Oxidative Interactions of Cholesterol with Triacylglycerols

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Triacylglycerols (TGs) accelerated the decomposition of cholesterol at 130°C. Addition of stearic and linoleic acids also accelerated cholesterol decomposition and produced characteristic cholesterol oxide profiles, qualitatively different from those produced in the presence of TGs. Milk fat accelerated cholesterol decomposition at 130°C and produced a cholesterol oxide pattern similar to that arising from the addition of pure TG. Not only did TGs affect cholesterol oxidation, but cholesterol influenced the decomposition of TGs. Addition of cholesterol accelerated the destruction of TGs at the beginning of heating while protecting them later. A similar pattern, *i.e.*, acceleration followed by protection, could also be seen when triacylglycerols were heated in the presence of other triacylglycerols. The results of this work demonstrate that the stability of lipid components in complex mixtures is influenced by interactions among these components and/or their decomposition products. Such interactions do not merely shift, *i.e.*, accelerate or delay, the oxidation rate, they may also modify the shape of the oxidation curve itself.

KEY WORDS: Cholesterol, interaction, milk fat, oxidation, triacylglycerols.

Although the presence of cholesterol oxides in food is a growing concern due to their reported toxicity, few quantitative data existed before 1980, mostly due to the difficulties associated with their isolation and detection. Recent developments in high-performance liquid and gas chromatography allowed more reliable analyses of these compounds.

Some cholesterol-containing foods are subjected to oxidizing conditions during processing, preparation and storage. The oxide levels in milk powders (low heat) were less than 0.1 ppm (1). However, 6–53 ppm of epoxides and minor 7-derivatives were found in dried chicken; and 15–27 ppm of epoxides, 8–20 ppm of 7-ketocholesterol and 0–18 ppm of 7-hydroxycholesterol in dried turkey (2). Up to 74.1 ppm of 7-hydroxycholesterols, 46 ppm of epoxides, 9.4 ppm of 7-ketocholesterol, 73 ppm of 25-hydroxycholesterol and a trace amount of triol were detected in dry egg yolk (3).

Heating of cholesterol-rich food also produced cholestereol oxidation products. When butter containing 2.6 ppm of 25-hydroxycholesterol was heated at 180°C for 5 min, 28 ppm of 7-hydroxycholesterol, 5.2 ppm of 7-ketocholesterol and 34 ppm of 25-hydroxycholesterol were produced (4). Butter also produced 7-hydroxycholesterol when exposed to light (5).

Cholesterol as it exists in food is surrounded by various other components. Interactions between these components and cholesterol may affect both its oxidative stability and its oxidation pathway. At high temperatures, as used in frying, the cholesterol present in the frying medium, *e.g.*, in tallow or released from food, may oxidize and produce oxidation products which in turn, can be absorbed by the food being fried. In this case, the neighboring molecules are mainly triacylglycerols. The objective of this work was to study the oxidative interactions between triacylglycerols and cholesterol at elevated temperatures. Milk fat was chosen as a model system containing cholesterol.

EXPERIMENTAL PROCEDURES

Materials. Milk fat was prepared from raw milk obtained from the University of Massachusetts farm by pasteurization (30 min, 63°C), cream separation, freezing-thawing of the cream, centrifugation (2,500 × *g*, 20 min) and washing the milk fat fraction with distilled water. It contained 68.1% (SD=2.2%, n=12) of saturated, straight-chain fatty acids and 19.1% (SD=1.5%, n=12) of oleate. Its cholesterol content was 0.35 mg/100 mg (SD=0.02 mg, n=5). Cholesterol, fatty acids, triacylglycerols (all 99%) and silicic acid were purchased from Sigma Chemical Co. (St. Louis, MO), cholesterol oxide standards were from Steraloid Inc. (Wilton, NH), and silylating agents were from Pierce Chemical Co. (Rockford, IL). Solvents were high-performance liquid chromatography (HPLC) or reagent grade.

Sample treatment. Chloroform solutions of milk fat, pure triacylglycerols, fatty acids, cholesterol or mixtures of these (1 mg each) were placed in 4-mL vials (15 mm dia., 45 mm long) and the solvent was evaporated with a nitrogen stream to make a thin lipid film. The samples were then heated for varying periods of time in air in an oven set at 130°C.

Cholesterol analysis. Analysis of cholesterol and its oxidation products was carried out as described previously (6). Cholesterol and its oxides were separated from the heated mixtures of cholesterol and TGs by silicic acid column chromatography. The non-polar lipids, mainly triacylglycerols, were eluted with hexane/diethyl ether (95:5, v/v). Cholesterol and its oxidation products were then eluted with 100% diethyl ether. The ether fraction was collected and silylated with N,O-*bis*-[trimethylsilyl]trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) at 80°C for 1 hr. The trimethylsilyl ethers were analyzed by gas chromatography (CG) on an Ultra-1 capillary column (50 m, 0.2 mm i.d., 0.33 μm film thickness; Hewlett Packard, Avondale, PA) with temperature programming at 100–300°C at 10°C/min. 5α-Androstan-3β-ol-17-one acetate was used as an internal standard.

Fatty acid analysis. Decomposition of triacylglycerols and fatty acids was monitored according to the loss of their acyl chains. Fatty acid analysis was done by methylating the lipid. The samples containing 1–2 mg lipid were saponified with 0.5 mL of 2N methanolic KOH at 65°C for 25 min, then methylated with 0.5 mL of 14% BF_3-methanol at 65°C. The residual methylating agent was then deactivated by adding 0.5 mL water. The methyl esters were extracted with hexane and analyzed by GC on a Supelcowax-10 capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness; Supelco Inc., Bellefonte, PA). Oven temperature was programmed from 40 to 245°C at

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2.5°C/min. Triheptadecanoin and heptadecanoic acid were used as internal standards for TGs and free fatty acids, respectively.

The Figures shown in this report represent averages of triplicate samples. The error bars (±, + or −) represent the standard deviations (SD) of the three observations.

RESULTS

When tristearin (TS), triolein (TO), trilinolein (TL) and milk fat were individually added to cholesterol at a 1:1 ratio (1 mg each), they accelerated cholesterol decomposition (Fig. 1). After 3 hr of heating at 130°C, tristearin was the least destructive, while triolein was the most destructive. Beyond 3 hr, less than 5% of the cholesterol remained. The effect of added milk fat was within the range of the pure TGs tested. When the concentration of milk fat in the mixture was increased, the oxidation of cholesterol was accelerated. No cholesterol remained after 20 hr of heating in the mixture containing more than 75% milk fat (data not shown). Stearic acid was more destructive than tristearin at the beginning of heating, but the difference between free fatty acids and TGs was not significant in prolonged heating.

Figure 2 shows characteristic oxide profiles for cholesterol heated alone, in the presence of a triacylglycerol (tristearin), and in the presence of a free fatty acid (stearic acid), after 20 hr at 130°C. When tristearin was added, the cholesterol oxide profile was qualitatively similar to that of cholesterol heated alone. The same was true when triolein or trilinolein was added (data not shown). In the presence of stearic acid, however, several oxide peaks were not detectable while peaks 2 (3,5-cholestadiene) and 5 (cholesta-3,5-dien-7-one) were relatively pronounced. The same was true when linoleic acid was added (data not shown). Milk fat showed the same effect on the resulting oxide profile as TGs, indicating that its influence was mainly due to its TG content. The chromatograms shown in Figure 2 are those obtained after 20 hr of heating. When the traces obtained after 3, 10, 20, 40, and 70 hr of heating were compared, it became evident that the progressive formation and subsequent breakdown patterns for various oxides were the same for cholesterol heated alone and when TGs or fatty acids (FAs) were added. The major difference was that the formation and breakdown of cholesterol oxides was faster in the presence of TGs or FAs. This is to be expected because the latter two classes accelerated the decomposition of cholesterol, as can be seen in Figure 1.

Not only did the addition of TGs and FAs influence cholesterol oxidation, but addition of cholesterol to TGs affected their decomposition. Figure 3 demonstrates the effect of adding cholesterol to milk fat at a 1:1 ratio on the loss of stearate and oleate during heating. Added cholesterol resulted in slight acceleration in fatty acid loss in the early stage of heating, followed by marked protection. This protective effect of cholesterol on the decomposition of triacylglycerol fatty acids appeared to be concentration dependent, as can be seen in Figure 4, where the remaining stearate and oleate in milk fat is plotted against cholesterol concentration in the mixture. A gradual increase in fatty acid stability was observed with increasing cholesterol concentration. As expected, the effects of cholesterol shown in Figures 3 and 4 on the

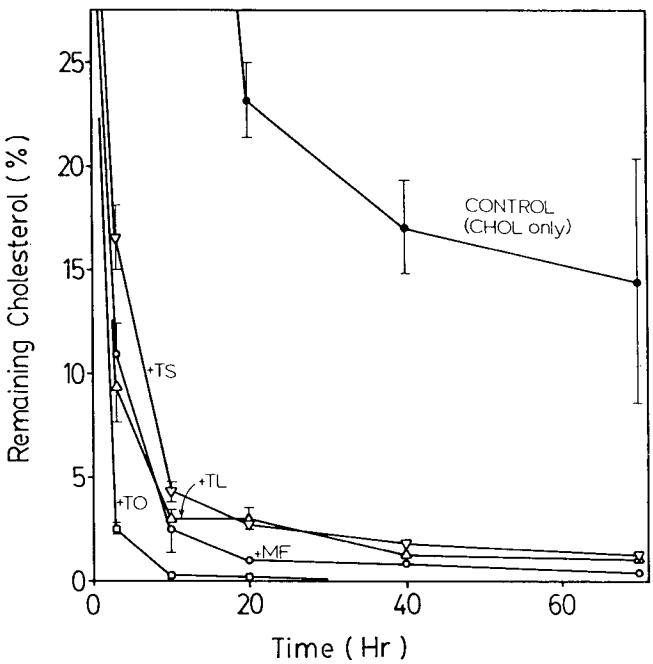

FIG. 1. Effect of added triacylglycerols on the stability of cholesterol at 130°C. CHOL, cholesterol; TS, tristearin; TO, triolein; TL, trilinolein; and MF, milk fat.

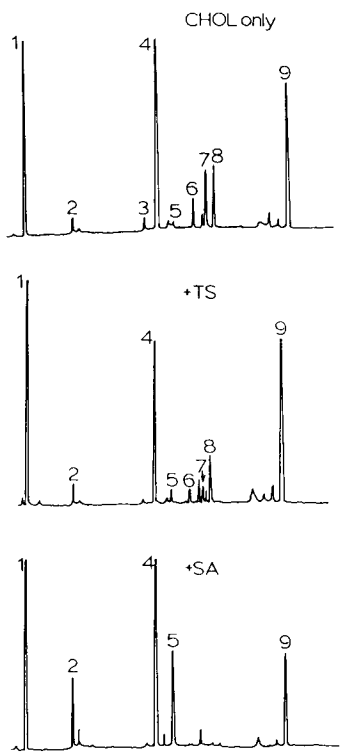

FIG. 2. Effect of added tristearin and stearic acid on the cholesterol decomposition products after heating at 130°C for 20 hr. CHOL, cholesterol; TS, tristearin; and SA, stearic acid. 1, 5α-Androstan-3β-ol-17-one acetate (internal standard); 2, 3,5-cholestadiene; 3, 7α-hydroxycholesterol; 4, cholesterol; 5, cholesta-3,5-dien-7-one; 6, 7β-hydroxycholesterol; 7, 5,6β-epoxy-5β-cholestan-3β-ol; 8, 5,6α-epoxy-5α-cholestan-3β-ol; and 9, 7-ketocholesterol.

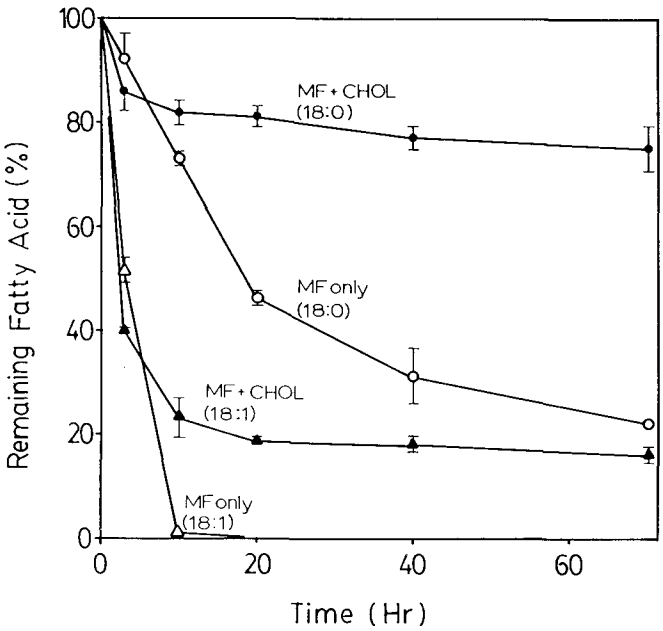

FIG. 3. Effect of cholesterol on the stability of stearate and oleate in milk fat at 130°C. CHOL, cholesterol; and MF, milk fat.

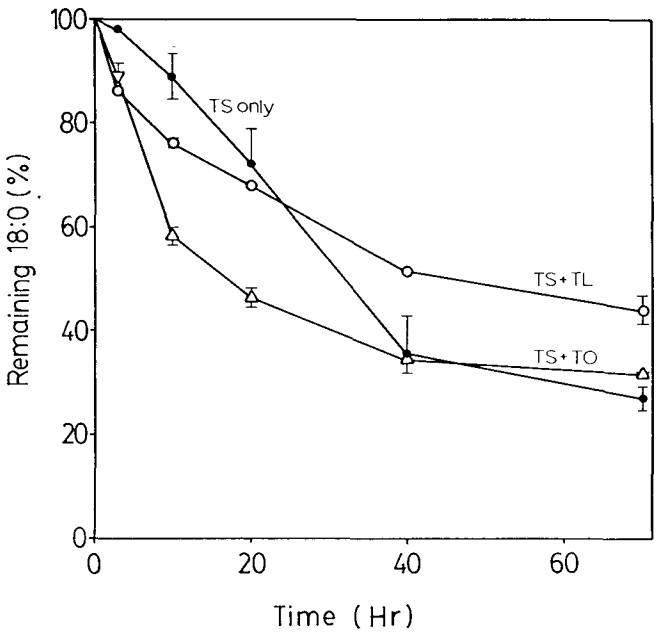

FIG. 5. Effect of added triolein (TO) and trilinolein (TL) on the stability of tristearin at 130°C. TS, tristearin; TO, triolein; and TL, trilinolein.

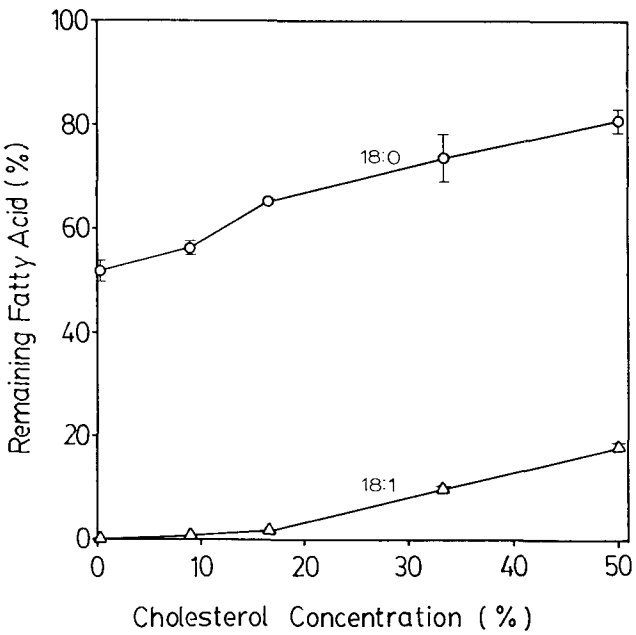

FIG. 4. Effect of cholesterol concentration on milk fat stability after 20 hr heating at 130°C.

stability of fatty acids in milk fat were also observed when cholesterol was added to pure triacylglycerols (data not shown).

The effect of cholesterol on the stability of TG fatty acids, *i.e.*, destruction at the beginning followed by protection, was not unique to cholesterol. A similar behavior could be observed when other triacylglycerols were added to tristearin (Fig. 5).

DISCUSSION

Oxidizing lipids interact in a complex manner. Under oxidative conditions, each lipid in a mixture may give rise to a variety of intermediates and end products which, in turn, may undergo further decomposition. The substrates themselves and/or their reaction products may accelerate or inhibit the reactions of each other. Such reactions and interactions may be significantly influenced by reaction conditions, *e.g.*, temperature and type and concentration of the components present. Influences of a physical nature may also be involved.

In the present study, two modes of interaction were observed. First, the addition of TGs to cholesterol accelerated its oxidation throughout the oxidation period, regardless of the stability of the added compound. Second, addition of cholesterol to triacylglycerols, or triacylglycerols to other triacylglycerols, produced a typical oxidation curve where decomposition of the TG fatty acids was accelerated at the beginning but inhibited later. Elucidation of the specific mechanisms responsible for these patterns is extremely difficult in view of the above-mentioned complexity.

A possible explanation for the accelerating effect of TGs when added to cholesterol is that TGs oxidize first, providing free radicals and peroxides, which trigger a faster oxidation of the cholesterol. This argument, however, is contradicted by the fact that cholesterol decomposed very rapidly when triacylglycerol was present (<5% cholesterol remained after 10 hr in the presence of TS, Fig. 1), while stearic acid in milk fat remained remarkably stable in the presence of cholesterol (>80% remained after 10 hr, Fig. 3). Furthermore, TO was more destructive to cholesterol than TL during the first 10 hr of heating, in spite of the

higher stability of the TO (Fig. 1). Another explanation for the accelerating effect of TGs may be their influence on the melting point of cholesterol. For example, when heated in the presence of cholesterol, TS melts first, thus turning the mixture into liquid at a temperature below the melting point of cholesterol. Cholesterol may oxidize faster when it exists in the liquid state. A third possibility may involve the influence of functional groups, such as the ester group, for example. No documentation, however, is available for such speculation at this time.

The second phenomenon, *i.e.,* acceleration at the beginning followed by protection, is equally difficult to explain. The fact that TO was more destructive than TL when added to TS (in the early stage of heating, Fig. 5) further supports the argument that acceleration is not due to instability of the added compound. The protective effect observed in the latter stage of heating may reflect an antioxidative effect of one or more of the added compound's decomposition products. In addition, physical inhibition may be another important factor. Certain reaction products, *e.g.,* polymers, may act as barriers impeding the mobility of the reacting molecules or their combination with oxygen.

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