Oxidative Decomposition of Cholesterol in Fish Products

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Cholesterol oxides in fish products popular in Japan, including salted and dried, boiled and dried and smoked products, were qualitatively and quantitatively determined as trimethylsilyl ether derivatives by gas-liquid chromatography and mass spectrometry. The level of total cholesterol oxides ranged widely between 8.3 ppm in boiled and dried shrimp and 188.0 ppm in boiled and dried anchovy. 7β-Hydroxycholesterol and 7-ketocholesterol were the most prominent oxidative decomposition products of cholesterol. The levels of epimeric epoxides, cholestane triol and 25-hydroxycholesterol were relatively low. To elucidate a mechanism of cholesterol oxidation proceeding during fish processing and subsequent preservation, four model systems, consisting of a mixture of purified cod liver triglycerides plus cholesterol, of a mixture of authentic triolein plus cholesterol, of triolein alone and of cholesterol alone, were stored separately at 25°C in dry air for up to 104 d. The residual fatty acids of the triglycerides, and the cholesterol oxides produced, were recovered and determined. Oxygen uptake remained almost unchanged for the mixture of triolein plus cholesterol. No detectable amount of cholesterol oxides was produced, and the fatty acid content of the residual oleic acid, measured by an internal standard, remained almost unchanged. For the mixture of cod liver triglycerides plus cholesterol, a remarkable increase in oxygen uptake was observed. A continuous increase in the amount of cholesterol oxides was observed, accompanied by a remarkable concurrent decrease in polyunsaturated fatty acid residues, as well as of the oleic acid naturally present. These results strongly suggest that cholesterol oxidation in fish products proceeds in conjunction with oxidative decomposition of the coexisting polyunsaturated fatty acids of fish oils.

KEY WORDS: Cholesterol, cholesterol oxide, fish oil, fish products, oxidation, polyunsaturated fatty acids, triglyceride.

Cholesterol oxides are known as powerful atherogenic reagents both *in vivo* and *in vitro* (1,2). The production of cholesterol oxides in many types of food and foodstuffs has been reported, including heated fats and oils (3–7), deep-fried foods (8), meat products (9,10) and milk and egg products (11–15). Salted-dried and boiled-dried fish products have been consumed for a long time in Japan as important sources of nutrients. They are now commercially produced and are readily available in local retail stores. In general, foods based on marine resources have a wide variety of constituent fatty acids with carbon chains from 14 to 22, and with up to 6 ethylenic bonds. Levels of certain polyunsaturated fatty acids (PUFA), especially 20:5(n-3) and 22:6(n-3), are high in marine resources (16). These PUFAs are markedly susceptible to peroxidation, even under mild ambient conditions, and they are easily incorporated into the accepted chain mechanism of lipid peroxidation to yield free radicals and peroxy radicals (17,18). It was logical, therefore, to suspect that the oxidation of cholesterol might be accelerated in the presence of peroxidized lipids. The goal of this study was to elucidate a mechanism for cholesterol oxidation in fish food products during processing and subsequent storage. For this purpose, the levels of cholesterol oxides in commercially available fish products were determined as trimethylsilyl (TMS) ether derivatives by gas-liquid chromatography (GLC). Possible mechanisms of cholesterol oxidation in conjunction with the peroxidation of polyunsaturated lipids in foods are discussed, based on the results of oxidation studies of model systems differing in degree of unsaturation of the lipids.

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EXPERIMENTAL PROCEDURES

Materials. Marine products used in this study were purchased from local fish retailers. Moisture contents of the fish products were relatively low, varying from 10.1 to 28.9%. Cholesterol was purchased from Sigma (St. Louis, MO). Authentic standards of cholesterol oxides, listed in Table 1, were from Steroids, Inc. (Wilton, NH). The chromatographic purities of the standard compounds were given as more than 99% upon purchase.

The total lipids were extracted from the fresh liver of Pacific cod (*Gadus macrocephalus*) by the Bligh and Dyer procedure (19) and subjected to column chromatography on Silica gel G (E. Merck, Darmstadt, Germany) and on Florisil (Floridin, Pittsuburg, OH) to isolate triglycerides. An authentic triolein of more than 99% purity (Sigma) was further purified by column chromatography on Florisil.

Preparation of model systems. For lipid oxidation studies, four different model systems were prepared; for each, a 220-g portion of microcrystalline cellulose (100–120 mesh, Funakoshi, Tokyo, Japan) was thoroughly mixed with one of the chloroform solutions of 1.1 g cholesterol, of 2.8 g triolein, of a mixture of 1.1 g cholesterol and 2.8 g triolein or of a mixture of 1.1 g cholesterol and 2.8 g of the purified fish oil triglycerides. Chloroform was evaporated *in vacuo*, and residual solvent in the lipid-coated microcrystalline cellulose was removed by continuous exposure to a rotary pump vacuum for 14 h. The model systems thus prepared were spread separately in glass petri dishes (20 cm in diameter) and allowed to stand in a desiccator at 25°C over phosphorus pentoxide to allow autoxidation.

TABLE 1

A List of Authentic Standards of Cholesterol and Its Related Compounds

Sterols	Common names
5α-Cholestane	5α-Cholestane
Cholest-5-en-3β-ol	Cholesterol
Cholest-5-en-3β,7β-diol	7β-Hydroxycholesterol
Cholest-5-en-3β,25-diol	25-Hydroxycholesterol
5,6-α-Epoxy-5α-cholestan-3β-ol	α-Epoxide
5α-Cholestane-3β,5,6β-triol	Cholestane triol
3β-Hydroxycholest-5-en-7-one	7-Ketocholesterol

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Extraction of lipids. For lipid extraction from fish products, aliquot amounts of water were added to the minced dried fish products to give a moisture content of 80%, followed by lipid extraction by a mixture of chloroform and methanol (1:2) according to the Bligh and Dyer procedure (19). For lipid recovery from the microcrystalline cellulose systems, 20 mL of the methanol/chloroform (2:1, vol/vol) mixture was added to 4 g of microcrystalline cellulose coated with lipids. Lipid extraction was repeated three times for each of the triplicate samples by adding fresh solvents and by continuous stirring. Solvents were removed as already described.

Cold saponification of lipids and derivatization of cholesterol oxides. An aliquot amount (*ca.* 125 μg) of 5α-cholestane in chloroform was added as an internal standard to the accurately weighed (*ca.* 500 mg) lipids extracted from fish products. To avoid formation of artificial secondary oxidation products (3,4) the lipids were submitted to cold saponification with 1 N methanolic KOH at 37°C for 18 h. Our preliminary examinations suggested that triglycerides were almost completely hydrolyzed and no additional amounts of cholesterol oxides were formed under the conditions of the alkaline hydrolysis described above. The saponified lipid sample was diluted with 10 vol of deionized water and extracted three times with diethyl ether. The ether extracts of unsaponifiable matter were pooled and washed with 0.05 N methanolic KOH. Cholesterol oxides and the unsaponifiable matter were dissolved in 1 mL dry pyridine and derivatized to TMS ethers by adding 0.5 mL hexamethyldisilazane (Tokyo Kasei, Japan) and 0.3 mL trimethylchlorosilane (Tokyo Kasei, Japan). The TMS derivatives of cholesterol oxides were dissolved in *n*-hexane, and an aliquot, usually 1 μL, of the hexane solution was subjected to GLC.

An excess amount of 6 N HCl was added to the residual aqueous layer (saponifiable matter) to liberate free fatty acids, followed by extraction with diethyl ether three times. Fatty acid methyl esters (FAME) were prepared from the free fatty acids extracted from the acidified saponifiable matter. Tricosanoic acid methyl ester of more than 99% purity (Nu-Chek-Prep, Elysian, MN) was used as an internal standard (20).

Determination of cholesterol oxide derivatives and fatty acids. A Shimadzu (Tokyo, Japan) GC-12A gas chromatograph equipped with a flame-ionization detector (FID), and a Shimadzu split injector SPL-G9 were used for the analysis of the TMS derivatives of cholesterol oxides. The outlet of an open tubular fused-silica column with a nonpolar methyl silicone liquid phase (0.25 mm i.d. × 25 m, 0.1 μm in film thickness, Quadrex, New Haven, CT) was connected directly to the FID jet. The oven temperature was programmed from 180 to 250°C at a rate of 3°C/min. The injector temperature was held at 250°C. Helium was used as carrier gas with a column inlet pressure at 2 kg/cm^2 and a split ratio of 1:1. A Shimadzu Chromatopack CR6A was used for peak integration. Determinations were repeated twice for each of triplicate extract samples.

To obtain calibration curves for the quantitative determination of cholesterol oxides, various amounts of cholesterol oxides were mixed with accurate amounts of 5α-cholestane, followed by derivatization to TMS-cholesterol oxides. Regression analyses for linearity of the calibration curves showed good correlation ($r^2 = 0.993–0.998$) between peak area ratio (0.10–3.8) *vs.* weight ratio (0.090–3.0).

Mass spectrometric analysis of cholesterol oxide TMS derivatives was carried out with a Shimadzu QP 1000 quadrupole mass spectrometer fitted with an electron ionization (EI) source, to which was admitted the outlet of a methylsilicone-phase open tubular fused-silica column (0.25 mm i.d. × 30 m, 0.1 μm in film thickness, Quadrex). The operating conditions were 70 eV electron beam energy, 3 KV accelerating energy and a source temperature of 210°C.

Quantitative analysis of FAME was carried out by gas chromatography with a Shimadzu GC 12A instrument equipped with a Supelcowax-10 fused-silica open tubular column (0.25 mm i.d. × 30 m, 0.25 μm in film thickness, Supelco Japan, Tokyo, Japan) and an FID (20). The column temperature was programmed from 170 to 230°C at a rate of 1°C/min. A Shimadzu SPL-G9 split injector was held at 250°C. Helium was used as carrier gas at a column inlet pressure at 2 kg/cm^2 and a split ratio of 1:50. This determination was repeated twice for each of the triplicate extract samples.

Determination of oxygen uptake. The accurately weighed samples (4 g each) of microcrystalline cellulose coated with the lipids were put into glass vials (68.7 mL in vol) and sealed tightly with a Teflon-lined septum. A 0.1-mL portion of the headspace air of the vial was analyzed for oxygen and nitrogen with a Shimadzu GC3BT gas chromatograph equipped with a glass spiral column (3 mm i.d. × 1.7 m) packed with molecular sieve 5A (80–100 mesh, Nihon Chromato, Tokyo, Japan) and a thermal conductive detector. This determination was repeated twice for each of triplicate samples.

RESULTS

Cholesterol oxides in fish products. A typical gas chromatogram of the TMS derivatives of cholesterol oxides obtained from the salted-dried anchovy is shown in Figure 1. Peak assignments were carried out by comparison of retention times with standards and by comparison of fragmentations in EI mass spectrometry. The components responsible for the peaks shown in the figure, except peak 3, were easily identified. The fragmentation of the peak 3 component was similar to that of the peak 4 component (α-epoxide). Because an authentic standard of β-epoxide was not available, direct comparison of retention time and fragmentation pattern was not carried out in this case. A study on the cholesterol oxides of heated tallow showed that the β-epoxide TMS derivative elutes just before the α-epoxide TMS derivative in a nonpolar liquid phase similar to the column used in this study (4). The epimeric epoxides yield similar fragmentations on EI mass spectrometry (4,21), just as was revealed for the analysis of epimeric 7-hydroxycholesterols (4). These published studies strongly suggest that the peak 3 component in the figure should be β-epoxide. The quantitative determination of β-epoxide was carried out provisionally by using the calibration curve of α-epoxide. Many previous studies have shown that α-hydroxycholesterol coexists with its isomeric β-hydroxycholesterol in food products (9–15). In the present study, however, α-hydroxycholesterol was not separated from cholesterol by GLC, because an isocratic derivatization with a large amount of unoxidized

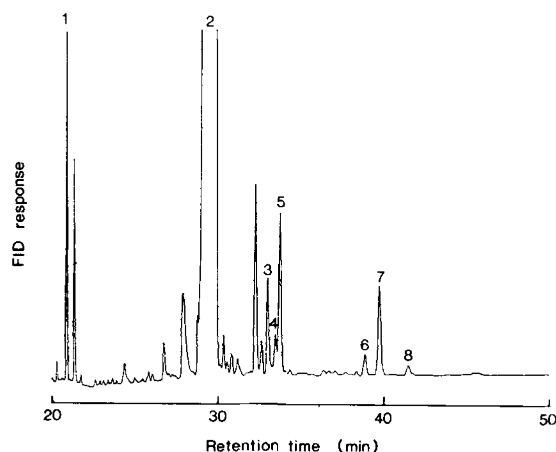

FIG. 1. A typical gas chromatogram of the trimethylsilyl ether derivatives of cholesterol oxides in salted and dried anchovy. An open tubular fused-silica column with methyl silicone phase (0.25 mm i.d. × 25 m, 0.1 μm in thickness) was used. Its temperature was programmed from 180 to 250°C at 3°C/min. Peak components were identified by mass spectrometry as: 1, 5α-cholestane (I.S.); 2, cholesterol; 3, β-epoxide; 4, α-epoxide; 5, 7β-hydroxycholesterol; 6, cholestane triol; 7, 7-ketocholesterol; and 8, 25-hydroxycholesterol. FID, flame-ionization detector.

cholesterol was carried out to avoid any selective losses of certain cholesterol oxides during previous screening. The results of mass spectrometry of the later-eluting part of peak 2 suggested the existence of α-hydroxycholesterol in the sample preparation (data not shown).

The levels of cholesterol oxides in the fish products are summarized in Table 2. Most of the samples contained varying levels of all cholesterol oxides. 7β-Hydroxycholesterol and 7-ketocholesterol were prominent, amounting to 2–55 ppm and 2–60 ppm in the edible portions, respectively. Salted-dried and boiled-dried products included relatively high levels of cholesterol oxides.

Changes in oxygen uptake of the model systems. Changes in oxygen uptake of the lipids coated on microcrystalline cellulose and stored in vials are illustrated in Figure 2. No detectable oxygen absorption were observed in cholesterol, in triolein or in a mixture of triolein plus triglyceride for up to 100 d of storage. For the mixture of fish oil triglycerides and cholesterol, a slight absorption of oxygen started after 24 d of storage, and subsequently a dramatic increase in oxygen uptake occurred after 38 d of storage. A slight decrease in oxygen absorption in the mixture of fish oil triglycerides plus cholesterol after 70 d storage might be due to a potential back-flash of the room air into a microsyringe when the needle of the syringe was withdrawn from the vial, in which the pressure of the headspace gas would be expected to be slightly lower than that of the outer air. These results confirm that oxidation of the mixture of fish oil triglycerides plus cholesterol, once started, proceed very rapidly compared to cholesterol, triolein or a mixture of triolein plus cholesterol. In autoxidation of ethyl eicosapentaenoic and ethyl docosahexaenoate, relatively shorter induction periods were recognized compared to oxidation of ethyl linoleate and ethyl linolenate (22,23).

Changes in residual fatty acids of model systems. The residual amounts of the most prominent constituent fatty

TABLE 2

Cholesterol Oxides in the Edible Portions of Commercial Fish Products

Sample	Total lipids (%)	μg/g (dry weight basis)						
		7β-OH[a]	7-Keto	α-Epoxide	β-Epoxide	Triol	25-OH[b]	Total
Salted-dried								
Anchovy *(Engraulis japonicus)*								
A	11.5[c]	37.1	46.4	11.9	33.3	3.5	5.8	138.0
B	7.1	30.5	48.8	13.1	18.7	5.3	0.2	116.6
Northern cod *(Eleginus gracilis)*								
A	4.8	6.8	9.7	2.9	5.5	1.5	0.9	27.3
B	2.4	3.6	4.3	1.6	3.4	1.2	0.2	14.3
Pacific cod *(Gadus macrocephalus)*								
A	1.9	3.7	3.8	3.1	5.9	1.8	2.5	20.8
B	1.6	2.9	2.3	1.0	2.3	0.9	0.2	9.6
Japanese whiting *(Sillago japonica)*	6.8	24.5	24.9	8.9	16.1	3.4	10.7	88.5
Pacific saury *(Cololabis saira)*	18.0	9.9	7.8	trace	trace	trace	trace	17.7
Pacific herring *(Clupea pallasii)*	27.0	8.4	7.5	3.5	5.4	3.5	5.6	33.9
Boiled-dried								
Anchovy *(E. japonicus)*								
A	11.2	55.8	60.6	18.0	43.3	8.5	1.8	188.0
B	10.7	18.1	20.8	6.4	14.9	3.9	5.3	69.4
Shrimp *(Sergestes lucens)*	6.2	3.7	4.0	trace	trace	trace	0.6	8.3
Anchovy *(E. japonicus)*	25.9	43.8	40.7	12.5	26.6	39.1	8.5	171.2
Smoked								
Keta salmon *(Onchorhynchus keta)*	8.7	7.3	6.3	2.4	3.3	2.7	4.8	26.8

[a]7β-Hydroxycholesterol. [b]25-Hydroxycholesterol. [c]The values are mean of duplicates.

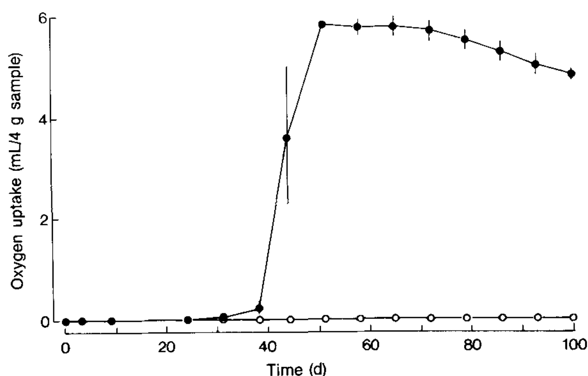

FIG. 2. Changes in oxygen uptake of the lipid samples coated on 4 g microcrystalline cellulose stored in vials at 25°C. ●-●, The mixture of cod liver oil triglyceride plus cholesterol; O-O, all of the following three sorts of lipids: cholesterol, triolein and the mixture of triolein plus cholesterol.

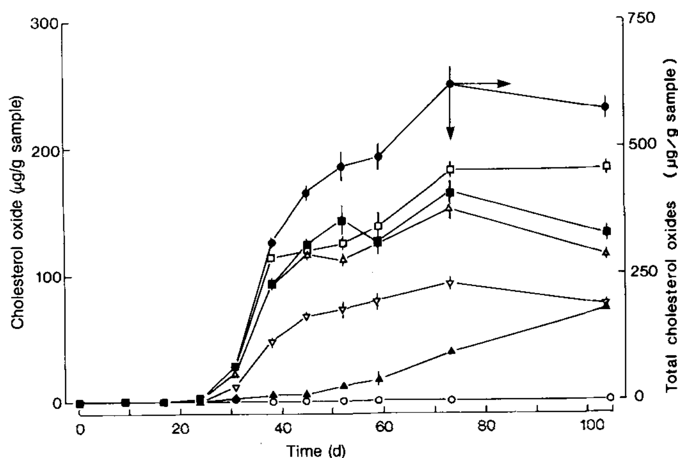

FIG. 4. Changes in the amounts of cholesterol oxides of the lipid samples coated on microcrystalline cellulose stored in dry air at 25°C. ●-●, Total cholesterol oxides; □-□, ■-■, △-△, ▽-▽ and ▲-▲ represent 7β-hydroxycholesterol, 7-ketocholesterol, β-epoxide, α-epoxide and cholestane triol, respectively, in the mixture of cod liver triglycerides plus cholesterol; O-O, the mixture of triolein plus cholesterol.

acids of a mixture of fish oil triglycerides plus cholesterol were compared to those of a mixture of triolein plus cholesterol and are illustrated in Figure 3. The residual amounts of oleic acid in the mixture of triolein and cholesterol remained almost unchanged during 104 d of storage. The residual amounts of oleic acids in triolein alone were also unchanged. Most prominent (more than 5%) constituent fatty acids of the cod liver oil triglycerides used in this study included 18:1(n-9), 18.76%; 22:6(n-3), 14.61%; 16:0, 13.04%; 20:5(n-3), 12.36% and 18:1(n-7), 7.68%. A minor component was 20:4(n-6), accounting for 1.07%. The residual amounts of PUFA, 20:5(n-3), 22:6(n-3) and 20:4(n-6), decreased after 9 d of storage, followed by a rapid decrease after 17 d of storage. After 38 d of storage, 20:5(n-3) and 22:6(n-3) almost completely disappeared due to oxidative decomposition. A remarkable decrease in the amount of residual oleic acids occurred at similar times of storage to the changes in the PUFAs. It is expected that a part of the oleic acid residues in the fish oil triglycerides were oxidized in conjunction with the oxidative deterioration of PUFAs, because the oleic acid residues of a mixture of triolein plus cholesterol were stable under similar storage conditions. This difference in the rate of decrease in the fatty acid residues between the mixture of fish oil triglycerides plus cholesterol and the mixture of triolein plus cholesterol was supported by the results of the oxygen uptake experiments (Fig. 2).

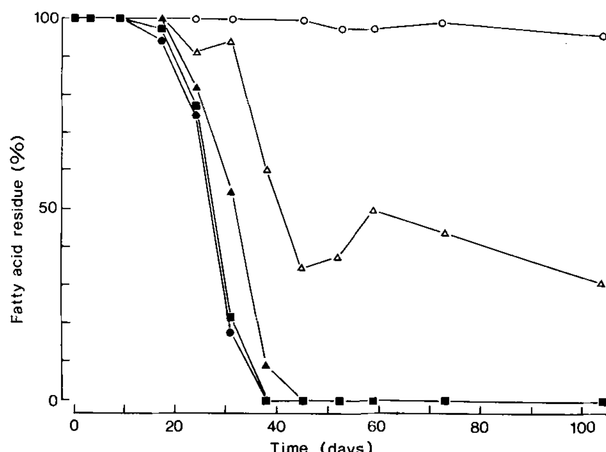

FIG. 3. Changes in the amounts of residual unoxidized fatty acids of triglycerides mixed with cholesterol and coated on microcrystalline cellulose stored in dry air at 25°C. O-O, 18:1(n-9) of the mixture of triolein plus cholesterol; △-△, ▲-▲, ■-■ and ●-● represent 18:1(n-9), 20:4(n-6), 20:5(n-3) and 22:6(n-3), respectively, in the mixture of cod liver triglycerides plus cholesterol.

Changes in cholesterol oxide content of model systems. Changes in the content of cholesterol oxides produced under atmospheric conditions in cholesterol alone, in the mixture of fish oil triglycerides plus cholesterol and in the mixture of triolein plus cholesterol are illustrated in Figure 4. No detectable amounts (below 0.1 µg/g) of cholesterol oxides were found in cholesterol alone or in the mixture of triolein plus cholesterol throughout 104 d of storage. For the mixture of fish oil triglycerides plus cholesterol, several cholesterol oxide species, including 7β-hydroxycholesterol, 7-ketocholesterol, epimeric epoxides and cholestane triol, were produced after 24 d of storage. Mass spectrometric analysis showed a large amount of unoxidized cholesterol that interfered with a quantitative analysis of 7α-hydroxycholesterol under the analytical conditions employed. These results strongly suggest that cholesterol is oxidized at a higher rate when it coexists with fish oil triglycerides, differing from the case of cholesterol alone, as well as from the mixture of triolein plus cholesterol. The time period for the initiation of a drastic increase in most cholesterol oxides, excluding cholestane triol (after 31 d of storage), was delayed slightly compared to the induction period in which the residual PUFAs began to decrease (Fig. 3).

DISCUSSION

Cholesterol oxides in the fish products. Park and Addis (4) reported that cholesterol was oxidized to isomeric 7-hydroxycholesterols, 7-ketocholesterol and epimeric epoxides in tallow heated at 155°C for 376 h. 7-Ketocholesterol is produced without a formation of epimeric 7-hydroxycholesterols as a decomposition product of

7-hydroperoxides, because 7-hydroxycholesterols are easily dehydrated in the absence of water at elevated temperatures, such as at 155°C. Many fish products subjected to the analysis for cholesterol oxides in the present study contained 7β-hydroxycholesterol (and possibly the isomeric 7α-hydroxycholesterol) as the dominant oxidation product. The 7α-hydroxycholesterol content was considered low, because many autoxidation studies showed the α/β ratios of the epimeric isomers are 1:8–11 in a mixture of oxidatively decomposed cholesterol (24) to 1:1–23 in commercially spray-dried egg products (25). Most dried fish products are intermediate-moisture foods, varying 10–30% in moisture contents. Under relatively high moisture content, therefore, 7-hydroperoxides seem to accumulate as the precursors of 7-ketocholesterol.

Many sorts of foods and foodstuffs have been investigated for cholesterol oxide contents. The levels of α- and β-epoxides in spray-dried egg were 17.4 and 31.8 ppm, respectively (14). The epimeric 5,6-epoxides and isomeric 7-hydroxycholesterols were dominant in grated, unbleached cheese packed in a clean glass bottle, amounting to 16 and 6 ppm, respectively (13). A cooked bratwurst contained large amounts of the B-ring oxidation products, *e.g.*, 7β-hydroxycholesterol (1640 ppm) and cholestane triol (1335 ppm), as well as the side-chain oxidation product, *e.g.*, 22-ketocholesterol (1869 ppm) (10). The levels of cholesterol oxides in other processed meats were remarkably high (10), including raw hamburger, cooked lean bacon and beef franks. For fish products, B-ring oxidation products were dominant in the determined total cholesterol oxides. 25-Hydroxycholesterol was the only side-chain oxidation product. Although oxidation in the side-chain of cholesterol yields a wide variety of 20-, 24-, 25- and 26-hydroxycholesterols (26,27); only one side-chain oxidation product, 25-hydroxycholesterol, was found in the fish products examined in this study. A typical gas chromatogram of the salted-dried anchovy (Fig. 1) reveals several unidentified peaks, and, therefore, side-chain oxidation products other than 25-hydroxycholesterol probably accumulated in the sample.

Cholesterol oxidation in the model systems. Generally, cholesterol autoxidation proceeds by radical processes similar to those in other lipids (28). Hence, cholesterol autoxidation yields epimeric 7-hydroxycholesterols and its dehydrated 7-ketocholesterol, as well as epimeric 7-hydroperoxides, as the primary oxidation products. In the other possible pathway, cholesterol epoxidation yields epimeric 5,6-epoxides as the primary oxidation products and cholestane triol as their hydrated product. Oxidation of the side-chain occurs as well. Thus, some 66 cholesterol decomposition products of air-aged cholesterol have been identified (29). 7β-Hydroxycholesterol (and possibly 7α-hydroxycholesterol) and 7-ketocholesterol produced in the mixture of fish oil triglycerides plus cholesterol are probably the oxidative decomposition products of cholesterol, although the determination of epimeric 7-hydroperoxides, the precursors of these cholesterol oxides, was not carried out. Cholesterol epoxidations in the mixture of fish oil triglycerides plus cholesterol are unquestionable because cholestane triol and epimeric 5,6-epoxides, the precursors, were accumulated. Thus, the oxidative decomposition products species in the mixture of fish oil triglycerides plus cholesterol matched those included in the fish products. Therefore, the cholesterol oxidation pathway of the model system reflected the pathway of cholesterol oxidation that occurred in the fish products.

The results of the oxygen uptake (Fig. 1) and of the analysis of the residual fatty acids (Fig. 2) suggest that during storage oxidative decomposition of fish oil triglycerides proceeded much more rapidly than that of triolein, due to the constituent PUFAs. The production of free radicals and peroxy radicals in the mixture of fish oil triglyceride plus cholesterol is, therefore, considered to proceed at much higher rates than in the mixture of triolein plus cholesterol. It is probably obvious that the radicals produced from PUFAs act as accelerators of cholesterol oxidation, because cholesterol itself is oxidized in a radical chain oxidation system (26). From comparisons of the degree of unsaturation of triglycerides and the rates of formation of cholesterol oxides within the model systems in the present study, it is concluded that cholesterol oxidative decomposition during fish processing and subsequent storage proceeded in conjunction with the oxidative degradation of constituent PUFAs of fish oils in the tissues. Cholesterol oxides are now emphasized as playing an important role in human diseases (30). Therefore, some effective procedure to prevent cholesterol peroxidation, such as wider use of antioxidants (3), should be established without delay in the field of fish processing and subsequent distribution.

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