Levels and Formation of Oxidized Cholesterols in Processed Marine Foods

Kyoichi Osada,† Takehiro Kodama,‡ Li Cui,§ Koji Yamada,‡ and Michihiro Sugano*,‡

Laboratory of Nutrition Chemistry and Laboratory of Food Science, Department of Food Science and Technology, Faculty of Agriculture 46–09, Kyushu University, Higashi-ku, Fukuoka 812, Japan, and Department of Food Science and Technology, Central China Agricultural University, Whhan 430070, People's Republic of China

The contents of oxidized cholesterols in uncooked and processed marine products (air-dried sardine, air-dried squid, canned squid, and pickled and spiced Alaskan pollack roe) were measured. Raw fish contained essentially no oxidized cholesterols, while the processed products examined contained 11.0–28.7 mg/100 g of oxidized cholesterols, and 7α-hydroxycholesterol (2.7–3.8 mg/100 g), 7β-hydroxycholesterol (2.8–9.8 mg/100 g), 5α-epoxycholesterol (0.2–5.8 mg/100 g), 5β-epoxycholesterol (0.7–4.9 mg/100 g), and 7-ketocholesterol (1.9–5.3 mg/100 g) were detected. To know the interaction of lipid oxidation with cholesterol oxidation, cholesterol was heated with or without various fats (tristearin, beef tallow, triolein, soybean oil, safflower oil, linseed oil, and sardine oil) at 100 °C for up to 24 h. Cholesterol was stable, and essentially no oxidized cholesterol was produced when it was heated alone. However, when fats were present simultaneously, cholesterol was unstable, in particular when heated with unsaturated fats; the content of cholesterol was readily reduced, and oxidized cholesterols were generated shortly after heating. The observations indicated that lipid peroxidation precedes cholesterol oxidation.

INTRODUCTION

Cholesterol readily undergoes autoxidation in air, either spontaneously or enzymatically, and more than 70 oxidized derivatives of cholesterol are produced (Smith, 1981). Oxidized derivatives of cholesterol (oxidized cholesterols) exert a wide range of biological activities such as disturbance of arachidonic acid metabolism (Seillan, 1990; Seillan and Dubuquoy, 1990), inhibition of cholesterol synthesis (Kandustsch and Chen, 1978; Peng et al., 1979; Baranowski et al., 1982), carcinogenesis (Wrensch et al., 1989; Morin et al., 1991; Kendall et al., 1992), mutagenicity (Smith et al., 1979; Sevanian and Peterson, 1986; Peterson et al., 1988), atherosclerosis (Guyton et al., 1990), and cytotoxicity (Jacobson et al., 1985; Peng et al., 1985, 1991; Matthias et al., 1987; Seillan and Dubuquoy, 1990). Analysis of the oxidized cholesterols has been hampered by the difficulties encountered in their isolation and separation. However, recent development of the capillary gas chromatography (GC) has made possible the analysis of individual oxidized cholesterols (Park and Addis, 1985), and the presence of oxidized cholesterols in processed animal foods such as butter (Luby et al., 1986; Pie et al., 1990), egg products (Herian and Lee, 1985; Tsai and Hudson, 1985; Nourooz-Zadeh and Appelqvist, 1987; van de Bovenkamp et al., 1988), heated tallow (Bascoul et al., 1986; Park and Addis, 1986), dairy products (Nourooz-Zadeh and Appelqvist, 1988; Sander et al., 1989), and meat products (Sander et al., 1989; Pie et al., 1991; Monahan et al., 1992) has been reported.

However, the available information regarding oxidized cholesterols in foods is still limited, and the effects of food processing on cholesterol oxidation are largely unknown. Kim and his colleagues (Nawer et al., 1991; Kim and Nawer, 1991, 1992) reported that triglyceride accelerates the decomposition of cholesterol and that the oxidized cholesterols are generated during peroxidation of triglyceride by heating. We have observed that cholesterol is stable for a relatively long period when cholesterol alone was heated at 100 °C (Osada et al., 1993). Therefore, it seems likely that cholesterol oxidation in foodstuffs proceeds accompanying the oxidation of coexisting lipids.

Although the Japanese people have commonly consumed various processed marine products for many years, little information is available on the occurrence of cholesterol oxidation products in these food items. In this study, we describe the quantitative determination of oxidized cholesterol products in processed marine foods and the influence of various triglycerides on cholesterol oxidation during heating.

MATERIALS AND METHODS

Reagents. 5α-Cholestane, cholest-5-en-3β-ol (cholesterol), 5,6α-epoxy-5α-cholestan-3β-ol (5α-epoxycholesterol), 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), cholestane-3β,5α,6β-triol (cholestanetriol), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), cholest-5-ene-3β,20α-diol (20α-hydroxycholesterol), cholest-5-ene-3β,22(S)-diol [22(S)-hydroxycholesterol], cholest-5-ene-3β,22(R)-diol [22(R)-hydroxycholesterol], and cholest-5-ene-3β,25-diol (25-hydroxycholesterol) were purchased from Sigma Chemical Co., St. Louis, MO. Cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) and 5,6β-epoxy-5β-cholestan-3β-ol (5β-epoxycholesterol) were obtained from Steraloids Inc., Wilton, NH. Trimethylchlorosilane (TMCS) and 1,1,1,3,3,3-hexamethyldisilazane (HMDS) were the products of Nacalai Tesque Co., Kyoto. Tristearin (stearic acid-rich triglyceride), beef tallow, triolein (oleic acid-rich triglyceride), soybean oil, and linseed oil were obtained from Wako Pure Chemicals, Osaka. Safflower oil and sardine oil were purchased from Rinoru Oil Co., Nagoya, and Nippon Chemical Feed Co., Hakodate, respectively. These oils were solvent extracted and refined products; Acid and peroxide values of these oils were low. Sardine oil contained less than 0.1% cholesterol. Other reagent grade chemicals were purchased from Wako.

Food Samples. Freshly prepared processed marine foods, which are the most typical items in Japan (air-dried sardine, air-dried squid, canned boiling squid, and pickled and spiced

* Author to whom correspondence should be addressed.
† Laboratory of Nutrition Chemistry.
‡ Laboratory of Food Science.
§ Department of Food Science and Technology.

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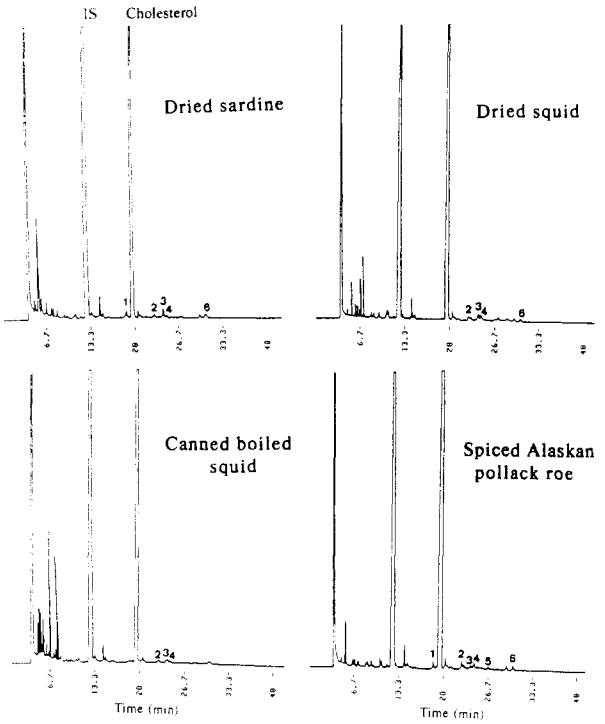


Figure 1. Gas chromatographic patterns of sterols in processed marine foods (dried sardine, dried squid, canned boiled squid, and spiced Alaskan pollack roe). The GC conditions are described in the text. IS, internal standard.

Alaskan pollack roe), and fresh raw sardine and squid were purchased from the local market. Fresh Alaskan pollack roe was donated by Hokkaido Wakkanai Fisheries Experimental Station. These samples were analyzed immediately after acquisition.

Lipid Extraction and Analysis. Each processed marine product (5 g) was minced and extracted with chloroform–methanol (Bligh and Dyer, 1959). Lipids were saponified by 1 N KOH in ethanol at 70 °C for 1 h (Metcalfe and Schmitz, 1961), and the unsaponifiable fraction was converted to trimethylsilyl ester in a mixture of TMCS, HMDS, and dried pyridine (1:3:9 v/v/v) at room temperature. The trimethylsilyl esters were applied to capillary gas chromatography (GC) using 5α-cholestane as an internal standard. Identification of individual oxidized cholesterols was carried out by comparison with GC–MS spectra and the retention time of the authentic standards. The fatty acid composition was measured by GC as the methyl ester prepared by interesterification of fatty acid in 2 N HCl–methanol at 50 °C (Prevot and Mordret, 1976).

Heating Cholesterol with Various Triglycerides. Cholesterol alone (50 mg/mL) and cholesterol (50 mg/mL) with one of the triglycerides (tristearin, beef tallow, triolein, soybean oil, safflower oil, linseed oil, and sardine oil, 50 mg/mL) dissolved in chloroform were placed in 10-mL tubes (10 mm × 70 mm). The solvent was evaporated under nitrogen gas to make a thin film and then heated for various periods of time (1, 3, 6, 12, and 24 h) at 100 °C in an electric oven. The heated samples were saponified as described above, and the unsaponifiable fraction was converted to trimethylsilyl ester and applied to capillary GC.

Gas Chromatography. Trimethylsilyl derivatives of cholesterol and the oxidized products were separated by capillary GC (GC-7AG, Shimadzu Co., Kyoto) with a flame ionization detector using a fused silica capillary ULBON HR-1 (phase: 100% methyl silicone) column with a liquid phase thickness of 0.25 μm (0.25 mm × 50 m, Shinwa Chemical Industries, Kyoto) and a Shimadzu C-R6A integrator. The oven and injector temperatures were 280 and 300 °C, respectively, and the flow rate of helium was 2.2 mL/min. Methyl esters of fatty acid were analyzed with a Shimadzu gas chromatograph (GC-8A) with a flame ionization detector using a 10% Silar 10C column (3 mm × 2 m) and a C-R5A integrator. The oven and injector temperatures were 210 and 250 °C, respectively. The flow rate of nitrogen gas was 25 mL/min.

Mass Spectrometry. GC–MS was performed to identify the major oxysterols using an Automass 50 mass spectrometer (JEOL Ltd., Tokyo) and a HP 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA) with a fused silica capillary DB-1 column (0.25 mm × 30 m, J&W Scientific, Folsom, CA; film thickness of 1.0 μm). The flow rate of helium was 1 mL/min. Oven temperature was raised from 240 to 300 °C (6 °C/min). The injector and interface temperatures were both 300 °C. Mass spectra were measured within the mass range *m/e* 30–650. Scan speed was 1 scan/s. Ionization energy was 70 eV.

RESULTS

Oxidized Cholesterols in Processed Marine Foods. The GC patterns of sterol trimethylsilyl esters in four processed marine products are shown in Figure 1. The *m/e* values of peaks 1 (546, 456, 441, 366, and 351), 3 (546, 456, 441, 366, and 351), 4 (474, 456, 384, and 366), 5 (474, 456, 384, and 366), and 7 (472, 457, 382, and 367) were identical to those of oxidized cholesterol standards (Park and Addis, 1985; Nourooz-Zadeh and Appelqvist, 1987; Pie et al., 1991). By comparison of the *m/e* value and the retention time of the oxidized cholesterol standards, each peak of Figure 1 was identified as follows: peak 1, 7α-hydroxycholesterol; peak 3, 7β-hydroxycholesterol; peak 4, 5β-epoxycholesterol; peak 5, 5α-epoxycholesterol; peak 6, cholestanetriol; and peak 7, 7-ketocholesterol.

Table I shows the content of each oxidized cholesterol in uncooked (raw) and processed samples. While no oxidized cholesterol was detected in raw samples, the processed products (air-dried sardine, air-dried squid, canned boiled squid, and pickled and spiced Alaskan pollack roe) contained 11.0–28.7 mg/100 g of sample of oxidized cholesterols. Among the oxidized cholesterols detected, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5β-epoxycholesterol, 5α-epoxycholesterol, cholestanetriol, and 7-ketocholesterol were identified. A relatively large amount of 7β-hydroxycholesterol occurred in all processed foods.

Table II shows the fatty acid composition of processed marine foods. Eicosapentaenoic acid (20:5*n*–3) and docosahexaenoic acid (22:6*n*–6) were found to be abundant, and squid and pollack roe contained the latter at a relatively high proportion.

Oxidation of Cholesterol Accompanying Fat Oxidation during Heating. Table III shows the fatty acid

Table I. Contents of Oxidized Cholesterols in Processed Marine Foods

		sterols, mg/100 g								
		cholesterol	7α-hydroxy-cholesterol	7β-hydroxy-cholesterol	5β-epoxy-cholesterol	5α-epoxy-cholesterol	cholestane-triol	7-keto-cholesterol	un-known	total oxidized cholesterol
sardine	fresh	193								
	dried	333	2.7	9.8	4.9	1.1		5.3	5.0	28.7
squid	fresh	799								
	dried	513		5.5	2.2	1.4		1.9	3.6	14.6
	canned boiled	356		2.8	0.7	0.2			7.3	11.0
Alaskan pollack roe	fresh	48.5								
	pickled and spiced	403	3.8	5.8	1.0	0.8	0.3	3.3	4.8	20.9

Table II. Fatty Acid Composition of Raw Fish and Processed Marine Foods

		total lipid content,[a] g/100 g	fatty acid, wt %												
			14:0	14:1	16:0	16:1	18:0	18:1	18:2n-6	20:1	20:5n-3	22:1	22:5n-3	22:6n-3	others
sardine	fresh	13.8	10.2	0.6	23.6	12.5	6.2	11.6	3.3	1.0	13.8	1.1	1.1	7.6	7.4
	dried	23.2	12.8	1.1	27.9	14.2	7.1	8.4	2.2	0.6	10.0	0.3	0.8	9.0	5.6
squid	fresh	1.0	2.6		34.2	2.5	4.0	6.5	3.5	1.9	12.5			25.5	6.8
	dried	4.4	1.4		30.3	1.3	4.2	11.8	4.2	2.1	10.8	0.1	0.1	25.7	8.0
	canned boiled	1.5	3.8		33.0	2.9	3.1	6.4	0.8		15.5			27.0	7.5
Alaskan pollack roe	fresh	1.5	2.5		18.2	5.9	3.8	15.0	1.5	2.4	16.6	0.4	2.2	24.2	7.3
	pickled and spiced	1.5	3.0		19.9	6.4	2.2	14.4	1.2	6.6	18.9	2.4	1.6	20.5	2.9

[a] Total lipid content was expressed in grams per 100 grams of samples (values were from Standard Tables of Food Composition in Japan, 1990).

Table III. Fatty Acid Composition of Triglyceride Vehicles

	fatty acid, wt %											
	14:0	16:0	16:1	18:0	18:1	18:2n-6	18:3	20:1	20:5n-3	22:1	22:5n-3	22:6n-3	others
tristearin[a]		26.8		64.9									8.3
beef tallow	3.3	24.4	6.0	13.0	47.5	3.1							2.7
triolein[b]	1.8	5.0	5.8	3.0	71.7	8.3	1.8						2.6
soybean oil		10.2		3.5	23.2	55.0	6.8						1.3
safflower oil		6.6		2.2	11.8	78.2							1.2
linseed oil		5.1	3.1	3.1	18.8	15.0	56.9						1.1
sardine oil	7.2	17.4	8.7	4.0	13.6	3.4		5.7	16.4	3.6	2.4	10.5	7.1

[a] Stearic acid-rich triglyceride. [b] Oleic acid-rich triglyceride.

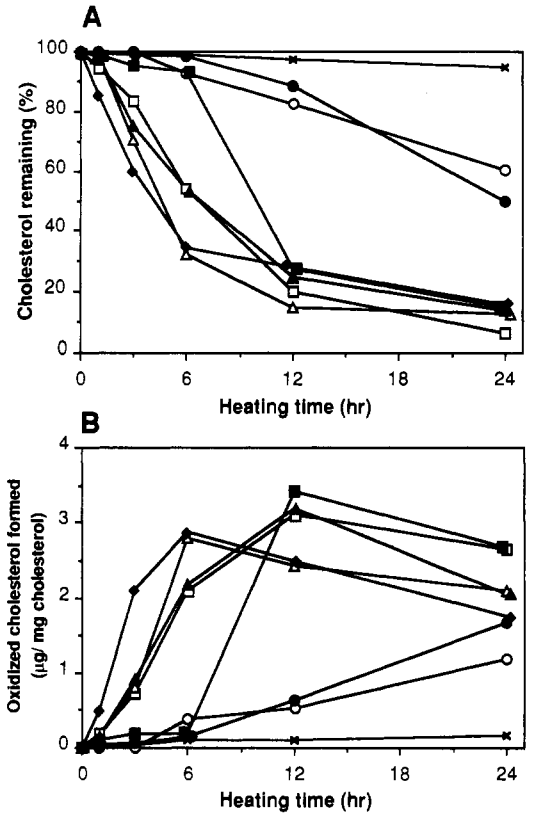


Figure 2. Time course of cholesterol remaining (A) and oxidized cholesterol production (B) during heating in different triglyceride vehicles heated at 100 °C for 24 h in an electric oven. The amount of oxidized cholesterol is the sum of each oxidized cholesterol. Added triglycerides: ×, cholesterol alone; ○, tristearin (stearic acid-rich triglyceride); ●, beef tallow; □, triolein (oleic acid-rich triglyceride); ■, soybean oil; △, safflower oil; ▲, linseed oil; ◆, sardine oil.

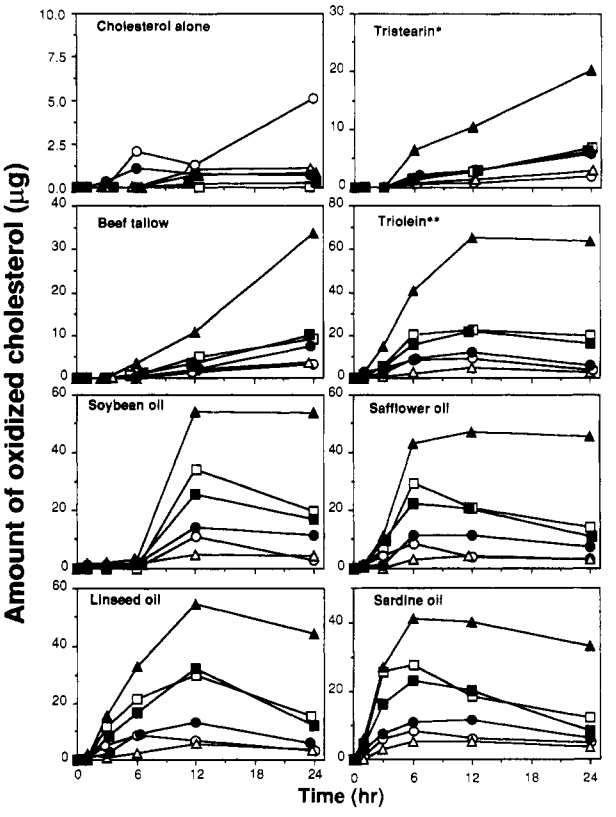
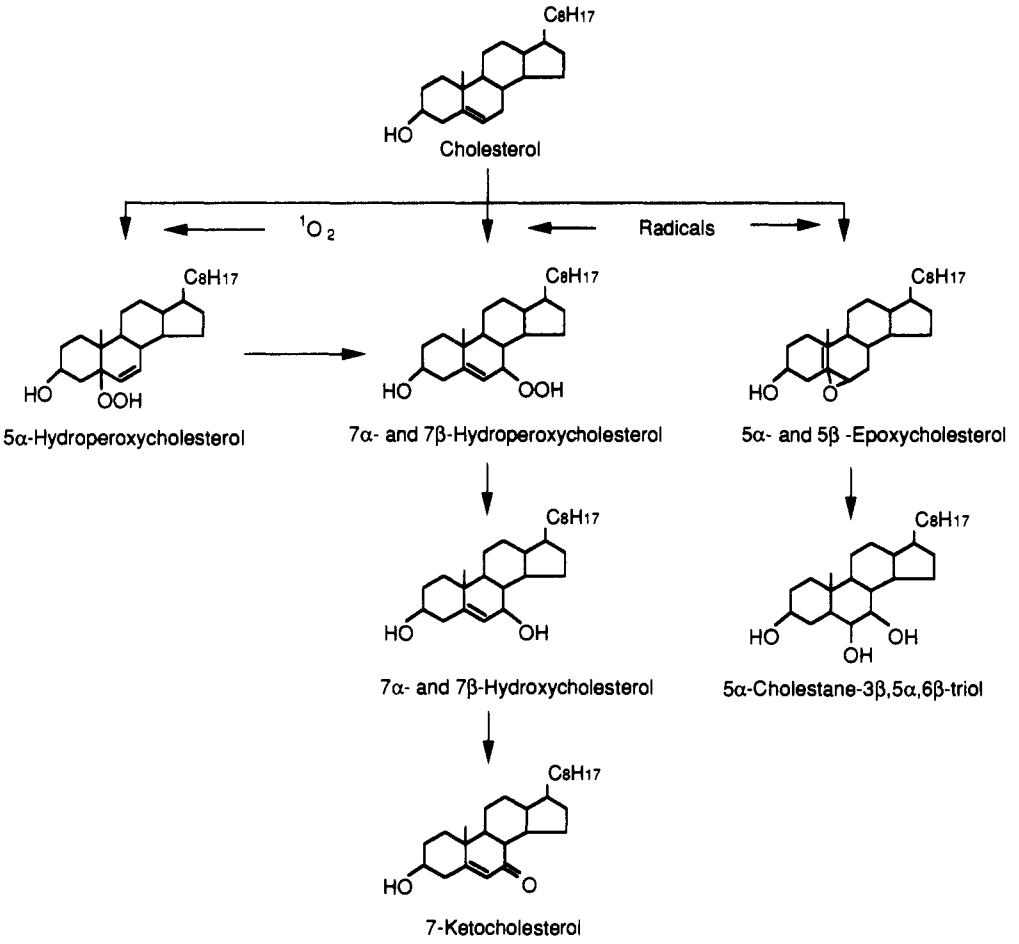


Figure 3. Production of major oxidized cholesterol in different triglyceride vehicles heated at 100 °C for 24 h. Cholesterol was heated at the same conditions as in Figure 2. Symbols: ○, 7α-hydroxycholesterol; ●, 7β-hydroxycholesterol; □, 5β-epoxycholesterol; ■, 5α-epoxycholesterol; △, cholestanetriol; ▲, 7-ketocholesterol.

composition of triglycerides used in this experiment. Cholesterol with or without various triglycerides was heated at 100 °C. The amounts of cholesterol remaining after heating with different triglycerides at 100 °C for 24 h are illustrated in Figure 2A. When cholesterol alone was heated at 100 °C, it was practically stable. However, when cholesterol was heated with triglycerides, there were variable degrees of decomposition. Thus, when cholesterol was heated with saturated fats such as tristearin and beef tallow, it was somewhat stable, and approximately 60 and 50%, respectively, of cholesterol remained unchanged after 24 h of heating. On the other hand, when heated with unsaturated fats, cholesterol was unstable and degraded almost completely after 24 h. When heated with poly-

Scheme I. Proposed Scheme of Cholesterol Oxidation



unsaturated fats, in particular sardine oil, cholesterol was readily decomposed even in 1 h of heating.

Figure 2B shows the time course of oxidized cholesterol formation in different triglyceride vehicles heated at 100 °C for 24 h. When cholesterol alone was heated, virtually no oxidized cholesterol was produced. The production of oxidized cholesterol was considerably low when heated together with saturated fats such as tristearin and beef tallow. When heated with sardine oil or safflower oil, the production of oxidized cholesterol reached a maximum after 6 h and then reduced thereafter. The peak value was around 3 μg/mg of cholesterol. On the other hand, when heated with soybean oil, linseed oil, and triolein, the level of oxidized cholesterol reached a maximum at 12 h, at around 3.3 μg/mg of cholesterol, and subsequently reduced.

The time course of the production of major oxidized cholesterols when heated with different fats is shown in Figure 3. The most predominant oxidized cholesterol detected was 7-ketocholesterol, while 5β-epoxycholesterol and 5α-epoxycholesterol were also produced to a comparatively high extent. Marginal amounts of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and cholestanetriol were produced.

DISCUSSION

In this study, it was found that processed marine foods contain various oxidized cholesterols. Since no oxidized cholesterol was found in uncooked fresh sardine and squid, it is likely that the oxidized cholesterols are formed during processing. Highly polyunsaturated fatty acids which are relatively rich in fish are unstable and are readily oxidized as compared with plant-type polyunsaturated fatty acids at room temperature and much more at high temperature (Cho et al., 1987a,b). Polyunsaturated fatty acids in seafoods are oxidized during processing such as heating at high temperature or drying under sunlight, and lipid peroxy radicals or singlet oxygen is formed. These radicals interact with cholesterol and promote oxidation of sterols (Ansari and Smith, 1979; Korytowski et al., 1992). Therefore, cholesterol alone was stable even when heated at 100 °C for 24 h, whereas when heated with sardine oil, cholesterol readily oxidized and various oxidized products were produced.

Triglycerides (tristearin, triolein, trilinolein, and milk fat) accelerated the decomposition of cholesterol at 130 and 180 °C (Nawer et al., 1991; Kim and Nawer, 1991); the effect was most marked with triolein. This observation was similar to our result. While there was no demonstratable difference in the decomposition of cholesterol after 24 h of heating between mono- and polyunsaturated fats, safflower oil, linseed oil, and sardine oil formed more oxidized sterols as compared with triolein during the initial stages (0–6 h). These results reflect the instability of unsaturated fatty acids to oxidation at high temperature.

Parts per million levels of oxidized cholesterol have been detected in long-term-stored and cooked egg and egg products such as spray-dried egg yolk powder, whole egg powder, and dried egg (Herian and Lee, 1985; Tsai and Hudson, 1985; Nourooz-Zadeh and Appelqvist, 1987). Since the major unsaturated fatty acids in egg are oleic acid and linoleic acid, it is likely that peroxy radicals of these unsaturated fatty acids attack cholesterol. Meat products and milk powder are also reported to contain

oxidized cholesterols (Nourooz-Zadeh and Appelqvist, 1988; Sander et al., 1989; Pie et al., 1991).

A possible pathway leading to oxidized cholesterol in processed foods is shown in Scheme I. The formation of fatty acid peroxy radicals is involved in this pathway. In addition, singlet oxygen produced by photooxidation and ultraviolet irradiation is also involved in the oxidation of cholesterol (Herian and Lee, 1985; Luby et al., 1986; Girotti, 1992). These radicals and singlet oxygen attack the 7- and 5-positions of cholesterol, and 7α- and 7β-hydroperoxycholesterols (Teng and Smith, 1976; Herian and Lee, 1985) and 5α-hydroperoxycholesterol (Kuling and Smith, 1973; Korytowski et al., 1992) are produced. Since these hydroperoxy derivatives are unstable, they readily change to 7α- and 7β-hydroxycholesterol (Lythgoe and Trippett, 1959; Teng et al., 1973). Further, they are converted to 7-ketocholesterol (Teng and Smith, 1976). Meanwhile, when fatty acid radicals and singlet oxygen attack the 5-position of cholesterol, 5α- and 5β-epoxycholesterols are also produced. They are then converted to cholestanetriol (Lythgoe and Trippett, 1959; Teng et al., 1973; Aringer and Eneroth, 1974; Watabe et al., 1980, 1981, 1984). This cholesterol oxidation pathway was surmised from the time course of major oxidized sterols shown in Figure 3, in which 7-ketocholesterol and 5α- and 5β-epoxycholesterols are the major oxidized sterols.

Oxidized cholesterol was not produced when cholesterol alone was heated. However, even when cholesterol was heated with relatively saturated fats like tristearin and beef tallow, oxidized cholesterol was produced after long-term heating. Since saturated fatty acid is stable and is not readily oxidized, the degree of radical formation seems to be low. Kim and Nawer (1992) reported that milk fat globule membrane protected cholesterol against oxidation, and α-tocopherol (Monahan et al., 1992) can reduce cholesterol oxide formation. In addition, phospholipid has been known to suppress lipid peroxidation (Komatsu et al., 1990). However, the present result indicates a possibility that cholesterol in foods is oxidized when it exists together with fats, even with relatively saturated fats.

In conclusion, the oxidation of cholesterol was stimulated when fats were present simultaneously. It was found that 6.0–28.0 mg/100 g of oxidized cholesterol exists in processed marine foods we are commonly consuming. Therefore, more systematic analysis of processed and cooked foods is necessary. Since there is an increasing trend in the consumption of processed animal foods, the physiological implication of ingesting oxidized cholesterol deserves further study.

ACKNOWLEDGMENT

We thank Dr. M. Shimoda of the Laboratory of Food Analysis for his assistance in GC–MS analysis, Dr. M. Hatano of Hokkaido University Faculty of Fisheries and Nippon Chemical Feed Co. for providing sardine oil, and Mr. A. Sugawara of the Hokkaido Wakkanai Fisheries Experimental Station for providing Alaskan pollack roe.

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Received for review June 1, 1993. Accepted August 31, 1993.*

* Abstract published in *Advance ACS Abstracts*, October 15, 1993.