Cholesterol Autoxidation–Current Status[1]

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Several oxidation products of cholesterol have been reported to have biological activity in animals. Cholesterol oxidizes readily in solution, in aqueous dispersion and in foods when it is exposed to air, elevated temperatures, free radical initiators, light, or a combination of these. The main outline of the pathway for cholesterol oxidation is fairly well understood and involves initial formation of allylic hydroperoxides. HPLC and GC techniques are available for measuring the concentration of cholesterol oxides in animal-derived lipids. Biologically active cholesterol oxides have been reported to be present in egg products, dairy products, frying oils and other foods. Their concentration may be subject to process control.

Cholesterol autoxidation has been recognized and studied for the past 90 years, but it wasn't until the 1960's that a systematic study of cholesterol autoxidation was undertaken (1), facilitated by the application of effective thin layer chromatography (TLC) procedures. More recently the interest in the oxidation of this sterol has intensified, spurred on by reports implicating cholesterol oxidation products in adverse human health effects (2). Most of the studies of biological effects of the oxides were carried out on animals; nevertheless, they created concerns about the possible presence of such products in human foods and therefore their effect on humans. Adverse effects reported include cytotoxicity (3–6), angiotoxicity (7), mutagenicity (8–10), carcinogenicity (11) and others (12–15). Several of these biological activities have led researchers to speculate that a link may exist between ingested cholesterol oxidation products and coronary heart disease (16). This notion was supported by the finding (17) that cholesterol oxides extracted from USP cholesterol produced angiotoxicity and arteriosclerosis in rabbits, while purified cholesterol did not.

Autoxidation of cholesterol, similar to that of other lipids, is enhanced by contact with oxygen (air) at elevated temperature. Early researchers seeking to identify cholesterol oxidation products in foods therefore concentrated their efforts on those animal-derived foods that were high in cholesterol and that had experienced exposure to heat and/or oxidizing agents during processing or storage. The research was made difficult by the unavailability of sensitive analytical methods, by the low concentrations in which even the principal oxidation product were present, and by the complexities of the food matrices.

A brief summary of the current knowledge of the chemistry of cholesterol oxidation will aid in the interpretation of the analytical findings.

The chemical structure of cholesterol (**1**) is seen in Figure 1 along with those of some closely related plant sterols. The common features of these molecules are a polycyclic nucleus with four fused rings, a branched aliphatic side chain attached to the D ring at C-17, a hydroxyl group that is attached to C-3 of the A ring and is β in configuration, and a Δ^5 double bond in the B ring. Of relevance to the question of autoxidation are the B ring unsaturation and positions allylic to it, and the presence of two tertiary carbons in the side chain. Cholesterol oxidation is initiated by hydrogen abstraction, predominantly at C-7. In solid phase or crystalline cholesterol, side chain oxidation also occurs at the tertiary C-25 position and results in the 25-hydroperoxy, and by degradation of the latter, in the 25-hydroxy derivative. Similarly, the 20-hydroxy derivative is seen when solid cholesterol oxidizes. By contrast, side chain oxidation is not observed in autoxidations carried out in solution or in aqueous dispersions. Most studies of the pathway of cholesterol oxidation have been carried out in solutions or dispersions. The current discussion will focus on these types of studies which provide a better model for the state of cholesterol in foods and in the aqueous environment of animal tissues than do those of solid phase cholesterol oxidation.

An examination of a molecular model of cholesterol reveals that the planar Δ^5 double bond affects the conformations of both the A and the B ring, bringing the allylic C-4 and C-7 positions into the plane of the double bond. One might expect, therefore, that oxidative attack should occur with almost equal facility at C-4 and C-7. In fact, attack at C-4 occurs rarely, possibly because of the influence of the neighboring hydroxyl group at C-3 and the trialkyl substituted C-5. Abstraction of a hydrogen atom at C-7 followed by attack by molecular oxygen gives rise to two epimeric hydroperoxides (**2a, 2b**) as shown in Scheme 1. The β-epimer (**2b**) is thermodynamically more stable than the α-form (**2a**), and ready interconversion occurs. When cholesterol in aqueous colloidal suspension dispersed with sodium stearate was stirred in the presence of molecular oxygen at 85 C and pH 8, the epimeric ratio of 7-hydroperoxides after three hours was found to be $\alpha{:}\beta$ = 1:2(18). The hydroperoxides are readily detectable in TLC by means of a spray with Wurster dyes such as N,N,N′N′-tetramethyl-p-phenylenediamine dihydrochloride (19). The thermal instability of the 7-hydroperoxides was recognized in the early 1940's (20,21), and the principal stable products of cholesterol oxidation under these conditions were found to be 7-ketocholesterol (**4**) and the epimeric 7-hydroxycholesterols (**3a, 3b**), the ratio of 7-ketone to combined hydroxy compounds being 2:1. The two epimeric C-7 hydroxides are also interconvertible (18), the equilibrium again favoring the equatorial 7β epimer (**3b**). Bergström (21) and Kimura (18) observed the formation of small amounts (6–7%) of 5,6-epoxides, and the generation of traces of other cholesterol derivatives when cholesterol dispersed with sodium stearate at pH 8 was oxidized, but no C-4 oxidation products were seen. Both researchers also noted that cholesterol oxidation ceased when 70–75% of the substrate had been consumed. This effect may be caused by changes in micellar structure due to the presence of the oxidation products. It has been reported that the solubility of cholesterol in aqueous media is enhanced by cholesterol oxidation products (22).

Reaction of cholesterol with singlet oxygen in pyridine solution and in the presence of hematoporphyrin as

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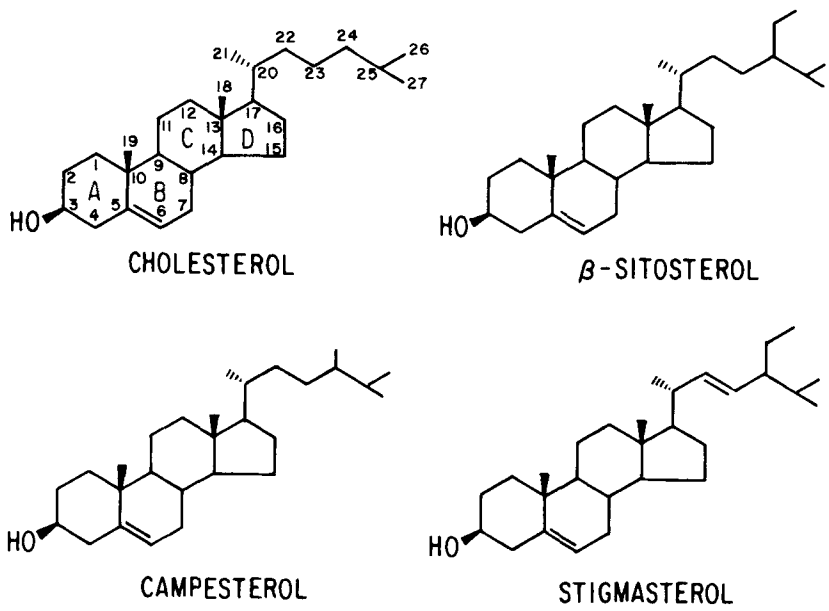


FIG. 1. Chemical structure of cholesterol and some closely related plant sterols.

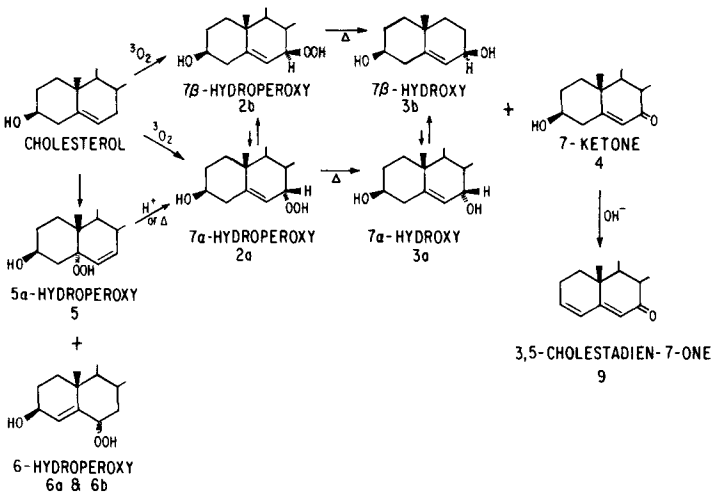
photosensitizer (23) led to the formation (74–75%) of the 5-hydroperoxide (5) in which the double bond has moved to the Δ^6 position. Also formed in very low concentration (1–2%) were both isomers of the 6-hydroperoxy-4-ene (**6a** and **6b**). The 6-hydroperoxides or their derived 6-hydroxy-4-enes are stable and not interconvertible. On the other hand, the 5-hydroperoxy-6-ene (5), in the presence of acid or on heating, isomerizes to the 7α-hydroperoxy-5-ene (**2a**) which then epimerizes to the 7β-hydroperoxide (**2b**) as mentioned above.

The epimeric 5,6-epoxides of cholesterol are formed as autoxidation products both in the crystalline state and in solution or dispersion. In the latter they account for about 6–7% of the oxidation products mixture, as pointed out above. Smith and co-workers (24) established that direct attack of either triplet or singlet oxygen leads only to hydroperoxides and not to the epoxides directly. On the other hand, treatment of cholesterol with either epimer of 7-hydroperoxycholesterol (**2a** or **2b**) or with the 5-hydroperoxy-6-ene (5) in chloroform gave low yields of both isomeric epoxides (**7a, 7b**, Scheme 2) with an α/β ratio of 1:8–11. Similar results were obtained in aerated aqueous dispersion with sodium stearate as dispersing agent. Recently it has been pointed out (25) that the relative amounts in which the isomeric epoxides are formed in aqueous dispersions are relatively independent of pH, temperature and dispersing agent, but that the final α/β ratio depends on the pH of the dispersion, since the β-epoxide (**7b**) hydrolyzes more rapidly than its epimer (**7a**) in acid media. Muto et al. (26) treated cholesterol with fatty acid hydroperoxides in benzene in the presence of ferric acetylacetonate and obtained about equal amounts of epoxides and C-7 oxidation products. The α/β ratio of the epoxides was about 1:3. From these experiments it is clear that the epoxides **7a** and **7b** are the products of attack by various hydroperoxides on the 5,6-double bond of cholesterol and therefore are secondary oxidation products.

It is instructive to speculate why hydration of either **7a** or **7b** (Scheme 2) results in the formation of the same 3β, 5α, 6β-triol (8), the most toxic of the cholesterol oxides tested to date. The hydration of epoxides in acid medium is initiated by protonation of the ring oxygen and is followed by approach of a nucleophile, in this case water, at the more highly branched carbon from the side opposite to that of the protonated oxygen. In the cholesterol 5,6-epoxides the C-5 position is dialkylated and thus best able to carry the transient positive charge. In the α-epoxide **7a** the rear side of C-5 is sterically hindered by the angular methyl C-19, and nucleophilic attack occurs not at C-5, but, reluctantly, at C-6 and results in the 3β, 5α, 6β-triol 8. In the β-epoxide, **7b** nucleophilic attack at C-5 is relatively unhindered and also results in the 3β, 5α, 6β-triol (8). If this interpretation is correct, the steric hindrance to the ring opening step in the α-epoxide should result in a slower rate of hydration for this epimer. Indeed it has been determined experimentally (25) that the rate of hydration of **7b** is about 2.5 times that of **7a**.

A prominent product of cholesterol autoxidation, 7-ketocholesterol (4), is highly sensitive to contact with base (27) which causes it to form 3,5-cholestadien-7-one (9) and several other products. It is common practice to isolate cholesterol and its oxidation products from lipid matrices by saponification and subsequent separation from the unsaponifiable residue. 7-Ketocholesterol (4) is lost in this procedure, and the dienone **9** is a poor measure of the amount 4 present before saponification, since **9** is only one of several products formed. It has been demonstrated (28) that 6-ketocholestanol, a compound that is sometimes used as an internal standard, is also affected by base and is partially destroyed by hot alkali. If this is not recognized by investigators, the quantitation of cholesterol oxides may incur serious errors when 6-ketocholestanol is used as internal standard.

Oxidation in the A ring of cholesterol has been observed to occur in cholesterol aged naturally in air, irradiated in air by ^{60}Co gamma radiation, or under other circumstances (29), but it has not been reported in autoxidations



SCHEME 1

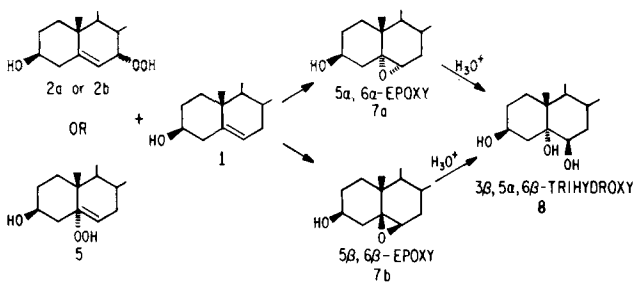
in solutions or dispersions. It involves dehydrogenation of 1 to cholest-5-en-3-one which isomerizes to cholest-4-en-3-one. It is mentioned here merely to round out the picture.

The advent of TLC in the early 1960's greatly simplified the detection and study of cholesterol oxidation products, particularly the principal products described above. Smith and co-workers (30) showed that TLC R_f values and colors of spots after spraying can be combined to provide a lead to the identity of components of a mixture. The usual procedure is to develop the mixture of oxides with solvent pairs such as heptane:ethyl acetate (1:1), to spray the developed TLC plate with 50% sulfuric acid and then to heat it cautiously at 110–120 C. The brilliant colors that develop range from magenta for cholesterol through blues for some of the diols and epoxides to yellow for the triol. Some of the colors are fairly fleeting, and it is customary to char the plates completely at higher temperatures. The visualization of hydroperoxides by sprays with Wurster dyes (19) already has been mentioned. It is extremely effective. A few compounds or groups of compounds are poorly resolved by TLC. Among these are 7-ketocholesterol (**4**) and the 5,6-epoxides (**7a** and **7b**). Their resolution requires an additional separation by other means such as high performance liquid chromatography (HPLC) or gas liquid chromatography (GC) (25).

HPLC has been used by a number of workers to analyze cholesterol oxidation products (31–33). Both reverse phase and normal phase HPLC systems have been used, but the latter have been more successful, and good separations have been obtained with hexane/isopropanol combinations in which the hexane phase comprised 90–99%. The procedure is highly effective for the separation of diols and hydroperoxides, but triol retention time is excessive, and this compound is often not determined in adsorption HPLC. Good resolution of the isomeric 5,6-epoxides has been reported (36).

Semipreparative HPLC is useful in separating cholesterol, triglycerides and other lipids from the cholesterol oxides and thereby enriching the oxide fraction when saponification is avoided (28). Certain cholesterol oxides that are less polar than cholesterol are lost in this procedure, as are antioxidants such as BHT that may have been added in a prior step.

GC has been used in the analysis of cholesterol oxidation products for some time, but resolution on packed columns has not been altogether satisfactory (34–36). The recent development of capillary GC, especially when combined with direct on-column capillary injection, has permitted better separations of the principal oxidation products. Several researchers (37–41) have prepared trimethylsilyl ether (TMS) derivatives of the cholesterol oxides because of the reported thermal instability of some of the diols. However, it has been shown that on-column injection on fused silica capillary columns permits the GC determination of nanogram amounts of unmodified cholesterol oxides without loss (28,42,43). As would be



SCHEME 2

expected, the order of elution of the derivatized and underivatized oxides differs, but in either case baseline separations are achieved in mixtures containing 10 or more of the major cholesterol oxidation products.

Even before the biological activity of various individual cholesterol oxidation products was appreciated, there were isolated reports of such compounds occurring in foods (1). Among the early reports was that of Chicoye et al. (27) who demonstrated that exposure of spray-dried egg yolk to low energy ultraviolet irradiation gave rise to a number of cholesterol oxides including the β-epoxide and the triol. Given the high cholesterol content of eggs and the common commercial practice of converting fresh eggs to a dehydrated powder for use by the food industry, it is not surprising to find that the cholesterol oxide content of spray-dried eggs and related products has been studied by a number of workers. Tsai and Hudson (44) found a range of 3–74 ppm of cholesterol 5,6-epoxides in a number of commercial dry whole egg samples and 3–166 ppm of the same oxides in dry yolk powders. One of the yolk samples contained 62 ppm of α-oxide, but most fell in the range of 1–10 ppm. The same authors found cholesterol epoxides in scrambled egg mix. Other workers (39,45) also have reported the presence of the two 5,6-epoxides in spray-dried whole egg as well as yolk. Missler et al. (40) determined that the cholesterol oxide content of an egg mix used by the military depended on the processing conditions under which the mix was produced. Dehydration with a direct heat source generated considerably greater amounts of oxidation products, including epoxides, than indirect heating. This was an important demonstration that control of processing conditions can strongly influence the degree to which foods are contaminated with these biologically active compounds.

Fresh egg yolk or spray dried egg yolk powder did not contain 25-hydroxycholesterol (46), but this compound as well as the epimeric 7-hydroxycholesterols was measured after egg yolk powder was heated at 100–110 C for four days (46,47). The 7-hydroxycholesterols were also found after dry egg nog mix had been exposed to fluorescent light for prolonged periods (48).

Food products other than eggs have been examined for evidence of cholesterol oxidation. Among these are various meats and sausages (39,49,50), dairy products (39,46,51,52), beef tallow (39,53), lard (46), french fries (54) and other foods that are wholly or partly animal derived. Cholesterol oxides were detected in many of these products after they had been exposed to heat, light, air or oxidizing agents, or a combination of these. Recently it was reported (55) that beef tallow that had been used in a commercial french frying operation for 60 hr contained substantial amounts (100–300 ppm) of cholesterol oxides, mostly the triol and C-7 oxidation products. The frying oil lost 25% of its cholesterol content during that period.

There is evidence that unprocessed foods or commodities contain minimal, if any, of these biologically active compounds. Hence, if the biological activities of cholesterol oxides reported to date are further confirmed and if their activity is found to apply to humans, it can be expected that processing conditions will be found that will minimize or eliminate cholesterol oxidation.

The methodology for the detection and measurement of cholesterol oxidation products has made great strides during the past 5–10 years, and further advances can be anticipated. A complicating problem is the possible presence of plant sterols and their oxidation products. Chemically, common plant sterols such as β-sitosterol, stigmasterol and campesterol (Fig. 1) are similar in structure to cholesterol, and the expected instability to air of Δ^5-sterols of plant origin has indeed been demonstrated (1). Indications are that some of the oxidation products of phytosterols, especially β-sitosterol, are similar in structure to those of cholesterol (54,56–61), and hence they must be expected to complicate the determination of cholesterol oxides in foods that contain materials derived from animal as well as vegetable sources.

Until the pathways and products of phytosterol oxidation are more clearly defined, it will be difficult to address another problem area in which further research is urgently needed. The questions that need answering have to do with the extent to which sterols and sterol oxides are exchanged during food processing steps. A practical example of such a situation involves the preparation of french fries by deep fat frying in beef tallow. To what extent are cholesterol oxidation products formed in the tallow absorbed by the potatoes, and to what degree are phytosterols from the potatoes leached into the tallow?

The current understanding of cholesterol oxidation and the techniques that have been developed to measure oxidation products form a solid basis for developing further information on the oxidation and transfer of food sterols.

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