of combustion processes. Morgan and Armstrong (1992) manipulated the levels of NO_x in the combustion gas of a direct gas-fired heating system and observed that the outlet temperatures and percent NO_x were the only conditions that affected OS formation in egg yolk powder. Lipid oxidation, including the generation of OS, was also high in spray-dried whole milk powder and tended to correlate with NO_x levels in the drying air (Chan *et al.*, 1993). Total OS formed in spray-dried whole egg using a direct-heating gas burner (which produced NO_x) was approximately 2–5 times greater than that in powders processed by an electric heating system (Lai *et al.*, 1995).

18.3.2. Oxysterols in High-Fat Products

Nielsen *et al.* (1996b) reported significant accumulation of OS in dairy spreads compared to butter. The concentration of OS was 4 times higher in dairy spreads than in butter after storage at 4°C for 13 weeks, and 7-keto was the dominant oxidation product, at 1.3 and 5.7 μg/g lipid in the stored butter and dairy spread, respectively. This difference in stability is undoubtedly related to the high content of PUFA in dairy spreads.

Home-made ghee, used extensively in traditional Indian cooking, has been estimated to contain up to 12% of total sterol as OS (Jacobson, 1987). However, several studies reported that OS were observed only when the product had been heated at a high temperature and where extensive lipid oxidation had occurred. Epoxides and triol were detected in home-made ghee when the fat was heated at 150°C for 20–25 min, but were absent in commercially processed ghee (Prasad and Subramanian, 1992). Kumar and Singhal (1992) studied the effects of processing conditions on the oxidation of CHOL in cow and buffalo ghee and observed that OS were formed when the samples were clarified at 120°C; the concentration of OS ranged from 0.7 to 0.9% of total CHOL. Intermittent frying increased the level of free fatty acids and the peroxide value (PV) of the ghee, which corresponded to the increase in OS in the samples. All the major sterols (7-keto, 7α-OH, 7β-OH, α-, β-epoxide) increased with frying time, and 20α-OH, 25-OH and triol were detected at low levels (8–16 mg/kg) only after the third frying cycle. Kumar *et al.* (1999) observed that commercial ghee samples contained a small amount of OS (1.32% of total sterol). However, when ghee was heated at 120°C until a PV of ~25 is reacted, OS levels increased to 17.6% of total sterol. Significant increases in the levels of 7-keto, total epoxide, 20α-OH and 3-keto-5-ene were observed.

Butter and ghee are also known to contain a high level of conjugated linoleic acid (CLA) (Sserunjogi *et al.*, 1998), and the content of this compound in ghee can be increased up to fivefold from the base level by increasing the

temperature of clarification from 110 to 120°C (Aneja and Murthi, 1991). Over the past two decades or so, numerous health benefits have been attributed to CLA in humans (Mougios *et al*., 2001) and experimental animals (Belury, 2002; see also Chapter 3).

18.3.3. Other Factors Involved in Oxysterol Formation

Irradiation is seldom used in the processing of milk and milk products. Nevertheless, it may be appropriate to comment on the effects of γ-irradiation on CHOL oxidation in food systems. In general, irradiation causes a significant increase in OS formation in raw and cooked muscle-based foods when stored under aerobic packaging conditions (Galvin *et al*., 1998a; Ahn *et al*., 2001). 7α-OH, 7β-OH and 7-keto were detected immediately after irradiation and other secondary products such as epoxides, triol and 20α-OH increased on storage. However, the sensitising effect of irradiation on triacylglycerol and CHOL oxidation is invariably overcome by vacuum packaging.

A strong positive correlation between lipid oxidation and the concentration of OS has been observed in whole milk powder (Chan *et al*., 1993; McCluskey *et al*., 1997). Similar relationships have also been observed for muscle-based foods (Monahan *et al*., 1992; Galvin *et al*., 1998b). The concentrations of OS and lipid oxidation products (thiobarbituric acid-reactive substances, TBARS) are closely linked to the content of PUFA in model systems (Li *et al*., 1994) and in meats (Li *et al*., 1996; Galvin *et al*., 1998a). The exclusion of oxygen inhibits the initiation of triacylglycerol oxidation, which probably inhibits the subsequent production of OS in food systems during storage for a prolonged period. Elevated α-tocopherol levels contributed to lower lipid and CHOL oxidation in whole milk powders during storage at an elevated temperature (McCluskey *et al*., 1997). In addition, antioxidant strategies, which improve the α-tocopherol status in foods (e.g., muscle-based foods), significantly reduced total OS and the levels of 7β-OH, 7-keto and β-epoxide (Monahan *et al*., 1992; Galvin *et al*., 2000).

18.4. Sources of Oxysterols *In Vivo*

Various OS have been detected in appreciable quantities in human tissue and fluids, including human plasma, atherogenic lipoproteins and atherosclerotic plaque. However, there is still considerable uncertainty regarding the origin of oxidatively-modified sterols (Vine *et al*., 1998; Brown and Jessup, 1999; Leonarduzzi *et al*., 2002). OS present *in vivo* may be of exogenous origin (i.e., derived from the diet), or generated endogenously through autoxidation (non-enzymatic oxidation of cholesterol), with a significant

contribution from enzymatic-driven conversion. Some OS of endogenous origin appear to be produced exclusively *via* enzymatic reactions (Bjorkhem *et al*., 1994).

18.4.1. Absorption of Dietary Oxysterols

Studies on humans (Linseisen and Wolfram, 1998) and animals (Vine *et al*., 1997, 1998) have demonstrated that dietary OS are efficiently absorbed, mainly in the upper intestinal tract, and then transported in the plasma within chylomicrons (circulating chylomicrons undergo conversion to CHOL-rich chylomicron remnants by the action of endothelial lipoprotein lipase) (Leonarduzzi *et al*., 2002). After lipolysis, the remnants are cleared rapidly by the liver. Estimates of the extent of OS absorption in rats, rabbits and humans vary greatly (from 93%, Bascoul *et al*., 1986 to 6%, Vine *et al*., 1997), which may relate to the dose, model and vehicle used to administer the dose (Brown and Jessup, 1999). There is a suggestion that some OS may be absorbed preferentially and transported by chylomicrons. For example, Emanuel *et al*. (1991) found that 7-keto was the major OS in the plasma of humans fed spray-dried egg powder even though it was not the OS present in the greatest quantity in the eggs. Vine *et al*. (1997) investigated the absorption rate of OS in lymph-cannulated rats and observed that 6% of the OS load was absorbed and incorporated into lymph chylomicrons. The incorporation of OS into lymph chylomicrons differed over time, with 7β-OH having peak absorption at 3 h, followed by 7-keto at 4 h and α-epoxide at 5 h. β-Epoxide was not detected in chylomicrons. In addition, the OS-treated group had a twofold increase in CHOL and triacylglycerol content compared to rats given purified CHOL. In a follow-up study, Vine *et al*. (1998) who examined the effects of feeding oxidized CHOL (containing 6% OS) or purified CHOL to rabbits over a 2-week period, observed that the group fed oxidized CHOL had 5-times the concentration of α-epoxide and double the level of 7-keto in triglyceride-rich lipoproteins compared to rabbits fed purified CHOL. The presence of 7-keto in LDL was exclusive to animals fed the oxidized CHOL-rich diet. The study showed that there may be selective absorption into plasma and lymph chylomicrons; in particular, β-epoxide, 3-keto-4-en and 25-OH were not observed in the plasma of the oxidized CHOL-fed rabbits, although these OS were in equal, if not greater, amounts in the feed compared to the α-epoxides. The concentrations of 7β-OH and 7-keto were similar in both cases, suggesting that a CHOL-supplemented diet may increase endogenous oxidative stress, thereby resulting in increased formation of OS, as suggested by Hodis *et al*. (1991). Differences in the bioavailability of individual OS were also observed by Linseinen and Wolfram (1998).

18.4.2. Oxysterols Formed Endogenously by Nonenzymatic Oxidation

Oxidative processes, similar to those that generate OS in some processed foods, are also likely to occur *in vivo*. Hodis *et al.* (1991) concluded that certain OS (7-keto, 25-OH, α- and β-epoxide) found at elevated levels in the plasma or aorta of CHOL-fed rabbits were unlikely to be of dietary origin since they could not be detected in the CHOL-containing feed. Hodis *et al.* (1994) studied the OS composition of LDL oxidized *in vivo* and reported very high levels of 7α- and 7β-OH, 7-keto, 7-keto-3,5-dien, α- and β-epoxide, triol and 25-OH. It is generally agreed that LDL undergoes oxidation *in vivo* when challenged by a variety of reactive oxygen and nitrogen species and that oxidized LDL is the component central to the initiation and/or progression of atherogenesis at the molecular and cellular level (Steinberg *et al.*, 1989; Ross, 1999). The mechanisms of CHOL oxidation in LDL are still debatable, but are likely to be similar to those that occur in the *in vitro* oxidation of LDL. The typical LDL particle (molecular weight $\sim 2.5 \times 10^6$ Da) contains ~1600 molecules of cholesteryl ester and ~600 molecules of free CHOL. About half of the total fatty acids are PUFAs (~1300 molecules), with the primary PUFAs being $C_{18:2}$ (86%) and $C_{20:4}$ (12%), and a small amount of $C_{22:6}$ (2%) (Esterbauer *et al.*, 1992). Vitamin E, mainly as α-tocopherol, is quantitatively the most important lipophilic antioxidant present in LDL particles. Each LDL particle is protected by 10 molecules of α-tocopherol (range 3–15 mol), 1 mol γ-tocopherol and small amounts of carotenoids (Esterbauer *et al.*, 1992; Carroll *et al.*, 2000). Vitamin E in the LDL particles acts as a chain-breaking antioxidant and prevents peroxidation of PUFAs and modification of proteins by reactive oxygen species (ROS).

Copper-catalyzed oxidation of LDL is often used to study the effects of oxidized LDL on cells. The oxidative modification of LDL can be divided arbitrarily into three consecutive phases: a lag phase, during which the LDL particles become depleted of α-tocopherol; a propagation phase, during which the lipid hydroperoxide content of LDL increases rapidly; and a decomposition phase, during which the unstable hydroperoxides are degraded. Chang *et al.* (1997) showed that in the early stages when isolated LDL was exposed to oxidative stress, vitamin E became progressively depleted and the content of conjugated dienes remained low. Small amounts of β-epoxide were formed during the lag phase and the level gradually increased into the propagation phase. Beyond the lag phase, no measurable levels of vitamin E were present, and the levels of conjugated dienes (''footprints'' of oxidative stress) increased rapidly, eventually reaching a maximum. As the propagation of lipid peroxidation increased, the accumulation of OS

paralleled the formation of conjugated dienes and OS become major products of the overall oxidation process. α-Epoxide and 7α-OH accumulated during the latter period of the propagation phase and the levels of 7-keto increased linearly during the entire oxidation period. There is some disagreement concerning the kinetics of OS formation relative to other lipid peroxidation products, as Dzeletovic *et al.* (1995) found that the disappearance of PUFAs and the formation of conjugated dienes preceded the appearance of OS during Cu^{2+}-induced oxidation and appreciable OS formation was detected later than conjugated dienes. Results, in general, show that $C_{18:2}$ and $C_{20:4}$ in LDL particles are consumed in the early stages of incubation with Cu^{2+} and the formation of conjugated dienes is more extensive than that of OS. The esterified rather than free CHOL is the more likely target for free radical attack (Dzeletovic *et al.*, 1995; Brown *et al.*, 1996). Thus, free CHOL is most resistant, followed by cholesteryl oleate ($C_{18:1}$), linoleate ($C_{18:2}$), arachidonate ($C_{20:4}$) and cholesteryl docosahexaenoate ($C_{22:6}$) was the most susceptible to oxidation. During the late final stage of the propagation phase, LOOH and related products with diene conjugated double bonds are substrates for further reactions with metal ions *via* a redox cyclic mechanism to yield $LOO^{\bullet}$ and $LO^{\bullet}$ (Morrissey *et al.*, 1998), that can further drive the OS formation pathways.

Endogenous α-tocopherol in LDL or supplementation with butylated hydroxytoluene has been shown to prevent (Patel *et al.*, 1996) or delay (Chang *et al.*, 1997) OS formation. This is consistent with previously reported studies where supplementation of diet for humans with vitamin E enhanced the ability of LDL to withstand oxidative stress *in vitro* and only minimal oxidation (as measured by production of conjugated dienes) of LDL occurred while antioxidants were present (Esterbauer *et al.*, 1993). Overall, as pointed out by several research groups (Dzeletovic *et al.*, 1995; Brown *et al.*, 1996, 1997; Patel *et al.*, 1996; Chang *et al.*, 1997), it may be concluded that the oxidation of PUFAs exposed to oxidative stressors *in vitro* or *in vivo* is the likely determining factor in the modification of LDL, and it appears that the oxidation of CHOL in LDL is a secondary and later oxidation event consequent on the attack by $LOO^{\bullet}$ or $LO^{\bullet}$ on the susceptible C-7 allylic position on the B-ring of CHOL. Irrespective of the initiation and propagation methods, the pattern of OS formation was essentially the same in all cases (Patel *et al.*, 1996; Chang *et al.*, 1997). The overall yield of products identified decreased in the order: 7-keto > 7β-OH > 7-α-OH > β-epoxide > α-epoxide, except in the case of peroxynitrite oxidation, where a higher yield of β-epoxide relative to 7-keto was found (Patel *et al.*, 1996). Side-chain oxidation products of CHOL, including 24-OH, 25-OH and 27-OH, are important components of LDL oxidation.

18.4.3. Oxysterols Formed Enzymatically

From a quantitative point of view, the most important OS found *in vivo* are enzymatic products of CHOL metabolism that are involved in the early steps in the conversion of CHOL to bile acids (Schroepfer, 2000). The products of these cytochrome P-450-mediated oxygenations are found in the circulation as dominating sterols, and are involved in the early stages of bile acid formation. The classical, and quantitatively the most important, pathway in the biosynthesis of bile acids in mammalian liver starts with the hydroxylation of CHOL at the 7α-position (Russell, 2000, 2003; Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002; Chiang, 2004). The biosynthetic pathway is under strict metabolic control (hormonal and dietary factors), and multiple mechanisms are involved in the regulation of the rate-limiting hepatic P-450 enzyme, cholesterol 7α-hydroxylase (CYP7A1). In addition to the 7α-hydroxylase pathway, there is an alternative pathway starting with the hydroxylation of cholesterol at C-27 by a mitochondrial cytochrome P-450 (CYP27A1) (Bjorkhem and Diczfalusy, 2002). Because CYP27A1 can oxidize the terminal methyl group not only to a CH_2OH group (27-OH), but also to a carboxylic acid (to form 3β-hydroxy-5-cholestenoic acid), this alternative route to bile acid synthesis is called the ''acid'' pathway. The 27-OH is converted to 7α-OH, 27-dihydroxycholesterol and the carboxylic acid is converted to 3β,7α-dihydroxy-5-cholestenoic acid by oxysterol 7α-hydroxylase, mainly in peripheral tissue. CYP27A1 also mediates the conversion of the C-27 steroid side-chain of CHOL to 24- and 25-OH in liver, brain and lung (Bjorkhem, 2002). One of the major OS in the circulation, cholest-5-ene-3 β,4β-diol (4β-OH), is formed from CHOL in the liver and possibly also in the intestine by the cytochrome P-450 species CYP 3A4. There is some evidence that 7-keto may be produced enzymatically in the liver. However, current thinking suggests that 7-keto present in atherosclerotic plaque is derived only from the diet or produced *in vivo* by free radical attack on CHOL (Lyons and Brown, 1999). The introduction of an oxygen atom into CHOL (formation of OS) drastically reduces its half-life and is a mechanism by which some cells direct excess CHOL to leave the body. OS generated in extrahepatic tissue and organs may be transported to the liver and metabolised to bile acids, that are then excreted into the intestine as water-soluble compounds (Lyons and Brown, 2000). In humans, approximately 400 mg of CHOL per day are converted to bile acids.

OS are able to regulate key enzymes in CHOL turnover at transcriptional and post-transcriptional levels (Wolf, 1999; Bjorkhem, 2000; Tall *et al*., 2002). CHOL biosynthesis and homeostasis are regulated by two transcriptional factors: steroid regulatory element-binding proteins (SREBP)-1 and -2. These become activated by proteolysis when the CHOL

supply to cells is low and interact with genes that increase enzymes involved in CHOL synthesis [e.g., hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase] (Wolf, 1999). The particular genes are affected by OS at the transcriptional level, HMG-CoA reductase is down-regulated, and CHOL synthesis is reduced (Bjorkhem, 2002; Bjorkhem and Diczfalusy, 2002). When the CHOL level is high, SREBPs become inactive, CHOL biosynthesis stops and the LDL receptor that facilitates the uptake of CHOL into cells is repressed (Wolf, 1999). At the post-transcriptional level, OS may also accelerate the degradation of HMG-CoA reductase because of the presence of high concentrations of Ca^{2+}. OS have been identified recently as important physiological activators of LXR and RXR transcription factors that regulate the catabolic degradation of CHOL by activation of the genes controlling cholesterol 7α-hydroxylase, the rate-limiting enzyme in the conversion of CHOL into bile (Wolf, 1999; Tall *et al.*, 2002). LXR and RXR work together to induce a battery of genes that mediate cellular CHOL efflux and transport and CHOL excretion in bile or intestinal lumen.

18.5. Biological Effects of Oxysterols

Despite being present at low concentrations, OS are considered to have potent biological effects and have been ascribed a number of important biological functions as metabolic intermediates (Russell, 2000; Schroepfer, 2000), regulators of CHOL homeostasis and other cellular processes (as discussed above), atherogenic agents (Brown and Jessop, 1999; Schroepfer, 2000; Panini and Sinensky, 2001), induction of apoptosis in cells (Panini and Sinensky, 2001) and modulators of cell permeability (Smondyrev and Berkowitz, 2001; Meaney *et al.*, 2002). These, and other issues, have been discussed comprehensively by the above authors and consequently only certain issues will be reviewed in this chapter.

18.5.1. Effects of Oxysterols on Cell Membranes

When CHOL is incorporated into a lipid bilayer, it preferentially orientates such that 3β-OH group interacts with nearby polar head-groups, with the rest of the CHOL molecule oriented roughly perpendicular to the plane of the membrane (Ohvo-Rekila, 2002). This configuration permits the maximum interactions between the non-polar regions of the CHOL molecule and the acyl chains of the bilayer lipids, while minimizing the exposure of the hydroxyl group to a nonpolar environment (Meaney *et al.*, 2002). The introduction of an additional polar moiety (e.g., an OS) into the hydrophobic region of lipid bilayers probably leads to a redistribution of the sterol in conjunction with a local reordering of the acyl chains (Meaney *et al.*, 2002).

The presence of ketocholesterols (Smondyrev and Berkowitz, 2001) or side-chain OS (Kauffman *et al*., 2000) leads to the movement of the OS towards the polar region with the net effect of increasing both the area of the membrane and acyl chain disorder (Smondyrev and Berkowitz, 2001). Increasing the area of the membrane is likely to facilitate rapid desorption of an OS from the membrane. For example, the transfer of unesterified 25-OH OS from red blood cells to plasma has been reported to occur about 2000-times faster than that of CHOL (Lange *et al*., 1995). In addition, the location of the additional oxygen function in OS is of critical importance for its rate of translocation in biological membranes and the rate of its elimination (Meaney *et al*., 2002). The rate of exchange between erythrocytes and plasma was found to be very high for 27-OH and 24-OH, and extremely low for 4β-OH (structurally similar to CHOL) and for CHOL (Meaney *et al*., 2002). The rate of transfer of OS from a monolayer to a lipoprotein particle has been shown to follow a clear rank order: 25-OH > 7β-OH > 7-keto (Theunissen *et al*., 1986). It has been suggested that where the distance between the 3β-OH group and the additional hydroxyl group is large, as in 25-OH or 27-OH, a high rate of OS movement between the inner and outer monolayers of the membrane occurs resulting in high local disordering and high permeability of the membrane (Theunissen *et al*., 1986; Meaney *et al*., 2002). On the other hand, when hydroxyl groups are close together in the nucleus (i.e., at positions 3 and 4 or 3 and 7), the disturbing effects on membrane lipids is low and membrane permeability is also low. The observed properties of side-chain oxidized OS may well be linked to their membrane-disturbing effects. Side-chain oxidized OS are cytotoxic and are very potent suppressors of CHOL synthesis (Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002). This effect may be due partly to the down-regulation of HMG-CoA reductase activity by the increased Ca^{2+} uptake in human smooth muscle cells (Zhou and Kummerow, 1994). It has been speculated that evolution has favoured metabolic systems that are able to minimize the risk for accumulation of the fast-moving membrane-disturbing OS. The possibility has been discussed that one of the major roles of oxysterol 7α-hydroxylase is to inactivate 25- and 27-OH by 7α-hydroxylation, thereby rendering them considerably less toxic.

18.5.2. Oxysterols and Apoptosis

OS have been shown to be cytotoxic *in vitro* (Aupeix *et al*., 1995) and most of the cytotoxicity of freshly isolated LDL is attributable to a minor fraction that has been oxidatively modified and highly enriched in OS (Sevanian *et al*., 1995). OS are considered to cause injury to endothelial cells, and atheromatous lesions represent "death zones" that contain toxic oxidized lipids and associated OS that cause dysfunction that ultimately

leads to programmed cell death or apoptosis (Li *et al*., 2001). The exact mechanism of OS-induced apoptosis has yet to be fully elucidated. However, lipid peroxides, OS and aldehydes may increase intracellular steady state levels of ROS, induce modification of cell proteins, and alter various signalling pathways and gene expression (Lizard *et al*., 2000; Rosenblat and Aviram, 2002; Salvayre *et al*., 2002; O'Callaghan *et al*., 2002; Leonarduzzi *et al*., 2004; Biasi *et al*., 2004). These events may participate in the toxic effect and trigger an intense, delayed and sustained calcium peak that elicits apoptosis (Salvayre *et al*., 2002). Disruption of calcium homeostasis is known to play a critical role in toxic cell injury by triggering activation of calcium-dependent degenerative "executioner" caspase enzymes, a family of cysteine-containing proteases, that is known to cause irreversible damage to cellular components and ultimately cell death (Coppola and Ghibelli, 2000; Salvayre *et al*., 2002).

A number of *in vitro* studies have characterized the potential pro-apoptotic effects of oxidized LDL and the major OS in various cell systems. Oxidized LDL has been shown to induce apoptosis in numerous cell lines, including smooth muscle cells (SMC), endothelial cells, macrophages and lymphoid cells (Auge *et al*., 2000). Among the OS of pathophysiologic interest, 7β-OH and 7-keto induce apoptosis in a variety of vascular cell lines (Lemaire *et al*., 1998; Miguet *et al*., 2001; Li *et al*., 2001) and in a dose-dependent manner (Nishio *et al*., 1996). Lizard *et al*. (1999) confirmed the effect of 7-keto using cultured SMC obtained from human artery and also observed 7β-OH and 7-keto to be toxic when added to cultures of human umbilical vein endothelial cells (HUVEC). Other OS (25-OH and α-epoxide) may also induce apoptosis in HUVEC (Lemaire *et al*., 1998) and apoptosis also occurred after treatment of human monocyte cell lines with either 25-OH or 7β-OH (Aupeix *et al*., 1995). When 7-keto was added to cells of a macrophage lineage, at a concentration range actually detectable in hypercholesterolemic patients, the pathways of apoptosis were stimulated strongly with cytochrome *c* release, caspase-9 activation and eventually caspase-3 activation (Biasi *et al*., 2004; Leonarduzzi *et al*., 2004). However, when identical concentrations of 7-keto were added to the same cells with another OS, namely 7β-OH, or in a mixture with other OS, also detectable in human LDL, the strong pro-apoptotic effect of 7-keto was attenuated markedly. The competition among OS in the mix apparently counteracted the ability of 7-keto given alone to increase greatly the steady levels of ROS in macrophage as well as the up-regulation of the pro-apoptotic factor p21 and the triggering of the mitochondria-dependent pathways of apoptosis (Leonarduzzi *et al*., 2004). During the very early phases of atherosclerotic lesion formation, various lipid oxidation products (probably including OS) contribute to the up-regulation and expression of inflammatory cytokines

and chemokines in endothelial and smooth muscle cells. In this way, slightly oxidized LDL (containing some OS) could possibly initiate subtle gene modulation within the cell wall without overt vascular cell toxicity and death (Leonarduzzi *et al*., 2002, 2004; Biasi *et al*., 2004). In the advanced stages, promotion and progression of atherosclerotic lesions are favoured by reactions that amplify the oxidative modification of LDL through cycles of cell toxicity, repair, proliferation and death (Leonarduzzi *et al*., 2004). However, the role of OS in this phase has not been defined and there is a need to reevaluate much of the earlier data obtained from *in vitro* studies on cell apoptosis where individual and non-physiological levels of OS were generally used. It is important to recognize also that a composite mix of OS is usually found in oxidized LDL, and is in the presence of a large excess (10^3–10^6) of CHOL.

18.5.3. Oxysterols and Atherosclerosis

It is generally accepted that the oxidation of LDL is an established hypothesis of atherogenesis and that accumulation of oxidized LDL in the vessel wall is an early event in disease progression (Steinberg *et al*., 1989; Parthasarathy *et al*., 1998). Through the oxidation of lipids, oxidized LDL acquire pro-inflammatory properties, that increase smooth muscle cell proliferation and play a critical role in the formation of foam cells (Lusis, 2000; Pryor, 2000; Hayden *et al*., 2002; Leonarduzzi *et al*., 2002; Steinberg, 2002). The association between atherosclerosis and OS has recently been extensively reviewed (Brown and Jessup, 1999; Schroepfer, 2000; Garcia-Cruset *et al*., 2002) and the reader is referred to these reports for detailed descriptions of the role of OS in the atherosclerotic process. OS have been implicated in atherogenesis due to their presence in human atherosclerotic plaque and their potent effect *in vitro* (Brown and Jessup, 1999). *In vitro*, OS activity could be related to atherogenic processes such as prostaglandin synthesis and platelet aggregation, their toxicity to vascular cells, their ability to modify LDL receptor function, their involvement in foam cell formation and advanced phases of atherosclerosis (see above reviews). Several authors have studied the effects of OS added to the diet of animals on the atherosclerotic process. According to Garcia-Cruset *et al*. (2002), the results from these studies seem to be conclusive about the role of OS on the progression of the atherosclerotic lesions. On the other hand, Lyons and Brown (2000) reviewed 16 oxysterol-feeding studies in the literature that used various animal models and concluded that seven were pro-atherogenic, eight anti-atherogenic and one showed no clear effect. Overall, the role of dietary OS in atherosclerosis in animals remains equivocal. Several studies have detected OS in human atherosclerotic plaque (e.g., Carpenter *et al*., 1995; Brown

et al., 1997), although only relatively few have presented quantitative data (Brown and Jessup, 1999). 27-OH is the major OS in advanced atherosclerotic lesions and was found to be approximately proportional to CHOL levels and increased with increasing severity of atherosclerosis (Carpenter *et al.*, 1995). The 7-oxygenated sterols (7β-OH and 7-keto) appear to be present in foam cells isolated from human atherosclerotic plaque (Mattsson-Hultin *et al.*, 1996) at concentrations at least two orders of magnitude higher than those of plasma (Brown and Jessup, 1999). Recently, Garcia-Cruset *et al.* (2002) reported that traces of OS, such as α- and β-epoxides, 7α- and 7β-OH, 7-keto and 27-OH, were present in normal human arteries, whereas total OS (standardized to CHOL) was much higher in fatty streaks and highest in advanced atherosclerotic plaque. Plasma OS have also been shown to be higher in smokers than in non-smokers and long-term vitamin E supplementation has been shown to be effective in reducing the plasma level of 7β-OH (Porkkala-Saratoha *et al.*, 2000).

Based on observational studies, Jacobson (1987) proposed that the markedly higher incidence of heart disease in Indians living in London compared with non-Indians in the same city may be related to their high consumption of ghee, which was reported to contain very high concentrations of OS (12.3% of total sterols). Recent studies in Britain have identified a greater degree of insulin resistance in children of South Asian ancestry compared with white children (Whincup *et al.*, 2002). Gupta *et al.* (2004) also observed that in Asian Indians there are increasing trends in the prevalence of metabolic syndrome, also known as cardiovascular dysmetabolic syndrome. Metabolic syndrome is a constellation of abnormalities characterized by central obesity, high triacylglycerol, hypertension or high normal blood pressure, low levels of high-density lipoprotein (HDL) CHOL and diabetes. Subjects with metabolic syndrome are at increased risk of developing diabetes mellitus and cardiovascular disease as well as increased mortality from other causes. Indian Asians living in the UK have approximately 50% higher rate of mortality from coronary heart disease than the native Caucasian population, which, essentially, can be attributed to metabolic syndrome (Brady *et al.*, 2004). Thus, the present knowledge points to the prevalence of various components of metabolic syndrome rather than dietary OS as the critical contributors to atherogenesis in Asian Indians living in the UK.

There is still no clear evidence that OS contribute directly to atherogenesis in humans. According to Brown and Jessup (1999), further studies are necessary to determine definitely the role of OS in atherosclerosis, and Bjorkhem and Diczfalusy (2002) concluded that the normal dietary intake of OS is probably of little or no importance in the development of atherosclerosis. It is possible that OS associated with modified LDL may be a marker or "footprint" of late events or may be an additional factor

accompanying, rather than causing, inflammation and fatty streak lesions in the arterial wall.

18.6. Conclusions

CHOL and its derivatives undergo autoxidation *via* a free radical mechanism and the first free radicals formed appear to be located at position 7. The process of CHOL oxidation is probably initiated in foods and other biological systems *via* free radicals generated from PUFA oxidation that abstract a hydrogen atom to form the initial CHOL radicals. Increasing the degree of unsaturation of coexisting triacylglycerols increases the susceptibility of CHOL to oxidation and reduces the induction phase. On the other hand, chain-breaking antioxidants, such as vitamin E, inhibit the oxidation of both PUFA and CHOL and innovative processing technologies and packaging and storage systems that help to maintain a favourable prooxidant-antioxidant balance are likely to prevent the formation of OS in foods. The available evidence indicates that the amount of OS ingested from conventionally processed foods is very low, and is unlikely to play a significant part in the development of atherosclerosis and other biological changes. OS are formed in LDL oxidized *in vitro*, and are also present in atherosclerotic plaque; however, there is no direct evidence yet that OS formed *in vivo* contribute to the development of atherosclerosis in humans. Differences have been reported on the degree of cytotoxicity from one OS to another and their ability to induce apoptosis in a variety of vascular cell types. These conclusions have been based mainly on data obtained from *in vitro* experiments with pure OS at levels of dubious pathophysiological relevance. The recent reports that competition among OS, present at normal physiological levels, diminish ROS induction and the triggering of apoptosis will no doubt provide a stimulus for further studies. Finally, OS are formed *in vivo* by enzymic mechanisms, and it has been reported that they may be important regulators of CHOL homeostasis. Understanding the mechanisms whereby OS regulate macrophage CHOL efflux and intestinal excretion may have potential for the treatment and/or prevention of atherosclerosis.

Bibliography

Ahn, D.U., Nam, K.C., Du, M., Jo, C. 2001. Effect of irradiation and packaging conditions after cooking on the formation of cholesterol and lipid oxidation products in meats during storage. *Meat Sci.* **57**, 413–418.

Aneja, R.P., Murthi, T.N. 1991. Beneficial effects of ghee. *Nature* **350,** 280.

Angulo, A.J., Romera, J.M., Ramirez, M., Gil, A. 1997. Determination of cholesterol oxides in dairy products. Effect of storage conditions. *J. Agric. Food Chem.* **45**, 4318–4323.

Appelqvist, L.-A. 1996. Oxidized sterols. Bulletin 315, International Dairy Federation, Brussels, pp. 52–58.

Auge, N., Negre-Salvayre, A., Salayre, R., Levade, T. 2000. Sphingomyelin metabolites in vascular cell signalling and atherogenesis. *Prog. Lipid Res.* **39**, 207–229.

Aupeix, K., Weltin, V., Mejia, J.E., Christ, M., Marchal, J., Freyssinet, J.-M., Bischoff, P. 1995. Oxysterol-induced apoptosis in human monocytic cell lines. *Immunobiol.* **194**, 415–428.

Bascoul, J., Domergue, M., Mourot, J., Dery, G., Crastes de Paulet, A. 1986. Intestinal absorption and fecal excretion of 5,6-α-epoxy- 5α-cholesta-3 β-ol by the male wistar rat. *Lipids* **21**, 744–747.

Beluny, M.A. 2002. Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action. *Ann. Rev. Nutr.* **22**, 505–531.

Biasi, F., Leonarduzzi, G., Vizio, B., Zanetti, D., Sevanian, A., Sottero, B., Verde, V., Zingaro, B., Chairpotto, E., Poli, G. 2004. Oxysterol mixtures prevent proapoptotic effects of 7-ketocholesterol in macrophages: implications for proatherogenic gene modulation. *FASEP J.* **18**, 693–705.

Bjorkhem, I. 2002. Do oxysterols control cholesterol homeostasis? *J. Clin. Invest.* **110**, 725–730.

Bjorkhem, I., Diczfalusy, U. 2002. Oxysterols. Friends, foes, or just fellow passengers? *Arterioscler. Thromb. Vasc. Biol.* **22**, 734–742.

Bjorkhem, I., Andersson, O., Diczfalusy, U., Sevastik, B., Xiu, R.-J., Duan, C., Lund, E.G. 1994. Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. *Proc. Natl. Acad. Sci.* USA **91**, 8592–8596.

Bortolomeazzi, R., Pizzale, L., Conte, L.S., Lercker, G. 1994. Identification of thermal oxidation products of cholestryl acetate. *J. Chromatogr.* **683**, 75–85.

Brady, L.M., Williams, C.M., Lovegrove, J.A. 2004. Dietary PUFA and the metabolic syndrome in Indian Asians living in the UK. *Proc. Nutr. Soc.* **63**, 115–125.

Brown, A.J., Jessup, W. 1999. Oxysterols and atherosclerosis. *Atherosclerosis* **142**, 1–28.

Brown, A. J., Dean, R.T., Jessup, W. 1996. Free and esterified oxysterol: formation during copper-oxidation of low-density lipoprotein and uptake of macrophage. *J. Lipid Res.* **37**, 320–335.

Brown, A.J., Leong, S.-L., Dean, R.T., Jessup, W. 1997. 7-Hydroxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque. *J. Lipid Res.* **38**, 1730–1745.

Carpenter, K.L.H, Taylor, S.E., van der Veen, C., Williamson, B.K., Ballantine, J.A., Mitchinson, M.J. 1995. Lipids and oxidized lipids in human atherosclerotic lesions at different stages of development. *Biochim. Biophys. Acta* **1256**, 141–150.

Carroll, Y.L., Corridan, B.M., Morrissey, P.A. 2000. Lipoprotein carotenoid profiles and the susceptibility of low density lipoprotein to oxidative modification in healthy elderly volunteers. *Eur. J. Clin. Nutr.* **54**, 500–507.

Chan, S.-H., Gray, J.I., Gomaa, E.A., Harte, B.R., Kelly, P.M., Buckley, D.J. 1993. Cholesterol oxidation in whole milk powders as influenced by processing and packaging. *Food Chem.* **47**, 321–328.

Chang, Y.H., Abdalla, D.S.P., Sevanian, A. 1997. Characterization of cholesterol oxidation products formed by oxidative modification of low density lipoproteins. *Free Radic. Biol. Med.* **23**, 202–214.

Chiang, J.Y.L. 2004. Regulation of bile acid synthesis: pathways, nuclear receptors, and mechanisms. *J. Hepatol.* **40**, 539–551.

Chien, J.T., Lu, Y.F., Hu, P.C., Chen, B.H. 2003. Cholesterol photooxidation as affected by combination of riboflavin and fatty acid methyl esters. *Food Chem.* **81**, 421–431.

Chien, J.T., Wang, H.C., Chen, B.H. 1998. Kinetic model of the cholesterol oxidation during heating. *J. Agric. Food Chem.* **46**, 2572–2577.

Coppola, S., Ghibelli, L. 2000. GSH extrusion and the mitochondrial pathway of apoptotic signalling. *Biochem. Soc. Trans.* **28**, 56–61.

Dutta, P.C., Savage, G.P. 2002. Harmonization of cholesterol oxidation product analysis. In: *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Bological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 114–123, AOCS Press, Champaign, IL.

Dutta, P.C., Carboni, M.F., Diczfalusy, U., Dionisi, F., Dzeletovic, S., Grandgirard, A., Guardiola, F., Kumpulainen, J., Lebovics, V.K., Pihlava, J.R., Rodriguez-Estrada, M.T., Ulberth, F. 1999. Measurement of cholesterol oxides in foods: results of an interlaboratory comparison study. In: *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Diseases* (J.T. Kumpulainen, J.T. Salonen, eds.), pp. 309–315, Royal Society of Chemistry, London.

Dzeletovic, S., Babiker, A., Lund, E., Diczfalusy, U. 1995. Time course of oxysterol formation during *in vitro* oxidation of low density lipoprotein. *Chem. Phys. Lipids* **78**, 119–128.

Emanuel, H.A. Hassel, C.A., Addis, P.B., Bergmann, S.D., Zavoral, J.H. 1991. Plasma cholesterol oxidation products in human subjects fed a meal rich in oxysterols. *J. Food Sci.* **56**, 843–847.

Esterbauer, H., Gebicki, J., Puhl, H., Jurgens, G. 1992. The role of lipid per- oxidation and antioxidants in oxidative modification of LDL. *Free Radic. Biol. Med.* **13**, 341–349.

Esterbauer, H., Puhl, H., Waeg, G., Krebs, A., Dieber-Rothender, M. 1993. The role of vitamin E in lipoprotein oxidation. In: *Vitamin E in Health and Disease* (L. Packer, J. Fuchs, eds.), pp. 649–671, Marcel Dekker, New York.

Evangelisti, F., Zunin, P. 2002. Formation and content of cholesterol oxidation products in other foods. In: *Cholesterol and Phytosterol Oxidation Products: Analysis*, *Occurrence, and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 204–216, AOCS Press, Champaign, IL.

Galobart, J., Guardiola, F. 2002. Formation and content of cholesterol oxidation products in egg and egg products. In: *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 124–146, AOCS Press, Champaign, IL.

Galvin, K., Morrissey, P.A., Buckley, D.J. 1998a. Effect of dietary α-tocopherol supplementation and gamma-irradiation on α-tocopherol retention and lipid oxidation in cooked minced chicken. *Food Chem.* **62**, 185–190.

Galvin, K., Morrissey, P.A., Buckley, D.J. 1998b. Cholesterol oxides in processed chicken muscle as influenced by dietary α-tocopherol supplementation. *Meat Sci.* **48**, 1–9.

Galvin, K., Lynch, A-M., Kerry, J.P., Morrissey, P.A., Buckley, D.J. 2000. Effect of dietary vitamin E supplementation on cholesterol oxidation in vacuum packaged cooked beef steaks. *Meat Sci.* **55**, 7–11.

Garcia-Cruset, S., Carpenter, K.L.H., Codony, R., Guardiola, F. 2002. Cholesterol oxidation products and atherosclerosis. In: *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 241–277, AOCS Press, Champaign, IL.

Gey, K.F., Moser, U.K., Jordan, P., Stahelin, H.B., Eicholzer, M., Lundin, E. 1993. Increased risk of cardiovascular disease at suboptimal plasma concentrations of essential antioxidants: an epidemiological update with special attention to β-carotene and vitamin C. *Am. J. Clin. Nutr.* **57,** 787S–797S.

Gey, K.G., Puska, P., Jordan, P., Moser, U.K. 1991. Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. *Am. J. Clin. Nutr.* **53**, 326S–334S.

Girotti, A.W. 2001. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxid effects, and cytoprotective mechanisms. *J. Photochem. Photobiol B: Biology* **63**, 103–113.

Guardiola, F., Boatella, J., Codony, R. 2002. Determination of cholesterol oxidation products by gas chromatography. In: *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 50–65, AOCS Press, Champaign, IL.

Guardiola, F., Codony, R., Addis, P.B., Rafecas, M., Boatella, J. 1996. Biological effects of oxysterols: current status. *Food Chem. Toxicol*. **34**, 193–211.

Gupta, R., Deedwania, P.C., Gupta, A., Rastogi, S., Panwar, R.B., Kothari, K. 2004. Prevalence of metabolic syndrome in an Indian urban population. *Int. J. Cardiol*. **97**, 257–261.

Halliwell, B., Gutteridge, J.M.C. 1999. *Free Radicals in Biology and Medicine*, 3rd edn, Oxford University Press, Oxford.

Hayden, J.M., Brachova, L., Higgins, K., Obermiller, L., Sevanian, A., Khandrika, S., Reaven, P.D. 2002. Induction of monocyte differentiation and foam cell formation *in vitro* by 7-ketocholesterol. *J. Lipid Res*. **43**, 26–35.

Hodis, H.N., Crawford, D.W., Sevanian, A. 1991. Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. *Atheroscler*. **89**, 117–126.

Hodis, H.N., Kramsch, D.M., Avogaro, P., Bittolo-Bon, G., Cazzoloto, G., Hwang, J., Peterson, H., Sevanian, A. 1994. Biochemical and cytotoxic characteristics of an *in vivo* circulating oxidized low density lipoprotein (LDL-). *J. Lipid Res*. **35**, 669–677.

Hu, P.C., Chen, B.H. 2002. Effects of riboflavin and fatty acid methyl esters on cholesterol oxidation during illumination. *J. Agric. Food Chem*. **50**, 3572–3578.

Jacobson, M.S. 1987. Cholesterol oxides in Indian ghee: possible cause of unexplained risk of atherosclerosis in Indian immigrant populations. *Lancet* **ii**, (8560), 656–658.

Kauffman, J.M., Westerman, P.W., Carey, M.C. 2000. Fluoro-cholesterols, in contrast to hydroxycholesterols, exhibit interfacial properties similar to cholesterol. *J. Lipid Res*. **41**, 991–1003.

Kerry, J.P., Gilroy, D.A., O'Brien, N.M. 2002. Formation and content of cholesterol oxidation products in meat and meat products. In: *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 162–185, AOCS Press, Champaign, IL.

Kromhout, D. 2001. Diet and cardiovascular diseases. *J. Nutr. Health Aging* **5**, 144–149.

Kumar, M.V., Sambaiah, K., Lokesh, B.R. 1999. Effect of dietary ghee – the anhydrous milk fat, on blood and liver lipids in rats. *J. Nutr. Biochem*. **10**, 96–104.

Kumar, N., Singhal, O.P. 1992. Effect of processing conditions on the oxidation of cholesterol in ghee. *J. Sci. Food Agric*. **58**, 267–273.

Kushi, L.H., Folsom, A.R., Prineas, R.J., Mink, P.J., Wu, Y., Bostick, R.M. 1996. Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. *New Engl. J. Med*. **334**, 1156–1162.

Lai, S.-M., Gray, J.I., Buckley, D.J., Kelly, P.M. 1995. Influence of free radicals and other factors on formation of cholesterol oxidation products in spray-dried whole egg. *J. Agric. Food Chem*. **43**, 1127–1131.

Lange, Y., Ye, J., Strebel, F. 1995. Movement of 25-hydroxycholesterol from the plasma membrane to the rough endoplasmic reticulum in cultured hepatoma cells. *J. Lipid Res*. **36,** 1092–1097.

Lebovics, V.K. 2002. Determination of cholesterol oxidation products by thin-layer chromatography. In: *Cholesterol and Phytosterol Oxidation Products: Analysis*, *Occurrence, and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 101–113, AOCS Press, Champaign, IL.

Lemaire, S., Lizard, G., Monier, S., Miguet, C., Gueldry, S., Volot, Gambert, P., Neel, D. 1998. Different patterns of IL-1 beta secretion, adhesion molecule expression and apoptosis induction in human endothelial cells treated with 7 alpha-, 7 beta-hydroxycholesterol, or 7-ketocholesterol. *FEBS Letts*. **440**, 434–439.

Leonarduzzi, G., Biasi, F., Chiarpotto, E., Poli, G. 2004. Trojan horse-like behaviour of a biologically representative mixture of oxysterols. *Mol. Aspects Med*. **25**, 155–167.

Leonarduzzi, G., Sottero, B., Poli, G. 2002. Oxidized products of cholesterol: dietary and metabolic origin, and proatherosclerotic effects (review). *J. Nutr. Biochem*. **13**, 700–710.

Lercker, G., Rodriguez-Estrada, M.T. 2002. Cholesterol oxidation mechanisms. In: *Cholesterol and Phytosterol Oxidation Products: Analysis Occurrence, and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 1–25, AOCS Press, Champaign, IL.

Li, N., Ohshima, T., Shozen, K., Ushio, H., Koizumi, C. 1994. Effects of the degree of unsaturation of coexisting triacylglycerols on cholesterol oxidation. *J. Am. Oil Chem. Soc*. **71**, 623–627.

Li, S.X., Ahn, D.U., Cherian, G., Chung, T.Y., Sim, J.S. 1996. Dietary oils and tocopherol supplementation on cholesterol oxide formation in freeze-dried chicken meat during storage. *J. Food Lipids* **3**, 27–42.

Li, W., Dalen, H., Eaton, J.W., Yuan, X.-M. 2001. Apoptotic death of inflammatory cells in human atheroma. *Arterioscler. Thromb. Vasc. Biol*. **21**, 1124–1130.

Linseisen, J., Wolfram, G. 1998. Absorption of cholesterol oxidation products from ordinary foodstuffs in humans. *Ann. Nutr. Metab*. **42**, 221–230

Lizard, G., Miguet, C., Bessede, G., Monier, S., Gueldry, S., Neel, D., Gambert, P. 2000. Impairment with various antioxidants of the loss of mitochondrial transmembrane potential and of the cytosolic release of cytochrome c occurring during 7-ketocholesterol-induced apoptosis. *Free Radic. Biol. Med*. **28**, 743–753.

Lizard, G., Monier, S., Cordelet, C., Gesquiere, L., Deckert, V., Gueldry, S., Lagrost, L., Gambert, P. 1999. Characterization and comparison of the mode of cell death, apoptosis versus necrosis, induced by 7β-hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. *Arterioscler. Thromb. Vasc. Biol*. **19**, 1190–1200.

Lusis, A.J. 2000. Atherosclerosis. *Nature* **407**, 233–241.

Lyons, M.A., Brown, A.J. 1999. 7-Ketocholesterol. *Int. J. Biochem. Cell Biol*. **31,** 369–375.

Lyons, M.A., Brown, A.J. 2000. Oxysterols in atherogenesis. In: *Atherosclerosis, Gene Expression, Cell Interactions, and Oxidation* (R.T. Dean, D. Kelly, eds.), pp. 348–370, Oxford University Press, Oxford.

Malavasi, B., Rasetti, M.F., Roma, P., Fogliatto, R., Allevi, P., Catapano, A.L., Galli, G. 1992. Evidence of the presence of 7-hydroperoxycholest-5-en-3β-ol in oxidized human LDL. *Chem. Phys. Lipids* **62**, 209–214.

Mattsson-Hulten, L., Lindmark, H., Diczfalusy, U., Björkhem, I., Ottosson, M., Liu, Y., Bondgers, G., Wilklund, O. 1996. Oxysterols present in atherosclerotic tissue decrease the expression of lipoprotein lipase messenger RNA in human monocyte-derived macrophages. *J. Clin. Invest*. **97**, 461–468.

McCluskey, S., Connolly, J.F., Devery, R., O'Brien, B., Kelly, J., Harrington, D., Stanton, C. 1997. Lipid and cholesterol oxidation in whole milk powder during processing and storage. *J. Food Sci*. **62**, 331–337.

McNamara, D.J. 2000. Dietary cholesterol and atherosclerosis. *Biochim. Biophys. Acta* **1529**, 310–320.

Meaney, S., Bodin, K., Diczfalusy, U., Bjorkhem, I. 2002. On the rate of translocation *in vitro* and kinetics *in vivo* of the major oxysterols in human circulation: critical importance of the position of the oxygen function. *J. Lipid Res*. **43**, 2130–2135.

Miguet, C., Monier, S., Bettaieb, A., Athias, A., Bessede, P., Laubriet, A., Lemaire, S., Neal, D., Gambert, P., Lizard, G. 2001. Ceramide generation occurring during 7β-hydroxycholesterol and 7-ketocholesterol-induced apoptosis is caspase independent and is not required to trigger cell death. *Cell Death Differ*. **8**, 83–99.

Monahan, F.J., Gray, J.I., Booren, A.M., Miller, E.R., Buckley, D.J., Morrissey, P.A., Gomaa, E.A. 1992. Influence of dietary treatment on lipid and cholesterol oxidation in pork. *J. Agric. Food Chem*. **40**, 1310–1315.

Morgan J.N., Armstrong, D.J. 1992. Quantification of cholesterol oxidation products in egg yolk powder spray-dried with direct heating. *J. Food Sci*. **57**, 43–45.

Morrissey, P.A., Buckley, D.J., Galvin, K. 2000. Vitamin E and the oxidative stability of pork and poultry. In: *Antioxidants in Muscle Foods. Nutritional Strategies to Improve Quality* (E.A. Decker, C. Faustman, C.J. Lopez-Bote, eds.), pp. 263–287, John Wiley, New York.

Morrissey, P.A., Sheehy, P.J.A., Galvin, K., Kerry, J.P., Buckley, D.J. 1998. Lipid stability in meat and meat products. *Meat Sci*. **49** (Suppl 1), 573–586.

Mougios, V., Matsakas, A., Petridou, A., Ring, S., Sagredos, A., Melissopoulou, A., Tsigilis, N., Nikolaidis, M. 2001. Effect of supplementation with conjugated linoleic acid on human serum lipids and body fat. *J. Nutr. Biochem*. **12**, 585–594.

Nielsen, J.H., Olsen, C.E., Skibsted, L.H. 1996a. Cholesterol oxidation in a heterogeneous system initiated by water-soluble radicals. *Food Chem*. **56**, 33–37.

Nielsen, J.H., Olsen, C.E., Jensen, C., Skibsted, L.H. 1996b. Cholesterol oxidation in butter and dairy spreads during storage. *J. Dairy Res*. **63**, 159–167.

Nishio, E., Arimura, S., Watanabe, Y. 1996. Oxidized LDL induces apoptosis in cultured smooth muscle cells: a possible role for 7-ketocholesterol. *Biochem. Biophys. Res. Commun*. **223**, 413–418.

O'Callaghan, Y.C., Woods, J.A., O'Brien, N.M. 2002. Characteristics of 7β-hydroxycholesterol-induced cell death in a human monocytic blood cell line, U937, and a human hepatoma cell line, HepG2. *Toxicol. in Vitro* **16**, 245–251.

Ohshima, T. 2002. Formation and content of cholesterol oxidation products in seafood and seafood products. In: *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 186–203, AOCS Press, Champaign, IL.

Ohshima, T., Li, N., Koizumi, C. 1993. Oxidative decomposition of cholesterol in fish products. *J. Am. Oil. Chem. Soc*. **70**, 995–600.

Ohvo-Rekila, H., Ramstedt, B., Leppimaki, P., Slotte, J.P. 2002. Cholesterol interactions with phospholipids in membranes. *Prog. Lipid Res*. **41**, 66–97.

Osada, K., Kodama, T., Yamada, K., Sugano, M. 1993a. Oxidation of cholesterol by heating. *J. Agric. Food Chem*. **41**, 1198–1202.

Osada, K., Kodama, T., Cui, L., Yamada, K., Sugano, M. 1993b. Levels and formation of oxidized cholesterols in processed marine oils. *J. Agric. Food Chem*. **41**, 1893–1898.

Paniangvait, P., King, A.J., Jones, A.D., German, B.G. 1995. Cholesterol oxides in foods of animal origin. *J. Food Sci*. **60**, 1159–1174.

Panini, S.R., Sinensky, M.S. 2001. Mechanisms of oxysterol-induced apoptosis. *Curr. Opin. Lipidol*. **12**, 529–533.

Parthasarathy, S., Santanam, N., Auge, N. 1998. Oxidized low-density lipoprotein, a two-faced Janus in coronary artery disease? *Biochem. Pharmacol*. **56**, 279–284.

Patel, R.P., Diazfalusy, U., Dzeletovic, S., Wilson, M.T., Darley-Usmar, V.M. 1996. Formation of oxysterols during oxidation of low-density lipoprotein by peroxynitrite, myoglobin and copper. *J. Lipid Res*. **37**, 2361–2371.

Porkkala-Sarataho, E., Salonen, J.T., Nyyssonen, K., Kaikkonen, J., Salonen, R., Ristonmaa, U., Diczfalusy, U., Brigelius-Flohe, R., Loft, S., Poulsen, H.E. 2000. Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2′-deoxyguanosine, serum cholesterol oxidation products and oxidation resistance of lipids in non-depleted men. *Arterioscler. Thromb. Vasc. Biol*. **20**, 2087–2093.

Prasad, C.R., Subramanian, R. 1992. Qualitative and comparative studies of cholesterol oxides in commercial and home-made Indian ghees. *Food Chem*. **45**, 71–73.

Pryor, W.A. 1994. Free radicals and lipid peroxidation: what they are and how they got that way. In: *Natural Antioxidants in Human Health and Disease* (B. Frei, ed.), pp. 1–24, Academic Press, London.

Pryor, W.A. 2000. Vitamin E and heart disease: Basic science to clinical intervention trials. *Free Radic. Biol. Med*. **28**, 141–164.

Rodriguez-Estrada, M.T., Caboni, M.F. 2002. Determination of cholesterol oxidation products by high-performance liquid chromatography. In: *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 66–100, AOCS Press, Champaign, IL.

Rosenblat, M., Aviram, M. 2002. Oxysterol-induced activation of macrophage NADPH-oxidase enhances cell-mediated oxidation of LDL in the atherosclerotic apolipoprotein E deficient mouse: inhibitory role for vitamin E. *Atheroscler*. **160**, 69–80.

Rose-Sallin, C., Huggett, A.C., Bosset, J.O., Tabacchi, R., Fay, L.B. 1995. Quantitation of cholesterol oxidation products in milk powder using [$^2H_7$] cholesterol to monitor cholesterol autoxidation. *J. Agric. Food Chem*. **43**, 935–941.

Ross, R. 1993. The pathogenesis of atherosclerosis, a perspective for the 1990s. *Nature* **362**, 801–809.

Ross, R. 1999. Atherosclerosis – an inflammatory disease. *New Engl. J. Med*. **340**, 115–126.

Russell, D.W. 2000. Oxysterol biosynthetic enzymes. *Biochim. Biophys. Acta* **1529**, 126–135.

Russell, D.W. 2003. The enzymes, regulation, and genetics of bile acid synthesis. *Ann. Rev. Biochem*. **72**, 137–174.

Salvayre, R., Auge, N., Benoist, H., Negre-Salvayre, A. 2002. Oxidized low-density lipoprotein-induced apoptosis. *Biochim. Biophys. Acta*. **1585**, 213–221.

Schroepfer, Jr., G.J. 2000. Oxysterols: modulators of cholesterol metabolism and other processes. *Physiol. Rev*. **80**, 361–554.

Sevanian, A., Hodis, H.N., Hwang, J., McLeod, L.L., Peterson, H. 1995. Characterization of endothelial cell injury by cholesterol oxidation products found in oxidized LDL. *J. Lipid Res*. **36**, 1971–1986.

Sevilla, C.L., Becker, D., Sevilla, M.D. 1986. An electron spin resonance investigation of radical intermediates in cholesterol and related products: relation to solid-state autoxidation. *J. Phys. Chem*. **90**, 2963–2968.

Shan, H, Pang, J., Li, S., Chiang, T.B., Wilson, W.K., Schroepfer, Jr., G.J. 2003. Chromatographic behaviour of oxygenated derivatives of cholesterol. *Steroids* **68**, 221–233.

Smith, L.L. 1987. Cholesterol autoxidation: 1981–1986. *Chem. Phys. Lipids* **44**, 87–125.

Smith, L.L. 1992. The oxidation of cholesterol. In: *Biological Effects of Cholesterol Oxides* (S-K. Peng, R.J. Morin, eds.), pp. 7–31, CRC Press, Boca Raton, FL.

Smith, L.L. 1996. Review of progress in sterol oxidations: 1987–1995. *Lipids* **31**, 453–487.

Smondyrev, A.M., Berkowit, M.L. 2001. Effects of oxygenated sterol on phospholipids bilayer properties: a molecular dynamics simulation. *Chem. Phys. Lipids* **112**, 31–39.

Sserunjogi, M.L., Abrahamsen, R.K., Narvhus, J. 1998. A review paper: current knowledge of ghee and related products. *Int. Dairy J.* **8**, 677–688.

Stanton, C., Devery, R. 2002. Formation and content of cholesterol oxidation products in milk and dairy products. In: *Cholsterol and Phytosterol Oxidation Products*: *Analysis,*

Occurrence and Biological Effects (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 147–161, AOCS Press, Champaign, IL.

Steinberg, D. 2002. Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nat. Med.* **8**, 1211–1217.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., Witztun, J.L. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *New Engl. J. Med.* **320**, 915–924.

Tall, A.R., Costet, P., Wang, N. 2002. Regulation and mechanisms of macrophage cholesterol efflux. *J. Clin Invest.* **110**, 899–904.

Theunissen, J.J., Jackson, R.L., Kempen, H.J., Demel, R.A. 1986. Membrane properties of oxysterols. Interfacial orientation, influence on membrane permeability and redistribution between membranes. *Biochim. Biophys. Acta* **860**, 66–74.

Ubhayasekera, S.J.K.A., Verleyen, T., Dutta, P.C. 2004. Evaluation of GC and GC-MS methods for the analysis of cholesterol oxidation products. *Food Chem.* **84**, 149–157.

Ulberth, F., Buchgraber, M. 2002. Extraction and purification of cholesterol oxidation products. In: *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects* (F. Guardiola, P.C. Dutta, R. Codony, G.P. Savage, eds.), pp. 26–49, AOCS Press, Champaign, IL. 387-392.

Van den Berg, J.J.M., Winterbourn, C.C., Kuypers, F.A. 1993. Hypochlorus acid-mediated modification of cholesterol and phospholipids: analysis of reaction products by gas chromatography-mass spectrometry. *J. Lipid Res.* **34**, 2005–2012.

Van Der Vliet, A., Smith, D., O'Neill, C.A., Kaur, H., Darley-Usmar, V., Cross, C.E., Halliwell, B. 1994. Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion *Biochem. J.* **303**, 295–301.

Vine, D.F., Croft, K.D., Beilin, L.J., Mamo, J.C.L. 1997. Absorption of dietary cholesterol oxidation products and incorporation into rat lymph chylomicrons. *Lipids* **32**, 887–893.

Vine, D.F., Mamo, J.C.L., Beilin, L.J., Mori, T.A., Croft, K.D. 1998. Dietary oxysterols are incorporated in plasma triglyceride-rich lipoproteins, increase their susceptibility to oxidation and increase aortic cholesterol concentration in rabbits. *J. Lipid Res.* **39**, 1995–2004.

Whincup, P. H., Gilg. J. A., Papacosta, O., Seymour, C., Miller, G. J., Alberti, K.G., Cook, D.G. 2002. Early evidence of ethnic differences in cardiovascular risk: cross sectional comparison of British South Asians and white children. *Br. Med. J.* **324**, 635–638.

Wolf, G. 1999. The role of oxysterols in cholesterol homeostasis. *Nutr. Rev.* **57**, 196–198

Zarev, S., Therond, P., Bonnefont-Rousselot, D. Beaudeux, J.-L., Gardes-Albert, M., Legrand, A. 1999. Major differences in oxysterol formation in human low density lipoproteins (LDLs) oxidized by $^{\bullet}OH/O_2^{\bullet}$ free radicals or by copper. *FEBS Letts.* **451**, 103–108.

Zhou, O., Kummerow, F.A. 1994. Alteration in Ca^{2+} uptake and lipid content in cultured human arterial smooth muscle cells treated with 26-hydroxycholesterol. *Artery* **21**, 182–192.