Antioxygenic Activity of Lecithin and its Hydrolysis Products. II. α- and β-Glycerophosphoric Acids*

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Since the last paper on the subject[1] was published, we have observed considerable variations in the activities of α- and β-glycerophosphoric acid with different batches of crude oleic acid obtained from beef tallow. Thus it became necessary to find some means of overcoming this difficulty. In the method of purification by low temperature crystallization, it was found difficult to remove acetone completely and we suspected that some impurities soluble in acetone might have been carried over to the final product to give these varied results. This difficulty was overcome, however, by crystallizing oleic acid as urea complex. The samples of the substrate purified in this way gave rather consistent results.

Further evidences were obtained as to the formation of oil-soluble products from both α- and β-glycerophosphoric acid during autoxidation, as suggested in the previous paper, by applying chromatographic techniques and measuring the ultraviolet absorption of the fractions obtained.

Experimental

Materials.—*Oleic acid.*—Crude oleic acid from beef tallow was supplied by the laboratory of Nihon Yushi Co., Ltd. Olive oil was treated according to the procedure described by Knight, et al.[2] to obtain crude oleic acid. These crude products were purified by the following two methods: *Method A*; The samples were purified by low temperature crystallization from acetone, as described in the previous paper. *Method B*;

* The preceding paper (This Bulletin **29**, 921 (1956).) will be designated as Part I of the series under this new title.

1) Chieko Urakami, Y. Kakutani and H. Okura, This Bulletin, **29**, 921 (1956).

2) H. B. Knight, E. F. Jordan, Jr., E. T. Roe and D. Swern, "Biochemical Preparation," Vol. 2, John Wiley & Sons, Inc., New York (1952). pp. 100.

The samples of oleic acid obtained in the above manner were treated with urea according to the procedure described by Swern and Parker[3]. The constants of these samples are summarized in Table I.

TABLE I
CONSTANTS OF SAMPLES OF OLEIC ACID

Source	Code No.	b. p.	n_D^{20}	Iodine No.	Acid No.
Beef tallow					
Method A	O-1	200~201/2	1.4596	90.2	197.9
〃	O-2	189~192/2	1.4589	91.8	198.3
Olive oil					
Method B	O-3	214/5	1.4600	90.0	198.2
Lit. or calcd. values		210/5	1.4585 1.4599	89.9	198.6

Methyl oleate.—Esterification of oleic acid was carried out by following the procedure described by Knight et al.[2] The samples of the ester prepared from the oleic acid which was purified by Method A and those prepared from the oleic acid which was purified by Method B are classed in the respective group in Table II.

TABLE II
CONSTANTS OF SAMPLES OF METHYL OLEATE

Source	Code No.	b. p.	n_D^{20}	Iodine No.	Acid No.
Beef tallow					
Method A	M-1	173~174/2	1.4520	84.5	194.2
〃	M-2	205/12	1.4352	88.6	196.1
Method B	M-3	160~164/3	1.4515	81.8	195.2
Olive oil					
Method B	M-4	166~169/1.8	1.4526	86.1	188.3
Lit. or calcd. values		172~173/2	1.4526 1.4522	85.7	189.2

α-Glycerophosphoric acid.—An ethanolic solution of the free acid was prepared from the sodium salt (procured from Daito Seiyaku Co., Ltd.) in the same manner as described in the previous paper[1]. This sample was found to contain about 21% β-glycerophosphoric acid and was used in the experiments with oleic acid. A sample containing 14% β-isomer was prepared by isomerization in an acid medium[4] and used in the runs with methyl oleate.

β-Glycerophosphoric acid.—An ethanolic solution was prepared from its sodium salt (obtained from Yashima Yakuhin Co., Ltd.) as described above. The sample was found to be 100% pure. The concentrations of the ethanolic solutions were determined by Burmaster's method[5].

Autoxidation.—The procedure employed was essentially the same as that described previously, except that the bath temperature was maintained at 45°C for the runs with methyl ester, instead of 50°C used for runs with the acid. The concentration of the glycerophosphoric acids in the substrate was 0.1%.

Peroxide determinations. — The method described in the previous paper was followed, except that the use of nitrogen gas was omitted. As shown in Table III, the values obtained by titration under nitrogen did not significantly differ from those obtained without bubbling nitrogen through the sample solution, as long as one to two drops of a 0.05% solution of hydroquinone in isopropanol had been added to each aliquot taken.

TABLE III
PEROXIDE VALUES OBTAINED BY TITRATION UNDER NITROGEN AND IN AIR

	Sample wt. g.	P. V. mM/100 g.
Under nitrogen	0.1824	2.54
	0.2107	2.53
In air	0.2180	2.52
	0.1926	2.58

Recovery test.—The recovery of the water-soluble glycerophosphoric acids from the substrates during autoxidation was examined in the same manner as described previously. The results are shown in Figs. 1 and 2 and Table IV.

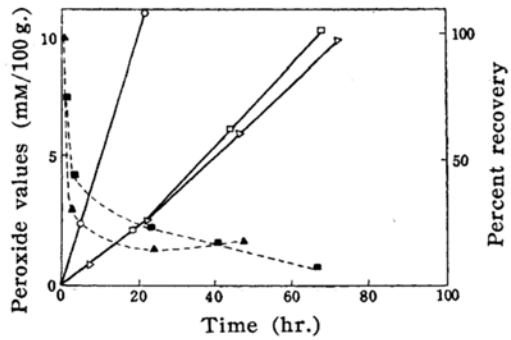

Fig. 1. Peroxide values and percent recovery against time.
○ Oleic acid, O-1, alone. △ Peroxide values for the same sample of oleic acid containing 0.1% α-glycerophosphoric acid (79% purity). □ Peroxide values for the same sample of oleic acid containing 0.1% β-glycerophosphoric acid. ▲ Percent recovery of the α-glycerophosphoric acid. ■ Percent recovery of the β-glycerophosphoric acid.

Chromatography. — Since the method described by Huennekens and his associates[6] affords separation of oleid acid from phosphorus containing substances of biological interest, we utilized the method in removing the substrate from the substance which seemed to be formed from the glycerophosphoric acids added initially. The

3) D. Swern and W. B. Parker, *J. Am. Oil Chemists' Soc.*, **29**, 614 (1955).
4) E. Chargaff, *J. Biol. Chem.*, **144**, 455 (1942).
5) C. F. Burmaster, ibid., **164**, 233 (1946).
6) F. M. Huennekens, D. J. Hanahan and M. Uziel, ibid., **206**, 443 (1954).

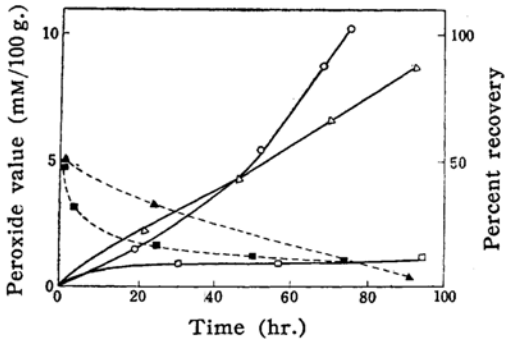

Fig. 2. Peroxide values and percent recovery against time
○ Methyl oleate, M-1, alone. △ Peroxide values for the same sample of methyl oleate containing 0.1% α-glycerophosphoric acid (86% purity). □ Peroxide values for the same sample of methyl oleate containing 0.1% β-glycerophosphoric acid. ▲ Percent recovery of the α-glycerophosphoric acid. ■ Percent recovery of the β-glycerophosphoric acid.

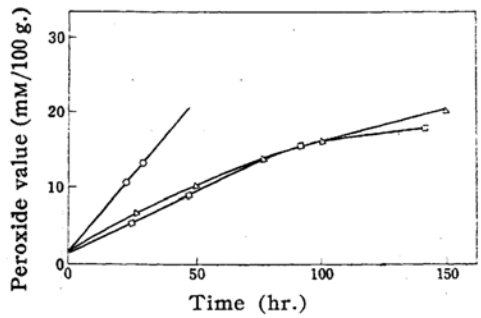

Fig. 3. Peroxide values against time
○ Oleic acid, O-2, alone. △ The same sample of oleic acid containing 0.1% α-glycerophosphoric acid (79% purity).
□ The same sample of oleic acid containing 0.1% β-glycerophosphoric acid.

TABLE IV
RECOVERY OF GLYCEROPHOSPHORIC ACIDS FROM SUBSTRATES

Time hr.	P. V. mM/100g.	Sample wt. g.	Recovery in water, mg. α	β	Total
0	0	0.1345[a]	10.03	4.11	14.15 (36.1)
87.5	1.18	0.1487[a]	0.104	0.045	0.149 (0.38)
39	15.07	1.3200[b]	0.0036 (1.5)		
82	23.85	1.7680[c]		0.0064 (2.0)	

a) Methyl oleate obtained from beef tallow containing 7% α-isomer. The ratio of the recovered α:β before and after oxidation remained the same, 70:30. b) Methyl oleate obtained from olive oil containing 0.1% α-isomer. c) Oleic acid from olive oil containing 0.1% β-isomer. The figures in the parentheses represent percent recovery.

last two samples shown in Table IV were subjected to chromatography, after removing any unreacted glycerophosphoric acids by repeated washing with water.

By a trial run with paper, using about 200 mg. each of the samples and a mixture of 1-propanol and water (8:1) as a developing solvent (at 21°C for 15.5 hr.), it was found that a significantly large amount of phosphorus was present in the upper half (toward the solvent front) of the chromatogram. This was somewhat more concentrated in the section 3/4 from the original point of application. Therefore, the upper half of the chromatogram was extracted successively three times with a 50 cc. portion of diethyl ether, twice with 25 cc. of ethanol and finally with 10cc. of a 0.1 N solution of sodium hydroxide. Each extract was tested for the presence of phosphorus and it was found to be present only in the alkaline extract. Therfore, the same sample was subjected to column chromatography in the same manner, using cellulose powder (Toyo-Roshi No. 50), except that a larger amount of diethyl ether was used in place of extracting with ethanol. A blank run was made in exactly the same way. The ultraviolet absorption curves obtained with the ether extract are shown in Fig. 9 and those of the alkaline extracts in Fig. 10 (corrected for the absorption of the blank).

Measurements of ultraviolet absorption.— A Beckman DU-spectrophotometer was used. For the purpose of comparison of the sample obtained from the incorporation of the α- and that from the incorporation of the β-isomer, the extinction coefficients were calculated on the basis of mg. %* of phosphorus since their molecular weights are unknown.

Results

In the present study with oleic acid obtained from two different batches of beef tallow and purified by Method A, both glycerophosphoric acids were found to be active in a comparable strength (see Figs. 1 and 3) but marked differences in activity were observed in the methyl ester prepared from the corresponding sample of oleic acid. In one sample the β-isomer was found to be strongly active, while the α-isomer almost inactive in the early stages of autoxidation as shown in Fig. 2. The activity shown in the later stages is considered to be due to the presence of some 14% β-isomer since the curve obtained by another run with the α-isomer containing 21% β-isomer runs closely parallel to the one shown here, except with an increased activity in the later stages. In

* This expression is used for a matter of convenience instead of the conventional expression, for the products have neither been isolated in pure form nor characterized. However, the estimated values for their molecular extinctions at certain characteristic wave lengths are given in the discussion section.

another sample of the substrate, on the other hand, both acids were found to be active in comparable strength as shown in Fig. 3, although some difference was observed in the later stages. These results are not only in disagreement with our previous finding but also are peculiar in that the activity of the same substance differs in oleic acid and in the methyl ester prepared from the corresponding sample of oleic acid. (Fig. 1 and 2).

The results of the recovery of the glycerophosphoric acids in the aqueous phase during the autoxidation (see Fig. 1 and 2) show, however, that a marked decrease in recovery occurs in the first several hours regardless of whether or not the activity is observed, although a somewhat gradual decrease is shown with the α-isomer during the period in which its activity is depressed. Therefore, it is considered that some minor impurities which could not be removed by low-temperature crystallization from acetone may have interfered so as to give the varied results observed, rather than the difference in the configurations of the glycerophosphoric acids as we suggested in the previous paper.

In the methyl oleate prepared from beef tallow and purified by Method B, both acids were found to be active in an equal strength, (Fig. 4). Comparable results were obtained in oleic acid and its methyl ester prepared from olive oil and purified by the same method, (Figs. 5 and 6). In the latter case, the α-isomer was found to be consistently somewhat more active than the β-isomer. When samples of the substrate obtained from olive oil purified by Method A were used (not presented here), on the other hand, a marked difference in activity was observed, the α-isomer being more strongly active than the β-isomer. It should be pointed out, however, that the β-isomer in methyl oleate did not show activity up to a peroxide value of 10 mM/100 g. but a strong activity in the later stages, Fig. 6. This might have been the case with the β-isomer in the previous report where the experiment was carried out up to this level of peroxide value.

From these results, it is probably certain that α- and β-glycerophosphoric acid are active antioxidants of a comparable strength at a concentration of 0.1%. It is also evident from these results that the purification by low-temperature crystallization from acetone is unsuitable for the study of this nature. Accordingly, those results obtained by using substrates which have been purified by merely repeating distillation of saponified products of fats should be subjected to re-examination.

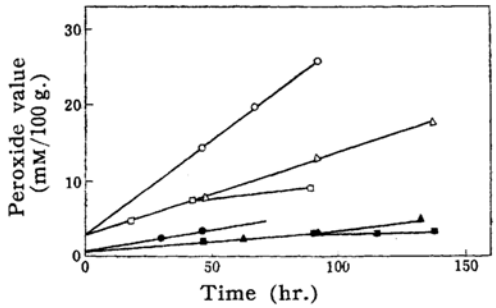

Fig. 4. Peroxide values against time.
○ Methyl oleate, M-2, alone. △ Methyl oleate, M-2, containing 0.1% α-glycerophosphoric acid (86% purity). □ Methyl oleate, M-2, containing 0.1% β-glycerophosphoric acid. ● Methyl oleate, M-3, alone. ▲ Methyl oleate, M-3, and 0.1% α-glycerophosphoric acid (86% purity). ■ Methyl oleate, M-3, containing (0,1%) β-glycerophosphoric acid.

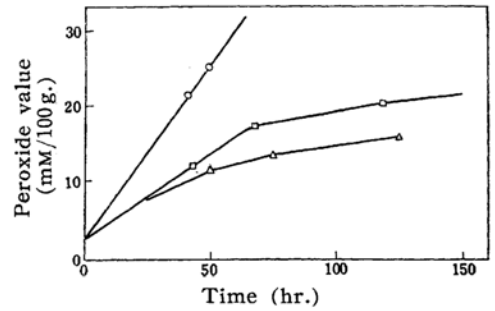

Fig. 5. Peroxide values against time.
○ Oleic acid, O-3, alone. △ The same sample of oleic acid containing 0.1% α-glycerophosphoric acid (79% purity). □ The same sample of oleic acid containing 0.1% β-glycerophosphoric acid.

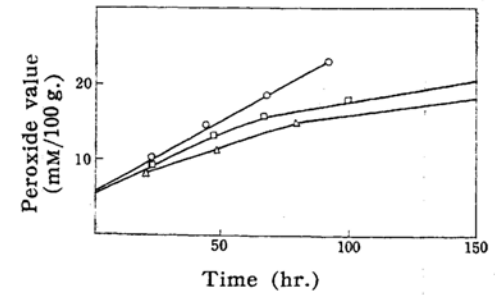

Fig. 6. Peroxide values against time.
○ Methyl oleate, M-4, alone. △ The same sample of methyl oleate containing 0.1% α-glycerophosphoric acid (86% purity). □ The same sample of methyl oleate containing 0.1% β-glycerophosphoric acid.

The absorption spectra observed in the region longer than 300 mμ in Fig. 7 are due to the presence of the α-isomer at a concentration of 7% since the *E* values calculated for the sample of glycerophosphoric acid employed (see Fig. 8) are much greater than those of the substrate before and after autoxidation (see Fig. 9). Therefore, the absorption spectrum observed after 87.5 hours' autoxidation indicates the presence of a substance other than the glycerophosphoric acid itself, probably a substance formed from it during the autoxidation.

With a low concentration of either α- or β-glycerophosphoric acid as actually used (0.1%) for testing their antioxygenic activities, it would be in possible to observe such an absorption in the ultraviolet region before autoxidation but it should be possible after autoxidation, only, however with the sample to which the α-isomer has been incorporated. This is shown in Fig. 9 A where the curve indicated by the solid line represents the absorption due to the presence of an intensely absorbing substance since the substrate isolated from it by the chromatographic means shows a lower intensity of absorption (dotted line). This sort of observation would not be possible with the

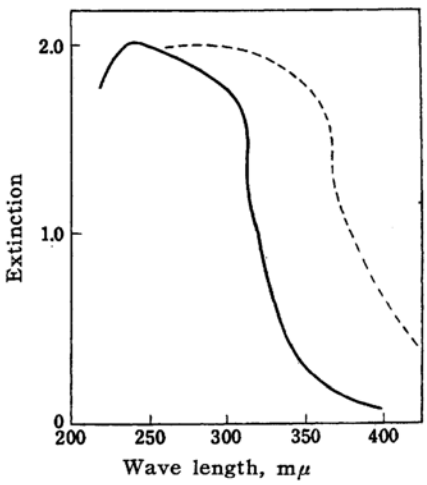

Fig. 7. Absorption curves of 1% ether solutions of methyl oleate containing 7% α-glycerophosphoric acid.
—— At zero time (in ether). ------ At the end of 87.5 hours of autoxidation (in ether).

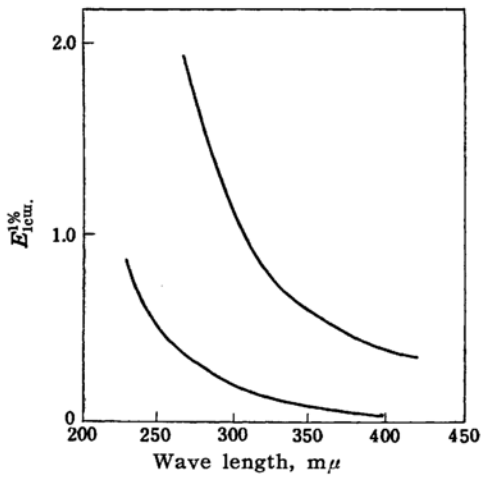

Fig. 8. Absorption curves of the glycerophosphoric acids (ethanol).
The upper curve, α-glycerophosphoric acid.
The lower curve, β-glycerophosphoric acid.

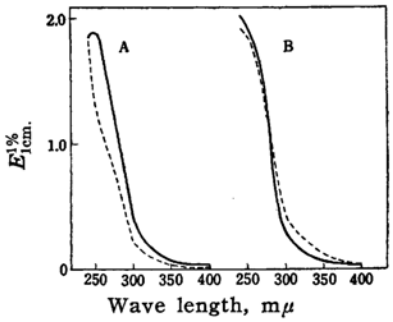

Fig. 9. Absorption curves of the ether solutions of: A, solid line, methyl oleate containing 0.1% α-glycerophosphoric acid (sample b, Table IV); dotted line, the ester isolated by the column chromatography. B, solid line, oleic acid containing 0.1% β-glycerophosphoric acid (sample c, Table IV); dotted line, oleic acid isolated by the column chromatography.

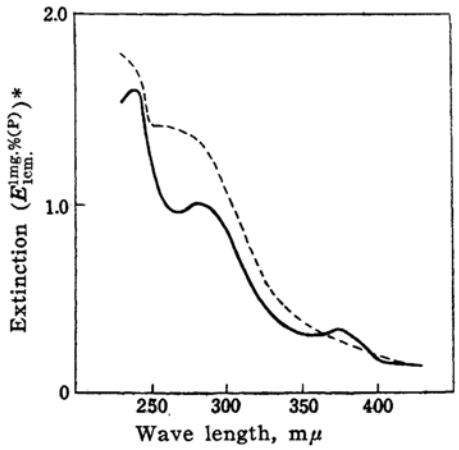

Fig. 10. Absorption curves of 0.1 N sodium hydroxide solutions of the phosphorus containing substances isolated by the column chromatography: dotted line, from the α-glycerophosphoric acid added sample (sample b, Table IV); solid line, from the β-glycerophosphoric acid added sample (sample c, Table IV).

β-isomer since the absorption of its ethanolic solution is much less intense than that of the α-isomer (see Fig. 8), and this found to be the case as shown in Fig. 9B where the absorption curve of the isolated substrate coincides with that of the substrate to which the β-isomer has been added.

Fig. 10 shows the absorption spectra of the phosphorus-containing substances found to be present in the alkaline extracts of the column chromatograms. They differ from those of the glycerophosphoric acids (see Fig. 8). The unknown substance which appears to be formed from β-glycerophosphoric acid shows $E_{1cm}^{1mg\%(P)}$ 1.6 at 240 mμ and 1.0 at 380 mμ and 0.96 at 270 mμ, an inflection. The substance believed to be formed from the α-isomer does not show a sharp maximum but an inflection at 250 mμ with $E_{1cm}^{1mg\%(P)}$ 1.42. This substance is probably a mixture of at least two compounds, one formed from the α- and the other from the β-isomer since the sample incorporated contained some 14% β-isomer and no isomerization is apparent as indicated in Table IV.

Discussion

Although varied reactivities of α- and β-glycerophosphoric acid were observed in different samples of the substrate, both must be active antioxidants since the consistent results could be obtained when highly purified oleic acid and methyl oleate were used. This may reasonably be supported by the isolation of the unknown substances with high intensity of absorption in the ultraviolet region from the substrates to which both α- and β-glycerophosphoric acid have been incorporated.

The antioxygenic activity of the glycerophosphoric acids may, however, be secondary in nature; that is, the unknown substances formed during autoxidation may be responsible for the activity observed, rather than the glycerophosphoric acids themselves. It is unlikely that the involvement of the glycerophosphoric acid molecule is analogous to that of hydroquinone where the molecule itself is ultimately oxidized to quinone after terminating free radical chain reactions by reacting with peroxy radicals[7]. Strong indications for this are that recovery of the glycerophosphoric acids drops sharply in the early stages of autoxidation and that the unknown substances with high intensity of absorption in the ultraviolet region can be isolated from samples which are still exhibiting antioxygenic activity, Table IV and Figs. 7 and 10.

Assuming the molecular weight of the unknown substances to be about 436, (which is the sum of the molecular weight of oleic acid and that of the glycerophosphoric acid minus a mole of water and is considered to be the lowest limit of those oxidized products retaining the original carbon chain length) their molecular extinctions are calculated to be about 42,000 at 270 mμ, at the inflection of the solid line (Fig. 10), and 70,000 at 240 mμ (the maximum). These values are probably higher than those calculated from absorption measured in non-polar solvents but they, nevertheless, indicate that the K-bands are involved. The absorption curve with λ_{max} at 240 and 380 and $\lambda_{infl.}$ at 270 resembles, in its general trend, those reported for oxidized products of ethyl oleate[8] and for the olefinic keto-acids[9]. Therefore, it appears that the glycerophosphoric acids have reacted with some highly conjugated form of compounds produced from the substrate molecule by a free-radical attack during autoxidation.

These results do not reveal, however, whether the phosphoryl group of the glycerophosphoric acid molecule is involved in such a reaction. Because compounds with the same absorption characteristics can possibly be formed, too, if a coordination takes place between a radical of a highly conjugated form (produced from the substrate molecule) and a radical of the glycerophosphoric acid involving the glycerol moiety, or between the former and inorganic phosphoric acid released from the glycerophosphoric acid, as a result of a free radical attack on the glycerol moiety, has been observed to take place in aqueous solutions by irradiation of ionizing radiations[10]. The latter case is least feasible, however, in the system under consideration. Some evidence for this can be provided by the results obtained here. If it had occurred, the absorption band indicated by the solid line in Fig. 9 A or the absorption spectrum observed after 87.5 hours of autoxidation (see Fig. 7) would not have been detected since the E value

7) J. L. Bolland and P. Ten Haave, *Trans. Faraday Soc.*, **43**, 2011 (1947).

8) R. T. Holman, W. O. Lundberg, W. M. Lauer and G. O. Burr, *J. Am. Chem. Soc.*, **67**, 1285 (1945).

9) G. King, *J. Chem. Soc.*, **1951**, 1980.

10) G. Scholes, W. Taylor and J. Weiss, ibid., **1957**, 235.

of orthophosphoric acid in ethanol at λ_{max} 250 mμ is 0.024(measured in our laboratory) and its extinction even at a concentration of 7% is less than 0.167, which is smaller than the E value of β-glycerophosphoric acid, 0.18 at 265 mμ. The β-isomer does not show absorption of the product in the presence of the substrate, (Fig. 9B). The question as to the former case will be treated in a later paper. A highly probable case is, however, participation of the phosphoryl group since phosphoric acid or its alkaline acid salts are known to add readily to ethylene oxide[11] or react with hydroperoxides obtained from lard[12].

Confirmation of the structures of the unknown substances containing phosphorus and exhibiting the high intensity of absorption in the ultraviolet region is of great importance in providing information as to requirements of molecular structures of phosphorus containing compounds of this nature for antioxygenic activity. It also appears to be important from the standpoint of elucidating free radical chain reaction mechanism which is yet obscure and of current interest to a number of workers[8,9,13-15]. The method employed in the present work for the isolation of the unknown substances may find its usefulness in the study of structures of products formed in early stages, if not primary products of a free-radical attack, of autoxidation of ethylenic compounds.

11) F. R. Atherton, H. T. Openshaw and A. R. Todd, ibid **1945**, 382.
12) F. Q. Quackenbush and O. S. Privett, *J. Am. Oil Chemists' Soc.*, **31**, 225 (1954).
13) R. W. Brauer and L. T. Steadman, *J. Am. Chem. Soc.*, **66**, 563 (1944).
14) L. Bateman and J. L. Bolland, *The British Rubber Producers' Association Publication*, No. 126 (1950); L. Bateman and A. L. Morris, ibid., No. 173 (1952); L. Bateman, G. Gee, A. L. Morris and W. F. Watson, ibid., No. 155 (1951).
15) D. Swern, H. B. Knight and J. E. Coleman, *J. Am. Oil Chemists' Soc.*, **28**, 498 (1951); **32**, 79, 221 (1955).

Summary

It has been demonstrated that the method of purification of the substrate by low temperature crystallization is not suitable for the study of antioxidant activity of the glycerophosphoric acids and that further purification by crystallization as the urea complex gives consistent results. In such a highly purified substrate, both acids have been found to be active in a comparable strength.

The study of ultraviolet absorption of the phosphorus-containing substances isolated by the column chromatography has shown that the glycerophosphoric acid molecule acquires a highly conjugated system during autoxidation. Discussions have been presented as to possibilities incurred in the formation of such highly conjugated compounds.

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