Effects of Phosphatidylcholines Containing Furan Fatty Acid on Oxidation in Multilamellar Liposomes

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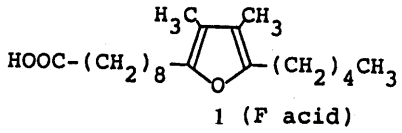
Several phosphatidylcholines (PCs) containing furan fatty acid (10,13-epoxy-11,12-dimethyloctadeca-10,12-dienoic acid, F acid) were synthesized, and their antioxidant activities were studied in the oxidation of soybean PC. The rate of oxidation in soybean PC liposomes initiated with 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), a lipophilic azo-initiator, was measured by following the oxygen uptake, the accumulation of conjugated diene, and the disappearance of unsaturated fatty acid moieties. These PCs containing F acid were found to retard the rate of oxidation in soybean PC liposomes, and the retarding effect increased in the following order: 1/2 mol of di-furanoacyl PC (di-F PC) < 1 mol of 1-furanoacyl-2-linoleoyl PC (F-18:2 PC) < 1 mol of 1-furanoacyl-2-arachidonyl PC (F-20:4 PC) < 1 mol of di-F PC (1:1:1:2, as molar ratios of F acid residue). This is the first report showing that the naturally occurring F acid has a retarding effect on lipid peroxidation.

Keywords furan fatty acid; phosphatidylcholine; liposome; antioxidant activity; retarder

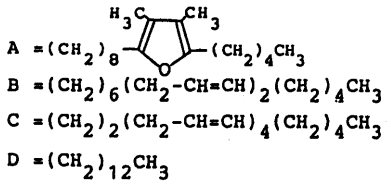
Furan fatty acids (F acids) have been found in several species of fishes.[1] We found F acids not only in fish but also in aquatic animals such as crustacean (crayfish),[2a] amphibian (bullfrog), and reptile (tortoise).[2b] These acids are generally found in cholesteryl esters, triacylglycerols, and phospholipids.

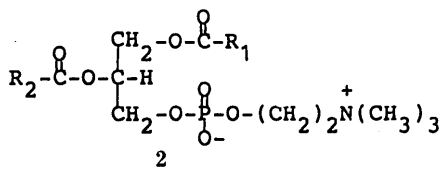
The biosynthetic study by Sand *et al.*[3] in fish showed that labeled acetic acid was not incorporated into the furan ring but only into the carboxylic side chain. Vertebrates such as man and rat easily metabolize such furans to produce dibasic furan derivatives that are excreted in the urine.[4] The biological function of naturally occurring substances containing a furan ring is still not clear in spite of extensive investigations.

It has been reported that phospholipids are major components of the cell membrane in fish and other aquatic animals, as in the case of terrestrial animals.[5] We have tried to clarify the role of the phospholipids containing F acid. This paper reports that phosphatidylcholines (PCs) containing F acid have antioxidant activity on the oxidation in multilamellar soybean PC liposomes.



1 (F acid)





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	R_1	R_2
a:	A	A (di-F PC)
b:	A	B (F-18:2 PC)
c:	A	C (F-20:4 PC)
d:	D	D (di-14:0 PC)
e:	B	B (di-18:2 PC)

Chart 1

Experimental

Fatty Acid Composition of Soybean PC Commercial soybean PC (Sigma Chemical Co., Type IV-S) was purified by silica gel column chromatography, using chloroform–methanol–H_2O (120:22:3, v/v). The fatty acid composition of the purified PC determined by gas chromatography (GC)[2c] after hydrolysis and esterification with trimethylsilyldiazomethane ($TMSCHN_2$)[6] was as follows: palmitic acid (16:0) (11.5%), stearic acid (18:0) (4.5%), oleic acid (18:1) (10.8%), linoleic acid (18:2) (62.8%), and linolenic acid (18:3) (6.4%).

Synthesis of Various PCs The structures of 10,13-epoxy-11,12-dimethyloctadeca-10,12-dienoic acid (F acid) (**1**) and various PCs (**2**) are shown in Chart 1. F acid was synthesized by the methods of Rahn *et al.*[7a] and Schödel and Spiteller.[7b] Di-F PC (**2a**) was synthesized by acylation of L-α-glycerophosphorylcholine with F acid anhydride, and of simple PCs (**2d**, **2e**) with the corresponding fatty acid anhydrides.[8] F-18:2 PC (**2b**) and F-20:4 (**2c**) were prepared by deacylation of **2a** by treatment with phospholipase A_2,[9] followed by reacylation with the corresponding fatty acid anhydrides. The crude PCs thus synthesized were purified by silica gel column chromatography, using chloroform–methanol–NH_4OH (120:22:3, v/v). The purified PCs showed only one spot on silica gel thin layer chromatography (TLC) with chloroform–methanol–H_2O (65:25:4, v/v) and contained 1 mol of phosphorus per mol.[10] In addition, the result of fatty acid positional analysis of **2b** and **2c** by phospholipase A_2 hydrolysis[9] indicated that the purity of each fatty acid at the *sn*-1 and *sn*-2 positions was 95—99 mol% (by GC).

Autoxidation of Liposomes Multilamellar liposomes were prepared by the method of Yamamoto *et al.*[11a] A chloroform solution of soybean PC and additives such as 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN. Wako Pure Chemicals), PC containing F acid and α-tocopherol were taken into a 10 ml or 100 ml conical flask. The solvent was removed *in vacuo* at about 20°C. An appropriate amount of 0.1 M NaCl solution was added to the dried lipid film, and then the dried PC residue was dispersed by shaking slowly. Liposomes thus prepared form multilamellar vesicles.[11a] Distilled, deionized and redistilled water was used for preparation of PC liposomes in 0.1 M NaCl solution. The lipid peroxidation induced with AMVN was carried out at 50 °C in the reaction vessel of a biological oxygen monitor, model YSI 53, under air. After the reaction vessel containing 2.5 ml of 0.1 M NaCl solution had reached thermal equilibrium, 0.5 ml of a liposome suspension containing soybean PC and additives was added. The quantities of components in the liposome suspension are described in the legends for each table and figure. The oxygen uptake in the liposome suspension was monitored by the use of an oxygen electrode.[11b]

Accumulation of conjugated diene was estimated by measuring the absorbance at 233 nm[12] after addition of 4 ml of ethanol to 50 μl of each liposome suspension. After oxidation, the samples were saponified by the method of Wu *et al.*[13] The resulting free fatty acids were esterified with $TMSCHN_2$,[6] followed by GC analysis.[2c]

Results and Discussion

The effects of newly synthesized PCs containing F acid (Chart 1) on the oxidation of unsaturated fatty acid (UFA) moieties of soybean PC liposomes induced by a radical initiator were studied. As the rate of spontaneous autoxidation of the UFA moieties was very small, a radical initiator, AMVN, was used to get a constant rate of initiation and, subsequently, of oxidation.[11a] The extent of oxidation was quantitatively measured in terms of the oxygen uptake,[11b] the accumulation of conjugated diene[12] and the disappearance of UFA moieties of soybean PC.[11a]

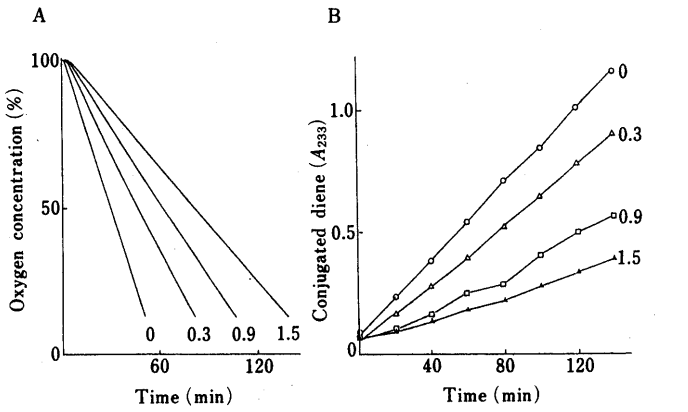

Fig. 1. Effect of Di-F PC (**2a**) on the AMVN-Initiated Oxidation in Soybean PC Liposomes in 0.1 M NaCl Solution

The reaction mixture consisted of soybean PC (30 μmol), AMVN (7.5 μmol), and di-F PC (**2a**) in 3 ml of 0.1 M NaCl solution. (A) Time course of oxygen uptake during oxidation at 37 °C. (B) Accumulation of conjugated diene during oxidation at 50 °C. The numbers in the figures show the amounts of di-F PC in μmol.

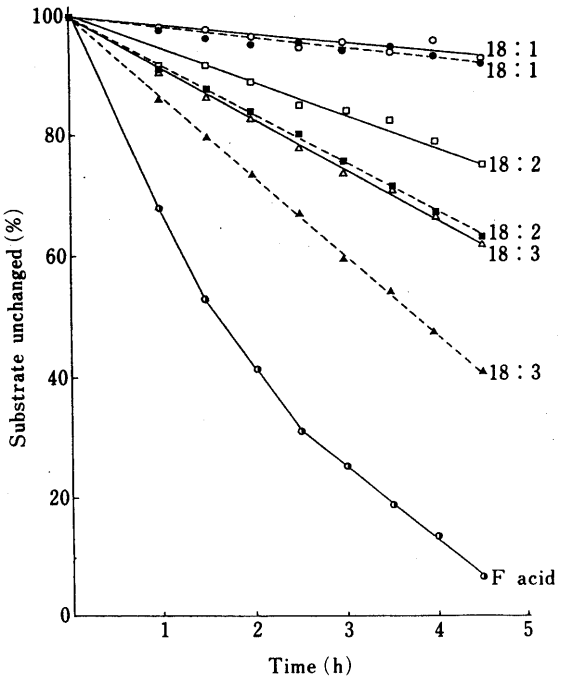

Fig. 2. Time Course of Residual Amounts of Fatty Acid Moieties of Soybean PC and F Acid of Di-F PC (**2a**) during Oxidation Initiated with AMVN at 50 °C

(○, □, △), with di-F PC; ------ (●, ■, ▲), without di-F PC; (◑), residual amount of F acid. The reaction mixture consisted of soybean PC (30 μmol), AMVN (7.5 μmol) and di-F PC (1.5 μmol) in 3 ml of 0.1 M NaCl solution.

Effect of Di-F PC (2a) on the Oxidation in Soybean PC Liposomes The time courses of oxygen uptake and the accumulation of conjugated diene during oxidation of soybean PC liposomes with various concentrations of di-F PC are shown in Fig. 1. In the absence of di-F PC the oxidation proceeded smoothly, and in its presence the oxidation was found to be retarded significantly. The extent of retardation increased with the concentration of di-F PC incorporated into the liposomal membrane. But di-F PC did not alter the induction period. Such substances, which decrease the rate of oxidation without causing any substantial alteration of the induction period, have been named retarders.[14] This effect of di-F PC on oxidation is similar to that of deoxyribonucleic acid (DNA)[15] and of benzo[*a*]-pyrene.[16]

The retarding effect of di-F PC on the oxidation was examined by measuring the residual amounts of UFA moieties of soybean PC and F acid component of di-F PC by GC (Fig. 2). The extent of oxidation of UFA moieties depended on the degree of unsaturation (18 : 1 < 18 : 2 < 18 : 3, Fig. 2). In the presence of di-F PC, the oxidation of UFA moieties was retarded with the consumption of the F acid component of di-F PC.

In order to investigate the role of di-F PC, the consumption of F acid during the oxidations of simple PC (such as di-14 : 0 PC (**2d**) and di-18 : 2 PC (**2e**)) liposomes with di-F PC was quantitated by GC (Table I). In the presence of AMVN, di-F PC incorporated into di-14 : 0 PC liposomal membrane was partly consumed (8% after 1 h), and in its absence, di-F PC was recovered quantitatively. This implied that the F acid component of di-F PC reacted with alkylperoxyl radicals (AOO·) originating from AMVN,[11] because the saturated PC, di-14 : 0 PC, was inert to oxidation under the experimental conditions. On the other hand, in the presence of AMVN, the consumption of F acid incorporated into di-18 : 2 PC liposomal membrane, which was susceptible to oxidation, was much greater (45% after 1 h) than that (8%) in the case of the saturated PC (**2d**) liposomal membrane. It was completely inhibited by α-tocopherol which can terminate a chain reaction by donating hydrogen atoms. The results described above suggested that the F acid component of di-F PC reacted with lipid peroxyl radicals (LOO·) generated from UFAs as well as with alkylperoxyl radicals (AOO·), and this reaction with lipid peroxyl radicals (LOO·) led to the retardation of

TABLE I. Consumption of F Acid during the Oxidation in Simple PC (**2d**, **2e**) Liposomes Containing Di-F PC[a]

Liposome[b]	Additive		Consumption of F acid (%)[d]
	AMVN (μmol)	α-Toc.[c] (μmol)	
Di-14 : 0 PC (30 μmol) + di-F PC (3 μmol)	—	—	0
Di-14 : 0 PC (30 μmol) + di-F PC (3 μmol)	7.5	—	8
Di-18 : 2 PC (30 μmol) + di-F PC (3 μmol)	7.5	—	45
Di-18 : 2 PC (30 μmol) + di-F PC (3 μmol)	7.5	0.3	0

a) The reaction was carried out at 50 °C with stirring under air for 1 h. *b*) In 3 ml of 0.1 M NaCl aqueous suspension. *c*) α-Tocopherol. *d*) Determined by GC.

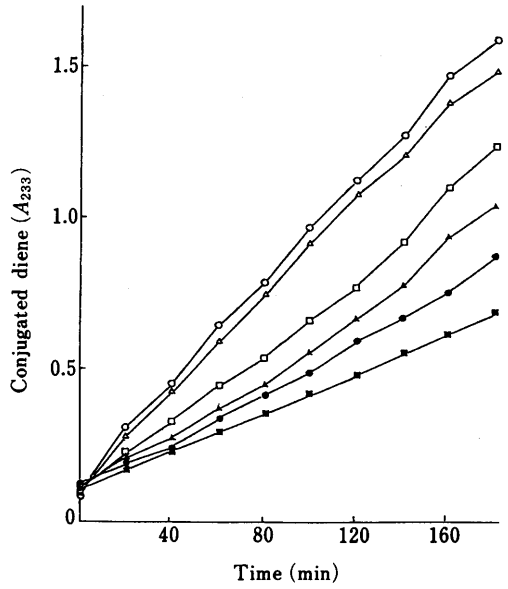

Fig. 3. Effects of F-18:2 PC (**2b**) and F-20:4 PC (**2c**) on the AMVN-Initiated Oxidation in Soybean PC Liposomes; A Comparison with That of Di-F PC (**2a**)

The reaction mixture consisted of soybean PC (30 μmol) and AMVN (7.5 μmol) with or without PCs containing F acids (**2**) (0.9 or 0.45 μmol) in 3 ml of 0.1 M NaCl solution. In the control experiment, the oxidation was carried out with 16:0–20:4 PC (0.9 μmol) in place of **2**. (○), none: (△), 0.9 μmol of 16:0–20:4 PC; (□), 0.45 μmol of di-F PC; (▲), 0.9 μmol of F-18:2 PC; (●), 0.9 μmol of F-20:4 PC; (■), 0.9 μmol of di-F PC.

oxidation in liposomes.

Effects of 1-Furanoacyl-2-Unsaturated Acylphosphatidylcholines (F-U PCs), F-18:2 PC (2b) and F-20:4-PC (2c), on the AMVN-Initiated Oxidation in Soybean PC Liposomes In the preceding paper,[2c] we reported that in salmon roe lipids F acids were exclusively esterified at the *sn*-1 position of PC and polyunsaturated fatty acids predominantly at the *sn*-2 position, as seen generally in fish and other animals.[17] The effects of 1-furanoacyl-2-unsaturated acylphosphatidylcholines (F-U PCs) (**2b**) (**2c**) (Chart 1) on the oxidation of UFA moieties in soybean PC liposomes were studied and compared with that of di-F PC. As shown in Fig. 3, the retarding effects of PCs containing F acid (**2a**—**c**) on the oxidation increased in the following order: 1/2 mol of di-F PC < 1 mol of F-18:2 PC < 1 mol of F-20:4 PC < 1 mol of di-F PC (1:1:1:2, as molar ratio of F acid residue). Antioxidative abilities of 1 mol of F-18:2 PC (**2b**) and of 1 mol of F-20:4 PC (**2c**) were higher than that of 1/2 mol of di-F PC, though 1/2 mol of di-F PC has the same molar amount of F acid residue as 1 mol of F-18:2 PC and of F-20:4 PC. Furthermore, F-20:4 PC had higher activity against the oxidation. It is interesting that the greater the number of double bonds of UFA at *sn*-2 in F-U PCs, the more the retarding effect of F-U PCs on the oxidation. The data in Fig. 3 were confirmed by experiments repeated several times. In addition, the effects of these PCs (**2a**—**c**) on the oxidation as examined by measuring the oxygen uptake showed the same phenomena as seen in Fig. 3 (data not shown). As described in the experimental section, the PCs (**2a**—**c**) were very pure. Therefore, these results can not be attributed to impurities. At this time, no definitive explanation of these results is possible.

This is the first report showing that the naturally occurring F acid has a ratarding effect of lipid peroxidation. The results of further studies of the mechanism of the antioxidant activity of F acids and their reaction products will be reported shortly.

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