

*John Dugan*

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EVALUATION OF THE FLAVOR CONTRIBUTION OF  
PRODUCTS OF THE MAILLARD REACTION

by

L. R. Dugan, Jr.

and

G. V. Raa

Michigan State University  
East Lansing, Michigan 48823

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from plasmalogens are usually long chain saturated ones or non-2-enals. pH of the model system had a profound effect on the products of the browning reaction of PE or phosphatidal ethanolamine. While greater production of Schiff base at pH 6 was observed, a greater quantity of unreacted amino component at pH 5 and a greater polymer formation at pH 8 were demonstrated.

The presence of myoglobin in the system affected the formation of carbonylamine reaction products through its pro-oxidant effect. PE reacted readily with glucose to form Schiff bases, secondary products and scission products.

Flavor evaluation studies indicated that the development of off-flavor may be related to carbonyl compounds formed in the system as well as the Schiff bases, scission products and polymer formed.

The reactions between PE and aldehydes on cellulose were compared with the reactions on a protein matrix (muscle fibers free of neutral and polar lipids). The carbonyl reactions proceeded with both amino groups from PE and from the protein matrix. Certain amino acids such as lysine, alanine, phenylalanine and tyrosine reacted with aldehydes sufficiently to reduce their quantity in the total amino acid content of the system. The reactions were competitive so that the presence of PE had a sparing effect on amino acids in the protein matrix. This observation may provide rationale to explain the changes in texture, color, flavor, and nutritive value of dried foods containing proteins and phospholipids.

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## FOREWORD

During food processing and storage, autoxidation of polyunsaturated fatty acid constituents of phospholipids followed by fission, intramolecular and intermolecular browning-type reaction with free amino groups, polymerization, etc., are liable to occur. Changes in these labile substances are known to be of great significance in freeze-dried meat. Phosphatidyl ethanolamine, phosphatidyl serine, ethanolamine plasmalogens and serine plasmalogens contain free amino groups which can apparently undergo Maillard-type reaction with carbonyl compounds. Glyceraldehyde, dihydroxyacetone, ribose, ribulose, lyxose, xylulose, erythrose, glucose, fructose and sedoheptulose are present in meat as phosphoric acid esters in the products of carbohydrate metabolism. L-Fucose is a constituent of blood group substances which contain galactose and mannose. These compounds can react with the free amino groups of phospholipids and/or with the free amino groups of meat proteins in a Maillard type reaction.

The purpose of this study is to investigate by means of model systems related to freeze-dried meat the Maillard-type reaction between the products of autoxidized polyunsaturated fatty acids and free amino groups of phospholipids and within meat proteins, and between the free amino groups of phospholipids and the monosaccharides present in meat. The reaction was elucidated and its products characterized and evaluated for its contribution to meat flavor.

This investigation was conducted at Michigan State University under Contract No. DAAG 17-68-C-0034 during the period from January 1969 to September 1971. Professor L. R. Dugan, Jr. was the Principal Investigator in association with Dr. G. V. Rao. The Project Officer for the U.S. Army Natick Laboratories was Dr. Ahmed F. Mabrouk. Dr. J. W. Giffey served as Alternate Project Officer.

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## ABSTRACT

Non-enzymatic browning reactions in freeze-dried foods involve compounds containing carbonyl and free amino groups resulting in flavor and color deterioration. Lipid-browning reactions proceed readily at low moisture level (2.5%) and ambient or elevated temperature. Inert matrix emulsion with polar lipid, carbonyl compound and carboxymethyl cellulose was used as a model system for this study.

Schiff bases formation was the dominant feature of nearly all the reaction systems. Secondary or consecutive reactions to form other products such as oxypolymers, other polymers, methyl phosphatidate and other less well-defined scission products took place to varying degree according to experimental conditions. Methyl phosphatidate was derived from systems containing phosphatidyl ethanolamine (PE) and either saturated aldehyde or non-vicinal-enal. No formation of methyl phosphatidate was observed during lipid-browning, when either 2-ene-1-al or 2,4-dienal were used in the model system. Furthermore, methyl phosphatidate was not present in the reaction products of systems containing PE and dialdehyde. No major difference was observed in the reaction of hydrogenated and non-hydrogenated PE in the amount of Schiff base formed. More carbonyls were observed in the presence of non-hydrogenated PE and at elevated temperature. In the presence of Phosphatidal ethanolamine, methyl phosphatidate formation was observed. This is due to the fact that carbonyls derived from plasmalogens are usually long chain saturated ones or non-2-enals. pH of the model system had a profound effect on the products of the browning reaction of PE or phosphatidal ethanolamine. While greater production of Schiff base at pH 6 was observed, a greater quantity of unreacted amino component at pH 5 and a greater polymer formation at pH 8 were demonstrated.

The presence of myoglobin in the system affected the formation of carbonyl-amine reaction products through its pro-oxidant effect. PE reacted readily with glucose to form Schiff bases, secondary products and scission products.

Flavor evaluation studies indicated that the development of off-flavor may be related to carbonyl compounds formed in the system as well as the Schiff bases, scission products and polymer formed.

The reactions between PE and aldehydes on cellulose were compared with the reactions on a protein matrix (muscle fibers free of neutral and polar lipids). The carbonyl reactions proceeded with both amino groups from PE and from the protein matrix. Certain amino acids such as lysine, alanine, phenylalanine and tyrosine reacted with aldehydes sufficiently to reduce their quantity in the total amino acid content of the system. The reactions were competitive so that the presence of PE had a sparing effect on amino acids in the protein matrix. This observation may provide rationale to explain the changes in texture, color, flavor, and nutritive value of dried foods containing proteins and phospholipids.

## PURPOSE OF STUDY

To investigate, by means of model systems related to freeze-dried meat, the Maillard type reaction between the products of autoxidized polyunsaturated fatty acids and free amino groups within the phospholipid molecules, and between the free amino groups of phospholipids and the monosaccharides present in animal cells.

## OBJECTIVES

To prepare, isolate and characterize compounds and substances formed by non-enzymatic browning reactions involving lipid components in dry meat systems or simulated meat systems. To evaluate whether lipid-browning reaction components contribute to flavor in systems where these components may be formed.

## INTRODUCTION

Non-enzymatic browning reactions involve a variety of components formed in food systems (1). A feature common to reactions involved in Maillard browning is the initial reaction between a free amino group and a carbonyl group in formation of a typical Schiff base (2). This is of particular importance to nutritional quality of foods if protein amino groups are blocked by reaction with carbonyls resulting from fat oxidation. The subsequent discoloration is based on the formation of polymers of the oxidized lipids whose mechanism of formation is not uniform (3). The browning products commonly result from molecular rearrangement, other inter- and intra-molecular reactions and polymerization.

Irrespective of the method of preparation, whether freeze-dried or air-dried, dehydrated meats of good quality all suffer deterioration to a greater or lesser degree during subsequent storage. Usually the dehydrated meat contains 30-40% fat which in the presence of air deteriorates rapidly, due to oxidative rancidity.

Phospholipids and lipoproteins can react, through their amino groups, with aldehydes and reducing sugars. Therefore, under oxidizing conditions, reactive carbonyl groups may be formed in the lipid moiety and these can participate in browning reactions. The darkening of oxidized phosphatidyl ethanolamine and the accompanying loss of free amino-nitrogen was described by Lea (4). He also revealed deteriorative reactions involving phospholipids and lipoproteins. The role of amino acids in non-enzymatic browning was studied by Spark and Eichner (3,5). Tappel (6) showed that fluorescent products were derived from reaction of malonaldehyde with amino acids. Reaction of cysteine and methionine with malonaldehyde was reported by Buttkus (7). All these

studies showing lipid-protein interactions established that they lead to a decrease in solubility, reduced enzymatic cleavability and some loss of nutritive value of protein.

The high concentration of polyunsaturated fatty acids frequently found in phosphoglycerides and other factors of composition, location relative to other components in cells and availability of oxygen in a dehydrated food system contribute to ready oxidation of phosphoglyceride containing components. The carbonyl compounds which derive from this are then available to participate in carbonyl-amine reactions with components such as phosphatidyl ethanolamine and phosphatidyl serine. Oxidation of phosphoglycerides may contribute secondary products through subsequent lipid-browning reactions which have effects on flavor.

## APPROACH

### System Used

Non-enzymatic browning reactions between phosphatidyl ethanolamine and various carbonyl compounds were studied in model systems simulating freeze-dried foods. An inert matrix was prepared by making a cellulose emulsion according to the method of Bishov (12). Cellulose was dispersed in borate buffer at a particular pH and then the pure phospholipids and saturated or unsaturated aldehydes were added separately by blending at high speed for 2 min. in a Waring blender after each component was added. The emulsion was frozen by holding overnight at -20C. It was then freeze-dried at 100 $\mu$  without added heat in a Stokes freeze dryer, Model 2003F-2, to a moisture level of 2.5%.

Special systems were established with each variance of components in order to best comply with the characteristics of the components and to best simulate conditions which could exist in a natural system.

### Method of Study

Each system was prepared, permitted to develop products under accelerated and ambient conditions and then the products were extracted. Various solvent systems were used for extraction. Separation of components was accomplished by thin-layer or column chromatography or a combination of these. Characteristics were determined by IR-spectra, UV spectra, fluorescence spectra and, in some instances, by mass spectra.

A. Materials Used.

- |   |  |
|---|--|
| 1. Total phospholipids<br>(from egg yolk)       | 9. Oleyl aldehyde                                |
| 2. Pure phosphatidyl-<br>ethanolamine           | 10. 10-Undecene-1-al                             |
| 3. Hydrogenated<br>phosphatidyl<br>ethanolamine | 11. Lauryl aldehyde                              |
| 4. Nonanal                                      | 12. D-Glucose                                    |
| 5. Hexanal                                      | 13. Myoglobin                                    |
| 6. 2-Hexene-1-al                                | 14. Phosphatidal ethanolamine<br>from beef brain |
| 7. 2,4-Hexadienal                               | 15. Purified pancreatic<br>lipase                |
| 8. Malonaldehyde                                | 16. Phospholipase C                              |
|   | 17. Cellulose Gum (CMC)                          |

B. Methods of Preparation.

1. Total phospholipids were prepared according to the procedure of Ansell and Hawthorne (8).

2. Phosphatidyl ethanolamine: A 5-step multibore column was used for better resolution of various phospholipids (9). Less overlap of components was observed in the fractions collected from a 5-step column.

Sixty-five (65) grams of activated silicic acid was slurried in an excess of chloroform and packed into the column. Elution was carried out at room temperature under a nitrogen atmosphere. Extracts of egg phospholipids in chloroform:methanol (1:1 by volume) dissolved in chloroform, were carefully applied to the top of the column in a ratio of 0.03g lipid/g of silicic acid. Elution was accomplished by various solvent systems and successive 25ml fractions were collected. Neutral lipids were eluted with chloroform and monitored by the Salkowski test until a negative reaction was obtained. Acetone was used as a scavenger for eluting oxidized matter and pigments from the column. The neutral lipids and pigments were discarded. The third fraction, which contained phosphatidyl ethanolamine was eluted with 12% methanol in chloroform. The purity of the phosphatidyl ethanolamine fraction was established by thin-layer chromatography and pure fractions were pooled for further study.

3. Hydrogenated phosphatidyl ethanolamine: The pure phosphatidyl ethanolamine fraction from the silicic acid column was hydrogenated using a Palladium-carbon catalyst in a Parr hydrogenator at 30 psi.

4. Nonanal was obtained from Aldrich Chemical Company.

5. Hexanal was obtained from Aldrich Chemical Company.

6. 2-Hexene-1-al was obtained from K & K Laboratories, Inc.
7. Malonaldehyde was prepared by hydrolyzing 8ml (0.036 moles) of 1,1,3,3-Tetraethoxypropane (Eastman Organic Chemicals) with 3ml of HCl. The solution was kept at 45-50C until miscible and clear (25 min.). After adding 2ml water, the hydrolysis mixture was held an additional 20 min. at room temperature. The solution was adjusted to pH 6 at room temperature with about 9ml 2N KOH. (If the acetal was not completely hydrolyzed, a cloudy solution was obtained during the neutralization step.)
8. 2,4-Hexadienal was obtained from Aldrich Chemical Company. The dienal was fractionally distilled under vacuo. A small fraction of 2,4-hexadienal boiling at 110-112C (10mm) was obtained (40% yield) and this fraction was stored under nitrogen at -20C.
9. Oleyl aldehyde: Reducing oleyl chloride with lithium aluminum hydride at -70C gave poor yields. The successful method involved tosylating the oleyl alcohol with p-toluene sulphonyl chloride in pyridine and heating the tosylate at 160C for 10 min. with sodium bicarbonate and dimethyl sulphoxide. The yield was only 25%. The aldehyde so obtained was purified by silicic acid column chromatography.
10. 10-Undecene-1-al was obtained from Aldrich Chemical Company.
11. Lauryl aldehyde was obtained from K & K Laboratories, Inc.
12. D-Glucose was obtained from Mallinckrodt Chemical Works, St. Louis, Missouri.
13. Myoglobin was obtained from Cal-Biochem, Los Angeles, California.
14. Concentrate of phosphatidal ethanolamine: Crude phosphatides were prepared by extracting fresh beef brain with 2:1 chloroform:methanol (10) under an atmosphere of nitrogen. A 5-step multibore column was used for better resolution of various phospholipids. The column was packed with silicic acid. After applying the sample onto the column, the column was eluted with chloroform to remove neutral lipids. Acetone was used as a scavenger for eluting oxidized matter, and finally the column was eluted with 10% methanol in water to isolate the concentrate of phosphatidal ethanolamine.
15. Purified pancreatic lipase: Lipase was purified by butanol extraction from fresh porcine pancreas (11). The lipase collected after purification was dissolved in deionized water and stored at 5C.

16. Phospholipase C was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

17. Cellulose Gum (CMC) type THF was obtained from Hercules, Incorporated.

C. Instruments used for Analysis.

1. Beckman IR-12 Infrared Spectrophotometer
2. Beckman IR-5 Infrared Spectrophotometer
3. Beckman DK-2A Ratio Recording Spectrophotometer
4. Beckman DU-2 Spectrophotometer
5. Aminco-Bowman Spectrophotometer  
(American Instrument Co., Inc.)
6. Stokes Freeze-Dryer, Model 2003F-2
7. Gilson Respirometer
8. Parr Hydrogenator
9. Beckman GC-5 Gas Chromatograph
10. Mass Spectrophotometer with attached  
Gas Liquid Chromatograph

LIPID BROWNING SYSTEMS INVOLVING PHOSPHOGLYCERIDES

I. Initial Studies with Total Phospholipids

An inert matrix emulsion was prepared with total phospholipids, carbonyl compound and cellulose according to the procedure of Bishov et al (12). Model freeze-dried systems prepared were:

- (a) Total phospholipids + Nonanal
- (b) Total phospholipids + Hexanal
- (c) Hydrogenated total phospholipids + Nonanal
- (d) Hydrogenated total phospholipids + Hexanal

Fractionation and Characterization of Components. The freeze-dried samples were stored at a temperature of 50 C overnight to enhance the rate of browning. The reaction product was first extracted with 80% ethanol, then the residue was extracted with chloroform. The two extracts were evaporated to dryness under an atmosphere of nitrogen and then tested for phosphorus and nitrogen. The alcohol solubles were tested for and contained both phosphorus and nitrogen. The chloroform solubles contained phosphorus but only traces of nitrogen. Both alcohol and chloroform solubles were analyzed by infrared and thin-layer chromatography. Alcohol solubles had a broad absorption band in the IR at 2.9-3.15  $\mu$  indicating a bonded amino group, at 9.5  $\mu$  for P-O-C, 8  $\mu$  for P=O and a peak at 10.35  $\mu$  showing substituted nitrogen. Absorption bands at 4.7  $\mu$  and 6.0-6.2  $\mu$  were observed for C=N and C=N, respectively.

Thin-layer chromatography was used to separate various phosphorus containing fractions. A number of absorbents and solvent systems were tried. Column chromatography was ultimately used for separating the various phosphorus containing fractions formed during browning reaction.

Activated silicic acid (25g) was slurried in an excess of chloroform and this slurry was poured into a column 2 x 45cm containing a coarse sintered glass disc with a teflon stopcock on the tube at the bottom. The silicic acid column was washed with methanol, chloroform and hexane to remove undesirable channels present in the column. A 1 - 2cm layer of anhydrous sodium sulphate was placed on top of the silicic acid to absorb any residual moisture in the sample. Elution was performed under a nitrogen atmosphere at ambient temperature.

The alcohol extract was evaporated to dryness under an atmosphere of nitrogen and then dissolved in chloroform prior to being applied to the top of the column. Elution was accomplished with various solvent systems. The fractions were monitored by tests for phosphorus, ninhydrin tests and by TLC. The various reactions eluted and solvent systems used are shown in Table 1.

Table 2 gives the IR spectra characteristics of fractions eluted from the column and the characteristics of alcohol and chloroform solubles from the browning reaction.

Table 1. Column Chromatography of Alcohol Soluble Components of Total Phospholipid - Aldehyde Reaction Products

Fraction	Solvent System	Volume (ml)	Component Characteristics
1	hexane:chloroform 100:5	100	Oxidized products (carbonyl compounds)
2	hexane:chloroform:methanol 100: 15 :5	200	Phosphorus present (methyl phosphatidate)
3	hexane:chloroform:methanol 100: 15 :12	200	Ninhydrin portion (unreacted PE)
4	hexane:chloroform:methanol 100: 15 :20	300	Phosphatidyl choline
5	chloroform:methanol 50:50	200	Reaction product (Schiff base)

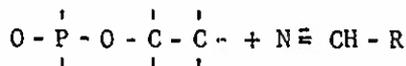
Table 2. Infrared Absorption Bands for Phospholipid - Aldehyde Reaction Products.

Sample	Absorption due to bonded -NH group (2.85-3.15 $\mu$ )	P - OH (3.95 $\mu$ )	C = N (5.95-6.2 $\mu$ )	C $\equiv$ N (4.9 $\mu$ )	Ester Carbonyl (5.85 $\mu$ )	P = O (8.0 $\mu$ )	P - O - C (9.35 $\mu$ )
1. Alcohol solubles of browning reaction product.	+	+	+	+	+	+	+
2. Chloroform solubles of browning reaction product.	-	-	-	-	+	+	+
<u>Fractions obtained from the column:</u>							
Fraction I	-	-	-	-	+	-	-
Fraction II	-	+	-	-	+	+	+
Fraction III	-	-	-	-	+	+	+
Fraction IV	-	-	-	-	+	+	+
Fraction V	+	+	+	+	+	+	+
(additional peaks at 10.35 $\mu$ )							

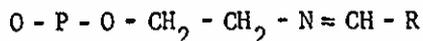
The chloroform soluble components from the residue of this model system contained phosphorus. A small portion of methyl phosphatidate and unreacted carbonyl compound was present in this fraction. The alcohol solubles had maximum fluorescence while the chloroform solubles had minimum fluorescence. Maximum fluorescence was observed in fraction V.

The chromatographic resolution of the alcohol soluble components of the reaction mixture revealed a multiplicity of products which indicate the nature of the reactions. The components of fraction I were relatively non-polar and apparently represented a heterogeneous mixture of secondary reaction products and lipid oxidation products.

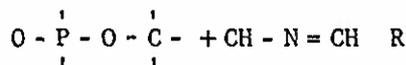
The components of fraction II had all the elements of a phosphoglyceride with the exception of the nitrogen moiety. Comparison of TLC development of this fraction with that of a standard methyl phosphatidate revealed a similar Rf. The IR spectra (Fig. 1) of the fraction also compared favorably with methyl phosphatidate. It is believed that this represents a molecular rearrangement and scission of the primary Schiff base compound as follows:



Ethyl phosphatidate



Schiff base



Methyl phosphatidate

Fraction III was ninhydrin positive and had all the characteristics of unreacted phosphatidyl ethanolamine. This could be unreacted phosphatidyl ethanolamine as well as a product of the reverse reaction from the initial Schiff base product as a consequence of temperature effects and pH.

Fraction IV was identified as phosphatidyl choline (PC), which was present as a consequence of using total phospholipids. Since PC has no free amino group, it would be expected to be unchanged except for oxidative changes in the fatty acid portion of the molecule or hydrolytic changes.

Fraction V had all the elements of phosphorus, nitrogen and IR spectra (Fig. II), which identified it as the primary reaction product present essentially as a typical Schiff base; in addition, the spectra bands for C = N and C ≡ N were present. These indicate the presence in

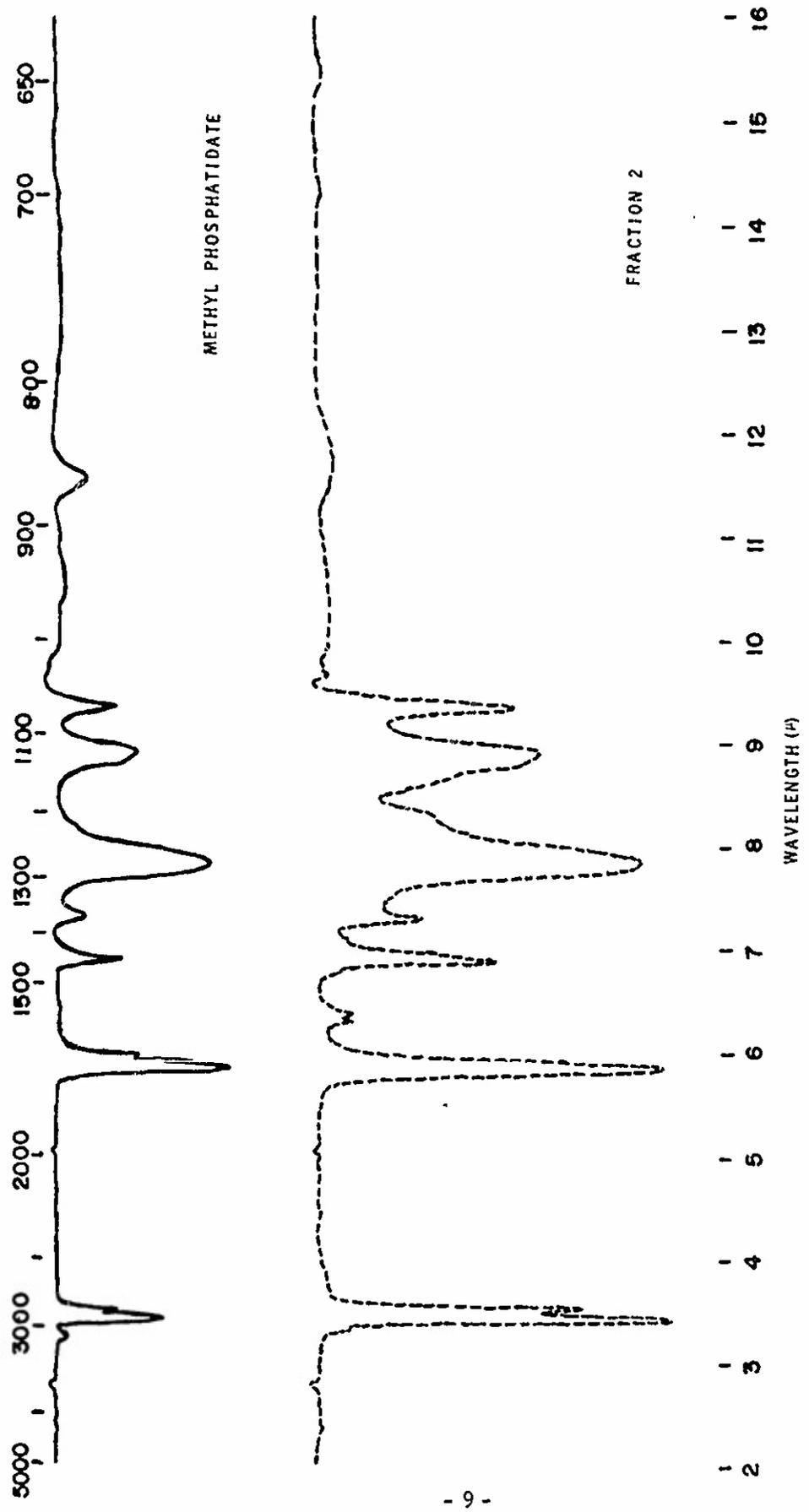
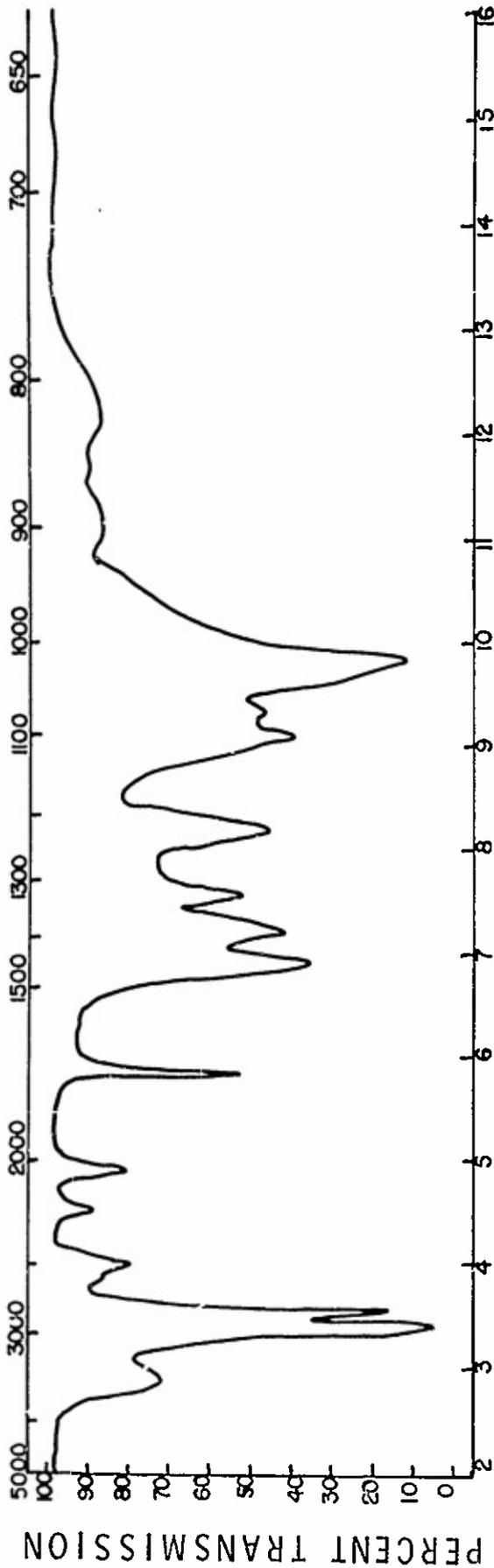


Fig. 1. IR spectrum of methyl phosphatidate and fraction 2 from reaction of total phospholipids with nonanal.

WAVENUMBER,  $\text{cm}^{-1}$



WAVELENGTH IN MICRONS

Fig. II. IR spectrum of fraction 5 isolated from reaction of total phospholipids with nonanal.

this fraction of nitrogen containing components, resulting from the scission of the primary product after molecular rearrangement.

The reactions involving hydrogenated phospholipids with saturated aldehydes were essentially identical to those of the non-hydrogenated phospholipids. The main differences were in the presence of more component in Fraction I (i.e., carbonyls) when non-hydrogenated substrate was used. The browning reaction would naturally be influenced by the input of additional carbonyl compounds from scission of hydroperoxides formed by autoxidation of the unsaturated fatty acid components in the non-hydrogenated phospholipids.

Reactions involving hexanal with the phospholipids gave results identical to those achieved with nonanal. This would be expected since no difference exists beyond a 3-carbon chain length in the aldehyde moiety.

These preliminary studies with phospholipids showed carbonyl-amine reactions yielding Schiff base, unreacted phosphatidyl ethanolamine and scission products. The Schiff bases have characteristic spectral absorption for C = N.

## II. Reaction of Pure Phosphatidyl Ethanolamine with Saturated Aldehydes

A. Reaction of PE with Nonanal. Model freeze-dried products were prepared in boric acid buffer at pH 6 in cellulose emulsion with pure reactants as shown below and stored at room temperature at RH 14% for 32 days.

- (a) Pure phosphatidyl ethanolamine + Nonanal
- (b) Pure hydrogenated phosphatidyl ethanolamine + Nonanal

The reaction product was first extracted with 80% ethanol, then the residue was re-extracted with chloroform. The alcohol soluble components contained both phosphorus and nitrogen and the chloroform solubles contained phosphorus.

The alcohol soluble substances were spotted on a silica gel G coated plate and developed with chloroform:methanol:hexane 5:5:90 (v/v). The non-phosphorus containing fraction remained at the base. The band which moved to the solvent front was isolated and again developed on a silica gel G coated plate with petroleum ether (40-60°):diethyl ether:acetic acid 90:10:1 (v/v). When sprayed with 2,4-DNP, the developed spots gave a positive test for carbonyl compounds. Standard saturated and unsaturated aldehydes were spotted for comparison. GLC of this carbonyl fraction revealed aldehydes with chain length C<sub>6</sub> - C<sub>12</sub>. IR spectra were recorded which had the typical characteristics of aldehydes.

Only unreacted nonanal was isolated in the alcohol soluble fraction from the model systems using hydrogenated phosphatidyl ethanolamine. This confirmed that oxidation of the polyunsaturated fatty acids present in the system were important factors during browning reactions involving the non-hydrogenated sample.

The phosphorus containing fraction which was at the base of the TLC plate was isolated and developed on a basic silica gel G coated plate with chloroform:methanol:water 65:25:4 by volume. The various phosphorus containing fractions were isolated from the developed plate and the percentages of each product formed (based on phosphorus analysis) are given in Table 3.

Table 3. Reaction Products of Nonanal with Phosphatidyl Ethanolamine.

System Components	Schiff Base	Non-Polymeric Reaction Product	Unreacted PE	Methyl Phosphatidate	Poly-mers
1. Phosphatidyl Ethanolamine + Nonanal	48.0	23.6	17.5	6.1	4.8
2. Hydrogenated Phosphatidyl Ethanolamine + Nonanal	46.0	19.9	19.5	4.9	9.4

The IR, UV and fluorescence spectra were determined on each fraction with the exception of the polymer fraction which, after isolation, became a thick film insoluble in all solvents.

The Schiff base fraction isolated from the plate had peaks in IR spectra for bonded -NH group (2.95 - 3.1  $\mu$ ), C = N (6.0 - 6.2  $\mu$ ); C  $\equiv$  N (4.9  $\mu$ ); P = O (8  $\mu$ ); and P - O - C (9.5  $\mu$ ); which are characteristic for such functional groupings.

The non-polymeric reaction product had no typical Schiff base characteristics, but it apparently resulted from a browning reaction because it gave a negative test for ninhydrin and therefore is not unreacted phosphatidyl ethanolamine.

The methyl phosphatidate fraction had characteristic IR spectra and an appropriate R<sub>f</sub> value on TLC.

The Schiff base fraction absorbed in the ultraviolet region at 280  $\mu$ . The other fraction failed to absorb at 280  $\mu$ .

The chloroform soluble components of these products had a very small amount of methyl phosphatidate as identified by IR and TLC. In addition to methyl phosphatidate, the chloroform soluble fraction contained carbonyl compounds which were detected on a TLC plate by spraying with 2,4-DNP reagent.

The Schiff base fraction was isolated and characterized in greater detail by passing the alcohol solubles through a Sephadex LH 20 column.

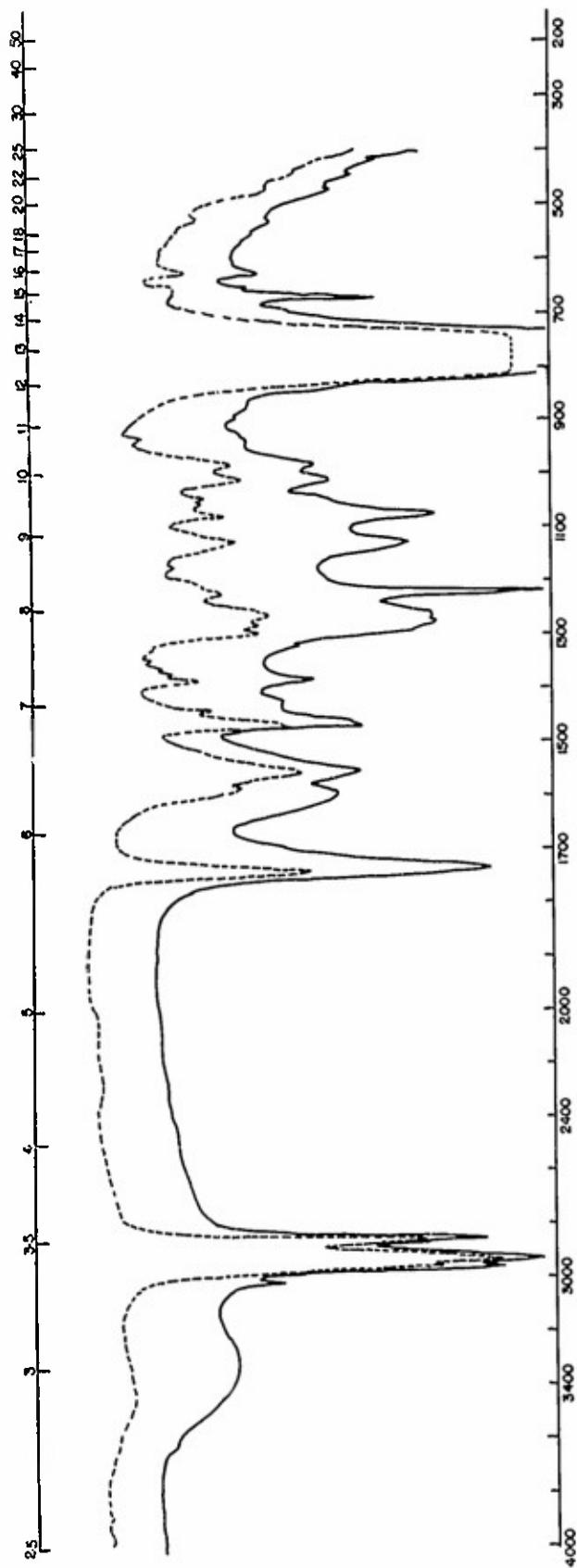
Isolation of Schiff bases by means of Sephadex LH 20 column:  
Sephadex LH 20, the lipophilic derivative of Sephadex, extends gel filtration to organic solvents. When using methanol as a solvent for column elution. Sephadex LH 20 is prepared from the dry state by adding 1.9ml methanol per gram (dry) of gel. After storing for 24 hr., the gel is kept under the same solvent in approximately a bed volume of 4.0 - 4.5ml per gram (dry) of gel.

The alcohol solubles, after being taken to dryness under nitrogen, were dissolved in a minimum amount of methanol and applied to a Sephadex LH 20 column. After the sample was absorbed into the column, a 10ml portion of methanol was added twice until all the sample was absorbed onto the column. The column was then eluted with methanol at a flow-rate of 60ml/hr. The various bands separated could be seen in the column and the fractions collected accordingly in separate receivers. The Schiff base fraction started to elute after 75ml solvent eluted. This fraction was followed by ninhydrin positive unreacted PE which was dark in color and this was followed by methyl phosphatidate. Other fractions eluted after collecting 200ml contained only nitrogen and no phosphorus.

The IR spectrum of the Schiff base (Fig. III) had a distinct absorption band at  $1620\text{cm}^{-1}$  for C = N and other characteristic peaks as cited above. The fluorescence spectrum had maximum fluorescence at 425  $\mu$  at an excitation of 360  $\mu$  (Fig. IV). Ultraviolet spectra had absorbance at 280  $\mu$  (Fig. V) typical for C = N and absorbance at 215  $\mu$  for the carbonyl fraction present in the sample.

To confirm the characteristics of the Schiff base, the isolated product was reduced with sodium borohydride and the C = N fraction was reduced to -CH<sub>2</sub>-NH-. The sample then lost its fluorescence and characteristic absorption in the IR and UV spectrum (13). Schiff base (0.15g in 25ml absolute methanol) was placed in a three-necked flask and NaBH<sub>4</sub> in dry methanol was added slowly over 2.5 hr. to excess. After addition of all the sodium borohydride solution, the mass was refluxed for 2 hr. on a water bath. The reduced product was placed in a 250ml separatory funnel and extracted with diethyl ether, washed with

WAVELENGTH IN MICRONS



WAVENUMBER,  $\text{cm}^{-1}$

Fig. III. IR spectra for Schiff base from phosphatidylethanolamine and nonanal.  
(Broken line represents the  $\text{NaBH}_4$  reduced sample.)

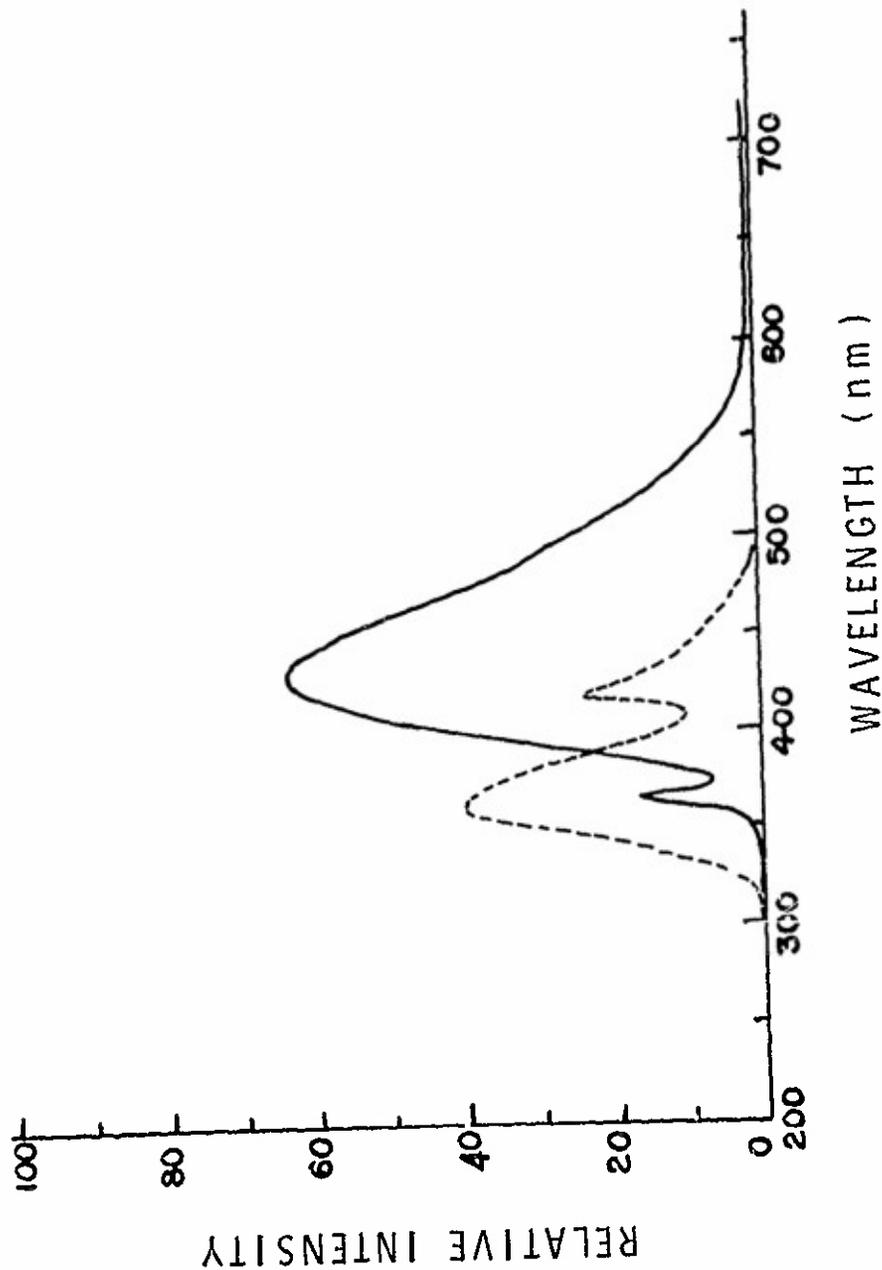


Fig. IV. Fluorescence spectra for Schiff base from phosphatidylethanolamine and nonanal. (Excitation - broken line; emission - solid line.)

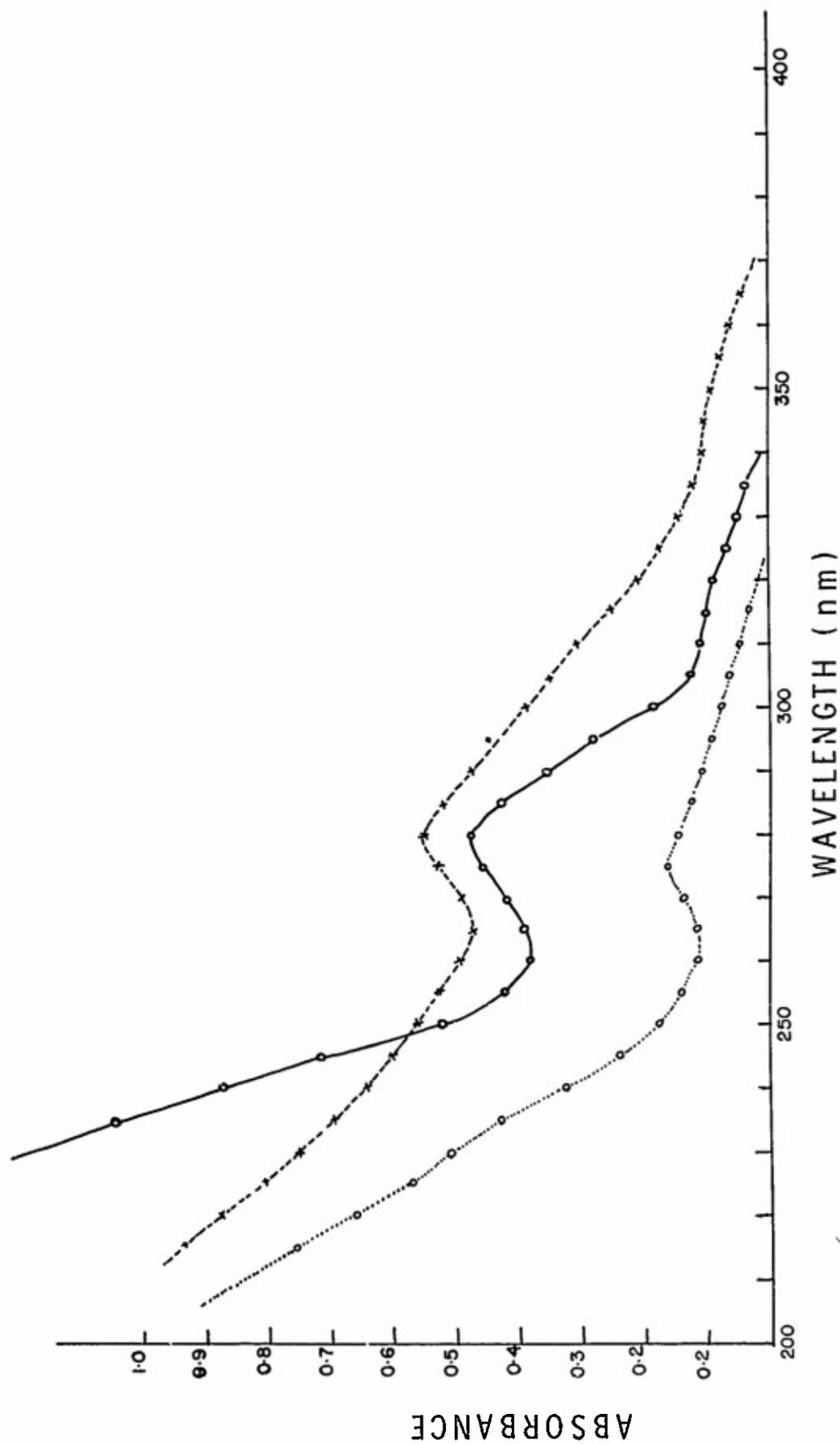


Fig. 5. Ultraviolet absorption spectra for Schiff base from phosphatidylethanolamine and aldehydes having no unsaturation conjugated with the carbonyl oxygen. (PE + nonanaldehyde, circles with solid line; PE + 10 undecene 1-al, x with broken line; PE + oleylaldehyde, circles with dotted line.)

WAVELENGTH IN MICRONS

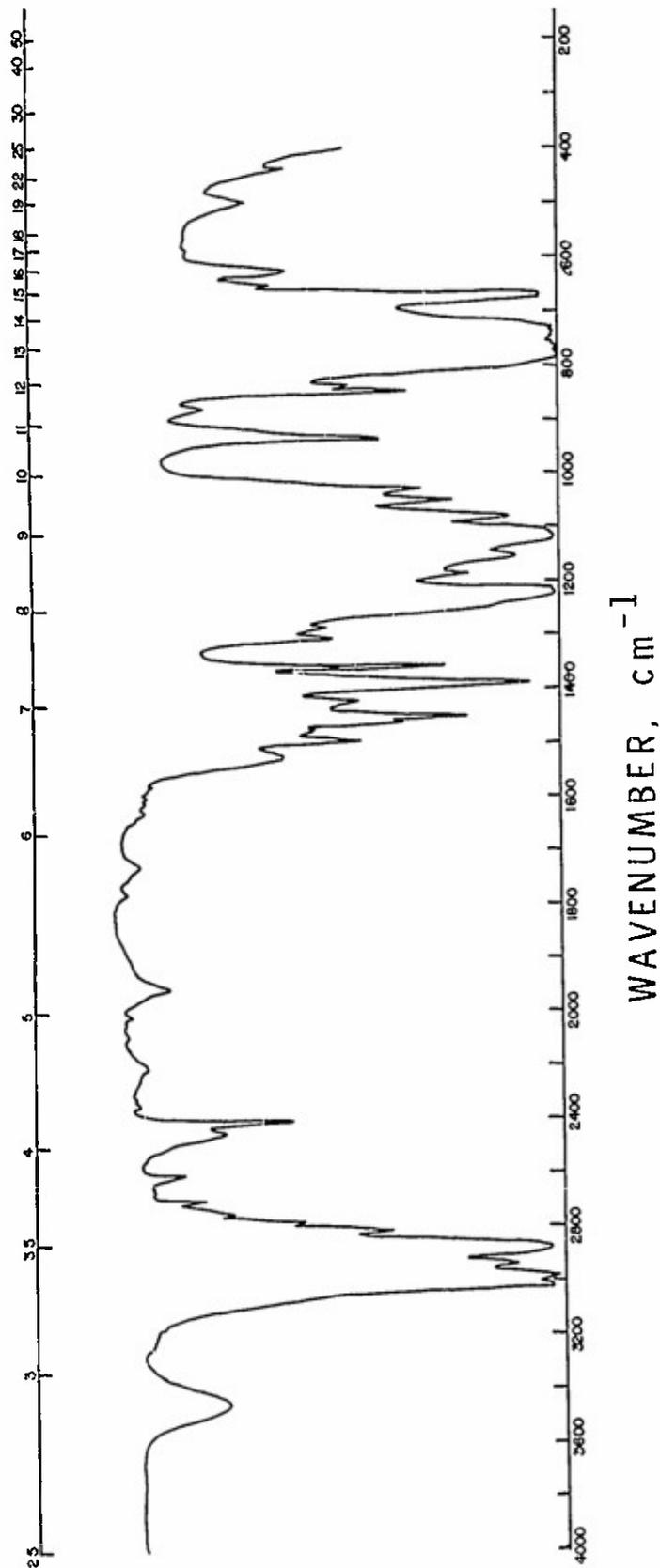


Fig. VI. IR spectra for nitrogen containing non-Schiff base products from reaction of PE with nonanal.

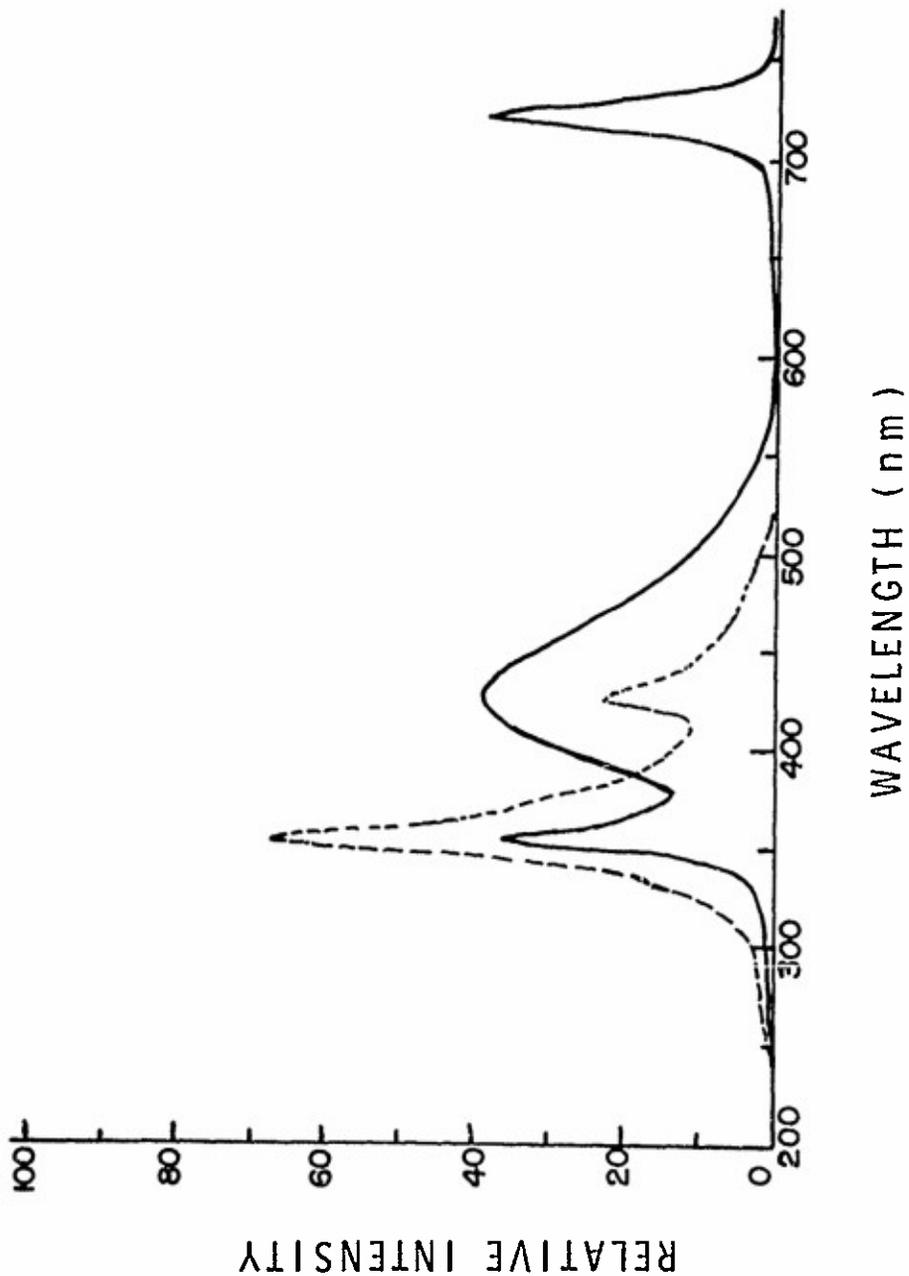


Fig. VII. Fluorescence spectra for nitrogen containing non-Schiff base products from reaction of PE with nonanal. (Excitation - broken line; emission - solid line.)

WAVELENGTH IN MICRONS

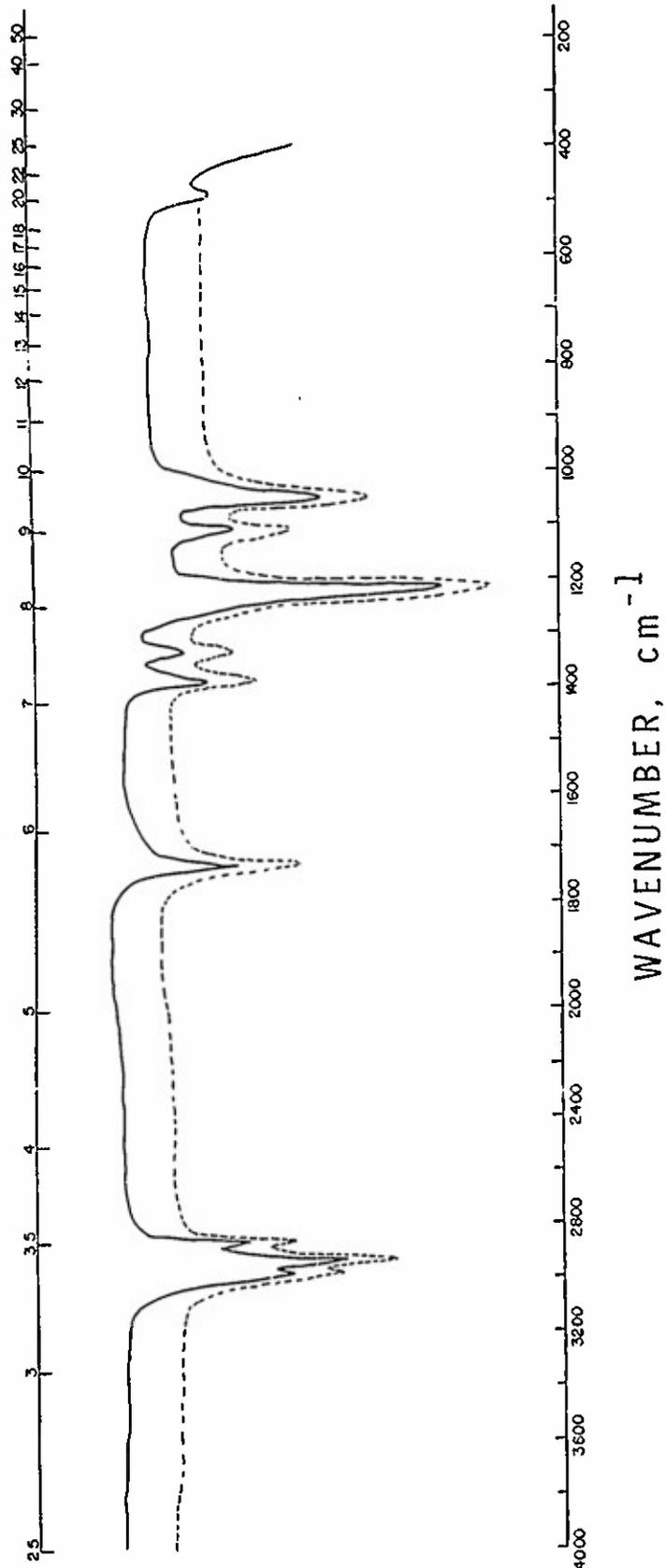
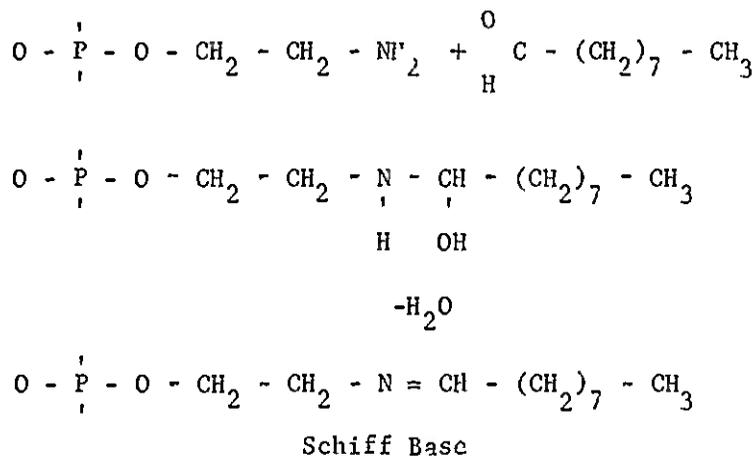


Fig. VIII. IR spectrum for methyl phosphatidate resulting from reaction of PE with nonanal (broken line).  
Spectrum for standard methyl phosphatidate (solid line).

distilled water and dried over anhydrous sodium sulphate. The IR spectra of the reduced product is shown by the broken line in Fig. III. The peak for C = N completely disappeared and the product no longer exhibited fluorescence and ultraviolet absorption.

The fraction collected after 200ml contained nitrogen and fluoresced strongly. The IR spectrum of this fraction is shown in Fig. VI. The fluorescence spectra (Fig. VII) had maximum fluorescence at 440 and 730 m $\mu$  at an excitation of 360 m $\mu$ . This fraction had characteristic emission at 730 m $\mu$ , whereas other Schiff bases failed to show this characteristic emission at this particular wavelength. It was not established whether this might be an overtone to excitation at 360 m $\mu$ . The mechanism of Schiff base formation is shown below:



The presence of methyl phosphatidate was shown by the IR spectrum for the fraction which eluted after the unreacted PE. The IR spectrum in Fig. VIII compares the fraction from the reaction (broken line) with a standard methyl phosphatidate (solid line).

B. Role of pH in Maillard-Type Browning Reaction Involving PE and Saturated Aldehydes. Previous studies showed the rate of loss of amino-nitrogen and the rate of browning to be greater with increasing pH (14); Mohammed *et al* (15) showed that the rate of browning of a solution of glucose and bovine serum albumin increased as the pH was increased; the plot of the logarithm of reaction rate against pH was linear, suggesting specific hydroxyl-ion catalysis. The present study was aimed to investigate the effect of pH in the browning reaction of pure phosphatidyl ethanolamine and carbonyl compounds.

Model systems were prepared on cellulose matrix using pure phosphatidyl ethanolamine and nonanal with a boric acid buffer at pH 5, 6 and 8. A portion of the freeze-dried system was stored at ambient temperature for 30 days at RH 14% and the remainder was heated at 50C for 10 hr.

to enhance browning. The intensity of the brown color after 30 days at ambient temperature was minimum at pH 5 and maximum at pH 8.

The system was first extracted with 80% ethanol and then the residue was extracted with chl oform. The alcohol solubles contained both phosphorus and nitrogen. As explained previously, the alcohol solubles were first extracted, fractionated on a silica gel G plate and then developed with hexane:chloroform:methanol 90:5:5 by volume. This eluted all the carbonyl compounds. The fraction which moved to the solvent front was isolated with chloroform:methanol 1:1 by volume and again developed with petroleum ether:diethyl ether:acetic acid 90:10:1 on a silica gel G coated plate. Standard saturated and unsaturated aldehydes were spotted as reference samples. After development, the plate was sprayed with 2,4-DNP and base to disclose the aldehydes as purple spots and then was charred with 50% sulphuric acid. The spots which contained carbonyls in the alcohol soluble fraction corresponded to nonanal and to the unsaturated aldehydes and dialdehydes which would have been produced by oxidation of polyunsaturated fatty acids present in phosphatidyl ethanolamine. In general, more of the carbonyl fraction was noticed in the sample at pH 8 and the least amount in the sample at pH 5.

The phosphorus positive fraction at the base of the development plate was spotted on a basic silica gel G coated plate and developed with chloroform:methanol:water 65:25:4 by volume. Various phosphorus containing fractions were isolated and the phosphorus content determined. The results are shown in Table 4 as percentage of each phosphorus containing product formed.

Table 4. Reaction Products of Nonanal with Phosphatidyl Ethanolamine\*.

pH of System	Schiff Base	Unreacted Phosphatidyl Ethanolamine	Non-polymeric Reaction Product	Methyl Phosphatidate	Polymers
	%	%	%	%	%
5	26.2	43.9	21.1	4.0	4.8
6	48.0	18.0	23.1	5.8	5.1
8	21.0	16.4	41.0	10.1	12.5

\*RH 14% and ambient temperature for 30 days.

The results show that the breakdown of the Schiff base is greater during browning at enhanced temperature than when stored at room temperature and constant relative humidity.

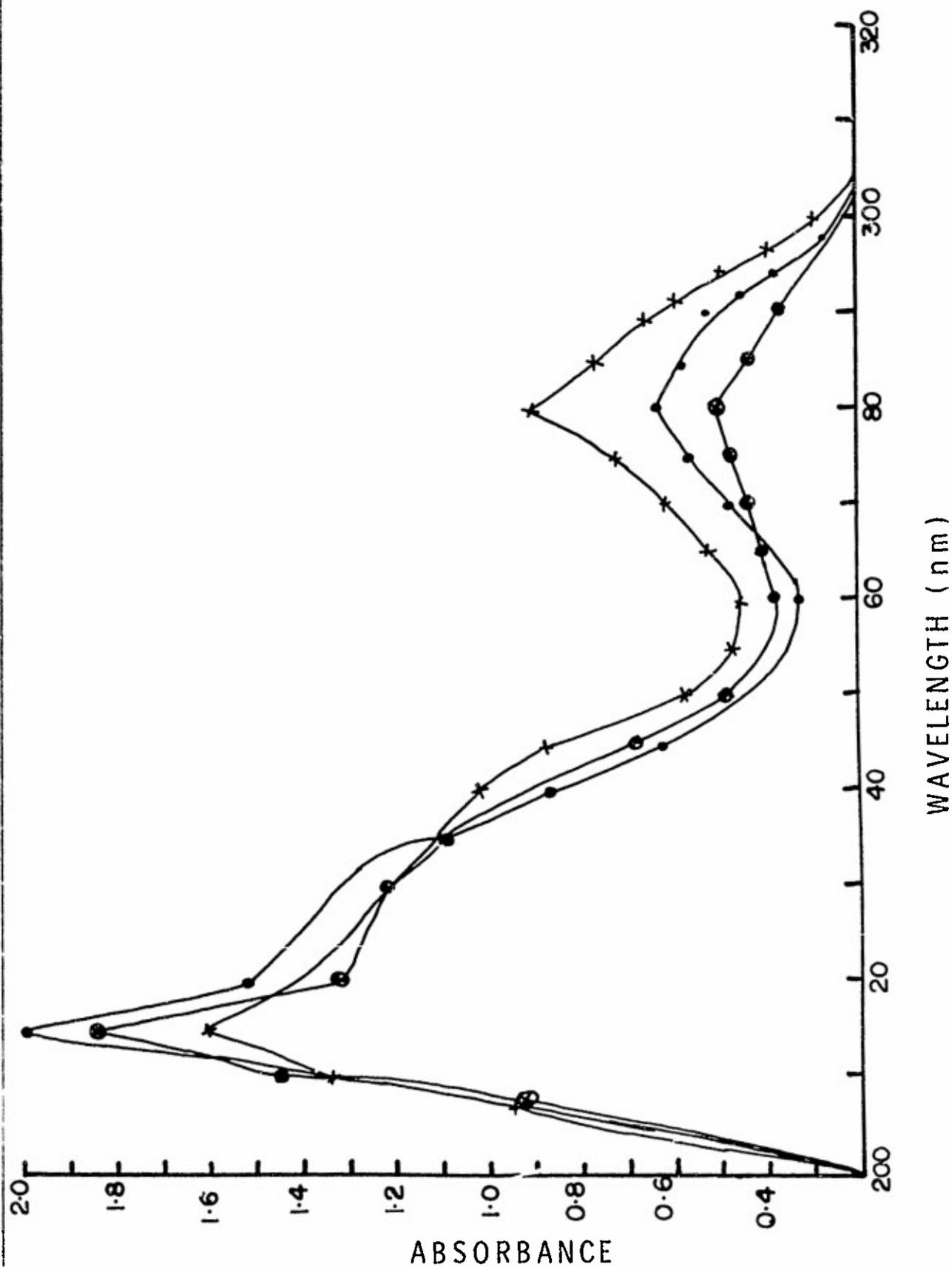


Fig. IX. UV spectra of Schiff bases formed from PE and noranal at different pH levels (x - pH 6.0; solid circles - pH 8.0; open circles - pH 5.0.)

At pH 8, the carbonyl fraction isolated by the first TLC fractionation with hexane:chloroform:methanol was greater than in the similar fractions from samples reacted at pH 5 and pH 6. This shows that at alkaline pH, oxidation of the polyunsaturated fatty acids is greater than at lower pH levels and browning was also greater as a consequence of all the side products formed.

The following information was derived from the samples isolated at constant temperature and RH 14%. The IR spectrum of the Schiff base fraction had characteristic absorption bands for bonded -NH group, P - O, P - O - C and C = N. It had maximum fluorescence at 425  $\mu$  at an excitation of 360  $\mu$ . The ultraviolet spectrum had maximum absorption at 280  $\mu$  and 215  $\mu$ , where absorption at 280  $\mu$  is characteristic of C = N formation. The UV absorbance of the Schiff base formed at various pH levels are shown in Fig. IX, all at the same concentration.

The IR spectrum of the fraction which was identified as methyl phosphatidate compared well with the IR spectrum of standard methyl phosphatidate. It also behaved similarly to standard methyl phosphatidate on a TLC plate.

Schiff base formation was at a maximum at pH 6 with less side products formed during the browning reaction than at either higher or lower pH levels. The rate of reaction of carbonyl compound with nitrogenous bases is pH dependent. As the pH was lowered from slightly alkaline to slightly acidic, the rate of Schiff base formation from the carbonyl compound increased. A further decrease in the pH then caused a decrease in the rate of reaction and enhanced the reversible reaction giving rise to the nitrogenous base and the carbonyl compound. At the alkaline pH the oxidation of the polyunsaturated fatty acid seemed to be greater, thus providing more reactants for browning reactions.

### III. Reaction of Phosphatidyl Ethanolamine with Non-vicinal En-als

The carbonyl compounds used were oleyl aldehyde and 10-undecene-1-al. When carbonyl-amine reactions occurred, these aldehydes reacted like saturated aldehydes forming Schiff bases and scission products. Scission of Schiff bases was not observed when unsaturated aldehydes like 2-hexene-1-al, 2,4-hexadienal and malonaldehyde were used in the model systems. The reason for not observing scission products when the above mentioned long chain aldehydes are used may be because the double bond in these carbonyl compounds is isolated from -C = N and no stability results from conjugation of double bonds.

#### A. Reaction of Phosphatidyl Ethanolamine with Oleyl Aldehyde.

The system was prepared using pure phosphatidyl ethanolamine and oleyl aldehyde in cellulose emulsion with boric acid buffer at pH 6. After freeze-drying to a moisture level of 2.5% the model system was stored

for 30 days at ambient temperature and RH 14%. The product was first extracted with 80% ethanol and then the residue re-extracted with chloroform. The chloroform soluble fraction contained unreacted carbonyl compounds and a small amount of methyl phosphatidate. The alcohol solubles contained both phosphorus and nitrogen.

A portion of the alcohol solubles was spotted on a silica gel G plate and developed with hexane:chloroform:methanol 90:5:5 by volume to remove the carbonyl compound. The phosphorus containing fraction at the base was further fractionated on a basic silica gel G plate and developed with chloroform:methanol:water 65:25:4 by volume. The phosphorus containing fractions formed (based on phosphorus analysis) are shown in Table 5.

Table 5. Reaction Products of Oleyl Aldehyde with Phosphatidyl Ethanolamine.

System Components	Schiff Base	Non-polymeric Reaction Product	Unreacted PE	Methyl Phosphatidate	Polymers
Phosphatidyl Ethanolamine + Oleyl Aldehyde	44.0	18.0	25.0	6.0	6.1

Isolation of Schiff base using Sephadex LH 20 column: The alcohol solubles were applied on the Sephadex LH 20 column, the column was eluted with methanol and 25ml fractions were collected. After eluting 90ml, the Schiff base was eluted. The following fraction was dark in color and ninhydrin positive indicating the presence of unreacted phosphatidyl ethanolamine. Methyl phosphatidate was eluted following the unreacted PE. The Schiff base isolated was purified on a basic silica gel G plate and the IR spectrum (Fig. X) had an absorption band for C = N at  $1565\text{cm}^{-1}$ . This fraction had fluorescence spectra excitation at  $365\text{m}\mu$  and emission at  $430\text{m}\mu$  as shown in Fig. XI. The ultraviolet spectrum had maximum absorption at  $280\text{m}\mu$  (Fig. V). The Schiff base fraction was reduced with sodium borohydride and the IR spectrum was as shown in Fig. X. The C = N absorption band at  $1565\text{cm}^{-1}$  was removed by the reduction.

Some of the Schiff base formed from phosphatidyl ethanolamine and oleyl aldehyde was degraded giving methyl phosphatidate and a nitrogen containing fraction just as was observed in the system when PE and nonanal were allowed to react.

WAVELENGTH IN MICRONS

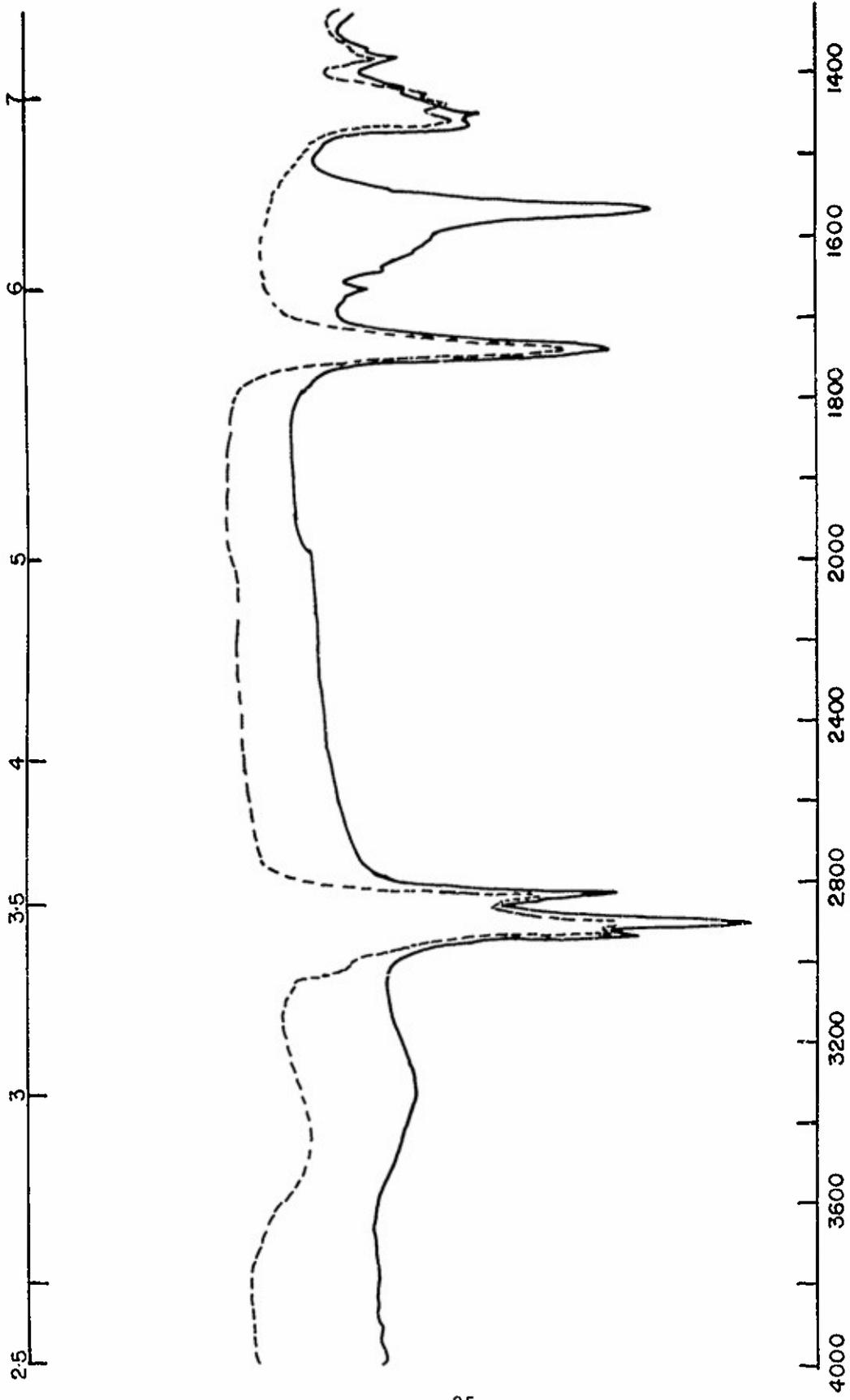


Fig. X. IR spectrum for Schiff base formed from PE and oleylaldehyde (solid line).  
Spectrum for NaBH<sub>4</sub> reduced product (broken line).

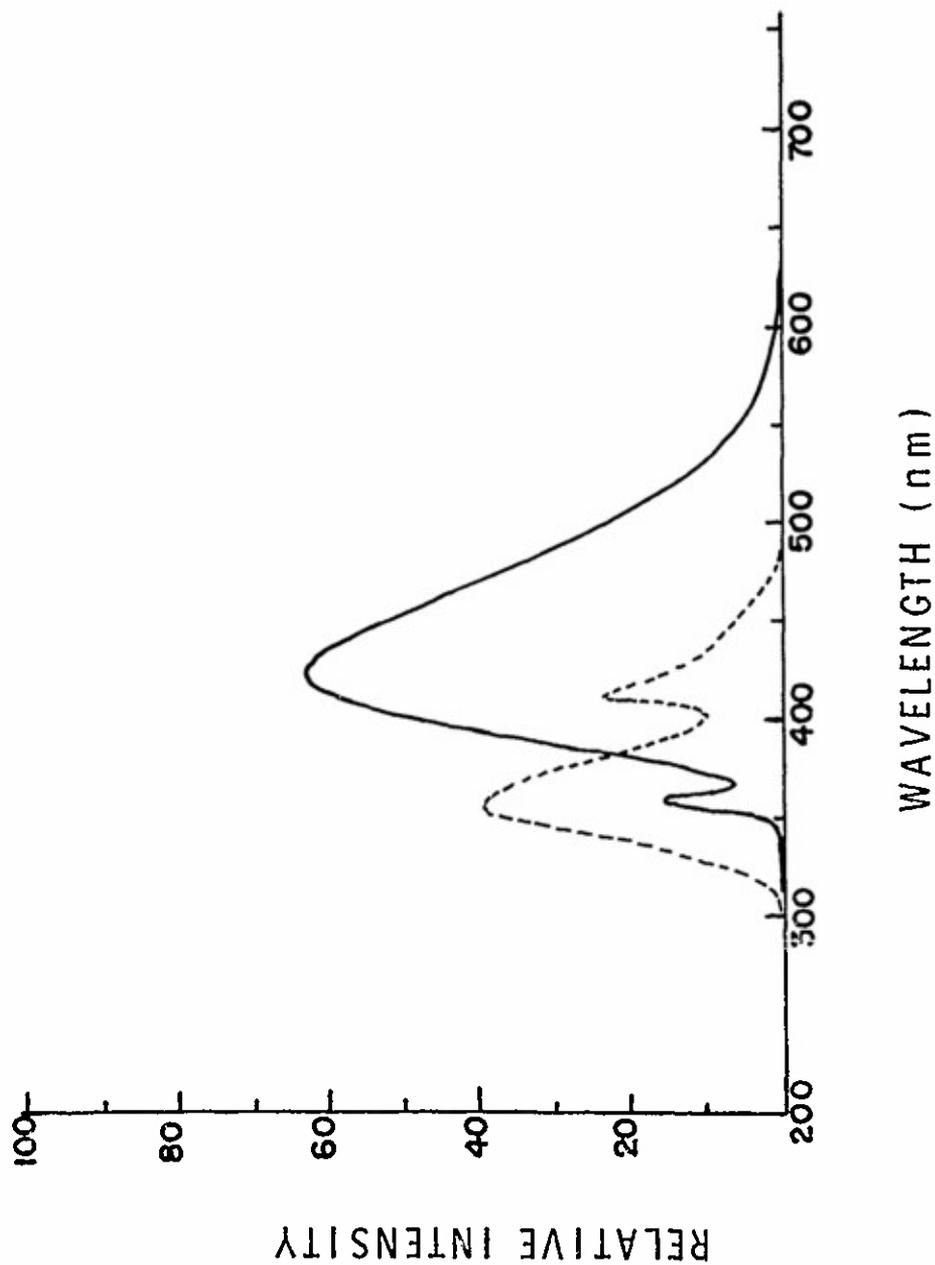


Fig. XI. Fluorescence spectra for Schiff base formed from PE and oleylaldehyde. (Excitation - broken line; emission - solid line.)

B. Reaction of Phosphatidyl Ethanolamine with 10-Undecene-1-al.

The systems were prepared with phosphatidyl ethanolamine and undecylenic aldehyde with and without myoglobin on a cellulose matrix with boric acid buffer at pH 6. After freeze-drying to a moisture level of 2.5%, the products were stored at RH 14% for 30 days at ambient temperature. The products were extracted with chloroform:methanol 1:1 by volume and the extract concentrated under an atmosphere of nitrogen.

A portion of the extract was applied to the silica gel G plate and developed with hexane:chloroform:methanol 90:5:5 by volume to eliminate the carbonyl fraction. The phosphorus containing fraction is isolated and respotted on a basic silica gel G plate and developed with chloroform:methanol:water 65:25:4 by volume. Various phosphorus containing fractions were isolated. The percentages of products formed (based on phosphorus analysis) are given in Table 6.

Table 6. Reaction Products of 10-Undecene-1-al and Phosphatidyl Ethanolamine.

System Components	Schiff Base	Non-polymeric Reaction Product	Unreacted PE	Methyl Phosphatidate	Polymers
1. Phosphatidyl Ethanolamine + Undecylenic Aldehyde	44.0	19.3	21.7	5.0	9.0
2. Phosphatidyl Ethanolamine + Undecylenic Aldehyde + Myoglobin	21.9	43.2	18.2	5.4	10.4

Isolation of Schiff base using Sephadex LH 20 column: The remaining extract was applied to the column and eluted with methanol. After eluting 75ml, a small quantity of Schiff base was eluted followed by unreacted phosphatidyl ethanolamine. After eluting 120ml, a fraction was eluted which had well defined Schiff base characteristics. This was followed by methyl phosphatidate.

All the fractions collected from the Sephadex column were further purified on a basic silica gel G plate by developing with chloroform:methanol:water 65:25:4 by volume.

IR spectrum of the Schiff base contained an absorption band characteristic of C = N at  $1685\text{cm}^{-1}$  and one at  $1645\text{cm}^{-1}$  for the  $\text{H}_2\text{C} = \text{CH}$  double bond system present in undecylenic aldehyde (Fig. XII). The ultraviolet spectrum had maximum absorbance at  $275\text{ m}\mu$  (Fig. V). Maximum fluorescence occurred at  $445\text{ m}\mu$  at an excitation of  $370\text{ m}\mu$  (Fig. XIII). Reduction of the Schiff base with sodium borohydride caused disappearance of the C = N absorption band at  $1685\text{cm}^{-1}$ , while the band at  $1645\text{cm}^{-1}$  was apparently unaffected (Fig. XII).

When myoglobin was present in the system, there was minimum formation of Schiff base while formation of non-polymeric and polymeric fractions was greater. The data in Table 7 clearly show that Schiff base formation was reduced in the model system when myoglobin was present. The formation of methyl phosphatidate in the system containing myoglobin indicated that scission of Schiff base occurred without unusual effect by myoglobin.

When, nonanal, oleyl aldehyde and undecylenic aldehydes were used in model systems together with PE, a fraction containing phosphorus but no nitrogen was observed and confirmed as methyl phosphatidate. This scission was observed when saturated carbonyl compounds were used in the model system. Oleyl and undecylenic aldehydes acted as saturated aldehyde in this sense, because the double bond in the carbonyl compound is too far removed from the C = N to contribute any stability by shifting double bonds.

Table 7 shows the various phosphorus containing fractions formed and the percentage of each product formed (based on phosphorus analysis) when nonanal, oleyl aldehyde and undecylenic aldehyde were used together with phosphatidyl ethanolamine in the model system. This summary of percentages of fractions formed during browning reactions reveals similar product formation indicating that unsaturated carbonyls in which the double bond is substantially distant in the carbon chain from the carbonyl function behave essentially as saturated carbonyls.

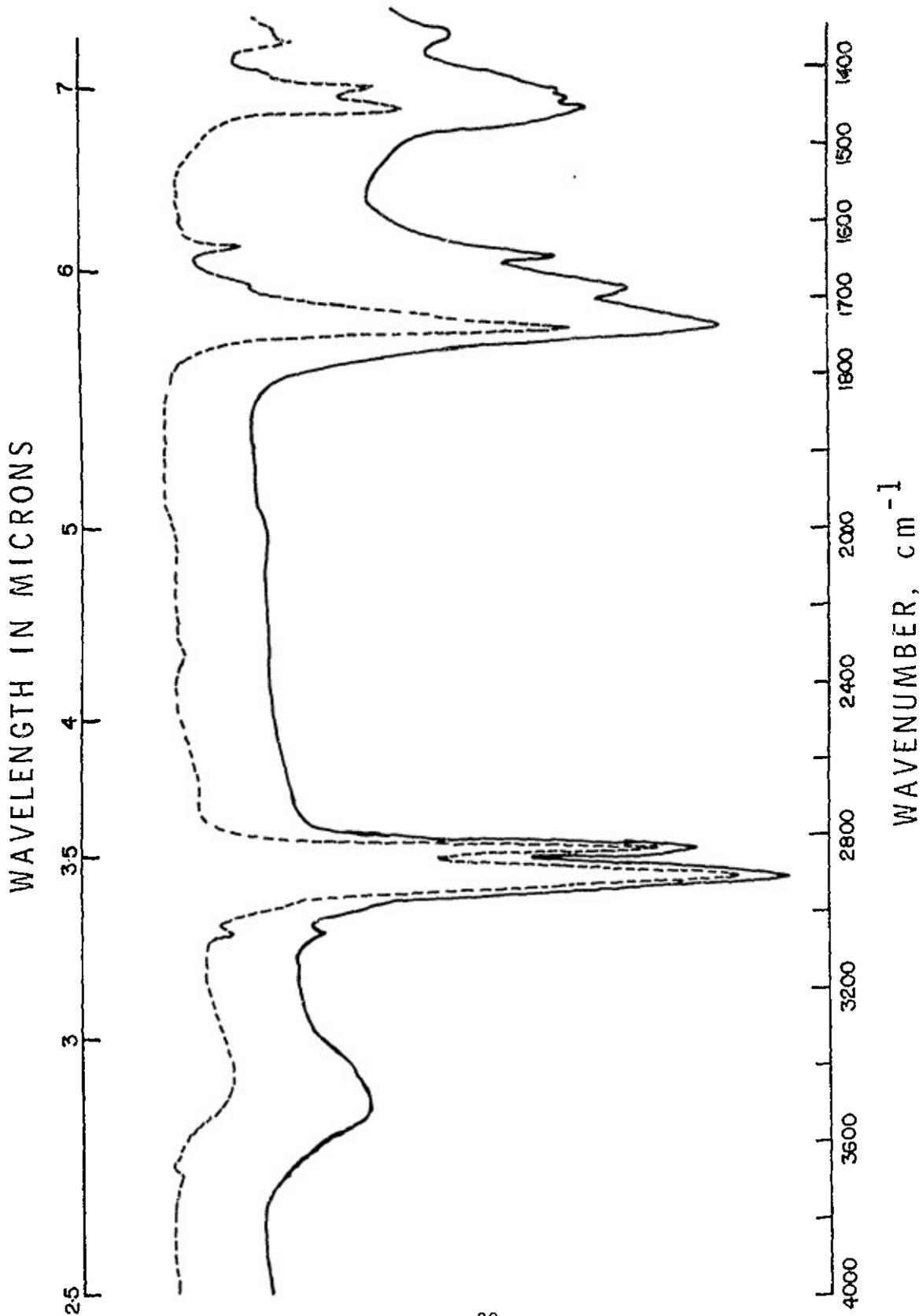


Fig. XII. IR spectrum of Schiff base formed from PE and 10-undecene-1-al (solid line).  
Spectrum for product reduced with NaBH<sub>4</sub> (broken line).

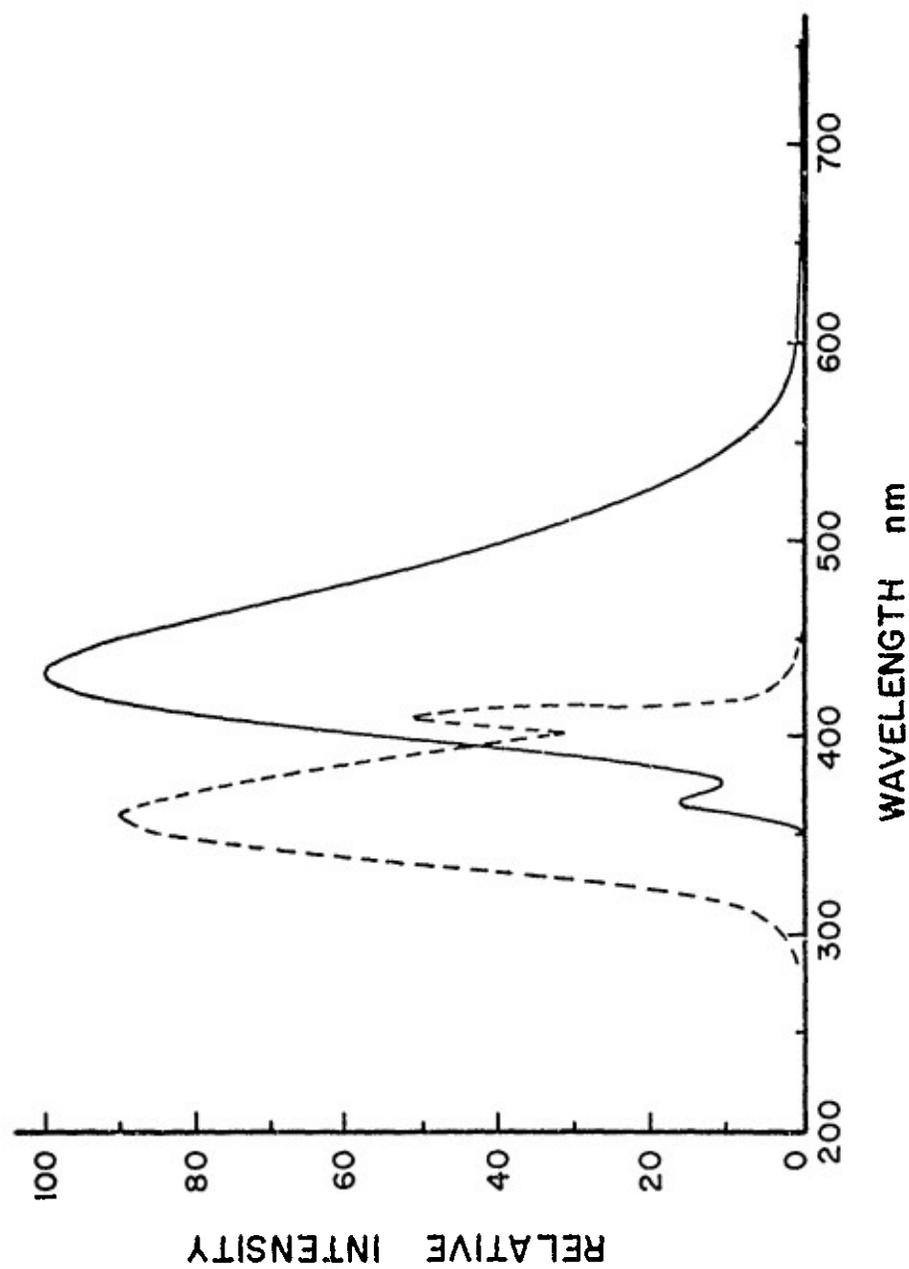


Fig. XIII. Fluorescence spectra of Schiff base formed from PE and 10-undecene-1-al.  
(Excitation - broken line; emission - solid line.)

Table 7. Reaction Products of Saturated Aldehydes and Phosphatidyl Ethanolamine\*.

System Components	Schiff Base	Unreacted PE	Non-polymeric Reaction Products	Methyl Phosphatide	Polymers
1. Phosphatidyl Ethanolamine + Nonanal	48.0	17.50	23.6	6.1	4.8
2. Hydrogenated Phosphatidyl Ethanolamine + Nonanal	46.0	19.5	19.9	4.9	9.4
3. Phosphatidyl Ethanolamine + Oleyl Aldehyde	44.0	25.0	18.0	6.0	6.1
4. Phosphatidyl Ethanolamine + Undecylenic Aldehyde	44.0	21.7	19.3	5.0	9.0
5. Phosphatidyl Ethanolamine + Undecylenic Aldehyde + Myoglobin	21.9	18.2	43.2	5.4	10.4

\*Stored at RH 14% and ambient temperature for 30 days.

#### IV. Reaction of Phosphatidyl Ethanolamine with Unsaturated Aldehydes

Oxidation of polyunsaturated fatty acids produces both saturated and unsaturated carbonyls. These can react with phosphatidyl ethanolamine present in a food system. The previous section reported studies on the reaction of PE with saturated aldehydes or those that behave similarly. This section reports reactions of PE with unsaturated aldehydes.

A. Reaction of Phosphatidyl Ethanolamine with 2 Ene als. The carbonyl compound used was 2-hexene-1-al. As previously described, the systems were prepared with pure hydrogenated and non-hydrogenated phosphatidyl ethanolamine and 2-hexene-1-al in cellulose emulsion with boric acid buffer at pH 6. A portion of the freeze-dried sample was stored at ambient temperature for 30 days at RH 14% and the other portion was heated at 50C for 10 hrs. to enhance browning. After

appropriate reaction time, the product was extracted with 80% ethanol and the residue was re-extracted with chloroform. The alcohol solubles contained both phosphorus and nitrogen and the chloroform solubles contained mostly carbonyls and a small quantity of PE. By spotting the chloroform solubles on a silica gel G plate and developing with petroleum ether:diethyl ether:acetic acid 90:10:1 by volume, spots were obtained which were purple with 2,4-DNP spray, indicating the presence of carbonyl compounds.

The alcohol solubles were first separated on an ordinary silica gel G plate by developing with hexane:chloroform:methanol 90:15:5 by volume to eliminate the carbonyl fraction. The phosphorus containing fractions were extracted from silica gel with chloroform:methanol 1:1. The extracts were spotted on a basic silica gel G plate and developed with chloroform:methanol:water 65:25:4 by volume. Various phosphorus containing fractions were isolated from the plate and the percentage of each product formed (based on phosphorus analysis) is shown in Table 8.

Table 8. Reaction Products of 2 Hexene-1-al and Phosphatidyl Ethanolamine.

System Components	Schiff Base	Non-Polymeric Reaction Product	Unreacted PE	Polymers
<u>1. Browning by heating at 50C for 10 hrs:</u>				
(a) Hydrogenated Phosphatidyl Ethanolamine + 2-Hexene-1-al	41.50	15.02	29.30	14.20
(b) Phosphatidyl Ethanolamine + 2-Hexene-1-al	40.91	19.50	29.79	9.80
<u>2. Storage at RH 14% for 30 days at ambient temperature:</u>				
(a) Hydrogenated Phosphatidyl Ethanolamine + 2-Hexene-1-al	43.65	18.26	24.60	13.49
(b) Phosphatidyl Ethanolamine + 2-Hexene-1-al	47.94	21.36	23.05	8.64

Isolation of Schiff base from Sephadex LH 20 column: The sample which was stored for 30 days at RH 14% at ambient temperature was extracted with chloroform:methanol 1:1 and the extract was applied to a Sephadex LH 20 column. After all the sample was absorbed on the column, the column was eluted with ethanol. The Schiff base fraction came out after eluting 75ml and this was followed by unreacted phosphatidyl ethanolamine.

The Schiff base fraction was further purified on a basic silica gel G plate and developed with chloroform:methanol:water 65:25:4 by volume. The IR spectrum of the Schiff base had an absorption band for C = N (substituted imine) at  $1582\text{cm}^{-1}$  and some absorption at  $1620\text{cm}^{-1}$  for C = C adjacent to the substituted imine group (Fig. XIV). The ultraviolet spectrum had absorption at 232 and 275  $\mu$  (Fig. XV). Maximum fluorescence occurred at 475  $\mu$  at an excitation of 400  $\mu$  (Fig. XVI). The formation of a Schiff base was confirmed by reducing with sodium borohydride; C = N to  $-\text{CH}_2 - \text{NH}-$ . The reduced product lost its IR absorption for C = N (Fig. XIV) and typical UV and fluorescence spectra.

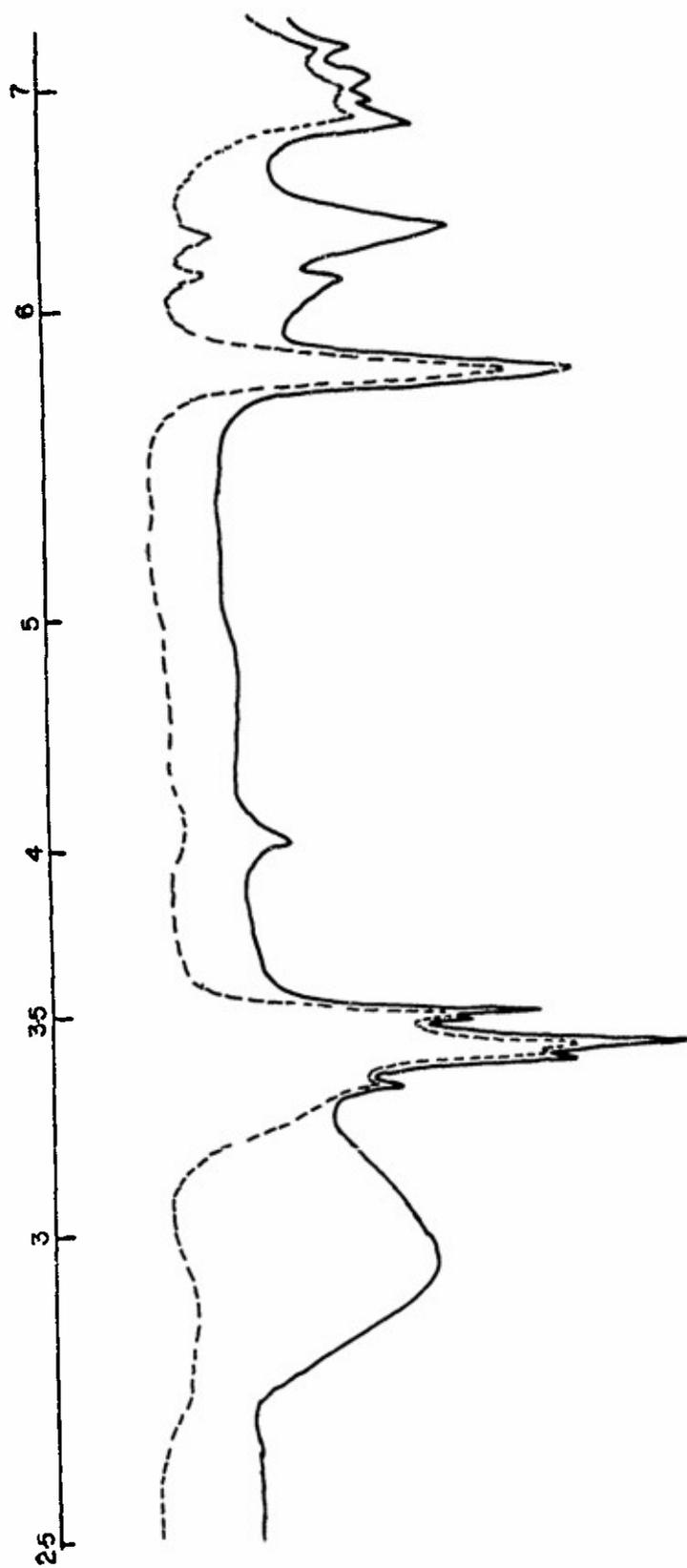
The following conclusions were made from this study:

(a) No major difference was observed in the reaction of hydrogenated and non-hydrogenated phosphatidyl ethanolamine in the percentage of Schiff base formed. The presence of a greater carbonyl fraction was observed in non-hydrogenated PE as a consequence of oxidation of the polyunsaturated fatty acids present in PE.

(b) More carbonyls were observed during browning at elevated temperatures when compared with samples stored at ambient temperature and constant RH. The non-polymeric reaction products and oxypolymers occurred in greater amounts at elevated temperatures.

(c) Formation of methyl phosphatidate was not observed during lipid browning when unsaturated aldehydes such as 2-hexene-1-al were used. The absence of scission products may be explained as follows. The strain in the Schiff base was neutralized by the shift of double bond in the product which then stabilized the Schiff base. The mechanism of Schiff base formation with PE and 2-hexene-1-al is shown below.

WAVELENGTH IN MICRONS



WAVENUMBER,  $\text{cm}^{-1}$

Fig. XIV. IR spectra for Schiff base formed from PE and 2-hexene-1-al. (Solid line - isolated fraction; broken line - product reduced with  $\text{NaBH}_4$ .)

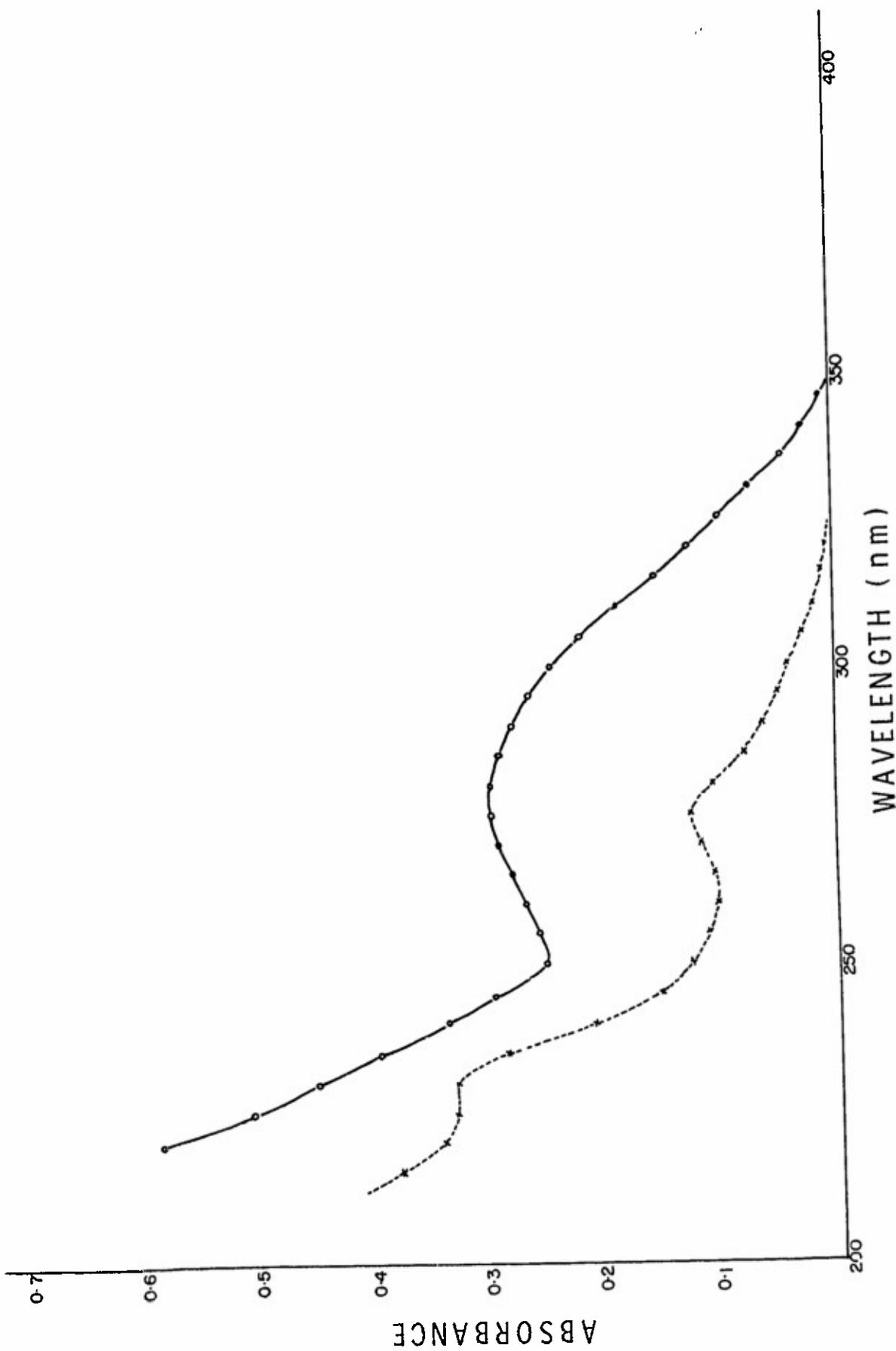
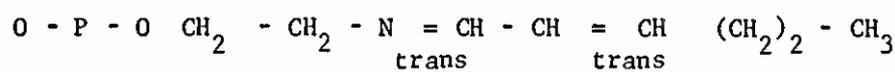
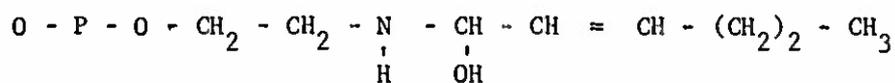
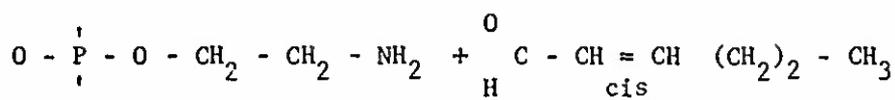


Fig. XV. UV spectra for Schiff bases formed from PE with unsaturated aldehydes.  
 (x - 2-hexene-1-al; circles - 2,4-hexadienal.)



Diene Conjugated Schiff Base

B. Reaction of Phosphatidyl Ethanolamine with 2,4 Dienals. The carbonyl compound used was 2,4-hexadienal. Model systems were prepared in cellulose emulsion with borate buffer at pH 6 using both hydrogenated and non-hydrogenated PE and 2,4-hexadienal with and without myoglobin. After freeze-drying to a moisture level of 2.5%, the products were stored at ambient temperature for 30 days at RH 14%.

The systems were then extracted with chloroform:methanol 1:1 by volume and the excess carbonyl compounds were eliminated by TLC as noted previously. The phosphorus containing band at the base was rechromatographed on a basic silica gel G coated plate and developed with chloroform:methanol:water 65:25:4 by volume. The various phosphorus containing fractions were isolated and the percentages of each product formed (based on phosphorus analysis) are reported in Table 9.

Less Schiff base was formed with 2,4-dienals than with other aldehydes. One possible reason may be that some of the 2,4-dienal itself was oxidized before the amine-carbonyl reaction. This would limit the availability of aldehyde for the reaction. No formation of methyl phosphatidate was observed when 2,4-dienals were used in the system. When myoglobin was present in the model system, it acted as a prooxidant so that Schiff base formation was minimal and polymer and other non-polymeric reaction products were increased.

Table 9. Reaction Products of 2,4-Hexadienal and Phosphatidyl Ethanolamine.

System Components	Schiff base	Non-Polymeric Reaction Products	Unreacted PE	Polymers
1. Hydrogenated Phosphatidyl Ethanolamine + 2,4-Hexadienal	21.50	12.75	56.05	9.70
2. Hydrogenated Phosphatidyl Ethanolamine + 2,4-Hexadienal + Myoglobin	12.59	19.81	54.10	14.5
3. Phosphatidyl Ethanolamine 2,4-Hexadienal	32.17	22.49	27.29	19.06
4. Phosphatidyl Ethanolamine + 2,4-Hexadienal + Myoglobin	19.50	32.45	19.50	28.55

Isolation of Schiff base using Sephadex LH 20 column: The chloroform:methanol extract was applied onto the Sephadex column and two 10ml portions of methanol were added to insure absorption of all the sample on the column. The column was then eluted with methanol, eluting at a flow rate of 60ml/hr. The various bands separated on the column could be observed and fractions were collected accordingly. A dark colored phosphorus containing fraction was eluted first which had no Schiff base characteristics. This was followed by Schiff base and then unreacted phosphatidyl ethanolamine. The dark colored fraction was more prominent in the sample containing myoglobin. In general, both systems had an appreciable quantity of a dark colored fraction which was probably composed of oxypolymers.

The IR spectrum of the Schiff base (Fig. XVII) had absorption bands for C = N at  $1585\text{cm}^{-1}$  and for C = C at  $1610\text{cm}^{-1}$ . A strong band at  $14.5\mu$  for a conjugated double bond system was also observed. The bands related to double bonds are  $3.3\mu$  (=CH stretching),  $6.1\mu$  (C = C stretching),  $7.2\mu$  (=CH - H planar bending) and  $14.5\mu$  (=CH non-planar bending). A sample was scanned from  $15\mu$  to  $9\mu$  in carbon disulphide. A typical spectrum was observed for a triene conjugated system with cis,trans,trans configuration having bands at  $10.4\mu$ ,

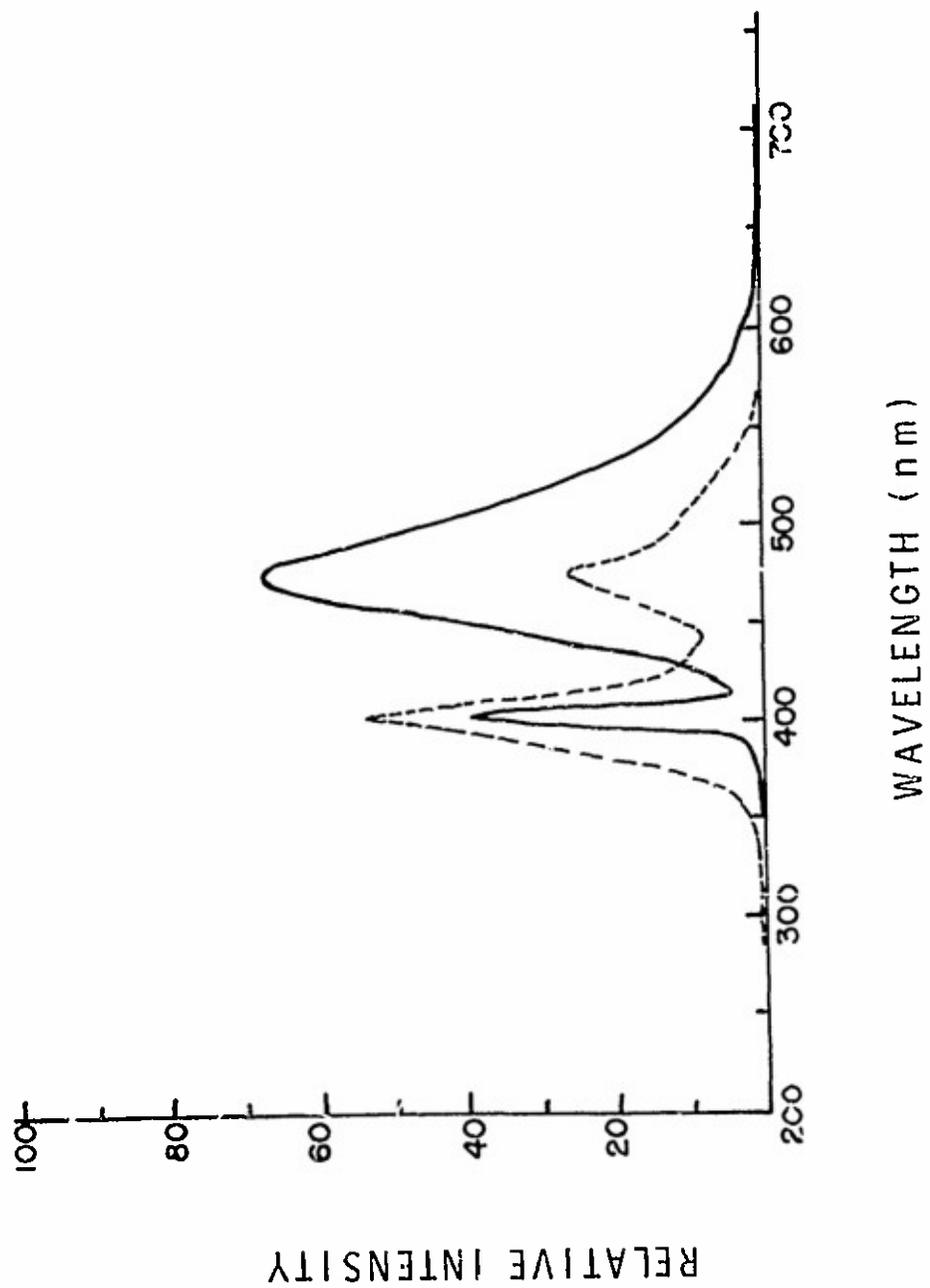


Fig. XVI. Fluorescence spectra of Schiff base formed from PE and 2-hexene-1-al. (Excitation - broken line; emission - solid line.)

WAVELENGTH IN MICRONS

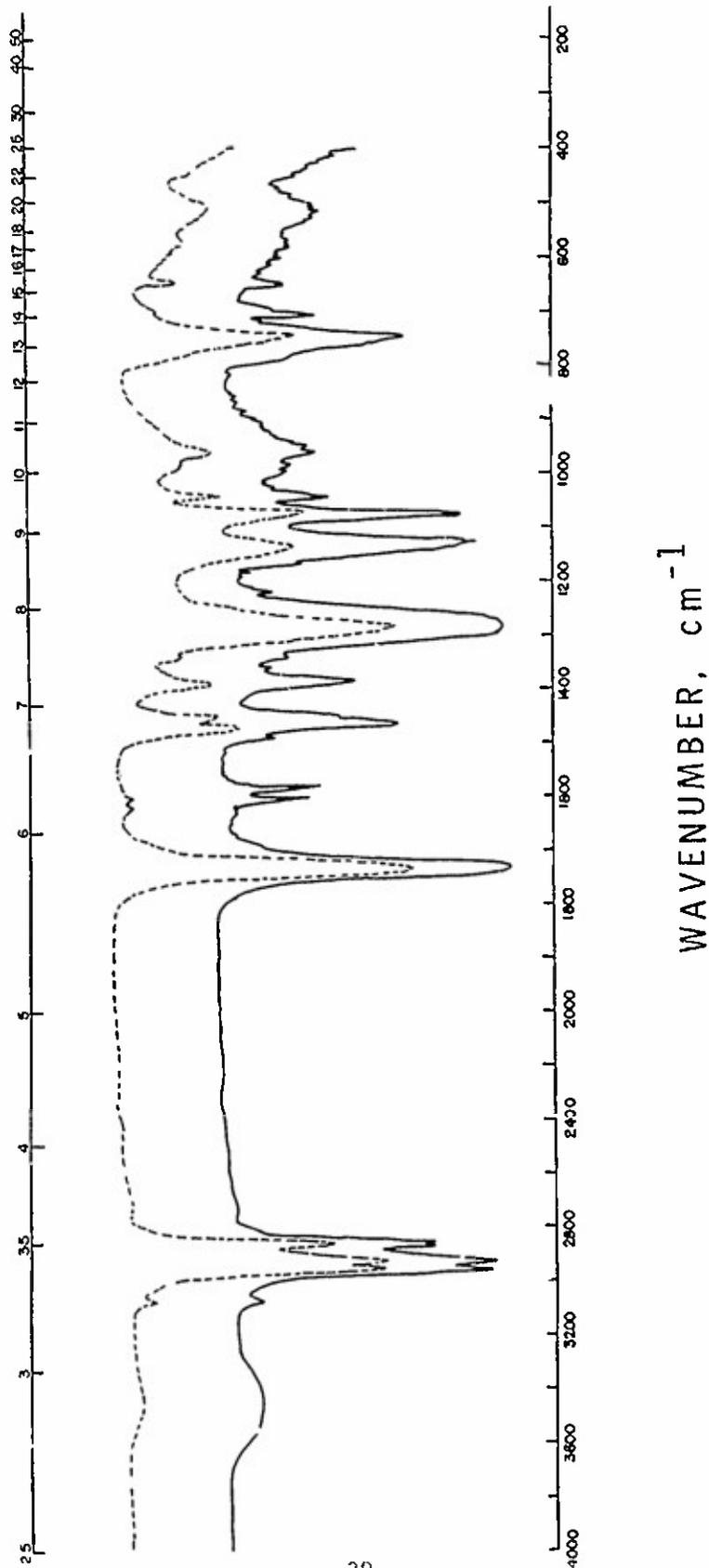
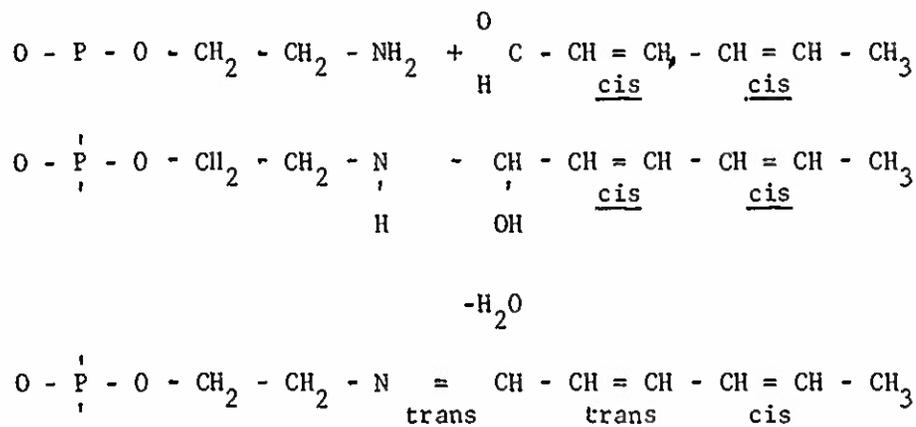


Fig. XVII. IR spectrum of Schiff base formed from PE and 2,4-hexadienal (solid line).  
Spectrum for product reduced with NaBH<sub>4</sub> (broken line).

10.8  $\mu$  and 10.9  $\mu$  (Fig. XVIII).

The ultraviolet spectrum had maximum absorbance at 280 m $\mu$ . The broad absorption band apparently derived from the contribution of both C = N and triene conjugation (Fig. XV). The Schiff base had maximum fluorescence at 455 m $\mu$  at an excitation of 375 m $\mu$  (Fig. XIX). Schiff base formation was confirmed by reducing it with sodium borohydride following which it lost its characteristic IR (Fig. XVII), UV and fluorescence spectra.

It has been shown that 2,4-dienals oxidize quite readily (21), giving breakdown products. This oxidation of dienals may limit the availability of the aldehyde for Schiff base formation and further increase the possibility of polymer formation. Myoglobin in the model systems acts as a pro-oxidant which readily oxidized the dienal, thus suppressing the carbonyl-amine interaction and increasing polymer formation. This was clearly observed from the percentage of Schiff base shown in Table 9. There is no formation of methyl phosphatidate when 2,4-dienals are involved in Schiff base formation. As noted earlier, this was observed only in the systems containing saturated aldehydes. The mechanism of Schiff base formation is postulated to be as shown below:



Triene Conjugated Imine Type Schiff Base

#### V. Reaction of Phosphatidyl Ethanolamine with Dialdehyde

The carbonyl compound used for this study was malonaldehyde. The model systems were prepared with pure hydrogenated or non-hydrogenated phosphatidyl ethanolamine and malonaldehyde in cellulose emulsion with boric acid buffer at pH 6. Samples were freeze-dried to a moisture level of 2.5% and stored at ambient temperature, RH 14% for 30 days. The product after storing was extracted with chloroform:methanol 1:1 by



Fig. XVIII. IR spectrum establishing trans, trans, cis configuration of double bonds in Schiff base formed from PE and 2,4-hexadienal.

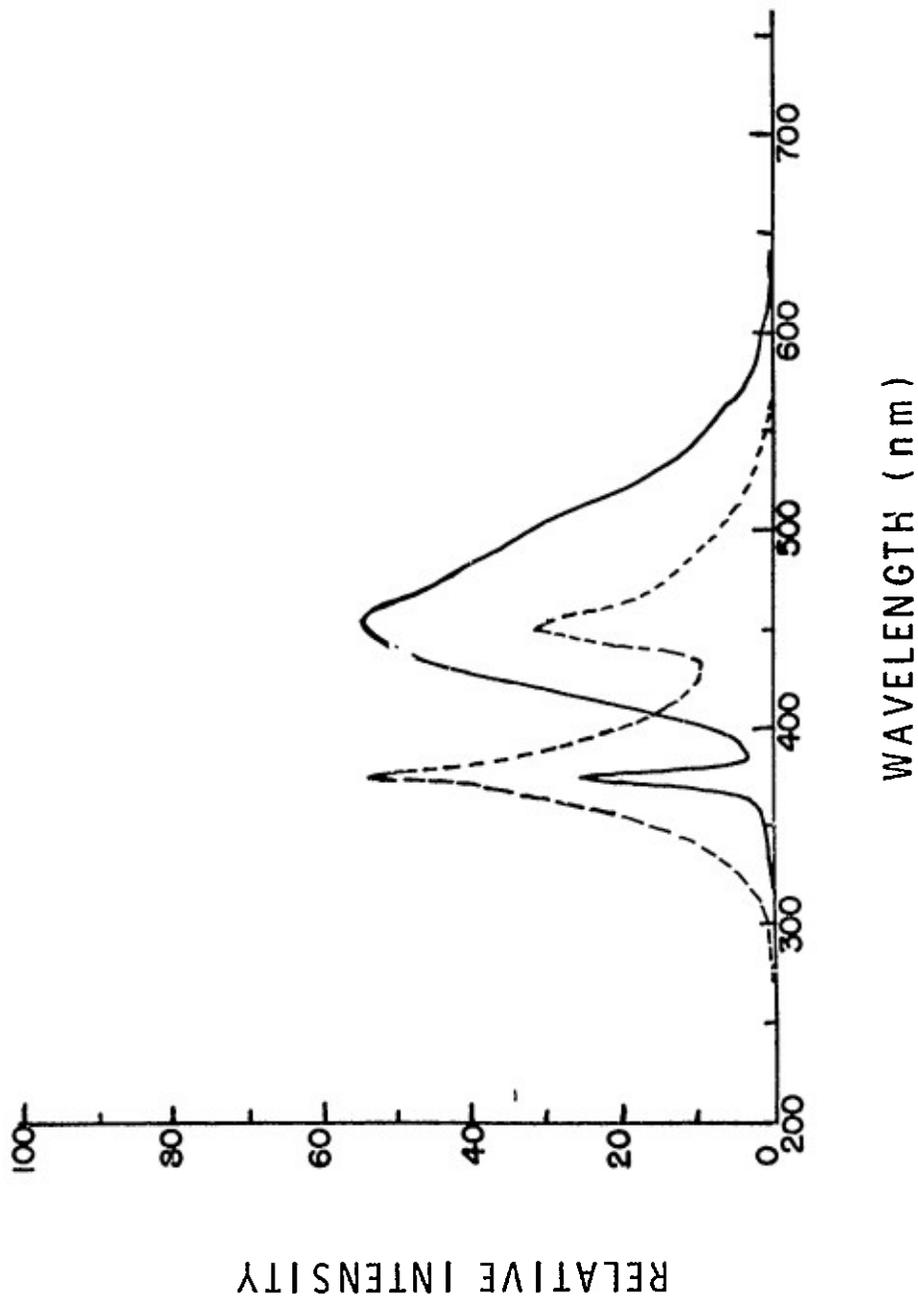


Fig. XIX. Fluorescence spectra for Schiff base formed from PE and 2,4-hexadienal.  
(Excitation - broken line; emission - solid line.)

volume. The extract was concentrated under an atmosphere of nitrogen. A portion of the concentrated extract was spotted on a silica gel G plate and developed with hexane:chloroform:methanol 90:5:5 by volume to remove all the excess carbonyl compounds. The phosphorus containing band at the base was respotted on a basic silica gel G plate and developed with chloroform:methanol:water 65:25:4 by volume. Various phosphorus containing fractions were isolated and the percentages of each product formed are reported in Table 10.

Reaction with malonaldehyde to form a Schiff base, probably of the enamine-imine type, apparently was regulated and stabilized by the availability of the two functional carbonyl groups in one compound, yielding a greater percentage of Schiff base when compared with other aldehydes. No formation of methyl phosphatidate was observed.

Table 10. Reaction Products of Malonaldehyde with Phosphatidyl Ethanolamine.

System Components	Schiff base	Non-Polymeric Reaction Products	Unreacted PE	Polymers
1. Hydrogenated Phosphatidyl Ethanolamine + Malonaldehyde	80.20	7.20	7.00	5.2
2. Phosphatidyl Ethanolamine + Malonaldehyde	76.4	10.40	8.6	4.8

Isolation of Schiff base using Sephadex LH 20 column: The remaining portion of the chloroform:methanol extract was taken to dryness under nitrogen and then dissolved in a minimum amount of methanol and applied onto the Sephadex column. After being absorbed into the column, two 10ml portions of methanol were added so that all the sample was absorbed onto the column. The column was eluted with methanol, eluting at a flow rate of 60ml/hr. The various bands separated on the column could be observed and fractions were collected accordingly. A dark colored product was eluted in the first 100ml and it had no Schiff base characteristics. This may be a polymer formed during oxidation. The fraction eluted after 120ml had maximum absorbance in the UV and strong fluorescence. This was followed by unreacted phosphatidyl ethanolamine.

The Schiff base isolated was further purified on a basic silica gel G plate. The IR spectrum of the Schiff base had absorption bands at  $1675\text{cm}^{-1}$  for C = N and at  $1628\text{cm}^{-1}$  for C = C adjacent to a C = N (Fig. XX). It had maximum fluorescence at  $455\text{ m}\mu$  at an excitation of  $340$  and  $410\text{ m}\mu$  (Fig. XXI) and maximum ultraviolet absorption at  $232$ ,  $265$ ,  $290$  and  $380\text{ m}\mu$  (Fig. XXII). To confirm Schiff base, it was reduced with sodium borohydride [HC = NH to (CH<sub>2</sub> · NH)]. The reduced product did not fluoresce or absorb in the ultraviolet and no C = N absorption was observed in the IR spectrum (Fig. XX).

The mechanism of Schiff base formation and the sodium borohydride reduction step is shown on the following page.

Table 11 summarized the various phosphorus containing fractions formed and percentage of each from reaction of unsaturated aldehydes with phosphatidyl ethanolamine in a model system. Table 12 summarizes the characteristics of Schiff bases isolated from systems produced from both saturated and unsaturated aldehyde reactions with phosphatidyl ethanolamine.

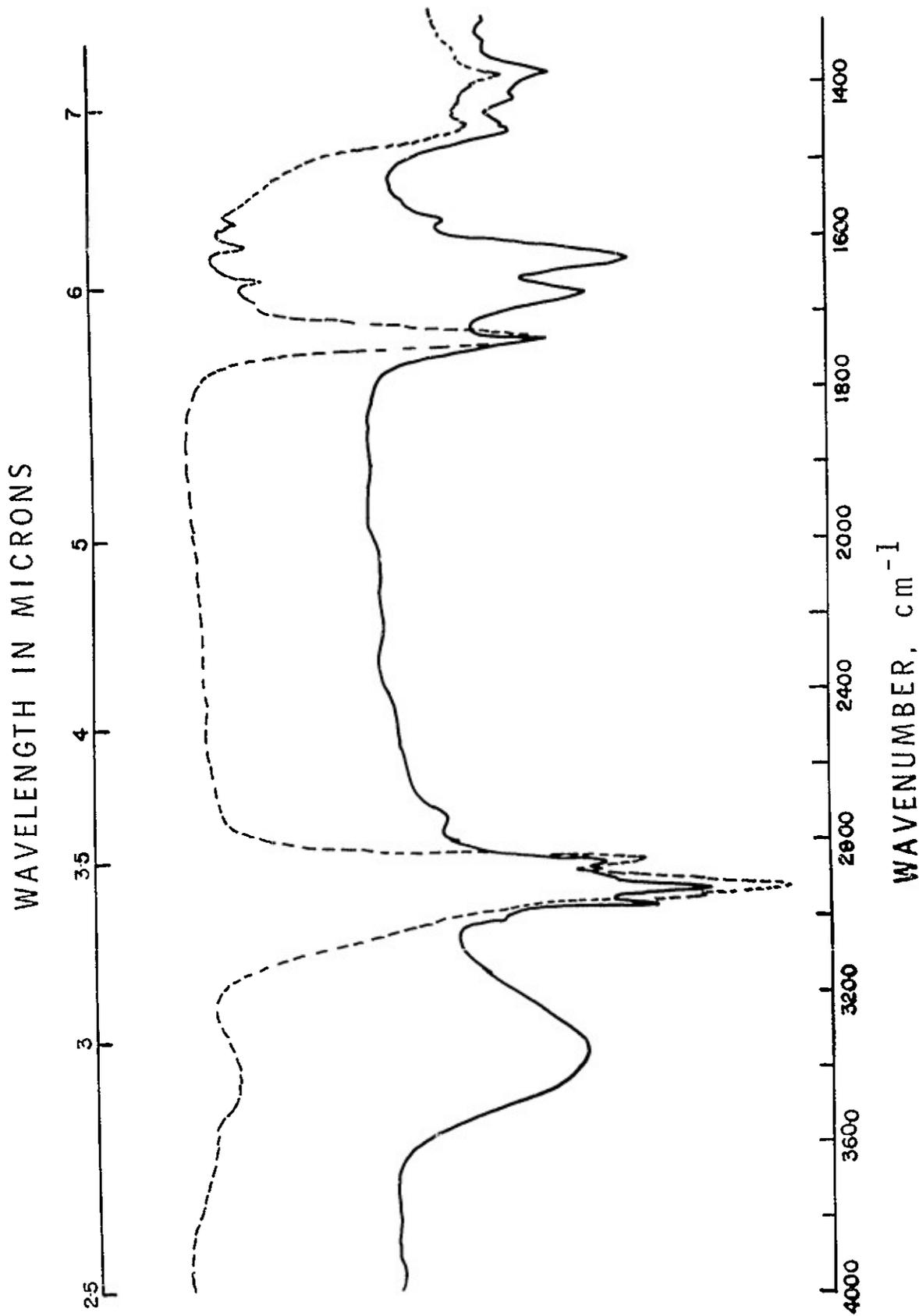


Fig. XX. IR spectrum for Schiff base formed from PE and malonaldehyde (solid line).  
Spectrum for NaBH<sub>4</sub> reduction product (broken line).

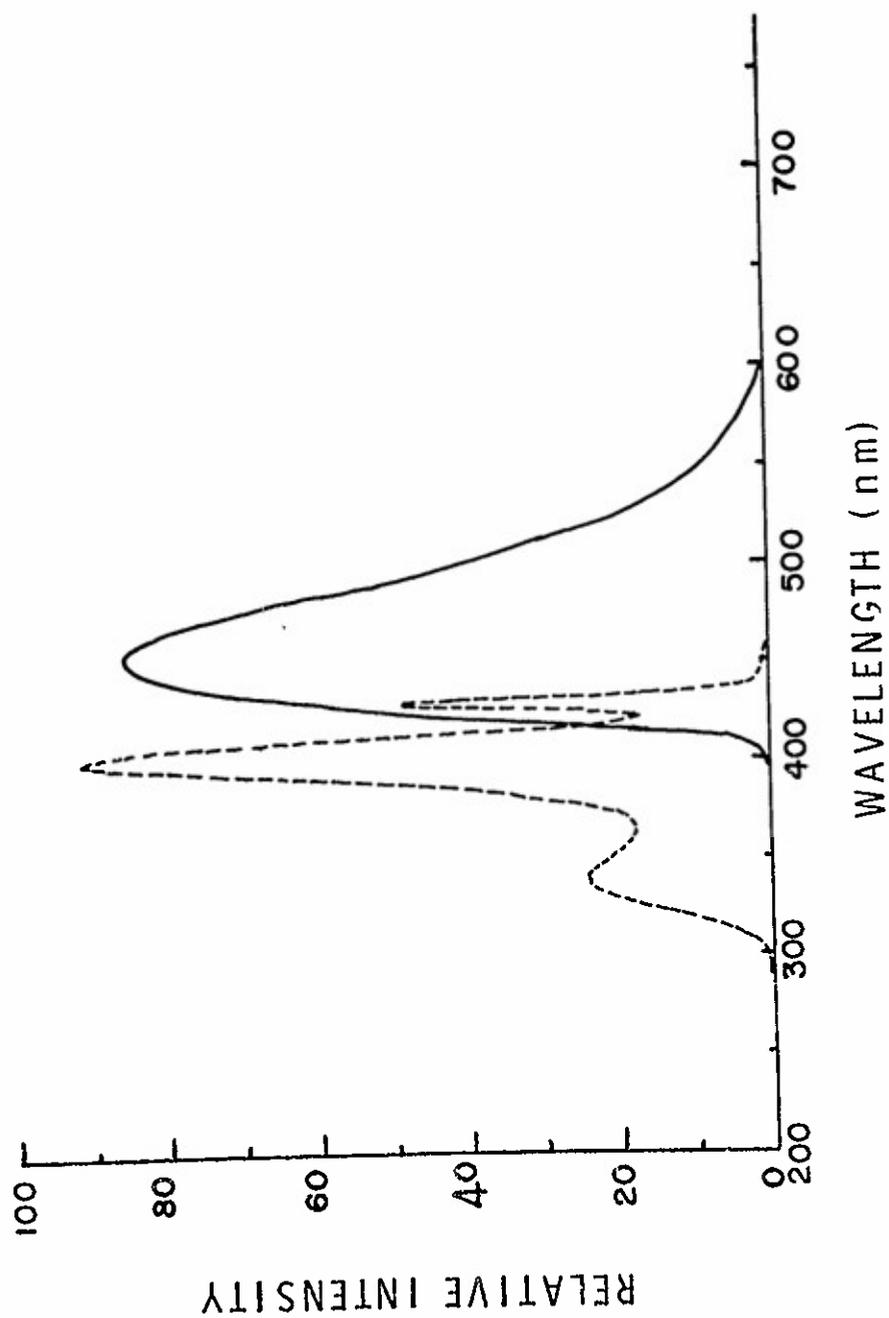


Fig. XXI. Fluorescence spectra for Schiff base formed from PE and malonaldehyde. (Excitation - broken line; emission - solid line.)

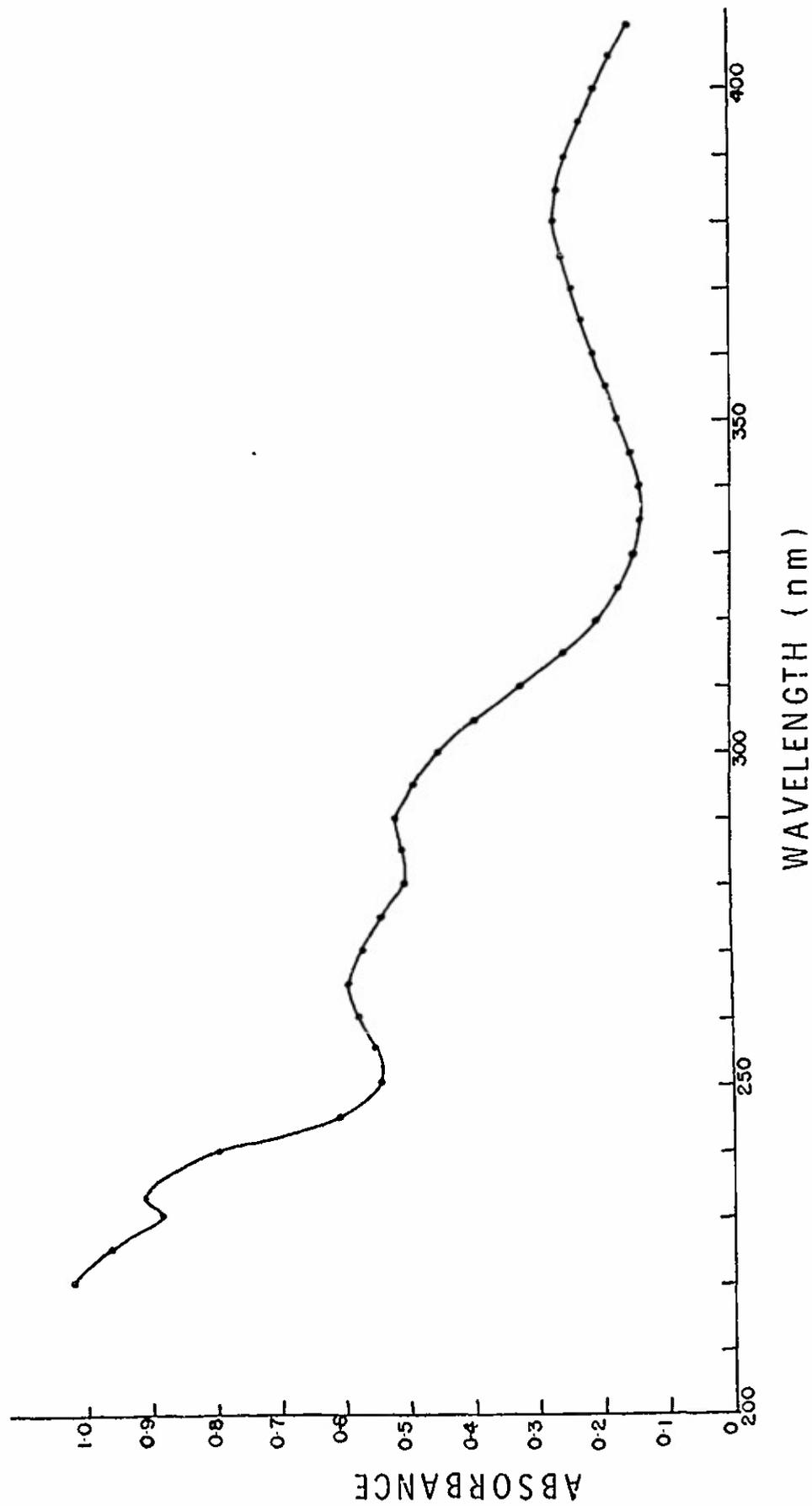
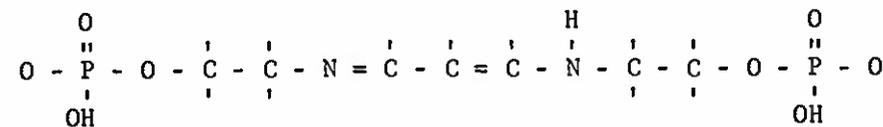
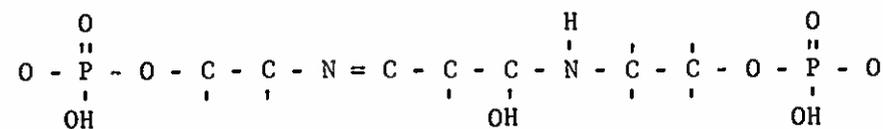
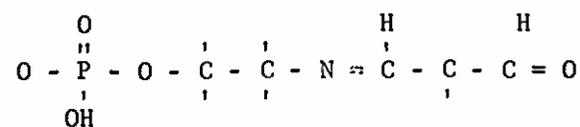
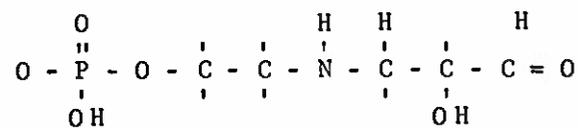
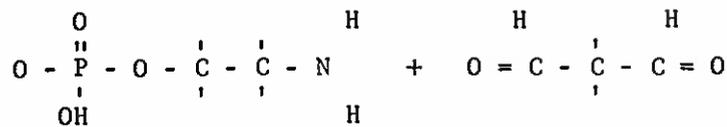
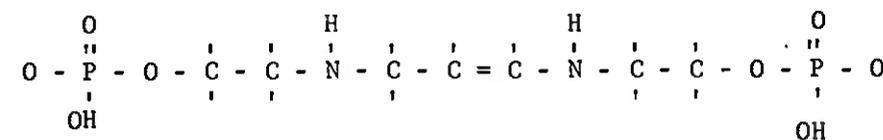
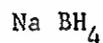


Fig. XXII. UV spectrum for Schiff base formed from PE and malonaldehyde.



Diene Conjugated Enamine - Imine Compound  
(Schiff Base)



REDUCED PRODUCT

Table 11. Reaction Products of Unsaturated Aldehydes and Phosphatidyl Ethanolamine\*.

System Components	Schiff base	Unreacted Phosphatidyl Ethanolamine	Non-Polymeric Reaction Products	Poly-mers
1 (a) Phosphatidyl Ethanolamine + 2-Hexene-1-al	47.94	23.05	21.36	8.64
(b) Hydrogenated PE + 2-Hexene-1-al	43.65	24.60	18.26	13.49
2 (a) Phosphatidyl Ethanolamine + 2,4-Hexadienal	32.17	27.29	22.49	19.06
(b) Hydrogenated PE + 2,4-Hexadienal	21.50	56.05	12.75	9.70
(c) PE + 2,4-Hexadienal + Myoglobin	19.50	19.50	32.45	28.55
3 (a) Phosphatidyl Ethanolamine + Malonaldehyde	76.40	8.60	10.40	4.80
(b) Hydrogenated PE + Malonaldehyde	80.20	7.00	7.20	5.20

\*Stored 30 days at RH 14% and ambient temperature.

Table 12. Schiff Base Characteristics.

Components Involved in Schiff Base Formation	Infrared Absorption (cm <sup>-1</sup> )		Ultraviolet Absorption Maxima (mμ)	Fluorescence Spectra (mμ)	
	C = N	C = C		Excitation	Emission
1. Phosphatidyl Ethanolamine + Nonanal	1620	-	280	360	425
2. PE + Oleyl Aldehyde	1565	-	280	365	430
3. PE + Undecylenic Aldehyde	1685	-	275	370	445
4. PE + 2-Hexene-1-al	1582	1620	232, 275	400	475
5. PE + 2,4-Hexadienal	1585	1610	280 (broad)	375	455
6. PE + Malonaldehyde	1675	1628	232, 265, 290, 380	340, 410	455
7. Phosphatidyl Ethanolamine + Nonanal	1625	1685 (C-O-C)	240, 280	365	440
8. Hydrogenated Phosphatidyl Ethanolamine + D-Glucose	1600	-	280 (broad)	350	475

## VI. Role of Phosphatidal Ethanolamine in Lipid Browning

In this section, model freeze-dried systems were prepared using plasmalogen PE and carbonyl compounds. The stability of the ether moiety was studied and the various reaction products formed during the browning reactions were investigated.

A. Reaction of Phosphatidal Ethanolamine with Nonanal. Model systems were prepared with pure phosphatidal ethanolamine with and without added aldehydes and myoglobin in cellulose matrix with borate buffer at pH 6.0. The emulsion was freeze-dried to a moisture level of 2.5% and stored at RH 14% and ambient temperature for 30 days. After storing, the components were extracted from the cellulose matrix with chloroform:methanol 1:1 by volume. The extract was concentrated and spotted on a silica gel G plate and developed with hexane:chloroform:methanol (90:5:5) by volume. The carbonyl fraction moved to the solvent front and the phosphorus containing fraction remained at the base. The band which moved to the solvent front was isolated and respotted on a silica gel G coated plate and developed with petroleum ether:diethyl ether:acetic acid (90:10:1) and sprayed with 2,4-DNP and a base which gave positive tests for carbonyl compounds. The phosphorus containing fraction at the base was isolated and respotted on a basic silica gel G plate and developed with chloroform:methanol:water (65:25:4) by volume. The various phosphorus containing fractions were isolated and the percentages of each product formed (based on phosphorus analysis) is shown in Table 13.

Browning reaction with phosphatidal ethanolamine and nonanal shows that aldehyde moieties of the plasmalogen are hydrolyzed off giving a lyso fraction, which reacts with the aldehyde from its own parent compound or with others formed in or added to the system forming a lyso Schiff base(s). Table 13 shows that the presence of myoglobin enhances polymer formation, increases the lyso fraction and decreases the Schiff base formation. Formation of methyl phosphatide was observed similar to the observations made when PE reacted with saturated aldehydes. This would be expected if the carbonyls involved came from the plasmalogen since the aldehyde moieties derived from these are usually long chain saturated or non-2-enal types of unsaturated aldehydes.

Isolation of Schiff base using Sephadex LH 20 column: The concentrate of chloroform:methanol extract was dissolved in 10ml methanol and applied on the Sephadex LH 20 column. After the sample was completely absorbed on the column, two 10ml portions of methanol were added to wash all the sample onto the column. The column was eluted with methanol and 25ml fractions were collected. A Schiff base fraction (I) was eluted after 125ml and this was followed by unreacted phosphatidal ethanolamine, Schiff base fraction (II), due to reaction between lyso PE and carbonyl compound, and finally unreacted lyso PE. All the fractions isolated from the column were further purified on a basic silica gel G plate and developed with chloroform:methanol:water 65:25:4 by volume.

Schiff base fraction (I) formed by reaction of phosphatidal ethanolamine with nonanal had IR absorption for C = N at  $1625\text{cm}^{-1}$ , for C - O - C (plasmalogen moiety) at  $1685\text{cm}^{-1}$  and other usual absorption bands (Fig. XXIII). The ultraviolet spectrum had absorption at 240 and 280  $\mu$  (Fig. XXIV) and maximum fluorescence at 440  $\mu$  at an excitation of 365  $\mu$  (Fig. XXV). The Schiff base fraction (II) was formed by reaction of carbonyl compounds present in the system with lyso phosphatidyl ethanolamine obtained by hydrolysis of the vinyl ether group in the plasmalogens. The IR spectrum of the lyso-Schiff base had absorption for C = N at  $1625\text{cm}^{-1}$  and no band for C - O - C (plasmalogen moiety), confirming the hydrolysis of the vinyl ether moieties during browning reaction (Fig. XXVI). The lyso-Schiff base had maximum fluorescence at 420  $\mu$  at an excitation of 365  $\mu$  (Fig. XXVII) and ultraviolet absorption at 240 (weak) and 280  $\mu$  (Fig. XXVIII). The presence of Schiff bases was confirmed by reduction with sodium borohydride. The IR spectra of the reduced products were appropriately different as noted in Figs. XXIII and XXVI.

Studies with phosphatidal ethanolamine showed that plasmalogens are not stable during the browning reaction; the lyso PE obtained by hydrolysis of plasmalogens react with carbonyls yielding lyso-Schiff bases. The presence of myoglobin gave enhanced oxidation yielding greater polymer and less Schiff base formation.

The proposed mechanism of formation of the Schiff bases is shown on page 58.

#### B. Effect of pH on Plasmalogen Hydrolysis during Browning Reaction.

Studies with phosphatidyl ethanolamine and nonanal in a model system showed maximum Schiff base formation at pH 6.0 with lesser quantities of side products formed during browning reaction than at either higher or lower pH levels. This study is concerned with the stability of plasmalogens in browning reactions at various pH levels.

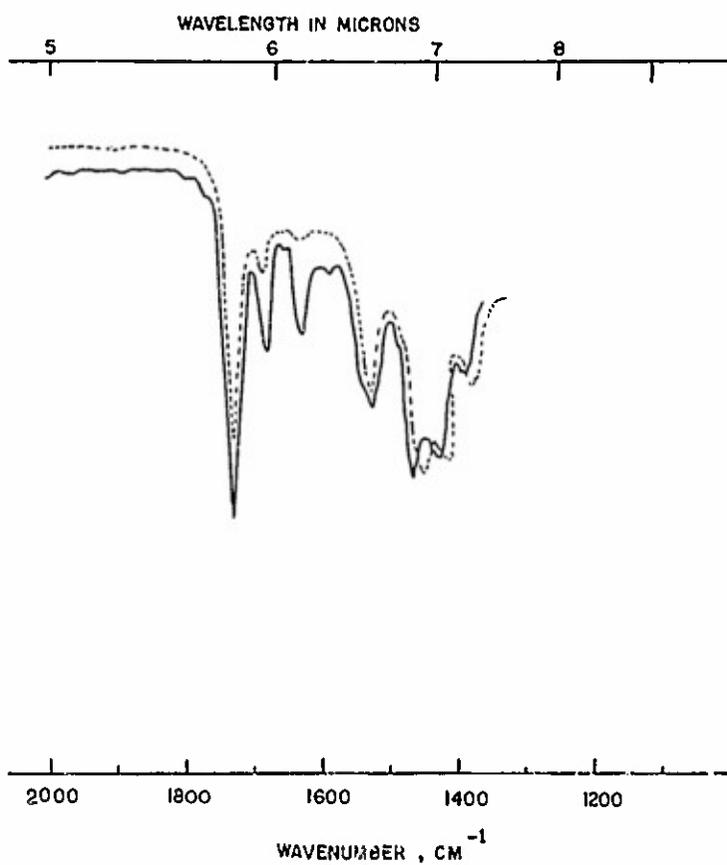


Fig. XXIII. IR spectrum of Schiff base formed from nonanal and phosphatidal ethanolamine.

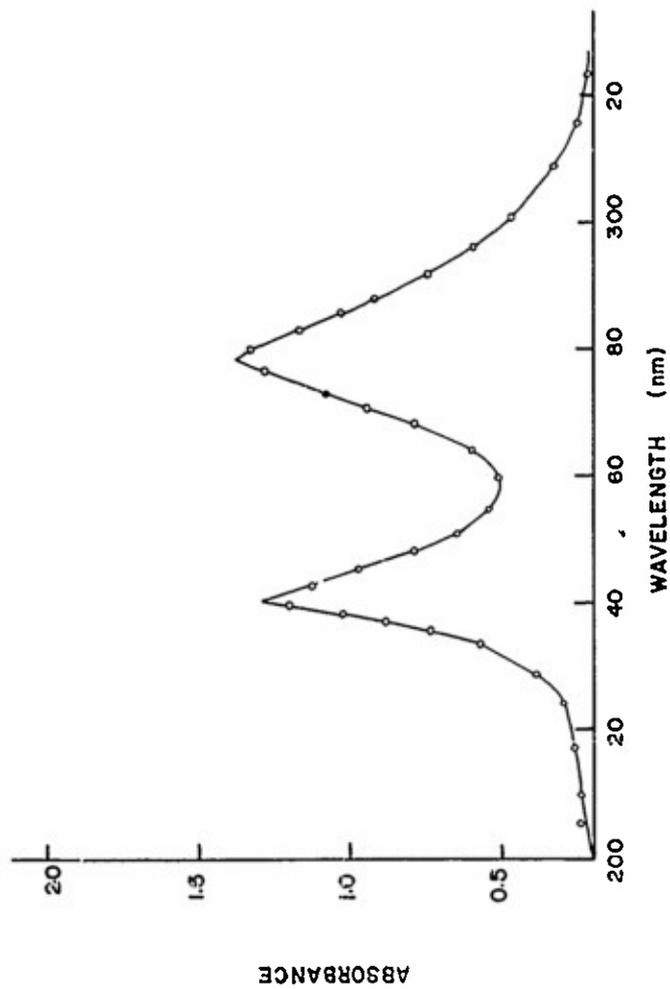


Fig. XXIV. Ultraviolet spectrum of Schiff base formed from nonanal and phosphatidal ethanalamine.

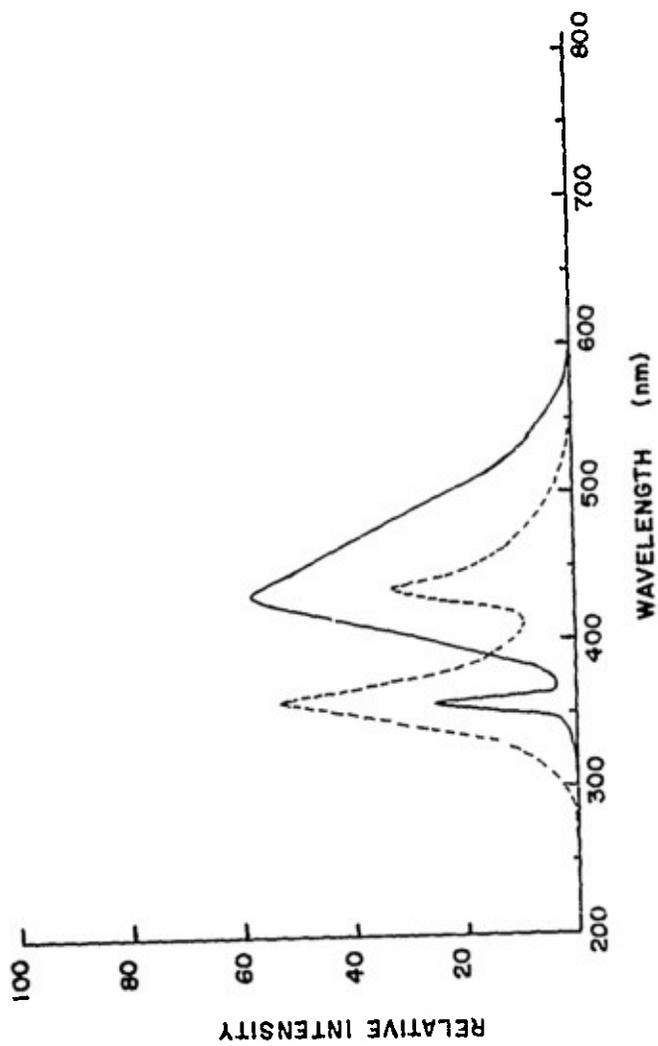


Fig. XXV. Fluorescence spectra for Schiff base formed from nonanal and phosphatidal ethanalamine.

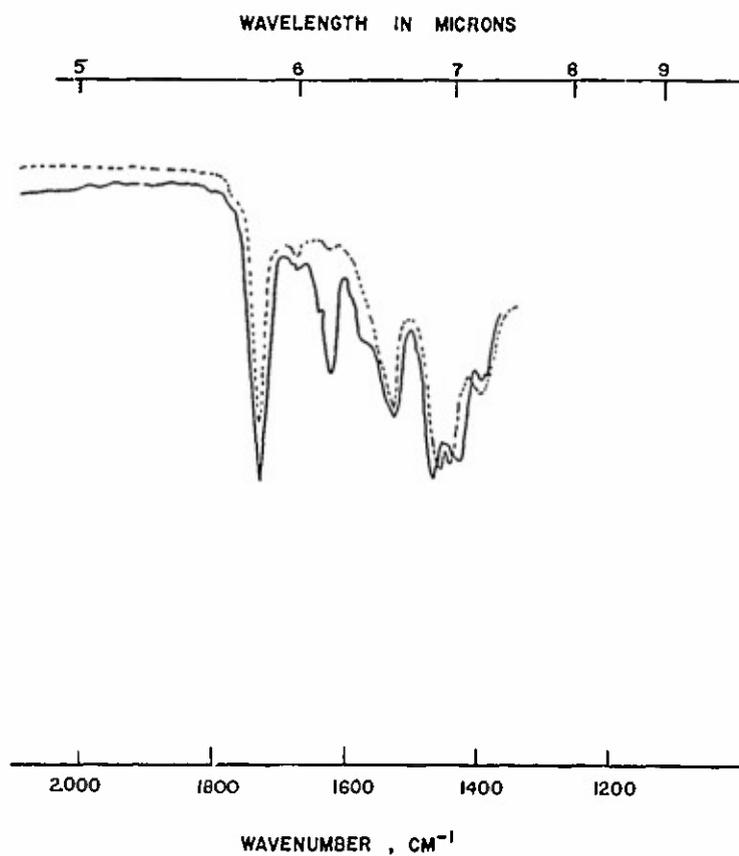


Fig. XXVI. IR spectrum of lyso-Schiff base formed from lyso-PE and nonanal.

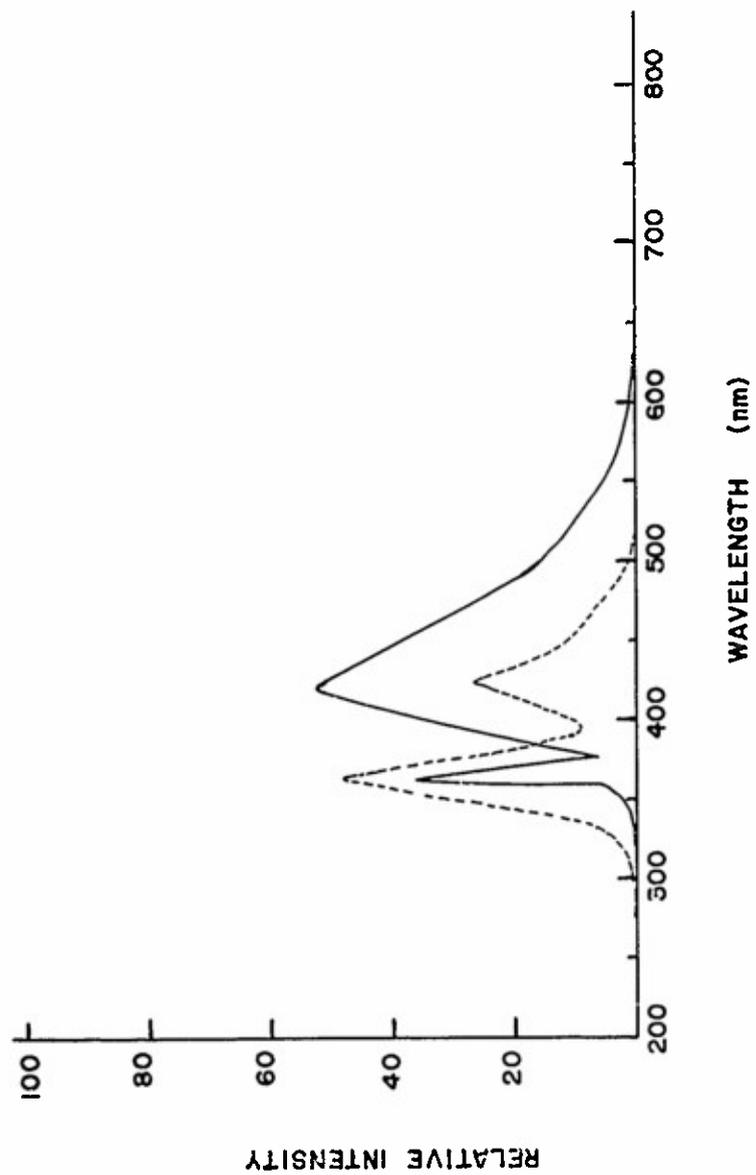


Fig. XXVII. Fluorescence spectra of lyso-Schiff base formed from lyso-PE and nonanal.

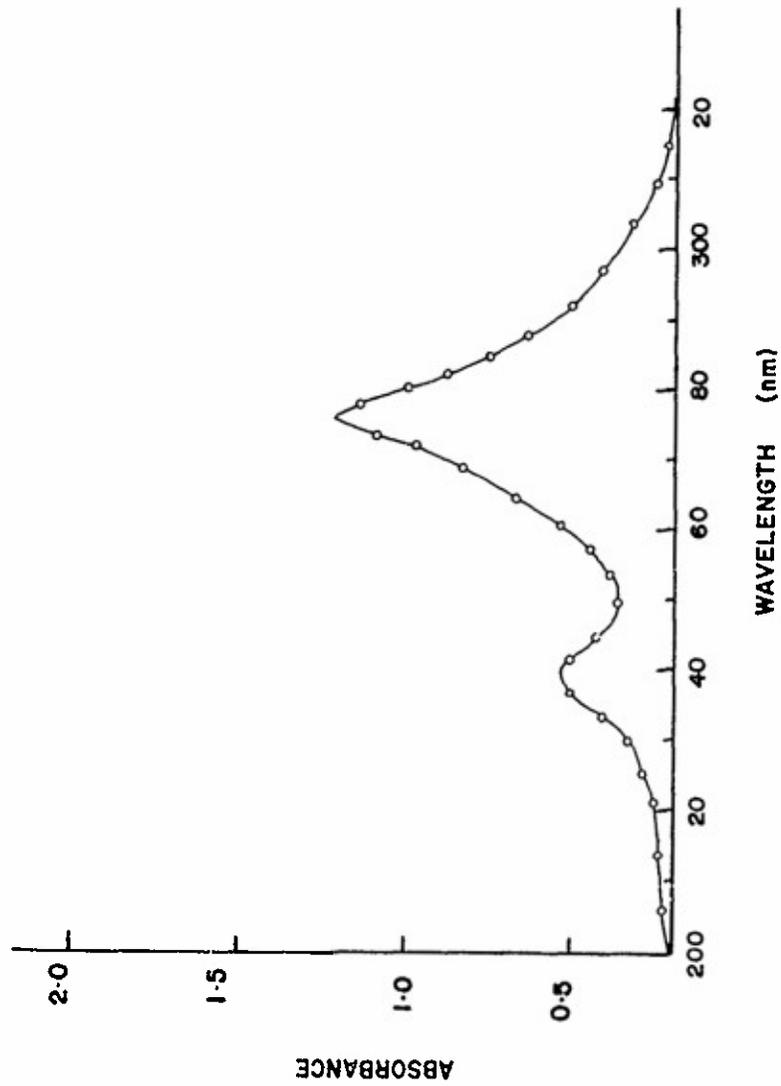
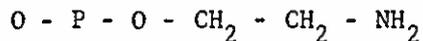


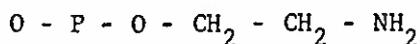
Fig. XXVIII. Ultraviolet spectrum of lyso-Schiff base formed from lyso-PE and nonanal.



phosphatidal ethanolamine

Hydrolysis

OH



Lyso PE

Reaction with  
carbonyl compound

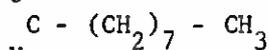
OH



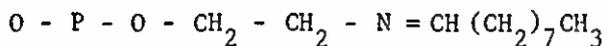
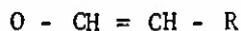
Lyso Schiff base (fraction II)

Reaction with added

O



H



Schiff base (fraction I)

\*\*\*

Model systems were prepared in cellulose matrix with borate buffer at pH 5, 6 and 8, using phosphatidal ethanolamine with and without nonanal at all three pH levels. After freeze-drying to a moisture level of 2.5%, they were stored at 14% RH and ambient temperature for 30 days. The reaction products were extracted with chloroform:methanol 1:1 by volume. The extract was concentrated and excess carbonyl eliminated by TLC. The phosphorus containing fractions were respotted on a basic silica gel G plate and developed with chloroform:methanol:water 65:25:4 by volume. The various phosphorus containing fractions were isolated from the plate and the percentages of each product formed (based on phosphorus analysis) are reported in Table 14.

The samples stored at 50C for 10 hr. to enhance browning contained more polymer. A slight excess of lyso fraction was observed at elevated temperatures at pH 8; but the samples prepared at pH 8 and stored at ambient temperature show the same percentages of lyso-fraction as the samples prepared at pH 5 and 6.

The effect of pH on phosphatidal ethanolamine participation in the browning reaction is similar in some respects to the effect noted when phosphatidyl ethanolamine reacted. Similarities include a greater production of Schiff base at pH 6, a greater quantity of unreacted amino component at pH 5 and a greater polymer formation at pH 8. These relations held when only phosphatidal ethanolamine was used in the system and also when competitive carbonyl functions participated through added nonanal.

When only the phosphatidal form was used in a system, pH had no apparent effect on formation and/or presence of lyso-PE. However, when the phosphatidal form was used with added nonanal, a substantial difference in lyso-PE was measured with the greater amount at pH 5 and the least amount at pH 6. The pH effect was apparent in the measured presence of lyso-Schiff base with nonanal in the system. Oddly enough, no lyso-Schiff base was found when added carbonyl was not in the system; although the presence of approximately 30% lyso-PE was shown in each pH system when plasmalogen alone was used. The Schiff base production in these same systems was not great. Lyso-Schiff bases are thus markedly less readily formed than Schiff bases even when availability of carbonyl reactants is not limiting as they may be when only plasmalogens are present.

C. Oxidative Stability of Vinyl Group. Some question has existed about whether the unsaturated linkage in the vinyl ether group in plasmalogen (phosphatidal ethanolamine) may react with oxygen to give measurable oxygen uptake and/or to provide oxidation products in oxidizing lipid systems.

To study the oxidative stability of vinyl group present in the plasmalogens (phosphatidal ethanolamine), a saturated plasmalogen PC was prepared. Since hydrogenation of a plasmalogen reduces the vinyl ether group, it was necessary to synthesize a saturated phospholipid having plasmalogen moieties intact. Synthesis of a saturated phosphatidal choline is reported in the section on Ancillary Studies.

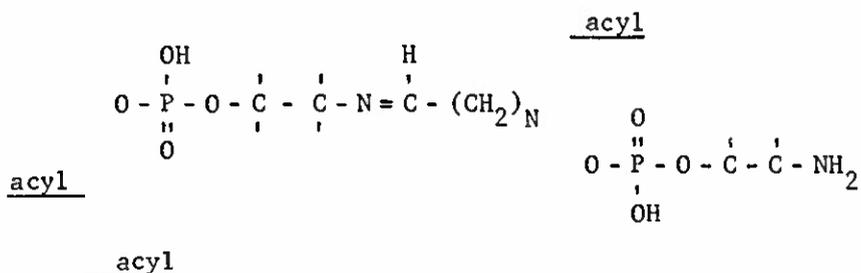
Oxygen uptake of the saturated phosphatidal choline was measured both in solution and a freeze-dried system to determine the relative susceptibility of the plasmalogen group to oxidation. For the dry systems, a cellulose emulsion was prepared in borate buffer at pH 6 using saturated phosphatidal choline and freeze-dried to a moisture level of 2.5%. For the solution system, saturated phosphatidal choline

was dissolved in 10% chloroform in methanol. The samples were placed in the flask of a Gilson Respirometer containing KOH pellets in the center well to absorb liberated carbon dioxide.

No oxygen uptake was observed in 60 hrs. at 24C indicating no apparent oxidation of the vinyl group in the plasmalogens.

### VII. Investigation of Polymer Formation

When only PE (or other class of phospholipid with a free -NH<sub>2</sub> group) was present in a model system during browning, nearly 40-45% polymers were found. Among the polymers that may be found may be those from reaction of PE and intact glyceride-carbonyl produced by oxidation and scission of the polyunsaturated fatty acids which occur chiefly at the β-position of PE. A typical molecule may be:



Other polymer molecules may result from reactions at other sites, between other functional units or by proliferation of reactions forming the typical dimer shown.

The studies reported were aimed at resolution of the polymer nature and to establish whether the model shown above may represent those isolated.

A. Isolation of Polymers from Model System containing Phosphatidyl Ethanolamine. A model system was prepared in borate buffer at pH 6.0 in a cellulose emulsion using pure phosphatidyl ethanolamine and was incubated at 50C for 10 hr. The reaction product was extracted with chloroform:methanol (1:1) by volume. The extract was concentrated and spotted on a basic silica gel G plate and developed with chloroform:methanol:water (65:25:4) by volume and the polymer fraction at its base was isolated. This was subjected to acid and base hydrolysis. Using 2N NaOH for 24 hr. gave good hydrolysis. The saponified fraction was extracted with ether and the extract respotted on a basic silica gel G plate with chloroform:methanol:water 65:25:4 by volume. Five phosphorus containing spots were observed containing 41.6% of a fraction at the base, 21.4% of a fraction having an R<sub>f</sub> value similar to that of a lyso-PE

Schiff base, 24.5% of a fraction having an Rf value equivalent to that of lyso-PE, 10.2% of a fraction having the Rf value of PE and 2.3% of a fraction having the Rf value of methyl phosphatidate. All percentages were calculated from phosphorus analysis.

B. Preparation of  $\beta$ -Carbonyl  $\alpha,\alpha'$ -Palmitostearate. It was decided to prepare a model polymer from a glycerol-aldehyde and PE and examine its behavior under the conditions employed for separation and study used heretofore for this purpose;  $\beta$ -carbonyl  $\alpha,\alpha'$ -palmitostearate was prepared for this purpose from  $\beta$ -oleo  $\alpha,\alpha'$ -palmitostearate.

(i) Preparation of  $\beta$ -oleo  $\alpha,\alpha'$ -palmitostearate: 100g cocoa butter was dissolved in 800ml acetone and kept at 18-19C for 15 hr. The precipitate containing tri-saturates was filtered off. The filtrate was concentrated (65g), dissolved in 525ml acetone and left overnight at 3C. The filtrate was discarded and the precipitate (50g) redissolved in 360 ml acetone and stored at 3C for 12 hr. The precipitate was filtered off and stored for analysis. The filtrate was discarded. A portion of the precipitate was converted into methyl esters at low temperature (-70C) with methanolic KOH. The fatty acid composition of the isolated product was: C<sub>16:0</sub> - 21.5%, C<sub>18:0</sub> - 35.7%, C<sub>18:1</sub> - 41.4% and C<sub>18:2</sub> - 1.5%.

(ii) Ozonolysis: 5g of  $\beta$ -oleo  $\alpha,\alpha'$ -palmitostearate was dissolved in 50ml of methanol and ozone was passed into the solution at 17C. The passage of gas continued for 5 hr. until the solution became blue, indicating complete ozonolysis. The flask was flushed with nitrogen to remove unreacted ozone in solution.

(iii) Reduction of ozonide: Hydrogenolysis of the ozonide in methanol with and without pyridine gave poor results.

5g ozonide was dissolved in 150ml glacial acetic acid and warmed to 30C. Zinc dust (10g) was added slowly in small quantities for one-half hour. Stirring was continued for an additional hour. The mixture was filtered and the methanolic solution was concentrated to one-half its volume and then extracted with diethyl ether. A pure carbonyl-glyceride was obtained by TLC purification on a silica gel G coated plate by developing with petroleum ether:diethyl ether:acetic acid (80:20:2) by volume. The carbonyl-glyceride fraction was identified by DNP-base spray.

C. Reaction of Phosphatidyl Ethanolamine with  $\beta$ -Carbonyl  $\alpha,\alpha'$ -palmitostearate. A model system was prepared using pure phosphatidyl ethanolamine and  $\beta$ -carbonyl  $\alpha,\alpha'$ -palmitostearate in cellulose emulsion with borate buffer. A portion was stored at ambient temperature and RH 14% for 30 days and another fraction was heated for 10 hrs. at 50C to enhance the rate of reaction. The products were isolated with chloroform:methanol (1:1) from cellulose and spotted on basic

silica gel G plates and developed with chloroform:methanol:water (65:25:4) by volume. Various phosphorus containing fractions were isolated and the percentages of each product formed (based on phosphorus analysis) are shown in Table 15. Schiff base was isolated from basic silica gel G plates, developed with chloroform:methanol:water 50:50:4 by volume. The Schiff base had the characteristic peak for C = N at  $1630\text{cm}^{-1}$  (broad peak) in the IR spectra (Fig. XXIX). The UV spectrum had a broad absorption in the range 235 - 255  $\text{m}\mu$  (Fig. XXX). The presence of Schiff base was confirmed by reducing with sodium borohydride. After confirming the Schiff base formation it was cold saponified with 2N NaOH for 24 hr. The saponified product was extracted with diethyl ether and applied on a basic silica gel G coated plate and developed with chloroform:methanol:water (65:25:4). The hydrolyzed product shows five phosphorus containing fractions as observed before. This suggests that the Schiff base from phosphatidyl ethanolamine and  $\beta$ -carbonyl  $\alpha,\alpha'$ -palmitostearate and the polymer derived from phosphatidyl ethanolamine on cellulose have similar structure and could have been formed from a similar sequence of reactions. The PE polymer and the Schiff base isolated from reaction of  $\beta$ -carbonyl  $\alpha,\alpha'$ -palmitostearate and PE had similar characteristics after alkaline hydrolysis.

D. Fatty Acid Analysis of Starting PE and Schiff Base Polymers. For further confirmation of the above polymer-type Schiff base, model systems were prepared with pure hydrogenated and non-hydrogenated phosphatidyl ethanolamine of known fatty acid composition with borate buffer at pH 6 in cellulose emulsion. After reaction (storing at 50C for 10 hr.) it was extracted with chloroform:methanol (1:1) and Schiff base was isolated by TLC as explained before. The fatty acid composition of the Schiff base was examined by converting into methyl esters at low temperature (-70C) with methanolic KOH. The fatty acid composition was determined by GLC. The fatty acid composition of starting phosphatidyl ethanolamine and the Schiff base isolated are reported in Table 16.

When hydrogenated phosphatidyl ethanolamine was used for isolating a polymer type of Schiff base, no major difference in fatty acid composition was observed before and after the reaction in the hydrogenated phospholipid. But, when non-hydrogenated phosphatidyl ethanolamine was used in a model system, the fatty acid composition of the polymer type Schiff base showed major differences in the fatty acid composition of 18:1, 18:2, 18:3 and 20:4. Besides the major change in the above mentioned fatty acids, extra fatty acids were observed of chain lengths 9 and 12 as was 20% of an unknown fraction. The observed loss of fatty acids was due to oxidation, which would have yielded an aldehyde in the intact glyceride, which in turn reacted with another molecule of phosphatidyl ethanolamine yielding a polymer type Schiff base. The variation of fatty acid composition and major loss of polyunsaturates indicates intermolecular reaction between the phosphatidyl ethanolamine and intact glyceride-carbonyl product produced during oxidation. This further supported the assumption of formation of a polymer type Schiff base.

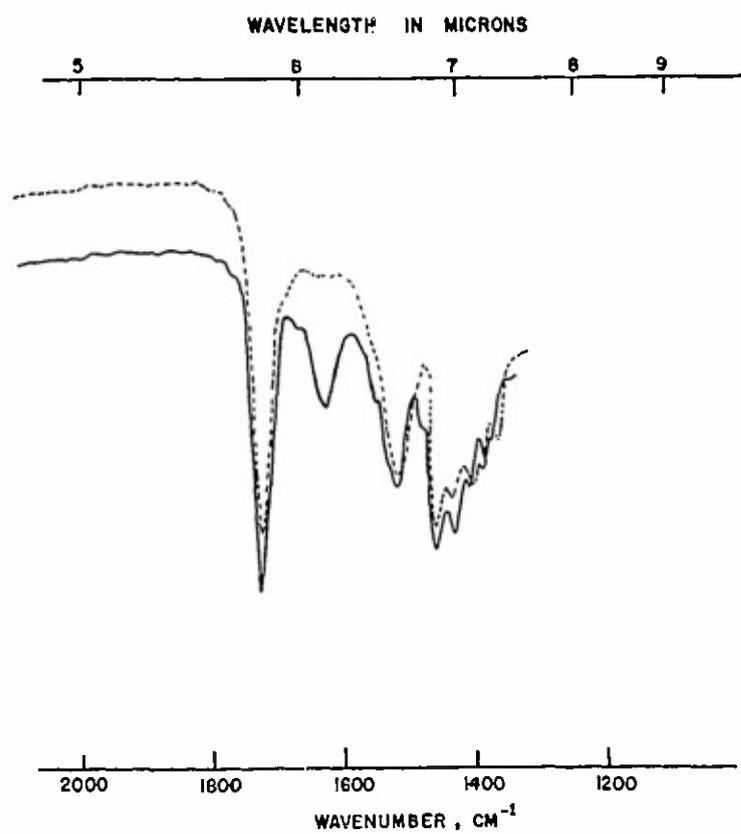


Fig. XXIX. IR spectrum of Schiff base formed from PE and  $\beta$ -carbonyl  $\alpha, \alpha'$ -palmitostearate.

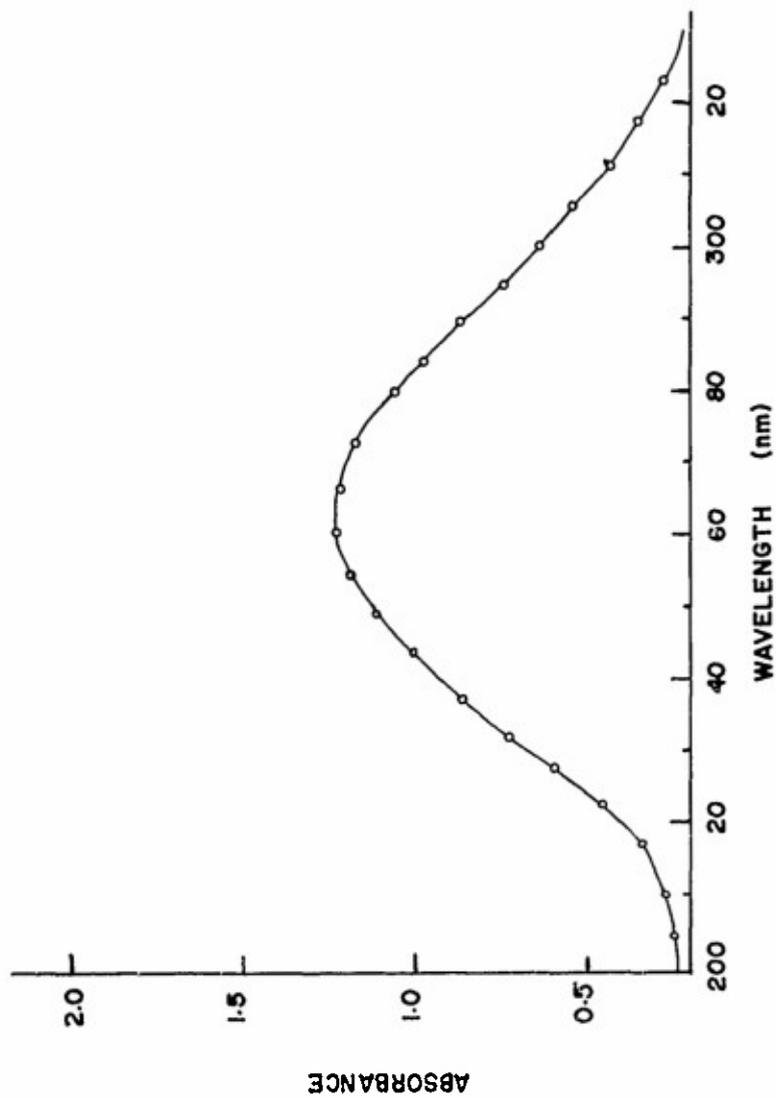


Fig. XXX. UV spectrum of Schiff base formed from PE and  $\beta$ -carbonyl  $\alpha,\alpha'$ -palmitostearate.

Table 13. Reaction Products of Nonanal and Phosphatidal Ethanolamine\*.

System Components	Schiff base	Other Reaction Products Lyso Schiff / Lyso PE base	Unreacted Phosphatidal Ethanolamine	Methyl Phosphate	Polymers	
1. Phosphatidal ethanolamine + Nonanal in Borate buffer (pH 6)	36.5	16.5	7.9	29.89	3.7	5.5
2. Phosphatidal ethanolamine in Borate buffer at pH 6	10.00	--	29.90	28.40	5.7	26.00
3. Phosphatidal ethanolamine + Nonanal + Myoglobin in Borate buffer (pH 6)	34.2	19.2	10.1	21.2	4.9	10.4

\*Stored at RH 14% and ambient temperature for 30 days.

Table 14. Browning Reaction Products of Phosphatidal Ethanolamine at Various pH Levels\*.

System Components	Schiff base	Other Reaction Products Lyso Schiff base	Lyso PE	Unreacted Phosphatidal Ethanolamine	Methyl Phosphate	Polymers
I(a) Phosphatidal ethanolamine (PE) in Borate buffer at pH 5	4.80	--	29.01	38.05	4.20	23.92
(b) PE + Nonanal in Borate buffer (pH 5)	20.50	10.00	12.00	34.90	8.50	5.90
II(a) PE in Borate buffer at pH 6	9.50	--	28.75	27.50	5.70	28.55
(b) PE + Nonanal in Borate buffer (pH 6)	38.10	16.40	9.10	24.60	5.00	7.80
III(a) PE in Borate buffer at pH 8	5.20	--	30.50	21.50	8.20	34.60
(b) PE + Nonanal in Borate buffer (pH 8)	26.71	15.98	11.54	20.60	9.70	15.56

\*Stored at RH 14% and ambient temperature for 30 days.

Table 15. Reaction Products from  $\beta$ -carbonyl  $\alpha,\alpha'$ -palmitostearate and Phosphatidyl Ethanolamine.

System Components	Schiff base	Unreacted Phosphatidyl Ethanolamine	Lyso PE	Schiff base from PE + Aldehyde*
1. $\beta$ -carbonyl $\alpha,\alpha'$ -palmitostearate+PE stored at 50C 8 hr. to enhance browning	59.9	21.00	14.2	4.9
2. $\beta$ -carbonyl $\alpha,\alpha'$ -palmitostearate+PE stored at ambient temperature and RH 14% for 30 days	56.6	24.5	9.5	9.4

\*Produced from oxidized fatty acids in phosphatidyl ethanolamine.

Table 16. Fatty Acid Analysis of the Polymer Type Schiff Base.

Carbon Chain Length	Fatty Acid Composition of Initial Reactants		Fatty Acid Composition of Schiff Base	
	Phosphatidyl Ethanolamine	Hydrogenated PE	Model System containing Phosphatidyl Ethanolamine	Model System containing Hydrogenated PE
C <sub>9:0</sub>	-	-	7.2	-
C <sub>12:0</sub>	-	-	5.9	-
C <sub>14:0</sub>	1.4	1.5	1.2	1.7
C <sub>16:0</sub>	20.5	23.0	19.2	22.1
C <sub>16:1</sub>	2.5	-	1.2	-
C <sub>18:0</sub>	28.2	63.4	20.5	59.4
C <sub>18:1</sub>	17.7	-	11.6	-
C <sub>18:2</sub>	15.4	-	9.1	-
C <sub>18:3</sub>	1.8	-	0.9	-
C <sub>20:0</sub>	-	12.1	-	10.9
C <sub>20:4</sub>	12.5	-	2.7	-
Unknown	-	-	20.5	5.9

### VIII. Reaction of Phosphatidyl Ethanolamine with Glucose

Reducing sugars that may be involved in non-enzymatic browning reactions include aldoses (pentoses, hexoses and disaccharides), uronic acids and ketoses. The interaction between an aldose and a primary or secondary amine may lead to browning, although there is considerable variation in the reactions of both sugar and amines. Since sugars occur in animal tissues in varying low levels, they may contribute to browning with PE with the consequent potential of affecting color and flavor of the system containing them.

A. Effect of Buffer on the Browning Reaction. Since borate reacts with carbohydrate, it was proposed to study the effect of citrate and phthalate buffers in the systems involving carbohydrates and phosphatidyl ethanolamine.

Model systems were prepared in cellulose emulsion as follows: (a) hydrogenated phosphatidyl ethanolamine and glucose in citrate buffer at pH 6; and (b) hydrogenated phosphatidyl ethanolamine and glucose in phthalate buffer at pH 6. The emulsions were frozen and then freeze-dried to a moisture level of 2.5%. The freeze-dried sample was heated at 50C for 20 hr. to enhance the rate of browning, then extracted with chloroform:methanol 25:75 by volume. The extract was concentrated under nitrogen and spotted on a basic silica gel C coated plate and developed with chloroform:methanol:water 65:25:4 by volume. Various phosphorus containing spots were isolated and the percentages of each product formed (based on phosphorus analysis) are shown in Table 17.

Table 17. Effect of Buffer on Browning Reaction involving hydrogenated Phosphatidyl Ethanolamine and Glucose\*.

System Components	Schiff base	Lyso PE	Unreacted Phosphatidyl Ethanolamine	Unknown Product
1. Hydrogenated Phosphatidyl Ethanolamine + Glucose in Citrate Buffer at pH 6.	57.17	10.65	24.54	7.64
2. Hydrogenated Phosphatidyl Ethanolamine + Glucose in Phthalate Buffer at pH 6.	48.47	8.95	35.28	7.30

\*Samples stored at 50C for 20 hr.

These now show that the kinds of products formed in the presence of each buffer were the same, but the percentage of each product formed varied. Greater Schiff base formation and less unreacted PE resulted in the systems using citrate buffer. Citrate buffer was used for the remainder of the studies with glucose.

B. Browning Reactions at Various pH Levels. The study reported here was initiated to learn the effect of pH in the browning reaction involving pure phosphatidyl ethanolamine and glucose. Glucose was chosen since it is a natural component of animal tissues and readily participates in carbonyl-amine reactions.

Model systems were prepared using hydrogenated phosphatidyl ethanolamine and glucose in cellulose emulsion with citrate buffer at pH 5, 6, 7, 8 and 9. Browning was induced by holding the freeze-dried systems at RH 14% and ambient temperature for 40 days at a moisture level of 2.5%. The intensity of the brown color in the system was minimal at pH 5 and maximum at pH 8 and above.

The browning products were extracted with chloroform:methanol (25:75) by volume. The extract was concentrated under nitrogen and spotted on a basic silica gel G plate and developed with chloroform:methanol:water (65:25:4) by volume. Various phosphorus containing spots were isolated and the percentages of each product formed are shown in Table 18.

Table 18. Reaction Products of D-Glucose and Hydrogenated Phosphatidyl Ethanolamine\*.

pH of System	Schiff Base	Unreacted Phosphatidyl Ethanolamine	Lyso Phosphatidyl Ethanolamine	Unknown
5	32.75	64.90	8.55	6.78
6	57.17	24.52	10.65	7.64
7	51.54	30.96	9.40	8.10
8	32.55	30.55	14.50	12.40
9	30.42	37.44	16.24	15.90

\*Stored at RH 14% and ambient temperature for 40 days.

Schiff base formation was at a maximum level at pH 6 with lesser quantities of side products being formed during the browning reaction than at either higher or lower pH levels. The rate of reaction between the carbonyl group and nitrogenous bases is pH dependent as was observed in the pH studies with other system components. As the pH was changed from slightly alkaline to acidic, the rate of Schiff base formation was increased; further acidification enhanced the reversible reaction giving back the sugar and phosphatidyl ethanolamine. At the higher pH level in the model system, the greatest amount of unknown product (a degradation product having phosphorus and nitrogen, but absence of ester carbonyl functionality) was formed and lesser Schiff base was formed.

Table 19. Reaction Products of D-Glucose and hydrogenated Phosphatidyl Ethanolamine\*.

System Components	Schiff base	Unreacted Phosphatidyl Ethanolamine	Lyso PE	Unknown
Hydrogenated Phosphatidyl Ethanolamine + D-Glucose on cellulose in citrate buffer at pH 6.	55.2	29.8	6.9	8.1

\*Stored at RH 14% and ambient temperature for 40 days.

C. Characteristics of Products. Hydrogenated phosphatidyl ethanolamine and glucose in citrate buffer at pH 6 was stored for 40 days at RH 14% and ambient temperature. This system was extracted with chloroform:methanol (25:75) by volume. The chloroform:methanol extract was applied onto a basic silica gel G plate and developed with chloroform:methanol:water (65:25:4) by volume and characterized (Table 19).

The IR spectrum of the Schiff base fraction gave a peak for C = N at  $1600\text{cm}^{-1}$  (broad), bonded -NH group at  $3400\text{cm}^{-1}$  (broad) and -OH absorption at  $3650\text{cm}^{-1}$  (broad), confirming the Schiff base formation (Fig. XXXI). The ultraviolet spectrum had maximum absorption at  $280\text{m}\mu$  (Fig. XXXII), and there was maximum fluorescence at  $475\text{m}\mu$  at an excitation of  $350\text{m}\mu$  (Fig. XXXIII). The presence of Schiff base was confirmed by reducing with sodium borohydride. After reduction of the Schiff base, the C = N characteristic and fluorescence were absent.

The unknown fraction mentioned in Tables 17 - 19 was isolated from the TLC plate. The IR spectrum contained a peak for bonded -NH group at  $3450\text{cm}^{-1}$  (strong), P - O - C at  $1050\text{cm}^{-1}$ , a weak absorption for C = N at  $1605\text{cm}^{-1}$  and absence of ester carbonyl absorption (Fig. XXXIV).

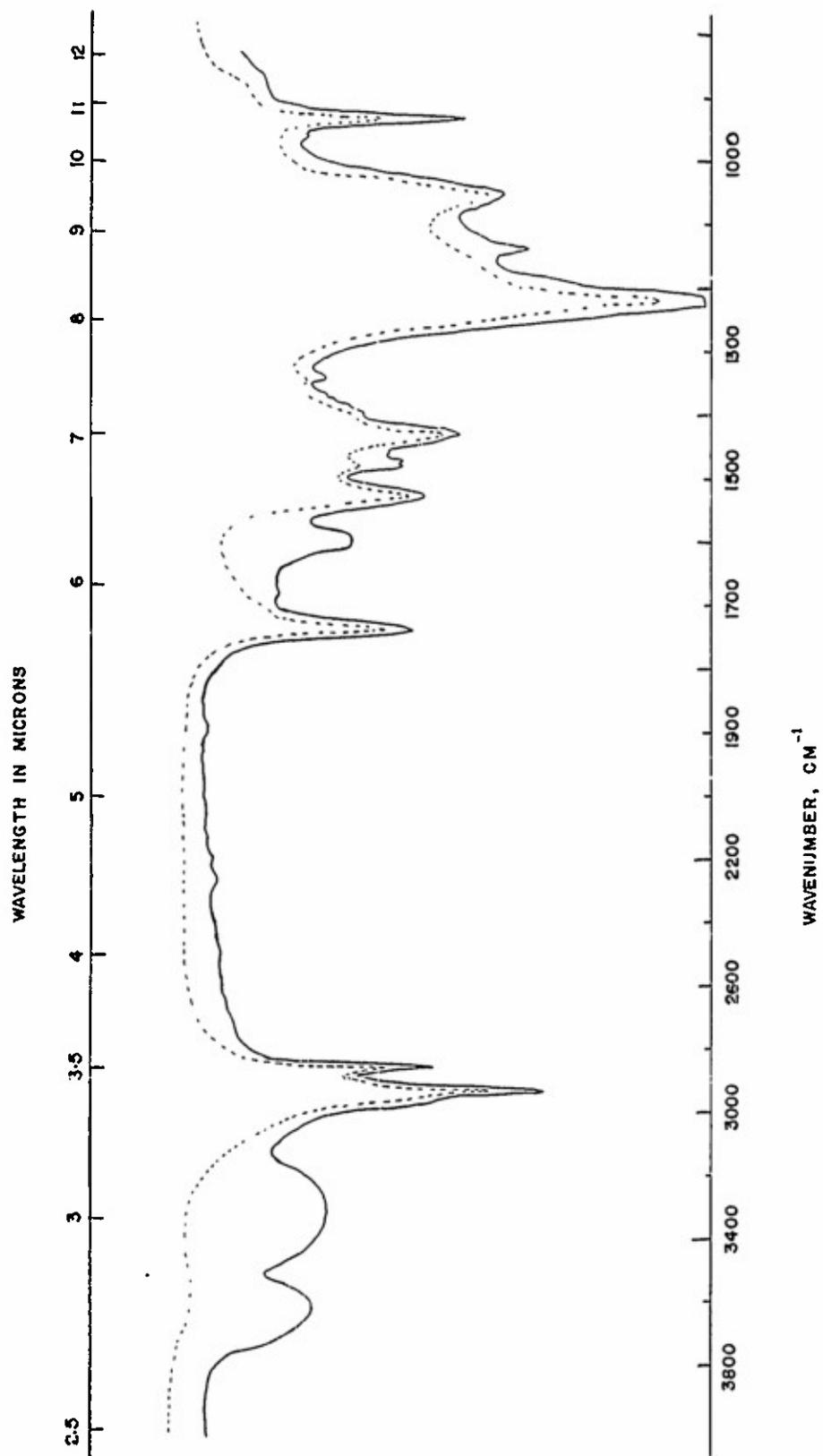


Fig. XXXI. IR spectrum of Schiff base formed from PE and glucose.

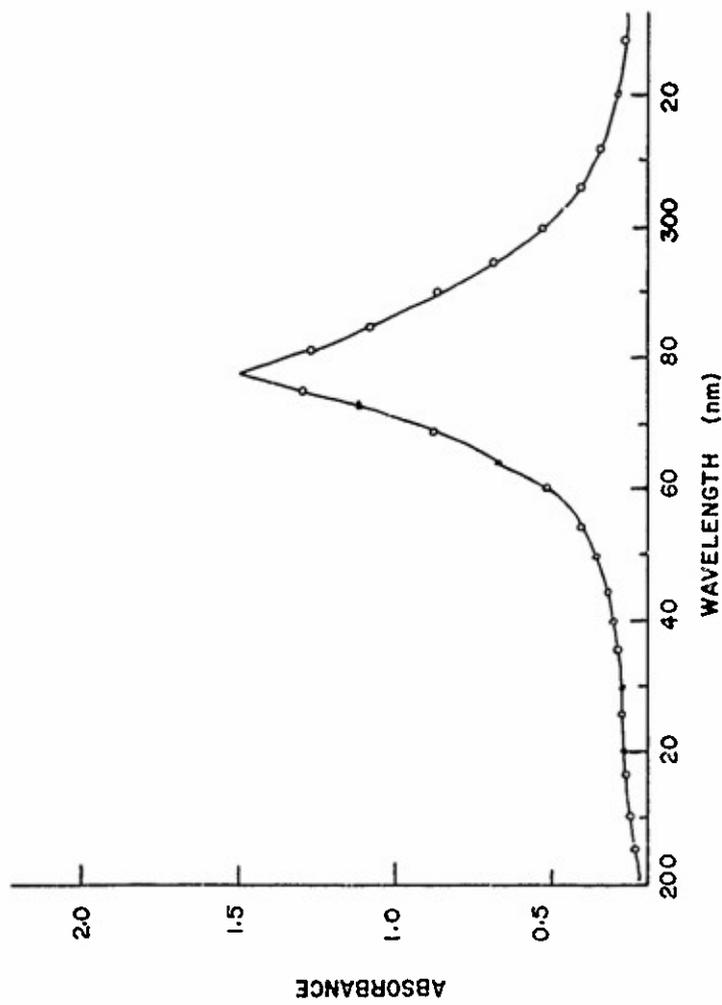


Fig. XXXII. UV spectrum of Schiff base formed from PE and glucose.

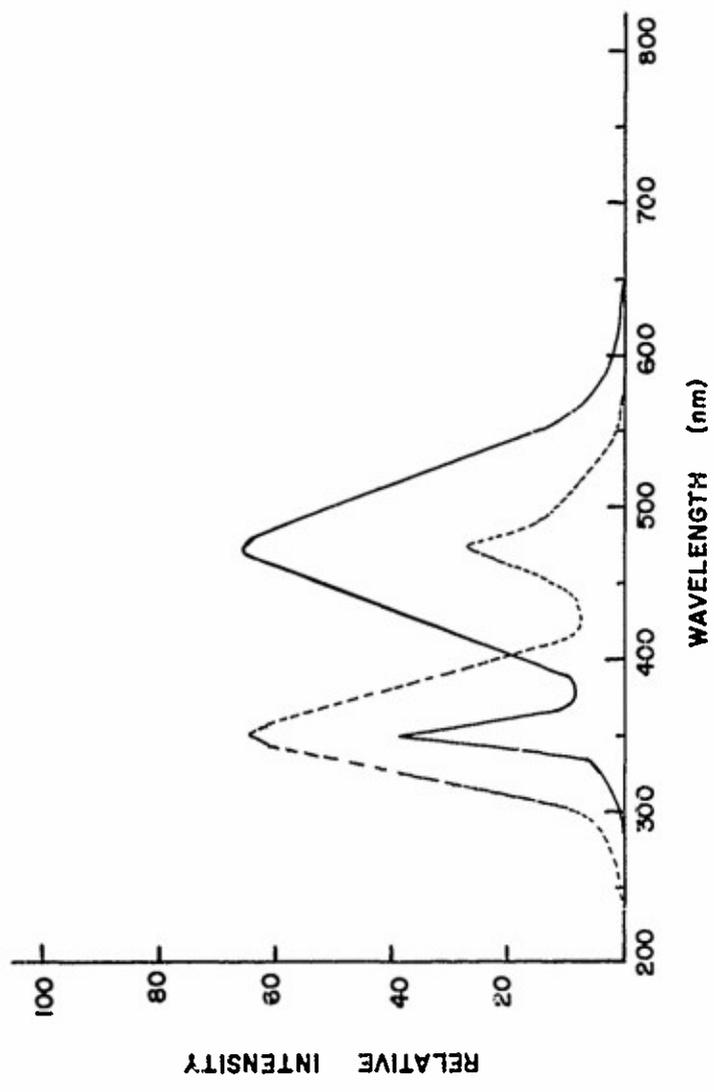


Fig. XXXIII. Fluorescence spectra of Schiff base formed from PE and glucose.

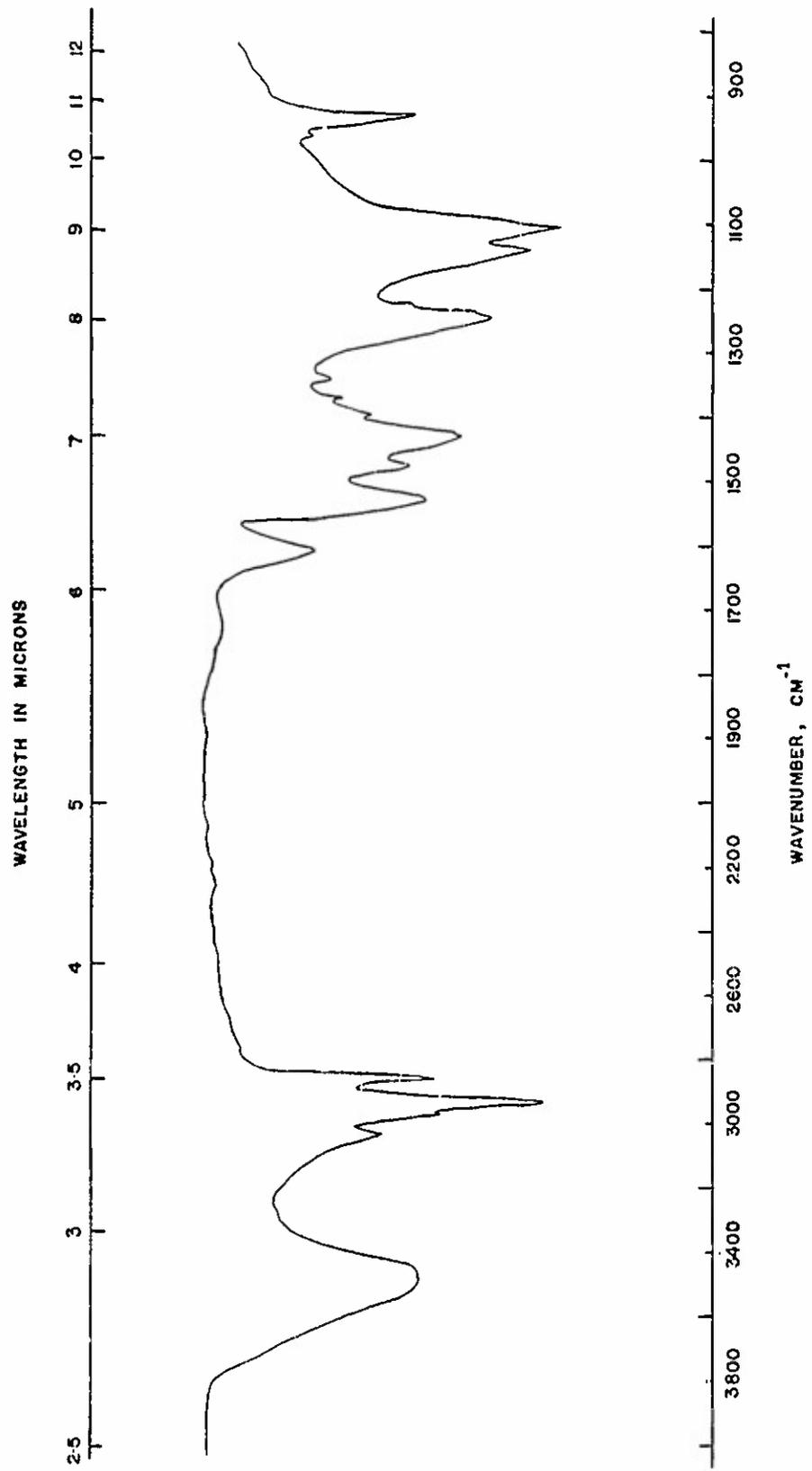
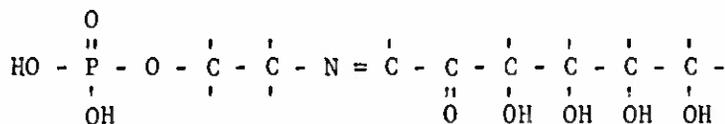


Fig. XXXIV. IR spectrum of unknown product from reaction of PE with glucose.

This indicates this fraction may be a degradation product from Schiff base. The possibility of this type of product formation is speculated as follows:

The Schiff base may be formed, Amadori rearrangement may occur and then a cyclic compound formed by hydrogen bonding which could cause a hydrolytic split of P - O - C bond at the  $\alpha'$ -glyceride position, yielding a product with a molecular weight of 301. The product envisioned would have both N and P but no ester carbonyl. The fraction had an Rf value equivalent to that of methyl phosphatidate, but no other characteristics of that compound.

Molecular weight determination, by passing through Bio-Beads S-X8, showed a molecular weight of 300 for a calculated MW of 301. Molecular weight determination by mass spectroscopy gave a molecular weight of 298. Details of molecular weight determination are discussed in the section on Ancillary Studies. The envisioned product may then be:



#### IX. Flavor Evaluation of Browning Reaction Product

Model systems were prepared in cellulose emulsion with borate buffer at pH 6 using:

- (a) Hydrogenated Phosphatidyl Ethanolamine + Nonanal
- (b) Phosphatidyl Ethanolamine + Nonanal
- (c) Hydrogenated Phosphatidyl Ethanolamine + 2-Hexene-1-al
- (d) Phosphatidyl Ethanolamine + 2-Hexene-1-al
- (e) Hydrogenated Phosphatidyl Ethanolamine + 2,4-Hexadienal
- (f) Phosphatidyl Ethanolamine + 2,4-Hexadienal
- (g) Hydrogenated Phosphatidyl Ethanolamine + Malonaldehyde
- (h) Phosphatidyl Ethanolamine + Malonaldehyde

After freeze-drying to a moisture level of 2.5%, the samples were stored at RH 14% and ambient temperature for 30 days and a portion was heated at 50C for 10 hrs. The model systems were examined for color, odor and flavor by a panel of judges. The results are shown in Table 20.

Portions of each sample not used for flavor evaluation were extracted with (1:1) chloroform:methanol to obtain for calculation the percentage of each phosphorus containing product formed in order to compare the products formed with the color, odor and flavor of the model system. The chloroform:methanol soluble fraction was concentrated and applied

onto a basic silica gel G plate and developed with chloroform:methanol:water 65:25:4 by volume. Various phosphorus containing spots were isolated and percentages of each product formed are reported in Table 21.

Systems incorporating the saturated aldehyde nonanal had enhanced flavor only when non-hydrogenated phosphatidyl ethanolamine was used. When unsaturated aldehydes were used with hydrogenated and non-hydrogenated phosphatidyl ethanolamine, all samples had some apparent flavor and color. Thus, the products obtained with unsaturated aldehyde contributed most to the flavor. When malonaldehyde was present in a model system, dark orange color and fishy odor was obtained. Phosphorus analysis of products from this model system revealed 80% Schiff base formation. This suggests that Schiff bases themselves may contribute to the distinctive flavors observed.

The beany flavors observed when the unsaturated aldehydes were used suggest the possibility of contribution of oxidized aldehyde components as well as contribution by the Schiff bases, scission products and polymers formed.

Table 20A. Color, Odor and Flavor Evaluation in Systems Composed of Phosphatidyl Ethanolamine and Saturated Aldehydes

Evaluation Test	Hydrogenated Phosphatidyl Ethanolamine + Nonanal	Phosphatidyl Ethanolamine + Nonanal
<u>1. Samples stored at 50C for 10 hrs.</u>		
A. COLOR	White	Slightly yellow
B. ODOR	Nutty	Off-flavor (nutty or burnt)
C. FLAVOR	No taste	Bitter
<u>2. Samples stored at RH 14% and ambient temperature for 30 days.</u>		
A. COLOR	White	Very light yellow
B. ODOR	Slightly nutty	Slight off-flavor
C. FLAVOR	No taste	Bitter

Table 20B. Color, Odor and Flavor Evaluation in systems composed of Phosphatidyl Ethanolamine and Unsaturated Aldehydes.

Evaluation Test	PE plus 2-Hexene-1-al	Hydrogenated PE plus 2-Hexene-1-al	PE plus 2,4-Hexadienal	Hydrogenated PE plus 2,4-Hexadienal	PE plus Malonaldehyde	Hydrogenated PE plus Malonaldehyde
1. <u>Samples stored at 50C for 10 hr.</u>						
A. COLOR	Yellow	White	Tan or Brown	Light Tan	Dark Orange	Light Orange
B. ODOR	Nutty	Burnt	Beany	Beany	Fishy	Beany
C. FLAVOR	Painty	None	Off-Flavor (rancid)	Off-Flavor (rancid)	Off-Flavor	Off-Flavor
2. <u>Samples stored at RH 14% and ambient temperature for 30 days.</u>						
A. COLOR	Light Yellow	White	Brown	Light Brown	Dark Orange	Light Orange
B. ODOR	Beany	Beany	Burnt	Beany	Fishy	Fishy
C. FLAVOR (slight off-flavor)	Bitter	Bitter	Off-Flavor (rancid)	Off-Flavor (rancid)	Off-Flavor (rancid)	Off-Flavor (rancid)

Table 21A. Browning Reaction Products of systems composed of Phosphatidyl Ethanolamine and Saturated Aldehydes

System Components	Schiff base	Unreacted PE	Lyso PE	Methyl Phosphate	Poly-mers
1. <u>Heated at 50C for 10 hrs.</u>					
a. Hydrogenated Phosphatidyl Ethanolamine + Nonanal in borate buffer at pH 6	35.7	40.2	10.4	4.5	9.2
b. Phosphatidyl Ethanolamine + Nonanal in borate buffer at pH 6	32.9	35.0	11.4	4.9	15.8
2. <u>Stored at RH 14% and ambient temperature for 30 days.</u>					
a. Hydrogenated Phosphatidyl Ethanolamine + Nonanal in borate buffer at pH 6	44.4	32.8	7.9	6.0	8.9
b. Phosphatidyl Ethanolamine + Nonanal in borate buffer at pH 6	40.1	33.70	9.2	7.2	9.8

Table 21B. Browning Reaction Products of systems composed of Phosphatidyl Ethanolamine and Unsaturated Aldehydes.

System Components	Schiff base	Non-Polymeric Reaction Product	Unreacted PE	Lyso PE	Polymers
<u>Storage at 50C for 10 hr.</u>					
1a. Phosphatidyl Ethanolamine + 2-Hexene-1-al	37.5	17.9	29.9	6.3	8.4
b. Hydrogenated PE + 2-Hexene-1-al	41.4	16.5	30.7	5.9	5.5
2a. Phosphatidyl Ethanolamine + 2,4-Hexadienal	29.5	23.2	25.8	7.9	21.5
b. Hydrogenated PE + 2,4-Hexadienal	32.7	25.9	17.0	4.9	19.5
3a. Phosphatidyl Ethanolamine + Malonaldehyde	65.5	10.4	11.2	4.7	8.2
b. Hydrogenated PE + Malonaldehyde	70.2	9.5	8.5	3.9	7.9

Table 21C. Browning Reaction Products of systems composed of Phosphatidyl Ethanolamine and Unsaturated Aldehydes.

System Components	Schiff base	Non-Polymeric Reaction Product	Unreacted PE	Lyso PE	Polymers
1a. Phosphatidyl Ethanolamine + 2-Hexene-1-al	45.74	20.42	19.84	4.80	9.20
b. Hydrogenated PE + 2-Hexene-1-al	46.21	18.40	21.79	5.20	8.40
2a. Phosphatidyl Ethanolamine + 2,4-Hexadienal	28.45	25.40	18.53	7.2	20.42
b. Hydrogenated PE + 2,4-Hexadienal	30.10	20.40	18.70	8.4	22.40
3a. Phosphatidyl Ethanolamine + Malonaldehyde	70.20	7.4	10.8	5.9	5.7
b. Hydrogenated PE + Malonaldehyde	74.12	8.2	4.5	6.9	7.2

\*Storage at RH 14% and ambient temperature.

## ANCILLARY STUDIES

### 1. Molecular Weight Determinations

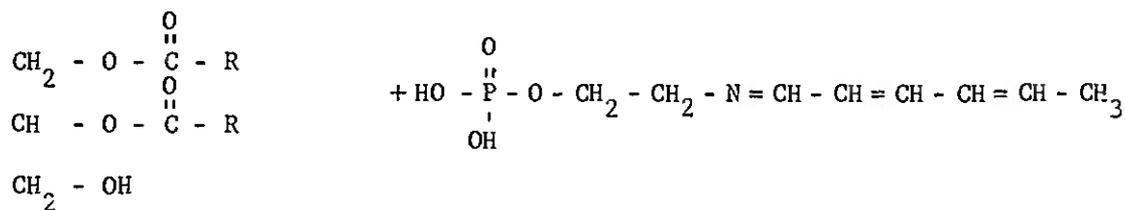
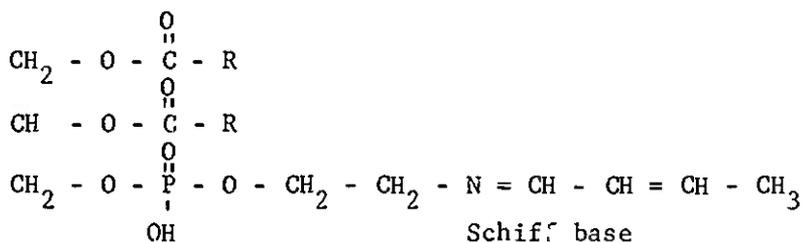
Molecular weight determinations were important to establish identity of some of the products formed. The mass spectrometer available on occasion was unable to define the mass of a Schiff base formed from PE and an aldehyde. It was necessary to modify some of the Schiff bases in order to provide fractions which could be analyzed and still maintain confidence that the original characteristics were retained.

To determine molecular weight of Schiff bases, a pure Schiff base was prepared from pure phosphatidyl ethanolamine and 2,4-hexadienal as described earlier. After confirming the Schiff base characteristics, a sample was injected into a GLC-mass spectroscopy unit to determine the molecular weight. This Schiff base was calculated to have an approximate molecular weight of 800; but the mass spectral determination indicated lower molecular weight fractions, suggesting the breakdown of the Schiff base on the column. It was decided to hydrolyze the Schiff base into two fragments using phospholipase C, and then determine the molecular weight of the two fractions individually.

Hydrolysis of Schiff base using phospholipase C: A 0.15g portion of the Schiff base was dispersed in 25ml of  $\text{NH}_4\text{Cl}/\text{NH}_4$  buffer at pH 7. Twenty five ml of 22% calcium chloride solution, 0.2ml of 25% bile salt solution and 50ml of diethyl ether were added. After an emulsion was formed, phospholipase C was added and the system was stirred for 3 hr. at ambient temperature. The pH was maintained at 7. After 3 hr., 50ml ethanol was added; the system was then extracted with 20% methanol in chloroform. The extract was dried over anhydrous sodium sulfate and the solvent was evaporated under an atmosphere of nitrogen.

The extract was spotted on a silica gel G plate and developed with petroleum ether:diethyl ether:acetic acid 80:20:2 by volume. The diglyceride fraction moved on the plate and the phosphorus containing portion remained near the origin. Both fractions were isolated from silica gel G and the mass spectra determined.

The mass determination of fraction I gave a molecular weight of 592 and of fraction II gave 227. The fatty acid composition of fraction I is not exactly known, so differences in molecular weight can be expected. In fraction II, a difference of 8 was observed, which does not vary widely from the molecular weight calculated. This procedure apparently then can be used for the determination of the molecular weight of Schiff bases. A more nearly exact molecular weight could be determined if the fatty acid composition of the phosphatidyl ethanolamine used for Schiff base formation were known before determination of the molecular weight.



I. Diglyceride

(Calc. Mol. Wt.--  
 if C<sub>18</sub>, C<sub>18</sub> = 605  
 if C<sub>18</sub>, C<sub>18:1</sub> = 603  
 if C<sub>18</sub>, C<sub>16</sub> = 577)

II. Phosphoryl Schiff base

(Calc. Mol. Wt. = 219)

A more rapid method for molecular weight determination was needed. A quick and reasonably satisfactory determination of molecular weight was achieved by gel filtration technique using Bio-Beads (polystyrene beads). Bio-Beads S-X8, mesh 200-400, (molecular weight exclusion limit 1,000), was swollen in carbon tetrachloride. After the gel beads were fully swollen, they were introduced into a chromatographic column (Sephadex column) and worked with the solvent in which they were swollen. During elution, 5ml fraction were collected and monitored by ultraviolet absorption and phosphorus analysis.

Three types of Schiff bases were eluted to determine the approximate molecular weight. The Schiff bases used for determination are listed below with the calculated and observed molecular weights. A linear correlation between the logarithm of molecular weight of the standard known compounds and the ratio of its elution volume (V) to the void volume (V<sub>0</sub>) of the column was found for Bio-Beads S-X8; a standard graph was plotted. The sample was applied onto the column, 5ml fractions were collected, and the elution volume was determined. The void volume of the column was determined every day. Knowing the elution volume the molecular weight of the compound under investigation was determined from the standard graph.

(molecular weight determinations for Schiff bases...)

- (a) Schiff base from a model system of phosphatidal ethanolamine + nonanal: calculated -- 801; observed from Bio-Bead column -- 785; (-16).
- (b) Lyso Schiff base from the above model system (a): calculated -- 535; observed from Bio-Bead column -- 510; (-25).
- (c) Schiff base from model system having  $\beta$ -carbonyl  $\alpha, \alpha'$ -palmitostearate + phosphatidyl ethanolamine: calculated -- 1700 (approx.). The elution of this fraction was obtained in the first 10ml, indicating the Bead (gel) used would not hold this higher molecular weight component on the column. Bio-Beads capable of separating higher molecular weight compounds were necessary for this sample.
- (d) An unknown fraction obtained from the model system made up from hydrogenated phosphatidyl ethanolamine + D-glucose: calculated -- 312 (approx.); observed from Bio-Bead column -- 300; (-12).

Determination of molecular weight of unknown compounds of the type encountered in this study by the Bio-Bead gel filtration technique appears to be quick, reproducible and reasonably satisfactory.

## 2. Preparation and Properties of Phosphatidal Ethanolamine

Many natural sources of phosphatidyl ethanolamine contain fairly large amounts of plasmalogens. These compounds are difficult to separate intact because their properties are very similar to those of phosphatidyl ethanolamine. Therefore, they are usually isolated together with the latter compounds.

The possibility of using controlled enzymatic hydrolysis for differentiating phosphatidal from phosphatidyl ethanolamine induced an examination of this system as a means of preparing high purity plasmalogens. It appeared to be possible to separate diacyl phosphoglycerides from plasmalogens by using pancreatic lipase hydrolysis.

Pancreatic lipase can hydrolyze 1,3 acyl groups in triglycerides and 1-acyl groups in phosphoglycerides. It would have no effect on the vinyl ether group of a plasmalogen; therefore, in a mixture of plasmalogen and diacyl phosphoglyceride only the 1-acyl group of the diacyl phosphoglyceride would be hydrolyzed leaving a 2-acyl lyso-compound which readily could be separated by TLC from the unchanged plasmalogen. Pancreatic lipase unless highly purified has substantial phospholipase A activity which could cause lyso-compound formation from each substrate at the 2-position.

It has been reported (16) that sucrose will suppress the phospholipase A activity in pancreatic lipase. Using this suppression made possible the development of an efficient procedure for preparation of phosphatidal ethanolamine by pancreatic lipase (butanol extracted) hydrolysis of a partially purified concentrate of cephalin from beef brain.

To study the effect of sucrose solution in the lipase hydrolysis (16), two experiments were set up; one using concentrated phosphatidal ethanolamine in ether, sucrose solution, calcium chloride, bile salt in buffer and lipase solution; and the other using the same substrates but excluding the sucrose solution. After one hour reaction time, the plasmalogen content of each system was determined. The results are shown in Table 22. This showed that the presence of sucrose during hydrolysis gave a higher yield of plasmalogen, thus supporting the concept that it suppressed the phospholipase A activity in the pancreatic lipase preparation. In the absence of sucrose, lipase catalyzed hydrolysis was not selective.

Hydrolysis at various time intervals: The sample size and reaction mixture were used as in the previous experiments and samples were taken after one-half hour, one hour, two hours and three hours. The plasmalogen content was determined with results as shown in Table 23. These show that maximum percentage of phosphatidal ethanolamine was achieved after a two-hour lipase catalyzed hydrolysis.

Table 22. Lipase Hydrolysis of Concentrated Phosphatidal Ethanolamine

Reactants Used for Hydrolysis	Percent Phosphorus Content	
	Phosphatidal Ethanolamine	Phosphatidyl Ethanolamine
1. Concentrated Phosphatidal Ethanolamine + 0.25 M Sucrose solution + CaCl <sub>2</sub> solution + Bile Salt + Lipase solution	86.6	13.4
2. Concentrated Phosphatidal Ethanolamine + CaCl <sub>2</sub> solution + Bile Salt + Lipase solution.	54.4	45.6

Table 23. Lipase Hydrolysis of Concentrated Phosphatidal Ethanolamine.

Time of Hydrolysis (hrs)	Phosphatidal Ethanolamine (%)	Phosphatidyl Ethanolamine (%)
0	53.0	47.0
0.5	82.7	17.3
1.0	92.7	7.3
2.0	92.8	2.2
3.0	94.0	6.0

Isolation of pure phosphatidal ethanolamine: A 0.5g portion of the concentrate of phosphatidyl and phosphatidal ethanolamine (from beef brain) dissolved in 150ml of diethyl ether was dispersed in 25ml of ammonium chloride-ammonium hydroxide buffer and 25ml of 0.25M sucrose solution. Twenty-five ml of 22% calcium chloride solution and 0.2ml of 25% sodium deoxycholate (bile salt) were added and the pH was adjusted to 9.0 by adding 1 N ammonium hydroxide. After an emulsion was formed, 7ml of lipase was added and the system was stirred at room temperature at pH 9. After 3 hr., 100ml of ethanol was added and the system extracted with 20% methanol in chloroform. The extract was dried over sodium sulphate and the solvent removed under an atmosphere of nitrogen. The hydrolyzed product was spotted on a silica gel G coated plate and developed with chloroform:methanol:water 65:30:4 by volume. A portion of the plate was sprayed with ammonium molybdate to visualize phosphorus containing spots; the lyso-phosphatidyl ethanolamine portion was discarded; and the phosphatidal ethanolamine portion was isolated from the silica gel C by extracting with chloroform:methanol (1:1) by volume. The plasmalogen content was determined according to the procedure of Rhee *et al* (18) and found to be 94.6, with 5.4 percent unchanged phosphatidyl ethanolamine remaining.

Properties of plasmalogen from beef brain: The infrared absorption spectrum of phosphatidal ethanolamine and phosphatidyl ethanolamine in chloroform solution was obtained. Although  $\alpha,\beta$ -unsaturated (vinyl) ethers show a characteristic strong absorption band at  $8.3 \mu$  ( $1200\text{cm}^{-1}$ ) attributed to [C - O - C] deformation, this is not adequate for identification since this band might be masked by P - O absorption at  $8.0 - 8.3 \mu$ . The absorption at  $6.0 \mu$  ( $1650\text{cm}^{-1}$ ) which is clearly seen in the spectrum is characteristic of the alkenyl ether group of plasmalogens (Fig. XXXV).

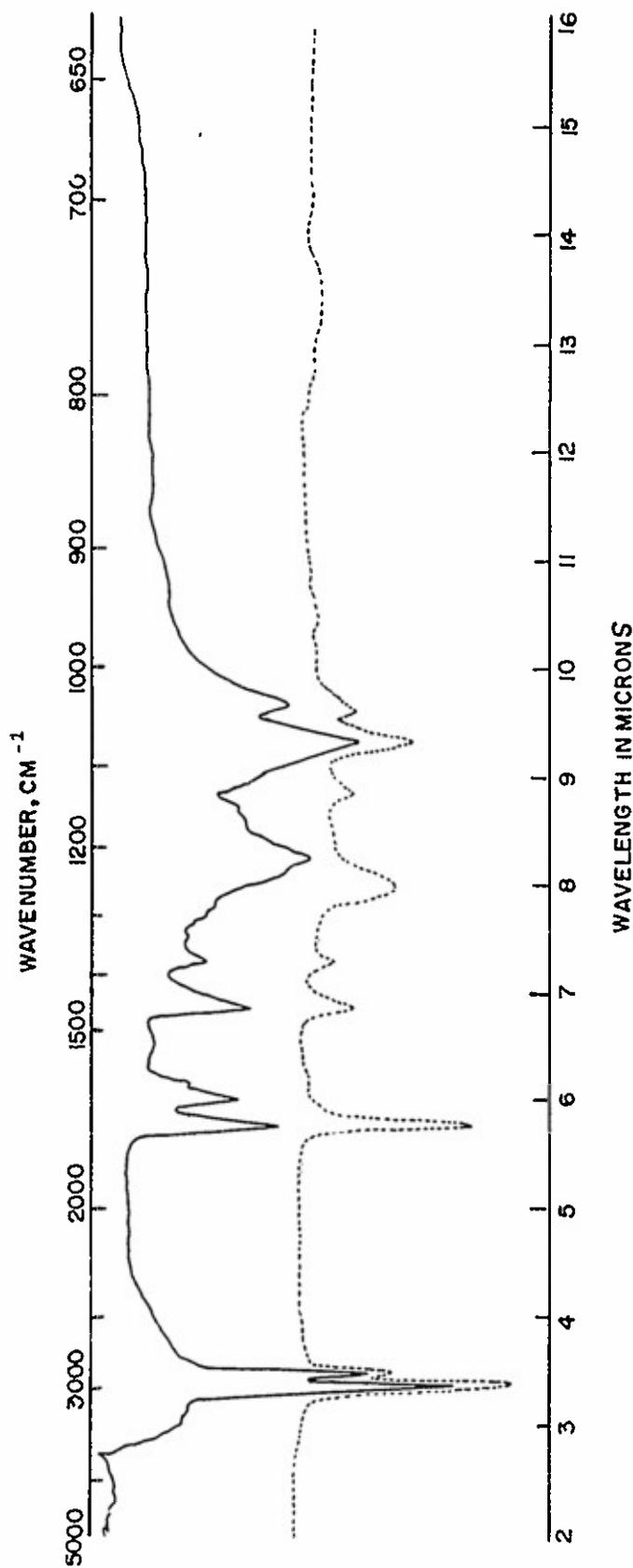


Fig. XXXV. IR spectrum of phosphatidal ethanalamine from beef brain.  
(Phosphatidal ethanalamine - solid line; PE - broken line.)

Aldehyde composition of the intact plasmalogen: A knowledge of the aldehyde moieties in the plasmalogen fraction was needed for studies in lipid browning involving reaction of the free amino group of phosphatidyl-ethanolamine with aldehydes. The aldehydes derivable from the plasmalogen fraction may participate in this reaction.

The aldehyde components of alk-1-enyl glycerol ethers and other aldehydogenic lipids are commonly analyzed as aldehydes after acid-catalyzed hydrolysis, or as dimethyl acetals followed by methanolysis. Acid-catalyzed hydrolysis of alk-1-enyl ethers can be effected by treatment with 90% acetic acid at 38C for 18 hr. Alternatively, the vinyl ether band in aldehydogenic lipids can be cleaved by exposing these lipids, in thin layers, to vapors of hydrochloric acid. The following procedure is based on the observation that alk-1-enyl ethers of ethanediol can be hydrolyzed completely by hydrochloric acid in diethyl ether.

Two mg of concentrate of phosphatidyl ethanolamine from beef brain was dissolved in 10ml of peroxide free ether and 1.5ml of concentrated hydrochloric acid was added. The mixture was stirred vigorously for 35 min. under an atmosphere of nitrogen. Two ml of ice cold water was added and then the mixture was extracted with hexane. The hexane extract was concentrated under an atmosphere of nitrogen and spotted on a silica gel G coated plate and developed with a solvent system of hexane:dimethyl ether 90:10 by volume. The lyso-phosphatidyl ethanolamine and phosphatidyl ethanolamine remained at the base and the released aldehydes moved near the solvent front on the plate. The aldehydes were identified by spraying a portion of the plate with 2,4-DNP reagent. The aldehyde positive band was scraped off and the aldehydes isolated from the silica gel by extracting with diethyl ether.

The aldehydes in the concentrated ether extract were reduced to the corresponding alcohols with lithium aluminum hydride; these were then acetylated with pyridine-acetic anhydride (2:1) and purified by thin-layer chromatography.

Gas-liquid partition chromatography was carried out with a Beckman GC-5 dual column, temperature programmed gas chromatograph. A stainless steel column (1/8" O.D. x 6') was used for separation of the alcohol acetates. The column was packed with 20% by weight DEGS and 1% by weight phosphoric acid on acid washed Chromasorb W 80/100 mesh as a support phase. The column was conditioned at 225C for 25 hr. before use. Operating conditions were: column temperature 175C; inlet temperature 230C; detector temperature 250C. The attenuator setting was usually  $5 \times 10^2$ . Peak identification was made through comparison of retention time with that of authentic samples of palmitoyl acetate, stearoyl acetate and oleoyl acetates prepared by reduction of the appropriate acid chloride with lithium aluminum hydride and then acetylated in pyridine-acetic anhydride. The percentage composition of the aldehydes as acetates calculated by triangulation is shown in Table 24.

Table 24. Fatty Aldehydes (as acetates) of Phosphatidyl Ethanolamine from Beef Brain.

Aldehyde Components	Percent
12:0	traces
13:0	0.56
14:0	1.00
15:0	1.00
16:0	24.62
17:0	2.80
18:0	39.17
18:1	30.82

The results show that the aldehydes in beef brain plasmalogen are predominantly saturated and the only unsaturated aldehyde is the monoene with 18 carbon atoms. Aldehydes with chain length greater than C<sub>18</sub> were not observed.

Identification of aldehydes as dimethyl acetals was also carried out, but was found to be less desirable and less reliable.

Analysis of free aldehydes as their acetates is preferred as it has been found that dimethyl acetals tend to form alk-1-enyl methyl ethers during gas chromatography, which may explain the observation of dimethyl acetal peaks of apparently unusual aldehydes in the GLC traces.

### 3. Oxygen Uptake Studies

Oxygen uptake was measured on a number of the model systems to determine relative susceptibility to oxidation of plasmalogen and phosphatidyl ethanolamine with and without aldehyde and myoglobin. Model systems were prepared in cellulose matrix in borate buffer at pH 6 using:

- (a) Hydrogenated Phosphatidyl Ethanolamine
- (b) Hydrogenated Phosphatidyl Ethanolamine + Myoglobin
- (c) Phosphatidyl Ethanolamine
- (d) Phosphatidyl Ethanolamine + Myoglobin
- (e) Phosphatidyl Ethanolamine
- (f) Phosphatidyl Ethanolamine + Oleyl Aldehyde
- (g) Phosphatidyl Ethanolamine + Nonanai + Myoglobin
- (h) Blank (only cellulose in borate buffer)

- (i) Schiff Base from Phosphatidyl Ethanolamine + Nonanal
- (j) Schiff Base from Hydrogenated Phosphatidyl Ethanolamine + Nonanal

To study the oxygen uptake of Schiff bases, Schiff bases were prepared under an atmosphere of nitrogen refluxing in 1:1 chloroform:methanol for 3 hr. After refluxing, the product was concentrated under an atmosphere of nitrogen and Schiff bases were purified by TLC. The isolated Schiff base was freeze-dried in a cellulose matrix with borate buffer at pH 6. After freeze-drying to a moisture level of 2.5% the samples were powdered well and placed in a Gilson Differential Respirometer flask containing KOH pellets in the middle cavity to absorb any liberated carbon dioxide. The manometer showed an increase in the initial reading, indicating an absorption of oxygen. Table 25 shows the rate of oxygen uptake in each of the model systems (Fig. XXXVI - XXXVIII).

This study showed that initiation takes place, in all the dry systems used, in about 5 hr. time. A greater rate of oxygen uptake was observed when added carbonyl compound was present in the model system, indicating rapid oxidation when both the reactive groups like  $\text{RNH}_2$  and  $\text{C} = \text{O}$  are present together. The rate of oxidation was slow and steady when only phospholipid was present, indicating limited oxidation. The absorption of oxygen was greater in the phosphatidyl ethanolamine (plasmalogens) when compared with phosphatidyl ethanolamine.

The presence of myoglobin in the model system enhanced oxygen uptake. The samples which contained myoglobin began to absorb oxygen within one-half hour and a maximum rate was measured after 2 hr., after which the absorption was reduced. As a possible explanation for this, myoglobin itself absorbs oxygen, so in the initial stages the uptake of oxygen may be due to myoglobin. In general, however, the rate of oxygen uptake was greater in the samples containing myoglobin, indicating enhanced oxidation.

Pure Schiff bases had minimal oxygen uptake, indicating very little oxidation. This uptake possibly can be attributed to oxidation at  $\text{C} = \text{N}$  in the Schiff base. The rate of uptake of oxygen was much greater with  $\text{C} = \text{C}$  groups present than when  $\text{C} = \text{N}$  groups were present. If an external stress were applied to  $\text{C} = \text{N}$ , it may be that it oxidized as a carbon, carbon double bond, but this assumption is subject to confirmation.

Oxidative Stability of Vinyl Group. It has never been established whether the unsaturated linkage of the vinyl ether group in plasmalogens may react with oxygen to give measurable oxygen uptake and/or to provide oxidation products in a lipid system other than those to be expected from oxidation of unsaturated fatty acids.

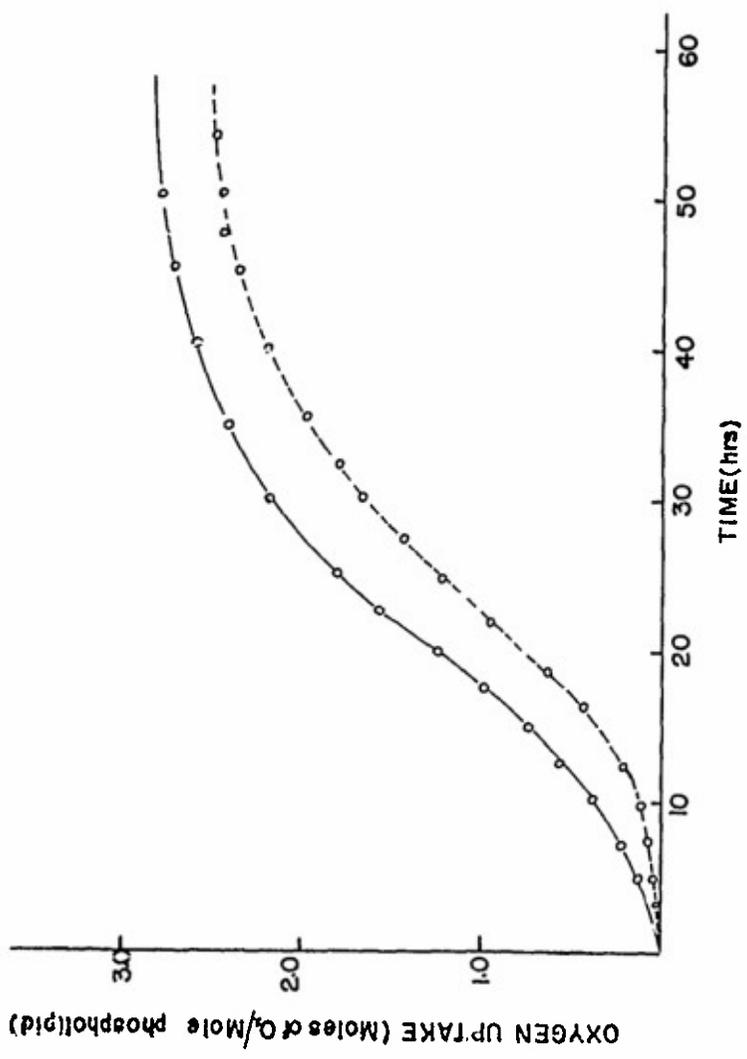


Fig. XXXVI. Oxygen uptake of PE.  
 (PE - circles/broken line; PE + myoglobin -  
 circles/solid line)

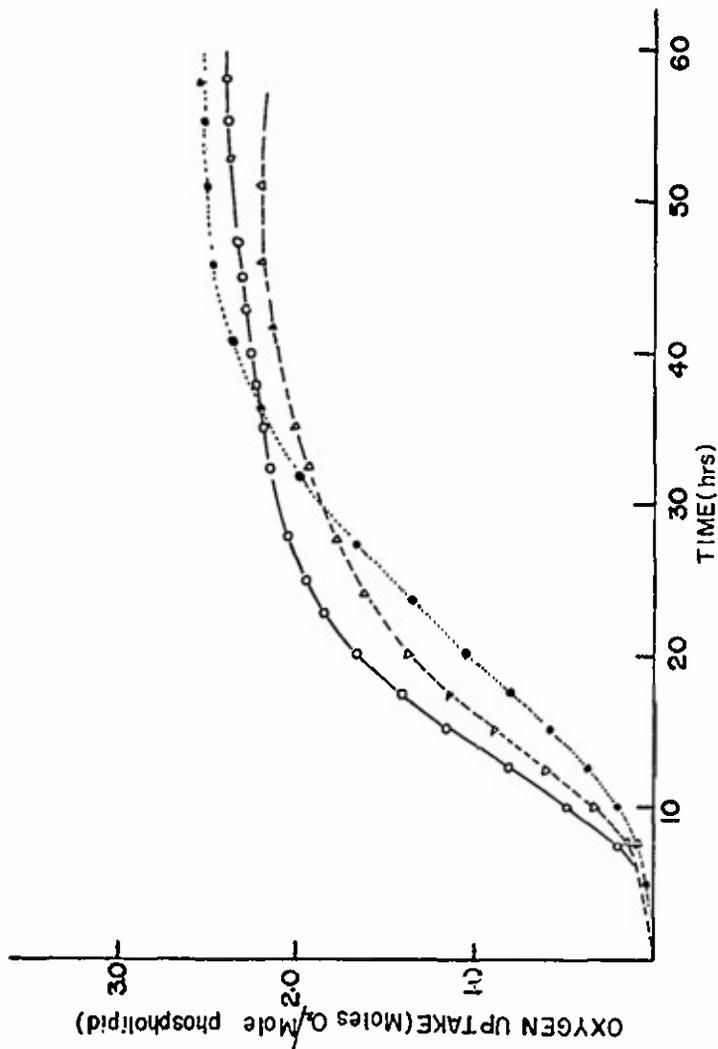


Fig. XXXVII. Oxygen uptake of Plasmalogen. (Phosphatidyl ethanolamine - solid circle/dotted line; phosphatidyl ethanolamine + nonanal + myoglobin - circle/solid line; phosphatidyl ethanolamine + oleyl aldehyde - triangle/dotted line.)

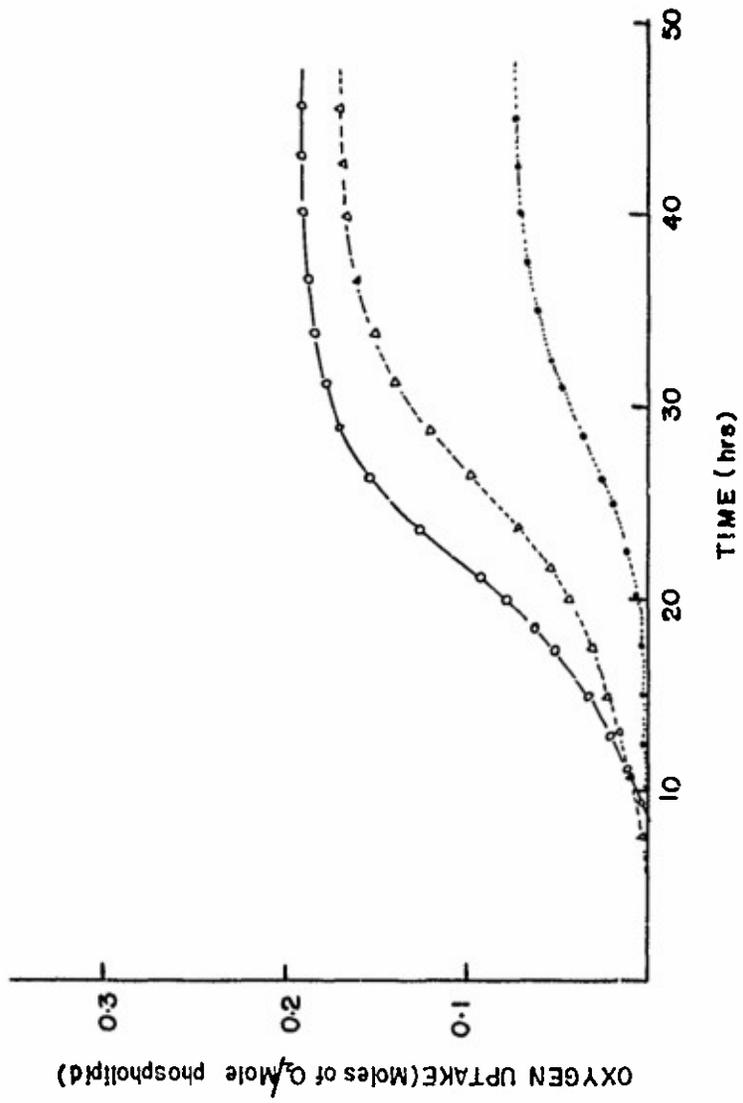


Fig. XXXVIII. Oxygen uptake of Schiff base. (Schiff base (soln) from PE + nonanal - circle/solid line; Schiff base (freeze-dried) - triangle/broken line; Schiff base (freeze-dried) from hydrogenated PE + nonanal.)

A saturated plasmalogen was prepared to study the oxidative stability of the vinyl group present in plasmalogens. Since hydrogenation of plasmalogens reduced the vinyl ether group, it was necessary to synthesize a saturated phospholipid having the vinyl ether group intact. The most inert plasmalogen, having most of the characteristics of a plasmalogen unchanged yet not otherwise susceptible to oxidation, was needed for this study. A saturated phosphatidyl choline was synthesized since it should have no groups that are readily oxidized and still have the vinyl ether grouping. It was prepared by enzymatic preparation of the  $\beta$ -acyl lyso-compound; hydrogenation of that compound so that the  $\beta$ -acyl group was saturated; condensation of the dimethyl acetal of lauryl aldehyde with this compound; halogen displacement of methoxyl; and then dehydrohalogenation with triethylamine to form the vinyl ether group. Details of the synthesis are given below.

(a) Preparation of hydrogenated lyso-phosphatidyl choline:

(i) Pure Phosphatidyl choline was prepared from egg yolk;  
(ii) Hydrolysis of phosphatidyl choline: 0.5g portion of pure phosphatidyl choline was dispersed in 25ml of 1.2M ammonium chloride: ammonium hydroxide buffer at pH 8.5. Ten ml of 22% calcium chloride solution and 0.2ml 25% bile salt solution were added together with 15ml of pancreatic lipase solution. The pH was maintained at 8.5 for 2 hr. by adding 1 N  $\text{NH}_4\text{OH}$  as needed. At the end of this time, 50ml of ethanol was added to suppress lipase activity and the system was extracted with 20% methanol in chloroform. After drying the sample under an atmosphere of nitrogen, it was spotted on a silica gel G coated plate and developed with chloroform:methanol:water 65:25:4. The phosphorous containing spots were visualized by spraying with ammonium molybdate. This showed complete hydrolysis of phosphatidyl choline into a lyso fraction. The purified lyso fraction was isolated and dissolved in 75ml of methanol with 10ml of chloroform. Palladium-carbon catalyst (0.4g) was added and hydrogenation performed in a Parr hydrogenator for 10 hr. at 30 psi. The catalyst was filtered off and the extract was concentrated. Fatty acid analysis showed that hydrogenation was complete.

(b) Preparation of dimethyl acetals: Two grams of lauryl aldehyde were dissolved in 100ml of dry peroxide free diethyl ether, cooled to  $10^\circ\text{C}$ , and 70ml of absolute methanol was added followed by 95ml of 35% methanolic-KOH. The mixture was stirred for 60 min. and poured onto crushed ice. The dimethyl acetal was extracted with ether and dried over anhydrous sodium sulphate. Dimethyl acetals were purified from lauryl aldehyde by TLC.

(c) Condensation of dimethyl acetal with hydrogenated lyso-phosphatidyl choline: Lyso-phosphatidyl choline (0.45g, hydrogenated) was dissolved in 175ml of dry benzene. Dimethyl acetal (0.75g) of laurylaldehyde and 0.5mg p-toluene sulphonic acid were added and the mixture refluxed for 2 hr. The volume was reduced to one-half under reduced pressure and the solution extracted with diethyl ether. The extract was washed three times with aqueous sodium bicarbonate, then dried over anhydrous sodium sulphate.

(d) Plasmalogen formation: Lauryl phosphatidyl acetal (0.47g) was dissolved in 40ml of freshly distilled acetyl chloride and the solution was kept at 22C for 4 days. The acetyl chloride was evaporated under anhydrous conditions and the residue was refluxed with freshly distilled triethylamine for 15 min. under anhydrous conditions. The mixture was poured into dry ether (200ml) and the triethylamine hydrochloride was removed by filtration. The filtrate was concentrated to give saturated phosphatidal choline (plasmalogen moiety intact). This was decolorized by boiling in 20% methanol in chloroform with activated charcoal. The IR spectrum of the synthesized product had absorption bands characteristic for the C - O - C group at  $1685\text{cm}^{-1}$ , for the ester group at  $1740\text{cm}^{-1}$ , and for the P - O - C group at  $1155\text{cm}^{-1}$ . The presence of plasmalogen was determined according to the procedure of Rhee et al (18) and found to be 98.4% phosphatidal choline.

Oxygen uptake was measured both in solution and in dry systems. For dry systems, a cellulose emulsion was prepared with boric acid buffer at pH 6 using the synthesized saturated acyl phosphatidal choline and freeze-dried to a moisture level of 2.5% for 30 hr. For a solution system, the phosphatidal choline was dissolved in 10% chloroform in methanol.

No oxygen uptake was observed in 40 hr. at 24C, indicating no apparent oxidation of or adjacent to the vinyl group in the plasmalogens at that temperature.

#### 4. Role of Myoglobin in the Browning Reaction

Myoglobin has been shown to act as a pro-oxidant in these model systems and also enhances polymerization. These effects were explored further by studying the effect of myoglobin in the following model systems. (The freeze-dried systems were prepared at pH 6, stored both at ambient temperature and RH 14% for 30 days, and at elevated temperature and 50C for 10 hr.)

- (a) Hydrogenated Phosphatidyl Ethanolamine
- (b) Hydrogenated Phosphatidyl Ethanolamine + Myoglobin
- (c) Phosphatidyl Ethanolamine
- (d) Phosphatidyl Ethanolamine + Myoglobin
- (e) Phosphatidal Ethanolamine
- (f) Phosphatidal Ethanolamine + Myoglobin
- (g) Oleyl Aldehyde
- (h) Oleyl Aldehyde + Myoglobin

The reaction products were extracted and the various phosphorus containing fractions were isolated. The percentages of each product formed (based on phosphorus analysis) are reported in Table 26.

Table 25. Oxygen Uptake in Dry Model Systems.

T	Moles of Oxygen Absorbed / Moles of Phospholipid												
	I	M	E	Blank	Hydro- genated PE	Hydro- genated PE + Myoglobin	PE	PE + Myo- globin	Phospha- tidal Ethanol- amine	Plas*PE + Oleyl Aldehyde	Plas*PE + Nonanal + Myoglobin	Schiff Base from PE + Nonanal	Schiff Base from Hydrog. PE + Nonanal
(hrs)													
5	-	-	-	-	0.12	0.02	0.09	0.02	0.08	0.09	-	-	-
10	-	-	-	-	0.09	0.095	0.34	0.20	0.32	0.50	0.009	-	-
15	-	-	-	-	0.11	0.33	0.52	0.60	0.86	1.14	0.024	-	-
20	-	-	-	-	0.15	0.73	0.22	1.04	1.37	1.62	0.045	0.004	0.004
25	-	-	-	-	0.17	1.20	1.80	1.46	1.66	1.96	0.08	0.019	0.019
30	-	-	-	-	0.19	1.62	2.18	1.88	1.88	2.10	0.13	0.032	0.032
35	-	-	-	-	0.20	1.98	2.40	2.18	2.00	2.19	0.164	0.051	0.051
40	-	-	-	-	0.22	2.20	2.59	2.37	2.12	2.24	0.170	0.062	0.062
45	-	-	-	-	0.20	2.36	2.72	2.46	2.20	2.32	0.170	0.064	0.064
50	-	-	-	-	0.20	2.42	2.80	2.50	2.22	2.36	0.170	0.064	0.064
55	-	-	-	-	0.18	2.42	2.81	2.48	2.22	2.38	0.170	0.062	0.062
60	-	-	-	-	0.20	2.42	2.80	2.50	2.22	2.38	0.170	0.064	0.064

\* Plas PE = plasmalogen PE or phosphatidyl ethanolamine.

Table 26. Role of Myoglobin in Browning Reaction\*.

System Components	Schiff base	Unreacted Phosphatidyl Ethanolamine	Lyso PE	Methyl Phosphatidate	Polymers
1. Hydrogenated Phosphatidyl Ethanolamine	---	29.35	5.25	---	65.40
2. Hydrogenated PE + Myoglobin	---	20.25	7.98	---	71.74
3. Phosphatidyl Ethanolamine	6.9	46.90	3.8	2.2	39.40
4. PE + Myoglobin	5.2	36.00	8.40	2.0	48.40

\*Samples stored at RH 14% and ambient temperature for 30 days.

When myoglobin was present with hydrogenated phosphatidyl ethanolamine, there were no major changes in reaction product formation. When non-hydrogenated phosphatidyl ethanolamine with myoglobin was present in the model system, polymer formation and lyso-fraction was greater when compared to the system containing only phosphatidyl ethanolamine.

The model systems (e) and (f) having phosphatidyl ethanolamine and myoglobin had a greater percentage of lyso-fraction and Schiff base due to lyso-fraction. The presence of myoglobin increased the amount of the lyso product. When plasmalogen was present in the model system during the storage period, there was hydrolysis of the vinyl ether causing liberation of aldehydes which participated in the carbonyl-amine reactions.

The chloroform:methanol extract of model systems (g) and (h) containing oleyl aldehyde and oleyl aldehyde + myoglobin was spotted on a silica gel G plate and developed with petroleum ether:diethyl ether:acetic acid 85:15:2 with pure oleyl aldehyde as a reference sample. After development, the plate was sprayed with 2,4-DNP reagent and a base to disclose the aldehydes as purple spots; the plate was then sprayed with 50% H<sub>2</sub>SO<sub>4</sub> and charred. The extracts from these model systems had different R<sub>f</sub> values when compared with standard oleyl aldehyde. These may be oxidized products having the aldehydes condensed by an aldol reaction. This could explain why extracts from model systems (g) and (h) gave negative response to 2,4-DNP spray and had R<sub>f</sub> values different from that of oleyl aldehyde. The extract from model systems (g) and (h) had R<sub>f</sub> value above that of oleyl alcohol and far below that of oleic acid. This indicates possible condensation of the aldehyde

Table 27. Lipid-type Amino-carbonyl Reaction Products on Protein Fibers\*.

System Components	Schiff Base	Other Reaction Products		Unreacted PE	Methyl Phosphatide	Polymers		
		Ninhydrin Positive	Ninhydrin Negative					
1. Muscle fiber + PE + Nonanal	35.4	5.9	4.2	37.5	8.1	8.9		
2. Muscle fiber + PE	10.5	10.5	2.1	46.9	1.9	29.9		
3. Muscle fiber + Nonanal	-	-	-	-	-	-		
4. Muscle fiber + Phosphatidyl ethanolamine	7.2	29.9	4.1	50.9	nil	7.9		
5. Muscle fiber + Hydrogenated phosphatidyl ethanolamine	-							
					Lyso PE 6.9	83.1	-	10.2

\*Storage at RH 14% and ambient temperature for 30 days.

Table 28. Amino Acid Analysis of Muscle Fiber after Storage with Phospholipids and/or Aldehydes.

Amino Acid	Gram Residue / 100 gram of Sample						
	Original Muscle fiber before Browning Reaction	Muscle fiber plus hydro-generated PE	Muscle fiber plus PE	Muscle fiber plus Phosphatidyl Ethanolamine	Muscle fiber plus PE and Nonanal	Muscle fiber plus Nonanal	Muscle fiber plus Nonanal
Lysine	1.74	1.77	1.21	1.00	0.71	0.39	0.39
Histidine	0.55	0.61	0.99	0.65	0.79	0.74	0.74
Arginine	1.24	1.20	1.11	1.02	1.09	1.20	1.20
Aspartic Acid	1.81	1.80	1.58	1.65	1.49	1.74	1.74
Threonine	1.08	1.05	1.00	0.89	1.10	0.89	0.89
Serine	0.68	0.71	0.76	0.68	0.71	0.67	0.67
Glutamic Acid	2.74	2.76	2.67	2.57	2.69	2.95	2.95
Proline	0.69	0.63	1.15	0.72	1.00	0.59	0.59
Glycine	0.72	0.69	0.92	0.89	0.90	0.86	0.86
Alanine	1.04	1.12	0.96	0.82	0.76	0.70	0.70
Cysteine	-	-	-	-	-	-	-
Valine	1.06	1.11	0.97	1.21	1.21	0.95	0.95
Methionine	0.23	0.25	0.00	0.05	0.00	0.00	0.00
Isoleucine	1.03	1.00	1.10	0.98	1.02	0.03	0.03
Leucine	1.55	1.49	1.51	1.62	1.58	1.55	1.55
Tyrosine	0.62	0.65	0.49	0.42	0.27	0.10	0.10
Phenylalanine	0.84	0.92	0.62	0.27	0.32	0.10	0.10

groups during oxidation, as no other reactive groups were present in the system. To examine the nature of these products, the extracts were dissolved in 50ml of diethyl ether and divided into two portions. To one portion was added 2N NaOH and to the other, 2N HCl. These stood over night at room temperature with stirring. The sample treated with HCl was partially hydrolyzed; but the sample treated with NaOH was completely hydrolyzed, liberating free aldehydes. The R<sub>f</sub> value, on a TLC plate, of the hydrolyzed product was similar to that of reference oleyl aldehyde. When only oleyl aldehyde was present, condensation occurred between carbonyl groups; but the presence of myoglobin had no apparent effect on the product formation.

The presence of myoglobin enhanced oxidation of unsaturated acyl groups in phospholipids, and increased polymer formation and decreased the carbonyl-amine interaction.

#### 5. Protein as a Matrix

A number of studies have shown that oxidizing fatty acids may induce changes in proteins and enzymes (19,20). Radicals derived from oxidizing lipids have been implicated in some of the damage. Other reactions involve carbonyl-amine reactions as in Maillard browning to form Schiff bases and secondary products. Studies with carbonyl-amine reactions in lipid systems have shown that Schiff bases are formed and other products such as lyso-compounds, scission compounds, polymers and reformed or unchanged phosphatidyl ethanolamine are present. The presence of appropriate functional groups should provide the possibility for competitive reactions when food systems involving proteins serve as the base instead of the cellulose, which has been used in this investigation. It was decided to use lipid-free protein fibers as the base for lipids and lipid derived components to examine whether the PE-carbonyl reactions were influenced by a different system.

Crude muscle fiber (free of lipids and phospholipids) was prepared as follows. Two pounds of fresh center cut beef round muscle was trimmed of fat and connective tissue and chopped into small pieces which were frozen. The frozen muscle was freeze-dried for 40 hrs. It was then ground and extracted with diethyl ether in a Soxhlet extraction apparatus. This process was continued until the extract gave a negative test for any type of lipids. Model systems were prepared on the muscle fiber dispersed in borate buffer at pH 6. The emulsion was frozen and then freeze-dried in a Stokes freeze dryer to a moisture level of 2.5%. The following model systems were prepared:

- (a) Muscle fiber + Hydrogenated phosphatidyl ethanolamine
- (b) Muscle fiber + Phosphatidyl ethanolamine
- (c) Muscle fiber + Phosphatidyl ethanolamine + Nonanal

- (d) Muscle fiber + Phosphatidyl ethanolamine
- (e) Muscle fiber + Nonanal

The freeze-dried model systems were stored at RH 14% and ambient temperature for 30 days. They were extracted with (1:1) chloroform:methanol. The extracts were concentrated under an atmosphere of nitrogen and spotted on a basic silica gel G plate and developed with chloroform:methanol:water 65:25:4 by volume. Various phosphorus containing products were isolated from the plate and the percentage of each product formed (based on phosphorus analysis) are given in Table 27.

Schiff bases were isolated from those systems in which they were formed by using Sephadex LH 20, as described in other studies. The isolated Schiff base fractions were further purified by thin-layer chromatography and characterized. The IR spectrum of the Schiff base (I) from PE and nonanal isolated from model system (c) had an absorption band for C = N at  $1600\text{cm}^{-1}$ , and for P - O - C at  $1050\text{cm}^{-1}$  (Fig. XXXIX). The ultraviolet spectrum had absorption at  $280\text{ m}\mu$  (Fig. XL) and maximum fluorescence was exhibited at  $450\text{ m}\mu$  at an excitation of  $350\text{ m}\mu$  (Fig. XLI).

Another Schiff base fraction (II) was isolated which presumably was a reaction product from added carbonyl compound with the protein. The IR spectrum of this fraction had absorption bands for C = N at  $1640$  and  $1685\text{cm}^{-1}$ , but the bands noted in phospholipid browning for P - O - C are absent (Fig. XLII). The ultraviolet spectrum had absorption at  $240\text{ m}\mu$  and  $280\text{ m}\mu$  (Fig. XL) and maximum fluorescence was exhibited at  $465\text{ m}\mu$  at an excitation of  $360\text{ m}\mu$  (Fig. XLIII). All these are characteristic of Schiff base. This Schiff base fraction gave a positive test for nitrogen and a negative test for phosphorus. Apparently then, this Schiff base is a product resulting from reaction between carbonyl compounds and protein amino groups present in the muscle fiber.

To confirm the interaction of added aldehydes with the protein moieties in the muscle fiber, amino acid analysis was made on the original muscle fiber before reaction and on chloroform:methanol extracted muscle fiber in all model systems. The amino acid analysis is shown in Table 28.

Amino acid analysis showed greater losses of amino acid in the model systems where only nonanal was present in the muscle fiber and least when phosphatidyl ethanolamine alone was present. When phosphatidyl ethanolamine was present together with nonanal, losses were greater when compared with a model system in which PE alone was present. The aldehydes present apparently contributed to Schiff base formation between phosphatidyl ethanolamine and proteins so that the interaction was distributed between the two reactive substrates. When plasmalogen PE was present, considerable losses in amino acid composition were observed. During incubation, the vinyl ether moiety apparently was hydrolyzed to yield aldehydes. The carbonyls produced by oxidation of the polyunsaturated fatty acids at the  $\beta$ -position in plasmalogen PE contributed also to

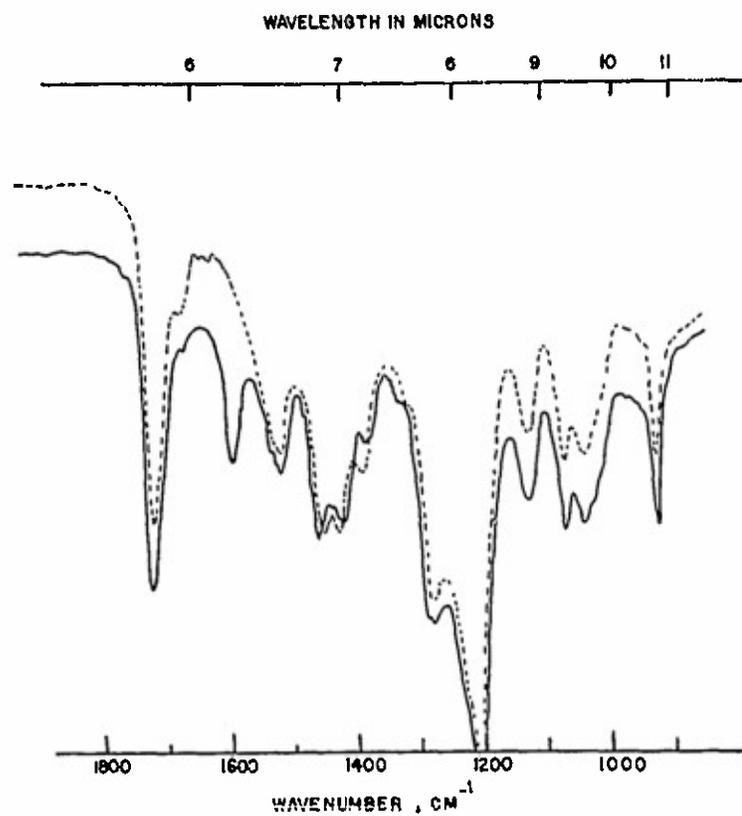


Fig. XXXIX. IR spectrum of Schiff base from PE and nonanal on muscle fiber.

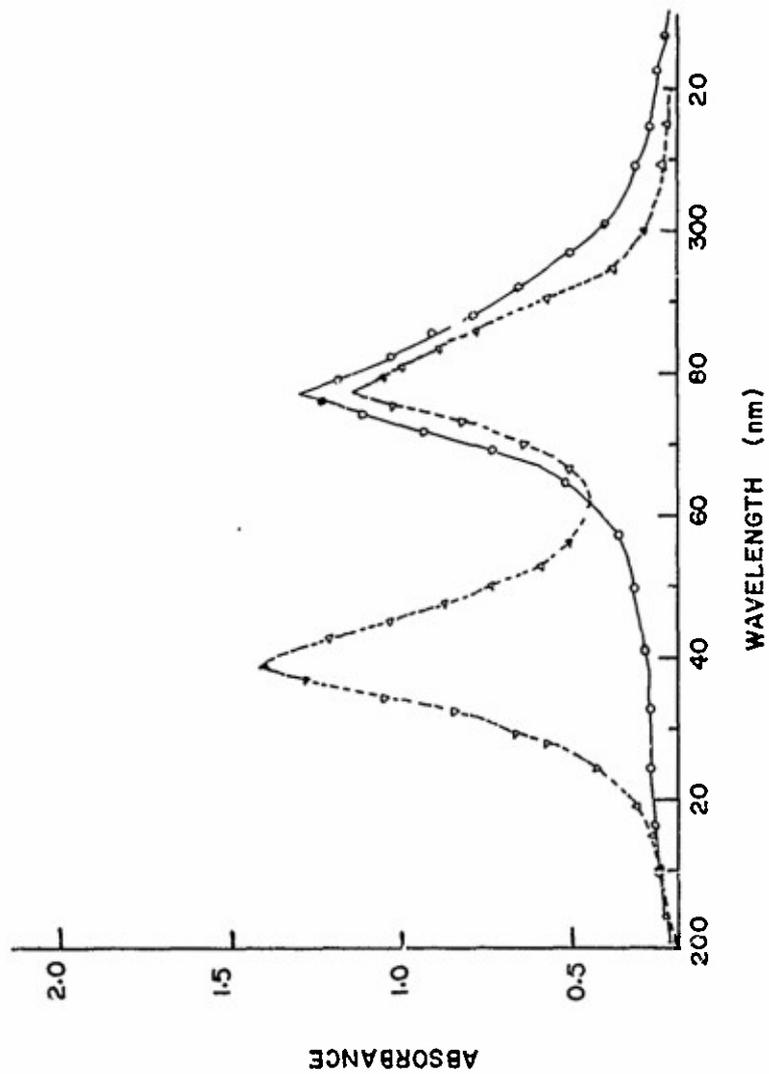


Fig. XL. UV spectrum of Schiff base from PE and nonanal on muscle fiber. (Schiff base I, PE + nonanal on muscle fiber - solid line; Schiff base II, amino acid of protein + aldehyde - broken line.)

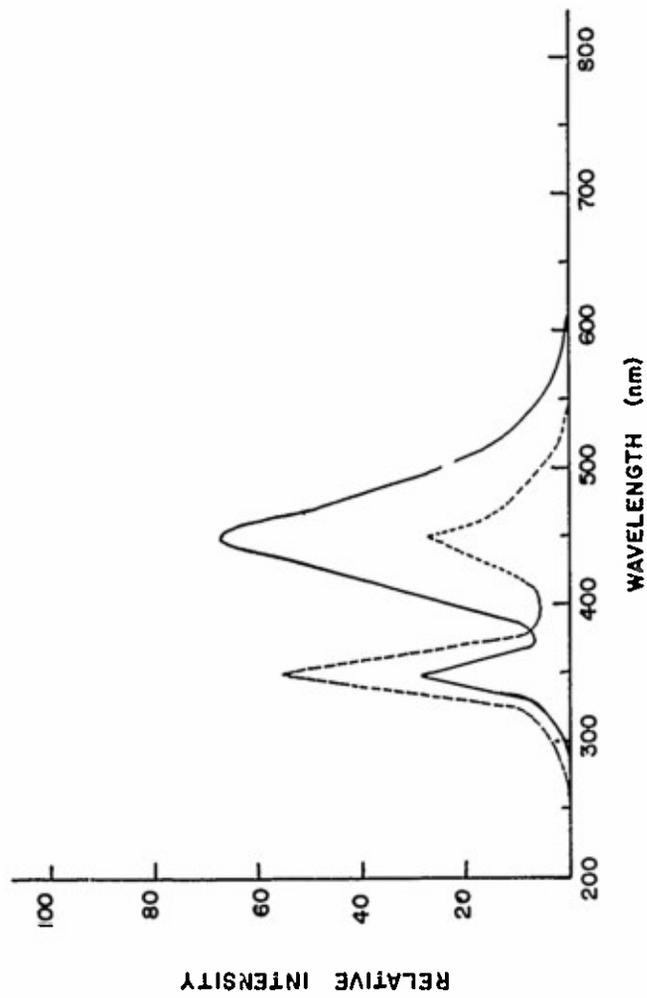


Fig. XLI. Fluorescence spectra of Schiff base from PE and nonanal on muscle fiber.

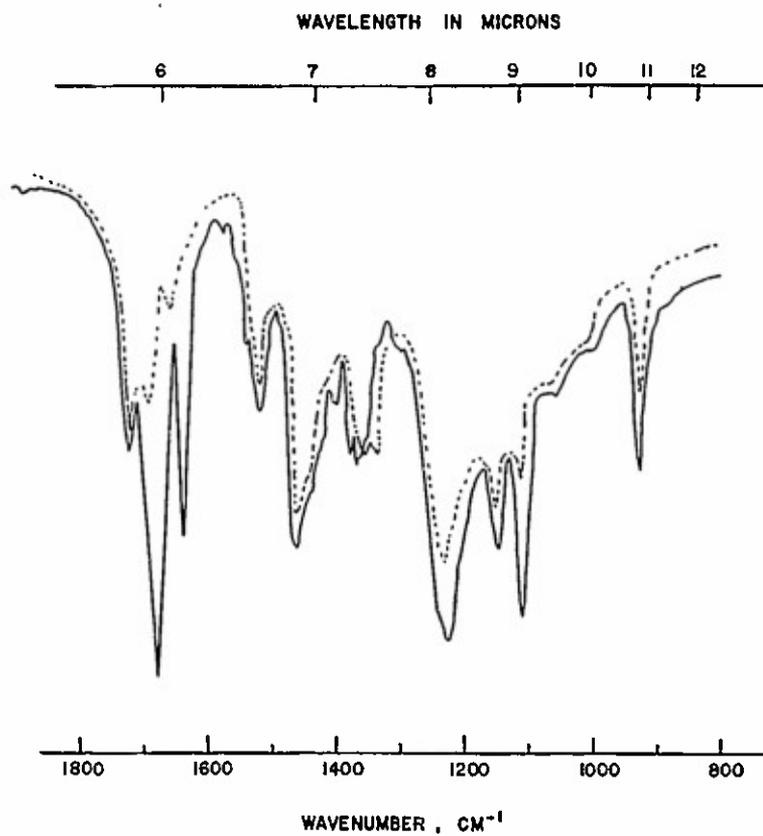


Fig. XLII. IR spectrum of Schiff base (II) from protein and nonanal.

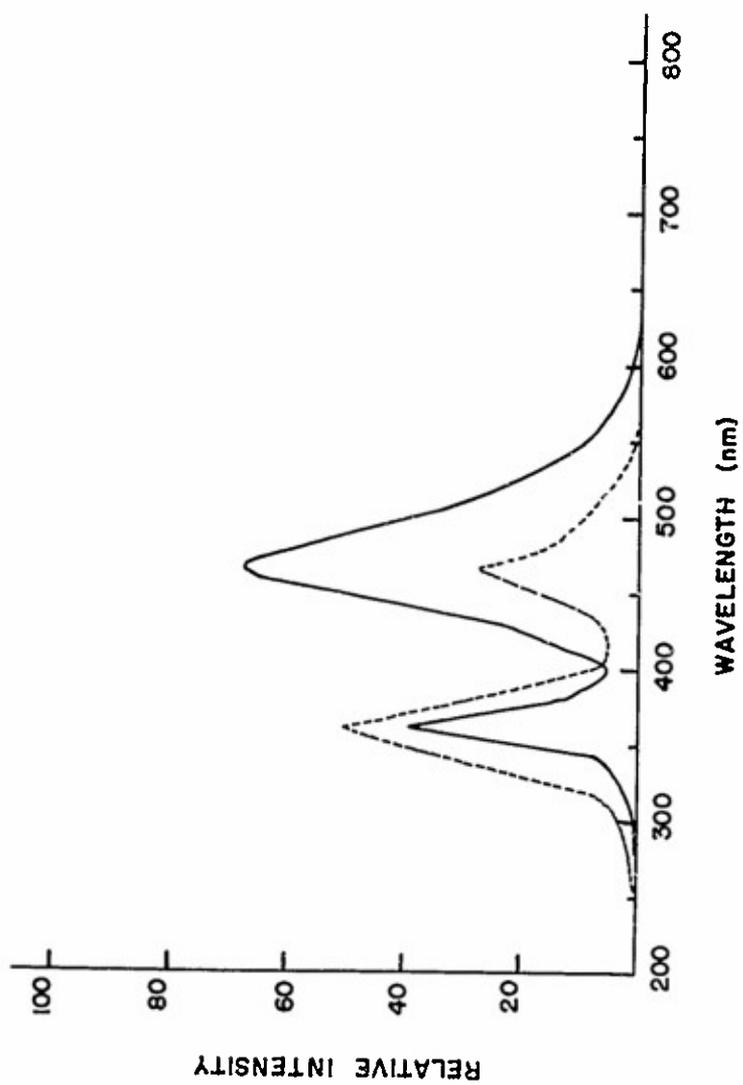


Fig. XLIII. Fluorescence spectra of Schiff base (iI) from protein and nonanal.

carbonyl-amine interaction in proteins. When carbonyl compounds were in the model system, a carbonyl-amine interaction occurred and losses of amino acids occurred. When only hydrogenated phosphatidyl ethanolamine was present, no losses of amino acid were observed. Losses were greatest in lysine, alanine, methionine, tyrosine and phenylalanine. The methodology available did not permit the estimation of cysteine on the samples.

A competitive reaction system was present when amino groups from PE in its various forms and from the protein matrix were available for reaction with carbonyl groups. This is evident from the differential loss of amino acids in the protein matrix when PE was present or absent. This could be a factor both in flavor quality, texture and color characteristics, and nutritional value of a dry food system in which phospholipids and proteins are present.

#### S U M M A R Y

The carbonyl-amine reactions in a simulated dry-food system involving phosphatidyl ethanolamine and carbonyl compounds, which occur naturally or can be derived readily from system components, have been investigated. The lipid-browning reactions proceed readily at low moisture level (2.5%) and at ambient or elevated temperature. Differences in product formation were detected with respect to (1) state of saturation of the phosphatidyl ethanolamine; (2) nature of the carbonyl compound; (3) the pH of the system; (4) the temperature, time and humidity conditions; (5) the presence or absence of myoglobin; and (6) the oxidative nature of the atmosphere.

Simulated dry food systems were prepared by dispersing the reactants in a cellulose emulsion at a buffered pH, freeze-drying the emulsion to a 2.5% moisture level, and holding under RH 14% at ambient temperatures or at 50C for shorter periods for reactions to take place.

Extraction, isolation, and separation systems were developed to make possible the study of the products of the reactions.

The formation of Schiff bases was the dominant feature of nearly all the reaction systems. Secondary or consecutive reactions to form other products such as oxypolymers, other polymers, methyl phosphatidate, and other less well defined scission products took place to varying degree according to different conditions imposed on the systems.

Saturated aliphatic aldehydes reacted with PE in the model systems to form Schiff bases, polymers and scission products. Among the scission products were components identified as methyl- or ethyl phosphatidate. This was proposed to be derived from a scission reaction of the prior formed Schiff base. Methyl phosphatidate was derived only from systems

in which saturated aldehydes participated or systems with unsaturated aldehydes in which the double bond was not conjugated to the carbonyl oxygen bond. Aldehydes such as oleyl aldehyde and 10-undecene-1-al behaved essentially as saturated aldehydes in this sense. The reactions were pH dependent and the relative amounts of products formed varied according to the pH. The greatest amount of Schiff base was found in the system held at pH 6.0. Scission and other secondary reactions were formed at pH 8.0. A stabilizing effect due to protonation of phosphatidyl ethanolamine resulted at pH 5.0 or lower pH and reduced the reactivity of PE with carbonyls or possibly caused reverse reactions to occur with a consequently greater amount of unreacted PE in these systems.

Unsaturated aldehydes of the en-al and dienal type participated in the carbonyl-amine reaction. When Schiff bases were formed, they were apparently capable of undergoing the scission reaction involving formation of methyl phosphatidate, due to the stabilizing effect of the double-bond conjugation. This suggests that the scission referred to may be a consequence of reactions which require a double bond migration. Schiff base formation was less with 2,4-dienals than with other aldehydes since dienals readily oxidize to other products and enhanced polymer formation results in the system.

Malonaldehyde reacted readily with PE to form products which were quite stable as a consequence of the enamine-imine type structure. A mixture of 2-hexene-1-al, 2,4-hexadienal, and malonaldehyde would provide a mixture of product Schiff bases in which those derived from malonaldehyde would be in greatest quantity and those from the dienal in least quantity.

The presence of myoglobin in the system affected the formation of carbonyl-amine reaction products through its pro-oxidant effect. The increased production of carbonyl compounds from oxidation of polyunsaturated fatty acids allowed more Schiff base and secondary product formation. The polymer content was enhanced and, in the systems containing plasmalogens, there was greater lyso-compound formation to permit the formation of lyso-Schiff bases.

Plasmalogens participated in the carbonyl-amine reactions in a variety of ways. The  $-NH_2$  of phosphatidyl ethanolamine reacted with added aldehydes to form Schiff bases and other products similarly to the behavior of phosphatidyl ethanolamine. The  $-NH_2$  of the lyso-compound resulting from hydrolytic cleavage of the vinyl ether group also participated in the reactions. The aldehyde formed by cleavage of the vinyl ether could react with the  $-NH_2$  of unchanged phosphatidyl ethanolamine or that in the lyso-compound.

Polymeric compounds were formed. Some may have been oxypolymers and some may have been Schiff base type polymers. The latter were postulated to be formed by reaction of  $-NH_2$  in an intact PE molecule

with the terminal aldehyde group at the 2-position in PE when oxidation of the unsaturated fatty acids resulted in scission products containing an aldehyde-ester. A triglyceride was synthesized containing a terminal aldehyde group on the ester at the 2-position. This reacted with PE to form compounds which behaved quite similarly to the polymers isolated from the various model systems studied.

PE reacted readily with glucose to form Schiff bases, secondary products, and scission products. Scission products contained a compound containing both nitrogen and phosphorus and a diglyceride. It was postulated that the Schiff base had undergone an Amadori rearrangement and other changes resulting in cleavage of the C - O - P bond. Molecular weight of the compound was determined at 298 and a calculated mol. wt. of 301 or 303 agreed quite well. Spectral data indicated typical Schiff base characteristics.

Oxygen uptake studies were utilized to examine oxidizability of the systems and their components. Major uptake of oxygen occurred with non-hydrogenated PE and with added aldehydes. The Schiff bases apparently were oxidizable to some degree. The vinyl ether group of plasmalogens was very stable to further oxidation.

Flavor evaluation studies made on various systems indicated that the development of off-flavors may be better related to the carbonyl compounds formed in a system than to the carbonyl-amine compounds or their derivatives which subsequently may be formed.

Preparation of a relatively pure phosphatidal ethanolamine derived from the requirement for suitable quantities to use in model system studies. The use of pancreatic lipase, with sucrose to suppress the phospholipase A activity, caused the hydrolysis of the  $\alpha'$ -acyl group in PE to form a lyso-compound which readily could be separated from the plasmalogen which was relatively unaffected by the enzymatic hydrolysis.

The reactions between PE and aldehydes on cellulose were compared to the reactions on a protein (meat fiber) matrix. The carbonyl reactions proceeded both with PE and free  $-NH_2$  groups of the protein. Certain amino acids such as lysine, alanine, phenylalanine, and tyrosine were reacted with by aldehydes sufficiently to reduce their quantity in the total amino acid pool of the products formed. The reactions were competitive so that the presence of PE had a sparing effect on amino acids in the protein. This observation may be important to understanding nutritive changes in dried foods containing protein and phospholipids.

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