Antioxygenic Activity of Lecithin and its Hydrolysis Products. III. Lecithin, Glycerophosphorylcholine, Phosphorylcholine, Phosphatidic Acids, Choline and Glycerol

By Chieko URAKAMI and Haru KAMEYAMA

(Received May 4, 1959)

It was reported that choline among other hydrolysis products of phosphatides such as glycerol, oleic and linoleic acids, glycerophosphoric acid, and aminoethyl alcohol was the only one active against oils of plant origin[1]. The opinion of other investigators[2,3], however, was that the active entity in the phosphatide molecule is the phosphoric acid residue and that lecithin in which the hydroxyl of the phosphoryl group is involved in zwitterionic combination should show no activity[2,4]. Olcott and Mattill[2], and also Hilditch and Paul[5] reported that purified lecithin was inactive. However, the purities of the substrates as well as those of the samples of lecithin which were employed by these investigators were doubtful.

It has been known that some minor impurities present in substrates or in samples of phosphatides may act synergistically with a compound which has no activity in itself or conversely inhibit activity of a truly active compound. As the authors have demonstrated with the glycerophosphoric acids[6], the method used for purification of the substrate is important in evaluating antioxygenic activity. In this connection, it is of interest to note the recent paper of Olcott[7]. He demonstrated that a minute amount of free fatty acids added to substrates influenced antioxygenic properties of various compounds. Therefore, those results obtained with substrates which had been purified by mere solvent fractionation and distillation would not be trustworthy.

In regard to purification of lecithin, it is fairly recent[8,9] that the separation of phosphatidylcholines from other components of phospholipids and impurities can be achieved by column chromatography, indicating inadequacy of purification by the acetone precipitation method. Therefore, reinvestigation of natural lecithin is desirable in order to ascertain the varied results reported in the literature. The study on its hydrolysis products such as phosphatidic acids, glycerophosphorylcholine, phosphorylcholine, choline and glycerol is also desirable in elucidating structural requirements of phospholipids for antioxygenic activity.

Experimental

Materials.—*Methyl oleate.*—The ester was prepared from olive oil (Nihon Yushi Co.) essentially in the same manner as described previously[6], except that the treatment with urea preceded the fractionation by low temperature crystallization from acetone. The fraction used as substrate had the following constants: b. p., 203~203.5°C/10 mmHg; n_D^{20} 1.4526; Saponification No., 188.9; Iodine No., 89.9. The ultraviolet absorption spectra of this sample in ether showed $E_{1cm}^{1\%}$ 0.930 at 270 mμ, indicating the presence of a small amount of unsaturated fatty acid esters other than the monoethenoid.

Orthophosphoric acid. — The analytical reagent grade (approximately 85%) of Mallinckrodt Chemical Works was dried over phosphorus pentoxide in a vacuum desiccator for four days or until a constant weight was attained.

Glycerol.—A fraction distilled at 140°C/3 mmHg was dried over phosphorus pentoxide in a vacuum desiccator.

Choline. — Free choline was prepared from choline chloride by shaking its aqueous solution with freshly precipitated silver hydroxide[10]. The yield was over 90% of the theoretical.

α- & β-Glycerophosphoric acids.—The free acids were prepared from the corresponding sodium salt in the same manner as described in the previous paper[6].

1) R. Strohecker et al., *Z. Untersuch. Lebensm.*, **79**, 23 (1940).
2) H. S. Olcott and H. A. Mattill, *Oil & Soap*, **13**, 98 (1936).
3) H. J. Dutton et al., *J. Am. Oil Chemists' Soc.*, **26**, 441 (1949).
4) V. P. Calkins, *J. Am. Chem. Soc.*, **69**, 384 (1947).
5) T. P. Hilditch and S. Paul, *J. Soc. Chem. Ind.*, **58**, 21 (1939).
6) C. Urakami et al., This Bulletin, **32**, 36 (1959).
7) H. S. Olcott, *J. Am. Oil Chemists' Soc.*, **35**, 597 (1958).
8) D. J. Hanahan et al., *J. Biol. Chem.*, **192**, 623 (1951).
9) D. N. Rhodes and C. H. Lea, *Biochem. J.*, **65**, 526 (1957).
10) H. W. Dudley, *J. Chem. Soc.*, **119**, 1260 (1921).

Phosphorylcholine chloride.—The barium salt of phosphorylcholine chloride was synthesized by H. Okura according to the method described by Baer[11]. The barium ion was precipitated as the sulfate and the clear supernatant liquid separated after removing the sulfate by centrifugation was concentrated to a small volume. The residue was treated with 95% ethanol and any precipitate formed was removed completely by centrifugation. The clear filtrate was concentrated to give syrupy material, which was dried over phosphorus pentoxide. The yield was approximately 83%.

Phosphorylcholine. — The chloride ion of the barium salt of phosphorylcholine chloride was removed first by precipitating as silver chloride with freshly prepared silver hydroxide (shaking was continued until the aqueous solution showed negative Beilstein test). The barium cation was removed in the manner described above for the preparation of phosphorylcholine chloride from its barium salt.

Lecithin. — Crude lecithin isolated from egg yolk by the conventional method of acetone precipitation was purified by slightly modified method of Rhodes and Led[9].

Phosphatidic acids.—The sample was prepared by M. Okada by interacting the lecithin prepared as above with lecithinase C (also called phosphatidase C or phospholipase D) of carrot roots in a manner similar to the method described by Kates[12]. Analysis showed that the sample contained about 70% phosphatidic acids and minor amounts of monoacylglycerophosphoric acids and glyceride-like substances.

DL-*α-Glycerophosphorylcholine.* — This compound was also synthesized by H. Okura according to the method of Baer and Kates[13]. A ratio of α-glyceryl ester to choline was found to be 1.03:1.0.

Autoxidation.—Each compound was added to the substrate at a concentration of 0.1% with the use of an appropriate solvent. The rest of the procedure employed was exactly the same as that described previously. The bath temperatures of 45 and 60°C were studied, the latter being chosen for comparison of the efficiencies of those found active.

Peroxide Determination. — The method previously described was employed.

Results

Purified ovo-lecithin, known to be L-α-lecithin, and DL-α-glycerophosphorylcholine were found to be inactive at 45°C as shown in Fig. 1. Both compounds were, however, found to be somewhat active at this temperature when methyl oleate purified by the method of low temperature solvent fractionation alone was used (Fig. 2.). Here again it should be emphasized that the method of purification of substrates is very important as the authors have advocated.

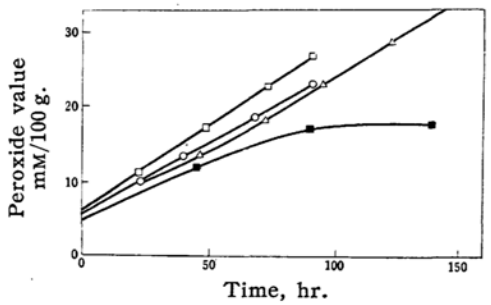

Fig. 1. Peroxide values against time at 45°C.
○: Methyl oleate alone. □: Methyl oleate containing 0.1% ovo-lecithin. △: Methyl oleate containing 0.1% DL-α-glycerophosphorylcholine. ■: Methyl oleate containing 0.1% α-glycerophosphoric acid.

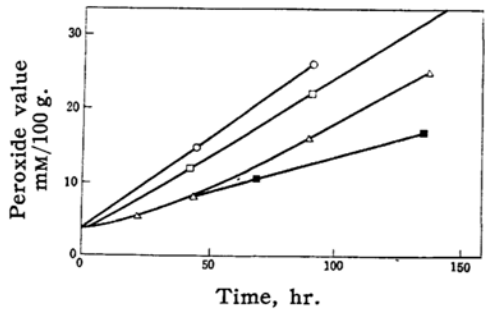

Fig. 2. Peroxide values against time at 45°C, using partially purified methyl oleate.
○: Methyl oleate alone. □: Methyl oleate containing 0.1% ovo-lecithin. △: Methyl oleate containing 0.1% DL-α-glycerophosphorylcholine. ■: Methyl oleate containing 0.1% α-glycerophosphoric acid.

Choline was found to be inactive both at 45 (Fig. 3) and 60°C (not shown here). It was also found to be inactive in oleic acid at 50°C. Consequently, choline itsef is definitely inactive contrary to the result reported by Strohecker et al.[1]

Glycerol tested at 60°C exhibited no activity and thus is considered to be an inactive compound in purified methyl oleate.

Phosphorylcholine chloride was found to be active both at 45 (Fig. 3) and 60°C (Fig. 4) and the free phosphorylcholine more active than the chloride at 60°C.

The sample of phosphatidic acids employed in this experiment showed an

11) E. Baer, *J. Am. Chem. Soc.*, **69**, 1253 (1947).
12) M. Kates, *Can. J. Biochem. Physiol.*, **33**, 575 (1955).
13) E. Baer and M. Kates, *J. Am. Chem. Soc.*, **70**, 1394 (1948).

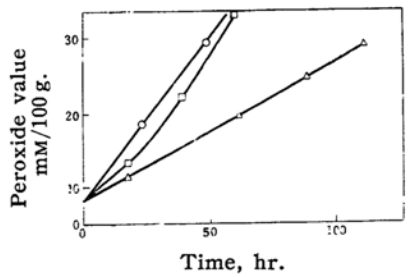

Fig. 3. Peroxide values against time at 45°C.
○: Methyl oleate alone. □: Methyl oleate containing 0.1% choline. △: Methyl oleate containg 0.1% phosphorylcholine chloride.

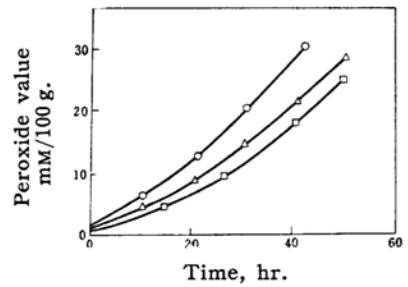

Fig. 4. Peroxide values against time at 60°C.
○: Methyl oleate alone. △: Methyl oleate containing 0.1% phosphorylcholine chloride. □: Methyl oleate containing 0.1% phosphorylcholine.

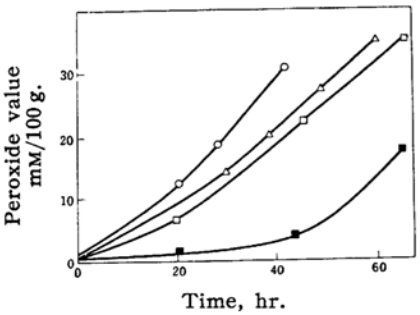

Fig. 5. Peroxide values against time at 60°C.
○: Methyl oleate alone. □: Methyl oleate containing 0.1% phosphatidic acid. △: Methyl oleate containing 0.1% β-glycerophosphoric acid. ■: Methyl oleate containing 0.1% orthophosphoric acid.

activity comparable to that of glycerophosphoric acids (Fig. 5). This result must, however, be considered with some reservation since the control of the purity of the natural free acids was very difficult, just as has been recorded in the literature, i.e. the acids easily lose the fatty acid residues when ethanol or a small amount of moisture is present[14].

The relative efficiencies of the active compounds were compared at 60°C and numerical values obtained by using the following expression: T_a/T_o, where T_a is the time required to give 20 mM in peroxide value when 0.1% of an active substance is present and T_o that required to show the same peroxide value by the substrate itself. This method of expression is a modification of that used by Emmanuel et al.[15] As shown in Table I, orthophosphoric acid is the most active one, followed by phosphorylcholine, phosphatidic acid, and phosphorylcholine chloride.

Discussion

Since glycerol was found to be inactive, the activity observed with the glycerophosphoric acids must be due to the phosphoryl moiety, apparently the glycerol moiety not participating. The absence of activity in DL-α-glycerophosphorylcholine also supports the fact that the glycerol moiety is not involved. This would mean that the alcoholic O–H or C–H linkage of the glycerol moiety is not readily attacked by free radicals, at least not as readily as the α-methylene group of the methyl oleate. This observation is in accord with the order of bond strengths of these three linkages. The bond strength of the O–H linkage is 110 kcal./mol.; that of the C–H of the α-methylene, 80 kcal./mol. or less than 64 when activated by resonance; and that of the C–H in the glycerol moiety, greater than 80 kcal./mol., since an effect produced by the alcoholic hydroxyl group on the C–H linkage is known to be much less than that by the α-double bond.

The activity shown by phosphorylcholine and its chloride is again attributed to the phosphoryl moiety since choline was found to be inactive. It appears that two methylene groups present in the phosphorylcholine molecule are less readily attacked than the α-methylene of the methyl oleate molecule.

It has been reported that ovo-lecithin purified by the method similar to the one the authors employed contained stearic acid in the β-position and an unsaturated acid in the α-position[16]. Since no activity was

14) J. Olley, *Chem. & Ind.*, **73**, 1069 (1954).
15) N. M. Emmanuel et al., *Myasnaya Ind. S. S. S. R.*, **29**, No. 2, 52 (1958); *Chem. Abstr.*, **52**, 17755*h* (1958).
16) D. J. Hanahan, *J. Biol. Chem.*, **211**, 321 (1954).

observed with the sample of lecithin used, it appears that the unsaturated fatty acid moiety in the lecithin does not compete with the α-methylene group of the substrate molecule. Thus, participation of the fatty acyl groups of ovo-lecithin can be excluded. However, the behavior of the fatty acids present in the sample of phosphatidic acids may differ from that of the lecithin fatty acyl group although the former was prepared from the same sample of lecithin used for the activity study. Kates[12] states that the isolated phosphatidic acids contain C_{18} fatty acids and about 20% of phosphatidic acid derived from the phospholipids present in the chromoplasts. Since the naturally occurring phosphatidic acids are said to be highly unsaturated, the fatty acid residue present in the sample of phosphatidic acids used is considered to be more highly unsaturated than those present in the lecithin originally subjected to the enzymatic action. Therefore, the activity observed with phosphatidic acids may partly be ascribed to autoxidation of the highly unsaturated fatty acyl group. However, it is highly probable that the phosphoryl residue has played a major role since the glycerophosphoric acids have been shown to be active, almost as active as phosphatidic acids (Fig. 5).

The foregoing discussion leads to the conclusion that the phosphoryl moiety is responsible for antioxygenic activity. A question arises, however, as to the absence of activity in ovo-lecithin and DL-α-glycerophosphorylcholine despite the presence of the phosphoryl group in both molecules, The authors tentatively attribute this to the zwitterionic forms of the molecules, as suggested earlier by Olcott and Mattill[2] and Calkins[4]. No definite conclusion concerning this can be drawn at this stage of investigation, for it is necessary to reveal true behavior of cephaline under identical conditions. It is said to be active according to the earlier investigators.

It would be of interest to examine whether the activity ratios, T_a/T_o, relate to some characteristics of the molecules of those found to be active such as acidity, the number of the free hydroxyl of the phosphoryl group or the ratio of the molecular weight of phosphoric acid to that of each of the active compounds.

As shown in Table I, an order of acidity is β-glycerophosphoric acid > α-glycerophosphoric acid > orthophosphoric acid phosphatidic acid > phosphorylcholine and its chloride, but this is not in accord with the order of activity ratio, orthophosphoric acid > phosphatidic acid and phosphorylcholine > phosphorylcholine chloride > α-glycerophosphoric acid > β-glycerophosphoric acid. Similarly, no significant relations can be found between the activity ratios and the number of the free hydroxyl of the phosphoryl group in each molecule, e. g. phosphorylcholine which is considered to have only one free hydroxyl, shows a stronger activity than does its chloride or β-glycerophosphoric acid although orthophosphoric acid with three free hydroxyl groups is exceptionally strongly active. Interaction of the alcoholic hydroxyl with the oxygen and hydroxyl of the phosphoryl group in the β-glycerophosphoric acid should, of course, be taken into consideration in this connection, but this will be dealt with in a later paper. The ratios of the molecular weight of phosphoric acid to that of each of the active compounds also bear no relation to the activity ratios.

Thus, it appears that other factors must be sought to understand how the phosphoryl group participates in exhibiting antioxygenic activity or in other words to

TABLE I. RELATION OF ACTIVITY RATIOS OF ACTIVE COMPOUNDS WITH SOME OF THEIR CHARACTERISTICS

	H_3PO_4	PA	PC	PCCl	β-GP	α-GP
T_a/T_o at 60°C	2.48	1.39	1.40	1.27	1.31	d
pK_{a1}	1.97	a	b	c	1.37	1.40
No. of free OH available	3	2	1	2	2	2
M. wt. of H_3PO_4/ that of compound	1	0.14	0.54	0.44	0.57	0.57

PA represents phosphatidic acids; PC, phosphorylcholine; PCCl, phosphorylcholine chloride; α- and β-GP, α- and β-glycerophosphoric acids. a) This is expected to be somewhat weaker than that of α-GP. b) and c) These are weaker than any of the rest of the compounds listed due to the presence of the choline base in their molecules. d) It is expected to be somewhat higher than 1.31 of β-GP according to the result obtained at 45°C[6].

understand mechanisms involved. For this, the authors have porposed that an oxygenated substrate molecule interact with the phosphoryl group to give highly conjugated compounds which undergo autoxidation much more readily than does the substrate molecule. Further investigation on this problem is underway.

Incidentally, Hanahan et al.[17] reported that phosphorylcholine and glycerophosphoric acids (in conjunction with choline) stimulated oxygen consumption of the isolated rat liver mitochondria, the former being particularly strongly active. Lecithin and glycerophosphorylcholine were found to stimulate some oxygen consumption but only under special conditions. The enzymatic system such as this is far more complex than the system under consideration and in no way should these two systems be compared. However, one can not deny a possibility that reactions taking place in both systems may be found to be related, in view of the recent finding that free radicals are present in biological systems[18,19].

17) M. Rodbell and D. J. Hanahan, ibid., **214**, 595 (1955).
18) B. Commoner et al., *Nature*, **174**, 689 (1954).
19) I. Miyagawa et al., *Proc. Natl. Acad. Sci. U. S.*, **44**, 613 (1958); *Chem. Abstr.*, **53**, 2325e (1959).

Summary

Purified ovo-lecithin, DL-α-glycerophosphorylcholine, choline and glycerol were found to exhibit no antioxygenic property against carefully purified methyl oleate. Phosphorylcholine, its chloride and natural phosphatidic acids, on the other hand, showed activity.

The order of activity efficiency at 60°C was found to be orthophosphoric acid > phosphatidic acid and phosphorylcholine > phosphorylcholine chloride > α-glycerophosphoric acid > β-glycerophosphoric acid. The discussion is presented to show that the phosphoryl moiety is predominantly responsible for antioxygenic activity.

The authors are indebted to Misses M. Ishida, J. Kondo, K. Yamaguchi and K. Sakamoto for their technical assistance.

Faculty of the Science of Living
Osaka City University
Nishi-ku, Osaka