Oxidative Stability of Sardine and Mackerel Lipids with Reference to Synergism Between Phospholipids and α-Tocopherol

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Relationships between oxidative stability and the compositions of sardine and mackerel lipids were investigated in view of possible synergism between phospholipids and α-tocopherol (α-Toc). The total lipids extracted from viscera were highly susceptible to autoxidation, compared with lipids of white and red muscles and of skin. This seemed to be due to lower concentrations of α-Toc and phosphatidylethanolamine (PE) in the tissue, but not to the level of polyunsaturated fatty acids. The synergistic effect of PE with α-Toc seemed to be slightly affected by the degree of unsaturation of its fatty acyl chains. The synergistic ability of *O*-phosphoethanolamine, the base moiety of PE, was higher than that of *O*-phosphoserine. *O*-Phosphocholine was only slightly effective. During the induction period of autoxidation, the α-Toc level decreased rapidly, and rapid lipid oxidation began only after α-Toc was almost exhausted.

KEY WORDS: α-Tocopherol, lipid oxidation, mackerel, oxidative stability, phospholipids, sardine, synergist.

When red meat fishes such as sardine and mackerel are stored at low temperature, their skin lipids undergo autoxidation at relatively higher rates than red muscle lipids and white muscle lipids (1). Similarly, lipids prepared from skin are more susceptible to oxidation than those from the other tissues (2–7). Oxidative stabilities of fish lipids are different from species to species and significantly higher in red meat species, such as sardine, mackerel and saury, than in white meat species, such as dusky sole, halibut and sablefish (8). The actual cause of the differences in lipid oxidation between species, as well as between tissues, has not yet been fully elucidated.

Most phospholipid fractions prepared from plant tissues are capable of protecting neutral lipids from oxidation (9–12). Olcott and Veen (13) found, however, that synthetic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) did not affect the oxidative stability of menhaden oil, although they acted as synergists with certain antioxidant against oxidation of that lipid. Hildebrand *et al.* (14) reported that additions of both phospholipids and α-tocopherol (α-Toc) resulted in a marked increase in oxidative stability of soybean oil. Similar results were reported by Yamaguchi and Toyomizu (15) who studied the higher oxidative stability of certain white meat fish lipids; phospholipids could act as synergists with endogenous α-Toc, a primary antioxidant, to protect lipids from oxidation. Recently, Kashima *et al.* (16) claimed that the stabilizing effect of phospholipids on perilla oil might be related to the antioxidant activity of coexisting α-Toc. Komatsu *et al.* (17) believed, however, that the inhibitory effect of the phospholipid fraction prepared from squid mantle muscle on peroxidation of sardine oil might not be due to synergism between the phospholipids and α-Toc because these lipids contained α-Toc in an extremely small quality.

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The present study deals with the relationship between the oxidative stability and the composition of fish lipids with reference to this synergism between phospholipids and α-Toc.

EXPERIMENTAL PROCEDURES

Lipid extraction and fractionation. Fresh sardines (*Sardinops melanosticta*) and mackerels (*Scomber japonicus*) were purchased from a local fish store and used immediately. White muscle, red muscle, viscera and skin tissues, including subcutaneous fat, were removed from sample fish, minced and treated separately with chloroform/methanol, according to the method of Bligh and Dyer (18) to extract total lipids (TL). TL were fractionated to polar lipids (PL) and nonpolar lipids (NL) by column chromatography on Bio-Beads S-X2 (200–400 mesh, Bio-Rad Laboratories, Richmond, CA), according to the method of Tipton *et al.* (19) and subsequent absorption chromatography on Sep-Pak Silica Cartridge (Waters, Milford, MA) after the method of Juaneda and Rocquelin (20). The PC and PE of the PL were separated from each other by silicic acid column chromatography (21).

Determination of the rate of oxygen absorption of lipids. Triplicate samples (50 mg each) were accurately weighed into glass vials (68.7 mL in volume), which were sealed with Teflon-lined septums. After sealing, a 0.1-mL portion of the headspace air of the vial was withdrawn by means of a gas-tight microsyringe and subjected to analysis on a Shimadzu gas chromatograph GC3BT, equipped with a glass column (3 mm i.d. × 1.7 m) packed with molecular sieve 5A (80–100 mesh, Nihon Chromato Co. Ltd., Tokyo, Japan) and a thermal conductivity detector. Helium was used as a carrier gas at an inlet pressure of 1.2 kg/cm^2. The vials were allowed to stand in an incubator at 37°C, and the ratio between N_2 and O_2 in the headspace air was determined at appropriate intervals.

Preparation of model system with microcrystalline cellulose. *O*-Phosphocholine was prepared from *O*-phosphocholine calcium chloride (Sigma Chemical Co., St. Louis, MO), according to the method of Tsai and Smith (22). An aqueous solution of each phosphoryl base, *O*-phosphocholine (prepared as described), *O*-phosphoethanolamine and *O*-phosphoserine (Sigma) was added to microcrystalline cellulose (Avicel, 100–120 mesh, Funakoshi Co., Tokyo, Japan) to give a concentration equimolar with 5 mg of sardine PE per 0.9 g of Avicel and was thoroughly mixed. After freeze-drying, 45 mg of the NL and 1.5 μg of α-Toc dissolved in *n*-hexane were added to the Avicel. Solvent in the mixture was completely stripped off in a rotary evaporator, and 0.9-g samples from the resulting solids were accurately weighed into glass vials (68.7 mL in vol) for oxidation tests in triplicate.

Determination of fatty acid composition. Aliquot sample of the lipids were saponified with 1 N alcoholic KOH under reflux for 1 h, and the resulting free fatty acids were converted to fatty acid methyl esters (FAME) by means of 14% BF_3 in methanol. FAME were subjected on a

Shimadzu GC 12APF gas chromatograph, equipped with a SUPELCOWAX-10 wall-coated open tubular column (0.25 mm i.d. × 30 m, Supelco, Japan, Tokyo, Japan) and a flame-ionization detector. The column oven and injection port were held at 195 and 250°C, respectively. Helium was used as a carrier gas with an inlet pressure of 2.0 kg/cm^2. A Shimadzu Chromatopak CR6A integrator was used for peak area calculation.

Hydrogenation of lipids. Hydrogenation of lipids or FAME was carried out over a catalytic amount of platinum oxide (Kojima Chemical Co. Ltd., Tokyo, Japan) in chloroform, by using a 50-mL round, flat-bottomed flask with standard taper neck. Hydrogen pressure was kept at 1.2 kg/cm^2 and the flask was stirred continuously by means of a Teflon-coated magnetic stirring bar (23).

Determination of α-Toc. The α-Toc content of lipid was determined by normal-phase high-performance liquid chromatography (HPLC) on a Lichrosorb Si 60 column (4 mm i.d. × 250 mm, E. Merck, Darmstadt, Germany) with *n*-hexane/tetrahydrofurane (95:5, vol/vol) mixture as the mobile phase at a flow rate of 1.2 mL/min. Tocol (>99% purity, Eizai Co., Tokyo, Japan) was used as an internal standard.

Determination of peroxide value. Peroxide value (POV) of lipids was determined according to the method of Buege and Aust (24). The result was expressed as mole of hydroperoxide/kg of lipid.

Determination of lipid class. Lipid class composition of the TL was determined by silica gel thin-layer chromatography (TLC) and subsequent densitometry as described previously (25).

RESULTS AND DISCUSSION

Oxidative stabilities of lipids extracted from different tissues. Oxygen absorption curves obtained with sardine TL prepared from white muscle, red muscle, viscera and skin tissues including subcutaneous lipids, during incubation, are shown in Figure 1. With TL from skin and viscera, rapid oxygen absorption occurred immediately after the start of incubation, while those of white and red muscles absorbed virtually no oxygen during incubation for 8 d.

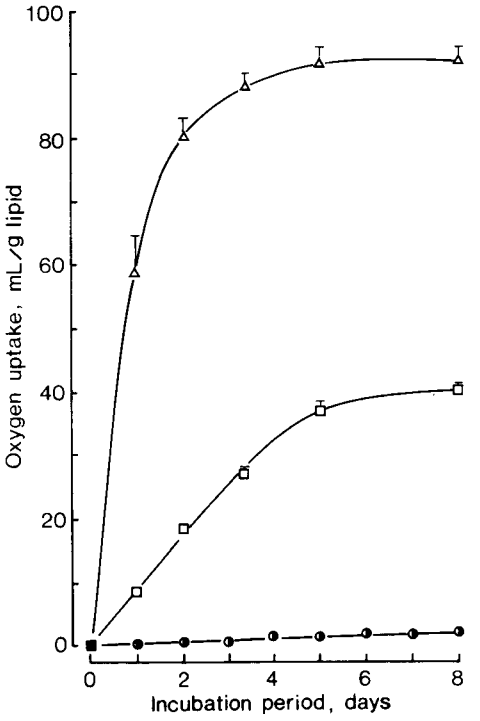

FIG. 1. Oxygen absorption curves of total lipids extracted from various parts of sardine during incubation at 37°C. ○, white muscle; ●, red muscle; □, viscera; and △, skin tissue. Symbols represent mean values and bars indicate range in triplicates.

As shown in Table 1, the TL contents of the sardine were highest in skin, followed by viscera, red muscle and white muscle, in that order. The concentrations of PL in the total lipids descended in the following order: white muscle (25.6%) > red muscle (25.4%) > skin (11.4%) > viscera (11.1%). α-Toc was detected at relatively high concentrations in the white and red muscle TL, 173 and 199 μg/g of lipid, respectively, while it was not detected in lipid of viscera and skin. The major lipid class of TL was triglyceride (TG), the highest percentage being in the skin (Table 2). The percentages of sterol (ST) and free fatty acid

TABLE 1

Lipid Contents in Various Tissues of Sardine and Mackerel

	Sardine				Mackerel			
Tissue	TL[a] (g/100 g)	NL[a] (%)	PL[a] (%)	α-Toc[a] (μg/g)	TL[a] (g/100 g)	NL[a] (%)	PL[a] (%)	α-Toc[a] (μg/g)
White muscle	1.37	74.5 (1020)[b]	25.6 (349)	173	1.34	67.6 (906)	32.4 (432)	—[d]
Red muscle	3.64	74.6 (2717)	25.4 (923)	199	4.71	74.2 (3494)	25.8 (1213)	167
Viscera	3.74	88.9 (3326)	11.1 (410)	ND[c]	4.42	82.3 (3639)	17.7 (776)	34.9
Skin	8.12	88.6 (7193)	11.4 (932)	ND	10.8	88.6 (9568)	11.4 (1230)	88.4

[a]TL, total lipids; NL, nonpolar lipids; PL, polar lipids; α-Toc, α-tocopherol.

[b]Values in parentheses are expressed as mg/100 g tissue.

[c]ND, not detected.

[d]Not determined.

TABLE 2

Lipid Class Compositions (%) of Total Lipids in Various Tissues of Sardine and Mackerel

Lipid[a] class	Sardine				Mackerel			
	White muscle	Red muscle	Viscera	Skin	White muscle	Red muscle	Viscera	Skin
SE	ND[b]	ND	6.6 (246)[c]	ND	ND	ND	1.1 (48)	ND
TG	69.1 (946)	69.7 (2539)	41.4 (1548)	85.4 (6934)	59.0 (790)	65.4 (3080)	68.8 (3040)	88.6 (9568)
DG	ND	ND	ND	ND	ND	ND	ND	ND
MG	ND	ND	ND	ND	ND	ND	ND	ND
ST	ND	ND	15.6 (583)	ND	0.9 (12)	4.6 (216)	5.2 (229)	ND
FFA	5.4 (74)	4.9 (178)	25.4 (949)	3.2 (259)	7.8 (104)	4.2 (198)	7.3 (322)	ND
PE	10.0 (137)	11.5 (418)	3.1 (115)	4.5 (365)	14.7 (196)	8.4 (395)	6.3 (278)	5.6 (604)
PI + PS	2.9 (39)	1.2 (43)	2.3 (86)	0.7 (56)	2.5 (33)	1.6 (75)	2.1 (92)	ND
SPM	ND	ND	ND	ND	ND	ND	ND	ND
PC	12.1 (165)	12.5 (455)	5.3 (198)	6.2 (503)	14.9 (199)	15.6 (734)	8.9 (393)	5.8 (626)
LPC	0.6 (8)	0.2 (7)	0.3 (11)	0.1 (8)	0.3 (4)	0.2 (9)	0.3 (13)	ND

[a]SE, steryl ester; TG, triglyceride; DG, diglyceride; MG, monoglyceride; ST, free sterol; FFA, free fatty acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPM, sphingomyelin; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

[b]ND, not detected.

[c]Values in parentheses are expressed as mg/100 g tissue.

TABLE 3

Fatty Acid Compositions (wt%) of Total Lipids in Various Tissues of Sardine and Mackerel

Tissue	Sardine			Mackerel		
	Saturate[a]	Monoene[a]	Polyene[a]	Saturate[a]	Monoene[a]	Polyene[a]
White muscle	32.3 (37.8)[b]	20.5 (21.0)	47.2 (41.2)	29.8 (35.9)	28.9 (30.3)	41.3 (33.8)
Red muscle	32.5 (33.7)	20.2 (21.2)	47.3 (45.1)	30.9 (33.0)	29.2 (31.7)	39.9 (35.3)
Viscera	31.1 (47.8)	24.5 (30.1)	44.4 (22.1)	31.4 (40.2)	34.2 (43.6)	34.5 (16.2)
Skin	30.3 (58.6)	25.6 (32.1)	44.1 (9.3)	30.4 (36.1)	36.8 (34.2)	32.8 (29.7)

[a]Most prominent fatty acids are revealed in the text.

[b]Figures in parentheses represent the percentages of fatty acids in the lipids after incubation at 37°C for 10 d.

(FFA) were high in viscera TL, while those of PE and PC were important in both white and red muscle TL. The fatty acid compositions of TL are summarized in Table 3. Saturated fatty acids consisted mainly of 16:0 (18.6–22.4% for sardine, 20.6–22.6% for mackerel), 14:0 (4.6–6.3% for sardine, 5.0–5.2% for mackerel) and 18:0 (3.0–4.1% for sardine, 4.6–5.5% for mackerel). Major monoenoic acids were 16:1(n-7) (6.3–8.5% for sardine, 5.0–5.7% for mackerel) and 18:1(n-9) (6.3–11.8% for sardine, 14.9–21.1% for mackerel); important polyenoic acids were 20:5(n-3) (11.7–16.5% for sardine, 8.1–9.2% for mackerel) and 22:6(n-3) (11.4–17.6% for sardine, 8.1–10.7% for mackerel). As mentioned above, the skin and viscera TL, which rapidly absorbed oxygen during incubation, did not contain α-Tbc. In addition, the concentrations of PC and PE in the skin and viscera TL were lower than in the white and red muscle TL, which barely absorb oxygen during incubation. The concentrations of PC and PE in the total lipids of sardine are: white muscle, 22.1%; red muscle, 24.0%; viscera, 8.4%; and skin, 10.7%. The lipids with both a higher concentration of PL in the TL and a higher level of α-Tbc revealed good stability against oxidation of TL. These results suggest strongly that PC, PE and α-Tbc are responsible for the stability of TL against oxidation.

A similar experiment was carried out with TL extracted from mackerel. Oxygen absorption curves of TL prepared from different tissues during incubation are shown in Figure 2. The viscera TL initiated oxygen absorption

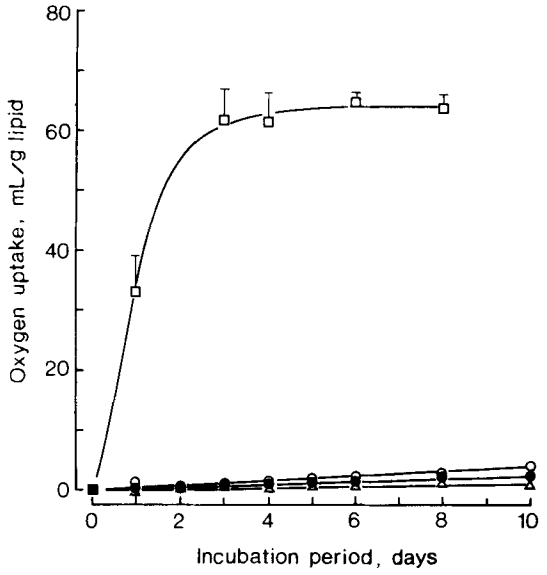

FIG. 2. Oxygen absorption curves of total lipids extracted from various parts of mackerel during incubation at 37°C. ○, white muscle; ●, red muscle; □, viscera; and △, skin tissue. Symbols represent mean values and bars indicate range in triplicates.

immediately after incubation, while oxygen absorption by TL extraction from the other tissues, including skin TL, did not occur for at least 10 d. As illustrated in Figure 3, changes in POV indicated that the viscera TL were less stable to oxidation that the other tissue TL. The results were in agreement with those obtained in the oxygen absorption experiment with sardine TL (cf. Fig. 2). The skin TL of mackerel are not always much more susceptible to oxidation than the muscle TL, as had been pointed out by Ke *et al.* (3). The lipids and α-Toc contents of mackerel tissues used in the present study are shown in Table 1. Although the white muscle TL of mackerel was not analyzed for α-Toc content, our supplemental experiments for four other mackerel specimens showed that the α-Toc levels in white and red muscles were 96.8–120 μg/g and 140–158 μg/g, respectively. The white muscle TL of

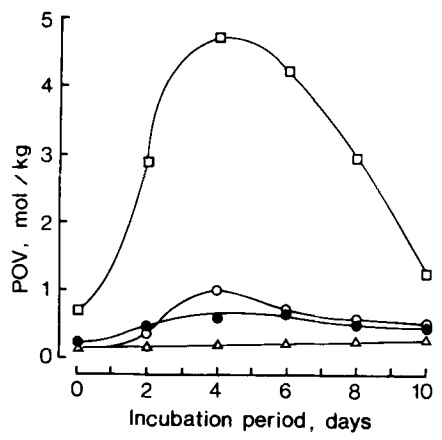

FIG. 3. Changes in peroxide values (POV) of total lipids extracted from various parts of mackerel during incubation at 37°C. ○, white muscle; ●, red muscle; □, viscera; and △, skin tissue.

mackerel were expected, therefore, to contain α-Toc at a level similar to that in the white muscle TL of sardine (173 μg/g TL) because α-Toc content of the red muscle TL of mackerel was almost comparable to that of the red muscle TL of the sardine used in the preceding experiment (167 and 199 μg/g lipid, respectively). The lipid class compositions of the mackerel TL are also shown in Table 2. In all TL prepared from the different tissues, TG was the major component. The concentrations of PC and PE in the total lipids were higher in the white and red muscle TL, while the skin TL showed the lowest percentage. Skin lipids revealed good stability against oxidation, although the concentrations of PC and PE in the total lipids are lower than those of viscera lipids. This does not mean discrepancy against the results of sardine lipids mentioned above because α-Toc content of the mackerel skin lipids was much higher than that of viscera lipids. α-Toc seems to act as a primary antioxidant in this case. As shown in Table 3, the percentages of polyunsaturated fatty acids (PUFA) in the white and red muscle TL were higher than those of viscera and skin TL. Therefore, the greater susceptibility to oxidation of the viscera TL seemed to be due to relatively lower concentrations of α-Toc, PC and PE, but not to the PUFA level.

Oxidative stabilities of NL and PL. The sardine skin TL was fractionated to NL and to PL, and their rates of oxygen absorption were compared (data not shown). The TL showed an induction period for 9 d, while that for NL was 5 d: removal of PL from TL resulted in shortening of the induction period by 4 d. The PL itself initiated rapid oxygen absorption, immediately after the start of incubation. TL contained α-Toc at 46.9 μg/g lipid, which was included in the nonpolar lipid fraction after separation of TL into NL and PL by column chromatography on Bio-Beads S-X2. Indeed, the supplemental examinations were repeated to confirm the recovery of α-Toc in NL by column chromatography under the present experimental conditions. As a result, α-Toc was eluted in NL with the recovery of 93–96%. Therefore, NL can be expected to contain α-Toc at a level similar to that of TL in this case. Nevertheless, the induction period of NL was remarkably shorter than that of TL. This clearly indicates that the oxidative stability of TL depends not only on α-Toc concentration but also on PL concentration. The lipid classes and fatty acid compositions of skin lipids are given in Tables 4 and 5, respectively. Major lipid classes of TL and NL were TG. The monoenoic acid in PL was lower than in NL. In both NL and PL, major PUFAs were 20:5(n-3) and 22:6(n-3); 18:1(n-9) and 16:1(n-7) were major monoenoic acids. The rapid oxidation of PL seemed to be due to higher percentage of PUFA and lack of α-Toc. These results suggest that both α-Toc and PL concentrations are closely associated with the oxidative stability of TL.

Sardine skin PL was added to sardine skin NL to give final concentrations of 5, 20 and 50%, which were incubated at 37°C. A set of typical oxygen absorption curves is illustrated in Figure 4. The NL gave an induction period of 5.0 d, which was not influenced by addition of PL at 5%. Addition of PL at 20% resulted in a fairly prolonged induction period (7.5 d) of the combined lipids. The α-Toc concentration in NL is somewhat diluted by adding PL: α-Toc content in the mixture is calculated to be 37.5 μg/g of lipid, based on the α-Toc content of NL (46.9 μg/g of lipid). Also, addition of PL certainly results in an

TABLE 4

Lipid Class Compositions (wt%) of Sardine Skin Lipids

Lipid class[a]	Total lipids	Nonpolar lipids	Polar lipids
SE	ND[b]	ND	—
TG	95.5 (23493)[c]	99.3 (23493)	—
DG	ND	ND	—
MG	ND	ND	—
ST	0.7 (172)	0.7 (172)	—
FFA	ND	ND	—
PE	1.9 (467)	—	50.0 (467)
PI + PS	0.2 (49)	—	5.3 (49)
SPM	ND	—	ND
PC	1.7 (418)	—	44.7 (418)
LPC	ND	—	ND

[a]SE, steryl ester; TG, triglyceride; DG, diglyceride; MG, monoglyceride; ST, free sterol; FFA, free fatty acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPM, sphingomyelin; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

[b]Not detected.

[c]Values in parentheses are expressed as mg/100 g tissue.

TABLE 5

Prominent Fatty Acid Compositions (wt%) of Sardine Skin Lipids

Fatty acids	Total lipids	Nonpolar lipids	Polar lipids
14:0	8.6	7.9	1.1
16:0	20.5	19.9	25.9
18:0	3.0	3.4	6.5
Σ saturates	33.5	32.6	34.9
16:1(n-7)	11.5	10.6	1.8
18:1(n-9)	13.8	14.7	6.7
Σ monoenes	30.5	31.7	13.4
18:4(n-3)	2.7	2.7	0.5
20:5(n-3)	13.4	14.1	13.4
22:5(n-3)	1.6	1.7	1.9
22:6(n-3)	3.9	3.8	28.8
Σ polyenes	36.0	35.7	51.7

increase in the percentage of PUFA in the lipids. Therefore, a significantly prolonged induction period seemed to be due to the synergistic action of PL with the antioxidant α-Toc, as had been suggested by Yamaguchi and Toyomizu (15). However, addition of PL at 50% resulted in acceleration of oxygen absorption of the lipids: no induction period was observed in the oxygen absorption curve of these lipids. A decrease in α-Toc concentration and an increase in PUFA percentage seemed to bring about rapid oxidation of the lipids.

Effects of PL, PC and PE on oxidative stability of NL. To identify synergists or antioxidant compounds present in PL, the respective effects of PE and PC, the major constituents of PL, on the oxidation rate of NL were investigated by using other samples of sardine skin lipids. PE (or PC) at a final concentration of 10% was added to NL,

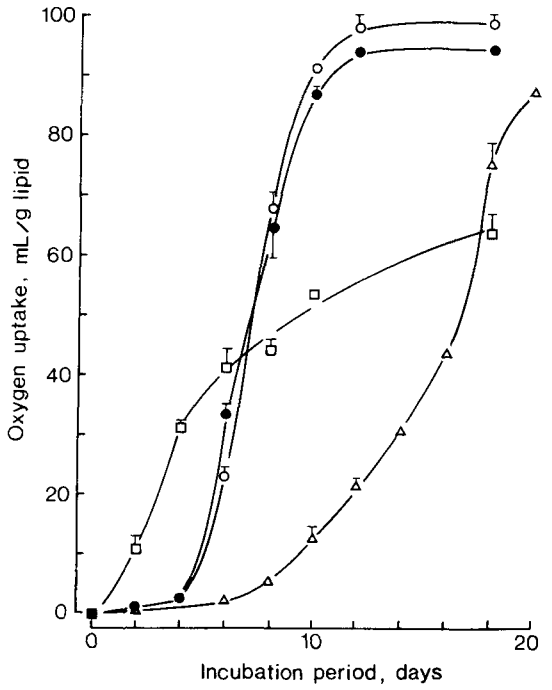

FIG. 4. Effects of added sardine phospholipids (PL) on autoxidation rate of sardine nonpolar lipids (NL) during storage at 37°C. ●, NL; ○, NL with 5% added PL; △, NL with 20% added PL; □, NL with 50% added PL. Symbols represent mean values and bars indicate range in triplicates.

and the resulting mixture was incubated at 37°C. α-Toc was also added to NL at 30 μg/g lipid before incubation because the NL was lacking in α-Toc in this case. Although oxygen absorption curves are not shown here, the lipids with added PC had a 1.2-d induction period and showed an oxygen absorption curve similar to that of the lipids without added PC (1.0-d induction period): PC did not affect the oxidation rate of the lipids. In contrast, addition of PE clearly prolonged the induction period of the lipids (3.5 d). Although detailed data on the lipid composition of the sample are not given here, the percentages of PUFAs of PE (43.2%) and PC (45.5%) were considerably higher than that of NL (34.8%).

The PE isolated from sardine white muscle was added to the same muscle NL at final concentrations of 5, 10 and 20% in the presence of added α-Toc at 30 μg/g lipid. The oxygen absorption curves are illustrated in Figure 5. The results obtained indicated that the higher the concentration of added PE, the longer the induction period of NL. In the absence of added α-Toc, however, PE had a slight prooxidant action (data not shown). From these results, it is suggested that PE acts as a synergist with α-Toc as the primary antioxidant, but is not itself an antioxidant.

Effects of acyl chains in PE and PC on oxidation rate of NL. The effects of PE and PC with acyl chains different in degree of unsaturation on oxidation rate of NL were investigated. For this purpose, synthetic dipalmitoyl PE (DPPE, Sigma) and dipalmitoyl PC (DPPC, Sigma) were used. Each of these phospholipids and α-Toc were added to the sardine skin NL at the final concentrations of 10% and 30 μg/g lipid, respectively (the results obtained are not shown here). An induction period of the lipids with

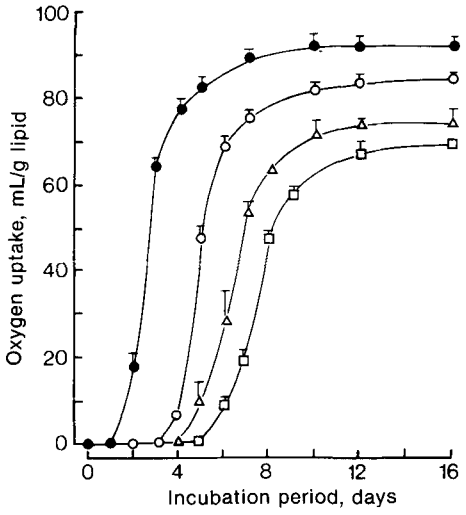

FIG. 5. Effects of added sardine phosphatidylethanolamine (PE) on autoxidation rates of sardine nonpolar lipids (NL) during incubation at 37°C. ●, NL; ○, NL with 5% added PE; △, NL with 10% added PE; □, NL with 20% added PE. Symbols represent mean values and bars indicate range in triplicates.

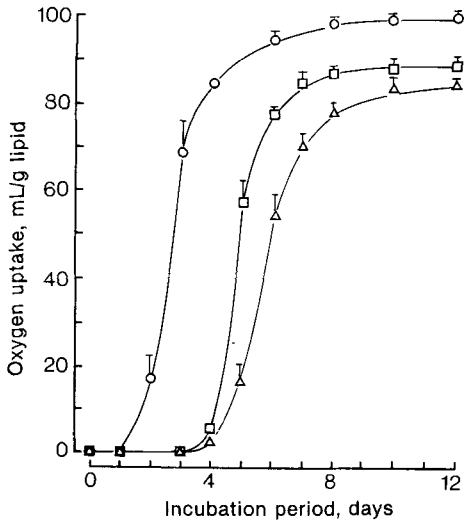

FIG. 6. Effects of added sardine phosphatidylethanolamine (PE) on autoxidation rates of sardine nonpolar lipids (NL) during incubation at 37°C. ○, NL; △, NL with 10% added PE; □, NL with 10% added hydrogenated PE. Symbols represent mean values and bars indicate range in triplicates.

added DPPC (2.0 d) was almost the same as that without added phospholipid (1.7 d). With the lipids containing added DPPE, however, the induction period was actually prolonged. This implies that the ethanolamine residue, the organic base portion of DPPE, may affect the oxidation rate of lipids as a synergist with α-Toc because DPPE contains the same acyl chains as those of DPPC, which in practice did not affect the length of induction period. These results coincide well with the published results of Chen and Nawar (26,27), in which they suggest that the free amino group of PE is responsible for the inhibitory effect against milk fat oxidation.

To examine the effects of the degree of unsaturation of the acyl chains in PE, a portion of the PE fraction from sardine white muscle was hydrogenated and added to NL from the sardine white muscle to give a final concentration of 10%. α-Toc was also added at 30μg/g lipid. As shown in Figure 6, addition of the hydrogenated PE resulted in a prolonged induction period for oxidation of the lipids, which was slightly shorter than that with added untreated sardine PE: both hydrogenated PE and untreated PE may act similarly as synergists with α-Toc to decelerate lipid oxidation. Bhatia *et al.* (10) postulated that the fatty acid chains of PE had no role in its antioxidant effect because PE prepared from sunflower, peanut, soybean and cottonseed were essentially the same in their antioxidant activities. However, the lipids tested by Bhatia *et al.* (10) were lacking in added Toc, a model differing from ours. The synergistic effect of PE with α-Toc on the oxidative stability of lipid seems to be slightly affected by the degree of unsaturation of its fatty acyl chains.

Effects of PE on oxidative stabilities of NL with fatty acyl chains different in unsaturation. The sardine PE, both fully hydrogenated and untreated, and DPPE, and sardine NL and trilinolein (Tri-Li, a synthetic triglyceride), were used as PE and NL in this experiment, respectively. NL and PE prepared from sardine white muscle were used. PE and α-Toc were added to NL to give final concentrations of 10% and 30 μg/g, respectively. Induction periods of these reaction systems during incubation are

TABLE 6

Comparison of Synergistic Effects of Phosphatidylethanolamine (PE) with Fatty Acyl Chains Different in Degree of Unsaturation with α-Tocopherol on Oxidative Stability of Nonpolar Lipids (NL)

Sample[a]	Induction period, days		Synergistic effect B/A[b]
	Without added PE (A)	With added PE (B)	
Trilinolein + dipalmitoyl PE	8.2	11.2	1.4
Trilinolein + hydrogenated sardine PE	6.0	9.1	1.5
Trilinolein + sardine PE	6.0	4.2	0.7
Sardine NL + dipalmitoyl PE	1.5	2.5	1.7
Sardine NL + hydrogenated sardine PE	1.2	3.5	2.9
Sardine NL + sardine PE	1.2	3.8	3.2

[a] α-Tocopherol was added to the samples at 30 μg/g before incubation.

[b] Synergistic effect was tentatively defined by B/A.

summarized in Table 6. The incubation period of Tri-Li was prolonged by addition of DPPE, while shortened by addition of sardine PE. When α-Toc was added to the lipids at 30 μg/g, oxidation of the lipids was nevertheless accelerated. However, addition of the hydrogenated sardine PE brought about an extension of the induction period. Hydrogenated PE showed a synergistic action similar to that of DPPE. With the sardine NL, all of the DPPE, the sardine PE and the hydrogenated sardine PE acted as synergists with α-Toc against NL oxidation. Of these PL, the sardine PE was the most effective synergist in reducing oxidation of the sardine NL, although it accelerated oxidation of Tri-Li to some extent. This seems to be due to the high susceptibility to oxidation of the PUFAs, such as 20:5(n-3) and 22:6(n-3), of the sardine PE. When the sardine PE is added to Tri-Li which is less susceptible to oxidation than the sardine NL, PUFA in the sardine PE may first be oxidized to produce some free radicals which initiate rapid oxidation of Tri-Li. However, the sardine PE was an effective synergist against oxidation of lipids that were highly susceptible to oxidation, such as the sardine NL.

Effects of phosphoryl bases on oxidation rate of NL. The role of the phosphoryl base portions in phospholipids on oxidative stability of NL was investigated in the presence of α-Toc (30 μg/g lipid). Each of *O*-phosphocholine, *O*-ethanolamine and *O*-serine was added to sardine NL and Tri-Li in *n*-hexane. The resulting solution was mixed with Avicel, and the *n*-hexane was evaporated completely from the mixture. Avicel, coated with the lipids, thus obtained was examined for induction period. The results obtained are summarized in Table 7. Both *O*-phosphoethanolamine and *O*-phosphoserine prolonged the induction periods of sardine NL and Tri-Li. The synergistic action of *O*-phosphoethanolamine was clearly greater than that of *O*-phosphoserine: *O*-phosphocholine was only slightly effective. Tsai and Smith (22) reported that *O*-phosphoserine effectively increased the oxidative stability of an aqueous emulsion of methyl linoleate. The inconsistency of the results in the present study seems to be due to the difference in the lipid used: the lipid used by Tsai and Smith (22) was lacking in added α-Toc. Differences of the three kinds of phosphoryl bases in their effectiveness as synergists with α-Toc may be due to differences in their organic bases. Hildebrand *et al.* (14) postulated that the mechanisms involved in synergism of PE, PC and phosphatidylinositol (PI) with Toc in the autoxidation of soybean oil were as follows: (i) Amino groups of organic bases in PE and PC molecules and reducing sugar in the PI molecule facilitate hydrogen or electron donation to Toc; and (ii) PE, PC and PI extend the antioxidant effectiveness of Toc by delaying the irreversible oxidation of Toc to tocopherylquinone.

Changes in fatty acid composition and α-Toc content during oxidation of TL. During oxidation of the white muscle TL of sardine, changes in fatty acid compositions of TL, NL and PE and in α-Toc content were determined. Results obtained are shown in Figures 7 and 8. The α-Toc content of TL decreased rapidly and α-Toc almost completely disappeared after 3 d of incubation. Once α-Toc was exhausted, rapid oxygen absorption for TL was initiated: the induction period of TL was estimated to be 3 d. During this induction period, the percentages of 20:5(n-3) and 22:6(n-3) in the TL decreased from 12.2 and 7.0% to 11.0 and 5.9%, respectively. Thereafter, more rapid decreases in these PUFA occurred: after incubation for 5 d, the 20:5(n-3) and 22-6(n-3) were considerably reduced (3.0 and 1.1%, respectively). The percentages of monoenoic acids, such as 16:1(n-7) and 18:1(n-9), apparently increased; however, the contents of these monoenoic acids in the lipids might decrease slightly. Figures 7 and 8 show that changes in the percentages of prominent fatty acids of NL during incubation were similar to those of TL. For PE, the percentages of 22:6(n-3) decreased rapidly from 37.1 to 27.6% during the first 3 d of incubation. Furthermore, immediately after the end of the induction period of the TL, a drastic decrease in the percentage of 22:6(n-3) was initiated; 22:6(n-3) almost disappeared after about 5 d of incubation. A similar decreasing pattern was observed for 20:5(n-3) in the PE, although to a lesser extent.

Oxidation of TL from sardine white muscle proceeded immediately after the end of an induction period, with accompanying rapid losses of the PUFA. After almost complete disappearance of the PUFA, the oxidation rate of

TABLE 7

Comparisons of Synergistic Effects of *O*-Phosphocholine (PC), *O*-Phosphoethanolamine (PE) and *O*-Phosphoserine with α-Tocopherol on Oxidative Stabilities of Nonpolar Lipids (NL)

Sample[a]	Induction period, days	Synergistic effect[b] B/A
Sardine NL	15.5 (A)	1.00
Sardine NL + *O*-phosphocholine	17.3 (B)	1.14
Sardine NL + *O*-phosphoethanolamine	32.0 (B)	2.06
Sardine NO + *O*-phosphoserine	23.6 (B)	1.52
Trilinolein	19.3 (A)	1.00
Trilinolein + *O*-phosphocholine	21.0 (B)	1.09
Trilinolein + *O*-phosphoethanolamine	28.0 (B)	1.45
Trilinolein + *O*-phosphoserine	24.3 (B)	1.26
Trilinolein + dipalmitoyl PE	24.2 (B)	1.25
Trilinolein + dipalmitoyl PC	20.6 (B)	1.07

[a] α-Tocopherol was added to the samples at 30μg/g before incubation.

[b] Synergistic effect was tentatively defined by B/A.

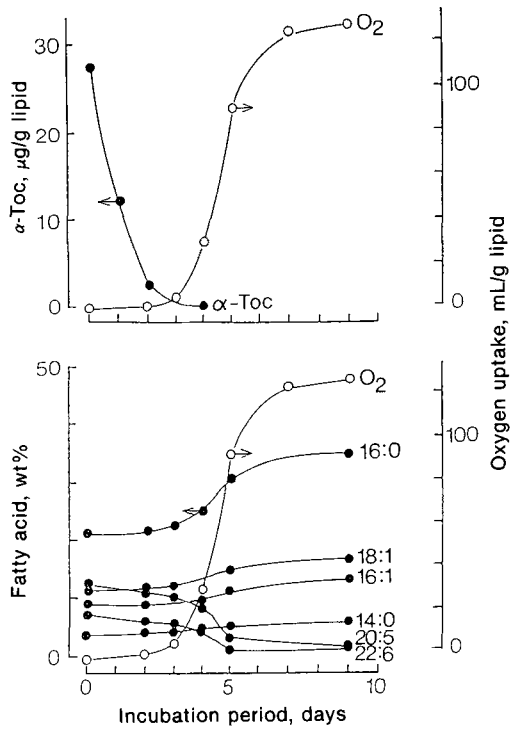

FIG. 7. Changes in added α-tocopherol (α-Toc) contents (top) and fatty acid compositions of sardine total lipids (bottom) during incubation at 37°C.

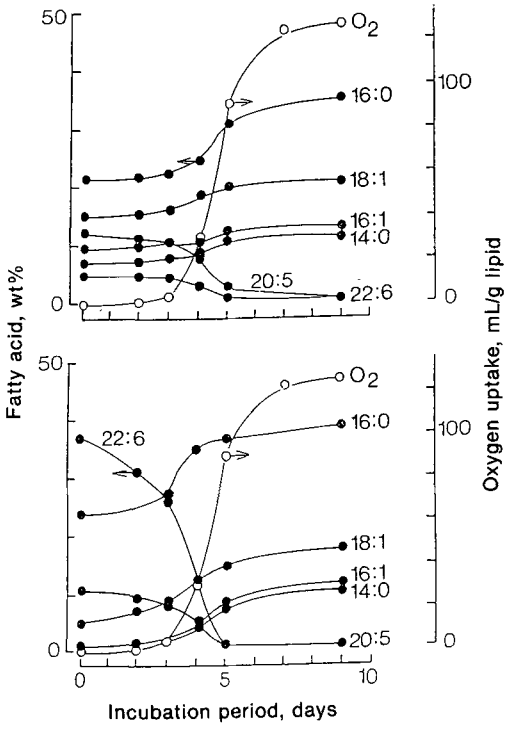

FIG. 8. Changes in fatty acid compositions and added α-tocopherol contents of sardine nonpolar lipids (top) and of sardine phosphatidylethanolamine (bottom) during incubation at 37°C.

the sardine TL gradually became lower. During the slow oxidation of TL, PUFA other than 22:6(n-3) and 20:5(n-3) seemed to be oxidized. After most of the PUFA were exhausted, oxygen absorption of TL almost ceased. Throughout the entire 9-d incubation period, losses of monoenoic acids such as 16:1(n-7) and 18:1(n-9) seemed to be only slight. The PUFA in the TL, especially in the PE, decrease at a relatively higher rate due to oxidation, even within the induction period. When free radicals produced from the PUFA are accumulated to some extent and α-Toc is exhausted, rapid oxidation of TL is initiated. Kashima *et al.* (16) postulated that autoxidation synergism between Toc and phospholipid is closely associated with the effects of phospholipid in suppressing the oxidative decomposition of Toc. However, the results of the present study suggest a selective role of unsaturated fatty acyl chains in NL and PE in autoxidation synergism.

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