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Iron-catalyzed Autoxidation of Liposomal Cholesterol[1)]

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The autoxidation of cholesterol in a benzene solution of egg lecithin and $Fe(acac)_3$, giving products variously oxygenated in the steroidal ring B, proceeded with consumption of the unsaturated long-chain fatty acid moieties, particularly $C_{18:2}$, in the lecithin molecule. The reaction showed a marked β-stereoselectivity of epoxidation and was inhibited by a radical scavenger (BHT).

Cholesterol was highly susceptible to ferric iron-catalyzed autoxidation within liposomes prepared by using egg lecithin. The oxidative degradation of the unsaturated moieties, $C_{18:1}$ and $C_{18:2}$, in the lecithin led to allylic oxidation as well as the β-stereoselective epoxidation of cholesterol. A radical scavenger inhibited both the degradation of these moieties and the oxygenation of cholesterol. The oxygenation was retarded in liposomes prepared by using saturated dipalmitoyl lecithin, and was dominated by allylic oxidation giving cholesteryl hydroperoxide as the main product. Cholesterol in the liposomes containing egg lecithin was, thus, assumed to be co-oxidized with the unsaturated fatty acid moieties by the radical pathway, when the liposomes were autoxidized in the presence of ferric catalyst.

Keywords——cholesterol; egg lecithin; liposome; liposomal cholesterol; autoxidation; stereoselective epoxidation; lipid peroxidation; co-oxidation; radical pathway; iron catalyst

The biological oxidation of cholesterol has been the subject of extensive investigation. The oxidation of cholesterol with the microsomal nicotinamide adenine dinucleotide phosphate (NADPH)-dependent lipid peroxidation system was reported to be a radical reaction catalyzed bymetal, particularly iron, and was well correlated to the peroxidation of lipid in the system.[2,3)] Cholesteryl epoxide, one of the oxygenation products, is known to be an inhibitor of cholesterol 7α-hydroxylase[3)] and may be related to the occurrence of skin carcinoma.[4)] We studied theoxygenation of cholesterol with the tris(acetylacetonato)iron(III), $Fe(acac)_3$,/alkylhydroperoxide system[5a)] as well as the autoxidation catalyzed by the same iron chelate in the presence of unsaturated long-chain fatty acid,[5b)] and reported the participation of alkylperoxy and alkoxy radicals in both the allylic oxidation and the epoxidation of the substrate. The radical epoxidation showed a marked β-stereoselectivity[5a,b,6)] that was in fair agreement with the results obtained in the microsomal lipid peroxidation system.[3)] In this paper, we report the$Fe(acac)_3$ or $Fe(ClO_4)_3$-catalyzed autoxidation of liposomal cholesterol, as a model for such biological oxygenation.

Results and Discussion

Autoxidation of Cholesterol in Benzene containing Egg Lecithin and $Fe(acac)_3$

A benzene solution of cholesterol (**1**), egg lecithin,[7)] and $Fe(acac)_3$ was bubbled through with molecular oxygen at 60°C for 24 h. Although the autoxidation of **1** proceeded to a lesser extent than in benzene solution containing an unsaturated long-chain fatty acid and $Fe(acac)_3$,[5b)] the C(5,6)-epoxide (**2**, 6%) and the C(7)-ketone (**3**, 2%) were similarly obtained; the marked β-stereoselectivity of epoxidation was also obtained, giving a ratio $\beta/(\alpha+\beta)$ of 0.75. The saturated fatty acid moieties such as palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids remained intact, whereas the unsaturated oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids were extensively

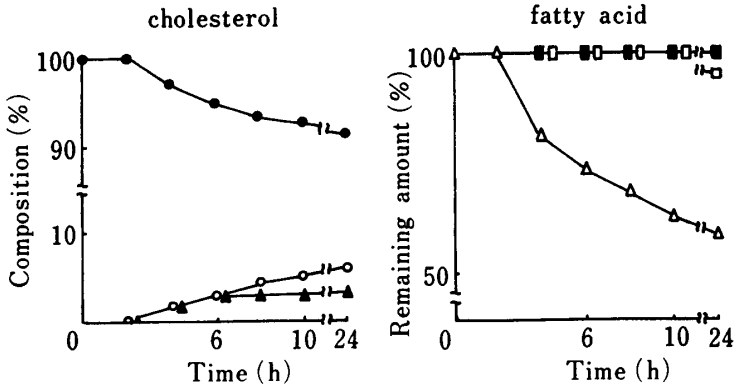


Fig. 1. Time Course of Oxygenation in Benzene at 60 °C
—●—: 1, —○—: 2, —▲—: 3, —■—: $C_{16:0}$ and $C_{18:0}$, —□—: $C_{18:1}$, —△—: $C_{18:2}$.

decomposed; the degradation of the latter was particularly rapid after a lag-time of 2 h. It is noteworthy that the oxygenation of **1** proceeded with the degradation of the $C_{18:2}$ moiety, as shown in Fig. 1, and no reaction occurred in the solution lacking $Fe(acac)_3$ or egg lecithin. The degradation of unsaturated fatty acid moieties and the oxygenation of **1** were also blocked or inhibited when a radical scavenger, 2,6-di-*tert*-butyl-*p*-cresol (BHT), was added to the reacting solution or to the initial reaction mixture. Thus, by analogy with the autoxidation of **1** in a benzene solution of $Fe(acac)_3$ and an unsaturated free fatty acid,[5b] it seems plausible that the unsaturated fatty acid moieties, particularly $C_{18:2}$, in lecithin molecules were initially peroxidized in the present reaction of **1** and the subsequently produced alkylperoxy- and alkoxyradical species then initiated the oxygenation of **1**, giving the epoxide **2**, the ketone **3**, *etc.*, as shown in Chart 1.

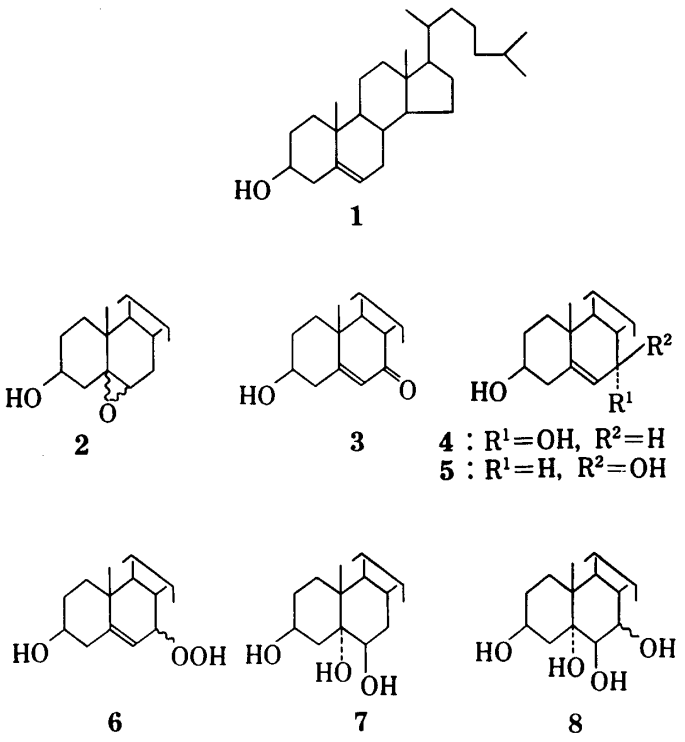
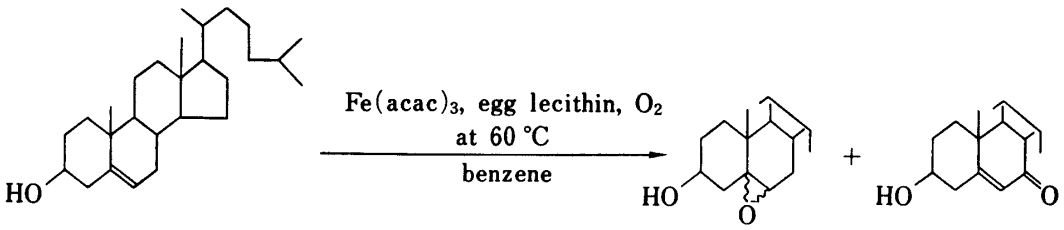


Chart 1



1 2 (6 %, $\beta/(\alpha+\beta)=0.75$) 3 (2 %)

Chart 2

TABLE I. Degradation of Fatty Acid and Distribution of Oxygenated Products

	Conversion (%)	Yield (%)							Degradation (%)			
		2	3	4	5	6	7	8	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Condition 1	69.5	3.5	26.5	7.8	6.5	8.0	13.5	9.7	0	0	35	99
Condition 2	74.2	2.6	22.8	7.7	5.5	5.2	18.4	12.0	0	0	34	98
Condition 3	9.0	4.0	2.3	0.5	0.5	0.6	0.2	0.9	0	0	9	61
Condition 4	16.0	1.0	1.5	1.0	1.0	11.0	0.2	0.3	0[a]			

a) Dipalmitoyllecithin.

Autoxidation of Liposomal Cholesterol in the Presence of Ferric Ions

Autoxidations of cholesterol (**1**) were carried out in vessels open to the air at 40°C for 24 h under four different conditions as follows. Condition 1: liposomes prepared with a mixture of **1**, egg lecithin, and $Fe(acac)_3$ were suspended in 0.2 M aq. NaCl. Condition 2: liposomes consisting of **1** and egg lecithin were suspended in a 0.2 M aq. NaCl solution of $Fe(ClO_4)_3$. Condition 3: a benzene solution of **1**, egg lecithin, and $Fe(acac)_3$ at the same concentrations as under the other three conditions. Condition 4: liposomes prepared with **1**, dipalmitoyl lecithin, and $Fe(acac)_3$ were suspended in 0.2 M aq. NaCl. The products were those known to be produced from **1** in various autoxidation systems, namely the epoxide **2**, the ketone **3**, the C(7)α- and C(7)β-alcohols (**4** and **5**), the C(7)-hydroperoxide (**6**), the C(3,5,6)-triol (**7**) that is known to be formed by the hydrolysis of **2**,[6] and the C(3,5,6,7)-tetrol (**8**) that was also produced from the autoxidation of **1** in a colloidal solution of sodium cholesteryl sulfate;[5c] their yields are summarized in Table I.

Under condition 1 or 2, the reaction proceeded to a large extent as compared to that under conditions 3 (Table I): **1** was also hardly autoxidized without an iron catalyst. The saturated fatty acid moieties, $C_{16:0}$ and $C_{18:0}$, remained intact and the unsaturated $C_{18:1}$ and $C_{18:2}$ were extensively decomposed; condition 3 gave rather similar results. The $C_{16:0}$ moiety also remained intact and the conversion of **1** was low (about 16%) under condition 4, giving mainly the hydroperoxide (**6**). In liposomes lacking the unsaturated fatty acid moieties, thus, allylic oxidation of **1** seemed to be dominant. The conversion of **1** was, in contrast, remarkably high under conditions 1 and 2. The unsaturated fatty acid moieties, therefore, probably participate in the autoxidation of liposomal cholesterol. In fact (Fig. 2), the $C_{18:2}$ moiety, which is susceptible to peroxidation, was rapidly consumed under condition 1 and the oxygenation of **1** proceeded together with the consumption of the $C_{18:1}$ and $C_{18:2}$ moieties, giving the maximum formation of cholesteryl hydroperoxide (**6**) after 6 h. Condition 2 gave similar results, though conditions 3 and 4 retarded these reactions, as shown in Fig. 3. Such high rates of both oxidative degradation of unsaturated fatty acid moieties and oxygenation of **1** under conditions 1 and 2 may be due to the concentration effect of the liposomal membrane.

A radical scavenger (BHT), on the other hand, increasingly retarded these high rates as its concentration was increased, as summarized in Table II. As to the C(5,6)-epoxidation in the title reaction, the total rate was also high under condition 1 or 2 and the marked β-stereo-

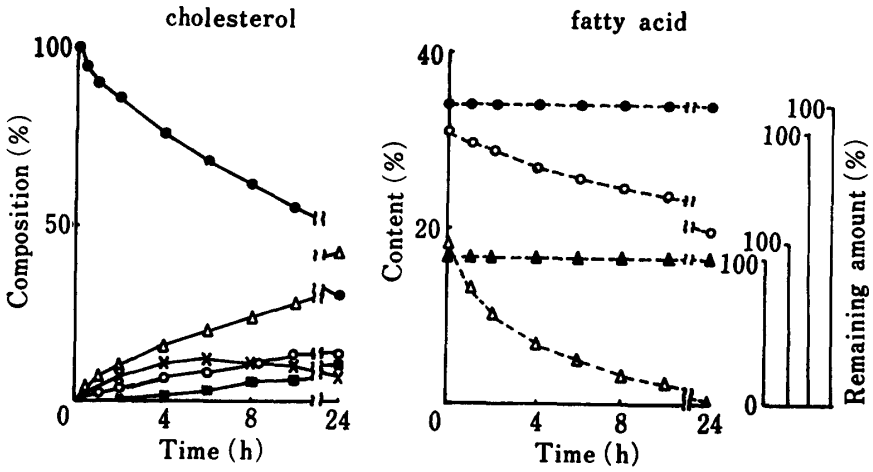


Fig. 2. Oxygenation of Liposomes under Condition 1

—●—: 1, —○—: epoxidation products (2+7), —△—: allylic oxidation products (3+4+5+6), —×—: 6, —■—: 8, --●--: $C_{16:0}$, --▲--: $C_{18:0}$, --○--: $C_{18:1}$, --△--: $C_{18:2}$.

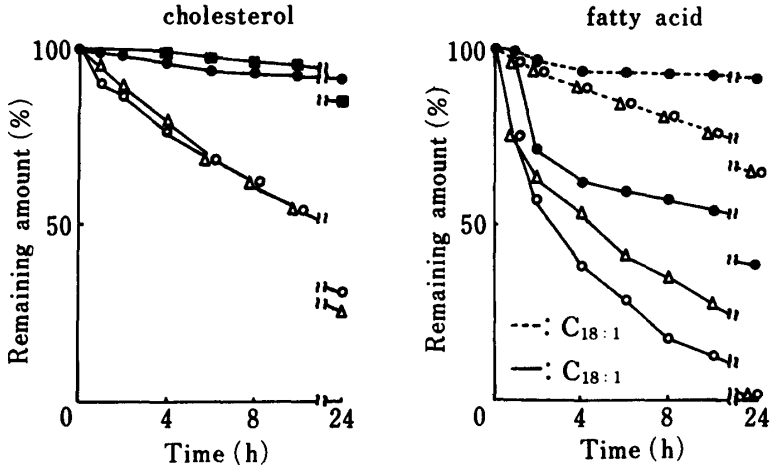


Fig. 3. Oxygenation of Cholesterol and Degradation of Fatty Acid

○: condition 1, △: condition 2, ●: condition 3, ■: condition 4.

TABLE II. Effect of Antioxidant on the Oxidation

Antioxidant	mol % to cholesterol	Oxidation (%) of cholesterol	Degradation (%) of fatty acid			
			$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
None[a]		69.5	0	0	34	99
BHT	0.2	69.5	0	0	34	96
	1	33.0	0	0	18	49
	2	4.0	0	0	0	2
	5	0	0	0	0	0

a) Condition 1.

selectivity[5a,b] ($\beta/(\alpha+\beta)$ ratio of about 0.8) after 2 h, when the formation of the triol (**7**) still remained negligible.

These results on the oxygenation of cholesterol (**1**) in liposomes containing egg lecithin are very similar to those obtained for the autoxidation of **1** in a benzene solution of $Fe(acac)_3$ and an unsaturated long-chain fatty acid, which also showed a marked β-stereoselectivity of

epoxidation and was elucidated to be a radical reaction involving attack of alkoxy as well as alkylperoxy radicals of the acid.[5b] The title reaction is, therefore, likely to be a radical reaction involving attacking species originated from the unsaturated long-chain fatty acid moieties. The mechanism may, however, be more complicated owing to the possibility that another radical species originated from cholesteryl hydroperoxide (**6**) may also attack the substrate (**1**), since the formation of **6** was maximum after 6 h and then decreased somewhat, as shown in Fig. 2. Further work remains necessary.

Experimental

General Methods——Proton nuclear magnetic resonance (PMR) spectra were measured with a JEOL JNM-FX 100 FT spectrometer at 100 MHz with tetramethylsilane as an internal standard in $CDCl_3$. Gas liquid chromatographic (GLC) data were obtained with a Shimadzu GC-4CM gas chromatograph equipped with a hydrogen flame ionization detector (FID) and a glass column (2 m × 3 mm i.d.) packed with 10% EGSS-X. Quantitative thin-layer chromatography (TLC–FID) was carried out on an Iatron TFG-10 Thinchrograph. Radioactivity was measured with a Packard Tri-Carb 2450.

Materials——[4-^{14}C]Cholesterol (52.2 mCi/mmol) was obtained from Japan Radioisotope Association. Egg lecithin and dipalmitoyl lecithin were purchased from E. Merck A.G., Darmstadt, and Sigma Chemical Co., respectively. The authentic specimens of **2**, **3**, **4**, **5**, **6**, **7**, and **8** were prepared by appropriate methods and purified by the ordinary methods. Other reagents were obtained from commercial sources.

Autoxidation of Cholesterol in Benzene containing Egg Lecithin and Fe(acac)$_3$ (Fig. 1)——A benzene solution (5 ml) of **1** (1.30×10^{-1} mmol), $Fe(acac)_3$ (1.35×10^{-2} mmol), and egg lecithin (1.28 mmol) was bubbled through with O_2 at 60°C for 24 h. At each specified time, the reaction mixture was sampled and the sample was worked up for product analysis as described below.

Reactions under the Four Different Conditions (Figs. 2 and 3)——Condition 1: Multilamellar liposomes were prepared as reported by Kinsky *et al.*[8] using egg lecithin (1.30×10^{-1} mmol), [4-^{14}C]cholesterol (7.80×10^{-2} mmol), and $Fe(acac)_3$ (7.80×10^{-3} mmol), and suspended in 0.2 M NaCl (10 ml). The liposomal suspension was stirred at 40°C for 24 h under the normal atmosphere. The reaction mixture was sampled at each specified time and samples were treated as described below for product analysis.

Condition 2: Multilamellar liposomes were prepared using egg lecithin (1.30×10^{-1} mmol) and [4-^{14}C]-cholesterol (7.80×10^{-2} mmol) as described above and suspended in 0.2 M NaCl (10 ml) containing $Fe(ClO_4)_3 \cdot 6H_2O$ (7.80×10^{-3} mmol). The liposomal suspension was treated in a similar manner to that in the case of condition 1 and samples were subjected to product analysis.

Condition 3: A benzene solution (10 ml) of egg lecithin, [4-^{14}C]cholesterol, and $Fe(acac)_3$ in the same amounts as those used under condition 1 was stirred at 40°C for 24 h under the normal atmosphere. The reaction mixture was sampled at each specified time and the samples were used for the product analysis.

Condition 4: Multilamellar liposomes were prepared using dipalmitoyl lecithin (1.30×10^{-1} mmol), [4-^{14}C]cholesterol (7.80×10^{-2} mmol), and $Fe(acac)_3$ (7.80×10^{-3} mmol) as described above and treated in a similar manner to that in the case of condition 1 for product analysis.

Determination of Substrate Consumption, Product Yield, and Fatty Acid Degradation (Table I)——Reaction in Benzene at 60°C: Ice-cold acetone (5 ml) and 10% $MgCl_2$–MeOH solution (5 drops) were added to the reaction mixture (0.1 ml), which was then allowed to stand at −30°C for 1 h in order to complete the precipitation. The acetone layer thus obtained was evaporated to dryness *in vacuo* and subjected to TLC-FID. The consumption of **1** and the product yield were determined according to the reported methods.[5d] The precipitates were dissolved in benzene (1 ml), 0.5 N NaOMe–MeOH solution (2 ml) was added, and the mixture thus obtained was allowed to stand at 60°C for 10 min under an Ar atmosphere. AcOH (0.1 ml) and H_2O (5 ml) were added to the mixture, which was then extracted with *n*-hexane (5 ml × 3). The organic layer was evaporated to dryness *in vacuo* to give the residue, which was redissolved in Me_2CO (0.1 ml) containing methyl *n*-pentadecanoate as an internal standard and subjected to GLC (column temperature: 170°C) for the determination of fatty acid degradation.

Reactions under the Four Different Conditions: The reaction mixture (25 μl) was added to MeOH (75 μl) containing the appropriate reference compounds as internal standards and subjected to TLC with Wako-gel B5F (Wako Pure Chem. Ind. Ltd.) as an adsorbent and Et_2O–cyclohexane (4: 1, v/v; triple developments) as a solvent; the internal standards were visualized with iodine vapor. The iodine was evaporated off at room temperature, and the appropriate zones were scraped into counting vials and assayed for radioactivity. Another portion (0.5 ml) of the reaction mixture was added to benzene (5 ml) and the solution was then evaporated to dryness *in vacuo* below 40°C. The residue thus obtained was washed with ice-cold Me_2CO (0.3 ml × 2) and treated as described above for the determination of fatty acid degradation.

Stereoselective Epoxidation: 1. A benzene solution (15 ml) of **1** (3.90×10^{-1} mmol), $Fe(acac)_3$ (4.05×10^{-2} mmol), and egg lecithin (3.84 mmol) was bubbled through with O_2 at 60°C for 24 h. After usual work-

up, the epimeric ratio of the epoxide was determined by the PMR method previously reported.[5d]

2. Multilamellar liposomal suspension was prepared under condition 1 and stirred at 40°C for 2 h under the normal atmosphere. A portion (0.5 ml) of the reaction mixture was subjected to TLC as described above and the epoxide fraction was divided into equal portions. Unlabelled cholesteryl α-epoxide was added to one of the portions and the β-epoxide to the other one, and then each product was recrystallized until its specific radioactivity reached a constant value, as listed below.

Recrystallization	Specific radioactivity (dmp/mg) α-Epoxide	β-Epoxide
No. 1	174×10^2	172×10^2
No. 2	101	134
No. 3	83	134
No. 4	49	
No. 5	44	
No. 6	44	

Effect of Radical Scavenger (Table II)——Multilamellar liposomes were prepared under condition 1 in the presence of BHT (0.2, 1, 2, and 5 mol% to **1**) and suspended in 0.2 M NaCl. The liposomal suspension was stirred at 40°C for 24 h under the normal atmosphere. After usual work-up, the substrate consumption, the product yield, and the fatty acid degradation were determined as described above.

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References and Notes

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