Comparison of the Binding of Metal Ions by the 11S and 7S Proteins. The measurements with 11S and 7S proteins indicated the following similarities and differences. Both the proteins bind Ca(II) and Mg(II) at pH 7.8 possibly at the imidazole groups of histidine residues. They bind Zn(II) at pH 6.5 also at the imidazole groups. A change in buffers (borate or Tris-HCl) had no effect on the binding of Ca(II) by 11S protein. However, the binding of Ca(II) or Mg(II) by 7S protein was higher in borate buffer than in Tris-HCl buffer. This is attributed to the increased negative charge on the protein due to the formation of a borate–carbohydrate complex; 7S protein is a glycoprotein. The Scatchard plot of Ca(II) binding by the 11S protein consisted of a single straight line. On the other hand the plot of Mg(II) or Zn(II) binding consisted of two straight lines suggesting nonequivalence of binding sites. In the case of 7S protein the binding of all the three metals gave Scatchard plots which consisted of single straight line. The addition of NaCl to the buffer had no effect on Zn(II) binding by 11S protein; it decreased Ca(II) binding and totally suppressed Mg(II) binding. In the case of Zn(II) binding by 7S, this was decreased by the addition of NaCl.

The addition of Ca(II) or Mg(II) to the EDTA-treated 11S protein caused changes in the sedimentation velocity pattern of protein which suggested reaggregation of the protein. In the case of 7S protein no such effect was observed. The addition of metal ions to 11S protein or 7S protein did not affect the rate of hydrolysis of protein by α-chymotrypsin. ORD and CD spectra suggested minor conformational changes in 11S protein due to the binding of Ca(II) or Mg(II). However, no such effect was observed in the case of 7S protein. The fluorescence spectrum of both the proteins was unaffected by the addition of metal ions. Ca(II) and Mg(II) enhanced the turbidity caused by heat coagulation of 11S and 7S proteins and Zn(II) enhanced precipitation. At room temperature addition of Ca(II), Mg(II), and Zn(II) at higher concentrations precipitated the 11S protein quantitatively. On the other hand, Ca(II) precipitated the 7S protein to 40%, Mg(II) 10%, and Zn(II) 90%. In all the cases addition of NaCl reduced precipitation.

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Metmyoglobin and Nonheme Iron as Prooxidants in Egg-Yolk Phospholipid Dispersions and Cooked Meat

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The influence of adding either metmyoglobin (MetMb) or ferrous, nonheme iron (Fe^{2+}) upon oxidation of egg-yolk phosphatidylethanolamine (PE) was studied at pH 5.5 and 7.0. Control (untreated) PE dispersions had greater oxygen uptake at pH 7.0 than at pH 5.5, whereas addition of either MetMb or Fe^{2+} caused little further oxidation at pH 7.0. On the other hand, both Fe^{2+} and MetMb accelerated oxidation of PE by three- to fourfold at pH 5.5. MetMb increased the production of fluorescent materials from PE at both pH 5.5 and 7.0, while Fe^{2+} accelerated production of fluorescent compounds only at pH 5.5 and inhibited fluorescence at pH 7.0. Tentative identification of *n*-hexanal in the headspace of oxidizing PE dispersions and of cooked meat by GLC (gas–liquid chromatography) suggested that it is one of the principal products of oxidation. Addition of 5 ppm of Fe^{2+} to water-extracted and heated meat produced an *n*-hexanal peak over twice the area of the control, whereas addition of tripolyphosphates decreased the quantity of hexanal by one-half. Results indicate that both Fe^{2+} and MetMb may act as prooxidants under certain conditions.

Phospholipids undergo rapid oxidation in many food products, including cooked meat, which results in serious flavor problems (Keller and Kinsella, 1973; Younathan and Watts, 1960). The speed of oxidation for phospholipids in cooked meat is at least partially due to their high content of unsaturated fatty acids (Luddy et al., 1970; O'Keefe et al., 1968; Giam and Dugan, 1965; Hornstein et al., 1961). The nature of the nitrogenous component bound in ester linkage to the phosphoric acid moiety may also influence the oxidation of the unsaturated fatty acids in the phospholipid molecule (Corliss and Dugan, 1970; Tsai and Smith, 1971).

Tsai and Smith (1971) studied the effects of ethanolamine, choline, and serine on the oxidation of methyl

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linoleate in aqueous emulsions. They reported that ethanolamine increased oxygen uptake by methyl linoleate at pH 7.9, whereas the choline had no effect upon the rate of lipid oxidation, and serine decreased oxygen consumption by methyl linoleate at pH 7.9. Similar results were obtained when phosphorylated bases were substituted. Corliss and Dugan (1970) reported that the ethanolamine moiety of phosphatidylethanolamine exerted a greater prooxidant effect than the choline portion of phosphatidylcholine. The rate of phospholipid oxidation during the steady state was reported to be a function of the unsaturation of the fatty acid components of phospholipids. In view of these facts, phosphatidylethanolamine would seem to be particularly susceptible to oxidation in meat, which was substantiated by Keller and Kinsella (1973) since they found more than a 25% loss of arachidonic acid from the phosphatidylethanolamine in cooked ground beef.

The speed with which muscle lipids oxidize indicates that substances promoting lipid oxidation are present in muscle. Hemoprotein muscle pigments have frequently been implicated as catalysts of lipid oxidation in meat (Hirano and Olcott, 1971; Kendrick and Watts, 1969). Recently, Love and Pearson (1974) and Sato and Hegarty (1971) have presented evidence showing that nonheme iron is the major prooxidant in cooked meat, while heme pigments have no catalytic effects on lipid oxidation in cooked meat systems.

A number of studies (Liu, 1970; Kendrick and Watts, 1969; O'Brien, 1969; Barber, 1966; Wills, 1965, 1966) investigating the prooxidant activities of nonheme iron have been carried out using either purified unsaturated fatty acids or fatty acid hydroperoxides as lipid substrates. Similar studies using phospholipids would be of interest since phospholipids undergo the most rapid oxidation in cooked meat (Younathan and Watts, 1960). Thus, this study was carried out to investigate the role of metmyoglobin (MetMb) and ferrous, nonheme iron (Fe^{2+}) in accelerating the oxidation of an aqueous emulsion of egg-yolk phosphatidylethanolamine (PE). Egg-yolk PE was used in the model system because of the similarity of its fatty acid composition to that of beef and pork (Love, 1972; Hornstein et al., 1961). Studies with the simple model system may help explain the effects of prooxidants, such as hemoproteins and nonheme iron, on lipid oxidation in meat and other lipid containing foods.

MATERIALS AND METHODS

Isolation of Egg-Yolk Phosphatidylethanolamine (PE). The lipid substrate used in the model studies was egg-yolk PE, which is highly unsaturated (Holub and Kuskis, 1969).

PE was prepared by a modification of the method of Olivecrona and Oreland (1971) by first extracting egg-yolk lipids with chloroform–methanol (2:1, v/v). The extracted lipids were then diluted to 50 ml with chloroform and added to 700 ml of cold (4 °C) acetone. The acetone-insoluble material was redissolved in chloroform and reprecipitated twice. Thin-layer chromatography on silica gel G using a solvent system of chloroform–methanol–water (65:25:4 by volume) showed the final precipitate contained mainly phospholipids. The purified PE was then isolated by preparative scale thin-layer chromatography on 20 × 20 cm glass plates coated with 0.75 mm of silica gel G. The developing solvent system was chloroform–methanol–water (65:25:4 by volume). Isolation procedures were carried out at low temperatures (4 °C) and in an inert atmosphere. The purified PE was dissolved in pentane and transferred to glass vials, which were flushed with nitrogen gas, sealed with Teflon screw caps, and stored at −30 °C.

Preparation of Aqueous Dispersions of PE. An aliquot of purified PE containing a known quantity of phosphorus, which had been determined by the procedure of Rouser et al. (1966), was removed from storage and the solvent was evaporated under nitrogen. Buffer (0.05 M Tris-maleate or borate, pH 5.5 or 7.0) containing 0.25% Tween 20 was added and the samples stirred on a magnetic stirrer until the PE was thoroughly mixed with the buffer. KOH (1.0 M) was added dropwise, while stirring was continued, until the lipid was dispersed. Buffer was added to give the desired volume, and the pH adjusted with 1 N HCl, if necessary. Aliquots of the PE dispersion were added to appropriate reaction vessels and buffer, MetMb, or Fe^{2+} was added. The reaction mixtures were then incubated at 38 °C in a constant temperature water bath with continuous shaking.

Measures of Lipid Oxidation. Oxygen uptake by the PE dispersions oxidizing at 38 °C was measured in a Gilson differential respirometer (Model GR14, Gilson Medical Electronics). Browning of the PE dispersions during incubation at 38 °C was assessed by measuring fluorescence of a 2:1 chloroform–methanol extract of the dispersion. An Aminco-Bowman spectrophotofluorometer and an Aminco x–y recorder were utilized to measure and record fluorescence. The wavelength for maximum excitation was 360 nm and that for maximum emission was 430 nm.

Losses in unsaturated fatty acids were followed using gas–liquid chromatography. The PE was extracted from the samples with chloroform–methanol (2:1, v/v) and esterified with 14% boron trifluoride in methanol as described by Morrison and Smith (1964). The methyl esters of the fatty acids were subjected to gas chromatography using a Varian Aerograph 200 gas chromatograph equipped with a flame ionization detector. The column was a $^1/_8$ in. (o.d.) 7 ft stainless steel column packed with 15% high-efficiency DEGS (diethylene glycol succinate) on acid-washed 80–100 mesh Chromosorb W. The temperature of the column was maintained at 180 °C, the injection port at 210 °C, and the detector at 195 °C. The flow rate of the prepurified nitrogen carrier gas was 40 ml/min. Samples of 2 μl were injected with a microsyringe. The emerging peaks were identified by comparing their retention volumes with those of standard mixtures of known methyl esters of fatty acids. Quantification was accomplished by multiplying peak height by peak width at half-height and expressing as percentage of total.

Analysis of Volatiles Produced by Oxidizing Phospholipids and Cooked Beef. Beef muscle (eye of round) was extracted with water as outlined by Love and Pearson (1974). The water extracted muscle tissue was heated to 70 °C and tripolyphosphate (TPP) was added to a 20-g aliquot at 0.5% by weight. Fe^{2+} (5 ppm) was added to a second 20-g portion, while a third 20-g portion without added prooxidants or inhibitors served as the control. In all cases, the additives (TPP and Fe^{2+}) were thoroughly mixed with the sample. The cooked samples and phospholipid dispersions were stored in Erlenmeyer flasks, which were sealed with rubber serum stoppers so that samples of volatiles could be removed without opening the flasks. The meat samples were held at 4 °C and the phospholipid dispersions at 38 °C.

Headspace samples (2.5 ml) were withdrawn from above the meat or the phospholipid dispersions using a gas-tight syringe at regular intervals of time. In order to get reproducible results, a standard sampling technique was

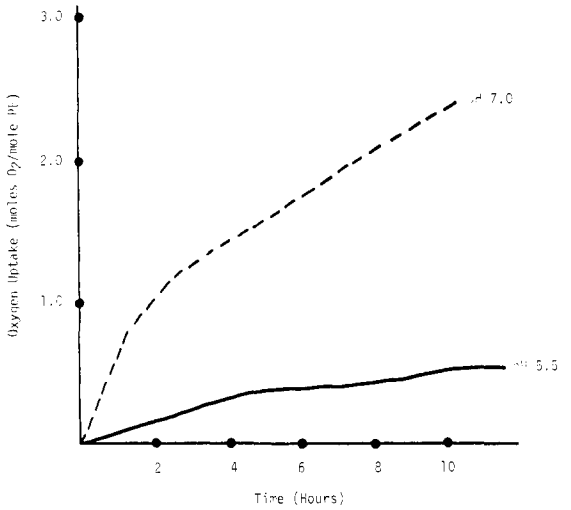

Figure 1. Rate of oxygen uptake of aqueous PE dispersions at pH 7.0 (- - -) and pH 5.5 (—) over a 10-h period at 38 °C.

Table I. Oxygen Uptake of Oxidizing PE Dispersions with and without MetMb and Fe^{2+} at pH 5.5 and 7.0

System	Oxygen uptake,[a] mol of O_2/mol of PE
PE, pH 5.5	0.4
PE, pH 7.0	2.3
PE + 1.5×10^{-4} M Fe^{2+}, pH 5.5	1.5
PE + 1.5×10^{-4} M Fe^{2+}, pH 7.0	2.0
PE + 5.0×10^{-6} M MetMb, pH 5.5	2.3
PE + 5.0×10^{-6} M MetMb, pH 7.0	2.1

[a] PE was at a concentration of 2.5×10^{-4} M and oxygen uptake is reported as the total for the 10-h period at 38 °C.

developed. The flasks containing the meat were held at 100 °C for 10 min with the serum stoppers vented to the atmosphere before sampling. The syringe was flushed with the headspace vapor before removing the sample to be analyzed.

Separation of the headspace volatiles was achieved on a Varian Aerograph 200 chromatograph equipped with a flame ionization detector and a $^1/_8$ in. (o.d.) 7 ft stainless steel column packed with Carbowax 20M on 60/80 mesh acid-washed Chromosorb W. Temperatures of 70, 150, and 140 °C were maintained for the column oven, injection port, and detector, respectively. The nitrogen carrier gas flow rate was 15 ml/min.

RESULTS AND DISCUSSION

Studies with PE. Oxidation of an aqueous dispersion of PE was more rapid at pH 7.0 than at pH 5.5 as shown in Figure 1. Oxygen uptakes of the PE dispersions upon the addition of MetMb and Fe^{2+} are shown in Table I. At pH 5.5 (the approximate pH of raw meat) addition of either Fe^{2+} or MetMb resulted in an increase in oxygen consumption. At pH 7.0, neither Fe^{2+} nor MetMb increased oxygen consumption over that of the samples containing only PE. This suggests that PE in fresh meat systems (pH 5.3–5.7) may be less susceptible to oxidation than in cooked meat systems (pH 5.9–6.2) where the higher pH may favor oxidation. This is further illustrated by the data in Table I, which shows oxygen uptake by PE is about sixfold greater at pH 7.0 than at pH 5.5.

Total oxygen uptakes for the PE system with added MetMb or Fe^{2+} at both pH 5.5 and 7.0 are also shown in Table I. At pH 5.5, both MetMb and Fe^{2+} accelerated oxidation by three- to fourfold, but at pH 7.0 neither MetMb nor Fe^{2+} caused any further increase in oxidation of PE. Fe^{2+} has been reported to have maximum prooxidant activity at pH 5.0–5.5 (Wills, 1965) and to cease functioning as a prooxidant above pH 6.4 (Liu, 1970). On the other hand, Liu (1970) reported that catalysis of lipid oxidation by myoglobin increased directly with pH in the range of 5.6–7.8. However, several other investigators (Ben-Aziz et al., 1970; Kendrick and Watts, 1969; O'Brien, 1969) have stated that myoglobin is a more effective prooxidant at acid pH values.

Aqueous dispersions of PE developed a yellow-brown discoloration after several hours of oxidation. The extent of browning appeared to be related to the amount of oxygen consumed by the PE dispersions. This observation is in agreement with Corliss and Dugan (1970) and Lea (1957), who have noted rapid development of brown discoloration in oxidizing PE.

The brown pigment was extractable from the PE dispersions with chloroform–methanol (2:1, v/v) and was found to contain fluorescent compounds. As shown in Figure 2, the extract exhibited maximum excitation at 360 nm, while the emission maximum occurred at 430 nm. No further attempts were made to characterize the fluorescent compounds, although their excitation and emission maxima were similar to those reported by Dillard and Tappel (1973) for fluorescent Schiff base compounds formed by the free amino groups from PE reacting with products from oxidizing lipids. As shown in Figure 3, fluorescent intensity increased during the first 6 h of oxidation at either pH 5.5 or 7.0, although less fluorescent material was formed at pH 5.5 than at pH 7.0. After 12 h of oxidation, the samples at pH 7.0 had a considerably greater fluorescent intensity than the samples at pH 5.5. By 24 h, the fluorescence of the PE samples at pH 7.0 had declined slightly, whereas the PE samples at pH 5.5 were still increasing in fluorescence.

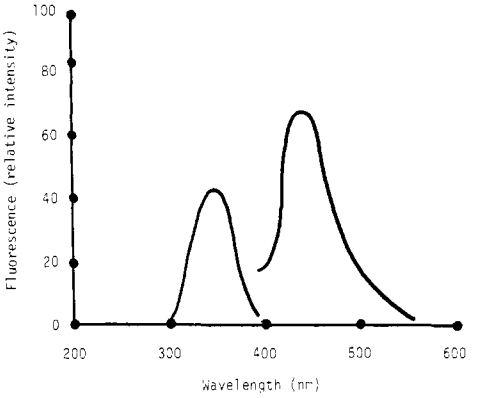

Figure 2. Fluorescence spectra of a chloroform–methanol extract of oxidizing PE dispersion.

Table II. Changes in Fluorescence of Oxidizing PE Dispersions Due to Addition of MetMb or Fe^{2+}

Sample[a]	Increase in fluorescence over PE alone
MetMb, pH 5.5	2.80
MetMb, pH 7.0	1.90
Fe^{2+}, pH 5.5	2.70
Fe^{2+}, pH 7.0	0.73

[a] After 24 h of oxidation at 38 °C using the following concentrations of reactants: PE = 3.0×10^{-4} M; Fe^{2+} = 1.5×10^{-4} M; and MetMb = 2.5×10^{-6} M.

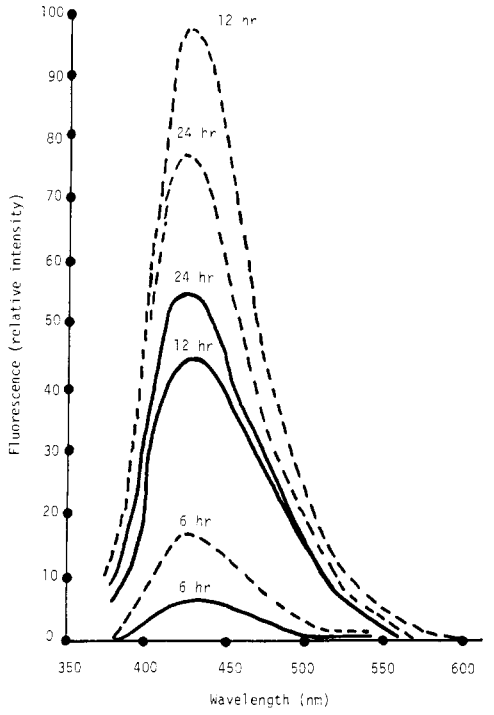

Figure 3. Emission spectra of phosphatidylethanolamine after 6, 12, and 24 h at 38 °C. Samples were incubated at either pH 5.5 (—) or pH 7.0 (- - -).

Table III. Ratio of Unsaturated Fatty Acids to Saturated Fatty Acids in Fresh PE and PE Held for 24 h at 38 °C

Sample[a]	Ratio of unsaturated to saturated fatty acids	
	pH 5.5	pH 7.0
PE, freshly prepared, no storage	1.60	1.60
PE stored 24 h at 38 °C	0.80	0.65
PE + Fe^{2+} stored 24 h at 38 °C	0.32	0.50
PE + MetMb stored 24 h at 38 °C	0.72	0.69

[a] Concentrations: PE = 3.0×10^{-4} M; $Fe^{2+} = 1.5 \times 10^{-4}$ M; MetMb = 2.5×10^{-6} M.

Incorporation of Fe^{2+} increased production of fluorescent intensity at pH 5.5 by two- to threefold (Table II). Addition of Fe^{2+} at pH 7.0 caused a decrease in fluorescence. Addition of MetMb increased the production of fluorescent materials at both pH 5.5 and 7.0. Blanks containing only buffer and MetMb or Fe^{2+} yielded insignificant amounts of fluorescent materials.

Data showing the losses in unsaturated fatty acids from PE at 24-h oxidation at 38 °C are shown in Table III. At pH 5.5, addition of Fe^{2+} caused the greatest amount of destruction of unsaturated fatty acids, whereas MetMb caused little or no loss as compared to the PE control at 24 h. At pH 7.0, the prooxidant effect of Fe^{2+} was less pronounced than at pH 5.5.

The destruction of MetMb incubated with PE dispersions at pH 5.5 for 24 h at 38 °C was indicated by the disappearance of the characteristic spectral peaks. However, the spectra of MetMb incubated at pH 7.0 for 24 h at 38 °C with PE dispersion were very similar to the spectra of freshly prepared samples or of MetMb in buffer held for 24 h at 38 °C. Apparently, the heme moiety of MetMb catalyzes the oxidation of fatty acids in PE at pH 5.5 and is degraded during the process. At pH 7.0, lipid oxidation proceeded rapidly in the PE dispersions, although MetMb did not accelerate oxygen uptake (Table I). Erikkson et al. (1970) have shown that acid treatment of hemoproteins results in increased prooxidant activity. They theorized that this is due to increased exposure of the heme group to the lipid substrate. These authors further reported that heating of hemoproteins increased the exposure of the heme to unsaturated fatty acids.

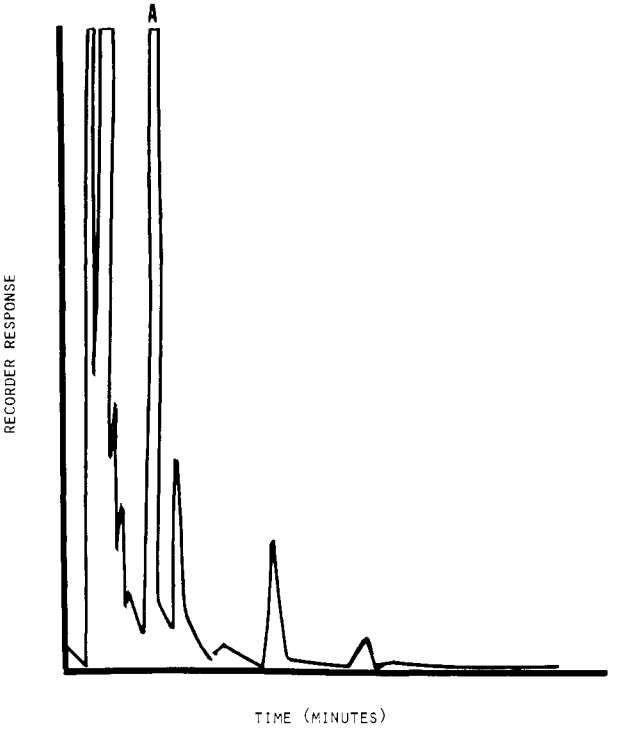

Figure 4. GLC chromatogram of volatiles produced by an oxidizing aqueous dispersion of PE. Peak A was tentatively identified as *n*-hexanal by comparison with the retention volume of an *n*-hexanal standard. Conditions are: Varian Aerograph chromatograph 200 with flame ionization detector; column, $^1/_8$ in. o.d. 7 ft stainless steel packed with Carbowax 20M on 60/80 mesh acid-washed Chromosorb W; temperatures, column oven, 70 °C; injection port, 150 °C; and detector, 140 °C; column flow rate, 15 ml/min nitrogen carrier gas.

Studies on Meat and Oxidizing Phospholipids. A characteristic oxidized odor was noted in some of the PE dispersions in these studies. In order to elucidate the nature of the oxidized odor, the volatiles from the headspace of aqueous dispersions of phospholipids were analyzed by gas–liquid chromatography and compared to the pattern of headspace volatiles from stored, cooked meat. Representative chromatograms are shown in Figures 4 and 5. Many of the same volatile components appeared to be present in the headspace above oxidizing PE dispersions and oxidized meat, yet only peak A (Figures 4 and 5) was tentatively identified by a retention time identical with standard *n*-hexanal and a column effluent odor that appeared to correspond to the *n*-hexanal standard.

Gaddis et al. (1961) reported *n*-hexanal to be a product of autoxidation of linoleate, while Evans (1961) proposed mechanisms that would explain its formation. Keller and Kinsella (1973) have also reported large increases in carbonyls (aldehydes) in oxidized ground beef. The area of the hexanal peak in the present study appeared to be related to the degree of oxidation occurring in the samples. For instance, the addition of tripolyphosphate to meat samples resulted in a 50% decrease in the volume of hexanal, while 5 ppm of Fe^{2+} produced a peak twice the area of the control (no additives) sample. El-Gharbawi and Dugan (1965) found that *n*-hexanal increased greatly during storage of freeze-dried beef. Cross and Ziegler

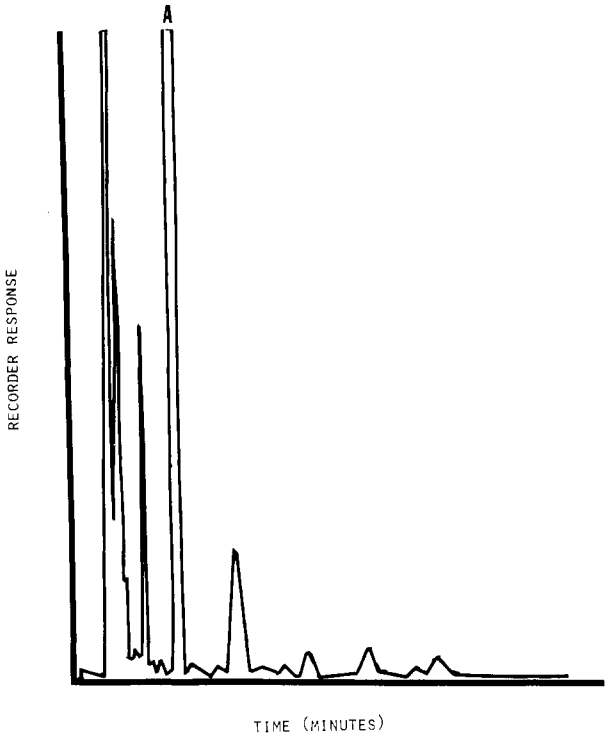

Figure 5. GLC chromatogram of volatiles produced by aqueous extracted meat residue containing 5 ppm of added Fe^{2+} ions. Peak A was tentatively identified as *n*-hexanal by comparison with the retention volume of a *n*-hexanal standard and odor characteristics. Conditions for operation of the chromatograph were identical with those given in Figure 2.

(1965) also observed that hexanal was present in greater quantities in uncured than in cured meat, presumably due to more extensive lipid oxidation in the uncured samples. These facts support the presence and probable role of hexanal to oxidized flavors in meat systems and suggest that PE may be a major contributor.

Yamauchi (1972) monitored lipid oxidation using TBA (2-thiobarbituric acid) numbers during heating of beef, pork, and mutton. They found development of rancidity was most rapid on heating to 70 °C for 1 h, whereas TBA values for the cooked meat decreased after heating above 80 °C. Studies on the mitochondrial, microsomal, myofibrillar, and sarcoplasmic fractions from chicken muscle showed that heating increased TBA values in all fractions except the sarcoplasmic, with the greatest increases occurring in the microsomal and mitochondrial fractions (Yamauchi, 1972). This suggests that denaturation of the lipoproteins in the microsomes and mitochondria causes the unsaturated fatty acids to be more susceptible to oxidation. In meat, the myoglobin is localized in the cytoplasm, while the phospholipids are integrated into the cellular structures, such as the cell wall, mitochondria, sarcoplasmic reticulum, and microsomes (Newbold et al., 1973; Kono and Colowick, 1961; Macfarlane et al., 1960; Holman and Widmer, 1959). During heating, myoglobin has been reported to coprecipitate with other proteins and to produce compounds possessing low spin characteristics (Ledward, 1971). Results suggest that heat denatured myoglobin-containing proteins are not effective as prooxidants in cooked meat, thus supporting earlier evidence showing that myoglobin does not promote oxidation in meat systems (Love and Pearson, 1974; Sato and Hegarty, 1971).

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