*Antioxygenic Activity of α- and β-Glycerophosphoric Acids**

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Introduction

Since Bollmann[1] discovered antioxygenic activity of crude lecithin, a number of other investigators[2] have reported similar findings. However, Hilditch[3] found that purified lecithin did not show any antioxygenic activity in the autoxidized esters of the distilled fatty acids of olive oil. For the study of relations of molecular structures of phosphatides to antioxygenic activity, their hydrolysis products have been investigated by a number of workers. Olcott and Mattill[4] reported that cephalin among other phosphatides had the strongest activity and suggested that the phosphoryl group in the molecule has some relation to the activity. Strohecker, et al.[5] obtained the result that only choline among other hydrolytic products of cephaline was active against oils of plant origin, whereas Dutton, Olcott, and Mattill[6] observed that the phosphoryl residue in the cephalin or lecithin molecules possessed activity. Altman[7] reported that phosphatides, choline and amino acids were effective in rubber latex. Recently, Desneulle et al.[8] have found that the phosphatidyl choline which has been obtained from eggs did not possess antioxygenic activity in the methyl esters of sunflower seed oil fatty acids but the phosphatidic acids obtained on treating the phosphatidyl choline with phosphatase prepared from carrots exerted the activity. Calkin[9] proposed the theory that cephaline with one free hydroxyl gorup should show some activity but lecithin which has no free hydroxyl group in its molecule should have no activity.

It is conceivable that nothing definite has been established as to the relation of molecular structures of the phosphatides to antioxygeninc activity. It is, however, reasonable to assume that the phosphoryl group in these molecules would possess the activity since a number of organic esters of phosphoric acid[10] are known to be antioxygenic to fat-containing materials.

The confusing results[3,5,6] on the phosphatidic acids may be due to difference in composition of the hydrolysis products with respect to the α and β-structures of the glycerophosphoric acids, since the isomerization of the phosphoryl group has been found to occur in either acid or alkaline hydrolysis[11], to a greater extent with the former. Therefore, we have called our attention to the relation of the structural difference of the phosphatidic acid, to antioxygenic activity and, as a preliminary work, studied α and β-glycerophosphoric acids, relatively stable hydrolysis products of the phosphatides, in purified oleic acid.

Conditions for autoxidation were selected so that least kinds and amounts of by-products would be produced during autoxidation, as demonstrated by Swern, et al.[12] As the rate of oxidation of oleic acid is slow compared to that of methyl oleate, we chose 50°C for the temperature of autoxidation and compared activities of the glycerophosphoric acids with that of phosphoric acid in an early stage, up to peroxide value of about 10 mM per 100 g. of the acid. It was found that the α-compound was just as active as orthophosphoric acid but the β-isomer did not have any activity.

We have also investigated recovery of the water soluble glycerophosphoric acids during autoxidation to obtain some clue as to mechanism involved in the antioxygenic activity. Preliminary results showed that the recovery of the water soluble gryceropho-sphoric acids from the autoxidized oleic acid

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1) H. Bollmann. *U. S. Pat* 1, 575, 529 (1926).

2) G. I. Evans, *Ind. Eng. Chem..*, 27, 399 (1935); H. N. Holmes, R. E. Corbet and R. A. Ragatz, ibid., 28, 133 (1936); M. R. Sahasrabudhe, *J. Sci. Ind. Research*, **12B** 63 (1953); H. J. Lips, *Food in Canada*, 12, No. 6, 9, 12 16 (1952).

3) T. P. Hilditch and S. Paul, *J. Soc. Chem, Ind* , 58, 21 (1939).

4) H. S. Olcott and H. A. Mattill, *Oil and Soap*, 13, 98 (1936).

5) R. Strohecker, W. Diemair and K. Reuland, *Z. Untersuch. Lebensm.*, 79, 23 (1940).

6) H. J. Dutton, H. S. Olcott, and H. A. Mattill, *J. Am. Oil Chemists' Soc.*, 26, 441 (1949).

7) R. F. A. Altman, *Trans. Inst. Rubber Ind.*, 23, 179 (1947).

8) P. Desneulle, R. Massoni and O. Benoit-Micaelli, *Bull. Soc. chim. France*, 1953, 595.

9) V. P. Calkins, *J. Am. Chem. Soc.*, 69, 384 (1947).

10) H. R. Kraybill and B. W. Beadle, *U. S. Pat.*, 2, 521, 856.

11) E. Baer and M. Kates, *J. Biol. Chem.*, 185, 615 (1950); E. Baer, H. C. Stancer and I. A. Korman, ibid., 200, 251 (1953).

12) H. B. Knight, J. E. Coleman and D. Swern *J. Am. Oil Chemists' Soc.*, 28, 498 (1951); ibid., 32, 135 (1955): D. H. Saunders, C. Ricciuti and D. Swern, ibid.,. 32, 79 (1955).

was very small, implying that a larger amount is associated with the fatty acid.

Experimental

Oleic Acid.—"Tokusei olein NTA 34" of the Nihon Yushi K. K.[13] was purified by recrystallization from acetone at low temperatures[14], first at −5° to remove stearic and other saturated acids present and then at −53° to removed linoleic and other highly unsaturated acids. The solvent was removed from the acetone solution containing chiefly oleic acid and the residue distilled three times. The sample thus prepared had the following constants: B.p., 220°/7 mm.and 221°/8 mm (220°/7 mm.).; Iodine number, 89.10 (89.9); Neutralization value, 199.7 (198.8); n_D^{20}, 1.4599, (1.4585, 1.4599). The figures given in the parentheses are those calculated or found in the literature.

Orthophosphoric Acid.—Analytical reagent (85%) of Mallinckrodt Chemical Works, U. S. A. was used. It was dehydrated in a vacuum desiccator over calcium chloride and then taken up in absolute alcohol. Its concentration was determined by Burmaster's method[15]. For the determination of absorption coefficients, Shimazu Model DF-11 with the filter no. 634 and 5 mm. cell were employed.

α-Glycerophosphoric Acid.—The barium ion of barium α-glycero-Phosphate[16] was removed in the form of sulfate by treating with 0.1 N sulfuric acid (adjusted to pH 1.5), followed by repeated washing with alcohol. The alcoholic solution was concentrated to 3 cc. in volume and the concentrate analyzed according to Burmaster's method. Analysis of 1 cc. of the alcoholic concentrate: inorganic P, 0.000 mg.; α-glycerophosphoric acid, 10.656 mg.; the β-isomer, 0.500 mg.

β-Glycerophosphoric Acid.—Sodium β-glycerophosphate suspended in alcohol was treated with 0.1N alcoholic HCl (adjusted to pH 1.5), the mixture concentrated under reduced pressure, and the residue washed several times with alcohol to remove the sodium chloride precipitated. The process was repeated several times to remove as much of the sodium chloride as possible, and the final alcoholic solution concentrated to give 3 cc. in volume. Analysis of 1 cc. of the solution: inorganic P, 0.000 mg.; α-glycerophosphoric acid, 0.600 mg.; the β-isomer, 20.960 mg.

Autoxidation of Oleic Acid.—After adding a sufficient amount of an alcoholic solution of the compound under consideration to 14 g. of oleic acid, the alcohol was removed at less than 40°C under reduced pressure and nitrogen atomosphere. The concentration of the compound in oleic acid was kept at 0.1%. The sample thus prepared was autoxidized at 50°C by passing purified air at a rate of 3l. per min. through a sintered glass funnel (a medium sized pore) without the sleeve. Aliquots were removed at intervals (into a weighing bottle containing 1–2 drops of a 0.05% solution of hydroquinone in isopropanol) and their peroxide values were determined according to Volz-Gortner's method[17], using 0.005N solution of sodium thiosulfate. The titration was carried out by using a 1 or 2 cc. pipette provided with a large injection needle at the tip end with the aid or rubber tubing and a long rubber tubing attached to a syringe at the other end. By manipulating the syringe, one cc. was cut into 70 drops, The method gave more accurate results than those obtained by using the microburette sold on the market. The error of average deviation involved was found to be 2% with peroxide values up to 1.5mM/100 g, 0.1% with 9 mM/100 g, and 0.5–1% for the intermediate values.

Recovery Test.—An aliquot of 0.1 cc. was removed at intervals during the autoxidation, weighed accurately, and dissolved in 2 cc. of ether. The ether solution was washed four times with 1 cc. portions of fresh water. The washing were combined, made up to a known volume and analyzed for the recovery of the glycerophosphoric acids. Blank tests were carried out in exactly the same manner with β-glycerophosphoric acid in non-autoxidized oleic acid.

Results

The effect of alcohol used as a solvent was observed, as shown in Tables I and II, higher peroxide values were obtained with the sample treated with alcohol. The results obtained with orthophosphoric acid, α and β-glycerophosphoric acids are shown in Tables III, IV and V, respectively. The maximum error

TABLE I

AUTOXIDATION OF OLEIC ACID AT 50°C

Time hr.	Peroxide Value m. mol./100 g.	Amount of Air Passed, Kl.
6.5	1.32±0.02	0.85
11.8	2.23±0.02	1.67
22.1	4.25±0.09	3.43
26.8	5.30±0.11	4.20
34.4	6.88±0.02	5.38
47.9	9.53±0.02*	7.68

* The determination was carried out 6 hr. after removing the sample from the autoxidation chamber.

TABLE II

AUTOXIDATION OF OLEIC ACID WITH TRACE OF ALCOHOL AT 50°C

Time hr.	Peroxide Value m. mol./100 g.	Amount of Air Passed, Kl.
5.9	1.56±0.02	0.95
18.4	4.00±0.06	3.15
23.0	5.01±0.02	3.86
28.1	6.20±0.04	4.66
43.8	9.70±0.04	7.28

13) We thank the Nihon Yushi K. K. Kenkyu-sho for the supply of the sample.

14) D. K. Kolb and J. B. Brown, *J. Am. Oil Chemists' Soc.*, **32**, 357 (1955).

15) C. F. Burmaster, *J. Biol. Chem.*, **164**, 233 (1946).

16) C. Urakami and Y. Kakutani, *Repts. Sci. Living*, Osaka City Univ., Series D. No. 1, 3 (1953).

17) F. E Volz and W. A. Gortner, *J. Am. Oil Chemists' Soc.*, **24**, 417 (1947).

involved in the rates of aeration of the samples were found to be 5% but the rates were very close to each other in the samples containing α and β-glycerophosphoric acids.

TABLE III
AUTOXIDATION OF OLEIC ACID IN THE PRESENCE OF H_3PO_4 AT 50°C

Time hr.	Peroxide Value m. mol./100 g.	Amount of Air Passed, Kl.
5.8	0.73±0.02	0.75
20.6	1.84±0.04	3.02
30.2	2.72±0.02	4.05
44.7	4.44±0.07	6.48
53.2	5.60±0.04	7.90

TABLE IV
AUTOXIDATION OF OLEIC ACID IN THE PRESENCE OF α-GLYCEROPHOSPHORIC ACID AT 50°C

Time hr.	Peroxide Value m. mol./100 g.	Amount of Air Passed, Kl.
6.0	0.69±0.01	1.00
19.1	1.20±0.01	3.33
29.0	1.97±0.01	4.95
43.6	4.27±0.08	7.51
51.6	5.50±0.00	8.81

As shown in Tables IV and V, there is a distinct difference in antioxygenic activity between the two isomers, α-glycerophosphoric acid has the activity somewhat better than phosphoric acid while the β-isomer has no activity at all. The peroxide values for the latter are somewhat higher than those of oleic acid alone but close to those of the blank run made with the addition of alcohol.

TABLE V
AUTOXIDATION OF OLEIC ACID IN THE PRESENCE OF β-GLYCEROPHOSPHORIC ACID AT 50°C

Time hr.	Peroxide Value m. mol./100 g.	Amount of Air Passed, Kl.
5.4	1.93±0.02	1.00
20.0	4.65±0.04	3.52
29.5	6.55±0.07	5.05
43.2	8.98±0.07	7.43
50.5	10.2 ±0.1	8.61

Recovery of water soluble β-glycerophosphoric acid from unautoxidized oleic acid was found to be 95%. However, there was a sharp drop in recovery of both α and β-glycerophosphoric acids from autoxidized oleic acid, 21–35% and 2–8%, respectively, as shown in Tables VI and VII. As the autoxidation progressed, a small amount of the α compound was detected in the β-sample.

Discussion

It has been demonstrated that α-glycerophosphoric acid has antioxygenic activity comparable to that of orthophosphoric acid in purified oleic acid but the β-isomer has no activity. According to Baer et al.[11] and Long and Maguire[18], alkaline or acid hydrolysis of the naturally occurring phosphatides results in migration of the phosphoryl radical. Consequently, the samples of glycero-

TABLE VI
RECOVERY OF α-GLYCEROPHOSPHORIC ACID FROM AUTOXIDIZED OLEIC ACID

Aliquot g.	Time of autoxidation, hr.	Absorbance* P			Microgram P				The Acids/0.2 g. Sample, μg.			Total recovery %
		H_3PO_4	α	Total	H_3PO_4	α	Total	β	α	β	H_3PO_4	
25 μl**		0.003	0.38	0.39	0.00	24.0	24.9	0.9	106.6 (96.4)	3.7 (3.4)		100
0.2408	29.0		0.113			6.9			63.6 (26.9)			
0.2421	〃			0.115			7.0			0.6 (0.25)		27.2
0.1907	43.4	0.008										
0.1899	〃		0.111			6.75			79.9 (33.8)			
0.1889	〃			0.115			7.0			2.3 (1.0)		34.8
0.2148	51.4	0.010			0.05						0.52 (0.2)	
0.1978	〃		0.062			3.5			39.3 (16.6)			
0.2164	〃			0.082			4.9			11.0 (4.7)		21.2

* The values were obtained by subtracting absorbance of blank from that of sample.
** Analysis of the alcoholic solution added to oleic acid. The figures in the parentheses are per cent recovery of the respective acid.

18) C. Long and M. F. Maguire, *Biochem. J.*, 54, 612 (1953).

TABLE VII

RECOVERY OF β-GLYCEROPHOSPHORIC ACID FROM AUTOXIDIZED OLEIC ACID

Aliquot g.	Time of Autoxidation, hr.	Absorbance* P			Microgram P			The Acid/0.2 g. Sample, μg.		Total recovery %
		H_3PO_4	α	Total	H_3PO_4	α	Tatal	α	β	
10 μl**		0.004	0.017	0.295		0.50	18.88	5.8	218.5 (97.4)	100
0.2382	19.6		0.007							
0.2368	〃			0.037			1.83		17.07	7.6
0.2164	29.3		0.010			0.05		0.51 (0.2)		
0.2269	〃			0.037			1.83		17.40 (7.8)	8.0
0.1027	43.1	0.00								
0.2110	〃		0.010			0.05		0.52 (0.2)		
0.2125	〃			0.022			0.86		8.36 (3.7)	4.0
0.2070	50.3	0.00								
0.2146	〃		0.010			0.05		0.25 (0.2)		
0.2206	〃			0.018			0.55		4.82 (2.2)	2.4

* The values were obtained by subtracting absorbance of blank from that of sample.
** Analysis of the alcoholic solution added to oleic acid. The figures in the parentheses are per cent recovery of the respective acid.

phosphoric acids or the hydrolysates of phosphatides studied earlier for their antioxygenic activity might have been a mixture of the isomers in various proportions and this might have been responsible for the confusing results found in the literature.

Concerning mechanisms of antioxygenic activity of orthophosphoric acid, Quackenbush[19] reported that at a higher temperature phosphoric acid reacted with the peroxides of lard to give an oil soluble polymer-like substance. On the other hand, Calkin[9] suggested that the activated oleic acid adsorbs phosphoric acid molecules to form an ester-like intermediate, which on dissipating its energy disintergrates into the original substances, oleic acid and phosphoric acid. Our preliminary results in the recovery of the glycerophosphoric acids indicate that they are either adsorbed on the oleic acid molecules or transformed into some kind of oil soluble products. Further investigation on this point is of great interest and we are extending the study.

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19) F. W. Quackenbush and O. S. Privett, *J. Am. Oil Chemists' Soc.*, 31, 225 (1954).