for preparing the computer-generated structure.

Registry No. Poly(C), 30811-80-4.

Supplementary Material Available: a description of the computer graphics system used to obtain a refined model of the poly(C) structure, tables of the backbone and base rotation torsion angles and sugar torsion angles in the proposed structure for poly(C) and cylindrical coordinates for a typical residue in poly(C), and figures that show the structure of the monomer unit of 5'-monophosphate cytidine and a stereoview of the left-handed poly(C) structure (6 pages). Ordering information is given on any current masthead page.

Autoxidation of Model Membrane Systems: Cooxidation of Polyunsaturated Lecithins with Steroids, Fatty Acids, and α-Tocopherol

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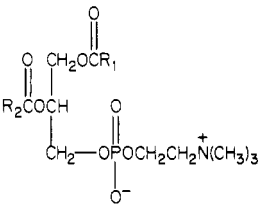
Abstract: The autoxidation of diL-PC and 1S,2A-PC in aqueous emulsion with several cosubstrates was investigated. Cholesterol, 7-dehydrocholesterol, linoleic acid, and α-tocopherol were cosubstrates in the autoxidation of dilinoleoylphosphatidylcholine (diL-PC). The distribution of the products, *tc* and *tt* diene hydroperoxides, was determined and evaluated. It was concluded that cholesterol has a lower H atom donating ability (K_p) and 7-dehydrocholesterol a much higher K_p than diL-PC. Linoleic acid when mixed with diL-PC, diP-PC, or a mixture of the two was found to behave analogous to a mixture of just the two lecithins. The cooxidation of diL-PC with α-tocopherol in the bilayer gave only *trans,cis* (*tc*) hydroperoxides, which can be ascribed to a very high k_{inh} for α-tocopherol, a very efficient antioxidant. 1-Stearoyl-2-arachidonoylphosphatidylcholine (1S,2A-PC) bilayer autoxidation gave a product distribution very similar to arachidonic acid neat autoxidation. However the products from cooxidation of a 1S,2A-PC bilayer with α-tocopherol unexpectedly did not include the 5-hydroperoxy eicosatetraenoic acid isomer (5-HPETE), although the 12, 15, 11, 9, and 8 isomers were present in almost equal amounts.

Lipid peroxidation is an important process leading to a class of compounds that play a central role in a variety of biological events such as inflammation,[1] platelet aggregation,[2] asthma,[3] and anaphylaxis.[4] Under normal conditions, enzymatic oxidation seems to be the predominant process; however, random autoxidation of polyunsaturated fatty acids also appears to be an important process[5] in vivo as evidenced by the expiration of pentane and ethane, known fatty acid oxidation products, by organisms under free-radical stress. Membrane damage induced by radiation[6] and carbon tetrachloride or ethanol poisoning[7-9] has been proposed to be the result of phospholipid destruction by molecular oxygen. In fact, a theory of aging has been proposed that rests in part on the free-radical oxidation of membrane lipid.[10] Reincorporation of an oxidized fatty acid formed enzymatically into human neutrophil membrane phosphatidylcholines (PC) in vivo suggests a biological role for PC oxidation products.[11] Lipid oxidation, both enzymatic and nonenzymatic, is also an important factor in the oxidative deterioration of food products,[12] which has been suggested to produce carcinogens.[13] A physiological role for the plant lipoxygenase pathway has not yet been established.

In light of the potential importance of phospholipid oxidation products, we have studied the autoxidation of the polyunsaturated lecithins **1** and **2** in the presence of some natural membrane



1, $R_1 = R_1$ = linoleate; diL-PC
2, R_1 = stearate, R_2 = arachidonate; 1S,2A-PC
3, $R_1 = R_2$ = palmitate; diP-PC

constituents such as steroids, fatty acids, saturated lecithin **3**, and α-tocopherol. We recently reported on the isolation and analysis of PC hydroperoxides[14] and on the factors controlling the stereochemistry of hydroperoxides produced by autoxidation of linoleic acid.[15] The proposed mechanism of *trans,cis* (*tc*) and *trans,trans* (*tt*) hydroperoxide formation is outlined in Scheme I.

Thus 13-peroxy-*trans,cis*-dienyl (13-*tc*) radical (conformers **5a** + **5b**) can lead to the 13-*tc* hydroperoxide **6** or (via conformer **5b**) can undergo β scission and eventually lead to the 9-*tt* hydroperoxide **8**. Similarly, a 9-*tc* peroxy radical can give the 9-*tc* hydroperoxide or (via conformer **5b**) can be converted to the 13-*tt* hydroperoxide. Analysis of the mechanism shown in Scheme I by using steady-state assumptions leads to eq 1, where [L-H] is

$$\frac{tc}{tt} = \frac{k_p[\text{L-H}]}{k_\beta(1-\alpha)} + \frac{\alpha}{1-\alpha} \qquad (1)$$

the concentration of linoleic acid, k_p is the rate constant for propagation, k_β is the rate constant for β fragmentation of peroxy radicals, and α and $(1-\alpha)$ refer to the partitioning of carbon radicals between the *tc* peroxy radical **5b** and the *tt* peroxy radical

(1) Kuehl, F. A. *Science (Washington, D.C)* **1980**, *210*, 978.
(2) Salomon, J. A.; Smith, D. R.; Flower, R. J.; Moncada, S.; Vane, J. R. *Biochim. Biophys. Acta* **1978**, *523*, 250.
(3) Parker, C. W. *Allergy Clin. Immunol.* **1979**, *63*, 1.
(4) Corey, E. J.; Barton, A. E.; Clark, D. A. *J. Am. Chem. Soc.* **1980**, *102*, 4278.
(5) Tappel, A. L.; Dillard, C. J. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1981**, *40*, 174.
(6) Wills, E. D. *Int. J. Radiat. Biol Relat. Stud. Phys., Chem. Med.* **1970**, *17*, 217.
(7) Weddle, C. C.; Hornbrook, K. K.; McKay, P. B. *J. Biol. Chem.* **1976**, *251*, 2973.
(8) Recknagel, R. O. *Pharmacol. Rev.* **1967**, *19*, 145.
(9) Litov, R. E.; Irving, D. H.; Downey, J. E.; Tappel, A. L. *Lipids* **1978**, *13*, 305.
(10) Tappel, A. L. *Geriatrics* **1968**, *23*, 97.
(11) (a) Stensen, W. F.; Parker, C. W. *J. Immunol.* **1980**, *124*, 2100. (b) Stenson, W. F.; and Parker, C. W. *Prostaglandins* **1979**, *18*, 285. (c) Bonser, R. W.; Siegel, M. I.; Chung, S. M.; McConnell, R. T.; Cuatrecasas, personal communication.
(12) Sessa, D. J. *J. Agric. Food Chem.* **1979**, *27*, 2.
(13) Shamberger, R. J.; Anderone, T. L.; Willis, C. E. *J. Natl. Cancer Inst.* **1974**, *53*, 1771.
(14) Porter, N. A.; Wolf, R. A.; Weenen, H. *Lipids*, **1980**, *15*, 163.
(15) Porter, N. A.; Weber, B. A.; Weenen, H.; Khan, J. A. *J. Am. Chem. Soc.* **1980**, *102*, 5597.

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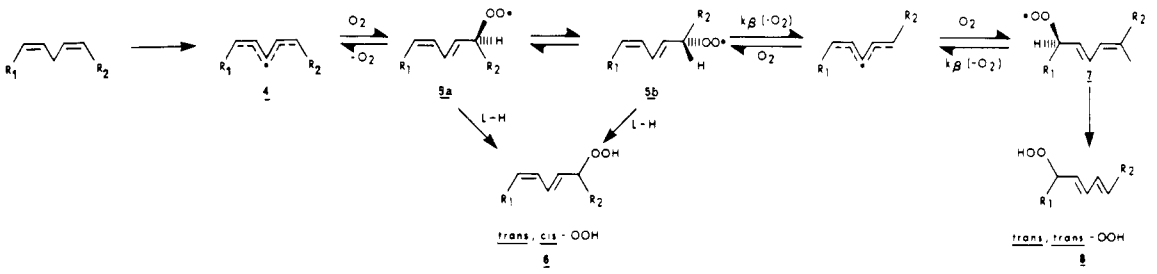
Scheme I



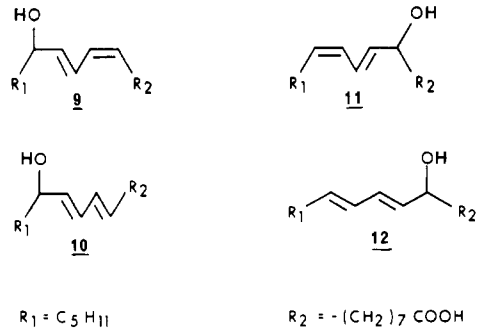
7. At a given temperature, the *tc/tt* ratio of the hydroperoxide formed in the autoxidation of linoleic acid (L-H) was found to be directly related to [L-H]. Also, in an aqueous mixed lecithin emulsion (diL-PC + diP-PC), the *tc/tt* ratio was found to be directly related to [diL-PC]/[DiP-PC + diL-PC], which is effectively the diL-PC concentration in the bilayer. Furthermore, in linoleic acid cooxidations with cosubstrates with high k_p, the *tc/tt* ratios were consistent with the proposed mechanism.[15,16] We herein report the effect of steroids, fatty acids, and α-tocopherol on the product distribution of the PC autoxidation.

We recently proposed a kinetic scheme for the oxidation of arachidonic acid[16] that is similar to that proposed for autoxidation of linoleate. Polyunsaturated fatty acids that contain three or more double bonds can undergo an additional competitive process, peroxy radical cyclization, that must be considered in the mechanism. Two of these processes, β scission and cyclization, are unimolecular events, while the third, H atom transfer, is critically dependent on the H donating ability of the medium (KP).[17] Thus, in a neat arachidonic acid autoxidation (KP $\approx$ 530 s^{-1}) cyclization and *tt* product formation compete with hydroperoxy eicosatetraenoate (HPETE) formation. The 15-hydroperoxy and 5-hydroperoxy compounds are the predominantly formed HPETE's, since they cannot undergo cyclization. At very high KP however, such as in the case of arachidonic acid in 97% cyclohexadiene (KP $\approx$ 3400 s^{-1}), the *tc*-HPETE's are the main products formed, while cyclization and *tt* product formation are very minor pathways. The 15- and 5-HPETE in this case were still predominant products, but the difference between these isomers and the others was much smaller than in the neat arachidonic acid oxidations.

We also report on the bilayer oxidation of arachidonic acid. Thus 1S,2A-PC and a mixture of 1S,2A-PC and α-tocopherol were autoxidized in aqueous emulsion, and the HPETE's formed were analyzed.

Results

Autoxidations of DiL-PC with DiP-PC, Cholesterol, and 7-Dehydrocholesterol. DiL-PC was oxidized in the presence of either diP-PC, cholesterol, or 7-dehydrocholesterol (7-DHC) at 37 °C as a homogeneous emulsion in 50 mM aqueous KCl. Products of the autoxidation were reduced with triphenylphosphine and hydrolyzed with phospholipase A2 (*Crotalus adamanteus*, snake venom) affording the hydroxy fatty acids **9–12**,[14] which were analyzed by normal-phase HPLC.



$R_1 = C_5H_{11}$ $R_2 = -(CH_2)_7COOH$

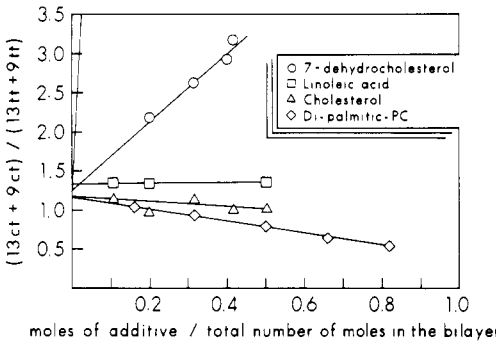


Figure 1. Product distribution of diL-PC hydroperoxides formed in bilayer cooxidations.

The oxidized phospholipid could also be hydrolyzed by base to give **9–12**. Transmethylation of the phospholipid with KOH/CH_3OH alternatively afforded the methyl esters of **9–12**, which could also be analyzed by HPLC. In emulsion oxidations of phospholipid in 50 mM KCl, essentially no difference in product distribution was observed in reactions worked up by the different methods described here. Oxidations were carried out to about 10% conversion as measured by UV absorption at 234 nm, corresponding to the diene chromophor. The ratio of C-9- to C-13-substituted products was essentially 1:1 in all cases. The (13-*tc* + 9-*tc*)/(13-*tt* + 9-*tt*) ratio, however, was substantially dependent on the nature and concentration of the medium as shown in Figure 1. Addition of not only diP-PC,[15] a completely saturated lecithin, but also cholesterol to the bilayer favors the formation of *tt* products.[17] Provitamin D (7-DHC), however, strongly enhances the formation of *tc* hydroperoxides. Thus a *tc/tt* ratio as high as 3.2 was found when a mixture of diL-PC + 7-DHC was oxidized for 19 h (5% diene formation) at 37 °C in 50 mM KCl.

Cooxidations of Lecithins and Linoleic Acid. Several physical studies (DSC, NMR, diffusion, permeability)[18-21] indicate that fatty acids can be incorporated into the phosphatidylcholine bilayer without disrupting its structure. Mabrey and Sturtevant[18] conclude from DSC experiments that only fatty acids with the same chain length give optimum incorporation into the bilayer. Although we could not measure a transition temperature for diL-PC over a temperature range of +10 to −60 °C, we did measure a transition of a 1:1 diL-PC + linoleic acid mixture at −27 °C. This mixture did not show a melting point for linoleic acid, as was observed for aqueous linoleic acid alone. Microscope studies (Nikon, 25–1000×, polarizers, phase contrast) showed no differences in this mixture when compared with aqueous diL-PC. To further investigate lecithin/fatty acid systems, we studied the cooxidation of linoleic acid and various lecithins in aqueous emulsion. Thus lecithin–linoleic acid mixtures were emulsified with a Vortex mixer, and in all cases reported here, a homogeneous emulsion was formed. A pH 7.0 phosphate buffer was used instead of aqueous KCl in analogy with Mabrey and Sturtevant's DSC experiments.

(16) Porter, N. A.; Lehman, L. S.; Weber, B. A.; Smith, K. J. *J. Am. Chem. Soc.* **1981**, *103*, 6447.
(17) Cholesterol is known to be incorporated in phospholipid bilayers.
(18) Mabrey, S.; Sturtevant, J. M. *Biochim. Biophys. Acta* **1977**, *486*, 444.
(19) Podo, F.; Blasie, J. K. *Biochim. Biophys. Acta* **1976**, *419*, 1.
(20) Jain, M. K.; White, H. B. *Adv. Lipid Res.* **1977**, *15*, 1.
(21) Koupanova, Y. A.; Antonov, V. F.; Vladimurov, Y. A. *Biofizika* **1975**, *20*, 740.

Table I. *tc/tt* Ratio of DiL-PC, DiP-PC, and L-H Cooxidation in pH 7.0 Buffer

lipids oxidized			*tc/tt* ratio[a]	
diP-PC	diL-PC	L-H	diL-PC[b]	L-H[c]
	8	1	1.4	1.4
	4	1	1.5	1.5
	1	1	1.4	1.4
4	4	1	0.9	0.9
1	1	1	0.9	0.9
1		1	0.9	

[a] Accuracy of reported numbers is approximately ±8%, on the basis of independently reproduced results in these and other experiments. [b] Ratio of products formed from linoleates attached to lecithin. [c] Ratio of products formed from free linoleic acid.

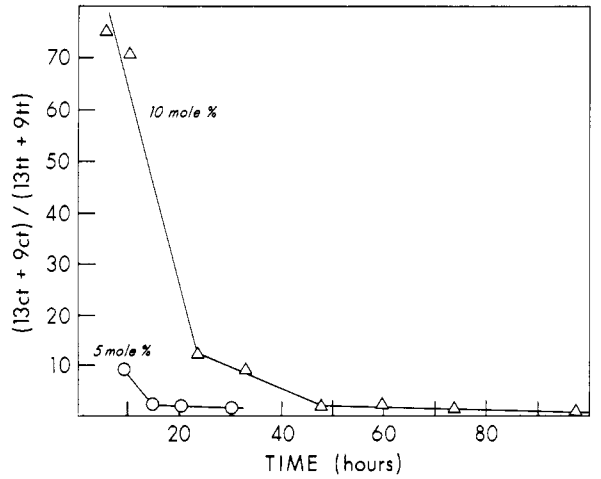


Figure 2. UV absorption at 234 nm as a function of time. 20-μL, 15-mM lecithin samples were diluted with 3.0 mL of EtOH adn their UV spectra recorded. *tc/tt* ratio of hydroperoxids formed in diL-PC + α-tocopherol cooxidation vs. time.

Table I presents *tc/tt* ratios of both L-H and diL-PC fractions of cooxidations of lecithins with linoleic acid. The fatty acids were separated from the lecithins by column chromatography (silica gel). The *tc/tt* ratio of the lecithins represents the 9 *tc/tt* ratio since the 13 *tt* percentage is unreliable when phospholiphase A-2 is used in combination with pH 7.0 phosphate buffer (unpublished results).

Cooxidations of Lecithins and α-Tocopherol. DiL-PC (15 mM) was cooxidized at 37 °C with α-tocopoherol (5 and 10 mol %) as a homogeneous emulsion. Doubly distilled water was used instead of aqueous KCl since the latter solvent was found to initiate autoxidation at a relatively fast rate, presumably due to impurities in the salt. The initial oxidation was much slower in doubly distilled water, and the reaction was therefore easier to monitor. Microscope studies did not show any difference between aqueous KCl emulsions and the same lecithin in doubly distilled water. Autoxidation was self-initiated and generation of conjugated diene products was followed over time by UV absorption at 234 nm.

At different time intervals, the lecithin hydroperoxides were converted to the corresponding methyl hydroxylinoleates,[22] and their product distribution was determined by HPLC. The unprecedented high *tc/tt* ratio of the products in the early stages of the cooxidation shows clearly the extremely efficient H-donating ability of α-tocopherol in the bilayer (Table II, Figure 2).

We note that the 13 *tc/tt* ratio does not significantly differ from that 9 *tc/tt* ratio, and thus the H-donating ability of α-tocopherol to the 9- and 13-peroxy radical must be essentially the same. The α-tocopherol/lecithin ratios were chosen this high for practical purposes (vide infra); however, high *tc/tt* ratios (2.5) could be detected at concentrations of α-tocopherol as low as 0.3%.

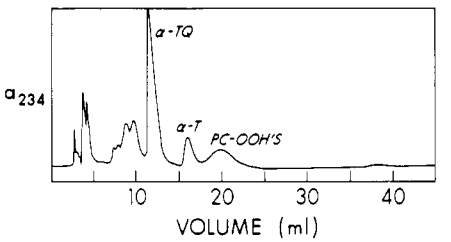


Figure 3. RP-HPLC trace of 1S,2APC + α-tocopherol cooxidation after 12 h, 234 nm.

Extreme care must be taken in experiments with added α-tocopherol to ensure that the starting lecithin is unoxidized. DiL-PC is oxidized extremely rapidly, and in these experiments the lecithin was purified by HPLC and collected directly into methanolic solutions of α-tocopherol followed by solvent removal in vacuo. This ensures that the starting lecithin is not preoxidized before it is mixed with α-tocopherol. High proportions of α-tocopherol relative to diL-PC allowed significant quantities of lecithin to be oxidized while α-tocopherol was present and made for easier analysis of products.

1S,2A-PC Autoxidation. 1S,2A-PC (15 mM) was autoxidized at 37 °C as a homogeneous emulsion in aqueous KCl (50 mM). Autoxidation was self-initiated and stopped after 5 h (5% diene formation, by UV absorbance at 234 nm). Shown in Figure 4a is the HPLC trace of the methyl hydroxyarachidonates (Me-HETE) obtained from 1S,2A-PC autoxidation. The position of hydroxy substitution on the 20-carbon chain is indicated in the figure. Each of the major products were found to have trans,cis stereochemistry for the conjugated diene. The 9- and 8-hydroxy-substituted isomers are not readily separated by HPLC as methyl esters.

1S,2A-PC + α-Tocopherol Cooxidation. 1S,2A-PC (15 mM) was cooxidized at 37 °C with α-tocopherol (25 mol %) as a homogeneous emulsion in doubly distilled water. Autoxidation was self-initiated and the reaction followed by reverse-phae high-pressure liquid chromatography (RP-HPLC, Figure 3). Under the conditions of the chromatography (95% MeOH, 2 mL/min), 1S,2A-PC elutes at 38 mL, the oxidized PC fraction at 19 mL, α-tocopherol at 16 mL, and α-tocopherolquinone at 12 mL.

When α-tocopherol was almost completely consumed, all fractions were analyzed by UV and the fraction with a UV absorption maximum at 234 nm collected, reduced and transmethylated. The HPLC trace of the product mixture obtained in this way is shown in Figure 4b.

One would expect all the isomers to be present in about equal amounts due to the extremely high H-donating ability (k_{inh}) of α-tocopherol, as reported in this paper for the cooxidation of diL-PC and α-tocopherol.[16] Figure 4b shows that this is essentially the case for the 12, 15, 11, 9, and 8 isomers; however 5-HETE methyl ester, which corresponds to the 5-hydroperoxy lecithin before workup, is surprisingly absent. In three independent cooxidations, the same distribution was found at the initial stage of the oxidation. Upon checking our methods we have not been able to find an error in methodology. Looking for a possible explanation, we worked up a crude cooxidation mixture directly before RP-HPLC purification. The normal-phase chromatogram obtained in this way again showed the absence of the 5-hydroxy isomer. We also found that the 5-O-lactone and the 5-keto derivative, both of which we independently synthesized,[23,24] had not been formed.

The largest peak in Figure 3 was found to be α-tocopherolquinone. It appears to be the main α-tocopherol oxidation product on the basis of refractive index detection. Other α-tocopherol

(22) Crawford, C. G.; Plattner, R. D.; Sessa, D. J.; Rackis, J. J. *Lipids* **1980**, *15*, 91.

(23) Corey, E. J.; Albright, J. O.; Barton, A. E.; Hashimoto, S. *J. Am. Chem. Soc.* **1980**, *102*, 1435.

(24) 5-HPETE was reacted with pyridine and a catalytic amount of acetyl chloride for 10 min at 0 °C.

Table II. Product Distribution of DiL-PC + α-Tocopherol (10 mol %) Cooxidation

time, h	9	10	11	12	*tc/tt*
5.5	50	0.6	49	0.7	74
10	51	0.7	48	0.7	70
24	45	3.7	48	3.7	13
33	33	16	45	4.9	9
48	33	15	35	16	2.1
60	35	17	32	18	1.8
98	28	20	29	23	1.3

products were not characterized but will be investigated subsequently.

Discussion

Autoxidation of DiL-PC with DiP-PC, Cholesterol, and 7-Dehydrocholesterol. As we reported earlier,[15] the *tc/tt* ratio of hydroperoxides formed in bilayer oxidations is dependent on the H-donating ability of the bilayer constituents. Thus, saturated diP-PC with a very low H-donating ability effectively lowers the concentration of diL-PC in the bilayer when the two are mixed in aqueous emulsion. The *tc/tt* ratio of the hydroperoxides formed in a mixture of the two lecithins decreases with increasing diP-PC/diL-PC ratio. A linear correlation was found when the *tc/tt* ratio was plotted against the diL-PC concentration in the bilayer. In the work described here, we report on a similar though less dramatic effect when cholesterol is added to diL-PC in aqueous emulsion (see Figure 1). However, the opposite effect was found when 7-DHC was cooxidized with diL-PC. A *tc/tt* ratio as high as 3.2 was found when 40 mol % of this steroid was present in the bilayer. We therefore conclude that 7-DHC is a much better H donor than diL-PC under the conditions described here. 7-DHC is known to be readily incorporated into phospholipid bilayers,[25] and the two tertiary axial hydrogens at C-9 and C-14 of the steroid nucleus would be easily abstracted.

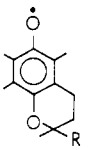
Cooxidation of Lecithins and Fatty Acids. It has been reported that palmitic acid is incorporated into a diP-PC bilayer without disrupting the liquid crystalline structure.[18] It was suggested that the bulkiness of the PC head group makes the incorporation of a fatty acid with equal chain length favorable, as suggested by a higher transition temperature for the mixture as compared to the PC above. Our results indicate that diL-PC + linoleic acid mixtures behave similarly. Thus the transition temperature of 1:1 dL-PC + L-H was found to be higher (−28 °C) than that for diL-PC alone (<−60 °C). Moreover, cooxidations of mixtures of diL-PC, diP-PC, and L-H show a very similar product distribution, as do diL-PC + diP-PC oxidations. In all cases the *tc/tt* ratio for the hydroperoxides formed from incorporated linoleic acid was essentially the same as the *tc/tt* ratio for the hydroperoxides formed from diL-PC alone. Furthermore, dilution with diP-PC has the same effect when mixed with L-H, L-H + diL-PC, or DiL-PC alone. Somewhat higher *tc/tt* ratios of the diL-PC + L-H cooxidations might be expected from the closer fatty acid chain packing and therefore more efficient H atom transfer.

Interestingly, a mixture of diP-PC and L-H appears to form a bilayer liquid crystalline structure, as suggested by its homogenous appearance macroscopically and microscopically and by its oxidation product distribution; however, neither diP-PC nor L-H forms a homogeneous emulsion alone at 37 °C.

Cooxidation of DiL-PC with α-Tocopherol. The autoxidation of polyunsaturated fatty acids has been shown to give a different product distribution depending upon the total H-donating ability, KP, of the medium.[16] Thus, linoleic acid cooxidation resulted in *tc/tt* ratios varying from 0.24 to 27.3 depending on the concentration and the cosubstrate present. Similarly, we now have found that in bilayer cooxidations, the product distribution can be varied from predominantly *tt* hydroperoxide formation (with diP-PC added) to almost exclusively *tc* product formation when α-tocopherol is added. It has been demonstrated that α-tocopherol readily incorporates into phospholipid bilayers,[26] and our data suggest it acts as an extremely good H atom donor in the bilayer. This observation is in agreement with the very high k_{inh} found for the antioxidant effect of α-tocopherol.[27] Because of the large difference between the k_p's of diL-PC and α-tocopherol, our data do not allow an accurate estimation of k_{inh} for α-tocopherol, since α-tocopherol will oxidize much faster than diL-PC, and therefore the α-tocopherol concentration during the reaction is unknown. Interestingly, in the highly ordered bilayer structure in which α-tocopherol can be expected to be located with its polar phenolic OH in the hydrophilic region of the bilayer and with its apolar tail in the hydrophobic region, there seems to be no difference between 13-linoleoyl peroxy radical–α-tocopherol and 9-linoleoyl peroxy radical–α-tocopherol interaction, as demonstrated by the 13 *tc/tt* and 9 *tc/tt* ratios, which are virtually the same. If peroxy radicals are polar enough to diffuse to the hydrophilic surface region of the bilayer where the active site of α-tocopherol is present, as suggested by Barclay and Ingold,[28] one might expect a difference between 13- and 9-peroxy radicals in the rate at which they diffuse to the surface region for reasons of conformational preference and steric hindrance. None of our diL-PC + α-tocopherol cooxidations showed this effect.

We suggest that the primary factor that influences product distribution in bilayer oxidations of diL-PC is the H-donating ability of the bilayer constituents. It should be noted that steroids, fatty acids, and α-tocopherol alter considerably the surface properties of lecithins. It is quite possible that the drastic changes in surface viscosity induced by other constituents in the bilayer could influence the partitioning between radicals **5** and **7** and thus influence the distribution of products. The dramatic differences in product distribution of linoleate oxidation observed with added cholesterol and α-tocopherol suggest, however, that the dominant factor controlling products is H-donating ability.

Cooxidation of 1S,2A-PC with α-Tocopherol. The distribution of products obtained in the autoxidation of arachidonic acid has been shown to be dependent on the H atom donating ability (KP) of the medium of oxidation.[16] Three competing processes determine the distribution of products obtained in arachidonic acid autoxidation. These processes include hydrogen atom abstraction,



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peroxy radical β scission, and peroxy radical cyclization. At low KP cyclization dominates, and little HPETE products are formed from peroxy radicals capable of undergoing cyclization. Thus, peroxy radicals substituted at positions 8, 9, 11, and 12 of the C-20 chain are homoallylic and therefore can cyclize if a good H atom donor is not present. The 5- and 15-substituted peroxy radicals, however, cannot cyclize since they are not homoallylic. Thus, at low KP, relatively more of these isomers are observed in comparison to the 8, 9, 11, and 12 isomers. This distribution of products, observed previously in bulk-phase fatty acid autoxidation,[29,30] is now also observed in bilayer oxidations of arachidonic acid. Compare, for example, the HPLC trace of 1S,2A-PC oxidation in Figure 4 shown with and without α-tocopherol present. As shown in Figure 4a, relatively little 8-, 9-, 11-, and 12-HPETE products are observed in oxidation of 1S,2A-PC with no added α-tocopherol. Cyclization of peroxy

(25) Hsia, J. C.; Long, R. A.; Hruska, F. E.; Gesser, H. D. *Biochem. Biophys. Acta* **1972**, *290*, 22.

(26) (a) Lucy, J. A. "Tocopherol, Oxygen, and Biomembranes"; de Duve, C., Hayaishi, O., Eds.; Biomedical press: Elsevier North Holland, Amsterdam, 1978; p 109. (b) Fukuzawa, K.; Hirohito, I.; Tokumura, A.; Tsukatami, H. *Chem. Phys. Lipids* **1979**, *23*, 13.

(27) Burton, G. W.; Le Page, Y.; Gabe, E. J.; Ingold, K. U. *J. Am. Chem. Soc.* **1980**, *102*, 7792.

(28) Barclay, L. R. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1980**, *102*, 7794.

(29) Porter, N. A.; Wolf, R. A.; Yarbro, E. M.; Weenen, H. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 1058.

(30) Terao, J.; Matsushita, S. *Agric. Biol. Chem.* **1981**, *45*, 595.

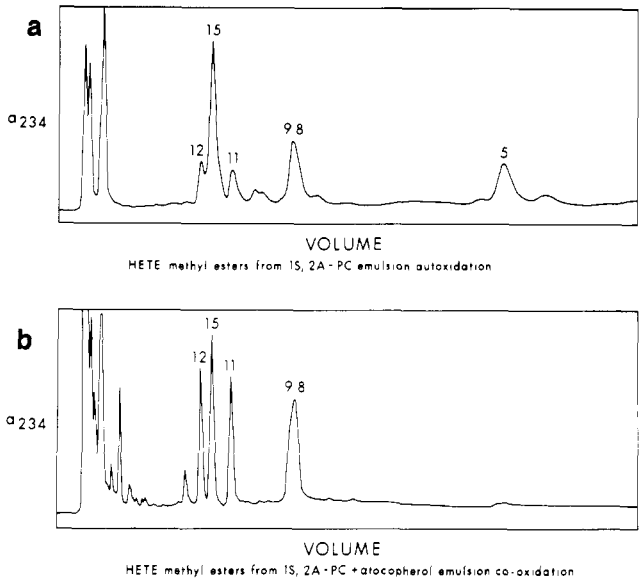
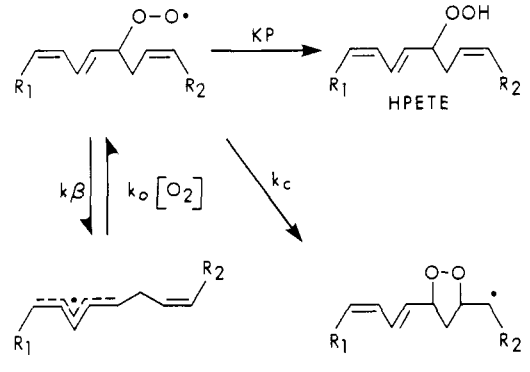


Figure 4. (a) HPLC trace of HETE methyl esters from autoxidation of 1S,2A-PC after 5 h. (b) HPLC trace of HETE methyl esters from cooxidation of 1S,2A-PC and α-tocopherol (25 mol %) after 16 h.

radicals that would otherwise lead to these products accounts for the fact that little of the acyclic hydroperoxides are formed. Most of the oxidation products of 1S,2A-PC in bilayer alone are due to cyclization of intermediate 8-, 9-, 11-, and 12-peroxy radicals. Only the 5- and 15-substituted-HPETE products that have no cyclization pathway available are formed in substantial amounts.

If the 1S,2A-PC emulsion oxidation is carried out with added α-tocopherol, a dramatic change in product distribution is observed from that noted in oxidations with no added tocopherol. First, the amounts of 8-, 9-, 11-, and 12-HPETE products are formed in amounts relatively equivalent to the 15- and 5-HPETE products. Figure 4b shows that indeed the 12-, 15-, 11-, 9- and 8-HPETE products are formed in almost equal amounts (12, 19%; 15, 26%; 11, 21%; 9 + 8, 35%). This patter fits the notion that α-tocopherol acts as a good H atom donor in the bilayer and traps the 8-, 9-, 11-, and 12-peroxy radicals before cyclization can occur. A second change in product distribution of 1S,2A-PC emulsion oxidation with added α-tocopherol is the virtual absence of 5-HPETE products. We have looked for a trivial explanation for this observation but have found none. For example, we reasoned that perhaps the 5-keto or 5-lactone products are somehow generated during oxidation or workup. Neither of these products was formed in the oxidation, however, as judged by HPLC comparison of the product mixture with authentic samples. Appropriate control experiments suggest that the lack of 5-substituted products in the α-tocopherol experiments was not due to its loss in the workup (see Experimental Section).

One possible explanation for the absence of 5-substituted arachidonic acid products in bilayer oxidations with added α-tocopherol is the selective trapping of arachidonyl peroxy radicals



by the tocopherol phenoxy radical. The α-tocopherol radical **13** formed by H atom donation would be expected to reside in the bilayer with the polar phenoxy oxygen near the hydrophilic head groups of the phospholipid. We suggest that the 5-substituted peroxy radical might be preferentially scavenged by **13** due to proximity of the radical (closest to the head group of any of the radicals generated) and **13**. Products would thus be diverted from the system presumably by preferential scavenging of the 5-substituted peroxy radical, presumably by addition at the para position to the phenoxy oxygen.

The lack of 5-substituted products with added α-tocopherol is of biological interest. The important class of compounds, the leukotrienes, are derived from 5-HPETE, and it has recently been reported that lecithins containing 5-hydroxy-substituted arachidonate esters are generated enzymatically. The specific inhibition of formation of 5-HPETE products by α-tocopherol in the membrane oxidations studied here may thus have some biological significance.

Experimental Section

Fatty acids were obtained from NuChekPrep (Elysian, MN), cholesterol, 7-dihydrocholesterol, and phospholipase A-2 from Sigma, and glycerophosphorylcholine–$CdCl_2$ complex and 1-stearoyllysolecithin from Serdary Labs (Canada) and were used without further purification.

RP High-Pressure Liquid Chromatography. A Waters 10 μBondak C_{18} column was used for reverse-phase chromatography (RP-HPLC) of the lecithins. The solvent used was 95:5 methanol/water. Detection was by UV absorption at 206 nm or refractive index (RI) for lecithins and by UV absorption at 234 nm for more sensitive analysis of the oxidized lecithins. In all cases, lecithins were purified immediately before starting the oxidations. Extreme care was taken to prevent oxidation during RP-HPLC purification by keeping the collected fractions cold (0 °C) and oxygen free (argon). DiL-PC and 1S,2A-PC treated this way showed no detectable peroxidation by RP-HPLC, determined by UV at 234 nm. The cooxidation of 1S,2A-PC and α-tocopherol was followed by RP-HPLC over a period of time, and when less than 20% of the original α-tocopherol was left unoxidized, samples were taken and the RP-HPLC fraction with a UV maximum at 234 nm collected and transmethylated to determine product distribution. All other cooxidation mixtures could be analyzed without the RP-HPLC isolation of the hydroperoxide fraction and were hydrolyzed or transmethylated after reduction of the unpurified reaction mixture. Appropriate control experiments assured us that this RP-HPLC purification did not cause the loss of the 5-substituted product in α-tocopherol cooxidations. RP-HPLC of 1S,2A-PC autoxidations carried out without added α-tocopherol led to normal product distribution, thus indicating that 5-HPETE products were not lost in the workup of the α-tocopherol reactions.

Analysis of the Lecithin Hydroperoxides. The product hydroperoxides were dried in vacuo at ambient temperature, reduced with triphenylphosphine, and hydrolyzed with phospholipase A_2.[14] The α-tocopherol cooxidations and the 1S,2A-PC autoxidation, however, were reduced with $NaBH_4$ in anhydrous methanol and transmethylated with KOH/MeOH.[22] The two methods are essentially equivalent, and only for practical reasons was one preferred over the other in certain cases. Thus all unpurified diL-PC autoxidations gave clean HPLC traces of the methyllinoleate hydroxides. The cooxidation products of 1S,2A-PC with α-tocopherol however had to be purified by RP-HPLC (vide infra). The reduced fatty acid + lecithin cooxidation mixtures were separated either by silica gel column chromatography or by extraction (MeOH + H_2O/hexane + ether) to give a fatty acid fraction and a lecithin fraction, which was then hydrolyzed. Both were characterized by HPLC.

HPLC Characterization of the Fatty Acid Hydroxides. A Whatman 5 μPartisil analytical silica column was used for analysis of the fatty acid hydroxides. The solvent used was 995:5 hexane/2-propanol for the hydroxy methyl esters and 989:10:1 hexane/2-propanol/acetic acid for the hydroxy fatty acids. Detection was by UV absorption at 234 nm. The product distribution was calculated on the basis of ε's for the individual methylhydroxylinoleates.[31] The ε's for the hydroxy linoleates were found to be essentially the same as the methyl esters.

Tocopherolquinone. The fraction containing α-tocopherolquinone (Figure 3) was found to be peroxide negative and had a faint yellow appearance as well as UV absorbance maxima at 262 (shoulder) and 268 nm. ^1H NMR spectroscopy was performed on a Bruker 250 MHz spectrometer; (CHCl) δ 3.33 (br s), 2.55 (m), 2.04 (s), 2.00 (s), 1.0–1.8 (complex mixture of multiplets), 0.86 (t).

Registry No. 1, 6542-05-8; **2**, 18892-74-5; **3**, 2644-64-6; (*E,Z*)-**5**,

(31) Chan, H. W. S.; Levett, G. *Lipids* **1977**, *12*, 99.

82707-37-7; (*E*,*Z*)-**6**, 23017-93-8; (*E*,*E*)-**6**, 63121-48-2; (*E*,*E*)-**7**, 82707-38-8; (*E*,*E*)-**8**, 63121-49-3; (*E*,*Z*)-**9**, 82707-39-9; (*E*,*Z*)-**10**, 5502-91-0; (*E*,*Z*,*Z*,*Z*)-5-HPETE, 70968-82-0; (*E*,*Z*,*Z*,*Z*)-8-HPETE, 70968-80-8; (*E*,*Z*,*Z*,*Z*)-9-HPETE, 70968-79-5; (ALL, *Z*)-10-HPETE, 82707-40-2; (*E*,*Z*,*Z*,*Z*)-11-HPETE, 70968-78-4; (*E*,*Z*,*Z*,*Z*)-15-HPETE, 69371-38-6; (*E*,*Z*,*Z*,*Z*)-12-HPETE, 71030-35-8; 7-DHC, 434-16-2; cholesterol, 57-88-5; α-tocopherol, 59-02-9; α-tocopherolquinone, 7559-04-8.

Carboxypeptidase A Catalyzed Hydrolysis of Thiopeptide and Thionester Analogues of Specific Substrates. An Effect on k_{cat} for Peptide, but Not Ester, Substrates

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Abstract: Carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.17.1) catalyzes the hydrolysis of *N*-(*N*-hippuryl-thioglycyl)-3-phenyl-L-alanine, *O*-(*N*-hippurylglycyl)-3-phenyl-L-lactic acid, and *O*-(*N*-hippurylthioglycyl)-3-phenyl-L-lactic acid, respectively the thiopeptide, ester, and thionester analogues of its specific tripeptide substrate *N*-(*N*-hippurylglycyl)-3-phenyl-L-alanine. Both k_{cat} and k_{cat}/K_m are equal for the ester and thionester substrates, reflecting the equal nonenzymic reactivities for these two compounds. However, k_{cat}/K_m for the thiopeptide is only 0.0009 as large as that for its peptide counterpart. This difference, which cannot be due to any inherent reactivity differences between amides and thioamides, lies in k_{cat}, since thiopeptide and peptide bind equally well. The controlling pK_a's of the k_{cat}– and k_{cat}/K_m–pH profiles match those previously observed for specific ester and peptide substrates. Since rotation about the thioamide bond is about 3 kcal mol^{-1} more difficult than rotation about a peptide bond, these data support a mechanism involving rate-determining bond rotation in peptidase, but not esterase, activity.

Carboxypeptidase A (CPA, peptidyl-L-amino-acid hydrolase, EC 3.4.17.1), a zinc-containing digestive protease, catalyzes the hydrolysis of peptide bonds adjacent to the C-terminal residue of a peptide chain.[1-3] X-ray determination of the structure of CPA and several of its complexes[1,4-7] has not sufficed to define clearly its mechanism of action, even when the myriad of kinetic data[2,8-27] on a variety of substrates is considered.

Of particular interest is the divergence of behavior of CPA toward peptide and ester substrates. The following differences have been noted. (1) For a peptide substrate, replacement of the essential zinc by other metals results in a change in k_{cat} while K_m remains unchanged. For an ester substrate, this behavior is reversed: k_{cat} is invariant as K_m changes.[13] (2) Inhibitors such as phenylpropionate, which presumably mimic a hydrophobic side chain of the C-terminal amino acid of a CPA substrate, are competitive against ester substrates, noncompetitive against peptides.[13] (3) The integrity of Tyr-248 is required for peptidase activity, but not for esterase activity.[1,18]

Cleland has attempted to account for all of the available data with a single mechanism, which differs for peptide and ester substrates only in the identity of the rate-determining step.[28] In this mechanism, the first enzyme–substrate complex (collision complex) is "strain free": a salt bridge is formed between the terminal carboxylate of the substrate and Arg-145, but the side chain of the terminal residue does not fill the enzyme's specificity pocket, nor does the carbonyl group adjacent to the scissile bond interact with the metal. Conversion to the precatalytic complex involves formation of these two latter interactions, which provide the energy for a rotation about the scisssile bond, straining it away from its preferred planar conformation into a less stable geometry that more closely resembles the transition state for the nucleophilic attack (eq 1).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES_2 \overset{k_5}{\rightarrow} E + P \qquad (1)$$

ES_1, collision complex; ES_2, precatalytic complex

For peptide substrates, rotation about the peptide bond, with its double-bond character, is difficult, so that k_3 is the rate-deter-

† Philadelphia College of Pharmacy and Science.
‡ New York University.

(1) Hartsuck, J. A.; Lipscomb, W. N. *Enzymes, 3rd Ed.* **1971**, *3*, 1–56.
(2) Kaiser, E. T.; Kaiser, B. L. *Acc. Chem. Res.* **1972**, *5*, 219–224.
(3) Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3875–3878.
(4) Lipscomb, W. N.; Hartsuck, J. A.; Reeke, G. N.; Quicho, F. A.; Bethge, P. A.; Ludwig, M. L.; Steitz, T. A.; Muirhead, H.; Coppola, J. C. *Brookhaven Symp. Biol.* **1968**, *21*, 24–90.
(5) Lipscomb, W. N. *Tetrahedron* **1975**, *30*, 1725–1732.
(6) Rees, D. C.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 277–280.
(7) Rees, D. C.; Honzatko, R. B.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3288–3291.
(8) Hall, P. L.; Kaiser, B. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1969**, *91*, 485–491.
(9) Kaiser, B. L.; Kaiser, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *64*, 36–41.
(10) Auld, D. S.; Vallee, B. *Biochemistry* **1970**, *9*, 602–609.
(11) Auld, D. S.; Vallee, B. *Biochemistry* **1970**, *9*, 4352–4359.
(12) Auld, D. S.; Vallee, B. *Biochemistry* **1971**, *10*, 2892–2897.
(13) Auld, D. S.; Holmquist, B. *Biochemistry* **1974**, *13*, 4355–4361.
(14) DeKoch, R. J.; West, D. J.; Cannon, J. C.; Chasteen, N. D. *Biochemistry* **1974**, *13*, 4347–4354.
(15) Bunting, J. W.; Murphy, J. *Can. J. Chem.* **1974**, *52*, 2640–2647.
(16) Nau, H.; Riordan, J. F. *Biochemistry* **1975**, *14*, 5285–5294.
(17) Kang, E. P.; Storm, C. B.; Carson, F. W. *J. Am. Chem. Soc.* **1975**, *97*, 6723–6728.
(18) Suh, J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1976**, *98*, 1940–1947.
(19) Makinen, M. W.; Yamamura, K.; Kaiser, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3882–3886.
(20) Breslow, R.; Wernick, D. *J. Am. Chem. Soc.* **1976**, *98*, 259–261.
(21) Breslow, R.; Wernick, D. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1303–1307.
(22) Bunting, J. W.; Chu, S. S.-T. *Biochim. Biophys. Acta* **1978**, *524*, 142–155.
(23) Bunting, J. W.; Kabir, S. H. *Biochim. Biophys. Acta* **1978**, *527*, 98–107.

0002-7863/82/1504-5221$01.25/0