PHOSPHOLIPID OXIDATION IN EMULSIONS

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
GLENN A. CORLISS
1968

This is to certify that the

thesis entitled

Phospholipid Oxidation in Emulsions

presented by

Glenn Arthur Corliss

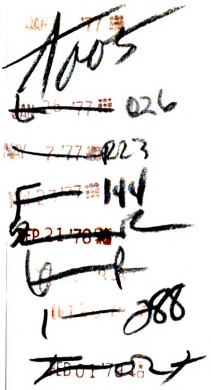
has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science

L. P. Hagan, Jr.
Major professor

Date September 12, 1968

O-169



ABSTRACT

PHOSPHOLIPID OXIDATION IN EMULSIONS

by Glenn A. Corliss

The autoxidation of emulsions consisting of purified phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC), extracted from egg and soybean lipids by silicic acid column chromatography, and 0.1M borate (pH 7.0) buffer was compared by measurement of oxygen uptake, TBA absorbance, ultraviolet absorbance and changes in the percentage composition of unsaturated fatty acids. Egg phospholipids were more reactive than soybean phospholipids and PE from egg and soybean oxidized more readily than egg and soybean PC respectively. All phospholipid fractions emulsified in 0.1M borate (pH 7.0)--Tween 20 buffer had comparable activation energies for oxidation. However, those fractions of a given phospholipid with the most unsaturation oxidized most rapidly. Although unsaturated fatty acid oxidation occurred in all fractions, phospholipid oxidation in emulsions was only partly a function of fatty acid composition. The ethanolamine and choline moieties influenced the induction period for the oxidation of PE and PC respectively. The effect of the nitrogen moiety

on the oxidation of unsaturated fatty acids was investigated in model systems with methyl linolenate emulsions. Ethanolamine was observed to exert a strong prooxidative effect in methyl linolenate emulsions compared to choline which had a slight antioxidative effect.

Specific changes in the phospholipid molecule during oxidation were measured by various physico-chemical tests. Of the two major polyunsaturated fatty acids in egg phospholipids, arachidonic acid disappeared at a more rapid rate during oxidation while the concentration of linoleic acid decreased to a level that was relatively stable. Considerable oxygen was absorbed by PE and PC after arachidonic acid had disappeared completely and linoleic acid had reached its minimum level. Higher TBA and diene and triene conjugation values were observed for PE than for PC. TBA and diene and triene conjugation attained their maximum levels for egg PC when the rate of oxygen consumption began to decrease. These values increased with oxygen uptake for egg PE and continued to increase after the rate of oxygen consumption had decreased. Although diene conjugation values increased as a result of the oxidation of soybean PE, triene conjugation was low at all stages of oxidation. The concentration of the free amino group of egg and soybean PE decreased and a brownish-yellow discoloration developed during oxidation.

Investigations were conducted on the effect of various pro- and antioxidative substances on the autoxidation of phospholipid emulsions. The reactivity of egg PE and PC was decreased by the addition of ethanolamine in 1:1 molar ratios of nitrogen moiety and phospholipid, which was in contrast to the prooxidative effect of the ethanolamine in methyl linolenate emulsions. Copper accelerated the oxidation of PE and PC. The antioxidative effect of ethanolamine and glycine was overcome by the addition of copper. The induction period for the oxidation of PC was less in the presence of ethanolamine plus copper and glycine plus copper than in the presence of copper alone. The greater reactivity which resulted when copper and an amino compound were added together was attributed to the formation of a complex between the metal and the nitrogen moieties. Glycine, lysine, and tryptophan increased the induction period of egg PE while histidine completely inhibited oxidation for the period of study as evaluated by oxygen uptake and TBA determinations. Variations in the antioxidative effect of glycine, lysine, tryptophan and histidine on the oxidation of PE were postulated to occur as a result of differences in the activation energy of the antioxidative reaction which depended upon the particular amino acid that was present. Butylated hydroxytoluene (BHT) and sodium tetra, ethylenediamine tetraacetate (Na EDTA) completely retarded

oxidation of egg PE as measured by oxygen uptake and the TBA test for the period of study. The ability of Na EDTA, a metal chelating agent, to inhibit phospholipid oxidation in emulsions indicated that trace metal impurities could have influenced oxidation. The strong antioxidative effect that was observed for BHT confirmed the work of others and suggested that its presence in lipid samples during their extraction, manipulation, and storage would retard the development of alterations as a result of autoxidation.

PHOSPHOLIPID OXIDATION IN EMULSIONS

By

Glenn A. Corliss

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science

1968

ACKNOWLEDGMENTS

Sincere appreciation is extended to Dr. L. R. Dugan, Jr. for his assistance in selecting the author's course of study and his constructive criticism and counsel during the various phases of the research work.

The author is grateful to Dr. B. S. Schweigert, Chairman of the Food Science Department, for his interest, advice and encouragement.

Appreciation and thanks are extended to the members of the guidance committee: Drs. C. L. Bedford, R. W. Luecke, C. M. Stine and D. E. Ullrey for their critical review of this dissertation.

To the Pillsbury Company, Minneapolis, Minnesota, the author is indebted for a Graduate Training Grant which partially supported this study.

Lastly, the author is especially grateful to his wife, Nan, and son, Kevin, for their understanding, encouragement and sacrifices during this graduate program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF APPENDICES

INTRODUCTION

Autoxidation of lipid materials is a complicated phenomenon even when it involves only the oxidation of an anhydrous polyunsaturated fatty acid under carefully controlled conditions. Autoxidation of fats in foods is further complicated since fat is present as a diverse mixture of many lipids. The fat is often in a finely divided or emulsified state, intimately associated with a mixture of solid components and a complex aqueous phase containing a variety of both soluble and colloidally dispersed materials. In such a system, numerous reactions, both pro- and antioxidative occur simultaneously.

Studies in a variety of food and natural products have shown that the early oxidation reactions are usually most dominant in the phospholipid moieties. The fatty acid composition of phospholipids vary with source but seem to follow a common pattern in that the phospholipid fatty acids are more unsaturated than those found in associated triglycerides and are more highly unsaturated at the β-position than at the α'-position. Since the phospholipids contain phosphorus, a nitrogen containing component such as choline, ethanolamine or serine, and unsaturated fatty acids, they offer a system far more

complicated for oxidation than the neutral triglycerides. One of the unexplained factors of basic interest is whether the tendency of phospholipids to oxidize is due simply to the high unsaturation of the fatty acids of the phospholipids or whether there is a different tendency and therefore rate of oxidation according to the specific molecular constitution of each individual phospholipid.

The purpose of this research was twofold. The major objective was to characterize the role of the nitrogen moiety during oxidation in order to learn whether the oxidation of phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) is related to the degree of unsaturation of the fatty acids or to the nitrogen containing component. Although published reports had indicated that unsaturated fatty acids and the nitrogen moiety are factors affecting the oxidation of phospholipids (1, 59, 77, 99), further study was needed in order to determine the relative importance of unsaturation and the nitrogen component on phospholipid oxidation. Another objective was to investigate the influence of various substances on the susceptibility of phospholipids to oxidation. The potential toxic effect of some commercial antioxidants has increased interest in the utilization of antioxidative substances which occur naturally in biological materials. In this study, the effect of amino acids, ethanolamine, choline, copper, butylated

hydroxytoluene and sodium tetra, ethylenediamine tetra-acetate on the oxidation of PE and PC was determined.

Egg yolks and a commercial soybean phosphatide preparation were utilized as sources of phospholipids. PE and PC were fractionated by silicic acid column chromatography and their purity was ascertained by thin-layer chromatography. Purified PE and PC were emulsified in borate (pH 7.0) buffer prior to the measurement of oxidation, since it was felt that oxidation under a neutral pH in the presence of moisture could be more readily controlled in terms of oxidation rate and would be more nearly typical of changes occurring in a number of natural food systems than oxidation in a dry system. Autoxidation of PE and PC emulsions was measured by oxygen uptake, TBA absorbance, ultraviolet absorbance and by changes in the percentage composition of unsaturated fatty acids.

LITERATURE REVIEW

Mechanism of Autoxidation

The spontaneous reaction between atmospheric oxygen and many types of organic compounds has been termed autoxidation. Autoxidation is an autocatalytic reaction in that the rate of reaction increases with time, due to the formation of products which themselves catalyze the reaction. Autoxidation may be beneficial or deleterious depending on the conditions and circumstances under which it occurs (115). It is advantageously employed in the production of blown oils and in various oxidation and polymerization products in the drying oil industry. Large quantities of organic chemicals are produced by the controlled oxidation of petroleum hydrocarbons (115).

Rancidification and other forms of oxidative deterioration of many foods result from autoxidation but are undesirable. Oxidative rancidity in foods has been traced to the lipid portions, in which both the simple triglycerides and the more complex phospholipids may be involved. The common feature of oxidative rancidity is the reactivity of the unsaturated fatty acid moieties in the lipids (28). In practice, the autocatalytic autoxidation of food lipids almost always involves the

presence of catalytic entities, such as prooxidants, antioxidants and light.

Autoxidation occurs in a series of reactions involving initiation, propagation and termination steps. These steps have been formulated as (28):

Initiation

$$RH + O_2 \longrightarrow R\bullet + \bullet OOH \qquad [1]$$

Propagation

$$R\bullet + O_2 \longrightarrow ROO\bullet \qquad [2]$$

$$ROO\bullet + RH \longrightarrow ROOH + R\bullet \qquad [3]$$

Termination

$$R\bullet + R\bullet \longrightarrow RR \qquad [4]$$

$$R\bullet + ROO\bullet \longrightarrow ROOR \qquad [5]$$

$$ROO\bullet + ROO\bullet \longrightarrow ROOR + O_2 \qquad [6]$$

RH refers to any unsaturated fatty acid in which the H is labile by reason of being on a carbon atom adjacent to a double bond. R• refers to a free radical formed by removal of a labile hydrogen. The over-all oxidation may be expressed as $RH + O_2 \longrightarrow ROOH$ up to a certain stage of oxidation. The oxidation process becomes more complex after the development of a quantity of hydroperoxides (ROOH), for the ROOH then decomposes, either through thermal instability or through reaction with other materials, to form more free radicals which then participate further in the chain reactions (28).

Several features of the autoxidation of common food fats have been summarized by Lundberg (68). They are:

1. The reaction involves, primarily, unsaturated acyl groups, and the hydroperoxide group formed as a result of autoxidation, appears in an alpha position relative to a double bond.
2. The rates of autoxidation are greatly dependent upon the degree of unsaturation of the fatty acids, increasing in an exponential manner with increasing unsaturation. Derivatives of saturated fatty acids autoxidize to form hydroperoxides of undetermined structures, although under ordinary conditions, the reaction is extremely slow.
3. Various extraneous influences, if present, can affect the rate of oxidation of any given food fat. Prooxidants, including trace metals and biological catalysts, accelerate the rate of oxidation. All forms of light radiation have marked accelerating effects. A multitude of substances, especially various phenolic compounds, exert an antioxidant or negative catalytic effect.
4. As with other chemical reactions, an increase in temperature increases the rate of autoxidation.
5. While the major products of fat autoxidation are hydroperoxides, certain secondary products, not derived from peroxides, are formed concurrently. In particular, with unsaturated fatty acids, small amounts of conjugated ketones are produced with the hydroperoxides.
6. The undesirable flavors and odors of autoxidized food materials are not due to the hydroperoxides themselves, but to secondary substances such as aldehydes, ketones, acids and polymeric compounds which are derived through various reactions and to further oxidation of the peroxides and their degradation products.

Lipid Oxidation in Aqueous Media

Several reactions occur when polyunsaturated fatty acids or their derivatives are dispersed for extended periods in water in the presence of air. As early as

1937, Bloor and Snider (8) discovered that unsaturated fatty acids become water-soluble upon autoxidation. Schulte and Schillinger (110), while studying the non-enzymatic oxidation of vitamin A acetate, established that the reaction catalyzed by oleic acid proceeded faster in a two-phase oil-water system than in a homogeneous nonaqueous phase. In contradiction, Spetsig (114) reported that the autoxidation of methyl linoleate was inhibited by water although, after the chain reaction had started, its course was not affected by the presence of water of pH between 5.1-9.2. Mabrouk and Dugan (70) observed that the oxidation rates of methyl linoleate emulsions increased markedly as the pH of the emulsion was raised from 6.0 to 8.0 while the oxidation rates of linoleic acid emulsions increased gradually from pH 4.5 to 5.5 and then decreased gradually to a minimum at pH 8.0.

The oxidation of lipids in water is strongly influenced by the degree of contact between the lipid and the oxygenated aqueous phase. The autoxidation of oil emulsified in water with and without detergents increased with the degree of dispersion of the oil and the effect of the detergents was greatest for oils with the most double bonds (52). Research on the oxidation of carotene

in linoleate soap solution has demonstrated that there is a critical middle concentration of linoleate where oxidation is minimal. When the linoleate concentration was below this middle concentration, the oxidation of carotene was not coupled to the oxidation of linoleate; however, the rate of autoxidation of both carotene and linoleate increased when the level of either was raised provided the linoleate concentration was above the critical middle concentration (66). Studies on the oxidation of methyl oleate after irradiation with UV light or saturation with oxygen at 50°C have shown that oxidation proceeded slower in water than in the dry state (117). These results were attributed to a higher solubility of oxygen in oil than in water. The addition of a surfactant or improved dispersion in the absence of surfactant increased the rate of oleate oxidation.

At one time, it was thought that the presence of water-soluble products in aqueous dispersions of polyunsaturated compounds resulted from the transfer of impurities from the original material to the aqueous phase (107). This possibility was eliminated by the finding that a higher yield of ultraviolet-absorbing water-soluble products were obtained when several successively smaller portions of highly purified starting material were dispersed in water (106). Infra-red spectroscopy of the dry residue from the clear filtered water phase

demonstrated the presence of hydroxyl groups and a reduction in the intensity of double-bond absorption (106). One result of the chemical interaction of linoleic acid and water is the formation of a saturated tetrahydroxy fatty acid (71). The identification of "active oxygen" in the aqueous phase (109) and the finding that the exclusion of oxygen from the dispersion prevented the formation of hydroxy compounds (108), strongly indicated that oxidation could occur when unsaturated lipids were dispersed in water.

The first major reaction in the aqueous oxidation of linoleic acid is believed to be identical with that which occurs in the autoxidation of dry linoleic acid, namely the formation of hydroperoxides. Measurement of the UV absorption of the aqueous phase after prolonged dispersion of linoleic acid in water has indicated the presence of conjugated double bonds (105). Hydroperoxides are known to be unstable and to react further with oxygen to yield secondary products such as aldehydes, ketones, dimers and polymers, and epoxides. A characteristic feature of the oxidation in aqueous suspension is that the water favors the formation of such tertiary products as hydrogen peroxide and carboxylic acids by the hydrolysis of secondary products (105). These products are formed only in minute amounts in the absence of water.

Baker and Wilson (4, 5) succeeded in separating the water-soluble products of UV-irradiated linoleic and linolenic acids into volatile and relatively non-volatile fractions, each of which reacted with thiobarbituric acid and peroxidase. The volatile fraction contained hydrogen peroxide and probably malonaldehyde and the non-volatile fraction had at least twelve compounds (approximate chain length, 7C-13C) containing α-β-unsaturated carbonyl groups. A number of compounds formed as a result of the aqueous oxidation of polyunsaturated acids have been identified by Schauenstein and co-workers (105). Since most of these compounds are short-chain products they are continuously eluted by the aqueous phase and become unavailable for further reaction in the oil phase. As a consequence, the oxidation of polyunsaturated lipids in water provides an opportunity for the isolation and identification of intermediate products from classical autoxidation. Many of these intermediate products have biological and medical significance in addition to being important in the deterioration of food lipids.

Lipid Autoxidation Tests

The oxidative degradation of unsaturated fatty acids can be followed by determining the total consumption of oxygen, by determining the amount of a product of lipid oxidation or by measuring the decrease in the concentration of unsaturated lipids.

Peroxide Test

The quantitative determination of peroxides is the most commonly used analytical method for following the course of autoxidation reactions (115). The peroxide test is based on the assumption that potassium, sodium or hydrogen iodide will liberate iodine in the presence of peroxidized product. The iodine, liberated in a stoichiometric ratio of two atoms of iodine for each atom of active oxygen, can be quantitated by titration with sodium thiosulfate. Although numerous peroxide methods have been proposed, results on a given sample, using different methods and different operators, are often quite erratic. Some investigators attribute this to errors in the methods or procedures, while others are inclined to suspect the peroxides themselves (65).

TBA Test

The 2-thiobarbituric acid (TBA) test has been applied to the estimation of rancidity in dairy products, meats, fishery products, cereal and baked products, and fats and oils (16). The pigment produced in this sensitive color reaction is known to be a condensation product of two molecules of TBA and one of malonic dialdehyde. A feature of the TBA test is its comparative specificity for oxidation products of polyunsaturated fatty acids. The TBA test can be applied directly to a lipid-containing material without prior extraction of the fat. The test

supposedly gives a more reliable measure of the extent of oxidation than the peroxide determination in cases where substances other than fat are present and cause decomposition of peroxides (67).

Ultraviolet Absorption

Oxidation of polyunsaturated fatty acids is accompanied by increased ultraviolet absorption due to the formation of conjugated diene and triene hydroperoxides. The infrared spectra of the mixed hydroperoxides from autoxidized linoleate, isolated by countercurrent distribution, indicated that the hydroperoxides assumed the cis-trans and trans-trans configurations (92). Cis-trans hydroperoxides predominated at low temperatures while trans-trans hydroperoxides were more prevalent at high temperatures and high levels of autoxidation. The magnitude of change of ultraviolet absorption is not easily related to degree of oxidation since the effects upon the various unsaturated acids (oleic, linoleic, linolenic, arachidonic, e.g.) are different in quality and magnitude (47). The spectral change for a given substance, however, can be used as a relative measure of oxidation and probably has its best application in the detection of oxidation rather than its measurement (115). The higher the absorption, the greater has been the exposure to oxygen.

Oxygen Absorption Tests

Manometric respirometers such as those used for biochemical respiration studies have been employed in many lipid laboratories to measure oxidation. In the usual set-up, a lipid sample in a closed glass chamber is agitated in a liquid bath at a constant temperature. The atmosphere in the chamber is either air or oxygen. As oxidation progresses, the amount of oxygen absorbed is indicated by a decrease in pressure which can be determined manometrically (67).

Miscellaneous Tests

Since there is no single approach to the problem of measuring lipid oxidation, various methods have been developed. Thin-layer chromatography has been used to detect and classify peroxides in complex lipid mixtures (82). Changes in the concentration of unsaturated fatty acids of phospholipids have been evaluated by gas-liquid chromatography (26, 99) and iodine values (59). The disappearance of the free amino group of phosphatidyl ethanolamine during oxidation has been measured by the ninhydrin reaction. Much attention has been directed toward the separation, estimation and identification of the total and volatile carbonyl degradation products from oxidizing fats. The most widely used method of determining carbonyl substances formed by the secondary degradation of hydroperoxides is probably the colorimetric

2,4-dinitrophenylhydrazine procedure of Henick et al. (45). A comprehensive review of carbonyl determinations is beyond the scope of this review. However, methods for the separation and identification of carbonyl compounds have been reviewed by Day (18) and Badings and Wassink (3).

All known oxidation tests have limitations, since no single procedure has proved adaptable to all lipid-autoxidizing systems. There is a definite need to devise more methods to characterize, objectively, accurately and rapidly, changes which occur during the autoxidation of lipid materials.

Phospholipids--As Antioxidants

Phosphatides are reported to have two opposing features when present in food products: participation in the protection of neutral lipid autoxidation and the acquirement of a fishy odor and off-flavor by decomposition and/or change. The debate over whether phospholipids are antioxidants or prooxidants has yet to be completely resolved. "Lecithin" was among the first of a number of naturally occurring edible products proposed as an antioxidant in foods (10). Most "lecithin" preparations have been crude fractions obtained from vegetable oils or animal fats on the basis of their solubilities in various solvents and have contained relatively large amounts of phosphatides other than phosphatidyl choline.

The conflicting reports in the literature on the antioxygenic activity of "lecithin" in various substrates probably results from the heterogeneity of the "lecithin" fractions utilized as well as the variety of substrates and testing methods employed (14).

In 1935 Evans (32) observed that crude vegetable lecithin appreciably lengthened the induction period of vegetable oils. Olcott and Mattill (85) reported that purified "lecithin" was inactive in lard while purified cephalin was a good antioxidant. Swift et al. (116) established by peroxide determinations that the antioxygenic activity of α-tocopherol in cottonseed oil was increased by the presence of a crude fraction of cottonseed cephalin. The antioxidant property of the cephalin molecule has been traced to the phosphoric acid group (85). The inactivity of phosphatidyl choline has been attributed to its existence in a zwitterion form involving the free hydroxyl group of the phosphoric acid radical and the nitrogen of the strongly basic choline (83). Since the nitrogen in ethanolamine is a much weaker base than the nitrogen in choline, it was suggested that the phosphoric acid group has a greater tendency to remain free in phosphatidyl ethanolamine (83).

The original description of antioxidants and synergists (86) differentiated between substances which were effective alone in relatively low concentration such

as tocopherols and phenolic compounds and those substances which had little activity by themselves but were effective in combination with the phenolic inhibitors. Citric, ascorbic and phosphoric acids and phospholipids have been classified as synergists. While phospholipids are usually regarded as synergists, certain phosphatides and glyceride acid phosphate esters may have true antioxidant activity (22). Privett and Quackenbush (93) observed that wheat germ phosphatides and phosphoric acid prevented the accumulation of peroxides in both fresh and partially oxidized wheat germ oil. This preventive action was attributed to the formation of polymeric substances by a reaction involving peroxides and the phosphoric acid group.

Patents proposing the use of phosphoric acid to retard the development of oxidative rancidity in fats were assigned to the Proctor and Gamble Company as early as 1934. At about the same time, Lea (57) observed that when lard was in contact with a phosphate buffer it oxidized more slowly than when it was in contact with other buffers, such as borate and acetate at the same pH. The effectiveness of phosphoric acid in vegetable oils but not in purified methyl esters was established by Olcott and Mattill (86). Golumbic (37) attributed its synergistic activity to its ability to regenerate phenolic antioxidants from their quinones. The activity of

phosphoric acid has also been ascribed to its ability to chelate heavy metals (31) and to decompose fatty peroxides (94).

Studies on the drying of linseed oil indicated that the mechanism of drying inhibition was antioxidant in nature (23). Phosphatides, containing the smallest percentage of nitrogen appeared to have the greatest inhibiting action as evidenced by the increase in drying inhibition for egg and soybean "lecithins" when choline was removed. The abnormally long drying time for a sample of linseed oil was related to the presence of 0.84% phosphatidic acid.

Recognition that the phosphate group of phospholipids could act as an antioxidant or synergist has encouraged studies to determine if the nitrogen moiety could contribute to the stabilization of unsaturated fatty acids. Choline has been reported to be prooxidative for ethyl oleate (24) and in lard emulsions (63). Urakami and Kameyama (120) found that choline failed to exhibit any antioxygenic action against purified methyl oleate, although phosphoryl choline, phosphoryl choline chloride and natural phosphatidic acid were active antioxidants. Olcott and Van der Veen (87) observed that saturated phosphatidyl choline was a more effective synergist for ethoxyquin in menhaden oil than was phosphatidyl ethanoamine. The latter finding was consistent with the

observation that tertiary and secondary amines were usually superior to primary amines as synergists for ethoxyquin (84).

Phospholipids--As Prooxidants

Even though most research efforts have studied the use of phosphatides as antioxidants or synergists, the prooxidative nature of phospholipids has been under investigation. In general, the antioxygenic mechanisms of phospholipids have been related to the phosphate or nitrogen moieties rather than to the preferential oxidation of unsaturated fatty acids. The fatty acid composition of polar and neutral lipids varies with the source but appears to exhibit a common pattern in that the fatty acids of phospholipids are more unsaturated than the fatty acids in associated triglycerides. The tendency of lipids to undergo oxidation is greatly enhanced by the degree of unsaturation.

According to Patton (88), the susceptibility of phospholipids to oxidation depends upon whether they are in water or fat. In the aqueous phase of milk, triglycerides are relatively stable and the phospholipids are preferentially oxidized. With butter, which represents an aqueous concentrate of phospholipid dispersed in fat, both fat and phospholipid are susceptible, the latter being most readily oxidized. When water is absent, such as in dried milk products, the triglycerides

are relatively susceptible to oxidation whereas the phospholipids are more stable and when present with the triglycerides, serve as an antioxidant. In contrast, El-Gharbawi and Dugan (30) reported that the losses of polyunsaturated fatty acids in freeze-dried raw beef were more pronounced in phospholipid than in neutral lipid fractions after storage. Younathan and Watts (126) observed that the tissue lipids remaining after extraction of cooked pork with a mixture of chloroform-methanol were more readily oxidized than the extracted lipid. Lea (60) fractionated egg yolk into crude fractions, stored them separately at 37°C and subsequently incorporated the fractions into fresh egg for tasting. In this way the off-flavors that developed were traced to the phospholipid fraction.

Experiments on the oxidative deterioration of cod flesh lipids indicated that triglycerides were highly resistant to autoxidation even when hemoglobin, a prooxidant was added. Phosphatidyl ethanolamine oxidized rapidly while phosphatidyl choline required a much longer period of time or a prooxidant before it began to absorb appreciable oxygen (99). Lea (59) reported that the phosphatidyl ethanolamine of egg, spread in thin films at 37°C, oxidized at a phenomenal rate while phosphatidyl choline, which constitutes the bulk of egg phosphatide, oxidized at a considerably lower rate. The rapid

oxidation of milk phospholipids has been attributed to fractions containing phosphatidyl ethanolamine and phosphatidyl serine (77). Acosta et al. (1) observed that phosphatidyl choline fractions oxidized more rapidly than other phospholipid fractions from turkey during early stages of oxidation. However, this anomaly was caused by phosphatidyl serine contamination since phosphatidyl serine was eluted with phosphatidyl choline in the elution scheme.

Mattsson and Swartling (77) have summarized those factors known to effect the rate of oxidation of phospholipids in aqueous dispersions. They are: (1) the degree of unsaturation of the phospholipid; (2) the nature of its nitrogenous constituent; (3) the presence or absence of oxidation catalysts or repressors; (4) the pH of the dispersion; and (5) the dispersibility of the phospholipid in solution which is related to the electrolytic state of phospholipid polar groups and to the pH. The importance of these factors singly and the manner in which they can interact to influence oxidation requires further study.

Metal Catalysis

Heavy metals possessing two or more valency states with a suitable oxidation-reduction potential between them (e.g., Co, Cu, Fe, Mn, Ni) generally increase oxidative deterioration of lipids by reducing the length

of the induction period and by increasing the maximum rate of oxidation. The metal catalyst appears to act by increasing the formation of free radicals since the removal of the metal catalyst from the substrate during the initial stages of oxygen uptake reduces the subsequent rate of oxidation (20). Furthermore, the addition of a strong free radical inhibitor to an autoxidizing system suppresses the chain propagating steps and prevents further oxidation for a period of time that is proportional to the concentration of the added inhibitor (21).

Oxidation catalyzed by metals in polar and non-polar systems has been observed to commence at the same time that the catalyst appears in its higher valency state (7). Free radicals are readily formed by the transfer of an electron from a metal ion to a hydroperoxide molecule. Metals can influence the autoxidation of unsaturated lipids in water by reacting in an aqueous phase with water-soluble radicals such as $\cdot OH$, $HO_2\cdot$ and hydroperoxides or by reacting at the oil-water interface with radicals, hydroperoxide and the substrate (49). Many of these radicals which are dissolved in solution can return to the organic phase and increase the rate of oxidation.

Many food lipids are present in aqueous emulsions along with hydrated metal ions. These metals, whether purposely added or supplied by metal activated enzymes

and their decomposition products, exist in the form of a coordination complex or as the salts of organic and inorganic acids (49). Metal salts dissolved in an aqueous phase in contact with a fat have been shown to have a detectable effect on the rate of autoxidation of the fat at one part in 1000 million (58). At low catalyst concentrations, the rate of oxidation is generally proportional to the concentration of the catalyst (49). However, the catalytic effect reaches a constant value at a specific catalyst concentration with the result that further increases in the amount of catalyst are unable to affect the oxidation rate (6).

The effectiveness of a chelating agent in reducing the prooxidant nature of metals is related to its concentration and the concentration of metals present. Several complexes can be formed from the combination of chelating agent-metal salt-hydroperoxide depending on the relative concentration of each species. These complexes often have different catalytic activity. Chelation may suppress, intensify or have no effect on the catalytic activity of metals depending on the nature of both the chelating agent, the metal and the complex (13). The action of a chelating agent in reducing the catalytic activity of a metal has been attributed to a steric or blocking effect on the formation of a complex between the metal and hydroperoxides (13). Also electrical conductance

determinations have indicated that chelation can modify the oxidation potential of metals (51).

Effect of Amino Acids on the Autoxidation of Unsaturated Fatty Acids and Esters

Amino acids have been mentioned as being both antioxidants and prooxidants. All amino acids appear to possess a potential antioxidative capacity and this capacity is not dependent on the presence of a primary antioxidant such as the tocopherols (76), although amino acids can behave as synergists in the presence of a primary antioxidant (75). The antioxidative effect of amino acids is only a potential one since they may be prooxidative, more or less ineffective, or antioxidative, depending upon the circumstances (76). Whether amino acids act as prooxidants or antioxidants has been shown to be a function of pH, concentration, and the presence or absence of metal ions, nonionic emulsifiers and phosphate (102).

Factors Affecting Oxidation of Lipid-Amino Acid Emulsions

The effect of glycine, histidine, and tryptophan on linoleic acid and methyl linoleate dispersed in water was exclusively prooxidative and increased with increasing concentration of amino acid (76). Histidine has been reported to catalyze the autoxidation of methyl oleate, a monounsaturated fatty acid ester (39). The threshold

concentration of histidine necessary for prooxidative action was observed to be greater than 0.0001M (102). In the presence of 0.1M phosphate buffer at pH 5, 6, and 7, the effect of glycine, histidine, tryptophan, alanine, serine, lysine and proline on methyl linoleate was exclusively antioxidative. Cysteine was prooxidative with and without phosphate buffer at pH 5, 6, and 7. However, it acted as an antioxidant at pH 9.5 (76).

Nonionic emulsifiers such as Span 20 and Tween 20 repressed the prooxidative action of histidine. Ionic emulsifiers such as potassium myristate, sodium palmitate and sodium dodecylsulfate supported the prooxidative action of histidine (15, 102). The effect of 0.7% Tween 20 on the rate of oxidation of amino acids was slightly retarding in water and in phosphate buffer. At higher concentrations, Tween 20 accelerated oxidation in the presence of phosphate and amino acids at pH 7 and retarded oxidation at pH 5 and 6 (76).

Trace metals accelerate methyl linoleate oxidation. The effect of ferrous or ferric ions was more than double that of cupric ions. Autoxidation as measured by oxygen uptake was immediate and rapid when ferrous or ferric ions were added with histidine and was greater than that of cupric ions and histidine. The linoleate peroxide content was increased more than three-fold by the addition of 1 ppm of Fe^{+2} and Fe^{+3}, approximately twenty-fold by

a 0.01M concentration of histidine and more than sixty-fold by the addition of both histidine and ionic iron (102).

Metal Ions

Histidine is known to complex with metal ions and oxygen (41). The catalyzing effect of ionic iron and histidine on the autoxidation of emulsified linoleate could result from the formation of an iron-histidine complex. The activity of histidine, when iron is not added, might be due to a complex between histidine and trace metals present as impurities. The catalytic action of high concentrations of amino acids on the autoxidation of methyl linoleate dispersed in water could thus result from an interaction between amino acids and trace metals (102). Linoleic acid (121) distilled in all-glass apparatus and purified methyl linoleate (122) have been reported to contain traces of copper, iron and cobalt. Also it is difficult to obtain amino acids containing less than 1-3 ppm of various trace metals, particularly if the amino acid has been prepared from biological sources (102). Trace metals can be contributed by glassware and by the lipid and amino acid samples.

Amino Acid Concentration

Histidine alone or added with iron has no appreciable prooxidative effect at 0.0001M (102). Studies on

the chelation of ferrous and ferric ions by histidine established that the ferrous-histidine chelate was unstable at low histidine concentrations because histidine was not able to compete successfully with the hydroxyl ion. In the presence of oxygen, the chelated Fe^{+2} was rapidly oxidized to Fe^{+3} and the competition of OH^- for Fe^{+3} was so strong that it prevented the formation of a ferric-histidine chelate at low ratios of histidine (41).

pH

The concentration of histidine and metal available for complex formation is probably related to the pH. With increasing pH, the concentration of OH^- increases, reducing the effective concentration of Fe^{+2} or Fe^{+3}. At pH values lower than 5.2 it has been reported that histidine was unavailable for chelate formation (41). Optimum catalysis would appear to occur when the product of the concentration of available free metal ion and free histidine is maximum, around pH 6.5 (102).

Emulsifiers

The effect of emulsifiers on the prooxidative and antioxidative action of amino acids is not understood. The reason for using an emulsifier is to decrease the size of lipid particles in an emulsion, thereby increasing surface contact between the lipid and the aqueous phases. However, the prooxidative action of ionic emulsifiers and

the antioxidative effect of nonionic emulsifiers in the presence of histidine suggests that more is involved than the size of the dispersed lipid particles.

An investigation by Coleman et al. (15) of factors affecting the autoxidation of methyl linoleate in emulsion provides some insight on the possible role of ionic and nonionic emulsifiers. In an emulsion polar groups such as carbmethoxy, hydroperoxide (initially present or formed in the reaction) and the hydrophilic group of the emulsifier are oriented at the interface toward the water phase. When an ionic emulsifier such as sodium dodecylsulfate is utilized, the linoleate boundary will have a negative electrical charge due to the ionic sulfate group. A second charged layer of positive ions (sodium and hydrogen) exist on the aqueous side of the interfacial boundary. In catalyzed reactions trace metals, present as impurities or deliberately added, and amino acids, oriented with the amino groups adjacent to metal ions, will be concentrated at the interface. With dissolved oxygen distributed uniformly in each phase and catalysts and reactive groups present at the interface in high concentrations, energy transfer could easily occur. A nonionic emulsifier cannot contribute an ionized charge to the lipid-aqueous interface. As a result metal ions and amino acids would be distributed randomly throughout the bulk aqueous phase, reducing the opportunity for them to react as a complex or singly at the interface.

Phosphate

The synergistic effect of phosphate on fat oxidation has been reported (12, 29, 37, 50, 78) and has been ascribed to metal binding, to the regeneration of primary antioxidants and to the retardation of the oxidation of primary antioxidants. Since phosphoric acid has been shown to react with fatty peroxides and to prevent their estimation by the usual iodometric methods (94), the phosphates may inhibit autoxidation by accelerating the decomposition of hydroperoxides. Thus the stronger antioxidant effect of amino acids in phosphate buffer probably can be attributed to the action of the phosphate group in complexing metal contaminants and in reacting with primary oxidation products, thereby permitting amino acids to function as antioxidants.

Effect of Amino Acids on Phospholipid Oxidation

An aqueous dispersion of phospholipids and amino acids might be expected to behave like unsaturated fatty acid esters and amino acids emulsified in phosphate buffer. Hirano (46), on the basis of manometric studies, reported that amino acids have an antioxidative effect on the autoxidation of phosphatidyl choline in the absence of nonionic emulsifier and phosphate buffer. Using phosphatidyl choline, methyl linolenate, amino acids, choline chloride and phosphate buffer he demonstrated that the phosphate segment was mainly responsible for the

antioxidative effect of amino acids in phosphatidyl choline autoxidation.

Mechanism of Antioxidation

Antioxidants are considered to function in two ways, either as inhibitors of free radical formation or as decomposers of peroxides. Since the formation of free radicals cannot be entirely suppressed, most research has been concentrated in the area of free radical acceptors or peroxide decomposers. Peroxide decomposers act as catalysts to decompose peroxides initially present as well as those that are formed during further oxidation. An important feature of this decomposition process is that the primary stable products are not free radicals (28).

Studies by Bolland and Ten Have (9) led to the proposal of a mechanism in which antioxidants acted as hydrogen donors or free radical acceptors. From the kinetics of the reaction it appeared that the free radical acceptors (XH_2) reacted primarily with $RO_2\cdot$ and not with R·, as follows:

$$RO_2\cdot + XH_2 \longrightarrow ROOH + XH\cdot$$

$$XH\cdot + XH\cdot \longrightarrow X + XH_2$$

Antioxidants that function as free radical inhibitors react with free radicals to form inert products as a termination step in the chain-reaction mechanism.

Peroxy radicals probably predominate in this termination step since hydrocarbon free radicals react readily with molecular oxygen (28).

The phenomenon of synergism in antioxidant action occurs when two or more compounds have a more pronounced antioxidant effect together than the sum of their individual effects. Synergism has been produced in fat by a combination of two phenolic antioxidants (27, 73) and by the use of an acid with a phenolic antioxidant (12, 37, 50). The increased action of two phenolic antioxidants in reducing autoxidation has been hypothesized to result because one of the two antioxidants has a lower activation energy (due to a lower bond dissociation energy) and a lower steric factor (due to a loss of rotational freedom in the transition state) than the other (123). Acid synergists are assumed to function by deactivating metals present as impurities.

Although a free radical acceptor appears to be essential for achieving protection against all prooxidant impurities, it can be supported by a metal-complexing reagent, capable of inactivating part of the trace metal content. Since free radicals are produced in an electron transfer reaction involving different valency states of the metal, those metal complexing reagents which reduce electron transfer would have metal-inactivating properties (123).

EXPERIMENTAL

Materials

Sample Preparation

Fresh, grade A, large eggs purchased from a local retail market were used as one source of phospholipids. Soybean phospholipid fractions were isolated from Centrolex P, a granular oil-free phosphatide preparation supplied by Central Soya, Chicago, Illinois.

Chemicals and Reagents

Solvents.--All solvents were A.C.S. reagent grade and were used directly from the container unless redistillation was specified in the procedure.

Lipids.--Methyl esters for GLC standards and methyl linolenate used for model lipid emulsion studies were obtained from the Hormel Institute, Austin, Minnesota.

Column Chromatography.--Silicic acid, employed for column chromatography of phospholipids, was purchased from Mallinckrodt Chemical Works, St. Louis, Missouri.

Thin-Layer Chromatography (TLC).--Silica Gel-G, supplied by Brinkman Instruments, Westbury, Long Island, New York, was used for all TLC work.

Gas-Liquid Chromatography (GLC).--Diethylene glycol succinate (DEGS), high temperature stabilized was made by Analabs Inc., Hamden, Connecticut. Chromosorb W, 80/100 mesh size, acid washed, was obtained from Applied Science Laboratories, State College, Pennsylvania. Gases for the chromatograph came from the Liquid Carbonic Division of General Dynamics and were supplied through Michigan State University scientific stores.

Specific Chemicals.--Amino acids were ordered from General Biochemicals, Chagrin Falls, Ohio. The various chemicals added to methyl linolenate dispersions to determine the effect of ethanolamine and choline bases on autoxidation were purchased from Sigma Chemical Company, St. Louis, Missouri. All other chemicals used and not mentioned here were of analytical reagent grade.

Methods

Extraction of Egg Phospholipids

Egg phospholipids were extracted by the procedure of Ansell and Hawthorne (2). Yolks from one dozen eggs were homogenized with 500 ml of acetone. The extract was filtered and the solid re-extracted in the same way. The residue was shaken with 300 ml of fresh chloroform-methanol (1:1 v/v) and filtered with suction. This extraction was repeated with 300 ml of fresh chloroform-methanol and the extracts were combined and stored under refrigeration until further use.

Silicic Acid Column Chromatography

Silicic acid (Mallinckrodt) was washed with deionized water until no turbidity was observed in the supernatant after coarse particles had settled to the bottom of the container. The silicic acid was then transferred to a Buchner funnel, washed with methanol, activated by drying for 24 hours at 120°C, and stored in a tightly stoppered bottle.

Two types of columns were used for chromatography: (1) a 2.5 cm o.d. x 30 cm column containing a 300 ml reservoir at the top of the column and a coarse sintered glass disc and Teflon stopcock at the bottom of the column; and (2) a 5 step multibore column (33) constructed of glass sections fused together and having the following dimensions:

o.d. (cm)	length of section (cm)
2.80	13.5
2.20	13.5
1.70	13.0
1.50	12.5
1.37	13.5

The 5 step column was designed with a 250 ml reservoir containing a side arm with Teflon stopcock to facilitate draining. Glass wool served to prevent the silicic acid from passing through the stopcock at the bottom of the column.

Better resolution of individual phospholipids was achieved with the 5 step multibore column than with the conventional column. Assessment of effectiveness of the resolution by the two columns was made on the basis of TLC of eluted fractions. Less overlap of components was observed for fractions collected from the 5 step column. The 5 step column had a larger sample capacity; however, more time and a larger volume of solvents were required to elute phospholipid fractions from the 5 step column than from the conventional column.

Activated silicic acid (50 g for the 2.5 x 30 cm column and 65 g for the 5 step column) was slurried in an excess of chloroform in a beaker with a magnetic stirrer. After stirring until the mixture was homogeneous and translucent, it was poured into the column in such a way that no air bubbles were trapped. The silicic acid was allowed to settle and washed respectively with acetone, methanol, and chloroform to determine whether undesirable channeling existed in the column. A 1-2 cm layer of powdered anhydrous sodium sulfate was then placed on the top of the packed column to absorb any residual moisture in the sample.

Column chromatography was performed at room temperature under nitrogen and with a fraction collector in a cold room at 2°C in order to minimize oxidation during preparative steps. When working at room temperature

nitrogen gas was introduced at the top of the column before the sample was added to condition the column and dry ice was used to create an inert atmosphere in and around the collection tubes. The nitrogen aided in packing the column and in removing some of the small air pockets that were present.

Extracts of egg phospholipids in chloroform-methanol (1:1 v/v) and soybean phospholipids from a granular, oil-free phosphatide preparation (Centrolex P), dissolved in chloroform, were carefully applied to the top of the column in a ration of 0.02 g lipid/g silicic acid. Elution was accomplished by various solvent systems and successive 20 ml (2.5 x 30 cm column) or 27 ml fractions (5 step column) were collected. Fractions were collected by hand from the conventional column and by a LKB RadiRac fraction collector, distributor type 3402B, from the 5 step column.

Neutral lipids were eluted by chloroform and monitored by the Salkowski test (55) until a negative reaction was obtained. Acetone, used as a scavenger for oxidized materials by Nelson and Freeman (80), also removed pigmented substances from the column. It was usually possible to follow the descent of the pigments visually as a brownish-yellow band. The effluents containing only neutral lipids and pigments were discarded. The third fraction which contained phosphatidyl ethanolamine (PE)

was eluted with 15% by volume of methanol in chloroform. This fraction was followed during the course of elution with a rapid ninhydrin test (100), consisting of equal volumes of eluate, ninhydrin (4 mg of ninhydrin/ml of 100% butanol), and 2,4-lutidine (20% 2,4-lutidine by volume in 100% butanol).

When all of the PE was removed from the column, as indicated by a negative ninhydrin test, the polarity of the solvent was raised by increasing the concentration of methanol to 25% by volume in chloroform to elute phosphatidyl choline. Elution of phosphatidyl choline (PC) was monitored by a molybdate test. The test was performed by heating a few drops of the sample in a test tube to dryness, adding a few drops of concentrated sulfuric acid before heating, cooling, and finally adding molybdate reagent. The molybdate reagent consisted of: 5 ml of 60% w/v perchloric acid, 10 ml of 1N HCl, and 25 ml of 4% w/v ammonium molybdate (89).

The volumes of solvent required to elute each fraction were variable, being dependent upon the size of the column, the packing of the column, the moisture in the solvents, and the amount of sample applied to the column. Since the purpose of fractionation by column chromatography was to obtain sufficient PE and PC for oxidation studies, collection was discontinued after all of the PC was eluted. Fractions were evaporated under nitrogen to 5-10

ml and stored at -20°C in capped vials until spotted on TLC plates to check for purity.

Thin-Layer Chromatography (TLC)

Thin-layer adsorption chromatography on Silica Gel-G was employed to check the identity and purity of phospholipid fractions eluted from the column. RSCO equipment consisting of a plastic mounting board, desiccator, and spotting template were used. Five clean glass plates (20 x 20 cm) were rinsed with ethanol and spread 0.50 mm thick with a Desaga variable thickness (0-2 mm) applicator distributed by Brinkmann Instruments, Westbury, Long Island, New York. Fifty g of Silica Gel-G containing approximately 13% calcium sulfate binder was shaken with 100 ml of deionized water in a 300 ml glass stoppered Erlenmeyer flask until the mixture was homogeneous.

After spreading, the plates were air dried at room temperature for 30 minutes and stored in laboratory drawers. The plates were activated at 100°C for one hour and cooled to room temperature in a desiccator before spotting. Better separation was obtained when plates were activated immediately before spotting rather than after storage of the activated plates for several days in a desiccator.

Phospholipid fractions were spotted on TLC plates with capillary pipettes and the plates were developed in

a solvent system consisting of chloroform: methanol: water (65:25:4 by volume). Samples of lipids methylated for GLC analysis were tested for completeness of methylation by spotting on TLC plates and chromatographing in petroleum ether: ethyl ether: acetic acid (90:10:1 by volume). The plate was removed from the chamber and allowed to dry at room temperature after the solvent had ascended at least 10 cm. Lipid component spots were detected by spraying with various indicators, e.g., ninhydrin solution for amino phosphatides (62), molybdic acid for phosphatides (25), and Dragendorf reagent for choline (113). Aqueous sulfuric acid (50%) was sprayed on the plate after first spraying with the other indicators. After 1-2 hours in an oven at 110°C, any previously undetected lipids and other impurities as well as phospholipid spots previously observed were visible as charred spots on the plate. The contents of vials containing more than one component were considered impure and were discarded. Pure fractions of PE were pooled in one container and fractions of PC were grouped in another. The phospholipd samples were flushed with nitrogen and stored at -20°C in capped vials for further study.

Phosphorus Determination

The phosphorus content of PE and PC was determined by the method of Rouser et al. (101). The phosphorus test was performed with equal success on phospholipid fractions

dissolved in organic solvents and on phospholipids dispersed in aqueous media. The importance of the phosphorus determination in these research studies cannot be overemphasized since it provided a means of quantitating all phospholipid fractions and dispersions. The phosphorus test was performed on aliquots of a monobasic potassium phosphate standard (Hartman Leddon Company, Philadelphia, Pennsylvania) ranging in concentration from 2-10 µg of phosphorus. The absorbance of these aliquots was read with a Beckman Model DU spectrophotometer at 820 mµ and a phosphorus standard curve (Appendix A) was obtained by plotting the absorbance values of the aliquots versus their concentration. Multiplication by the factor, 11.3, calculated from the standard curve, permitted a direct conversion of an absorbance reading to µg of phosphorus in a sample. Rhee and Dugan (97) demonstrated that the method of Rouser et al. could be extended to give a linear curve up to 17.44 µg of phosphorus. However, the concentration of most of the phospholipid samples analyzed were diluted to give readings within the 2-8 µg range (.182-.728 absorbance).

Dispersion of Phospholipids in Aqueous Media

Phospholipids were emulsified in three ways. In the first method, purified PE and PC from egg and soybean dissolved in chloroform-methanol were pipetted into separate cell homogenizer flasks. After the chloroform-

methanol was evaporated from the homogenizer flasks by a stream of nitrogen, 50 ml of 0.1M borate (pH 7.0) buffer* and 0.1 ml of Tween 20 (polyoxyethylene sorbitan monolaurate) were added to the dry phospholipid residues which remained in the flasks. The atmosphere within the flasks was purged with nitrogen and emulsification was achieved with a Braun Cell Homogenizer (Bronwill Scientific, Inc., Rochester, New York) at a speed of 4,000 rpm for 1-2 minutes. Carbon dioxide liquified under pressure was employed to keep the contents of the flask below room temperature while the emulsion was being formed. A control consisting of equivalent amounts of borate buffer and Tween 20 was prepared in the same manner.

No difficulty was encountered in dispersing PC. PE had a much greater tendency to remain together in clumps and to adhere to the sides of the homogenizer flask than phosphatidyl choline, especially if no Tween 20 was present to facilitate the formation of an emulsion. The inability of PE to emulgate in buffer solution was reported by Mattsson and Swartling (77). When glass beads were added to the homogenizer flask and the time of agitation was increased beyond 1-2 minutes, the solution turned slightly brown, indicating that degradation of PE had occurred. Consequently neither technique was

---

*Boric acid was dissolved in redistilled deionized water and adjusted to pH 7.0 with 0.1N NaOH.

utilized. In order to minimize pipetting errors resulting in an uneven transfer of PE from the cell homogenizer flask to respirometer vessels, phosphorus determinations were performed on aliquots from individual vessels after the termination of oxygen uptake measurements. PE was observed to disperse in buffer at about the same time that it began to absorb measurable amounts of oxygen and was always well emulsified before the oxygen uptake curve reached its maximum level.

A second method of emulsifying phospholipids was adopted for studies of the optimum pH for the oxidation of PE and PC. Because of difficulties in dispersing phosphatidyl ethanolamine over a rather wide range of pH, even with the aid of Tween 20, PE and PC, dissolved in chloroform-methanol in amounts corresponding to 40-50 µg phosphorus (101), were introduced directly into respirometer flasks. Solvents were removed by nitrogen and 4 ml of 0.1M borate buffer* was added.

The third technique of emulsifying phospholipids was similar to the second method but was necessitated by an entirely different reason. When an aliquot of Tween 20 (polyoxyethylene sorbitan monolaurate) was methylated and injected into the gas chromatograph, the major fatty acid ester was laurate but palmitate, stearate and oleate

---

*Boric acid was dissolved in redistilled deionized water and adjusted to the desired pH with 0.1N NaOH or 0.1N HCl.

were also present. Since their presence would interfere with subsequent GLC determinations of the changes in the fatty acids of PE and PC during oxidation, another means of forming a stable emulsion was needed. Sonication was considered but it has been shown to produce many unknown peaks from PE and PC as evidenced by GLC traces of fatty acid methyl esters (38). A small amount of phosphatidyl serine has been used to disperse PE in a buffer solution (77). Phosphatidyl serine is rapidly oxidized, however (1, 77). The addition of 10% PC to PE was observed to reduce the time required to disperse PE. Accordingly, 10% PC was added to each preparation of PE utilized in studies of phospholipid alteration resulting from phospholipid oxidation. A homogeneous emulsion was needed to minimize pipetting errors for ultraviolet absorption spectra, 2-thiobarbituric acid and free amino group determinations. Egg PC was used with egg PE and soybean PC with soybean PE. PE and PC were pipetted directly into respirometer flasks as in the second method of emulsifying phospholipids.

Preparation of Methyl Linolenate Dispersions

A 0.0133M dispersion of methyl linolenate (Hormel Institute, Austin, Minnesota) in 0.1M borate (pH 7.0) buffer was prepared by agitation for 1 minute with the Braun Cell Homogenizer. Three ml of the dispersion was

pipetted into respirometer flasks and 1 ml of solution was added in the following combinations:

1. 0.5 ml of 0.08M DL-α-glycerophosphate (sodium salt) and 0.5 ml of 0.08M ethanolamine.
2. 0.5 ml of 0.08M DL-α-gylcerophosphate (sodium salt) and 0.5 ml of 0.08M choline chloride.
3. 0.5 ml of 0.08M DL-α-glycerophosphate (sodium salt) and 0.5 ml of 0.1M borate (pH 7.0) buffer.
4. 1.0 ml of 0.1M borate (pH 7.0) buffer.

All solutions were made in 0.1M borate (pH 7.0) buffer. The final concentration of methyl linolenate and the various additives in the 4 ml of borate buffer was 0.01M.

Introduction of Additives to Phospholipid Dispersions

The most convenient method of maintaining the borate buffer concentration at 0.1M in the phospholipid emulsions was to make up all solutions in borate buffer. The level of amino acid, choline chloride and ethanolamine in borate solution was such that the introduction of 1 ml to 3 ml of phospholipid emulsion resulted in a 1:1 molar ratio of phospholipid to additive. Sodium tetra, ethylenediamine tetraacetate and $CuSO_4$ were pipetted in 1 ml amounts which reduced their initial concentrations four-fold to 0.0025% and 1×10^{-4}M respectively. One ml of 0.01% BHT dissolved in chloroform was introduced to a respirometer flask immediately after the addition of the phospholipid

in chloroform-methanol. Solvents were removed by nitrogen and 4 ml of 0.1M borate buffer was added.

Oxygen Uptake Measurements

Oxidation of phosphatidyl ethanolamine, phosphatidyl choline and methyl linolenate was studied by measuring oxygen uptake in a Differential Respirometer (36) (Model GR 14, Gilson Medical Electronics, Middleton, Wisconsin). The volume of borate buffer transferred to each respirometer flask was 4 ml, either pipetted in the form of a lipid emulsion from a cell homogenizer flask or added to PE and PC after these phospholipids had been freed of the organic solvents in which they were introduced to the reaction flasks. The amount of phospholipid in each reaction flask was 40-50 µg of phosphorus. The 15 ml capacity respirometer vessel was conical and made of Pyrex glass. Before it was fastened to the flask-holder, a ground joint standard taper 7/15 stopper was fitted to the vessel's sac and 0.2 ml of 5% KOH was added to its center well. The vessels were placed in the water by lowering the flask-holders and allowed to equilibrate 15 minutes before starting measurement. A minimum of 3 duplicates were run against at least 3 controls of borate buffer. Experiments were conducted at temperatures ranging from 15 to 50°C in an atmosphere of air with a shaking rate of 150 oscillations of 5 cm per minute.

Preparation of Methyl Esters for Gas-Liquid Chromatography

Methylation of the fatty acids in the phospholipids was performed by the low temperature method of McGinnis and Dugan (79) modified by deletion of the 2 ml of sulfuric acid. This modification was based on the work of Zook (127) who found that almost complete methylation of soybean and egg phospholipids can be accomplished by omitting sulfuric acid and reducing the amount of base to 2-4 g of KOH/15 ml of methanol. Evidence for this modification was based on TLC results of methyl ester preparations chromatographed in petroleum ether: diethyl ether: acetic acid, 90:10:1 by volume (127).

Methyl esters were prepared from phospholipid fractions derived from silicic acid columns and from dispersions of phospholipids in aqueous media. Identical samples of PE and PC in solvent and in buffer were methylated and their fatty acid compositions were determined by GLC. Good agreement was obtained between samples in solvent and samples in buffer (Appendix B). Methylation of phospholipid emulsions during oxidation provided a means of following changes in the percentage of specific unsaturated fatty acids in relation to oxygen uptake.

Samples of PE and PC in organic solvent and in borate buffer, usually in volumes of 1-3 ml, were pipetted into 125 ml Erlenmeyer flasks containing 20 ml of diethyl ether. The flasks were placed in dry ice-acetone baths

and stirred by magnetic stirrers. When the contents of the flask had cooled to -60°C, 15 ml of absolute methanol was added and the mixture was stirred for 10 minutes. The temperature was adjusted to -60°C and methanolic-KOH (2 g of KOH dissolved in 15 ml of absolute methanol) was added. After stirring the mixture for 20 minutes at -60°C, it was allowed to reach room temperature while stirring was continued. The mixture was quantitatively transferred to a 500 ml separatory funnel by rinsing the flask with 150 ml of water. Methyl esters were extracted once with 30 ml of petroleum ether (30°-60°C B.P.) and twice with 15 ml of petroleum ether. All samples were dried by filtration through anhydrous sodium sulfate before being concentrated to a volume of 0.2 ml in graduated 12 ml centrifuge tubes.

Gas-Liquid Chromatography (GLC)

Gas-liquid partition chromatography was accomplished with a Beckman GC-5 dual column, temperature programmed gas chromatograph. The unit was equipped with thermal conductivity and flame ionization detectors, and a recorder. All methylated samples were chromatographed using the flame ionization detector.

Two coiled stainless steel columns (1/8 in. o.d. x 6 ft.) were used for methyl ester separation. Both columns were packed with 20% diethylene glycol succinate

(DEGS) and 1% by weight phosphoric acid on acid washed chromosorb W 80/100 mesh as a support phase. Approximately 2.5 g of column material were packed into each of the columns. The columns were conditioned at 220°C for 24-48 hours before being used. Operating conditions for this study were: column temperature, 184°C; inlet temperature, 230°C; flow rates for air, helium and hydrogen, 250, 30, and 32 ml/min. respectively; tank pressures for air, helium and hydrogen, 16, 70 and 30 psi respectively. The attenuator settings ranged from 5×10^3 (2.5×10^{-10} amperes, fullscale deflection) to the higher sensitivity of 5×10^2 (2.5×10^{-11} amperes, fullscale deflection).

Fatty acid methyl esters on the chromatogram were identified by direct comparison of major peaks with those of chromatographic standards (99% pure) passed through the same column under identical conditions. Peak areas were calculated by the triangulation method of multiplying the peak height by one-half the base (11).

Spectrophotometric Determinations as Indices of Phospholipid Oxidation

These determinations were based on aliquots containing 5 µg phosphorus as assayed by the procedure of Rouser *et al.* (101). The aliquots were made up to a volume of 1.0 ml with 0.1M borate (pH 7.0) buffer.

Ultraviolet Absorption Spectra.--Conjugation of oxidized and non-oxidized PE and PC was determined by diluting 1.0 ml of emulsified sample with 2 ml of Fisher certified A.C.S. spectranalyzed methanol. Ultraviolet absorption spectra were recorded with a Beckman Model DU spectrophotometer at 232 mμ and 268 mμ against 1.0 ml of 0.1M borate (pH 7.0) buffer similarly diluted with methanol.

2-Thiobarbituric Acid (TBA) Test.--Malonaldehyde formation was measured by the TBA test of Placer et al. (90) designed to accommodate emulsions of polyunsaturated fatty acids. In this procedure, the trimethine colored substance, resulting from the reaction of TBA reagent with the lipid peroxidation product, malonaldehyde, was estimated in alkaline solution. The method involved incubating 1.0 ml of phospholipid emulsion in a stoppered vial for 30 minutes at 37°C. Following incubation, 1.0 ml of TBA reagent was added and the mixture was heated for 10 minutes in a boiling water bath using glass beads as a condenser. The TBA reagent consisted of 2 volumes of a 0.8% TBA solution and 1 volume of 7.0% perchloric acid. The TBA solution was prepared by dissolving TBA in a small amount of 1N NaOH before neutralizing with 7% perchloric acid. After the TBA-emulsion mixture had cooled, 0.7 ml of 1N NaOH was added and the solution was mixed by shaking. The absorbance of the reaction

mixture was measured at 548 mμ versus 1.0 ml of borate buffer similarly treated. The original procedure utilized 2.0 ml of pyridine/n-butanol (3/1, v/v) to dissolve any proteins or lipids that might precipitate or become opalescent as a result of the reaction. Since the solutions in this study were clear after the addition of 1N NaOH the addition of pyridine/n-butanol was omitted.

Free Amino Group Quantitation.--Reaction with ninhydrin has been utilized for the detection and analysis of lipids contianing free amino groups (19, 61, 72). Ninhydrin reagents are difficult to use since reproducible color yields are obtained only after careful control of all conditions. Also color yields vary with different substances. In contrast, reactions with trinitrobenzene sulfonic acid (TNBS) yield uniform, reproducible and stable color intensities for free amino groups of a variety of substances, both lipid and non-lipid. The procedure described below is based on the method of Siakotos (111). One ml of phospholipid emulsion was pipetted into a 12 ml graduated centrifuge tube followed by 1 ml of neutralized Hyamine hydroxide (Packard Instrument Company, La Grange, Illinois) and 1 ml of 0.1% TNBS (Nutritional Biochemicals Corporation, Cleveland, Ohio) in distilled water. The Hyamine hydroxide [p-(diisobutylcresoxyethoxyethyl) dimethyl

benzyl ammonium hydroxide] was neutralized to pH 8.0 with glacial acetic acid and diluted to 0.5M with methanol. After mixing thoroughly, the samples were incubated at 40°C for 2 hours in a water bath and covered with aluminum foil to exclude light. One ml of methanolic 1N hydrochloric acid (8.33 ml concentrated HCl diluted to 100 ml with absolute methanol) was added to quench the unreacted TNBS color and the volume was made up to 5 ml with methanol. The color intensity was read at 340 mμ using a reagent blank as zero. A standard curve for ethanolamine was constructed by using 2/1 chloroform-methanol aliquots of 2-aminoethanol (Sigma Chemical Company, St. Louis, Missouri) ranging in concentration from 0.05 to 0.50 μM (Appendix C). Good agreement was obtained when aliquots of PE (non-oxidized) were quantitated by the phosphorus determination and the TNBS free amino group determination.

RESULTS AND DISCUSSION

The Effect of pH on Oxygen Consumption

Preliminary oxygen uptake measurements on phospholipids in organic solvents, in aqueous dispersions and in a dry state indicated that the rate of oxidation could be most readily determined under controlled pH in emulsified systems. An additional reason for measuring oxidation under these conditions was that any changes that occurred would be more typical of changes occurring in a number of natural food systems.

Figure 1 illustrates the influence of pH on the oxygen consumption of egg phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC). Oxygen consumption after 50 hours was greatest for PC at pH 7.0 and PE at pH 8.0. Mattsson and Swartling (77), working with butter phospholipids in phosphate buffers, reported that PE was reluctant to take up oxygen except at pH values in the alkaline range, while PC had a maximum probably below pH 2 and reacted fairly slowly around pH 7.0.

Subsequent oxygen uptake determinations were conducted at pH 7.0 since it represented the maximum pH for the less reactive PC and a near maximum for PE, and

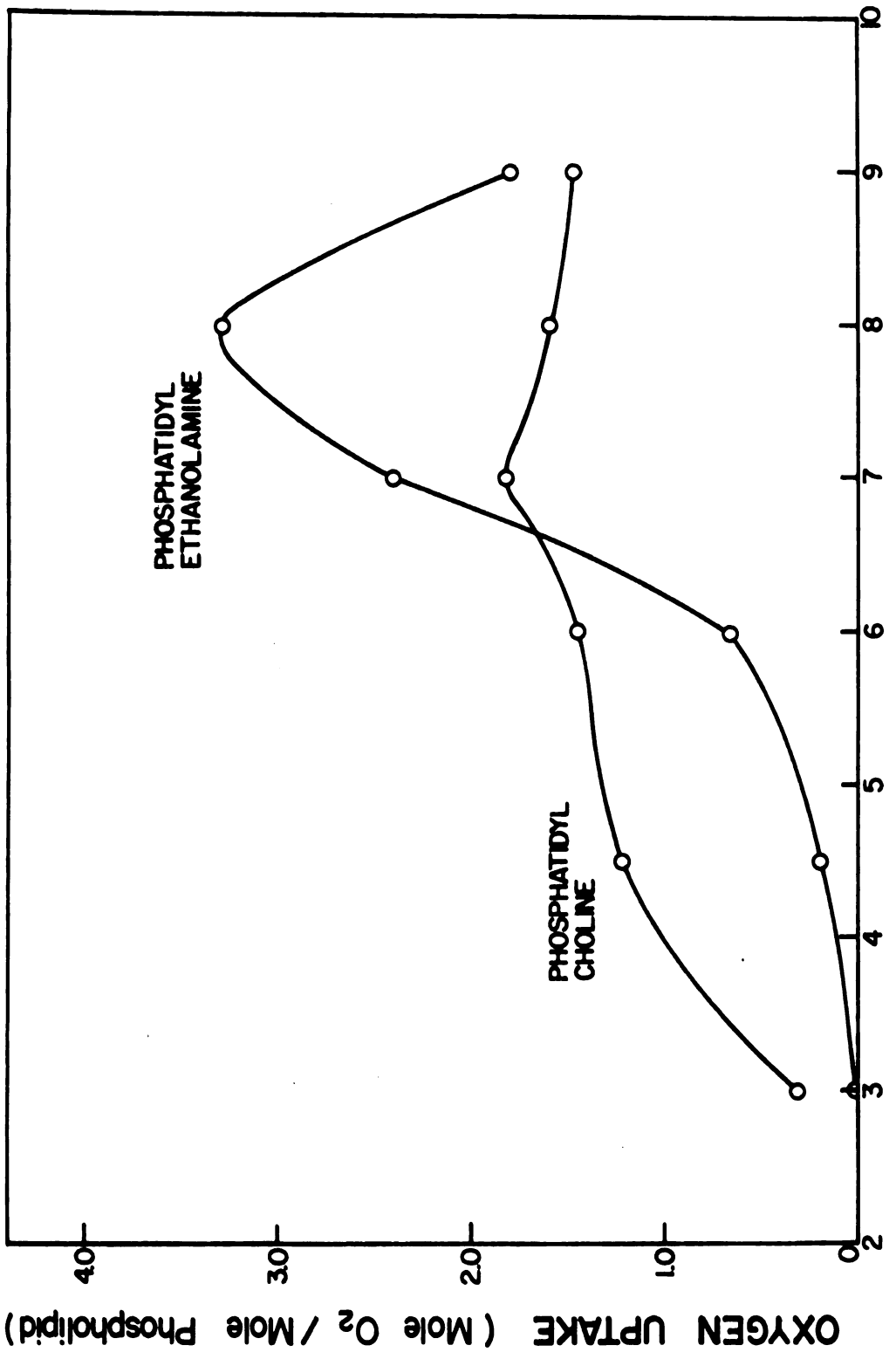


FIGURE 1.--The effect of pH on oxygen consumption of phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) in 0.1M borate buffer after 50 hours. Temperature of reaction: PE-25°C, PC-40°C.

because it approximated the physiological pH of most animal and vegetable products. Consequently, studies on the oxidation of phospholipids could possibly provide information on the mode of autoxidation of foods during processing and storage.

Reactivity of Egg and Soybean Phospholipids

The relative rate of oxidation of both PE and PC was temperature dependent, increasing with increasing temperature. PE from egg or from soybean absorbed oxygen in a much more rapid manner than did PC from the same sources (Figures 2, 3). Lea (60) reported that thin films of egg PE at 37°C oxidized at phenomenal rates. Roubal (99) observed that PE from the flesh lipids of cod fish exhibited a high rate of oxidation while PC required an added prooxidant before it would react with oxygen.

Variations in experimental techniques among individual researchers studying the oxidation of phospholipids make it difficult to compare results with regard to the relative reactivity of phospholipids from different sources. When phospholipids from egg and soybean were compared by the same method of oxidation, egg phospholipids were found to be more reactive. Egg PE and PC oxidized more rapidly than did soybean PE and PC respectively (Figures 4, 5).

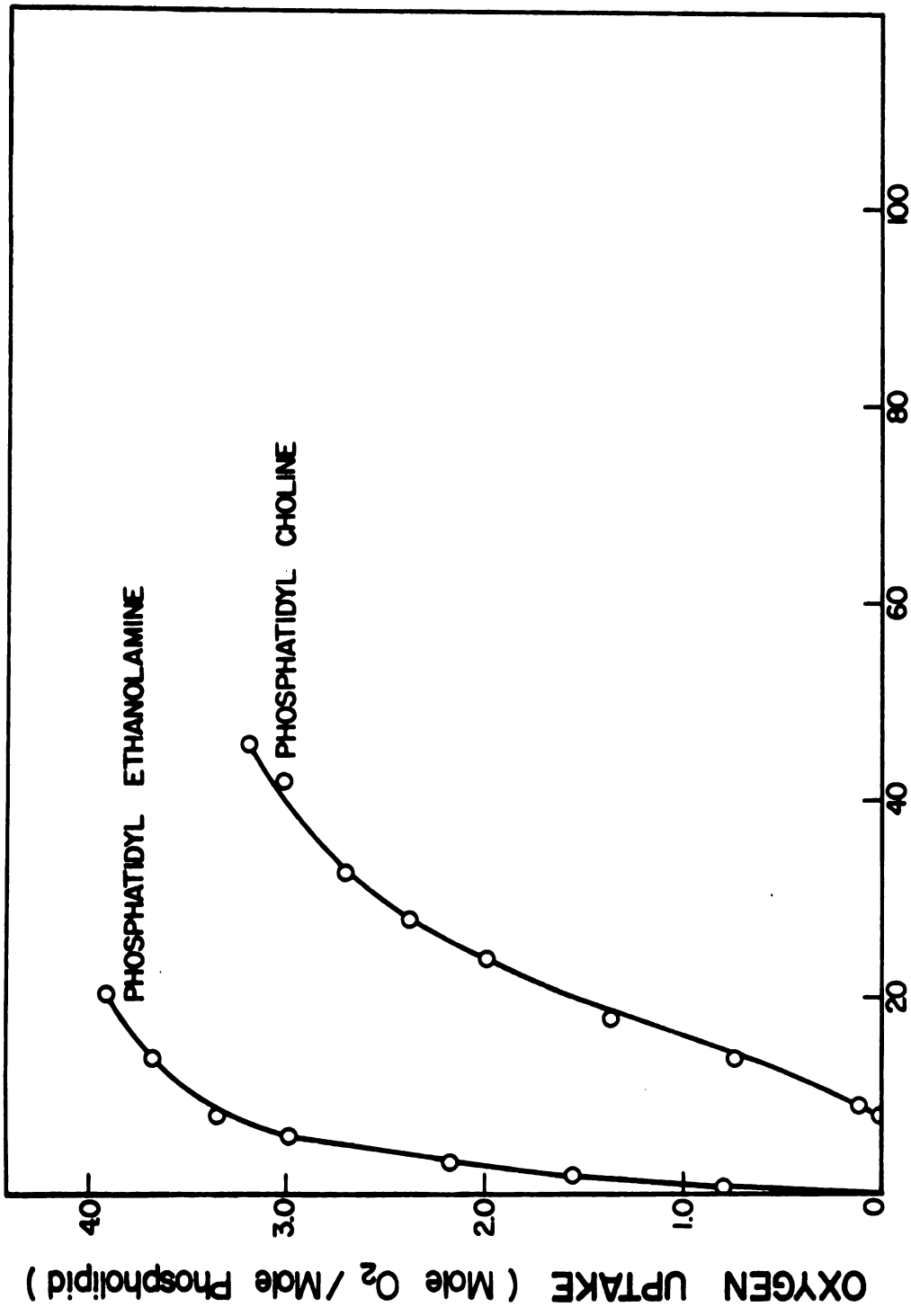


FIGURE 2.--The oxygen consumption of egg phosphatidyl ethanolamine and phosphatidyl choline emulsified in 0.1M borate (pH 7.0)-Tween 20 buffer at 25°C.

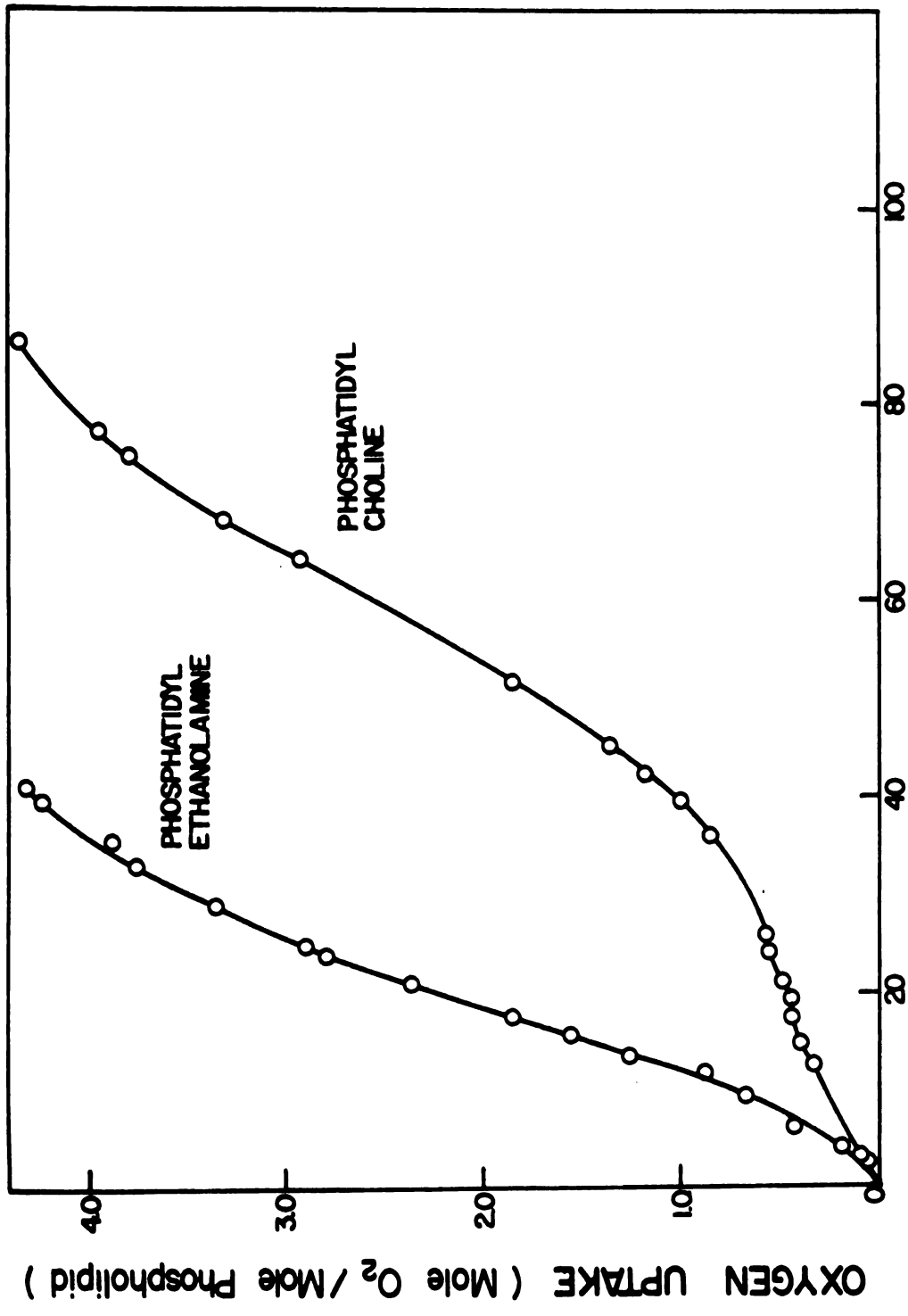


FIGURE 3.--The oxygen consumption of soybean phosphatidyl ethanolamine and phosphatidyl choline emulsified in 0.1M borate (pH 7.0)-Tween 20 buffer at 40°C.

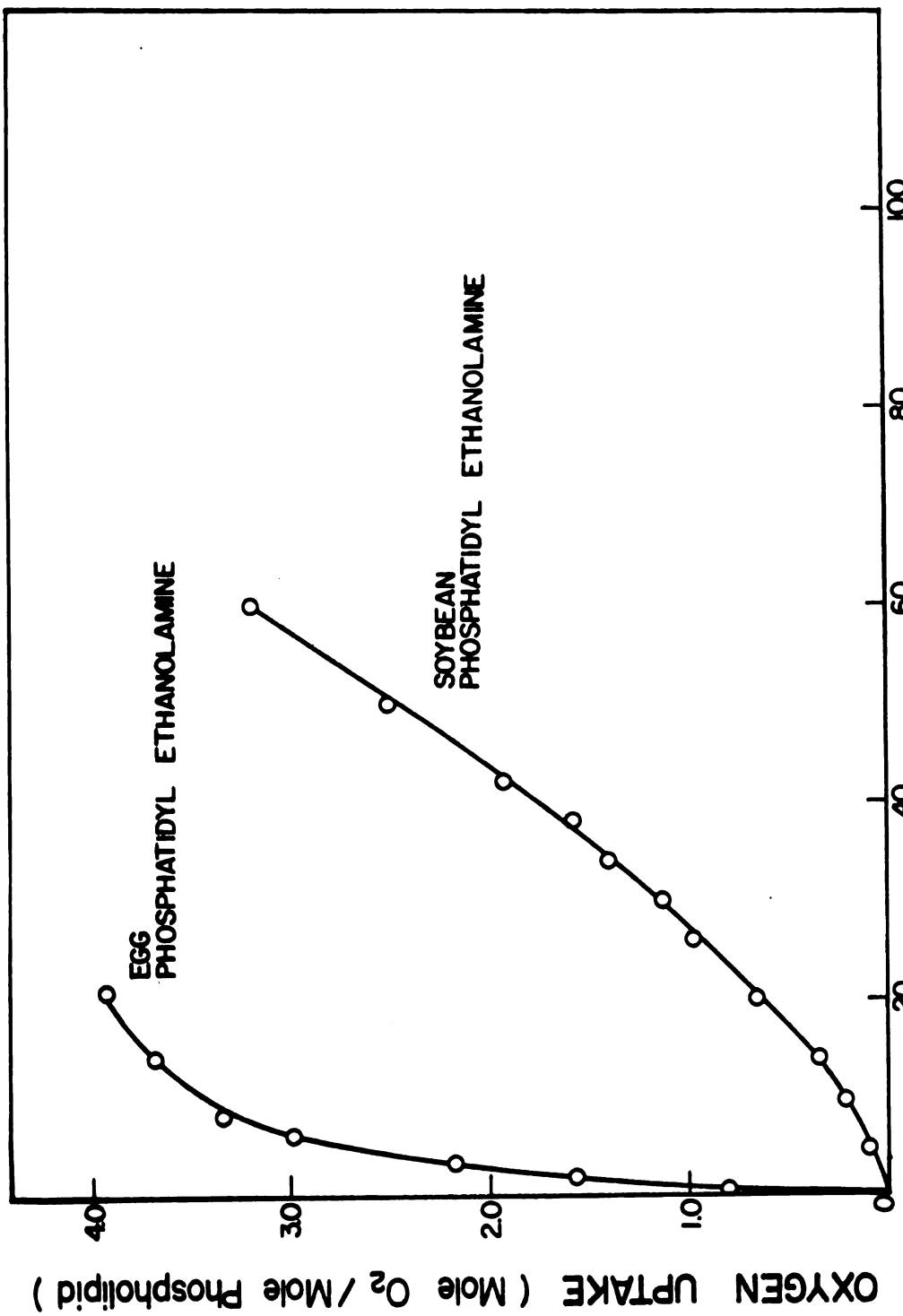


FIGURE 4.--The oxygen consumption of egg and soybean phosphatidyl ethanolamine emulsified in 0.1M borate (pH 7.0)-Tween 20 buffer at 25°C.

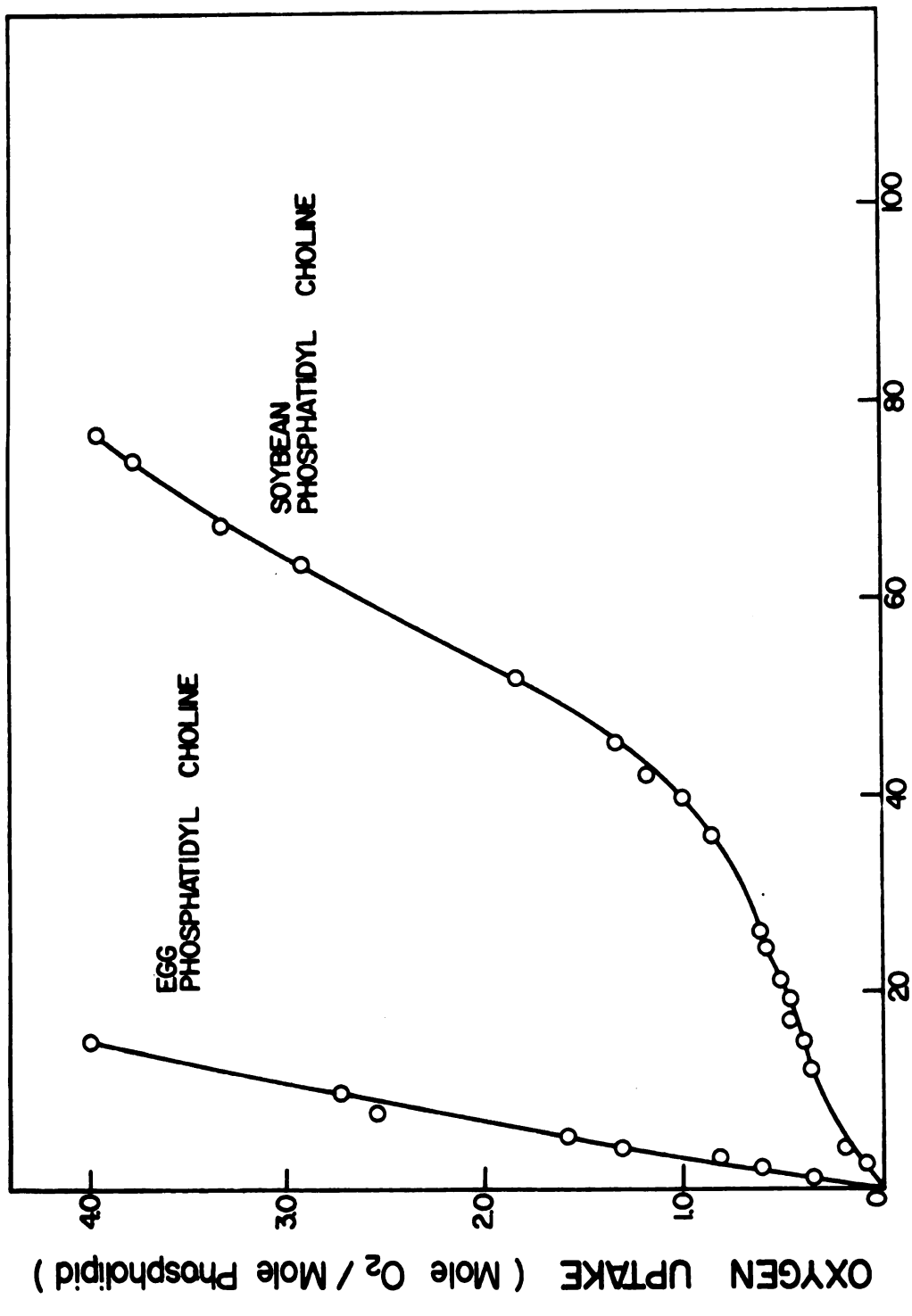


FIGURE 5.--The oxygen consumption of egg and soybean phosphatidyl choline emulsified in 0.1M borate (pH 7.0)-Tween 20 buffer at 40°C.

A partial explanation for the differences in oxidation for egg and soybean PE and PC can be traced to their fatty acid compositions. Lea (60) concluded that a relationship existed between the oxidation rate and the degree of unsaturation of individual phospholipids. Hawke (40) reported that egg PE contained a higher percentage of polyunsaturated fatty acids than egg PC. The fatty acid composition of the phospholipids used in this study is presented in Table 1. The concentration of arachidonic acid is markedly higher in egg PE (13.3%) than in egg PC (3.8%). The amount and degree of polyunsaturated fatty acids are also higher in soybean PE than in soybean PC with nearly twice as much linolenic acid present in soybean PE.

The greater reactivity of egg phospholipids compared to soybean phospholipids cannot be explained solely by the percentage of polyunsaturated fatty acids since the degree of unsaturation is higher for soybean phospholipids. Of perhaps greater significance is the presence of arachidonic acid in egg phospholipids. Holman and Elmer (48) reported that the rate of oxidation of methyl arachidonate is about twice that of ethyl linolenate. These workers concluded that the introduction of one additional double bond into a fatty acid at least doubled the oxidation rate. Since the fatty acid with the most unsaturation in both egg phospholipids and soybean phospholipids is

TABLE 1.--Fatty acid composition of purified phospholipids.

Fatty Acid	Egg PC %	Egg PE %	Soybean PC %	Soybean PE %
C_{14}	trace	trace	1.2	4.4
C_{16}	30.2	19.5	21.7	18.8
$C_{16:1}$	1.5	3.6	--	--
C_{18}	16.4	30.0	4.1	1.9
$C_{18:1}$	32.1	16.9	17.0	8.2
$C_{18:2}$	16.0	16.6	50.4	56.0
$C_{18:3}$	trace	trace	5.7	10.6
$C_{20:4}$	3.8	13.3	--	--
polyunsaturates	19.8	29.8	56.1	66.1

PC = phosphatidyl choline
PE = phosphatidyl ethanolamine

oxidized before fatty acids with lesser unsaturation, egg phospholipids containing arachidonic acid should be more susceptible to oxidative attack than soybean phospholipids with linolenic acid.

Activation Energies of PE and PC

The rates of oxygen uptake at temperatures ranging from 15 to 50°C were utilized to determine activation energies for the autoxidation of PE and PC from egg and soybean. The oxidation rate for a phospholipid was measured at 4-5 temperatures and was obtained from the straight line portion of the uptake curve after the onset of initiation and other early autoxidation reactions. Thus the rates reflect the oxidizability of the unsaturated fatty acids of PE and PC. Oxidation rates were expressed as the number of moles of O_2 absorbed per mole of phospholipid per hour. The logarithm of each rate was plotted against the reciprocal of the absolute temperature to give a straight line (Figure 6). According to the Arrhenius treatment of kinetic data the slope of this line is related to the activation energy by the following equations (17):

$$\frac{\log K_1}{\log K_2} = \left(\frac{Ea}{2.303R}\right) \frac{T_2 - T_1}{T_1 T_2} \qquad \text{and}$$

$$\text{slope of } \log K \text{ vs } 1/T = \frac{-Ea}{2.303R} \qquad \text{where}$$

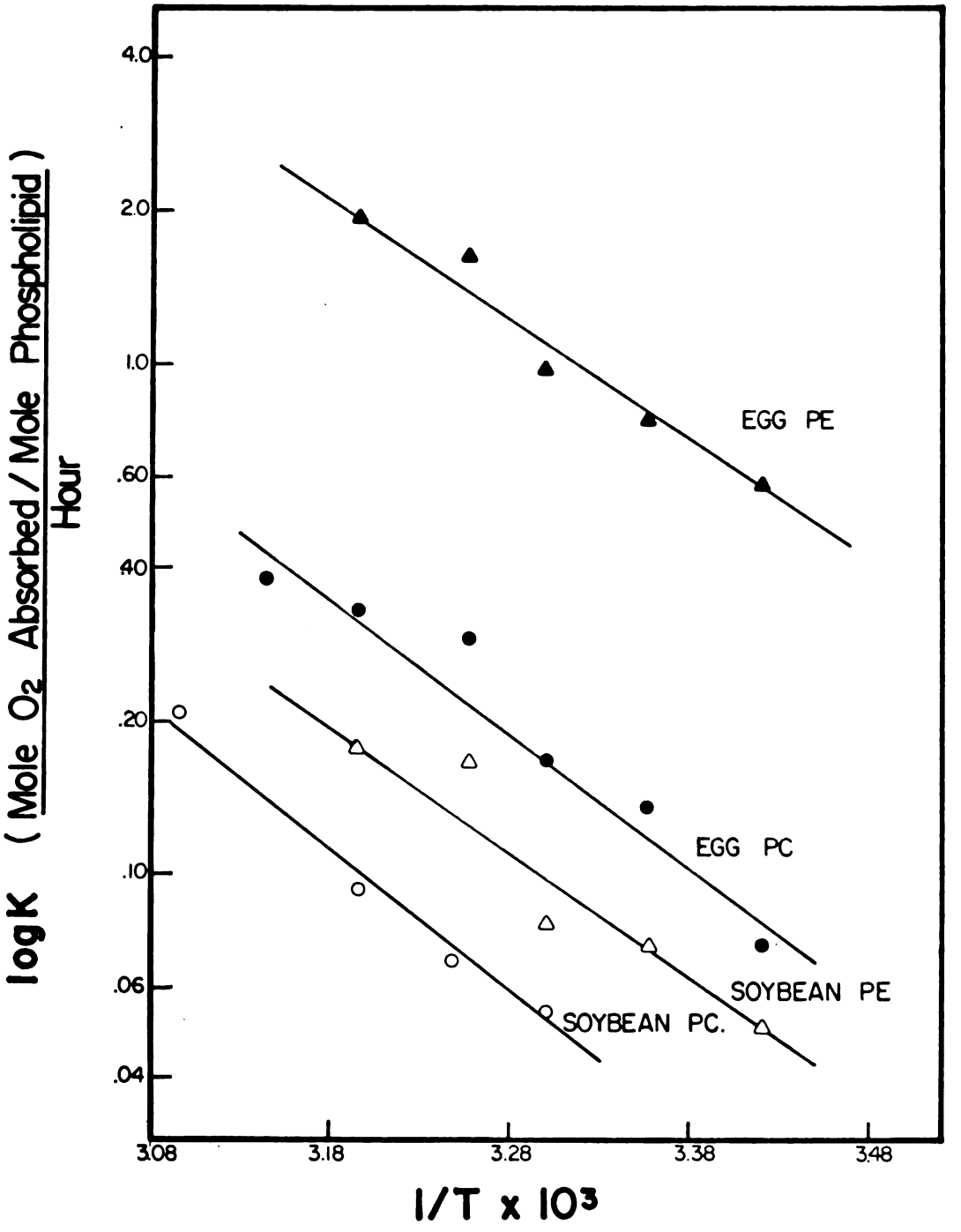


FIGURE 6.--Arrhenius plot of the autoxidation of egg and soybean phospholipids relating the rate of oxidation to the reciprocal of temperature.

Ea = activation energy in K calories per mole

log K = logarithm of oxidation rate at T

log K_1 = logarithm of oxidation rate at T_1

log K_2 = logarithm of oxidation rate at T_2

T_1 = Absolute temperature at which K_1 was determined

T_2 = Absolute temperature at which K_2 was determined

R = Gas constant (.001987 K calories per °C per mole).

The activation energies presented in Table 2 were found to be quite comparable, which suggests that the mode of oxidation, at least during the period of steady state oxidation, was the same for the egg and soybean phospholipids utilized in this study. The values obtained were approximately one-half of the values reported for the autoxidation of methyl linoleate and linoleic acid emulsions (70) indicating the greater reactivity of the phospholipids.

The Effect of Fatty Acid Unsaturation on Phospholipid Oxidation

Silicic acid column chromatography (35, 98) and thin-layer chromatography (69) have been used to obtain natural phosphatidyl choline with varying degrees of unsaturation. The faster-moving PC subfractions were found to have higher proportions of stearic and arachidonic

TABLE 2.--Energy of activation for the autoxidation of phospholipids emulsified in 0.1M borate (pH 7.0)-Tween 20 buffer.

Source and Phospholipid	K cal./mole
Egg PE	11.7
Soybean PE	11.8
Egg PC	12.2
Soybean PC	12.8

acids than slower moving fractions. The fatty acid composition of PC fractions that were initially recovered from the column or that moved the farthest on TLC resembled the fatty acid composition of PE (35, 69). When egg PC was chromatographed with chloroform-methanol (25-35% methanol by volume) on a silicic acid column, the first fractions contained a higher degree of unsaturated fatty acids, particularly arachidonic acid, than did later fractions (Table 3). In fact, the percentage of arachidonic acid in egg PC first recovered from the column was almost identical with the percentage of arachidonic acid in egg PE (Table 1).

The oxygen uptake of the high arachidonic acid-containing PC was compared with PC from succeeding fractions and with egg PE at 40°C and 25°C (Figures 7, 8). This high arachidonic acid PC was more reactive than typical PC which has approximately 4% of its fatty acid composition as arachidonic acid; however, it was less reactive than PE. The oxygen uptake pattern of the third PC fraction is not shown in either figure because its induction period was more than double the time scale used for the other phospholipid fractions. The third PC fraction containing 1.3% arachidonic acid did not begin to absorb any measurable amount of oxygen at 40°C until 116 hours had elapsed.

TABLE 3.--Fatty acid composition of three egg phosphatidyl choline fractions separated on a silicic acid column.

Fatty Acid	First Fraction %	Second Fraction %	Third Fraction %
C_{14}	trace	trace	trace
C_{16}	26.5	34.6	34.0
$C_{16:1}$	1.4	1.9	3.3
C_{18}	28.8	16.5	11.3
$C_{18:1}$	18.9	28.4	37.7
$C_{18:2}$	12.0	14.4	12.4
$C_{18:3}$	trace	trace	trace
$C_{20:4}$	12.4	4.2	1.3

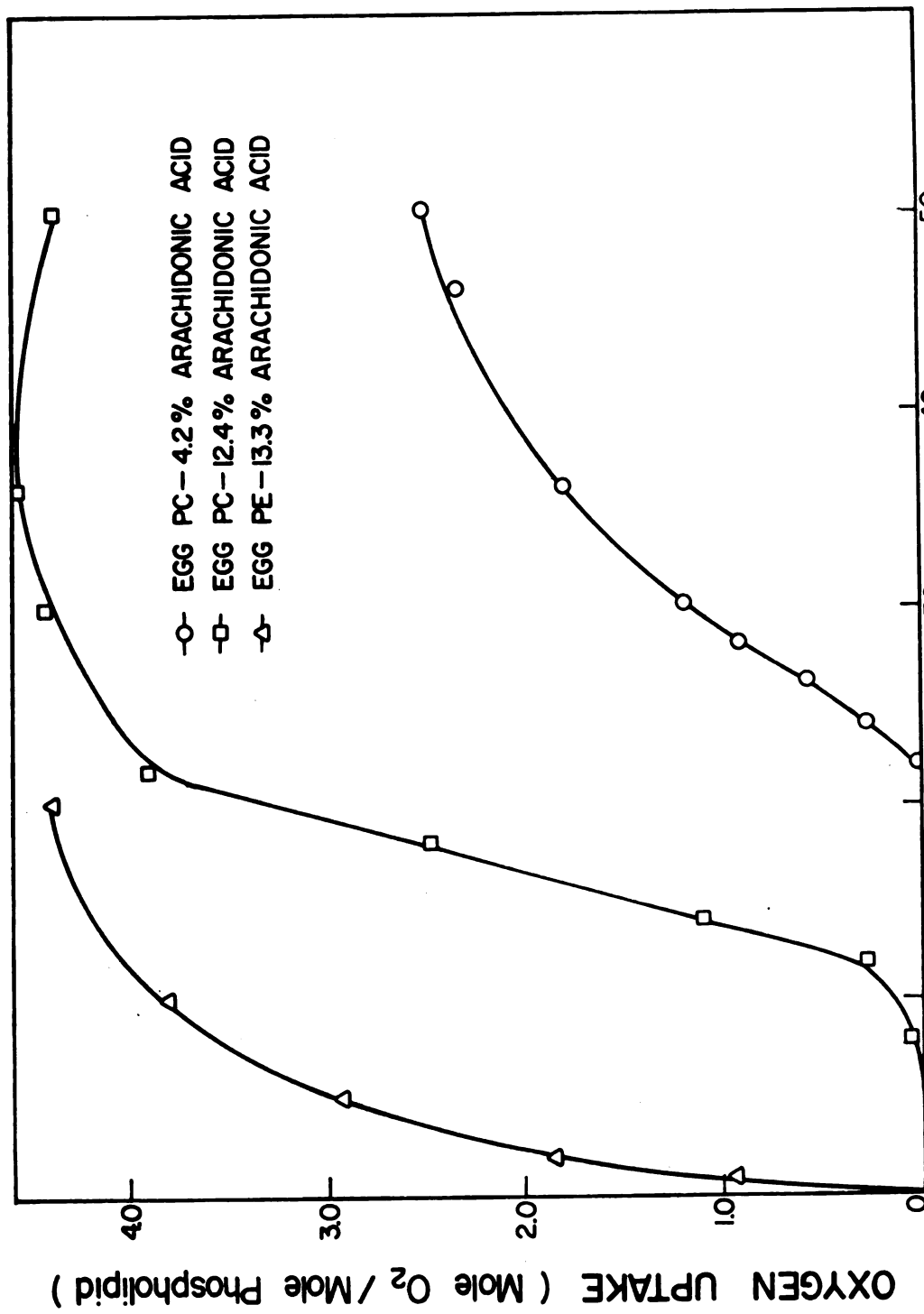


FIGURE 7.--The effect of arachidonic acid composition on the oxygen consumption of egg phospholipids emulsified in 0.1M borate (pH 7.0)-Tween 20 buffer at 40°C.

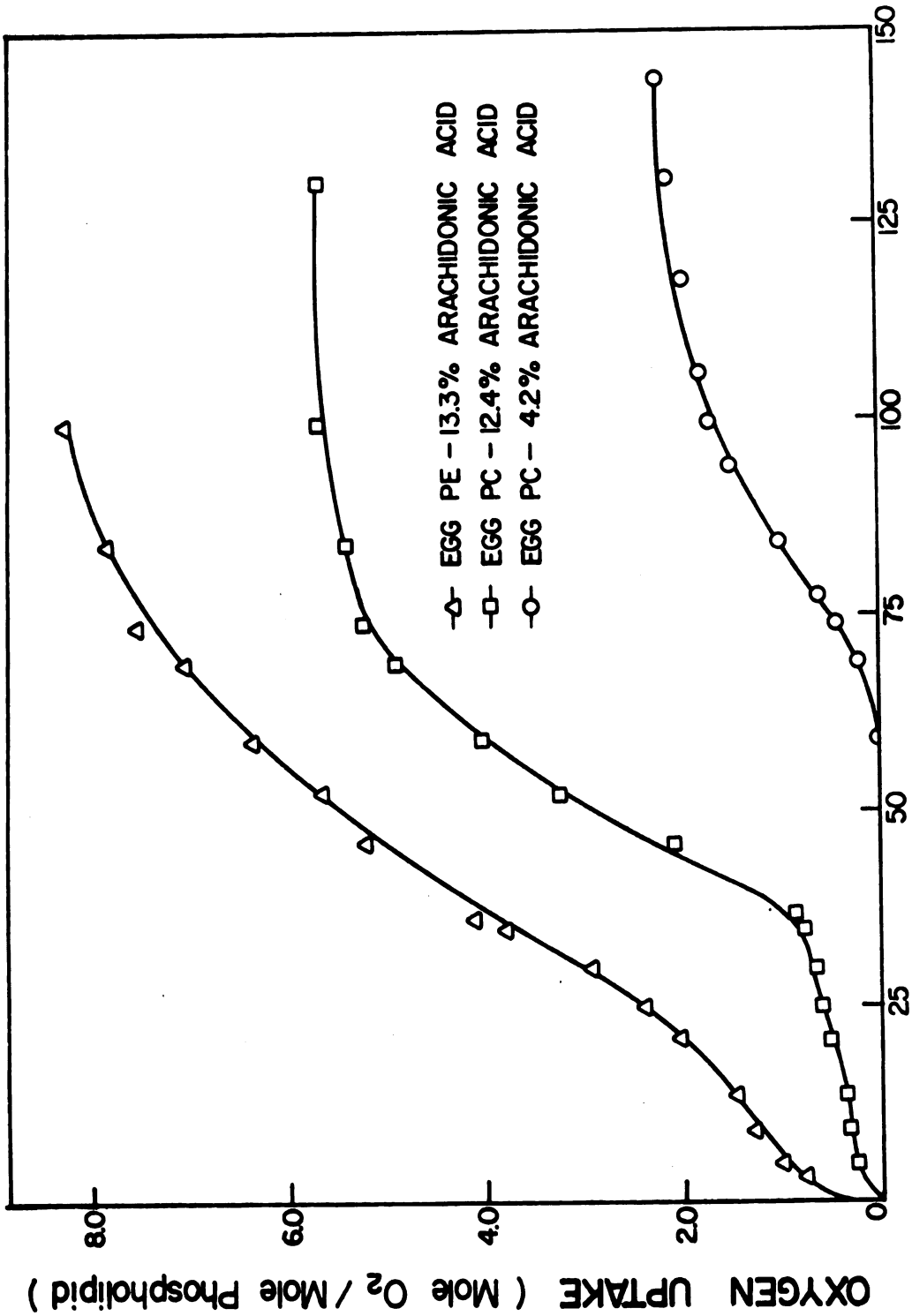


FIGURE 8.--The effect of arachidonic acid composition on the oxygen consumption of egg phospholipids emulsified in 0.1M borate (pH 7.0)-Tween 20 buffer at 25°C.

The greater reactivity of PE as compared to the high arachidonic acid-containing PC was characterized by a shorter induction period. The maximum rates of oxidation for the high arachidonic acid PC and PE were measured and found to be almost equal (Table 4, next section). The similarity in rates for the high arachidonic acid PC and PE suggests that the straight line portion of the oxygen uptake curve (representing the maximum rate of oxidation) for a particular phospholipid is regulated primarily by the composition of unsaturated fatty acids.

Comparison of Oxidation Rates Obtained by Experimentation and Calculation

Oxidation rates for egg and soybean phospholipids were compared by two methods to determine quantitatively the influence of fatty acid composition on the reactivity of a given phospholipid. In the first method, maximum rates of oxidation were obtained from the straight line portion of each oxygen uptake curve. The values for egg phospholipids are presented in Table 4 and those for soybean phospholipids in Table 5.

The second method was based on data published by Holman and Elmer (48). These workers reported the maximum rates of oxidation of various acids and esters. The maximum rate of oxidation of ethyl linolenate was 2.4 times that of ethyl linoleate, and the rate of oxidation

TABLE 4.--Comparison of the oxidation rates of egg phospholipids obtained by experimentation and calculation.

Experimental Rates

Phospholipid Oxidized	Oxidation Rate (40°C) mole O_2/mole P. hr	Ratio of Rates
Egg PC--normal arachidonic acid	.185	$\frac{.185}{.185} = 1.0$
Egg PC--high arachidonic acid	.360	$\frac{.360}{.185} = 1.9$
Egg PE	.383	$\frac{.383}{.185} = 2.1$

Calculated Rates

Phospholipid Oxidized	% Fatty Acid	Oxidation Rate Relative to $C_{18:2}$	Product	Fatty Acid Summation Oxidation Rate	Ratio of Rates
Egg PC--normal arachidonic acid $C_{18:2}$	14.4%	(1.0) =	14.4		$\frac{34.6}{34.6} = 1.0$
$C_{20:4}$	4.2%	(4.8) =	20.2		
				34.6	
Egg PC--high arachidonic acid $C_{18:2}$	12.0%	(1.0) =	12.0		$\frac{71.5}{34.6} = 2.1$
$C_{20:4}$	12.4%	(4.8) =	59.5		
				71.5	
Egg PE $C_{18:2}$	16.6%	(1.0) =	16.6		$\frac{80.4}{34.6} = 2.3$
$C_{20:4}$	13.3%	(4.8) =	63.8		
				80.4	

NOTE: The bracked numbers (1) and (4.8) are based on Holman and Elmer's data (48). The rate of oxidation of arachidonic acid ($C_{20:4}$) was reported to be 4.8 times greater than linoleic acid ($C_{18:2}$) at 37°C.

TABLE 5.--Comparison of the oxidation rates of soybean phospholipids obtained by experimentation and calculation.

Experimental Rates

Phospholipid Oxidized	Oxidation Rate (40°C) mole O_2/mole P. hr	Ratio of Rates
Soybean PC	.092	$\frac{.092}{.092} = 1.0$
Soybean PE	.138	$\frac{.138}{.092} = 1.5$

Calculated Rates

Phospholipid Oxidized	% Fatty Acid	Oxidation Rate Relative to $C_{18:2}$	Product	Fatty Acid Summation Oxidation Rate	Ratio of Rates
Soybean PC					
$C_{18:2}$	50.4%	(1.0) =	50.4		$\frac{64.1}{64.1} = 1.0$
$C_{18:3}$	5.7%	(2.4) =	13.7		
				64.1	
Soybean PE					
$C_{18:2}$	56.0	(1.0) =	56.0		$\frac{81.4}{64.1} = 1.3$
$C_{18:3}$	10.6%	(2.4) =	25.4		
				81.4	

NOTE: The bracketed numbers (1) and (2.4) are based on Holman and Elmer's data (48). The rate of oxidation of linolenic acid ($C_{18:3}$) was reported to be 2.4 times greater than linoleic acid ($C_{18:2}$) at 37°C.

of methyl arachidonate was 4.8 times that of ethyl linoleate. Ethyl oleate in comparison to ethyl linoleate was highly resistant to oxidation at 37°C, the temperature at which autoxidation was measured.

In the second or calculated method, the percentage of each polyunsaturated fatty acid for a given phospholipid was multiplied by its rate of oxidation relative to linoleate. The products were totaled to give oxidation summation rates for egg phospholipids (Table 4) and soybean phospholipids (Table 5). Thus in Table 4 the oxidation summation rate of 80.4 for egg PE was calculated by adding the product of linoleic acid, 16.6 (derived by multiplying its percentage of 16.6 times its rate of oxidation of 1) and the product of arachiodonic acid, 63.8 (derived by multiplying its percentage of 13.3 times its rate of oxidation of 4.8, relative to linoleic acid).

The oxidation summation rates in themselves represent nothing. However, they assume a measure of significance when compared to the experimental rates by ratios. In the ratio of rates column in Table 4 the oxidation rate of each phospholipid is divided by the oxidation rate of egg PC containing the normal quantity of archidonic acid. The ratio of rates for egg PC with a high amount of arachidonic relative to normal PC was 1.9 by the experimental method and 2.1 by the calculated

method (Table 4). Experimental and calculated rates by ratio were quite similar for egg PE and soybean PE (Table 5). In the case of soybean PE the comparison of the two methods was relative to soybean PC.

An interesting aspect of the closeness between the two methods is that it lends further support to the conclusion that the rate of autoxidation of a phospholipid is regulated specifically by the kind and amount of polyunsaturated fatty acids it contains. However, comparisons between egg and soybean phospholipids in the same manner would seem to negate this conclusion. Thus it would appear that if comparisons are to have any validity, they should be made between individual egg phospholipid fractions and between soybean phospholipids.

Other workers have tried similar comparisons on the relative oxidative rates of various oils without obtaining any apparent correlation. Sims (112) was unable to find a correlation between the flavor stability of partially hydrogenated soybean oils containing 0.0-8.0% linolenic acid and their oxidative stabilities or content of polyunsaturated glycerides. Pyriadi and Mason (95) reported that the stability of pecan oils was dependent upon the concentration of tocopherols and the degree of unsaturation. The presence of natural prooxidant and antioxidant materials in these oils could account for the lack of correlation between oxidative stability and

unsaturation while the absence of these materials in purified phospholipids could explain the relationship between the rate of oxidation of a phospholipid and its concentration of unsaturated fatty acids. The reason the ratio of rates for egg phospholipids was larger than the ratio of rates for soybean phospholipids might be related to the percentage of the most unsaturated fatty acid in each phospholipid (Table 1), since it is the fatty acid that is oxidized first and is primarily responsible for the rate of oxidation. Egg PE has 3.5 times more arachidonic acid than egg PC while soybean PE has only 1.9 times more linolenic acid than soybean PC. This rather large amount of arachidonic acid in egg PE could also explain why it had a much higher rate of oxidation than the other phospholipids for the temperatures at which the rate was measured (Figure 6).

Effect of the Nitrogen Moiety on Phospholipid Oxidation

The differences in the amount of time which elapsed before the egg PC fractions in Figures 7 and 8 reached their maximum rate of oxidation can probably be attributed to the content of arachidonic acid in these fractions. The induction period of the three PC fractions increased as the percentage of arachidonic acid decreased. Since the arachidonic acid concentration of the first egg PC fraction was almost identical with the arachidonic

acid concentration of egg PE, an additional factor, possibly the influence of the nitrogen moiety, could explain the different induction periods for PE and the high arachidonic acid PC. To test this hypothesis egg PC was purposely contaminated with 1% and 5% PE (Figure 9). The rate of oxidation of PC was not altered appreciably by the addition of a small amount of PE, which was not unexpected in view of the finding that the straight line portion of the oxygen uptake curve is regulated primarily by the composition of unsaturated fatty acids. However, there was a definite shortening of the induction period with the greatest reduction occurring for the PC with 5% added PE, indicating either that the ethanolamine base has a greater prooxidant effect than choline or that the choline base has a greater antioxidant capability than ethanolamine.

Effect of the Nitrogen Moiety on Methyl Linolenate Oxidation

The influence of the nitrogen moiety of phospholipids on the oxidation of neutral lipids and phospholipids is readily apparent but not completely understood. Although PE has been regarded as a more effective antioxidant than PC (83, 85), the greater susceptibility of PE toward oxidation has also been reported (1, 77, 99). In an effort to investigate the role of the nitrogen moiety during the oxidation of unsaturated fatty acids,

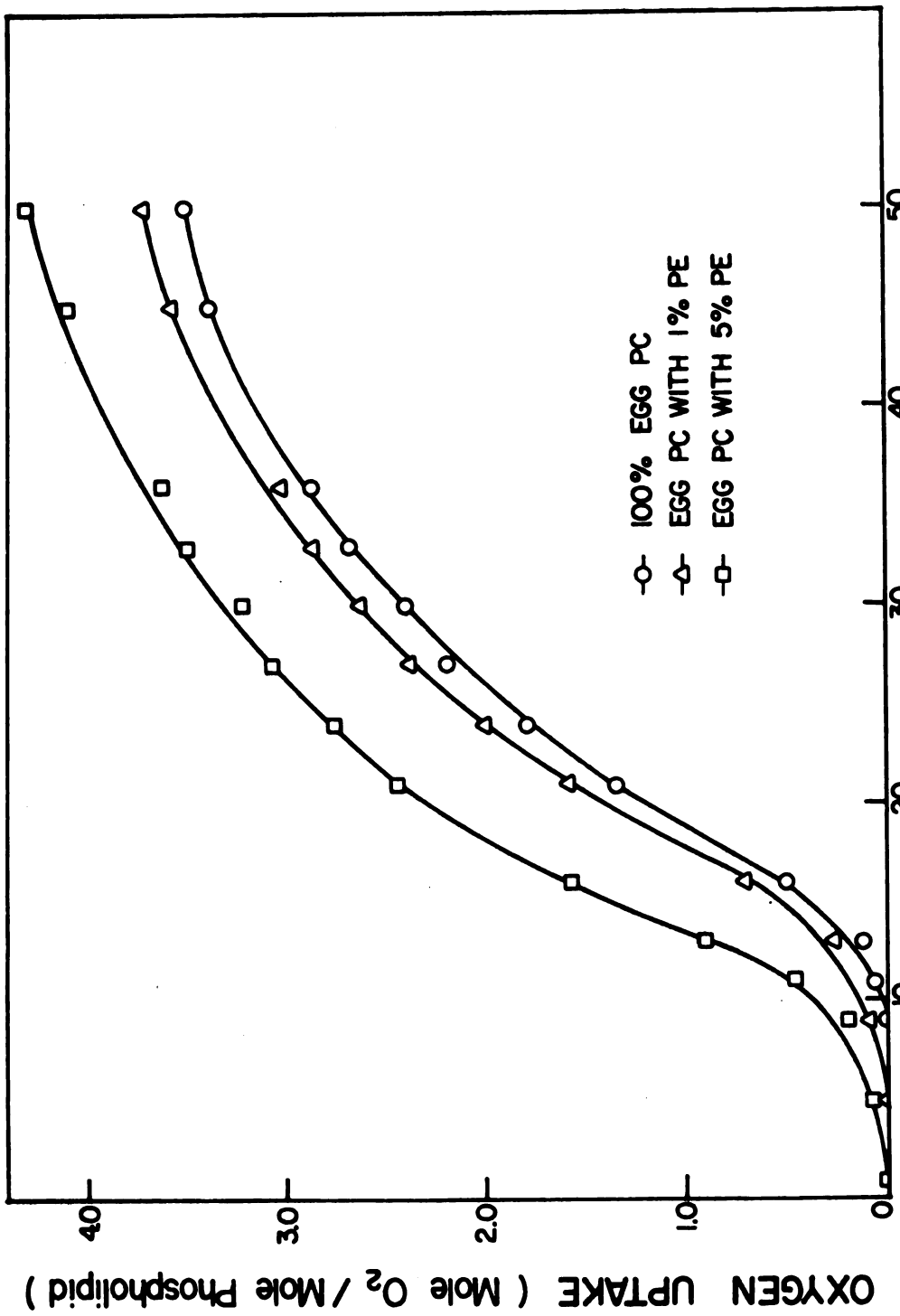


FIGURE 9.--The effect of phosphatidyl ethanolamine (PE) contamination on the oxygen consumption of egg phosphatidyl choline (PC). PC was emulsified in 0.1M borate (pH 7.0)-Tween 20 buffer and oxidized at 40°C.

model systems were prepared by dispersing methyl linolenate in borate (pH 7.0) buffer. Aliquots of glycerophosphate, choline chloride and ethanolamine were added to approximate the composition of phosphatidic acid, PC and PE. Figure 10 summarizes the effect of these additives on the autoxidation of methyl linolenate. The induction period for the methyl linolenate dispersion was significantly reduced when glycerophosphate and ethanolamine were added. The effect of glycerophosphate and glycerophosphate + choline chloride was slightly antioxidative while that of glycerophosphate + ethanolamine was definitely prooxidative as compared to methyl linolenate without additives.

After the onset of oxidation, a brownish-yellow color developed in the methyl linolenate dispersion containing glycerophosphate + ethanolamine. This color served to distinguish the dispersion containing ethanolamine from the other methyl linolenate treatments presented in Figure 10. The brownish-yellow color resembled the color of aqueous PE dispersions which had undergone oxidation. The readiness with which some phospholipids decompose and become brown has been noted by other investigators. Folch (34) observed that a PE preparation from brain, although initially white, acquired a tan color after 2 weeks in an evacuated desiccator. Lea (59) reported that browning occurred as a result of the autoxidation

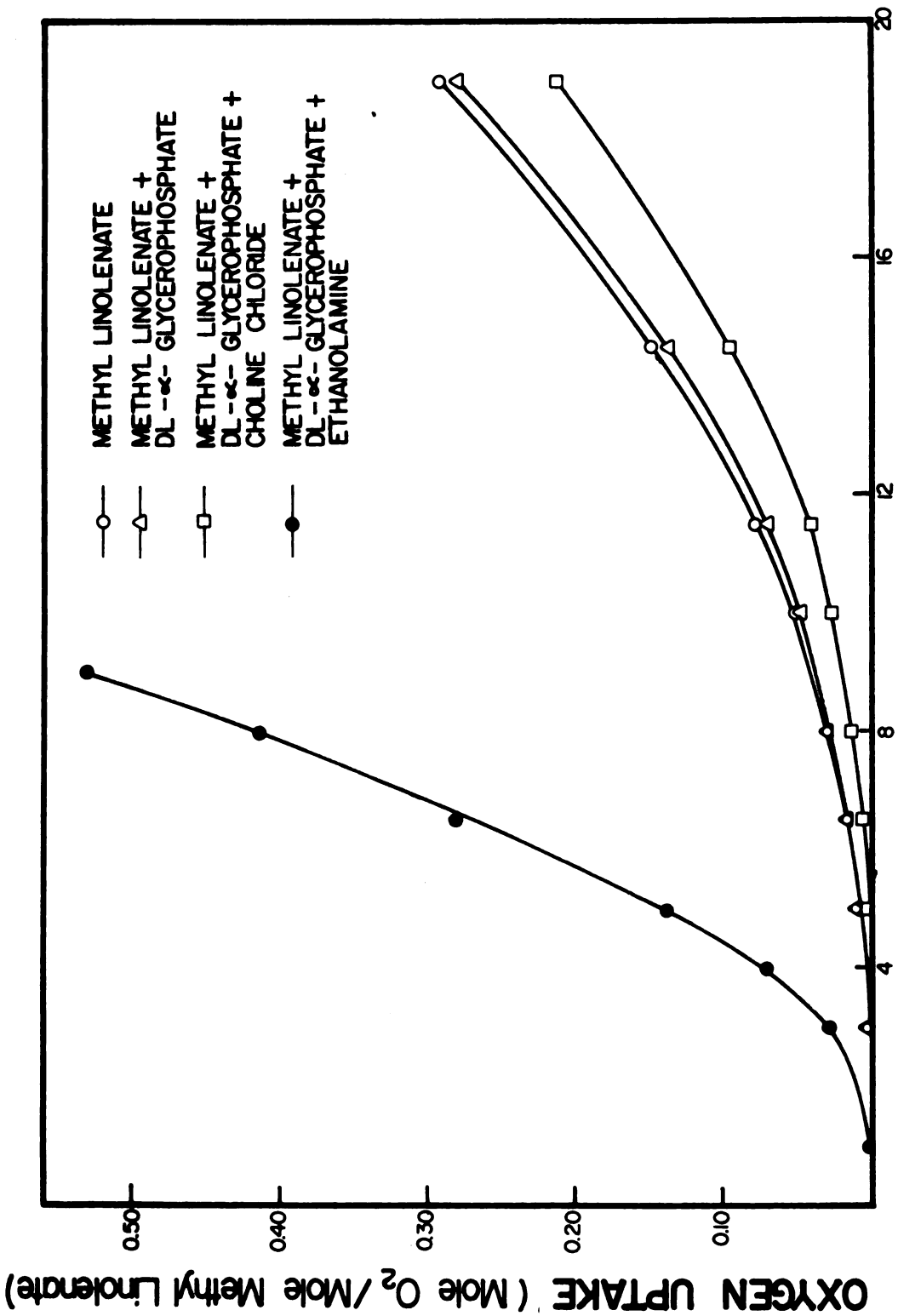


FIGURE 10.--The effect of ethanolamine and choline chloride on the autoxidation of methyl linolenate dispersed in 0.1M borate (pH 7.0) buffer at 40°C.

of PE; however, browning in relation to oxygen absorption was considerably less for PC than for PE.

The browning of many food products has been traced to Maillard-type reactions between the amino group of proteins and the aldehydic group of sugars. During the autoxidation of polyunsaturated fatty acids carbonyl compounds are formed. Carbonyl compounds possess an aldehydic group which is capable of reacting with the free amino group of ethanolamine or PE in a Maillard-type reaction, analogous to the reaction between sugars and proteins. In fact, the amino-containing phospholipids of egg yolk have been implicated in the Maillard reaction in dried eggs, although glucose instead of autoxidation products was thought to be the aldehyde mainly responsible for browning (59).

Since the only difference between the model system containing ethanolamine and the one containing choline was in the nitrogen moiety, the data suggest that the free amino group exerts a definite prooxidant effect on the autoxidation of unsaturated fatty acids. Furthermore, the brownish-yellow color which developed during oxidation was observed in the methyl linolenate dispersion containing ethanolamine but not in the one containing choline. The prooxidant effect of ethanolamine might be related to the participation of its free amino group in the development of the brownish-yellow color

by a Maillard-type reaction. In contrast, the quaternary nitrogen of choline did not react with the carbonyl group of oxidation products at the temperatures used in this study to produce a brownish-yellow discoloration.

Relative Rates of Autoxidation of Polyunsaturated Fatty Acids in Phospholipids

El-Gharbawi and Dugan (30) reported that a marked loss of polyunsaturated fatty acids occurred in the phospholipid fractions of freeze-dried raw beef after a short time of storage. The susceptibility of arachidonic and linoleic acids in egg PE and PC to oxidative attack is illustrated in Figures 11 and 12. Oxygen uptake and the percentage of the two unsaturated fatty acids remaining, during the period of oxidation, are plotted as functions of time. The pattern was similar for both phospholipids. Arachidonic acid disappeared at a more rapid rate and was totally absent midway during the period of oxidation. The percentage of linoleic acid decreased to a minimum level that was relatively stable.

The observation that arachidonic acid oxidized more rapidly than linoleic acid was predictable from data published by Holman and Elmer (48). These workers reported that the rate of oxidation was 4.8 times greater for methyl arachidonate than for ethyl linoleate. The more rapid disappearance of arachidonic acid compared to

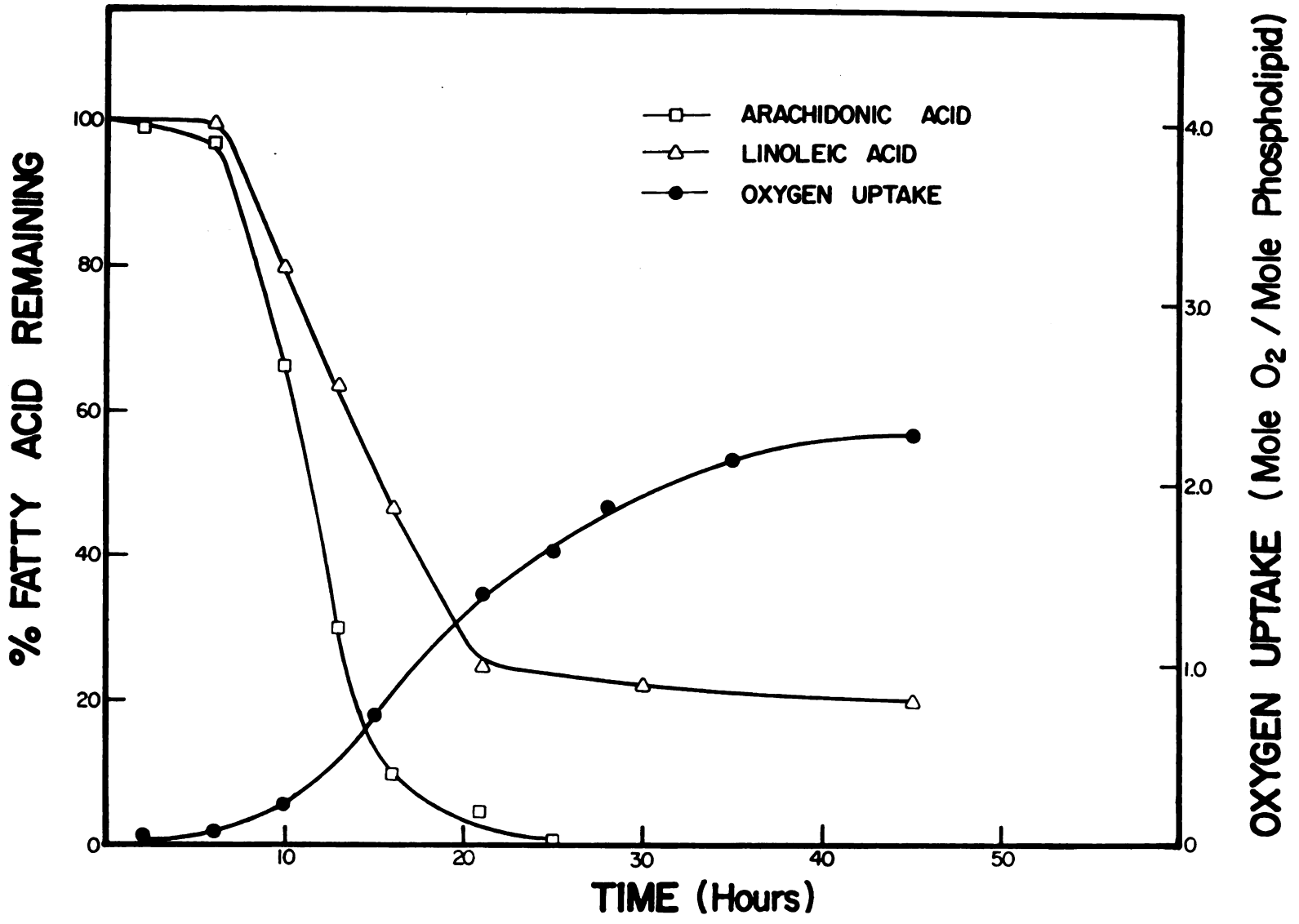


FIGURE 11.--Relationship between the disappearance of the two major polyunsaturated fatty acids of egg phosphatidyl choline emulsified in 0.1M borate (pH 7.0) buffer and oxygen uptake at 40°C.

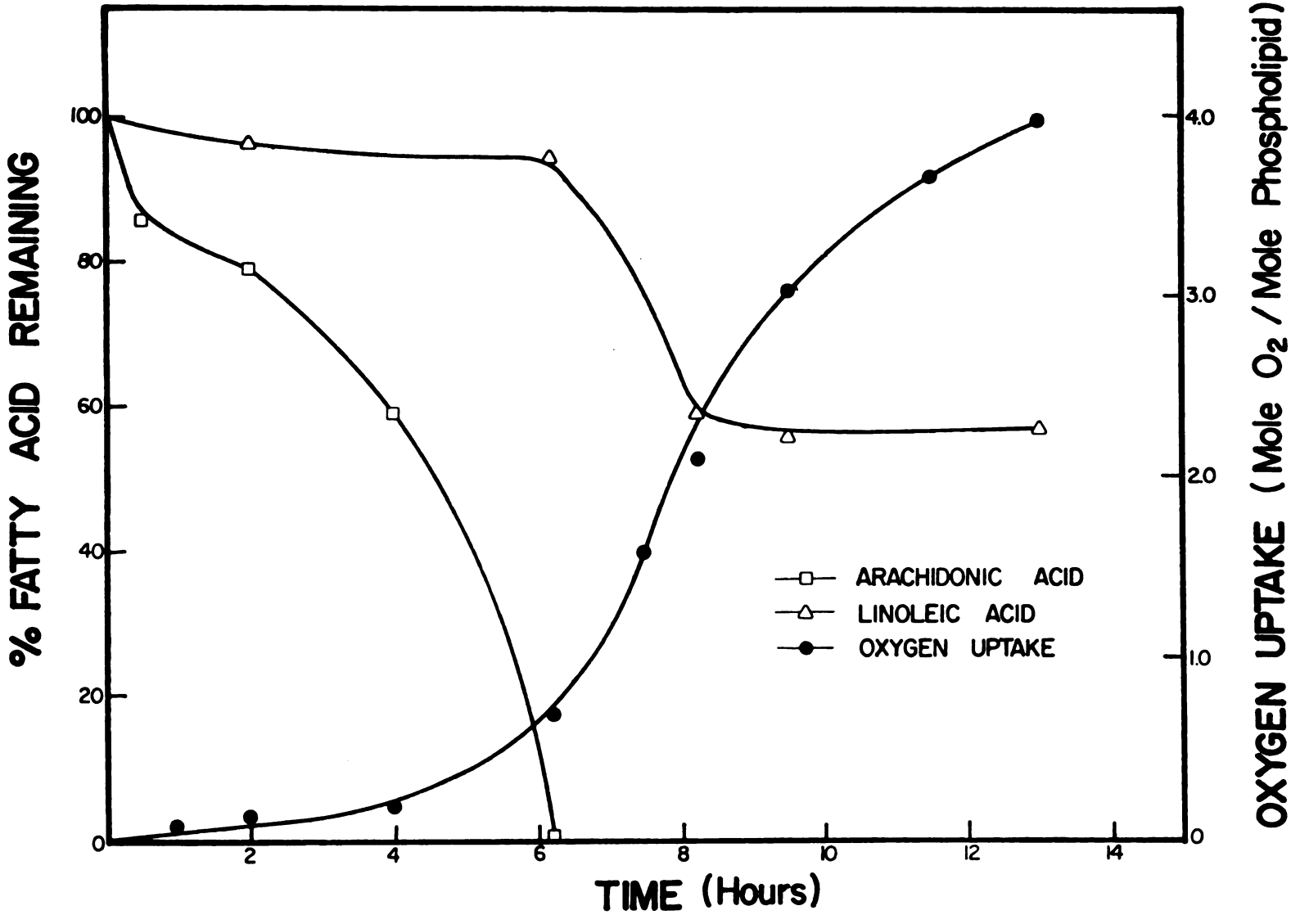


FIGURE 12.--Relationship between the disappearance of the two major polyunsaturated fatty acids of egg phosphatidyl ethanolamine emulsified in 0.1M borate (pH 7.0) buffer and oxygen uptake at 25°C.

linoleic acid was consistent with the finding that egg PC containing 12.4% arachidonic acid was considerably more reactive than egg PC containing 4.2% arachidonic acid (Figures 7, 8) and supported the conclusion that the rate of oxidation of a phospholipid is regulated by its polyunsaturated fatty acid content.

During the 45 hour reaction period for PC and the 13 hour reaction period for PE, the concentration of the other unsaturated fatty acids, 16:1 and 18:1 and the saturated fatty acids, 16:0 and 18:0 remained fairly constant relative to each other. They increased in amount concurrent with the disappearance of arachidonic and linoleic acids. Similar results were reported for the autoxidation of cod PE (99) and human red cells (26).

The greater reactivity of PE as compared to PC has been well documented (59, 77, 99). Not only does egg PE oxidize at a more rapid rate, it also has the capacity to react with more oxygen, undoubtedly because it has more unsaturation than egg PC. The curves in Figures 11 and 12 support and in part explain the differences in reactivity for PE and PC. Although the arachidonic acid concentration is much higher in PE than in PC (Table 1), the time required for the total disappearance of arachidonic acid was approximately 6 hours at 25°C for PE and about 25 hours at 40°C for PC. The

arachidonic acid disappeared so rapidly and selectively in PE that none was present after 6 hours, while almost all of the linoleic acid was still present. However, the disappearance of linoleic acid followed more closely the disappearance of arachidonic acid in PC and reached its minimum for the period of oxidation at about the same time that the percentage of arachidonic acid dropped to zero. The difference in the disappearance of linoleic acid in PC and PE could have resulted because of a higher rate of reaction at the higher temperature. Also the longer time and higher temperature (40°C) that were required to completely eliminate arachidonic acid from the PC reaction mixture may have allowed products of the oxidation of arachidonic acid more opportunity to catalyze the disappearance of linoleic acid.

Considerable oxygen was absorbed, especially by PE but also by PC, after arachidonic acid was totally absent and linoleic acid had reached its minimum level. This observation is probably related to the manner in which lipids oxidize. The primary products of the reaction of oxygen with unsaturated lipids are hydroperoxides, while alcohols, aldehydes and ketones are secondary products formed by degradation of primary hydroperoxides. The latter compounds, particularly if they are unsaturated, are highly susceptible to further oxidation (53). Lillard and Day (64) have reported that unsaturated

aldehydes oxidize at a much faster rate than either methyl linoleate or linolenate. Thus, the degradation compounds from the autoxidation of PE and PC would be expected to be highly reactive and as a consequence capable of increasing the total amount of oxygen consumed by the reaction mixture.

Relationship Between Spectrophotometric Determinations and Oxygen Uptake

Since various degradation compounds are produced by the autoxidation of phospholipids, an attempt was made to correlate oxygen absorption with specific changes which may be evaluated by spectrophotometric determinations. Figures 13-15 illustrate the relationship between oxygen uptake and the development of alterations in egg PC, egg PE and soybean PE respectively, as measured by the TBA test, ultraviolet absorbance of diene and triene conjugation, and changes in the amount of the free amino group of ethanolamine.

TBA and diene and triene conjugation followed a similar pattern for egg PC (Figure 13). They attained their maximum levels when the rate of oxygen consumption began to decrease. Tarladgis and Watts (119) measured malonaldehyde production with the TBA test during the oxidation of unsaturated fatty acids. They observed that malonaldehyde production followed oxygen uptake, reaching a peak at the same time that oxygen uptake

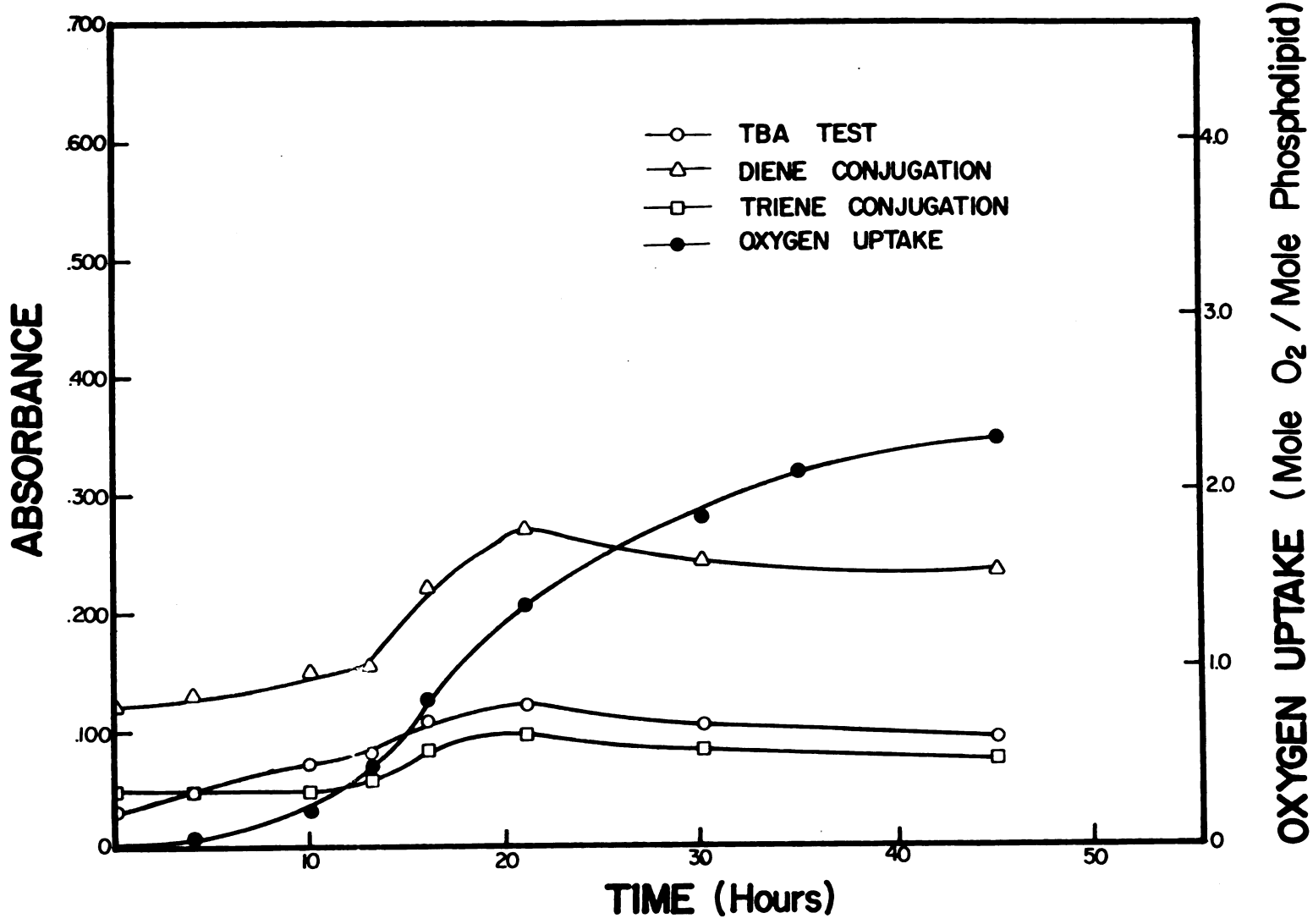


FIGURE 13.--Alterations of egg phosphatidyl choline emulsified in 0.1M borate (pH 7.0) buffer during oxidation at 40°C as evaluated by various physico-chemical tests.

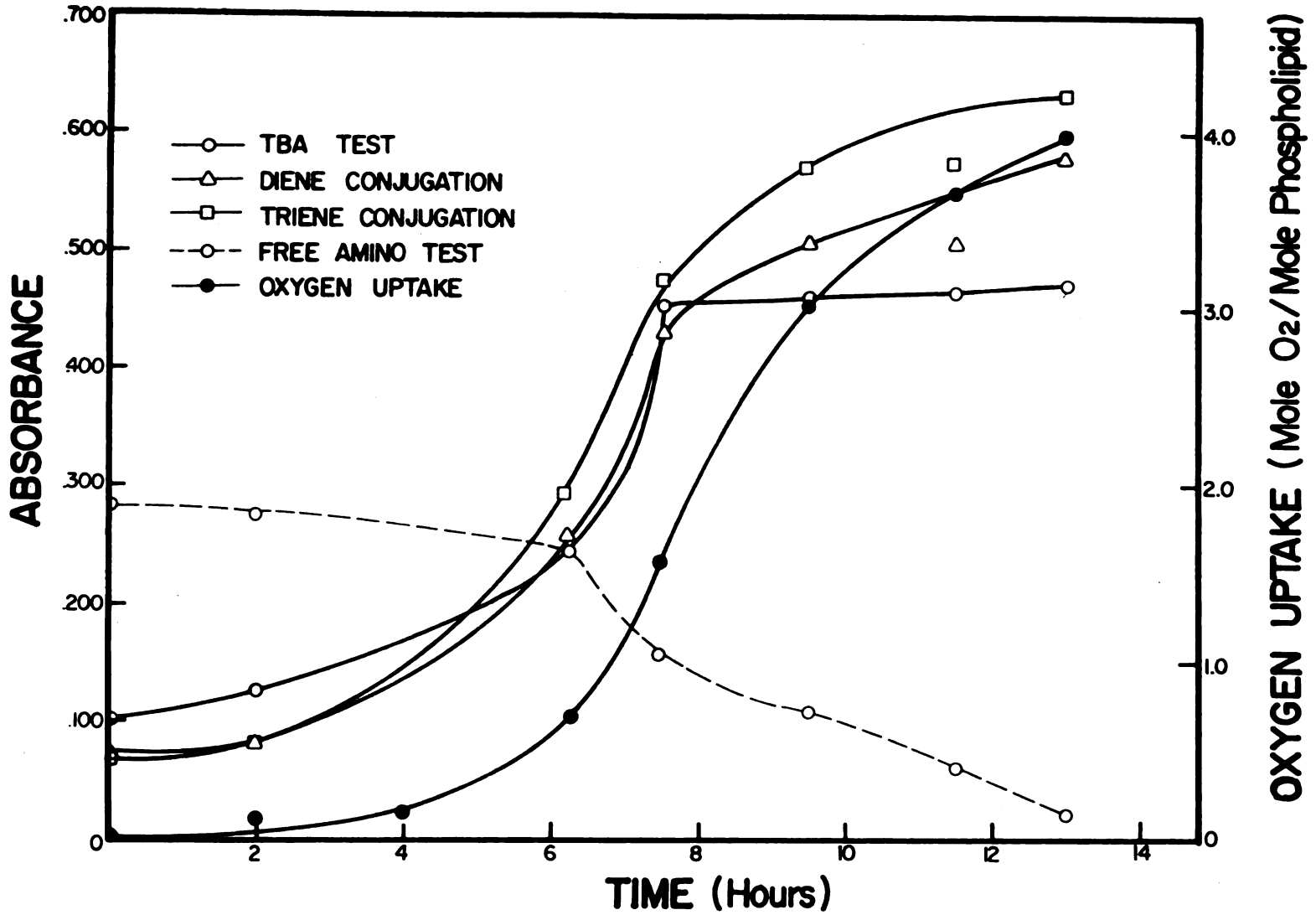


FIGURE 14.--Alterations of egg phosphatidyl ethanolamine emulsified in 0.1M borate (pH 7.0) buffer during oxidation at 25°C as evaluated by various physico-chemical tests.

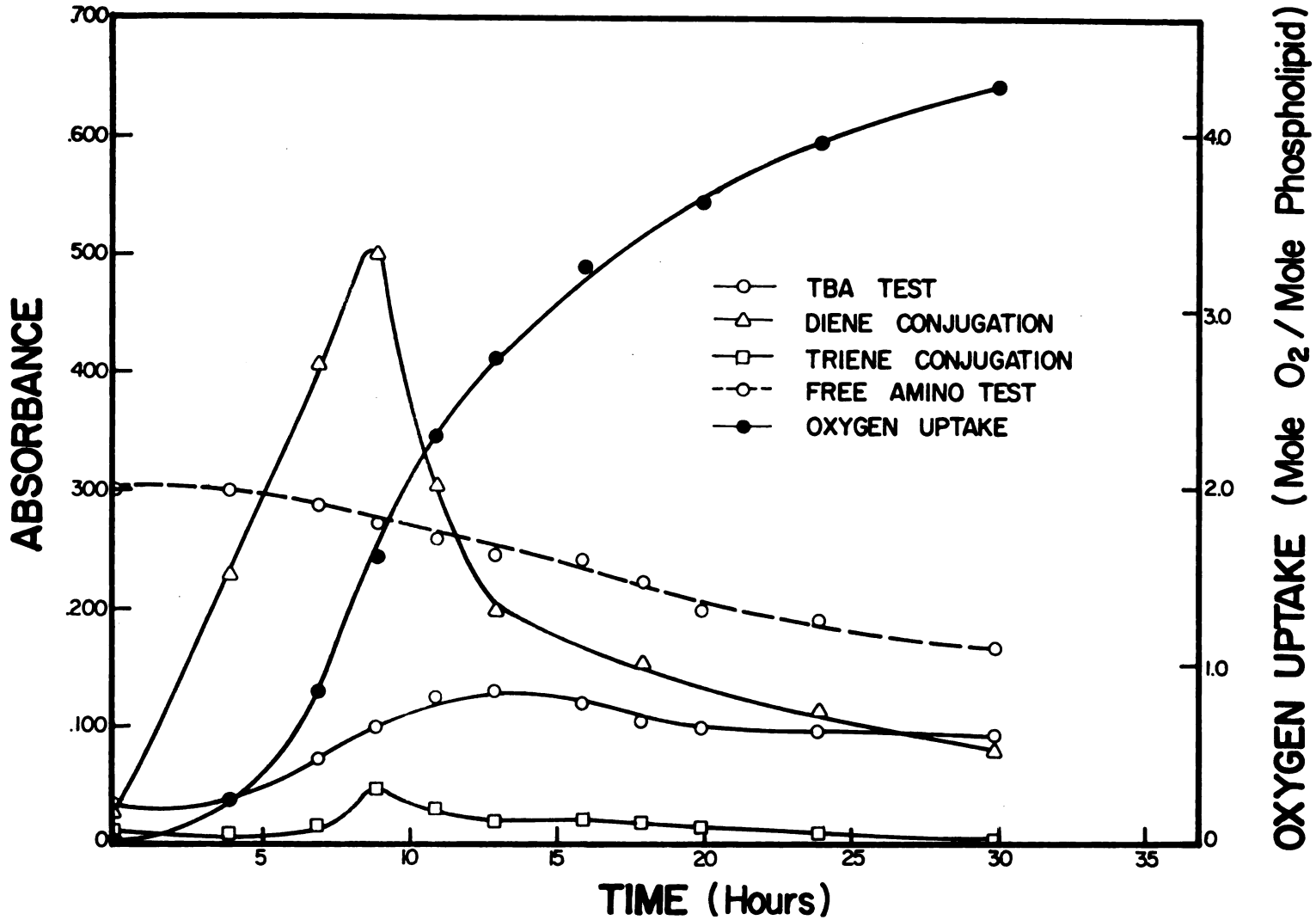


FIGURE 15.--Alterations of soybean phosphatidyl ethanolamine emulsified in 0.1M borate (pH 7.0) buffer during oxidation at 40°C as evaluated by various physico-chemical tests.

started to decline. These workers concluded that the malonaldehyde precursor was not accumulating as a stable end-product. After reaching the peak, more malonaldehyde precursor was destroyed than produced. The relationship between oxygen uptake and the extent of the ultraviolet absorption (UV) of autoxidized methyl linoleate has been demonstrated (91). At the end of the induction period, the UV absorption of methyl linoleate increased sharply with the formation of stable (conjugated diene) hydroperoxides (91).

Higher TBA and diene and triene conjugation values for egg PE (Figure 14) reflect the more rapid oxidation of PE as compared to PC. These values followed oxygen uptake but continued to increase even though the rate of oxygen consumption has decreased when this study was terminated. Triene conjugation was slightly higher than diene conjugation for egg PE. The greater amount of arachidonic acid in PE as compared to PC (Table 1) undoubtedly accounted for the higher triene conjugation in the former.

TBA absorbance reached a maximum soon after diene and triene conjugation values were at their highest levels and then decreased somewhat for the duration of the autoxidation study of soybean PE (Figure 15). Thus an increase in the production of TBA reactables resulted from the oxidation of dienes and trienes, although the TBA reactables

did not accumulate as stable end-products. Diene conjugation increased rather rapidly for soybean PE during the early phase of oxidation as measured by oxygen uptake. Only a slight amount of triene conjugation was observed. The large amount of diene conjugation compared to the small amount of triene conjugation is probably related to the fatty acid composition of soybean PE (Table 1). Approximately two-thirds of the fatty acids in soybean PE are linoleic and linolenic acids, which contain 2 and 3 bonds. Considerable diene conjugation of soybean PE could occur by the shift of one double bond in a small percentage of the fatty acids containing 2 or 3 double bonds. Although 10.6% of the fatty acids of soybean PE are linolenic, the autoxidation of soybean PE did not result in the formation of triene conjugation to the extent that occurred by the autoxidation of egg PE and PC, particularly PE. The data indicates that secondary oxidation products were produced from the diene conjugates of soybean PE prior to the formation of appreciable triene conjugates. The presence of 4 double bonds in arachidonic acid increased the opportunity for a shift of two double bonds and probably accounted for the greater triene conjugation of egg PE and PC compared to soybean PE.

The real value of TBA and ultraviolet absorption measurements for the analysis of unsaturated lipids has

frequently been questioned. The magnitude of change of TBA and UV readings is not as easily related to the degree of oxidation as the determination of oxygen absorption or the measurement of decreases in specific unsaturated fatty acids. When considered separately, the significance of TBA and UV measurements is small, since the products they measure do not increase proportionally to the extent of lipid oxidation. Even though TBA and UV measurements are not always effective indicators of the precise stage of lipid oxidation, they contribute to a more thorough understanding of the complexities of the autoxidation of unsaturated lipids when they are utilized in combination with the quantitative determination of oxygen absorption and with changes in the concentration of specific unsaturated fatty acids.

Influence of the Free Amino Group on Phospholipid Oxidation

The high reactivity of PE (59, 77, 99) and phosphatidyl serine (1, 77) in comparison to PC suggests that the free amino group of ethanolamine and serine influences phospholipid oxidation. PC fractions with the same iodine value as PE were reported to oxidize less rapidly (59). The result was similar for PE and a PC fraction containing equivalent amounts of polyunsaturated fatty acids (Figures 7, 8). PE absorbed oxygen more rapidly than the high arachidonic containing PC.

In this study, the free amino test was performed on PE to determine if changes in its concentration* might be related to the reactivity of PE. Lea (59) reported that the free amino group of PE disappeared during oxidation in a degree approximately proportional to the amount of oxygen absorbed. The free amino absorbance value of egg PE was significantly reduced during the period of oxidation (Figure 14). The disappearance of the free amino group of soybean PE (Figure 15) was not as pronounced as that for egg PE, even though both phospholipids consumed nearly equivalent amounts of oxygen.

The disappearance of the free amino group of PE provides support for the contention that a Maillard-type browning reaction can occur as a result of the autoxidation of PE. Malonaldehyde and other 2-thiobarbituric-acid-reactive substances, produced during the oxidation of polyunsaturated fatty acids, can react with amino acids and proteins (56). The decrease in the free amino group of PE probably resulted from its interaction with specific autoxidation products of unsaturated fatty acids such as carbonyl compounds (60). Hydroperoxides, epoxides and aldehydes have been mentioned as substances which could contribute to the disappearance of the free

---

*The concentration of ethanolamine can be obtained from a standard curve once the absorbance of the reaction between trinitrobenzene sulfonic acid and PE has been determined (111).

amino group of PE (59). During oxidation, the brownish-yellow color which developed in dispersions of methyl linolenate + ethanolamine, was also observed in PE emulsions. Characteristic features which serve to distinguish the oxidation of PE from the oxidation of PC are the development of the brownish-yellow color, a decrease in the concentration of the free amino group and a shorter induction period. The development of the discoloration and the decrease in the concentration of the free amino group suggest that Maillard-type browning reactions have occurred. The relationship between browning and the shorter induction period of PE is not readily apparent. Maillard-type browning reactions could accelerate phospholipid oxidation or else be incidental and occur as a result of the presence of carbonyl and amino groups. Nevertheless, the possibility exists that intermediate products from the reaction of free amino and carbonyl groups might accelerate phospholipid oxidation, perhaps by promoting the formation of free radicals or other readily oxidizable substances, and thereby account for the greater reactivity of PE compared to PC.

Influence of Added Nitrogen-Containing Compounds on Phospholipid Oxidation

Since the nature of the nitrogen moiety is an important factor affecting phospholipid oxidation, experiments were conducted to determine if the addition

of ethanolamine and choline would have any influence on the autoxidation of purified phospholipids. Figure 16 illustrates the effect of ethanolamine and choline on the oxidation of PE when both nitrogen moieties were added in a 1:1 molar ratio of nitrogen moiety and phospholipid. Compared to the PE control, the free nitrogen moieties caused a slight reduction in the reactivity of PE. Both ethanolamine and choline (added as choline chloride) were somewhat antioxidative. When ethanolamine was added to PC in a ratio of 1 mole ethanolamine to 1 mole phospholipid the result was similar (Figure 17). Ethanolamine retarded the autoxidation of PC. The antioxidative effect of the added ethanolamine in phospholipid emulsions was in contrast with the prooxidative effect of ethanolamine in methyl linolenate emulsions. The added ethanolamine could function in the same way as amino acids which are often prooxidants in neutral lipid emulsions (76) and antioxidants in phospholipid emulsions (46). The antioxidative effect of ethanolamine or amino acids in phospholipids, as opposed to neutral lipids, might then be related to the phosphate group in phospholipids which under certain conditions has been reported to have antioxidant activity (22, 93). Furthermore, amino acids are known to act as antioxidants for neutral lipid emulsions in the presence of phosphate buffer (76, 102). Thus, the prooxidative effect which

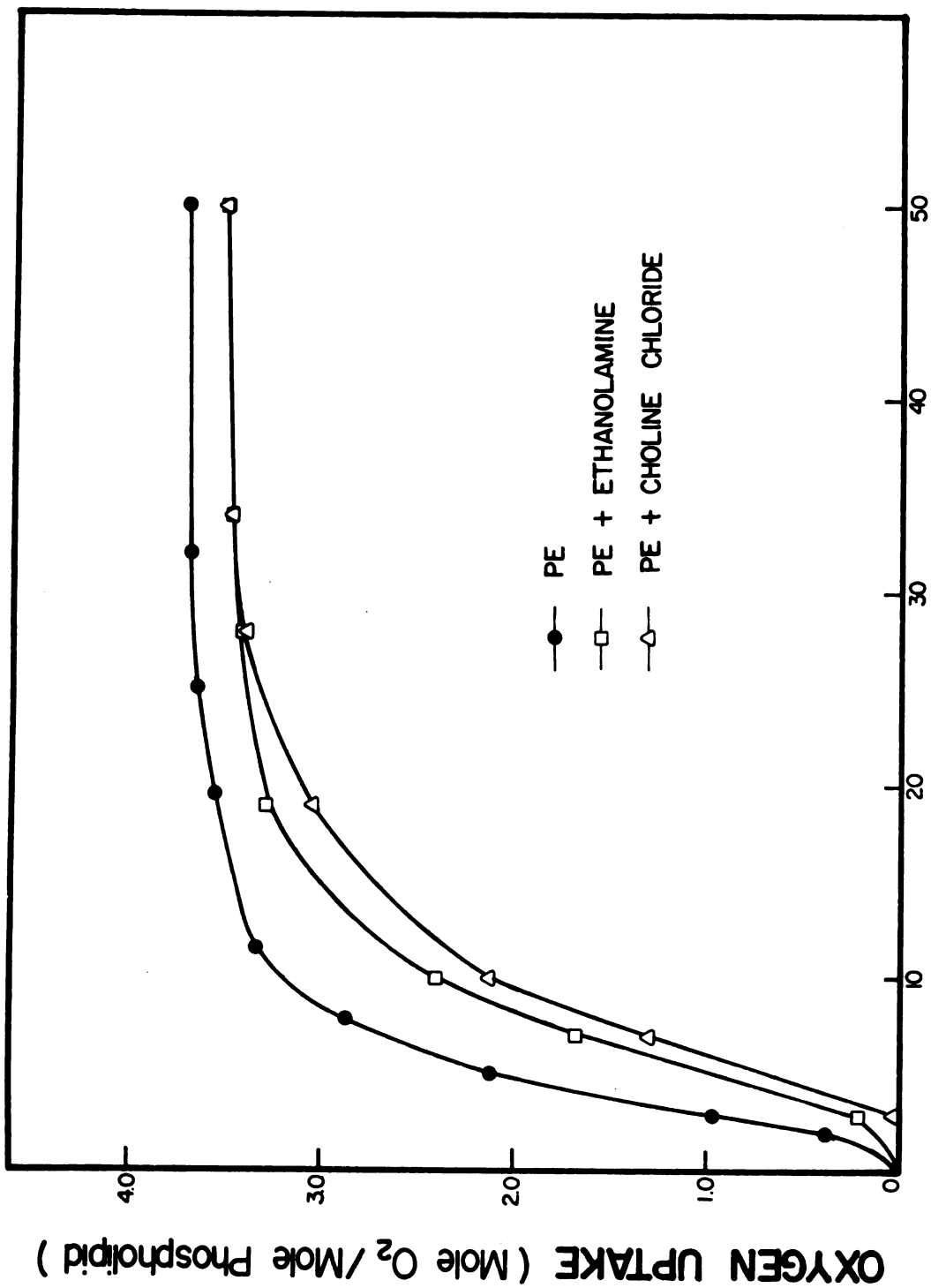


FIGURE 16.--The effect of choline chloride and ethanolamine on the autoxidation of egg PE dispersed in 0.1M borate (pH 7.0) buffer at 25°C.

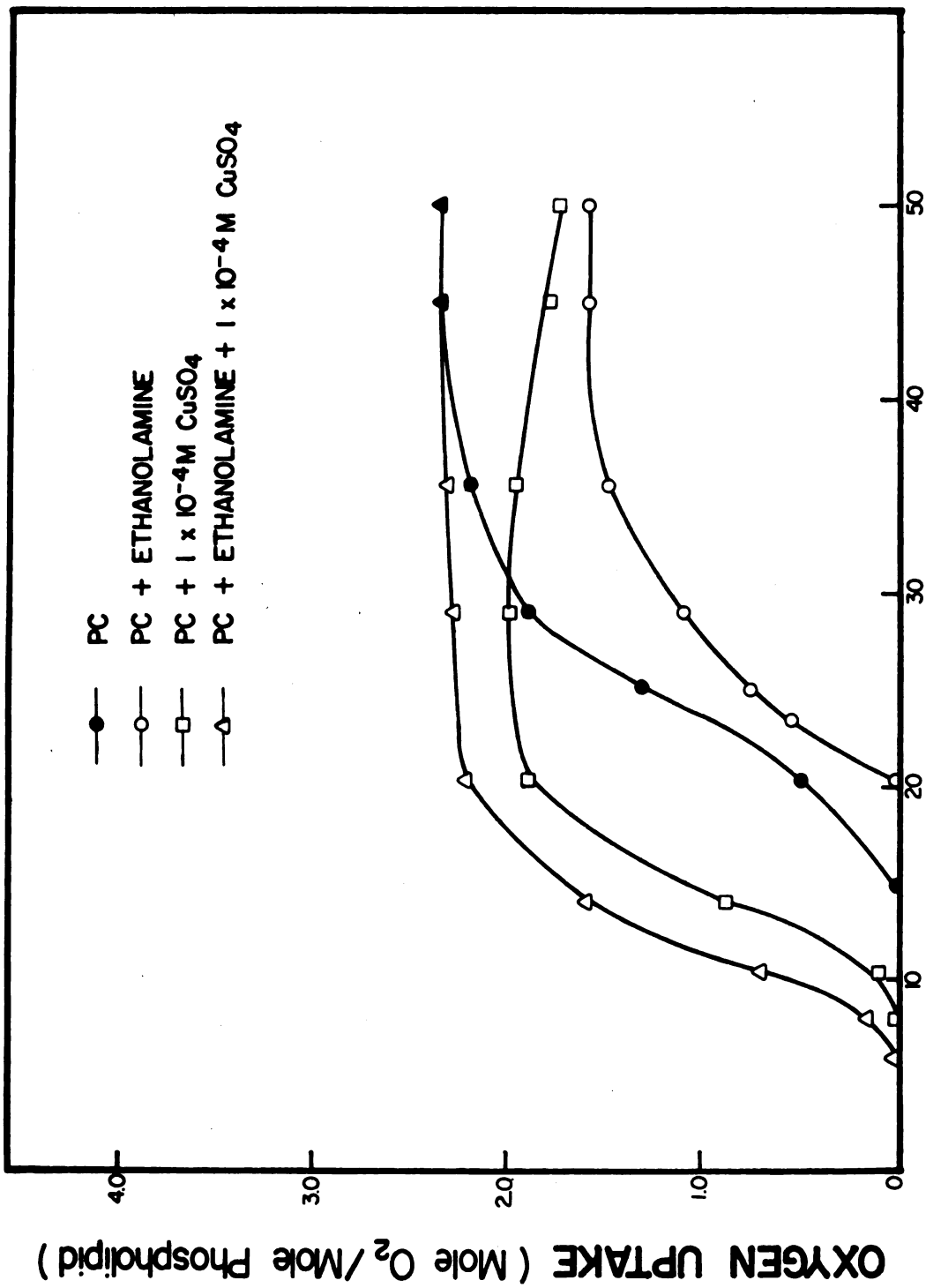


FIGURE 17.--The effect of ethanolamine and $CuSO_4$ on the autoxidation of egg PC dispersed in 0.1M borate (pH 7.0) buffer at 40°C.

the nitrogen moiety of PE has been shown to exert during the oxidation of PE must depend upon the attachment of ethanolamine to the phospholipid molecule.

Heavy metals, particularly those possessing two or more valency states with a suitable oxidation-potential between them (e.g., Co, Cu, Fe, Mn, Ni, etc.), generally increase the rate of oxidative deterioration of lipids (49). These metals reduce the length of the induction period and increase the maximum rate of oxidation (49). The presence of 1×10^{-4}M $CuSO_4$ in an aqueous dispersion of egg PC increased the oxidation rate and reduced the length of the induction period (Figure 17). Of interest was the finding that the reactivity of PC was greater for ethanolamine and $CuSO_4$ together than for $CuSO_4$ alone, despite the fact that ethanolamine exerted an antioxidative effect on PC.

The results were similar when the amino acid, glycine, was substituted for ethanolamine (Figure 18). Glycine decreased and $CuSO_4$ increased the susceptibility of PC to oxidation. When added together, glycine and $CuSO_4$ caused an increase in the reactivity of PC. The curves in Figures 17 and 18 indicate that together Cu^{++} and possibly the amino group of ethanolamine and glycine influence the autoxidation of PC by reducing the induction period and increasing the rate of oxidation.

Other workers have observed that lipid oxidation is accelerated by a combined effect of copper and amino

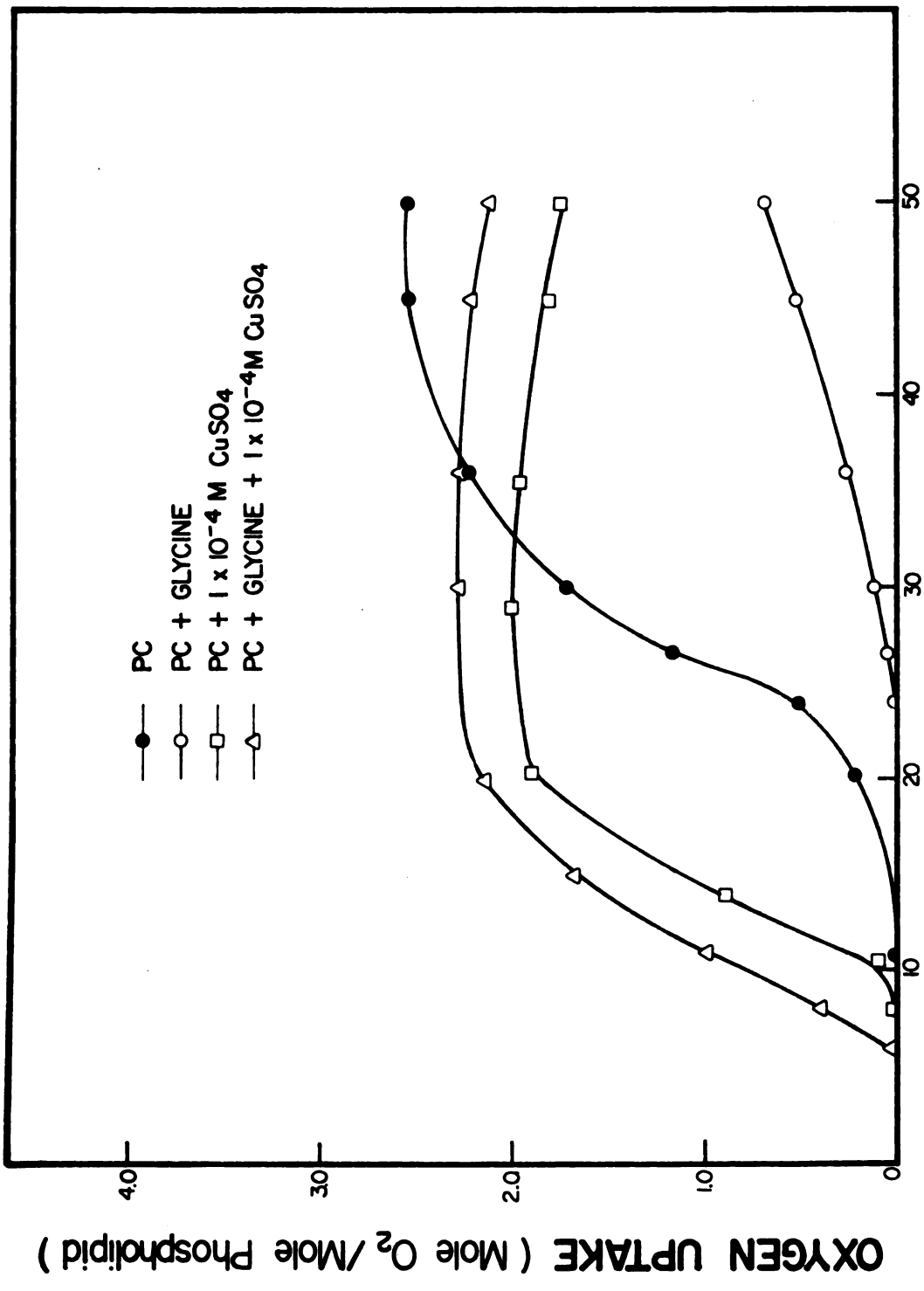


FIGURE 18.--The effect of glycine and $CuSO_4$ on the autoxidation of egg PC dispersed in 0.1M borate (pH 7.0) buffer at 40°C.

compounds. Copper-protein complexes were shown to be more effective catalysts for linoleate oxidation than was copper alone (118). Mattsson and Swartling (77) reported that PE which was poorly dispersed in buffer oxidized more readily in the presence of copper even though the copper did not improve its emulsification. Saunders et al. (102) studied the effect of histidine and trace metals on the oxidation of linoleate esters. The catalytic action these workers observed was attributed to an interaction between histidine and trace metals, since histidine had been shown to complex with metal ions and oxygen (41).

The accelerated oxidation illustrated in Figures 17 and 18 could result from an interaction between copper and ethanolamine and copper and glycine. Copper was shown to bind amino acids, peptides and proteins and to exert a strong catalyzing effect on the oxidation of ascorbic acid (103, 104). Compounds containing more than one donor N atom formed more than one type of complex with copper (103). Those complexes in which copper atoms were firmly bound tended to be the best catalysts (104). Part of the catalytic activity of copper-protein complexes in methyl linoleate oxidation has been ascribed to the combined effect of copper and protein in forming chelate bridges and in stabilizing the activated complex of linoleate peroxide-copper-protein (54). On

the basis of the data presented, the mechanism(s) by which a complex between copper and an amino compound could enhance lipid oxidation can only be speculated. The complex could conceivably increase the prooxidative effect of copper by changing its orientation at the aqueous-lipid interface. The formation of a complex might make copper, oxygen or both more available for oxidation reactions at or near the double bonds of unsaturated fatty acids. A copper-amino complex could accelerate the decomposition of hydroperoxides to form new chain-promoting radicals or to paraphrase a mechanism previously mentioned (54), the combined effect of copper and an amino compound might promote phospholipid oxidation by stabilizing the active complex of unsaturated fatty acid peroxide-copper-amino compound.

The Effect of Amino Acids on Phospholipid Oxidation

Amino acids have been shown to act as both pro- and antioxidants for the autoxidation of unsaturated fatty acids and esters (76, 102). Amino acids have been reported to exert an antioxidative effect on the oxidation of PC in the absence of nonionic emulsifier and phosphate buffers (46). The influence of glycine, lysine, tryptophan and histidine on the oxidation of egg PE dispersed in borate (pH 7.0) buffer is illustrated in Figure 19. Glycine, lysine and tryptophan increased the induction

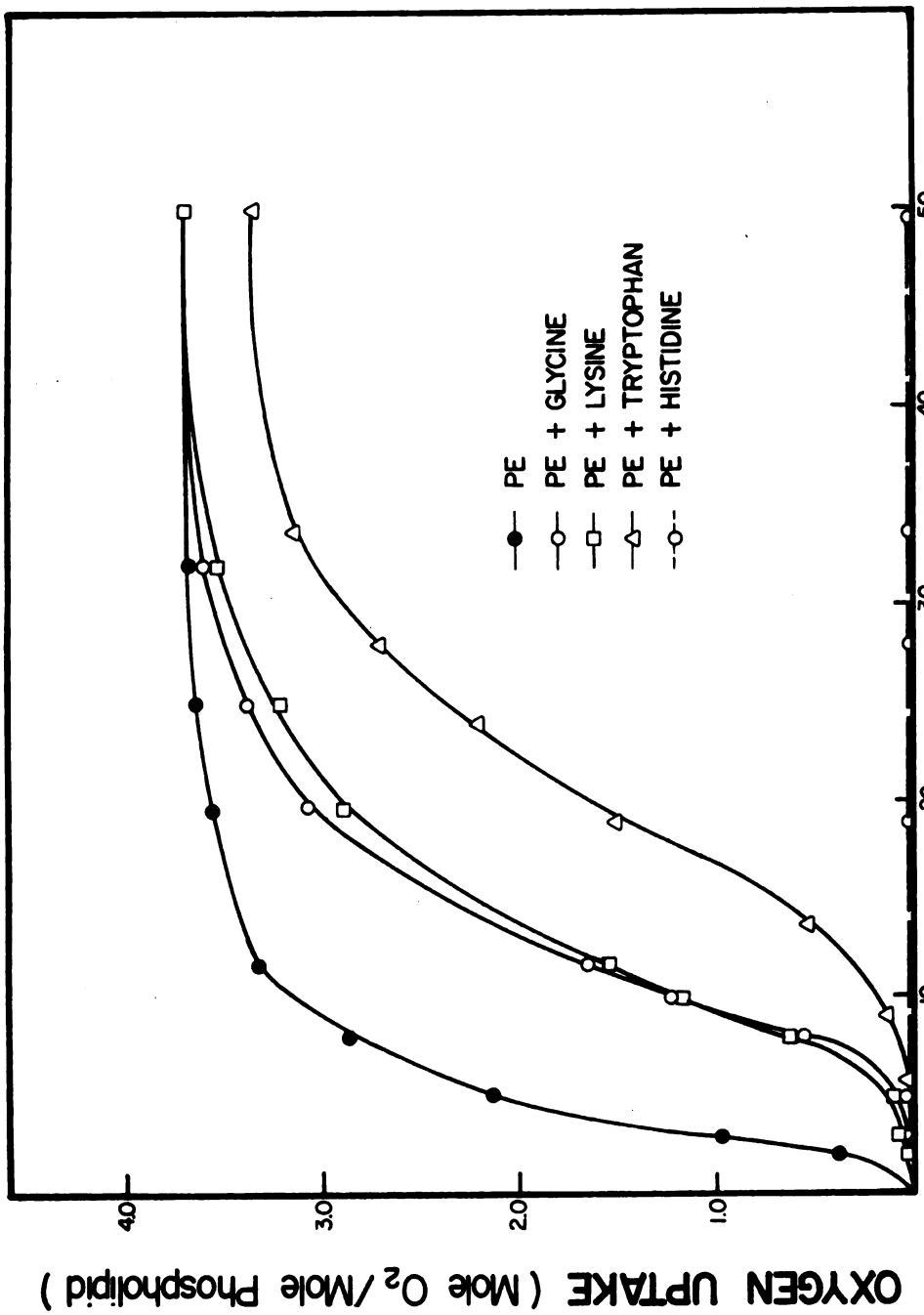


FIGURE 19.--The effect of various amino acids on the autoxidation of egg PE dispersed in 0.1M borate (pH 7.0) buffer at 25°C.

period for the oxidation of PE. Histidine completely inhibited the oxidation of PE as evaluated by oxygen uptake measurements. Confirmation for the inhibition of PE oxidation by histidine was obtained by TBA determinations. The TBA absorbance of PE with histidine was virtually unchanged after 50 hours as compared to the original TBA value of PE before measurements of uptake were initiated (Table 6). In contrast, the TAB absorbance of the PE samples containing the other three amino acids increased considerably.

The variability of the antioxidative capacity of different amino acids on linoleic acid and its methyl ester was observed by Marcuse (76). Histidine and tryptophan exerted a relatively strong antioxidative effect, which was especially pronounced under certain conditions such as a low amino acid concentration, a pH of 7 or higher, and the presence of phosphate buffer and/or nonionic emulsifier. A good explanation for the varied antioxidative effect of different amino acids on an unsaturated lipid has not been provided.

At higher amino acid concentrations, histidine and tryptophan inverted from an antioxidative to a prooxidative effect (76). Such inversion has been demonstrated for phenolic primary antioxidants (14) and ascorbic acid (74, 124). According to Heimann and Pezold (42, 43, 44), three reactions could be responsible for the final

TABLE 6.--Analysis, by the TBA test, of the extent of oxidation of PE samples[a] emulsified in 0.1M borate (pH 7.0) buffer after 50 hours at 25°C.[b]

Sample	TBA Absorbance
PE	.534
PE + $CuSO_4$	.574
PE + BHT	.009
PE + Na EDTA	.075
PE + Glycine	.544
PE + Lysine	.521
PE + Tryptophan	.557
PE + Histidine	.061

[a]TBA absorbance of PE before oxidation was .060.

[b]Determinations were made on aliquots containing 5.0 µg phosphorus as assayed by the procedure of Rouser et al. (101).

result in a system containing substances capable of being prooxidative as well antioxidative. They are: (1) the normal oxidation of the substrate; (2) the antioxidative reaction; and (3) the prooxidative reaction. In the system that Heimann and Pezold examined, containing lard and tocopherol, the prooxidative reaction had a high activation energy and only prevailed when a large amount of tocopherol was present. In contrast, only a small amount of tocopherol was needed to induce the antioxidative reaction.

The same explanation given by Heimann and Pezold for lard as substrate and tocopherol as a pro- or antioxidant could be valid for unsaturated lipids and amino acids. By analogy then, a small concentration of amino acids could protect against lipid oxidation by encouraging the antioxidative reaction, while the prooxidative reaction, having a high activation energy, would be enhanced by a high amino acid concentration. The variability of the antioxidative capacity of different amino acids when present at the same concentration could be related to their individual activation energies for the prooxidative reaction and the antioxidative reaction. The greater antioxidative effect of histidine in preventing the autoxidation of PE (Figure 19) could indicate that histidine has a lower activation energy for the antioxidative reaction or a higher activation energy

for the prooxidative reaction than glycine, lysine and tryptophan. If the antioxidative effect of an amino acid was dependent upon its activation energy for the antioxidative reaction, histidine would have the lowest activation energy for the system illustrated in Figure 19, followed by tryptophan, lysine and glycine respectively.

The significance of the antioxidative action of amino acids toward phosphatides is considerable since both exist in continuous interaction with each other in an aqueous system in many food and agricultural products. A better understanding of the prooxidative, antioxidative and synergistic relationships that exist between amino acids and phospholipids can provide information useful in protecting foods from lipid oxidation.

Phospholipid Antioxidation

Information on the autoxidation of egg PE in the presence of a metal, an antioxidant and a chelating agent is presented in Figure 20. Copper accelerated the oxidation of PE as evidenced by an increase in the oxidation rate and a decrease in the induction period. Butylated hydroxytoluene (BHT) and sodium tetra, ethylenediamine tetraacetate (Na EDTA) completely retarded oxidation as measured by oxygen uptake and the TBA test. The TBA absorbance of PE + histidine was less after 50 hours than the value recorded for PE prior to the start of oxygen uptake measurements (Table 6).

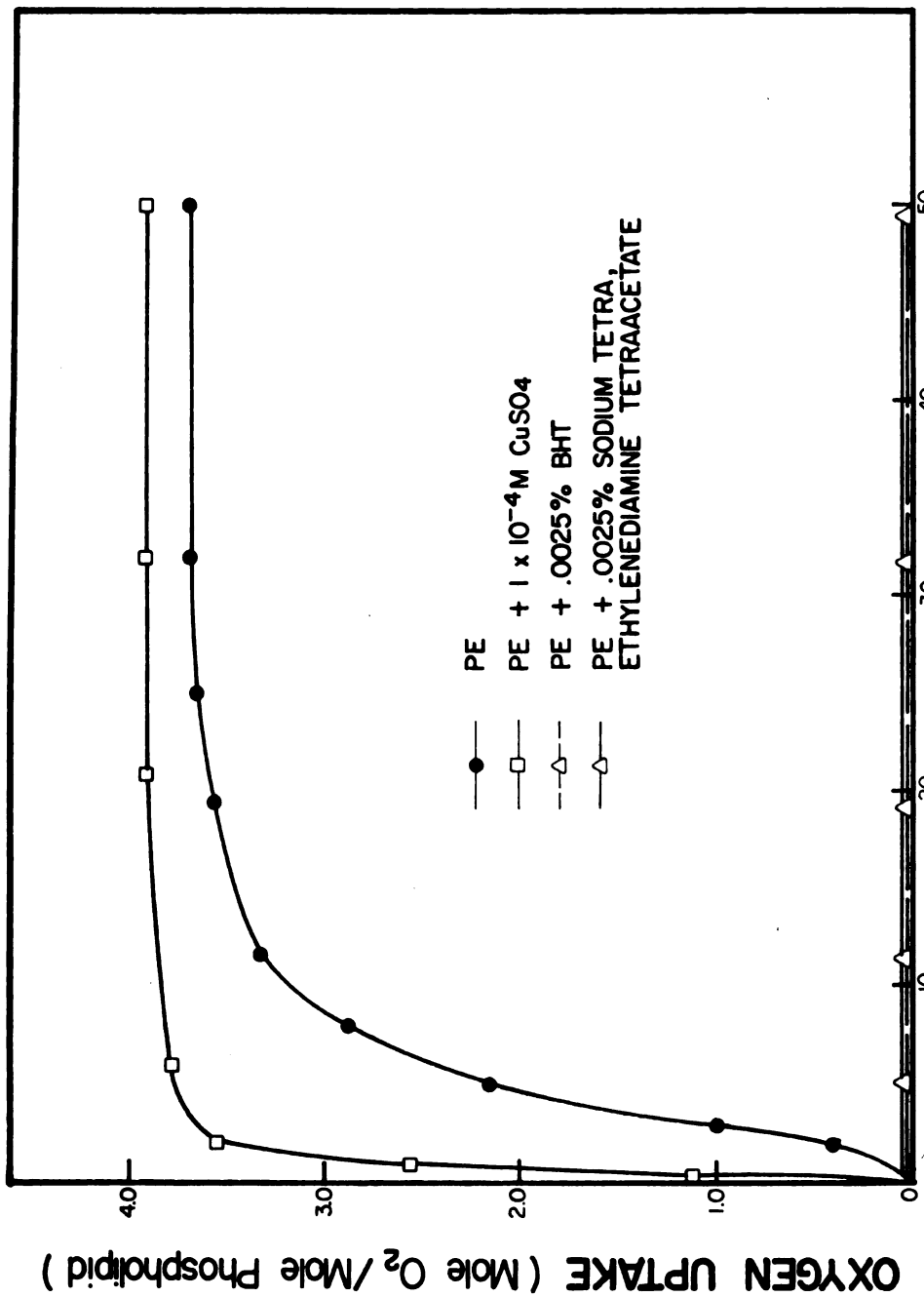


FIGURE 20.--The effect of a metal, an antioxidant and a chelating agent on the autoxidation of egg PE dispersed in 0.1M borate (pH 7.0) buffer at 25°C.

The ability of Na EDTA to retard oxidation suggests that trace metals may play a significant role in lipid oxidation. Uri (121, 122) has reported that lipid materials contain traces of copper, iron and cobalt. By complexing metal impurities, Na EDTA could aid in reducing the formation of free radicals and thereby provide a partial inactivation of oxidation in an autoxidizing system.

A free radical acceptor such as BHT is considered to be essential in achieving protection against all pro-oxidant impurities (123). The addition of BHT to organic solvents at the 0.005% level permitted safe storage of egg PE almost indefinitely at -20°C and during preparative work by column and thin-layer chromatography (81, 125). Research on various aspects of phospholipid oxidation has revealed that phosphatides are highly susceptible to autoxidation. A search of the literature will indicate that numerous studies are being conducted to characterize the role(s) of phospholipids in biological systems. Because changes in the chemical, physical and biological properties of phospholipids can arise through degradation, precautions should be taken to prevent oxidation during extraction, manipulation and storage of phospholipids.

General Discussion

This thesis research, which was designed to investigate the autoxidation of phospholipids, had a two-fold

purpose. One purpose was to relate the oxidation of phospholipids to their specific molecular constitution and the other was to examine the influence of various extraneous substances on phospholipid oxidation. Individual phospholipids have been observed to vary in their susceptibility to oxidation. Several workers (1, 59, 77, 99) have reported that phosphatidyl ethanolamine (PE) oxidizes more rapidly than phosphatidyl choline (PC). Unsaturated fatty acids and the nitrogen moiety are factors known to affect the oxidation of phospholipids. Substances which could be considered extraneous in model systems can act as either pro- or antioxidants in the presence of phospholipids and thus are important in natural food systems where their proximity to phospholipids may permit interaction.

Oxidation studies were performed on purified phospholipids which were emulsified in borate buffer. After the optimum pH for the autoxidation of PE and PC was shown to approximate neutrality, subsequent studies were conducted at pH 7.0. Autoxidation of egg and soybean phospholipids was followed by oxygen uptake determinations, ultraviolet absorption measurements, the TBA test and by changes in the concentration of polyunsaturated fatty acids.

Oxygen uptake determinations emphasized that phospholipids from different sources can vary in their susceptibility to oxidation. Egg phospholipids oxidized more

rapidly than soybean phospholipids. The differences that were noted were ascribed to the fatty acid compositions of egg and soybean phospholipids. Arachidonic acid, the most unsaturated fatty acid in egg PE and PC, contains four double bonds. Soybean PE and PC have as their most unsaturated fatty acid, linolenic acid with three double bonds, one less than arachidonic acid. The relationship between the unsaturation of fatty acids and their oxidation rate has been reported by Holman and Elmer (48). These workers measured the oxidation rate of several unsaturated fatty acids and their esters and concluded that an increase of one double bond in an unsaturated fatty acid or ester caused the rate of oxidation to increase by a factor of two or more. An increase in the number of double bonds also reduced the length of the induction period. Thus, the higher rate of oxidation and shorter induction period for arachidonic acid compared to linolenic acid could explain why egg phospholipids are more susceptible than soybean phospholipids to autoxidation.

The greater reactivity of PE compared to PC, observed by numerous workers, was confirmed in studies on the oxidation of egg and soybean phospholipids. An examination of the fatty acid composition of these phospholipids verified a finding reported by several lipid investigators. The finding, consistent for both

egg and soybean phospholipids, was that PE contained a higher percentage of unsaturated fatty acids than PC. The higher content of polyunsaturated fatty acids, particularly arachidonic acid in egg PE and linolenic acid in soybean PE, provides a partial explanation for the greater reactivity of PE compared to PC.

Corollary measurements which were made at intervals during the course of phospholipid oxidation were the TBA test, ultraviolet absorption of diene and triene conjugation and changes in the percentage composition of specific unsaturated fatty acids. The results of these tests were consistent with data obtained through oxygen uptake determinations and provided additional information on characteristics of phospholipid oxidation. Higher TBA and diene and triene absorption values for egg PE as compared to egg PC supported the oxygen uptake studies which indicated the greater reactivity of PE. Low triene conjugation values for soybean PE at all stages of oxidation suggested that the formation of triene conjugates was more likely to result from a phospholipid containing a 4 double bonded fatty acid than from one with three double bonds. The susceptibility of the arachidonic acid in egg phospholipids to oxidation was shown by GLC determinations, which permitted a quantitation of the decrease in its composition in relation to oxygen uptake. By comparison, the amount of linoleic acid in egg PE and PC decreased at a considerably slower rate.

The separation of egg PC by silicic acid column chromatography into three fractions which differed in their unsaturated fatty acid compositions provided an additional means of illustrating the importance of unsaturated fatty acids on phospholipid oxidation. The induction period increased as the percentage of unsaturated fatty acids, particularly arachidonic acid, decreased. The finding that the first egg PC fraction had a fatty acid composition which approximated the percentage of arachidonic acid in PE, permitted an examination of the influence of the nitrogen moiety in phospholipid oxidation. The importance of the nitrogen moiety was established by the observation that the induction period for PE was significantly less than that for the PC with the high arachidonic acid content. The maximum rate of oxidation for PE and the high arachidonic acid PC, after the end of the induction period, was identical. On the basis of oxygen uptake determinations on PE and the high arachidonic acid PC, it would appear that the maximum rate of oxidation (measured from the straight line portion of the oxygen uptake curve at the end of the induction period) for a phospholipid was regulated by the kind and amount of unsaturated fatty acids.

After further examination of the oxygen uptake curves for PE and the high arachidonic acid PC, the following hypothesis was advanced: the nitrogen moiety

of phospholipids influences the length of the induction period. Confirmation for this hypothesis was obtained by the finding that the length of the induction period of PC was reduced through contamination by minor amounts of PE. The reduction in the induction period appeared to be proportional to the amount of PE added. Experiments in model systems of methyl linolenate emulsions demonstrated that ethanolamine was prooxidative while choline was slightly antioxidative. A brownish-yellow color in the methyl linoleate-ethanolamine emulsion, after the onset of oxidation, suggested that a Maillard-type reaction had occurred between the amino group of ethanolamine and oxidation products of methyl linolenate. Since this discolaration, which had also been observed in oxidized PE emulsions, indicated that the free amino group of ethanolamine probably was reacting with compounds containing a carbonyl group, a study was initiated to measure the free amino group of PE at various intervals during autoxidation. Measurements of the free amino group of egg and soybean PE demonstrated that it decreased during oxidation and therefore supported the contention that a Maillard-type browning reaction occurred.

The second purpose of this research, which consisted of a study on the influence of various extraneous substances on phospholipid oxidation, was not as well defined as the first which related the oxidation of

phospholipids to their specific molecular consitution. Although the second phase of this research assumed the nature of a general survey and may have raised as many questions as it answered, part of the information that was obtained provided an insight on the effect of various substances on phospholipid oxidation. Substances that were investigated included ethanolamine, choline, amino acids, copper, butylated hydroxytoluene (BHT) and the sodium salt of ethylenediamine tetraacetic acid.

The gap between the first and second phases was bridged by oxygen uptake studies on PE in the presence of added ethanolamine and choline chloride. The presence of additional ethanolamine, the nitrogen moiety shown previously to accelerate oxidation, had a depressing effect on the autoxidation of PE. Choline chloride also acted as an antioxidant. Ethanolamine and glycine decreased the susceptibility of PC to oxidation as evaluated by oxygen uptake measurements. Copper accelerated the oxidation of PC. A greater prooxidative effect was observed for copper and ethanolamine or copper and glycine than for copper alone. Since complexes between metals and amino compounds are known to occur (41, 103, 118), and to accelerate oxidation (102, 104, 118), the increased prooxidative effect of copper + ethanolamine and copper + glycine could be attributed to the formation of a complex.

Amino acids were observed to exert an antioxidative effect on the oxidation of phospholipids. Considerable differences existed in the amount of protection afforded PE by individual amino acids. Histidine had a strong inhibitory effect as evaluated by oxygen uptake and TBA absorbance measurements. In contrast, glycine, lysine and tryptophan were less inhibitory than histidine. The differences in the antioxidative effect for each amino acid were thought to relate to a competition between pro-oxidative and antioxidative reactions, since under certain conditions amino acids have been shown to accelerate lipid oxidation. A low activation energy for the anti-oxidative reaction could explain why histidine exerted a stronger antioxidative effect than glycine, lysine and tryptophan.

The phenolic antioxidant, butylated hydroxytoluene (BHT), and sodium tetra, ethylenediamine tetraacetate (Na EDTA), a metal chelating agent, completely retarded the oxidation of PE as measured by oxygen uptake and the TBA test. The antioxidative effect of Na EDTA was unexpected even though its ability to chelate metal ions is known. The possibility exists that the effectiveness of Na EDTA in preventing oxidation could be related to a chelation of metal impurities present in phospholipid emulsions. The strong antioxidant effect, observed for BHT, confirmed previous reports of phospholipid anti-oxidation (81, 125) and provided a way to protect

phospholipids from oxidative degradation during their extraction, manipulation and storage.

A study of the influence of various extraneous substances on phospholipid oxidation was undertaken to determine which substances promoted and which substances retarded the oxidation of phospholipid emulsions. Experiments with added nitrogen containing substances provided additional information of the importance of the nitrogen moiety in phospholipid oxidation. Ethanolamine was observed to accelerate the oxidation of methyl linolenate emulsions but to retard the oxidation of phospholipids. The effect of the free amino group in the presence of a metal was definitely prooxidant. These results indicate that phospholipid oxidation is a complicated phenomenon and is influenced by various factors. The objective of the second phase of the thesis was not unrelated to the study of the oxidation of phospholipids in relation to their specific molecular constitution. In both instances, the goal was the same, namely to induce and measure oxidation in order to obtain information which could be utilized to reduce the susceptibility of phospholipids to oxidation.

SUMMARY AND CONCLUSIONS

Phospholipids from egg yolk and a commercial soybean phosphatide preparation (Centrolex P) were separated into fractions by silicic acid column chromatography. The purity of individual fractions was ascertained by thin-layer chromatography. Purified phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) were emulsified in a Tween 20-borate buffer by a Braun Cell Homogenizer and their oxygen uptakes were followed manometrically with a Gilson Differential Respirometer.

Oxygen consumption after 50 hours was greatest for PE at pH 8.0 and PC at pH 7.0. Since uptake was greater for PE than for PC at all temperatures studied, subsequent oxygen uptake measurements were conducted at pH 7.0. Egg phospholipids were more reactive than soybean phospholipids. The rates of oxygen uptake at different temperatures were utilized to determine activation energies for PE and PC. The activation energies of PE and PC from egg and soybean were comparable; however, the values obtained for PE and PC were approximately one-half of the values reported for the autoxidation of methyl linoleate and linoleic acid emulsions.

Egg PC was separated into fractions with varying degrees of unsaturation by silicic acid column chromatography. Of the three fractions collected, the PC fraction with the highest percentage of arachidonic acid was the most reactive while the fraction with the least amount of arachidonic acid exhibited the lowest rate of oxidation. The fatty acid composition of the first PC fraction eluted from the column, containing the highest percentage of arachidonic acid, resembled the fatty acid composition of egg PE. However, this high arachidonic acid PC had a longer induction period than egg PE. When egg PC was purposely contaminated with a small amount of PE the rate of oxidation was not altered appreciably, although the induction period was reduced. The addition of ethanolamine to emulsions containing methyl linolenate and α-glycerophosphate significantly reduced the induction period for autoxidation while the addition of choline (as choline chloride) produced a slight antioxidative effect.

Various physico-chemical measurements were made of specific changes in the phospholipid molecule during autoxidation. Of the two major polyunsaturated fatty acids in egg phospholipids, arachidonic acid disappeared at a more rapid rate during oxidation while the concentration of linoleic acid decreased to a level that was stable. Higher 2-thiobarbituric acid (TBA) and diene and triene conjugation values were observed for PE

than for PC. The concentration of the free amino group of egg and soybean PE decreased during oxidation.

The effect of various prooxidative and antioxidative substances on the autoxidation of aqueous emulsions of PE and PC was investigated. Ethanolamine and choline decreased the reactivity of egg PE. Copper accelerated the oxidation of PE and PC. Ethanolamine and glycine exerted an antioxidative effect on PC; however, when either ethanolamine or glycine was added with copper, PC was observed to oxidize faster than PC with copper alone. Glycine, lysine and tryptophan increased the induction period of egg PE. Histidine, butylated hydroxytoluene and sodium tetra, ethylenediamine tetraacetate completely retarded the autoxidation of PE for the period of study as evaluated by oxygen uptake and TBA determinations.

The conclusions reached as a result of this study are summarized below:

1. The activation energies of egg and soybean PE and PC are comparable, suggesting that the mode of oxidation during the period of steady state oxidation is identical.
2. The greater reactivity of egg phospholipids compared to soybean phospholipids is related to the presence of arachidonic acid in egg phospholipids.

3. The rate of phospholipid oxidation during the period of steady state oxidation is regulated by the unsaturated fatty acids.
4. The induction period for a phospholipid is a function of the nitrogen moiety.
5. The ethanolamine moiety of PE has a greater prooxidant effect than the choline moiety of PC.
6. The concentration of the free amino group of PE decreases during autoxidation, and is probably related to the development of a brownish-yellow discoloration by a Maillard type reaction.
7. The autoxidation of emulsified phospholipids is promoted by copper, retarded by glycine, lysine, tryptophan and histidine and almost completely inhibited by butylated hydroxy-toluene and sodium tetra, ethylenediamine tetraacetate.

PROPOSALS FOR FURTHER RESEARCH

Nearly every study, and this one was no exception, raises questions which cannot be adequately explained. Moreover, there are always topics, existing in the fringe areas of any study, which seemingly tantalize the researcher and attempt quite innocently to make him detour from his original objectives. Some unanswered questions and tantalizing topics which developed as a result of this study are listed below.

1. Silicic acid column chromatography and thin-layer chromatography have been used to obtain phospholipids with varying degrees of unsaturation in their fatty acids. It would be interesting to learn if plasmalogens could be fractionated in an analogous manner. Such a study on the fractionation of plasmalogens could be performed in conjunction with the fractionation of a pure phospholipid to determine the extent of unsaturated and saturated fatty acids in plasmalogens.
2. No attempt was made to relate phospholipid oxidation to the effect of plasmalogens. The plasmalogen content of egg yolk phospholipids

has been reported to be 1% (2) and the amount of plasmalogen in soybean PE and PC was shown to range from 4-7% and 8-9% respectively depending upon the method of determination (96). Although the plasmalogen content of egg and soybean phospholipids is low, its effect on oxidation could be considerable since the vinyl ether linkage can be readily hydrolyzed in aqueous solution to yield aldehydes. The latter are known to be susceptible to oxidation. Measurement of the oxidation of different plasmalogen fractions could provide information to enhance that obtained on the oxidation of various phospholipid fractions.

3. Preliminary experiments were conducted to determine the effect of the enzyme, lipoxidase, on the oxidation of phospholipids. Indications were that lipoxidase retarded the oxidation of PC at 15°C in a borate (pH 7.0) buffer. Studies could be undertaken to explain this phenomenon and to learn if there are conditions in which lipoxidase can accelerate the oxidation of phospholipids.
4. Many intermediates of classical autoxidation can be isolated and identified when oxidation experiments are carried out in aqueous systems.

This type of a study would probably require the use of sophisticated instrumentation such as IR, UV, and mass spectrometers, NMR spectroscopy, GLC and the polarograph. Product isolation and identification could be performed at various temperatures on PE and PC as different oxidation products are obtained at high temperatures compared to low temperatures. Methodology relative to such a problem has been considered in a review article by Schauenstein (105).

5. Because the free amino group of phosphatidyl ethanolamine has been shown to decrease during oxidation, special attention should be given to products containing ethanolamine and its derivatives. Such an emphasis could help to define the exact changes in the free amino group in phospholipid oxidation and to characterize browning reactions.

6. The success of Na EDTA in preventing phospholipid oxidation bears further study. An important question that should be answered is: does the apparent antioxidative effect of Na EDTA relate entirely to its ability to chelate metals?

7. The increased prooxidative effect of ethanolamine + copper and glycine + copper as compared to copper alone needs to be better understood. If a complex is formed, what is its structure and could it cause the copper ion to function more efficiently at or near the double bonds of unsaturated fatty acids?

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Acosta, O. S., W. W. Marion and R. H. Forsythe. 1966. Total lipids and phospholipids in turkey tissues. Poultry Sci. 45:169-184.

2. Ansell, G. B. and J. N. Hawthorne. 1964. Phospholipids--Chemistry, Metabolism and Function. 1st ed. Elsevier Publishing Co., New York, p. 88.

3. Badings, H. T. and J. G. Wassink. 1963. Separation and identification of aliphatic aldehydes and ketones by thin-layer chromatography of the 2,4-dinitrophenylhydrazones. Neth. Milk & Dairy J. 17:132-149.

4. Baker, N. and L. Wilson. 1966. Water-soluble products of uv-irradiated, autoxidized linoleic and linolenic acids. J. Lipid Res. 7:341-348.

5. Baker, N. and L. Wilson. 1966. Water-soluble inhibitor(s) of tumor respiration formed from ultraviolet-induced oxidation of linoleic and linolenic acids. J. Lipid Res. 7:349-356.

6. Bawn, C. E. H. 1953. Free radical reactions in solution initiated by heavy metal ions. Discussions Faraday Soc. 14:181-190.

7. Bawn, C. E. H., A. A. Pennington and C. F. H. Tipper. 1951. The catalyzed oxidation of trimethylethylene in solution. Discussions Faraday Soc. 10:282-291.

8. Bloor, W. and R. Snider. 1937. Phospholipids as oxygen carriers. Proc. Soc. Exptl. Biol. Med. 36:215-217.

9. Bolland, J. L. and P. Ten Have. 1947. Kinetic studies in the chemistry of rubber and related materials. IV. The inhibitory effect of hydroquinone on the thermal oxidation of ethyl linoleate. Trans. Faraday Soc. 43:201-210.

10. Bollmann, H. 1925. Preserving vegetable oils. British patent 260, 108. Assigned Nov. 9, 1925. Through Chem. Abst. 21:3477 (1927).

11. Burchfield, H. P. and E. E. Storrs. 1962. Biochemical Applications of Gas Chromatography. Academic Press, New York, p. 122.

12. Calkins, V. P. 1947. The mechanism of the antioxygenic synergism of quinones and quinols with phosphoric acid and other acids in fat systems. J. Am. Oil Chem. Soc. 69:384-388.

13. Chalk, A. J. and A. H. Smith. 1957. Catalysis of cyclohexene autoxidation by trace metals in non-polar media. II. Metal salts in the presence of chelating agents. Trans. Faraday Soc. 53:1235-1245.

14. Chipault, J. R. 1962. Antioxidants for use in foods. In: Autoxidation and Antioxidants. Vol. 2. Ed. by W. O. Lundberg. Interscience Publishers, New York, pp. 477-542.

15. Coleman, J. E., J. W. Hampson and D. H. Saunders. 1964. Autoxidation of fatty materials in emulsion. II. Factors affecting the histidine-catalyzed autoxidation of emulsified methyl linoleate. J. Am. Oil Chem. Soc. 41:347-351.

16. Corliss, G. A. 1963. The influence of cooking and storage on oxidative changes in freeze-dried beef as evaluated by the 2-thiobarbituric acid test. M. S. Thesis, Michigan State University.

17. Daniels, F. and R. A. Alberty. 1961. Physical Chemistry. 2nd ed. John Wiley & Sons, Inc., New York, pp. 315-318.

18. Day, E. A. 1965. Some properties of carbonyl compounds encountered in the flavor isolates of dairy products--a review. Food Technol. 19:129-134.

19. DeKoning, A. J. 1966. The determination of ethanolamine and serine in phospholipids. Analyst. 91:523-525.

20. Denisov, E. T. and N. M. Emanuel. 1956. Kinetic characteristics of the oxidation of cyclohexane in the presence of cobalt stearate (in Russian). Zhur. Fiz. Khim. 30:2327-2336. Through Chem. Abst. 51:9274d (1957).

21. Denisov, E. T. and N. M. Emanuel. 1960. Catalysis by salts of metals with variable valence in reactions of liquid-phase oxidation (in Russian). Uspekhi Khim. 29:1409-1438. Through Chem. Abst. 55:7005d (1961).

22. Desnuelle, P., R. Massoni and O. Benoit-Micaelli. 1953. Sur le pouvoir antioxygène des phosphates et des sulfates acides liposolubles. Bull. Soc. Chim. France No. 120:595-599.

23. Desnuelle, P. and R. Massoni. 1953. Phosphatides as drying inhibitors for linseed oil (in French). Peintures, Pigments, Vernis. 29:390-392. Through Chem. Abst. 47:9628e (1953).

24. Diemair, W., H. Ludwig and K. Weise. 1943. Study of the antioxidative effect of biological material on fat spoilage (in German). Fette u Seifen 50:349-354. Through Chem. Abst. 38:3498-6 (1944).

25. Dittmer, J. C. and R. L. Lester. 1964. A simple specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. 5:126-127.

26. Dodge, J. T. and G. B. Phillips. 1966. Autoxidation as a cause of altered lipid distribution in extracts from human red cells. J. Lipid Res. 7:387-395.

27. Dugan, L. R., Jr., L. Marx, C. E. Weir and H. R. Kraybill. 1954. Butylated hydroxytoluene. A new antioxidant for food use. Am. Meat Inst. Foundation Bull. No. 18.

28. Dugan, L. R., Jr. 1961. Development and inhibition of oxidative rancidity in foods. Food Technol. 15:10-18.

29. Dutton, H. J., A. Schwab, H. A. Moser and J. C. Cowan. 1949. The flavor problem of soybean oil. V. Some considerations in the use of metal scavengers in commercial operations. J. Am. Oil Chem. Soc. 26:441-444.

30. El-Gharbawi, M. I. and L. R. Dugan, Jr. 1965. Stability of nitrogenous compounds and lipids during storage of freeze-dried raw beef. J. Food Sci. 30:817-822.

31. Evans, C. D., E. N. Frankel and P. M. Cooney. 1959. Tocopherol oxidation in fats. Hydrogenated soybean oils. J. Am. Oil Chem. Soc. 36:73-77.

32. Evans, E. I. 1935. Antioxidant properties of vegetable lecithin. Ind. Eng. Chem. 27:329-331.

33. Fischer, G. A. and J. J. Kabara. 1964. Simple, multibore columns for superior fractionation of lipids. Anal. Biochem. 9:303-309.

34. Folch, J. 1949. Brain cephalin, a mixture of phosphatides. Separation from it of phosphatidyl serine, phosphatidyl ethanolamine and a fraction containing an inositol phosphatide. J. Biol. Chem. 146:35-44.

35. Getz, G. S., W. Bartley, F. Stirpe, B. M. Notton and A. Renshaw. 1962. The lipid composition of rat-liver mitochondria, fluffy layer and microsomes. Biochem. J. 83:181-191.

36. Gilson, W. E. Differential respirometer of simplified and improved design. Science. 141:531-532.

37. Golumbic, C. 1942. The antioxygenic action of phosphoric acid in association with tocopherols and hydroquinones. Oil & Soap. 19:181-182.

38. Goodman, L. P. 1968. Sonication studies on the hydrolysis of triglycerides and egg phospholipids by lipolytic enzymes. Ph.D. Thesis, Michigan State University.

39. Hampson, J. W., S. F. Herb and P. Magidman. 1968. Autoxidation of fatty materials in emulsion. III. Application of GLC and TLC to studies of the histidine-catalyzed autoxidation of methyl oleate. J. Am. Oil Chem. Soc. 45:443-447.

40. Hawke, J. C. 1959. The fatty acids of phosphatidylethanolamine and phosphatidylcholine from hen's egg. Biochem. J. 71:588-592.

41. Hearon, J. Z., D. Burk and A. L. Schade. 1949. Physicochemical studies of reversible and irreversible complexes of cobalt, histidine, and molecular oxygen. J. Natl. Cancer Inst. 9:337-377.

42. Heimann, W. and H. Pezold. 1957. Proöxygen effects of antioxygens (in German). Fette, Seifen Anstrichm. 59:330-338. Through Chem. Abst. 53:18513c (1959).

43. Heimann, W. and H. Pezold. 1958. Über die wirkungsinversion bei fett-antioxygenen. I. Mitteilung. Vergleich zwischen dem verhalten tierischer und planzlicher fette gegenüber antioxygenen. Zeitschr. f. Lebensm. Unters. 108:317-322.

44. Heimann, W. and H. Pezold. 1960. Über die wirkungsinversion bei fett-antioxygenen. III. Mitteilung. Einfluß der temperatur auf induktionsperiode, schutzfaktor und wirkungsinversion bei der autoxydation von fetten. Zeitschr. f. Lebensm. Unters. 112:463-470.

45. Henick, A. S., M. F. Benca and J. H. Mitchell. 1954. Estimating carbonyl compounds in rancid fats and foods. J. Am. Oil Chem. Soc. 31:88-91.

46. Hirano, Y. 1964. The significance of the phosphoryl segment in lecithin autoxidation. Presented before a meeting of the Agricultural Chemical Society of Japan at Nagoya, Japan, October 14, 1964.

47. Holman, R. T. and G. O. Burr. 1946. Spectrophotometric studies of the oxidation of fats. VI. Oxygen absorption and chromophore production in fatty esters. J. Am. Oil Chem. Soc. 68:562-566.

48. Holman, R. T. and O. C. Elmer. 1947. The rates of oxidation of unsaturated fatty acids and esters. J. Am. Oil Chem. Soc. 24:127-129.

49. Ingold, K. U. 1962. Metal catalysis. In: Lipids and Their Oxidation. Ed. by H. W. Schultz, E. A. Day and R. O. Sinnhuber. Avi Publishing Co., Inc., Westport, Conn., pp. 93-121.

50. Issidorides, A. 1951. The antioxygenic synergism of various acids with α-tocopherol. J. Am. Chem. Soc. 73:5146-5148.

51. Jacini, G., E. Fedeli and W. C. Schaefer. 1966. Effect of synthetic chelates on the autoxidation of unsaturated fatty acid esters. Agric. Food Chem. 14:650-653.

52. Jauslin, H. and K. Leupin. 1960. Autoxidation of emulsified oils (in German). Pharm. Acta Helv. 35:148-167. Through Chem. Abst. 54:21792i (1960).

53. Keeney, M. 1962. Secondary degradation products. In: Lipids and Their Oxidation. Ed. by H. W. Schultz, E. A. Day and R. O. Sinnhuber. Avi Publishing Co., Inc., Westport, Conn., pp. 79-89.

54. Klotz, I. M. 1954. Mechanism of Enzyme Action. The John Hopkins Press, Baltimore, Md., pp. 257-285.

55. Kuchmak, M. and L. R. Dugan, Jr. 1963. Phospholipids of pork muscle tissues. J. Am. Oil Chem. Soc. 40:734-736.

56. Kwon, T. W., D. B. Menzel and H. S. Olcott. 1965. Reactivity of malonaldehyde with food constituents. J. Food Sci. 30:808-813.

57. Lea, C. H. 1935. Antioxidants and the preservation of edible fats. Dept. Sci. Ind. Research (British), Report of the Food Investigation Board 1934:38-43. Through Chem. Abst. 30: 310-9 (1936).

58. Lea, C. H. 1939. Rancidity in Edible Fats. Chemical Publishing Co., Inc., New York.

59. Lea, C. H. 1956. Some observations on the preparation and properties of phosphatidyl-ethanolamine. In: Biochemical Problems of Lipids. Ed. by G. Popjak and E. Le Breton. Interscience Publishers, Inc., New York, pp. 81-90.

60. Lea, C. H. 1957. Deteriorative reactions involving phospholipids and lipoproteins. J. Sci. Food Agric. 8:1-13.

61. Lea, C. H. and D. N. Rhodes. 1955. The ninhydrin reaction of unhydrolysed phospholipids. Biochim. Biophys. Acta. 17:416-423.

62. Lea, C. H., D. N. Rhodes and R. D. Stoll. 1955. Phospholipids. 3. On the chromatographic separation of glycerophospholipids. Biochem. J. 60:353-363.

63. Lew, Y. T. and A. L. Tappel. 1956. Antioxidant and synergist inhibition of hematin-catalyzed oxidative fat rancidity. Food Technol. 10:285-289.

64. Lillard, D. A. and E. A. Day. 1964. Degradation of monocarbonyls from autoxidizing lipids. J. Am. Oil Chem. Soc. 41:549-552.

65. Link, W. E. and M. W. Formo. 1961. Analysis of autoxidation mixtures. In: Autoxidation and Antioxidants. Ed. by W. O. Lundberg. Interscience Publishers Inc., New York, Vol. I, pp. 367-416.

66. Lohmar, R. L. and H. L. Tookey. 1959. Autoxidation in aqueous carotene-linoleate systems. Arch. Biochem. Biophys. 84:297-304.

67. Lundberg, W. O. 1961. Oxidative rancidity and its prevention. In: Autoxidation and Antioxidants. Ed. by W. O. Lundberg. Interscience Publishers Inc., New York, Vol. II, pp. 451-476.

68. Lundberg, W. O. 1962. Mechanisms. In: Lipids and Their Oxidation. Ed. by H. W. Schultz, E. A. Day and R. O. Sinnhuber. Avi Publishing Co., Inc., Westport, Conn., pp. 31-50.

69. Lyman, R. L., J. Tinoco, P. Bouchard, G. Sheehan, R. Ostwald and P. Miljanich. 1967. Sex differences in the metabolism of phosphatidyl cholines in rat liver. Biochim. Biophys. Acta. 137: 107-114.

70. Mabrouk, A. F. and L. R. Dugan, Jr. 1960. A kinetic study of the autoxidation of methyl linoleate and linoleic acid emulsions in the presence of sodium chloride. J. Am. Oil Chem. Soc. 37: 486-490.

71. Mabrouk, A. F. and L. R. Dugan, Jr. 1961. Solubility of linoleic acid in aqueous solutions and its reaction with water. J. Am. Oil Chem. Soc. 38:9-13.

72. Magee, W. L., R. W. R. Baker and R. H. Thompson. 1960. The identification and quantitative estimation of ethanolamine and serine in lipid hydrolysates. Biochim. Biophys. Acta. 40: 118-123.

73. Mahon, J. H. and R. A. Chapman. 1953. The relative rates of destruction of propyl gallate and butylated hydroxyanisole in oxidizing lard. J. Am. Oil Chem. Soc. 30:34-39.

74. Marcuse, R. 1956. The effect of ascorbic acid on the autoxidation of fats (in German). Fette, Seifen, Anstrichm. 58:1063-1066. Through Chem. Abst. 52:9470h (1958).

75. Marcuse, R. 1961. Zur wirkung von aminosäuren bei der fett-oxydation II: Untersuchungen mit linoleat. Fette, Seifen, Anstrichm. 63:940-944.

76. Marcuse, R. 1962. The effect of some amino acids on the oxidation of linoleic acid and its methyl ester. J. Am. Oil Chem. Soc. 39: 97-103.

77. Mattsson, S. and P. Swartling. 1963. Oxidation of milk phospholipids. Milk and Dairy Res., Alnarp, Sweden, Report 68, pp. 1-23.

78. Morris, S. G., J. S. Meyers, M. L. Kip and R. W. Riemenschneider. 1950. Metal deactivation in lard. J. Am. Oil Chem. Soc. 27:105-107.

79. McGinnis, G. W. and L. R. Dugan, Jr. 1965. A rapid low temperature method for preparation of methyl esters of fatty acids. J. Am. Oil Chem. Soc. 42:305-307.

80. Nelson, G. T. and N. K. Freeman. 1959. Serum phospholipide analysis by chromatography and infrared spectrophotometry. J. Biol. Chem. 234:1375-1380.

81. Neudoerffer, T. S. and C. H. Lea. 1966. Antioxidants for the (thin-layer) chromatography of lipids. J. Chromatog. 21:138-140.

82. Oette, K. 1965. Identification of some lipid peroxides by thin-layer chromatography. J. Lipid Res. 6:449-454.

83. Olcott, H. S. 1941. Antioxidants for edible fats and oils. Oil and Soap. 18:77-80.

84. Olcott, H. S. 1962. Marine products. In: Lipids and Their Oxidation. Ed. by H. W. Schultz, E. A. Day and R. O. Sinnhuber. Avi Publishing Co., Inc., Westport, Conn., pp. 173-189.

85. Olcott, H. S. and H. A. Mattill. 1936. Antioxidants and autoxidation of fats. IV. Lecithin as an antioxidant. Oil and Soap. 13:98-100.

86. Olcott, H. S. and H. A. Mattill. 1936. Antioxidants and the autoxidation of fats. VII. Preliminary classification of inhibitors. J. Am. Chem. Soc. 58:2204-2208.

87. Olcott, H. S. and J. Van der Veen. 1963. Role of individual phospholipids as antioxidants. J. Food Sci. 28:313-315.

88. Patton, S. 1962. Dairy products. In: Lipids and Their Oxidation. Ed. by H. W. Schultz, E. A. Day and R. O. Sinnhuber. Avi Publishing Co., Inc., Westport, Conn., pp. 190-201.

89. Peng, C. Y. and L. R. Dugan, Jr. 1965. Composition and structure of phospholipids in chicken muscle tissues. J. Am. Oil Chem. Soc. 42:533-536.

90. Placer, Z. A., L. L. Cushman and B. C. Johnson. 1966. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal. Biochem. 16:359-364.

91. Privett, O. S. and M. L. Blank. 1962. The initial stages of autoxidation. J. Am. Oil Chem. Soc. 39:465-469.

92. Privett, O. S., W. O. Lundberg, N. A. Khan, W. E. Tolberg and D. H. Wheeler. 1953. Structure of hydroperoxides obtained from autoxidized methyl linoleate. J. Am. Oil Chem. Soc. 30:17-21.

93. Privett, O. S. and F. W. Quackenbush. 1954. Studies on the antioxygenic properties of wheat germ phosphatides. J. Am. Oil Chem. Soc. 31:169-171.

94. Privett, O. S. and F. W. Quackenbush. 1954. Reactions of phosphoric acid in the autoxidation of fats. J. Am. Oil Chem. Soc. 31:225-227.

95. Pyriadi, T. M. and M. E. Mason. 1968. Composition and stability of pecan oils. J. Am. Oil Chem. Soc. 45:437-440.

96. Rhee, K. S., R. R. Del Rosario and L. R. Dugan, Jr. 1967. Determination of plasmalogens after treating with a 2,4-dinitrophenylhydrazine-phosphoric acid reagent. Lipids. 2:334-338.

97. Rhee, K. S. and L. R. Dugan, Jr. 1967. The implication of acid concentration in lipid phosphorus determinations. Anal. Biochem. 19:157-165.

98. Rhodes, D. N. and C. H. Lea. 1956. Chromatographic separation of glycerophospholipids. In: Biochemical Problems of Lipids. Ed. by G. Popjak and E. Le Breton. Interscience Publishers Inc., New York, pp. 73-80.

99. Roubal, W. T. 1967. Oxidative deterioration of flesh lipids of pacific cod (Gadus macrocephalus). J. Am. Oil Chem. Soc. 44:325-327.

100. Rouser, G., J. S. O'Brien and D. Heller. 1961. The separation of phosphatidyl ethanolamine and phosphatidyl serine by column chromatography. J. Am. Oil Chem. Soc. 38:14-19.

101. Rouser, G., A. N. Siakotos and S. Fleischer. 1966. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. Lipids. 1:85-86.

102. Saunders, D. H., J. E. Coleman, J. W. Hampson, P. A. Wells and R. W. Riemenschneider. 1962. Autoxidation of fatty materials in emulsions. I. Pro-oxidant effect of histidine and trace metals on the autoxidation of linoleate esters. J. Am. Oil Chem. Soc. 39:434-439.

103. Scaife, J. F. 1959. The combination of copper with amino acids, peptides, and proteins. Can. J. Biochem. Physiol. 37:1033-1048.

104. Scaife, J. F. 1959. The catalysis of ascorbic acid oxidation by copper and its complexes with amino acids, peptides, and proteins. Can. J. Biochem. Physiol. 37:1049-1067.

105. Schauenstein, E. 1967. Autoxidation of polyunsaturated esters in water: chemical structure and biological activity of the products. J. Lipid Res. 8:417-428.

106. Schauenstein, E. and J. H. Biheller. 1956. Über die reaktion ungesättigter verbindungen mit dem wasser. 2. Mitteilung: Versuche mit reinster 9.12-linolsäure. Montash. Chem. 87:158-163.

107. Schauenstein, E., O. Gold, and B. Pibus. 1956. Über die reaktion ungesättigter verbindungen mit dem wasser. 1. Mitteilung: Orientierende vorversuche mit leinölfettsäuren und oleum citri. Montash. Chem. 87:144-157.

108. Schauenstein, E. and G. Schatz. 1959. Über die biologische bedeutung der reaktion mehrfach ungesättigter fettsäuren und deren ester in wasser. Fette, Seifen, Anstrichm. 61:1068-1076.

109. Schauenstein, E., G. Schatz and G. Benedikt. 1961. Über die reaktion von $\Delta^{9,12}$-linolsäureäthylester in wasser, 4. Mitt: Bildung wasserlöslicher fettsäureester-hydroperoxyde mit atmungs- und gärungshemmender wirkung auf tumorzellen und hefe. Montash. Chem. 92:442-462.

110. Schulte, K. E., A. Schillinger and F. Lynen. 1953. Nonenzymic oxidation of vitamin A. acetate (in German). Fette, Seifen, Anstrichm. 55: 705-710. Through Chem. Abst. 50:1144e (1956).

111. Siakotos, A. N. 1967. Rapid determination of lipids containing free amino groups with trinitrobenzene sulfonic acid reagent. Lipids. 2:87-88.

112. Sims, R. J. 1952. Variations in the chemical, physical, and organoleptic properties of soybean oil hydrogenated under widely varying conditions. J. Am. Oil Chem. Soc. 29:347-350.

113. Skidmore, W. D. and C. Entenman. 1962. Two-dimensional thin-layer chromatography of rat liver phosphatides. J. Lipid Res. 3:471-475.

114. Spetsig, L. O. 1954. The influence of water on the autoxidation of methyl linoleate. Acta Chem. Scand. 8:1643-1645.

115. Swern, D. 1961. Primary products of olefinic autoxidations. In: Autoxidation and Antioxidants. Ed. by W. O. Lundberg. Interscience Publishers Inc., New York, Vol. I, pp. 1-54.

116. Swift, E. C., W. G. Rose and G. S. Jamieson. 1942. Factors affecting the stability of cottonseed oil. A study of the antioxygenic activity of alpha-tocopherol. Oil and Soap. 19:176-178.

117. Tadros, B. and K. Leupin. 1965. Autoxidation of emulsifiers and emulsions (in German). Pharm. Acta Helv. 40:407-420. Through Chem. Abst. 63:6794c (1965).

118. Tappel, A. C. 1955. Catalysis of linoleate oxidation by copper-proteins. J. Am. Oil Chem. Soc. 32:252-254.

119. Tarladgis, B. G. and B. M. Watts. 1960. Malonaldehyde production during the controlled oxidation of pure, unsaturated fatty acids. J. Am. Oil Chem. Soc. 37:403-406.

120. Urakami, C. and H. Kameyama. 1960. Antioxygenic activity of lecithin and its hydrolysis products. III. Lecithin, glycerophosphorylcholine, phosphorylcholine, phosphatidic acids, choline and glycerol. Bull. Chem. Soc. Japan. 33:29-33.

121. Uri, N. 1956. Metal ion catalysis and polarity of environment in the aerobic oxidation of unsaturated fatty acids. Nature. 177:1177-1178.

122. Uri. N. 1958. Essential Fatty Acids. Ed. by H. M. Sinclair. Butterworths Scientific Publications, London, pp. 30-35.

123. Uri, N. 1961. Mechanisms of antioxidation. In: Autoxidation and Antioxidants. Ed. by W. O. Lundberg. Interscience Publishers, Inc., New York, Vol. I, pp. 133-169.

124. Watts, B. M. and R. Wong. 1951. Some factors affecting the antioxidant behavior of ascorbic acid with unsaturated fats. Arch. Biochem. 30:110-120.

125. Wren, J. J. and A. D. Szczepanowska. 1964. Chromatography of lipids in presence of an antioxidant, 4-methyl-2,6-di-tert-butylphenol. J. Chromatog. 14:405-410.

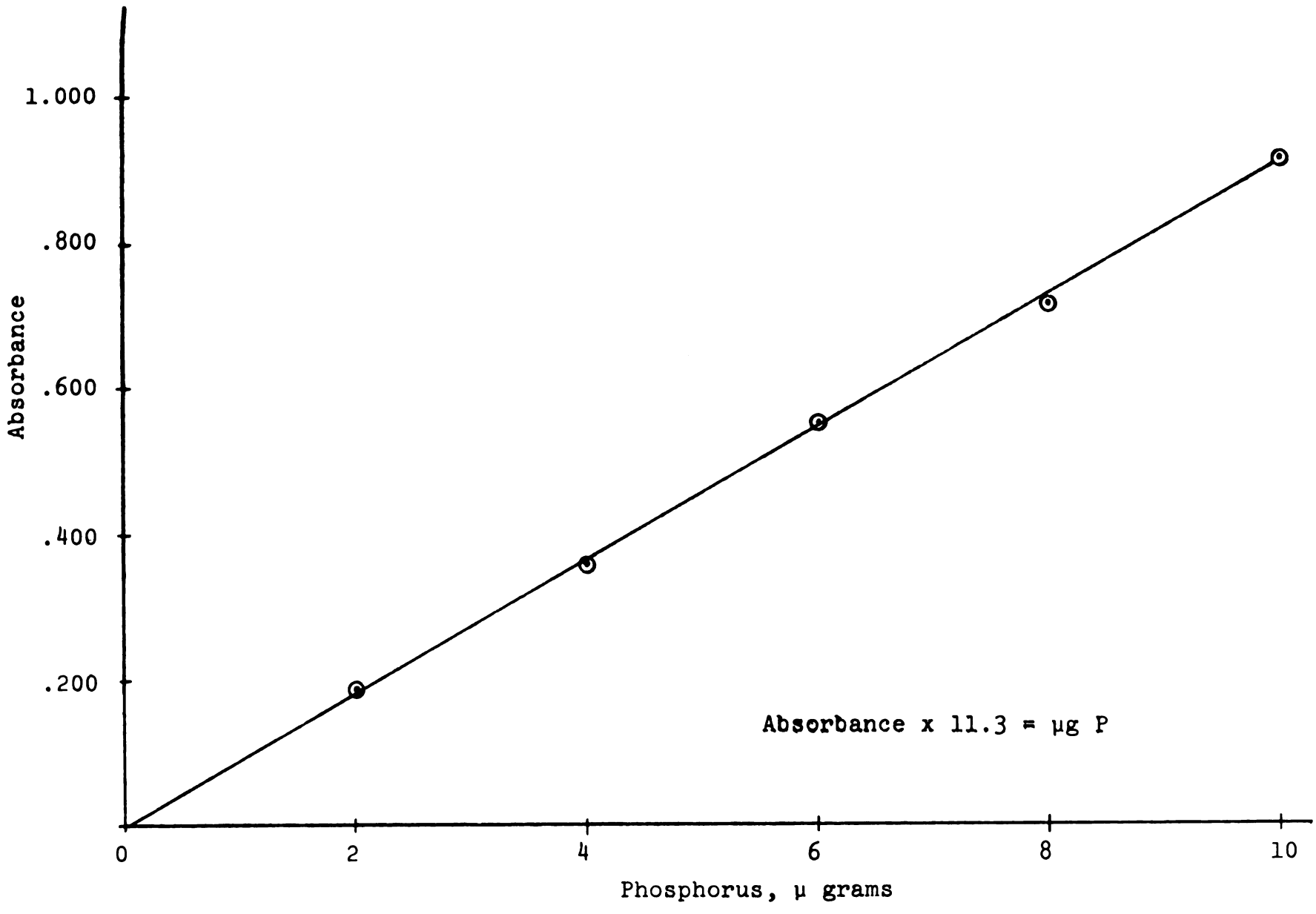
126. Younathan, M. T. and B. M. Watts. 1960. Oxidation of tissue lipids in cooked pork. Food Research. 25:538-543.

127. Zook, B. J. 1968. Some reactions for methylation of triglycerides and phospholipids. M. S. Thesis, Michigan State University.

APPENDICES

APPENDIX A

STANDARD CURVE FOR PHOSPHORUS DETERMINATION


1.000
.800
.600
.400
.200
Absorbance
0
2
4
6
8
10
Phosphorus, µ grams
Absorbance x 11.3 = µg P

APPENDIX B

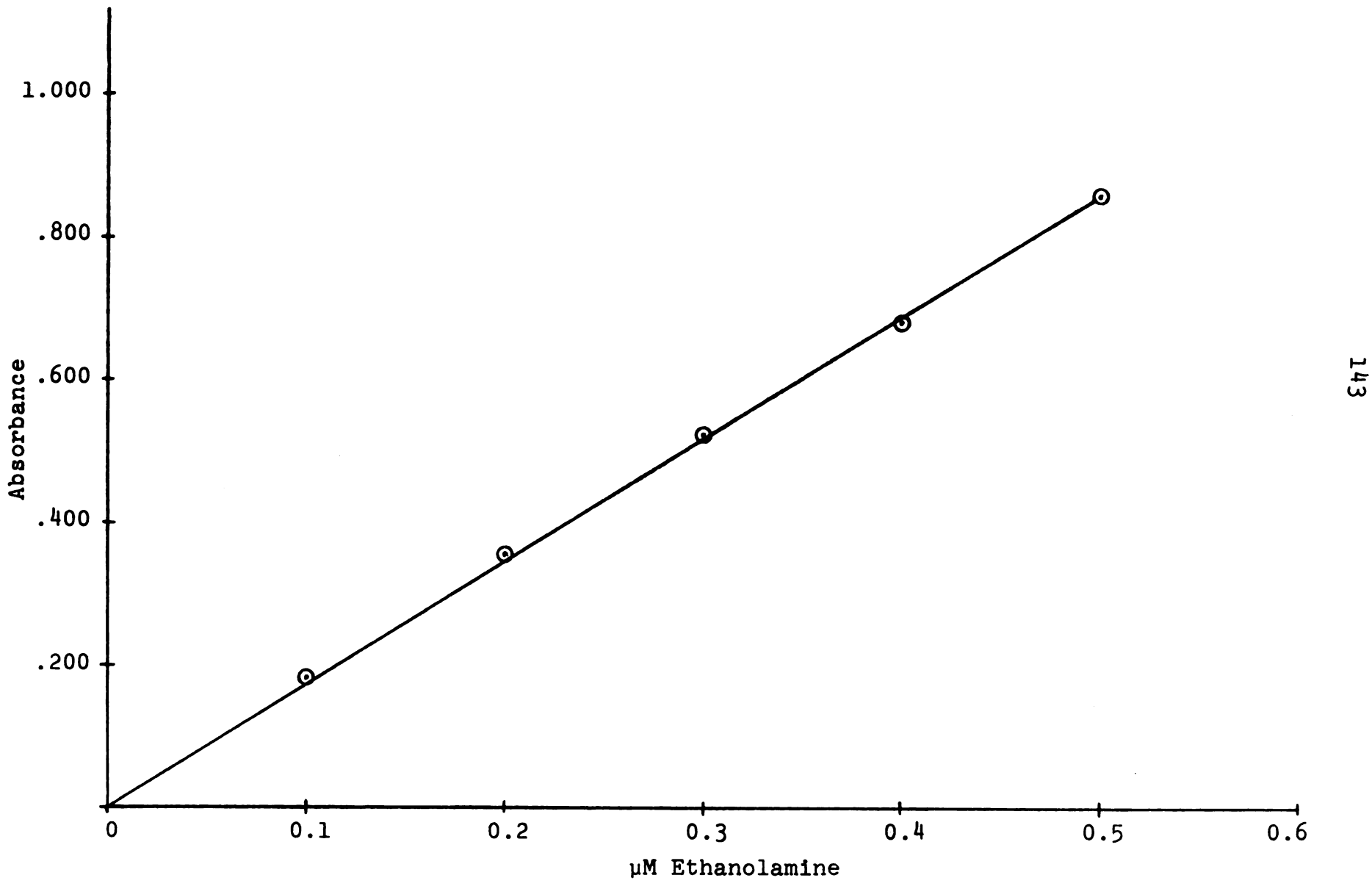
FATTY ACID COMPOSITION OF PURIFIED PHOSPHOLIPID FRACTIONS DISSOLVED IN SOLVENT AND DISPERSED IN BUFFER

Fatty acid composition of purified phospholipid fractions dissolved in solvent and dispersed in buffer.

Fatty Acid	Egg PE		Egg PC	
	In Solvent %	In Buffer %	In Solvent %	In Buffer %
C_{16}	19.8	20.0	33.3	36.3
$C_{16:1}$	1.6	2.1	--	--
C_{18}	34.2	34.8	14.6	14.0
$C_{18:1}$	14.5	14.3	34.4	33.4
$C_{18:2}$	9.1	9.9	15.6	14.4
$C_{18:3}$	3.2	3.3	--	--
$C_{20:4}$	17.6	15.7	2.1	2.0

APPENDIX C

STANDARD CURVE FOR ETHANOLAMINE


1.000
.800
.600
.400
.200
Absorbance
0
0.1
0.2
0.3
0.4
0.5
0.6
µM Ethanolamine