A COMPARISON OF MYOGLOBIN AND NON-HEME IRON AS PROOXIDANTS IN COOKED MEAT AND DISPERSIONS OF PHOSPHOLIPID

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ABSTRACT

A COMPARISON OF MYOGLOBIN AND NON-HEME IRON AS PROOXIDANTS IN COOKED MEAT AND DISPERSIONS OF PHOSPHOLIPID

By

Jane Davis Love

Oxidation of tissue phospholipids occurs readily in cooked meat, resulting in the development of off-flavors and odors. Hemoprotein muscle pigments and non-heme iron have been implicated as the major prooxidants in cooked meat. While both hemoproteins and non-heme iron have been shown to be effective in accelerating the oxidation of unsaturated fatty acids in model systems, their role in promoting lipid oxidation in meat is not clearly defined.

The present investigation was designed to study the relative importance of myoglobin and ferrous iron as prooxidants in cooked meat. In the first phase of the study, beef and pork muscle were water-extracted and the aqueous extract was subjected to extensive dialysis. The aqueous muscle extract and the dialyzable and non-dialyzable portions of the extract were added back to the water-extracted muscle, which was heated to 70°C and stored at 4°C. The 2-thiobarbituric acid (TBA) test was used to assess the extent of lipid oxidation in the stored samples.

Results indicated that the prooxidant substances in muscle tissue were removed by water extraction. While some of the prooxidant activity appeared to be located in the non-dialyzable portion of the aqueous muscle extract, a major portion of the prooxidants could be removed from the aqueous extract by dialysis. These results indicated that non-heme iron plays a major role in accelerating lipid oxidation in cooked meat.

To test the hypothesis that non-heme iron rather than hemoproteins was the active prooxidant in the muscle extract, model systems were devised. Purified myoglobin and ferrous iron were added to the water-extracted beef and pork muscle, the samples were heated to 70°C and stored at 4°C until analyzed using the TBA test. The addition of myoglobin at levels ranging from 1 to 10 mg/g failed to accelerate the oxidation of the heated muscle. Ferrous iron was an effective prooxidant at levels as low as 1 ppm. Low levels of ascorbic acid enhanced the activity of Fe^{+2}.

The next phase of the study was designed to assess the ability of non-heme iron and hemoproteins to catalyze the oxidation of a purified phospholipid, phosphatidyl ethanolamine (PE). Phospholipids are known to be the components of cooked meat that oxidize most rapidly. The model systems provided a less variable and less complex environment for studying their oxidation than meat. A variety of methods was used to assess the effects of ferrous iron and metmyoglobin on the oxidation of PE in aqueous dispersions buffered at pH 5.5 and 7.0. Both metmyoglobin and ferrous iron increased oxygen uptake by PE dispersions at pH 5.5. A corresponding increase in TBA reactive material and fluorescent products of lipid oxidation was also noted. Due to increased oxidation in the presence of metmyoglobin and ferrous iron at pH 5.5, a greater loss of unsaturated fatty acid residues was apparent in the PE containing metmyoglobin or Fe^{+2} than in the control samples.

At pH 7.0, neither metmyoglobin nor ferrous iron accelerated oxygen uptake in aqueous PE dispersions. Ferrous iron did not increase the production of fluorescent materials under the same conditions, which confirmed the lack of prooxidant activity. Metmyoglobin did increase the fluorescence at pH 7.0, in spite of its apparent lack of prooxidant activity. Addition of ferrous iron slightly increased the loss of unsaturated fatty acids at pH 7.0, although the effect was much greater at pH 5.5. Addition of metmyoglobin at pH 7.0 failed to increase the oxidative degradation of unsaturated fatty acids.

Spectral studies indicated that the heme group of metmyoglobin was destroyed after 24 hr of incubation in a pH 5.5 PE dispersion. At pH 7.0, metmyoglobin was not degraded under identical conditions.

The volatiles found in the headspace above oxidizing phospholipid dispersions were similar to the headspace volatiles in a sample of oxidized cooked meat. Hexanal

was a major component in the volatiles of both oxidizing phospholipids and cooked meat. The concentration of n-hexanal increased as a result of oxidation and apparently is related to the development of off-odors in cooked meat.

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TABLE OF CONTENTS

Page

INTRODUCTION. 1

REVIEW OF LITERATURE. 3

Mechanisms of Lipid Oxidation. 3

Lipid Autoxidation. 3
Metals as Prooxidants 5
Heme Compounds as Prooxidants 7
Heme Compounds as Antioxidants. 8

Oxidation of Lipids in Meat. 10

Composition of Muscle Lipids. 10
Phospholipid Oxidation in Cooked Meat . 11
Catalysts of Lipid Oxidation in Meat. . 13
Lipid Oxidation in Tissue Homogenates and Model Systems 16

Lipid Oxidation and Meat Flavor. 19

Measures of Lipid Oxidation. 20

2-Thiobarbituric Acid (TBA) Test. . . . 20

EXPERIMENTAL. 23

Materials. 23

Samples 23
Solvents. 23
Thin-Layer Chromatography (TLC) 23
Gas-Liquid Chromatography (GLC) 23
Myoglobin 24
Chemicals 24

Experimental Systems 24

Methods. 25

Extraction of Muscle Tissue 25
Fractionation of the Water Extract. . . 26
Preparation of Model Systems to Study Lipid Oxidation in Cooked Meat. . . . 26

Page

TBA Analysis of Lipid Oxidation in Meat Systems 26
Isolation of Egg Yolk Phosphatidyl Ethanolamine (PE) 27
Preparation of Aqueous Dispersions of PE 30
Phosphorus Determination. 31
TBA Analysis of PE Dispersions. 32
Fluorescence of Oxidizing PE Dispersions 32
Oxygen Uptake by Oxidizing PE Dispersions 33
Gas-Chromatographic Analysis of the Fatty Acid Composition of Phosphatidyl Ethanolamine. 34
Gas-Chromatographic Analysis of Volatiles Produced by Oxidizing Phospholipids 35
Gas-Chromatographic Analysis of Volatiles Produced by Cooked Meat . . 35

RESULTS AND DISCUSSION. 37

Prooxidant Activity of Muscle Extract, Dialyzate and Diffusate. 37

Prooxidant Activity of Myoglobin, Ferrous Iron and Ascorbic Acid 42

Oxidation of Aqueous, Buffered Dispersions of Phosphatidyl Ethanolamine 52

Oxygen Uptake by Aqueous, Buffered PE Dispersions. 53

TBA Values of Oxidizing Aqueous Dispersions of PE. 58

Fluorescence of Oxidized Dispersions of PE . . 60

Fatty Acid Composition of Fresh and Oxidized PE. 66

Volatiles Produced by Oxidizing Phospholipid Dispersions. 69

Volatiles Produced by Oxidized Meat. 69

SUMMARY AND CONCLUSIONS 74

BIBLIOGRAPHY. 76

LIST OF TABLES

Table Page

1 The effect of adding various fractions of aqueous muscle extracts on TBA values of water-extracted, heated beef muscle stored for 18 hr at 4°C 38

2 The effect of adding various fractions of aqueous muscle extracts on TBA values of water-extracted heated beef muscle stored for 48 hr at 4°C 39

3 The effect of myoglobin, ferrous iron and myoglobin plus ferrous iron on TBA numbers of cooked, water-extracted beef stored for 48 hr at 4°C 44

4 The effect of myoglobin, ferrous iron and ferrous iron plus ascorbic acid on TBA numbers of cooked, water-extracted pork samples stored for 24 hr at 4°C. 50

5 The effect of PE concentration on oxygen consumption during 10 hr oxidation at 38°C . . 53

6 The effect of Fe^{+2}, metmyoglobin and ascorbic acid on the production of TBA reactants in PE dispersions held at 38°C for 24 hr. 59

7 The change in fluorescence of an oxidizing PE dispersion due to the addition of Fe^{+2} or metmyoglobin. 64

8 The fatty acid composition of PE from egg yolk and pork and beef muscle phospholipids. . 67

9 Ratio of unsaturated fatty acids to saturated fatty acids in fresh PE and PE held for 24 hr at 38°C. 68

LIST OF FIGURES

Figure Page

1 The effect of increasing levels of extract, dialyzate and diffusate on the TBA numbers of water-extracted beef stored for 18 hr at 4°C after heating to 70°C 41

2 The effect of myoglobin on the TBA numbers of cooked, water-extracted beef stored for 24 hr at 4°C 43

3 The effect of Fe^{+2} on the TBA numbers of cooked, water-extracted beef stored for 24 hr at 4°C. 45

4 Changes in TBA numbers of water-extracted, cooked pork loin muscle stored at 4°C. 51

5 Oxygen uptake of an oxidizing PE dispersion at 38°C. 55

6 Oxygen uptake of an oxidizing PE dispersion containing Fe^{+2} at 38°C. 56

7 Oxygen uptake of an oxidizing PE dispersion containing metmyoglobin (MMb) at 38°C. 57

8 Excitation and emission curves for the chloroform extract of an oxidizing PE dispersion 62

9 Fluorescent intensity in a PE dispersion as a function of hours of oxidation at 38°C 63

10 Gas-liquid chromatogram of headspace volatiles from oxidized phospholipid dispersions . 70

11 Gas-liquid chromatogram of headspace volatiles from oxidized meat containing 5 ppm Fe^{+2} 72

INTRODUCTION

Lipid oxidation is a major cause of deterioration in the quality of meat and meat products. Fresh and frozen meat undergo a gradual deterioration due to lipid oxidation, while rapid acceleration in oxidation of lipids occurs in cooked meat. With greater commercial production and increased consumption of precooked meat items for institutional and home use, elucidation of the mechanisms involved in lipid oxidation has become increasingly important.

Rapid deterioration in flavor occurring in cooked meat has been linked to oxidation of the highly unsaturated tissue phospholipids. Heme pigments have traditionally been considered the catalysts of lipid oxidation in meat. Recent evidence indicates that non-heme iron may play a major role in accelerating the oxidation of meat lipids (Sato and Hegarty, 1971).

Both hemoproteins and non-heme iron have been shown to be effective in accelerating the oxidation of unsaturated fatty acids in model systems. Ferric hemes are more active prooxidants than ferrous hemoproteins, thus, metmyoglobin in raw meat and the ferric denatured hemichromes in cooked meat have been considered the catalytically active forms of the muscle pigments. Non-heme iron in the ferrous oxidation state is a more active prooxidant than ferric iron. Ascorbic acid has been shown to enhance the prooxidant activity of non-heme iron.

While model system studies indicate that either heme or non-heme forms of iron are capable of accelerating lipid oxidation under specified experimental conditions, in the complex and more variable meat system the prooxidant roles of heme and non-heme iron are less clear. Consequently the present investigation was designed to study the relative importance of myoglobin and ferrous iron in catalyzing lipid oxidation in cooked meat. The prooxidant activity of heme and non-heme iron was evaluated in systems containing cooked, water-extracted muscle tissue or purified phospholipids.

REVIEW OF LITERATURE

Mechanisms of Lipid Oxidation

Lipid Autoxidation

The spontaneous reaction between lipids and atmospheric oxygen has been termed autoxidation. Lundberg (1962) has pointed out that autoxidation of lipids involves a free radical chain mechanism. Dugan (1961) described the early stages of the reaction by the following simplified scheme:

Initiation

$$(1)\quad RH + O_2 \longrightarrow R^{\cdot} + {}^{\cdot}OH$$

Propagation

$$(2)\quad R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}$$

$$(3)\quad ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$$

The reaction is initiated when a labile hydrogen is abstracted from a site on the lipid (RH), with the production of lipid radicals ($R^{\cdot}$) as shown in step (1). Reaction with oxygen (step 2), which yields peroxyl radicals ($ROO^{\cdot}$), is followed by the abstraction of another hydrogen (step 3). A hydroperoxide (ROOH) and another free radical ($R^{\cdot}$), which is capable of perpetuating the chain, are formed.

Decomposition of the ROOH species forms more free radicals, which participate further in the chain reactions (Dugan, 1961). Lundberg (1962) stated that the hydroperoxides may exist in an equilibrium as follows:

$$2\ \mathrm{ROOH} \rightleftharpoons (\mathrm{ROOH})_2$$

At low total peroxide concentrations, hydroperoxide decomposition is mainly monomolecular (Lundberg, 1962) and may be illustrated by $\mathrm{ROOH} \longrightarrow \mathrm{RO}^{\cdot} + {}^{\cdot}\mathrm{OH}$ (Dugan, 1961). At high hydroperoxide concentrations a bimolecular decomposition occurs (Lundberg, 1962). The decomposition may be schematically illustrated by $2\mathrm{ROOH} \longrightarrow \mathrm{ROO}^{\cdot} + \mathrm{RO}^{\circ} + \mathrm{H_2O}$ (Dugan, 1961).

Lea (1962) indicated that the hydroperoxides possess very little "off-flavor." Dugan (1961) has suggested that a variety of hydroxy and carbonyl compounds and shorter-chain fatty acids are responsible for the odors and flavors that are characteristic of rancidity.

Ingold (1962) presented the following mechanism to summarize reactions resulting in termination of the lipid oxidation chain:

$$\mathrm{RO_2}^{\cdot} + \mathrm{X} \longrightarrow \text{inactive products}$$

He stated that X may be either a free radical ($\mathrm{RO_2}^{\cdot}$, RO° etc.) or a free radical inhibitor.

While an autocatalytic mechanism may describe the oxidative processes occurring in a highly refined fat or oil, the situation in a food, such as meat, is more complex. In meat, both neutral lipids and phospholipids are present, and either class may undergo oxidation (Watts, 1962). The reactivity of lipids is, of course, influenced by the degree of unsaturation of the constituent fatty acids, as well as by the presence of activating or inhibiting substances (Lundberg, 1962). The speed with which undesirable rancid odors and flavors develop in animal tissues indicates the presence of substances promoting the oxidation of the unsaturated lipid components (Dugan, 1961). Hemoprotein muscle pigments and metals, especially iron, have been implicated as prooxidants in meat (Tappel, 1962; Watts, 1962; Liu and Watts, 1970; Sato and Hegarty, 1971). The proximity of lipids to muscle catalysts of lipid oxidation may influence the extent of oxidation (El-Gharbawi and Dugan, 1965).

Metals as Prooxidants

Ingold (1962) has summarized the activity of heavy metals in increasing the rate of oxidation of food lipids. He pointed out that metals such as iron, cobalt and copper, possessing two or more valency states with a suitable oxidation-reduction potential between them, are particularly important catalysts. He also stated that the effect of metals can be reflected in an altered rate of chain initiation, propagation or termination, as well as by an altered rate of hydroperoxide decomposition.

The basic function of the metal catalyst is to increase the rate of formation of free radicals (Ingold, 1962). Heaton and Uri (1961) have shown that metal ions in their higher valency state will react directly with lipid substrates. Ingold (1962) has proposed the following equation for a reaction involving metal catalysis of this type:

$$M^{(n+1)+} + RH \longrightarrow M^{(n+1)+}H + R^{\cdot}$$

Heaton and Uri (1961) have shown that metals in their lower valency states may also initiate lipid oxidation chain reactions directly. Uri (1956) and Heaton and Uri (1961) suggested that the first stage in this process may be represented as an activation of dissolved oxygen:

$$M^{n+} + O_2 \rightleftharpoons M^{(n+1)+} \; . \; . \; . \; O_2^-$$

A subsequent reaction with the organic substrate then generates free radicals (Brown *et al.*, 1963).

Uri (1956) has described a commonly accepted mechanism for metal catalysis involving the oxidation of a metal ion with hydroperoxide decomposition resulting as follows:

$$M^{+n} + ROOH \longrightarrow M^{+(n+1)} + {}^{-}OH + RO^{\cdot}$$

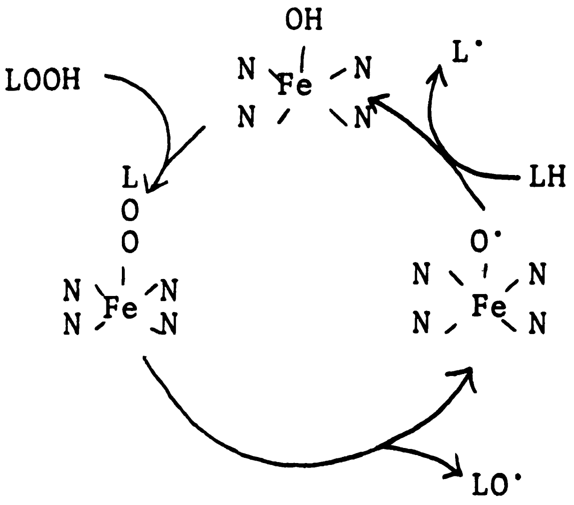
Ferrous iron has been shown to have greater prooxidant activity than iron in the ferric oxidation state (Brown *et al.*, 1963; Wills, 1965; O'Brien, 1969). Bawn (1953) noted that oxidation commences at the same time as metal first appears in its higher valency state. A number of investigators (Wills, 1965; Barber, 1966; Sato and Hegarty, 1971) have reported that low levels of ascorbic acid increase the efficiency of iron as a catalyst for lipid oxidation, presumably by regenerating the active ferrous ion.

Ingold (1962) has pointed out that metals exist as hydrated ions in an aqueous lipid system. He proposed that the water soluble radicals and products of lipid oxidation enter the aqueous phase as lipid oxidation proceeds. He then suggested that metals may react with the water soluble species in the aqueous phase or with the substrate, radicals or hydroperoxides at the oil-water interface.

Heme Compounds as Prooxidants

The catalytic effect of iron porphyrins on the oxidative decomposition of polyunsaturated fatty acids was first described by Robinson (1924). The heme catalyzed oxidation of unsaturated fatty acids has been extensively studied, and acceleration of lipid oxidation due to a variety of heme compounds is now a generally accepted phenomenon. Tappel (1962) has reviewed some of the voluminous literature dealing with heme catalyzed lipid oxidation.

According to Tappel (1962), catalysis by iron porphyrins is characterized by rapid initiation and propagation of the lipid oxidation chain reaction. He stated that the catalytic homolytic cleavage of the O-OH bond of the hydroperoxide is a general property of hematin catalysts. He suggested the following mechanism as the most probable one for hematin-catalyzed unsaturated lipid oxidation:



Tappel (1962) also suggested that a direct attack on the lipid by the heme compound could result in generation of lipid radicals according to the following mechanism:

$$\text{LH} + \text{Hematin-Fe}^{+3} \longrightarrow \text{L}^{\cdot} + \text{Hematin-Fe}^{+2} + \text{H}^{+}$$

Tarladgis (1961) attributed the catalytic activity of ferric hemoproteins to the paramagnetic character of the porphyrin bound iron. He suggested that the presence of five unpaired electrons in metmyoglobin produces a strong magnetic field that would favor the initiation of free radical formation. He further reported that the decomposition of hydroperoxides by a ferric porphyrin was mediated through the donation of an electron from the π cloud of the porphyrin ring.

Heme Compounds as Antioxidants

While the prooxidant activity of hemes has been known for many years, it has been recognized more recently that heme compounds can also act as antioxidants. Maier and Tappel (1959), using a fixed heme concentration, observed that when the linoleate concentration dropped below a specified level, lengthy induction periods occurred. Banks *et al.* (1961) found acceleration of fatty acid oxidation with increasing cytochrome c concentrations up to a maximum, while further increases resulted in inhibition. Lewis and Wills (1963) have also reported that the pro- or antioxidant activity of a heme compound is determined by the ratio of heme to unsaturated fatty acids.

Linoleate:heme ratios for maximum catalysis of lipid oxidation were determined by Kendrick and Watts (1969). They reported optimum linoleate: heme ratios of 100 for hemin and catalase, 250 for metmyoglobin, 400 for cytochrome c and 500 for methemoglobin. At heme concentrations of two to four times the optimum catalytic amount, they noted that oxidation did not occur. They theorized that a stable lipid hydroperoxide-heme derivative was formed at inhibitory heme concentrations. At lower heme concentrations, it was postulated that the heme may be unable to contain the lipid radicals and oxidation results, with eventual destruction of the hemes.

Nakamura and Nishida (1971) reported that association of fatty acids with hemoglobin was responsible for the observed dependence of lipid oxidation on hemoglobin concentration. As the hemoglobin concentration in a linoleic acid emulsion increased, they noted that an increasing amount of linoleic acid was associated with the hemoglobin. When more than 77% of the linoleate was bound to the hemoglobin, a lengthy induction period was observed. The authors reported that the visible spectra of the hemoglobin indicated that it existed in a low spin ferric form during the induction period. A carboxylate ion and a *cis* double bond in the fatty acid structure were also reported to be required for the binding of the fatty acid to the hemoglobin.

Oxidation of Lipids in Meat

Composition of Muscle Lipids

Lipids found in meat can be classified as intermuscular or depot lipids and intramuscular or tissue lipids. The depot lipids are generally stored in specialized connective tissues in relatively large deposits. The tissue lipids are widely distributed throughout the muscle tissue, where they are integral parts of cellular structures. The intracellular lipids exist in close association with proteins and contain a large percentage of the total phospholipids (Watts, 1962).

According to Hornstein *et al.* (1961) the phospholipids in muscle contain a larger percentage of unsaturated fatty acids than the neutral lipids. They found that 19% of the fatty acids in beef phospholipids had four double bonds, while only 0.1% of the triglyceride fatty acids showed this degree of unsaturation. Particularly high levels of linoleic and arachidonic acids are present in the phospholipids (Giam and Dugan, 1965).

Phospholipids contribute less than 1% of total muscle weight, while the triglyceride fraction is about five times as large (Hornstein *et al.*, 1961). The amount of phospholipid has been shown to be relatively constant in muscles from different animals or carcass locations, while the amounts of total and neutral lipid are more variable (Hornstein *et al.*, 1967; O'Keefe *et al.*, 1968).

Several researchers (Kuchmak and Dugan, 1965; Hornstein *et al.*, 1967; O'Keefe *et al.*, 1968) have reported that the fatty acid composition of phospholipids varies with carcass location. Luddy *et al.* (1970) reported that phospholipid fatty acids from muscles classified as light colored had a predominance of monoenes, while polyunsaturated fatty acids were present in greater amounts in phospholipids from dark muscles.

Giam and Dugan (1965) suggested that the tendency of pork muscle to undergo oxidative deterioration may be due to the high levels of linoleic and arachidonic acids in the "free" and "bound" lipids. Compositional differences in the fatty acids of the phospholipid fraction certainly might be expected to result in varying susceptibilities to oxidative rancidity in different species or in cuts from different carcass locations.

Phospholipid Oxidation in Cooked Meat

Early studies of rancidity in meat were concerned with the oxidation of adipose tissue lipids (Watts, 1954; 1961). Timms and Watts (1958) first noted that the rapid deterioration in the flavor of cooked meat did not correlate with measures of the oxidation of the neutral lipids. Later work by Younathan and Watts (1960) showed that more oxidation had occurred in the phospholipid fraction from rancid, cooked pork than in the neutral lipids.

Hornstein *et al.* (1961) also observed that the phospholipid fraction and the total lipids from pork and beef became rancid quickly when exposed to air. These authors concluded that the neutral fats developed off-flavors less readily.

Thus, even though phospholipids are present in relatively small amounts in muscle tissue, they are apparently oxidized rapidly and contribute significantly to stale flavors in meat (Watts, 1962). Their rapid oxidation reflects the highly unsaturated nature of the phospholipid fatty acids (Lea, 1957). Oxidized flavors may be particularly intense in lean meat, since less neutral fat is present to trap volatile decomposition products of polar lipids (Watts, 1962).

On heating, the neutral lipids are lost from the meat more readily than the phospholipids (Campbell and Turkki, 1967). The relative concentration of phospholipids in meat thus increases as a result of cooking. Campbell and Turkki (1967) have reported that cooking pork or beef by a dry heat method fails to appreciably change the fatty acid composition of the phospholipids. Giam and Dugan (1965) also observed that there was little difference in the fatty acid content of free or bound lipids in freeze dried raw or cooked meat. Chang and Watts (1952) had previously noted that the fatty acid composition of ether-extractable lipids of meat and poultry was not changed by cooking.

Catalysts of Lipid Oxidation in Meat

The rapidity with which animal tissue lipids undergo oxidation has stimulated efforts to identify the catalysts involved in the reaction. The accelerating effect of iron porphyrins on the oxidation of lipids is a generally accepted phenomenon. Meat contains significant quantities of hemoproteins, consisting of some hemoglobin from the blood and larger amounts of the muscle pigment, myoglobin (Craig *et al.*, 1966).

Fox (1966) has reviewed the chemistry of meat pigments. He stated that in fresh meat myoglobin exists in three interconvertible forms. Oxymyoglobin imparts a desirable bright red color to meat. Reduced myoglobin is purplish-red in color and metmyoglobin is responsible for the undesirable brown to black discoloration in fresh meat. Watts *et al.* (1966) stated that the balance between the different pigment forms is affected by the activity of enzymatic reducing systems in the meat and the oxygen concentration of the surrounding atmosphere.

During cooking, the pigments are irreversibly converted to denatured ferric hemichromogens (Fox, 1966). Ledward (1971) has studied the nature of the cooked meat

pigment. His study indicates that myoglobin coprecipitates with other muscle proteins during heating. Increasing temperature was presumed to result in conformational changes in the hematin environment. Ledward (1971) also postulated that denatured proteins may then attack the hematin, resulting in replacement of apomyoglobin by other proteins. He suggested that the pigments then aggregate and precipitate with other unreacted denatured proteins to form a range of denatured hemoproteins.

It has been generally accepted that the heme compounds of meat are catalysts of lipid oxidation (Watts and Peng, 1947; Tappel, 1952; Younathan and Watts, 1959; Watts, 1961; 1962; Tappel, 1962; Liu and Watts, 1970). Brown and co-workers (1963) reported that ferric hemes were more active catalysts of lipid oxidation than ferrous hemes. The rapid oxidation of lipids in cooked meat has been attributed to catalysis by the denatured ferric hemichromes (Younathan and Watts, 1959; Liu and Watts, 1970).

The ferric heme, metmyoglobin, has been implicated as a catalyst of lipid oxidation in fresh meat (Greene *et al.*, 1971). Hutchins *et al.* (1967) have reported a positive correlation between metmyoglobin accumulation and lipid oxidation in raw meat. Ledward and MacFarlane (1971) observed that high TBA values corresponded to high metmyoglobin concentrations in stored, frozen beef; however, apparently both TBA values and metmyoglobin accumulation were dependent on pre-freezing treatment. Interestingly enough, however, high initial metmyoglobin concentrations did not affect the rate of lipid oxidation during freezer storage.

Even though heme pigments have traditionally been implicated as the major prooxidants in meat, there is evidence that non-heme iron may play an important role in accelerating oxidation of muscle lipids (Moskovits and Kielsmeier, 1960; MacLean and Castell, 1964; Sato and Hegarty, 1971). Moskovits and Kielsmeier (1960) demonstrated that contaminating iron functions is a prooxidant in sausage. MacLean and Castell (1964) found that trace amounts of iron added to cod muscle produced a rancid odor. Sato and Hegarty (1971) showed that non-heme iron accelerated the oxidation of lipids in water extracted cooked meat. They also reported that myoglobin and hemoglobin failed to act as prooxidants in cooked meat.

If non-heme iron is the major prooxidant in muscle tissue, the effectiveness of polyphosphates as inhibitors of lipid oxidation in meat (Timms and Watts, 1958) is easily explained. Presumably phosphates do not inhibit heme-catalyzed reactions, but act by sequestering trace metals (Liu and Watts, 1970).

Kesinkel *et al.* (1964) observed that raw muscle was subject to more extensive lipid oxidation at lower pH values. Since non-heme iron is more active at acid pH values (Wills, 1965; 1966), the observations of Kesinkel *et al.* (1964) support a major prooxidant role for non-heme iron.

Lipid Oxidation in Tissue Homogenates and Model Systems

Many studies have focused on the rapid non-enzymatic lipid oxidation taking place in tissue homogenates and particulate fractions exposed to atmospheric oxygen (Bernheim, 1964; Robinson, 1965; Wills, 1965; 1966; Barber, 1966). Lipid oxidation may occur in animal tissues deficient in natural antioxidants (Desai and Tappel, 1963), in tissues subjected to irradiation (Kokatnur *et al.*, 1966) or in tissues in which other cell damage has occurred (Nishida and Nishida, 1965).

Wills (1966) attempted to assess the relative importance of hemoprotein and non-heme catalysts of lipid oxidation in various animal tissues incubated in air. He compared the activity of tissue homogenates in promoting lipid oxidation of unsaturated fatty acid emulsions to the results obtained in model systems containing only unsaturated fatty acids and heme or non-heme iron. He reported that both heme and non-heme iron were present in most tissue fractions, and were capable of catalyzing the oxidation of added unsaturated fatty acids. His results indicated that non-heme iron was a more active prooxidant at acid pH values, whereas hemoproteins were reported to be less pH sensitive. He also reported that non-heme iron was apparently more important than heme proteins in catalyzing the oxidation of the endogenous lipids in tissue homogenates. Barber (1966) has also suggested that non-heme iron and ascorbic acid constitute the normal prooxidant system in animal tissues exposed to atmospheric oxygen.

Liu (1970) reported that a 1:1 complex of Fe (II)-EDTA accelerated oxidation of a linoleic acid emulsion at pH values lower than 6.4, while myoglobin catalysis increased directly with pH from pH 5.6-7.8. This is in contrast to several other studies (O'Brien, 1969; Kendrick and Watts, 1969; Ben-Aziz *et al.*, 1970) which have reported that while myoglobin catalysis is less pH sensitive than iron catalysis, it may be enhanced at acid pH values.

O'Brien (1969) studied the decomposition of linoleic hydroperoxides by metal ions and heme compounds. He observed a pH maximum for the catalytic activity of iron between pH 5.0 and pH 5.5. He also reported that the activity of hemoproteins was enhanced at acid pH values, presumably due to increased exposure of the heme moiety of the hemoproteins.

A number of studies (Wills, 1965; 1966; Barber, 1966; O'Brien, 1969; Kendrick and Watts, 1970; Liu, 1970) of heme and metal catalyzed lipid oxidation have been conducted using purified unsaturated fatty acids as the lipid reactant. Similar studies with phospholipids would be of interest, since phospholipids are the lipid component most rapidly oxidized in cooked meat (Younathan and Watts, 1960), in freeze-dried beef (El-Gharbawi and Dugan, 1965) and in cod muscle (Roubal, 1967). While an aqueous dispersion of phospholipids might be expected to behave like an emulsion of unsaturated fatty acids, the phospholipids offer a more complex oxidation system than the neutral lipids. The phospholipids contain phosphorus and a nitrogenous moiety as well as unsaturated fatty acids.

The tendency of phospholipids to oxidize very rapidly is at least partially due to their high content of unsaturated fatty acids (Lea, 1957). The phosphorylated bases may also affect the oxidation of the unsaturated fatty acids in the phospholipid molecule (Corliss, 1968; Lee Shin and Smith, 1970).

Lee Shin and Smith (1970) studied the effects of the functional groups of the phosphoryl bases of phosphatidyl ethanolamine and phosphatidyl choline on the oxidation of methyl linoleate in aqueous emulsions. They reported that ethanolamine and o-phosphoethanolamine increased oxygen uptake by methyl linoleate at pH 7.9 and decreased oxygen consumption at pH 10.2. The choline containing groups were reported to have no effect on the rate of lipid oxidation.

Corliss (1968) has reported that the induction period for oxidation of phospholipids is a function of the nitrogen containing moiety. He found that the ethanolamine moiety of phosphatidyl ethanolamine exerts a greater prooxidant effect than does the choline portion of phosphatidyl choline. He then concluded that the rate of phospholipid oxidation during the steady state was a function of the unsaturation of the fatty acid components of phospholipids.

Lipid Oxidation and Meat Flavor

The flavor of cooked meat changes rapidly during storage. Various terms such as "warmed over", "stale" or "rancid" have been used to describe the characteristic flavors developing in cooked, stored meat (Timms and Watts, 1958). The compounds responsible for the undesirable flavor changes are presumably degradation products of the unsaturated phospholipid fatty acids, since these lipids are rapidly oxidized in cooked meat (Younathan and Watts, 1960).

In recent years a number of attempts have been made to characterize the compounds involved in meat flavor. Herz and Chang (1970) summarized much of this research in a recent review. Some studies of meat flavor have dealt with the contribution of lipids to flavor. Hornstein *et al.* (1960) and Hornstein and Crowe (1960) have shown that depot fat plays an important role in the development of species-specific aromas of cooked beef, pork and lamb. However, they did not investigate the possible contribution of intramuscular fat to either desirable or undesirable flavors.

The most numerous members of any class of compounds identified in meat flavor concentrates are the carbonyl compounds (Herz and Chang, 1970). Hexanal has been identified as a major component of meat flavor concentrates (Herz and Chang, 1970). Hexanal, as well as a variety of other carbonyls, is a degradation product of oxidized linoleic fatty acid (Gaddis *et al.*, 1961).

There is some evidence that hexanal is a product of lipid oxidation occurring in cooked meat (Cross and Ziegler, 1965). The volatile fractions isolated from cured and uncured pork were compared by Cross and Ziegler (1965). They reported that hexanal and valeraldehyde were present in uncured, but not in cured, pork. The failure of cured, cooked meat to undergo the extensive lipid oxidation observed in cooked, uncured meat has been noted by Younathan and Watts (1959). Thus, certain of the carbonyl compounds identified in meat flavor concentrates may result from lipid oxidation and contribute undesirable instead of desirable flavors to cooked meat.

Measures of Lipid Oxidation

Oxidative deterioration of lipids can be followed by measuring the oxygen consumption, by determining the concentration of products of the reaction or by assessing the decrease in the concentration of unsaturated fatty acids. All known measures of lipid oxidation have limitations and cannot be applied with equal success in all systems of oxidizing lipids.

2-Thiobarbituric Acid (TBA) Test

The 2-thiobarbituric acid (TBA) test has been used extensively in the study of lipid oxidation (Lea, 1962). Sinnhuber *et al.* (1958) indicated that the TBA test measures the pink color produced by reacting TBA with malonaldehyde. Malonaldehyde and/or substances closely resembling it occur in foods as end products of the oxidation of polyunsaturated fatty acids (Kwon *et al.*, 1965). Malonaldehyde may be a secondary oxidation product derived from unsaturated aldehydes resulting from the cleavage of hydroperoxides (Evans, 1961). According to Tarladgis *et al.* (1960) malonaldehyde itself does not contribute to typical rancid odors, although a high correlation between malonaldehyde content and rancid odors has been noted (Zipser *et al.*, 1964). The relationship may be limited to moist foods, especially animal tissues (Kwon and Watts, 1964).

The TBA test can be applied directly to a lipid-containing material without prior extraction of the fat (Lea, 1962). However, substances other than lipid oxidation products can react with the TBA reagent and give the red colored compound measured spectrophotometrically in the TBA test (Kwon *et al.*, 1965). Alternatively, the product can be acidified and steam distilled and the TBA reaction can be carried out on the distillate (Tarladgis *et al.*, 1960).

The presence of trace amounts of Fe^{+2} or Fe^{+3} has been reported to cause spurious increases in TBA values (Wills, 1964; Castell *et al.*, 1966; Castell and Spears, 1968). Presumably this is due to iron catalysis of additional lipid oxidation during blending, heating or distillation procedures (McKnight and Hunter, 1965). Castell *et al.* (1966) have recommended that EDTA be added during the blending process. McKnight and Hunter (1965) suggested that samples containing iron be heated or distilled anaerobically. Ascorbic acid has also been reported to result in high TBA values (Wills, 1966).

EXPERIMENTAL

Materials

Samples

Fresh, U.S. Choice grade beef round and fresh, grade A hens' eggs were obtained from the Michigan State University Food Stores. Pork shoulder and loin samples were obtained from the Meats Laboratory at Michigan State University.

Solvents

All solvents were freshly redistilled before use. Methanol was refluxed with 2,4-dinitrophenylhydrazine and trichloroacetic acid prior to distillation.

Thin-Layer Chromatography (TLC)

Silica Gel G, obtained from Brinkmann Instruments, Westbury, New York, was used for all TLC work.

Gas-Liquid Chromatography (GLC)

Chromosorb W, acid washed (80-100 mesh), coated with 15% diethylene glycol succinate (DEGS), and mixtures of fatty acid methyl esters were obtained from Applied Science Laboratories, Inc., State College, Pennsylvania. Acid washed, 60-80 mesh Chromosorb W coated with 20% Carbowax

20M was obtained from Varian Aerograph, Walnut Creek, California.

Myoglobin

Whale skeletal muscle myoglobin, A grade, was purchased from Calbiochem, Los Angeles, California.

Chemicals

All other chemicals were of reagent grade, unless otherwise specified.

Experimental Systems

The effect of heme and non-heme iron on lipid oxidation was assessed in various systems. Initial studies were designed to determine whether the prooxidants occurring in muscle were low-molecular weight, dialyzable substances (non-heme iron) or non-dialyzable proteins (myoglobin and hemoglobin). Water-extracted, ground beef or pork muscle was the basis of the systems used in this phase of the study. The water extract contained prooxidant substances. The activity of the dialyzate and diffusate from the extract in accelerating lipid oxidation in the extracted meat samples was evaluated.

The next series of experiments involved model systems designed to ascertain whether purified metmyoglobin and non-heme iron would exhibit prooxidant activities similar to those observed for the dialyzate and diffusate. Purified heme proteins, ferrous iron (Fe^{+2}) or Fe^{+2} and ascorbic acid were added to samples of the water-extracted muscle, and the extent of lipid oxidation was evaluated.

Since phospholipids are the components of cooked meat undergoing the most rapid oxidative deterioration, model systems containing purified phospholipids (phosphatidyl ethanolamine) and heme and non-heme iron were devised. While model systems do not duplicate the environment found in meat, the activity of heme and non-heme iron in these systems may help to elucidate the prooxidant mechanisms in meat.

Methods

Extraction of Muscle Tissue

After removing all visible fat and connective tissue, meat samples were ground through a 1/8 in plate of a Hobart grinder. Weighed samples of ground meat were extracted overnight at 4°C with 3 volumes of distilled, deionized water containing a few drops of chloroform to retard microbial growth. Samples were stirred occasionally during extraction. The meat slurry was filtered through cheesecloth and the residue re-extracted until it appeared to be devoid of any myoglobin or hemoglobin pigment. Ten gram portions of the extracted meat were placed in Kapak bags, flushed with nitrogen gas, sealed and stored frozen at -25°C. The samples of water-extracted tissue were used in the model systems devised to study the acceleration of oxidation in cooked meat.

Fractionation of the Water Extract

The water extracts were combined and freeze dried. The concentrated extracts were dialyzed against distilled, deionized water at 4°C, with several changes of water. Cellulose dialysis tubing with an average pore diameter of 48 Å was obtained from the Arthur H. Thomas Company, Philadelphia, Pennsylvania. The muscle extract was also concentrated by ultrafiltration at 4°C, using an Amicon Model 402 ultrafiltration cell (Amicon Corporation, Lexington, Massachusetts) with a UM10 membrane, which retains substance with a molecular weight greater than 10,000.

Preparation of Model Systems to Study Lipid Oxidation in Cooked Meat

Frozen, 10 g samples of meat were thawed, mixed with fractions of the meat extracts, myoglobin, ferrous iron or distilled water and heated to 70°C in a boiling water bath. Samples were cooled rapidly and stored at 4°C for specified periods of time prior to analysis for TBA reactive material.

TBA Analysis of Lipid Oxidation in Meat Systems

The distillation method of Tarladgis *et al.* (1960) was used to analyze for thiobarbituric acid reactive material. A 10 g sample of meat was homogenized with 45 ml of distilled, deionized water for 2 min in a Waring blender. EDTA was added at a level of 0.2% to minimize iron-catalyzed oxidation during the blending process. The

blended sample was transferred to a macro-Kjeldahl flask by washing with 47.5 ml of distilled, deionized water. The pH of the meat slurry was lowered to 1.5 by adding 2.5 ml of 4 N HCl. The samples were steam distilled at the highest setting of a macro-Kjeldahl apparatus until 50 ml of distillate was collected.

The distillate was mixed and 5 ml were transferred to a 50 ml test tube. Then 5 ml of TBA reagent (0.02 M 2-thiobarbituric acid in 90% glacial acetic acid) were added, the tubes were stoppered and the contents were mixed. Samples were heated in a boiling water bath for 35 min. After cooling to room temperature, absorbance was read at 538 nm, against a blank containing only distilled water and TBA reagent. Results are expressed as mg malonaldehyde per 1000 g of meat.

Isolation of Egg Yolk Phosphatidyl Ethanolamine (PE)

To study the ability of heme and non-heme iron to catalyze lipid oxidation in a less variable and complex system than meat, model systems containing purified phospholipids were devised. While it is not feasible to isolate muscle phospholipids in sufficient quantities to use in model systems, phospholipids can be readily isolated from egg yolks (Holub and Kuskis, 1969). Egg yolk phosphatidyl ethanolamine (PE) has a relatively high proportion of linoleic and arachidonic acids (Holub and Kuskis, 1969), as do muscle phospholipids (Hornstein *et al.*, 1961; Giam and Dugan, 1965). Egg yolk phosphatidyl ethanolamine has been observed to oxidize rapidly in aqueous emulsions (Corliss, 1968).

Egg yolk lipids were extracted by a modification of the method of Olivecrona and Oreland (1971). Egg yolks and whites were separated and the membranes removed from the yolks prior to filtration through cheesecloth. Fifty grams of filtered yolk were homogenized with 300 ml of chloroform:methanol (2:1, v/v) for 3 minutes at high speed on a Waring blender. The homogenate was filtered through Whatman #1 filter paper on a Büchner funnel. The residue was re-extracted with chloroform. The combined extracts were mixed with 150 ml of water, and the phases were allowed to separate at 4°C. The water layer was removed by aspiration, and the chloroform layer was dried over anhydrous sodium sulfate prior to concentration under vacuum on a Büchi rotary evaporator. Final traces of the solvent were removed under nitrogen and the residue was dissolved in chloroform.

An aliquot was taken to determine total lipid content before the concentrated lipids were added to cold (4°C) acetone. Twenty-five grams of total lipid were made up to 50 ml volume with chloroform and added to 700 ml of acetone. The acetone insoluble material was redissolved in chloroform and reprecipitated twice. Thin-layer chromatography on Silica Gel G with a solvent system of chloroform:methanol:water (65:25:4, by volume) indicated that the final

precipitate contained mainly phospholipids with only a trace of neutral lipids.

Phosphatidyl ethanolamine was isolated by thin-layer chromatography. A 0.75 mm thick layer of Silica Gel G was applied to methanol rinsed, 20 x 20 cm glass plates using a Desaga spreader. The plates were air dried for 24 hr, activated for 1 hr at 105°C and cooled to room temperature in desiccator cabinets. Approximately 20 mg of lipid were streaked on each plate, under nitrogen, using a narrow tipped pipette. The plates were developed in chloroform:methanol:water (65:25:4, by volume) and the PE band was identified by spraying one edge of the plate with 0.25% ninhydrin in butanol. The PE zone was scraped from each plate and eluted with 50 ml of chloroform: methanol (4:1, v/v). Samples were evaporated to dryness under nitrogen and redissolved in chloroform or pentane. After centrifuging for 5 min at 1500 x g in an International Refrigerated Centrifuge, Model PR-6, the solvent containing PE was transferred to glass vials, which were flushed with nitrogen gas, sealed with teflon-lined screw caps and stored at -30°C.

Before use in model systems, the PE was checked for purity by TLC. Thin-layer plates were also sprayed with ferrous thiocyanate, which is capable of detecting low levels of lipid peroxides (Stahl, 1969). Purified PE was used shortly after preparation, before detectable levels of peroxides had accumulated.

Preparation of Aqueous Dispersions of PE

Several authors (Corliss, 1968; Carraway and Huggins, 1972) encountered difficulty in dispersing PE in aqueous systems. Since the size of the dispersed lipid micelles affects the oxidation of lipids, it is important to be able to form uniform, reproducible dispersions.

Several methods of dispersing PE in buffer (0.05 M borate or 0.05 M tris-maleate buffer) were used in the model system studies. In one method, an aliquot of the solvent containing a known quantity of phosphorus was pipetted into a small beaker and the solvent was evaporated under nitrogen. Buffer containing 0.25% Tween 20, a non-ionic emulsifier, was added to the PE, and the mixture was stirred magnetically until the PE was mixed with the buffer. Potassium hydroxide (1.0 M) was added drop-wise, while stirring was continued, until the PE was uniformly dispersed. Buffer was added to the desired volume and the pH was adjusted with 1N HCl, if necessary. Aliquots were then transferred to the reaction vessels.

Alternatively, an aliquot containing a known quantity of phosphorus was pipetted directly into the flasks in which the study was to be conducted. The desired volume of buffer containing 0.25% Tween 20 was added, and the samples were placed in a water bath shaker at 38°C. After about 20 min of shaking, the PE was uniformly dispersed in the buffer. Then solutions of myoglobin or Fe^{+2} were added and various measures of lipid oxidation were initiated.

Since Tween 20 interfered with the gas chromatographic analyses of fatty acid methyl esters, it could not be used for dispersing PE in these studies. The PE was dispersed in buffer by stirring, raising the pH with potassium hydroxide then readjusting to the desired pH and volume with buffer. Lowering the pH to 5.5 resulted in the formation of a turbid dispersion. However, the PE dispersion became somewhat clearer as shaking and incubation at 38°C proceeded.

Phosphorus Determination

The PE content of lipid samples and model systems was quantitated by analyzing for phosphorus content using the colorimetric procedure described by Rouser *et al.* (1966). A standard curve was prepared using aliquots of monobasic potassium phosphate solution (Hartman-Leddon Company, Philadelphia, Pennsylvania) containing from 2-10 μg of phosphorus.

Organic solvents were completely evaporated from lipid samples prior to digestion in order to minimize the danger of explosion (McClare, 1972). Samples were digested by heating with 0.9 ml of 72% perchloric acid for 20 min at the lowest setting of an Aminco micro-Kjeldahl rack. A glass fume cover connected to a water aspirator removed fumes from the perchloric acid digest. After digestion was complete, the sides of the flask were rinsed with 5 ml of distilled water and 1 ml of 2.5% ammonium molybdate was added. After swirling the flask, 1 ml of freshly prepared

10% ascorbic acid was added. Two milliliters of distilled water were added, and the contents of the digestion flask were transferred to a test tube and heated in a boiling water bath for 5 min. After cooling the samples to room temperature, absorbance was read at 820 nm with a Beckman DU-2 spectrophotometer. Water was used as a blank and a control reading was subtracted from the absorbance of the samples. The absorbance at 820 nm was multiplied by a factor of 11.0, which was calculated from a standard curve, to convert the readings to µg of phosphorus.

TBA Analysis of PE Dispersions

Aqueous, buffered dispersions of PE were held in a water bath shaker maintained at 38°C. At intervals, 0.5 ml aliquots were withdrawn and mixed with 1 ml of TBA reagent (0.02 M thiobarbituric acid in 90% glacial acetic acid) in a 15 ml test tube. The stoppered tubes were heated at 100°C for 20 min. After cooling to room temperature, 1.5 ml of acetone was added, the samples were mixed thoroughly and the absorbance at 538 nm was read against a blank containing only TBA reagent and buffer. Results are expressed as absorbance units (A) at 538 nm.

Fluorescence of Oxidizing PE Dispersions

Fluorescent material was extracted from oxidizing PE by shaking 4 ml of the PE dispersion with 7 ml of chloroform:methanol (2:1, v/v). Samples were then centrifuged for 5 min at 1500 x g in an International Refrigerated

Centrifuge, Model PR-6. The aqueous (upper) phase was removed by aspiration and the lower phase was dried over 3 g of chloroform rinsed, anhydrous sodium sulfate. The final volume of all samples was adjusted to 6.5 ml.

Fluorescence measurements were made on a 1 ml aliquot of the extract using an Aminco-Bowman spectrophotofluorometer and an Aminco X-Y recorder (American Instrument Company, Silver Springs, Maryland).

Oxygen Uptake by Oxidizing PE Dispersions

Phosphatidyl ethanolamine and buffer or the buffered PE dispersion were pipetted into a 15 ml respirometer flask and a few potassium hydroxide pellets were added to the center well. Buffer or buffer containing myoglobin or Fe^{+2} were added to the side arm of the flask. After 20 min of equilibration at 38°C, the contents of the sidearm was added to the reaction mixture and measurement of oxygen consumption began. The final volume of reaction mixture in each flask was 5 ml. All experiments were conducted at 38°C, in an air atmosphere with constant shaking.

Blanks consisting of buffer alone, buffer containing myoglobin or buffer containing Fe^{+2} were run with each experiment. Micrometer readings were converted to moles dry gas at STP, and results are expressed as moles of oxygen absorbed per mole of PE.

Gas Chromatographic Analysis of the Fatty Acid Composition of Phosphatidyl Ethanolamine

PE was extracted from aqueous, buffered dispersions with chloroform:methanol in the same manner as the fluorescent materials. The solvent was evaporated from the PE under nitrogen. Then 1 ml of 14% boron trifluoride in methanol was added under nitrogen to the lipid residue in a 10 x 125 mm centrifuge tube. The tubes were sealed with teflon-lined screw caps and heated for 10 min at 100°C, as suggested by Morrison and Smith (1964). After cooling the samples to room temperature, 2 ml of pentane and 1 ml of water were added to each tube. The contents were mixed, then the samples were centrifuged at 1500 x g for 5 min. The upper pentane layer was removed and concentrated under nitrogen.

Gas chromatographic analyses were performed using a Varian Aerograph 200 chromatograph, equipped with a flame ionization detector. A 1/8 in o.d., 7 ft stainless steel column was packed with 15% high efficiency DEGS on acid-washed, 80-100 mesh Chromosorb W. The column oven temperature was 180°C, the injection port was maintained at 210°C and the detector at 195°C. The nitrogen flow rate through the column was 40 ml/min. Flow rates of hydrogen and air were 20 ml/min and 250 ml/min, respectively. Samples of 2 µl were injected, and the emerging peaks were identified by comparing retention times to those of standard mixtures of known fatty acid methyl esters. Peak areas were calculated by multiplying peak height times peak width at half-height.

Gas Chromatographic Analysis of Volatiles Produced by Oxidizing Phospholipids

A 20 ml volume of an aqueous dispersion of phospholipids, buffered at pH 7.0, or 20 ml of buffer were placed in 50 ml Erlenmeyer flasks, which were sealed with rubber serum stoppers. The samples were incubated in a 38°C water bath, with constant shaking. At intervals, a 2.5 ml sample of the headspace vapors above the oxidizing lipid dispersion or the buffer control was withdrawn with a gas-tight syringe for gas chromatographic analysis. A standard technique for sampling (including flushing of the syringe with headspace vapors) was necessary to obtain reproducible results.

Separation of the headspace volatiles was accomplished on a 1/8 in by 10 ft stainless steel column packed with 20% Carbowax 20 M on 60/80 mesh, acid washed, Chromosorb W. A Varian Aerograph Model 200 gas chromatograph equipped with a flame ionization detector was used in the analyses. Column oven temperature was 70°C, the injection port was maintained at 150°C and the detector at 140°C. Nitrogen flow rate through the column was 15 ml/min. Flow rates for hydrogen and air were 20 ml/min and 200 ml/min, respectively.

Gas Chromatographic Analysis of Volatiles Produced by Cooked Meat

Water-extracted muscle tissue was heated to 70°C. Tripolyphosphate was added to a 20 g portion of the cooked meat at a level of 0.5%. Ferrous iron (5 ppm) was added to a second 20 g sample, while the control sample contained no added prooxidants or inhibitors. The samples of cooked meat were stored in Erlenmeyer flasks sealed with rubber serum stoppers. They were held at 4°C for 72 hours prior to headspace sampling. Before sampling, the samples were heated at 100°C for 5 minutes. The serum stoppers were vented to the atmosphere during the heating period. A 2.5 ml volume of the headspace vapor was withdrawn with a gas-tight syringe and was analyzed in the same manner as the headspace samples for oxidizing phospholipids.

RESULTS AND DISCUSSION

Prooxidant Activity of Muscle Extract, Dialyzate and Diffusate

The first series of experiments was designed to determine whether the prooxidants in muscle tissue could be extracted with water, and if so, whether they were located in the dialyzable or non-dialyzable fraction of the extract. The aqueous extract and the dialyzable and non-dialyzable fractions of the extract were added back to the water-extracted muscle tissue at the approximate levels present in the unextracted meat. A control sample was also prepared using water-extracted muscle plus deionized distilled water. Samples were then heated to 70°C and stored at 4°C for specified periods of time prior to TBA analysis.

The TBA values presented in Table 1 show the effect of the muscle extract, the dialyzate and the diffusate on the production of TBA reactive material in the water-extracted meat samples. Addition of the aqueous extract resulted in a 2.8 fold increase in the TBA values as compared to control samples. Apparently substances capable of accelerating lipid oxidation in cooked meat were removed from the muscle tissue during water extraction. Lipids capable of undergoing oxidation apparently remained in the extracted muscle tissue as indicated by an increase in the TBA values for the control sample during storage.

Table 1. The effect of adding various fractions of aqueous muscle extracts on TBA values of water-extracted, heated beef muscle stored for 18 hr at 4°C

Sample	TBA Number (mg malonaldehyde/1000 g meat)
Control--extracted muscle + water	0.34
Extracted muscle + aqueous extract	0.96
Extracted muscle + dialyzate	0.49
Extracted muscle + diffusate	0.54

Both the dialyzate (containing hemoproteins) and the colorless diffusate were capable of accelerating lipid oxidation in cooked meat (Table 1). Apparently, a prooxidant substance in the muscle extract was diffusing through the dialysis tubing. The initial experiments failed to isolate the prooxidant activity in either the dialyzate or diffusate. The prooxidant activity of the muscle extract appeared to be approximately equally divided between the dialyzate and the diffusate. This suggests that either a non-diffusing catalyst was present in the dialyzate, a low-molecular weight catalyst was partially bound to a non-diffusable protein or else dialysis was incomplete.

In subsequent experiments, dialysis was carried out for longer periods of time. The diffusate was collected and concentrated in two fractions. Each fraction consisted of 5 volumes of distilled, deionized water containing the material dialyzed from 1 volume of muscle extract. The two fractions of the diffusate, as well as the aqueous extract and the dialyzate, were added back to the water-extracted muscle. The samples were then heated to 70°C and stored at 4°C for 48 hr prior to TBA analysis. Table 2 shows that both fractions of the diffusate were capable of accelerating the oxidation of lipids in water-extracted cooked meat. In spite of the longer dialysis period, some

Table 2. The effect of adding various fractions of aqueous muscle extracts on TBA values of water-extracted heated beef muscle stored for 48 hr at 4°C

Sample	TBA Number (mg malonaldehyde/1000 g meat)
Control--extracted muscle + water	1.23
Extracted muscle + aqueous extract	5.21
Extracted muscle + dialyzate	1.66
Extracted muscle + first diffusate fraction	1.48
Extracted muscle + second diffusate fraction	1.40

prooxidant activity still remained in the dialyzate. The longer dialysis period decreased the prooxidant activity of the dialyzate relative to the combined activities of the diffusate fractions. The combined diffusate fractions caused a 2.4 fold increase over control TBA values. Addition of the dialyzate resulted in TBA numbers 1.4 times controls.

TBA values have been reported to correlate with the development of off-flavors in cooked meat products (Zipser *et al.*, 1964). A TBA value of 1-2 has been reported as the threshold level for the detection of objectionable odors in cooked meat products (Watts, 1962). In the present study, the control samples developed off-flavors very slowly. A comparison of the data in Tables 1 and 2 shows an increase of 0.89 TBA units for the control samples when the length of storage was increased from 18-48 hours. In contrast, addition of the aqueous extract caused rapid increases in TBA numbers and the development of rancid odors. Increasing the storage time from 18 to 48 hr (Tables 1 and 2) resulted in an increase of 4.25 in the TBA numbers of the water-extracted sample plus the aqueous extract.

The data in Figure 1 show that TBA values increased as the amount of muscle extract, dialyzate or diffusate added to the sample was increased. The concentrated extract or fractions added in volumes ranging from 1-5 ml produced TBA values that were greater than control values in all cases.

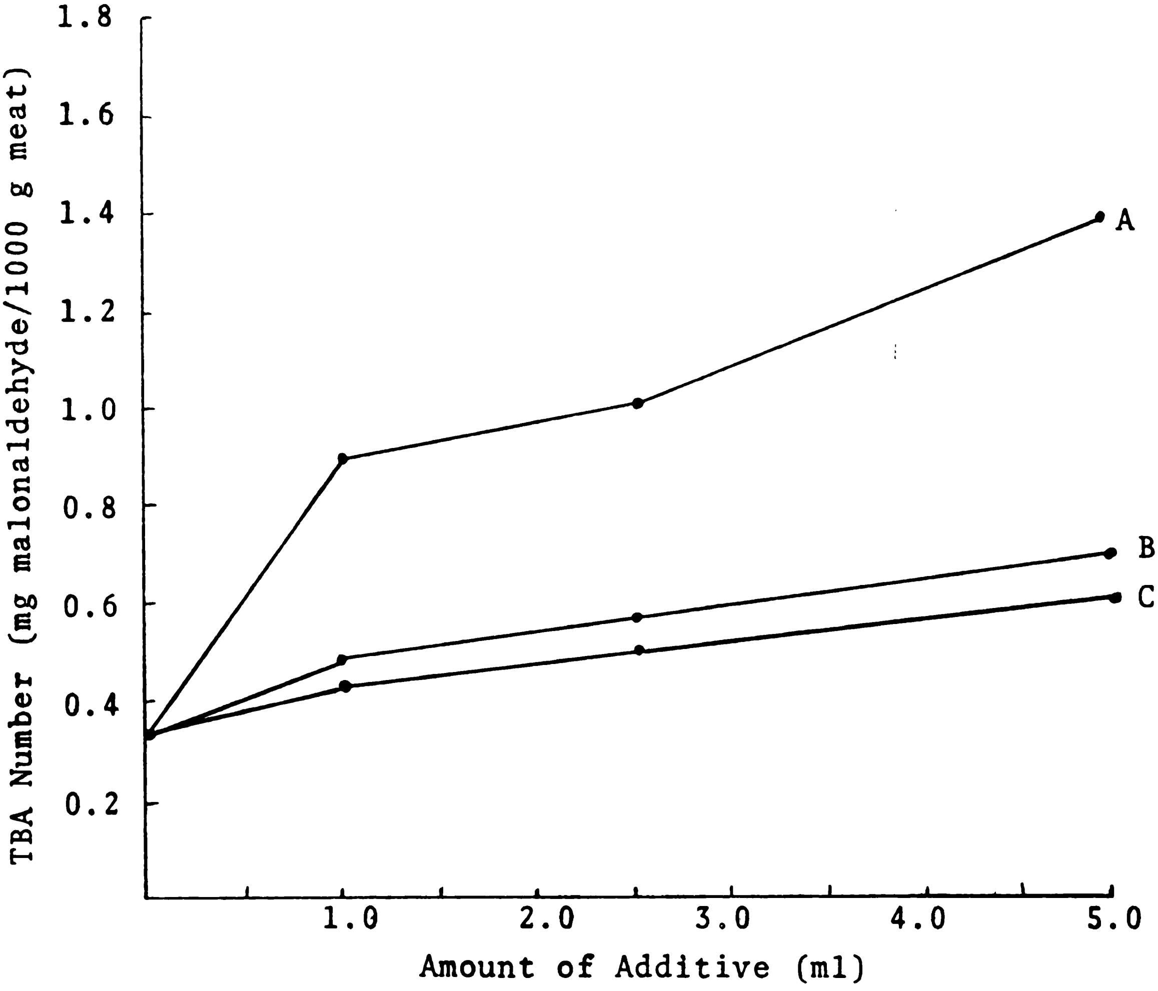


Figure 1. The effect of increasing levels of extract, dialyzate and diffusate on the TBA numbers of water-extracted beef stored for 18 hr at 4°C after heating to 70°C. A = water-extracted muscle plus aqueous extract; B = water-extracted muscle plus diffusate; C = water-extracted muscle plus dialyzate.

Lewis and Wills (1963) reported that the hemes in tissue homogenates were effective catalysts of the oxidation of added unsaturated fatty acids in dilute (1.0%, w/v) suspensions. At higher concentrations (5%, w/v), they reported that the tissue homogenates had an inhibitory effect. If hemoproteins served as active prooxidants in the muscle extract or dialyzate in the current study, no inhibitory effect was apparent. Either an inhibiting concentration of hemoproteins was not reached or the heme compounds did not influence the extent of lipid oxidation.

Prooxidant Activity of Myoglobin, Ferrous Iron and Ascorbic Acid

In the initial experiments, it was established that prooxidant substances in muscle tissue could be extracted with water, and that a significant amount of the prooxidant activity could be removed from the aqueous muscle extract by dialysis. This would indicate, contrary to traditional views, that non-heme iron may be a more important catalyst of lipid oxidation in cooked meat than the denatured muscle pigments. To test the hypothesis that non-heme iron rather than hemoproteins was the prooxidant substance in the muscle extract, a series of experiments with model systems was designed.

Myoglobin was added to water-extracted muscle at levels ranging from 1.0-10.0 mg/g. The samples were heated to 70°C and stored at 4°C for 18 hr prior to TBA analysis. The results shown in Figure 2 demonstrated that addition of myoglobin to beef muscle did not increase TBA values.

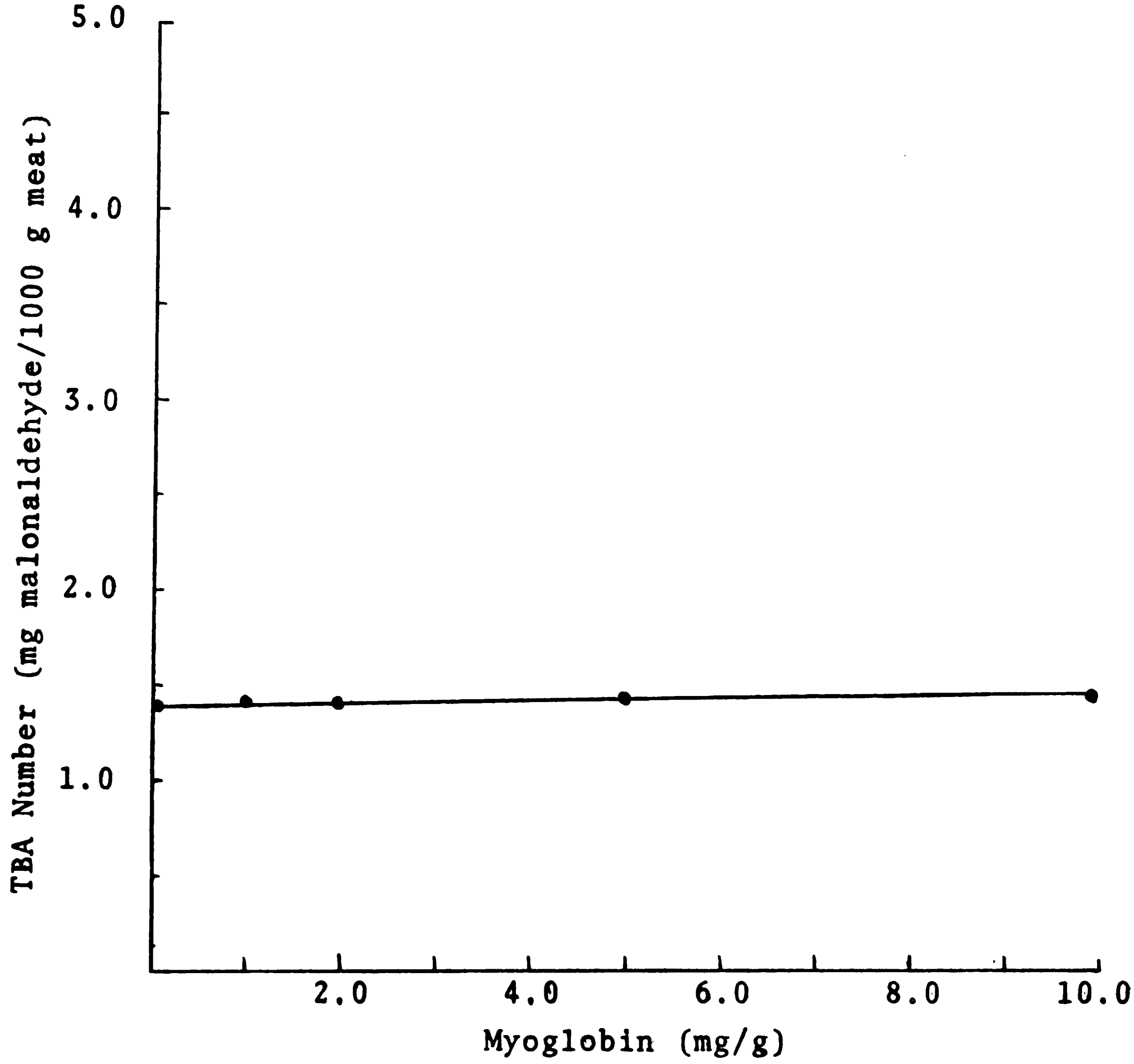


Figure 2. The effect of myoglobin on the TBA numbers of cooked, water-extracted beef stored for 24 hr at 4°C.

Ferrous iron was added to samples of water-extracted muscle at levels ranging from 1-5 ppm. The samples were treated in the same manner as those containing purified myoglobin. As shown in Figure 3, the TBA numbers of the water-extracted muscle tissue showed a linear increase as the concentration of Fe^{+2} increased from 1-5 ppm. When both 1 ppm Fe^{+2} and 5 mg/g of myoglobin were added to a sample of water-extracted muscle tissue, the TBA values were not significantly greater than those for samples containing only 1 ppm Fe^{+2} (Table 3) suggesting that addition of myoglobin did not catalyze lipid oxidation.

Table 3. The effect of myoglobin, ferrous iron and myoglobin plus ferrous iron on TBA numbers of cooked, water-extracted beef stored for 48 hr at 4°C

Sample	TBA Number (mg malonaldehyde/1000 g meat)
Control--extracted meat	2.10
Extracted meat + 1 ppm Fe^{+2}	2.50
Extracted meat + 5 mg/g myoglobin	1.97
Extracted meat + 1 ppm Fe^{+2} + 5 mg/g myoglobin	2.62

The levels of myoglobin and ferrous iron were chosen to approximate the concentrations of heme proteins and non-heme iron reported to occur in beef muscle. Myoglobin, the oxygen-binding muscle pigment, and hemoglobin, from blood

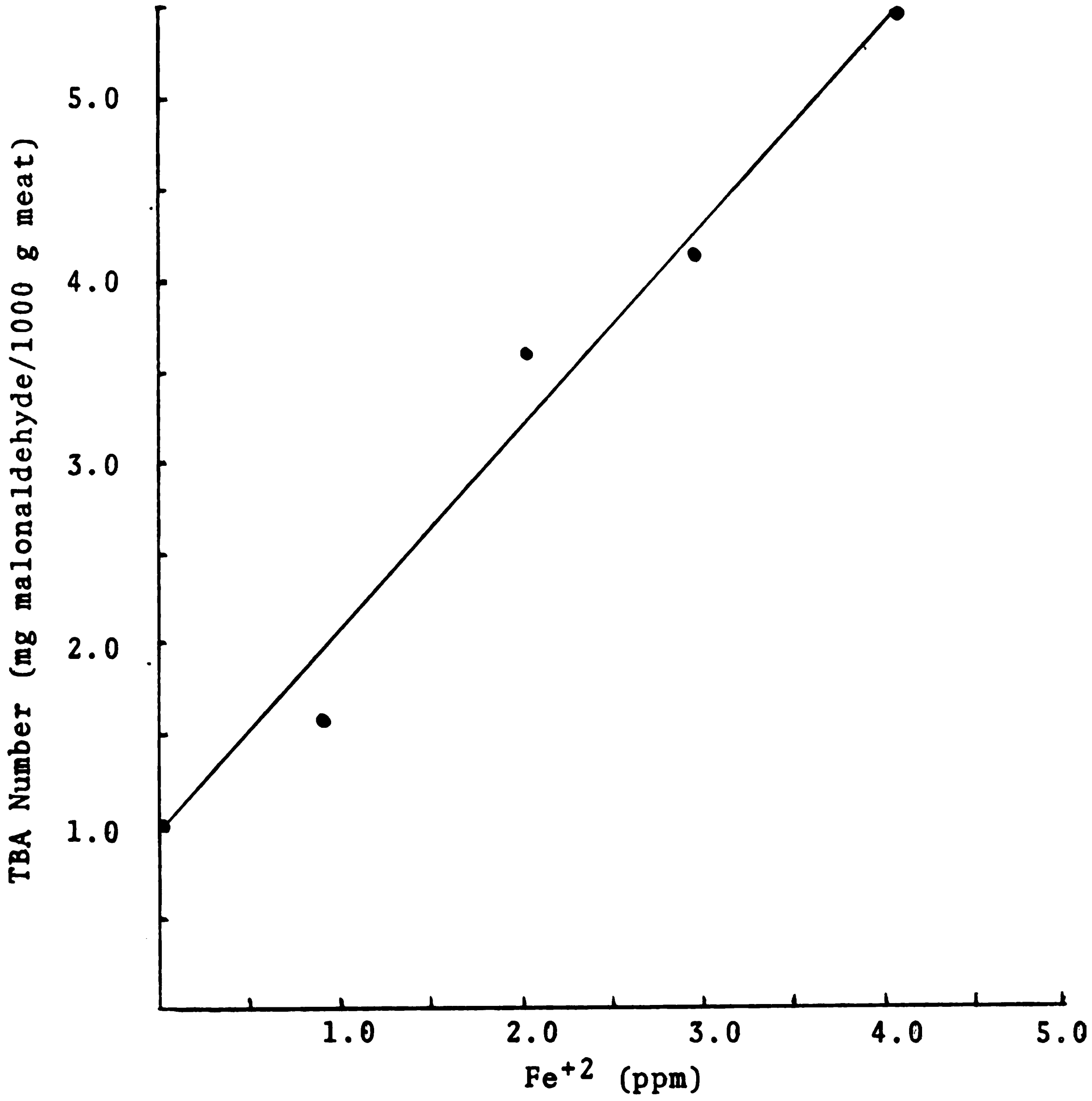


Figure 3. The effect of Fe^{+2} on the TBA numbers of cooked, water-extracted beef stored for 24 hr at 4°C.

trapped in muscle tissues, are the major sources of iron in muscle. Craig *et al.* (1966) reported that the total heme pigment content in the *longissimus dorsi* muscle of beef animals is about 3.79 mg/g. In beef foreshank, these authors reported the myoglobin and hemoglobin content to be 4.25 mg/g.

In undenatured muscle, myoglobin is in solution in the cytoplasm. The highly unsaturated phospholipids are located in the membranes of cellular structures. Thus, the contact between myoglobin and the unsaturated fatty acids of the phospholipids may be limited by cellular structure.

It is known that when the concentration of heme to polyunsaturated fatty acids at a particular site exceeds a fixed ratio, the heme acts as an antioxidant rather than a prooxidant (Lewis and Wills, 1963; Kendrick and Watts, 1969). Thus, it is possible that the soluble hemoproteins may be out of contact with unsaturated lipids in some portions of the cell and be present in inhibiting concentrations in other areas.

Sato and Hegarty (1971) reported that myoglobin did not accelerate lipid oxidation when it was added to water-extracted muscle tissue at a level of 5 mg/g. This level of myoglobin approximates the total amount of hemoprotein present in muscle tissue. However, grinding and extracting muscle tissue may alter the structure so that added myoglobin does not make the same degree of contact with the lipids as in unextracted tissue. Therefore, it was decided to examine the effect of both high and low levels of myoglobin on production of TBA reactive materials in muscle tissue. The data presented in Figure 2 and Table 3 support the hypothesis of Sato and Hegarty (1971) that myoglobin does not cause the oxidation of lipids in cooked meat, while non-heme iron is an effective catalyst of lipid oxidation, even at very low concentrations.

Ledward (1971) stated that on heating, hemoproteins in muscle tissue are denatured and form aggregates with other denatured muscle proteins. Denaturation of hemoproteins has been reported to increase their prooxidant activity (Banks, 1961; Eriksson *et al.*, 1971). Banks (1961) proposed that undenatured cytochrome-c may actually prolong the induction period during oxidation of unsaturated fat. He suggested that undenatured hemoproteins may break down hydroperoxides so that they are not capable of initiating oxidation. He advanced the hypothesis that a denatured form of the heme compound accelerates oxidation following the induction period.

Eriksson *et al.* (1971) reported that heating the hemoproteins, catalase and peroxidase, increased their efficiency as heme catalysts of linoleic acid oxidation. Spectral analysis indicated that there was no change in the oxidation state of the hemoprotein iron. The authors attributed the observed increase in prooxidant activity to a possible unfolding of the protein to cause a greater exposure of heme groups to the substrate. Koch (1962) cited Bishov and Henick (unpublished data) as showing that heat denaturation of heme pigments reduced their prooxidant activity. They observed that cooked dehydrated meat was more stable than raw dehydrated meat. Lipid oxidation rates in model systems containing heated muscle myoglobin extracts were also reduced.

While denaturation of myoglobin makes the heme group more available, apparently the exposed heme group has an affinity for other proteins. If other proteins are present, as would be the case in meat or meat extracts, other denatured proteins apparently co-precipitate with myoglobin to form denatured hemoprotein aggregates. This could decrease the exposure of the heme groups and thus decrease their prooxidant activity. Another possible explanation for the reduced prooxidant activity of denatured hemoproteins is the observation by Ledward (1971) that the ferric hematin in cooked meat possesses low-spin characteristics. Generally, iron-porphyrins which have low spin states are poor catalysts of lipid oxidation.

While most of the iron in animal tissue occurs in hemoproteins, a number of substances in the animal body contain non-heme iron. Iron-protein complexes (ferritin, hemosiderin and transferrin) function in the storage and transport of non-heme iron. Small amounts of non-heme iron proteins appear to perform key functions in electron transport (Mahler and Cordes, 1966). Enzymes such as succinic dehydrogenase, DPNH-cytochrome reductase and xanthine oxidase contain non-heme iron. Wills (1966) has demonstrated that when non-heme iron is released from ferritin, it becomes an active catalyst of lipid oxidation.

Ascorbic acid can function in the release of non-heme iron from iron-containing proteins (Wills, 1966). This may partially explain why low levels of ascorbic acid enhance tissue lipid oxidation.

Sato and Hegarty (1971) reported that the non-heme iron content of beef muscle was about 1 ppm. The data in Figure 3 indicate that very low levels of ferrous iron can result in significant acceleration of lipid oxidation. The non-heme iron containing proteins, which function in electron transport, would be located in close proximity to the phospholipids in the mitochondrial membranes. Heat denaturation of muscle tissue may cause cellular disorganization and thereby release non-heme iron from proteins. Degradation of hemoproteins during cooking and storage could also make non-heme iron available. In the present study TBA values in cooked, water-extracted meat samples were about twice the values observed for unheated, water-extracted samples of muscle stored for 24 hours. Several authors have noted that a more rapid increase in TBA values takes place in heat-treated muscle than in unheated muscle tissue (Timms and Watts, 1958; Sato and Hegarty, 1971). This suggests that heating decreases the stability of the muscle phospholipids, perhaps by increasing the activity of muscle catalysts of lipid oxidation.

The studies reported previously were carried out with eye of round (*semitendinosus*) muscle from beef. Similar results were obtained with water-extracted pork muscle. Ferrous iron at a level of 1 ppm was observed to increase the production of TBA reactive material relative to control values (Table 4). Ascorbic acid (5 ppm) increased the prooxidant activity of added Fe^{+2}. The addition of 5 mg/g of myoglobin had no effect upon the production of TBA reactive material.

Table 4. The effect of myoglobin, ferrous iron and ferrous iron plus ascorbic acid on TBA numbers of cooked, water-extracted pork samples stored for 24 hr at 4°C

Sample	TBA Number (mg malonaldehyde/1000 g meat)
Control--water-extracted muscle	0.80
Water-extracted muscle + 1 ppm Fe^{+2}	1.16
Water-extracted muscle + 1 ppm Fe^{+2} + 5 ppm ascorbic acid	1.61
Water-extracted muscle + 5 mg/g myoglobin	0.77

A typical pattern of change in TBA values over a 3-day period is shown in Figure 4. A combination of ferrous iron and ascorbic acid resulted in the most rapid rate of oxidation, particularly during the first 24 hr period. Ferrous iron alone accelerated oxidation to a lesser extent than a combination of Fe^{+2} and ascorbic acid. Myoglobin failed to accelerate oxidation of the lipids in cooked pork.

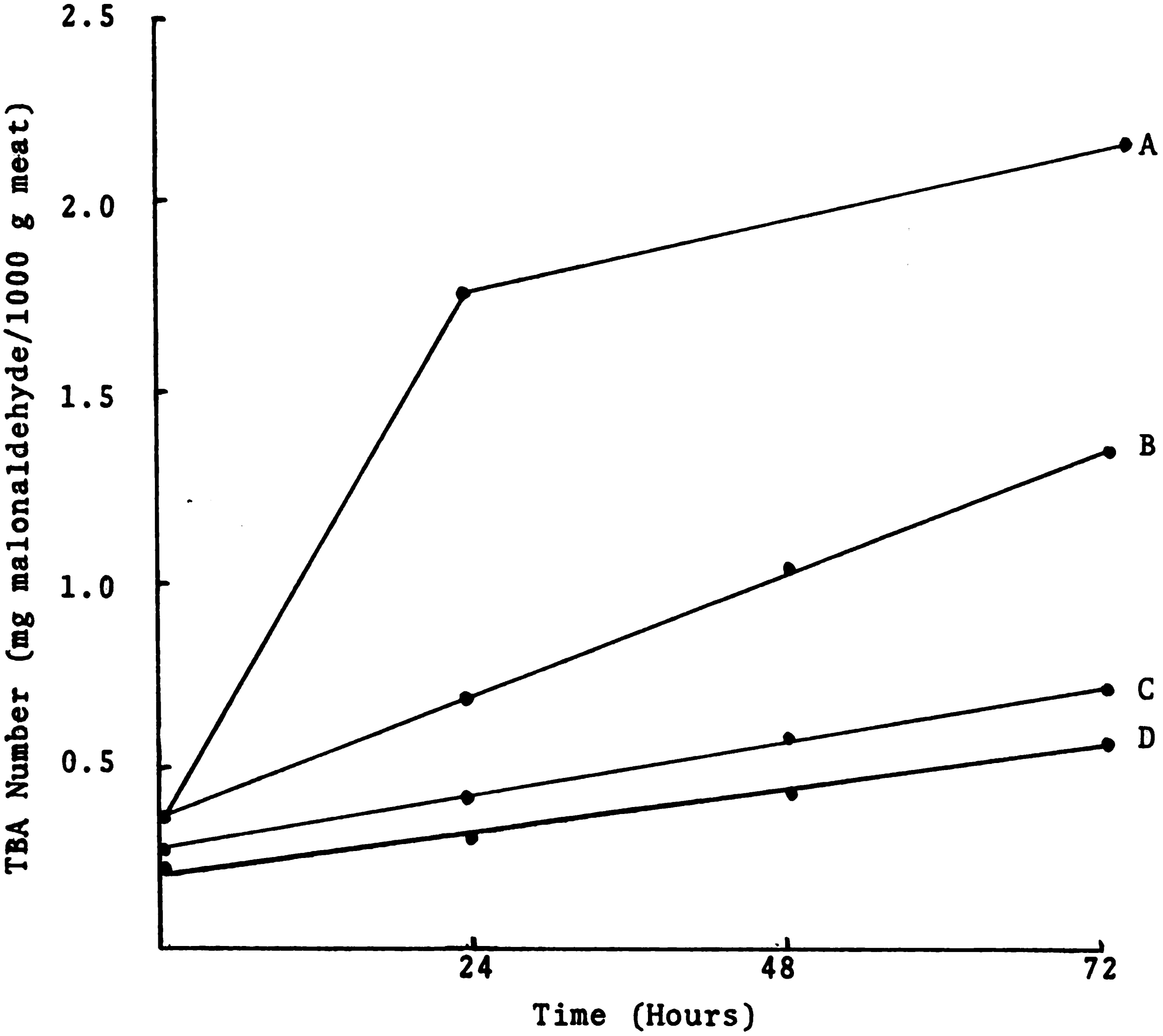


Figure 4. Changes in TBA numbers of water-extracted, cooked pork loin muscle stored at 4°C. A = water-extracted muscle + 1 ppm Fe^{+2} + 5 ppm ascorbic acid; B = water extracted muscle + 1 ppm Fe^{+2}; C = water-extracted muscle + distilled water; D = water-extracted muscle + 5 mg/g myoglobin.

Muscles classified as dark-colored have been reported to contain more highly unsaturated phospholipids than light-colored muscles (Luddy *et al.*, 1970). Thus, dark-colored muscles would be expected to undergo more extensive lipid oxidation than light muscles. During the course of the present study TBA values of light and dark beef and pork muscles, which had been water-extracted, cooked and stored at 4°C, were compared. Dark muscles had about 1.7 times higher TBA values than light muscles. Although only a limited number of samples were used, results indicate that dark-colored muscles undergo more lipid oxidation than light muscles. This could be of practical significance, since the proportions of light and dark muscle incorporated into precooked, processed products may influence the extent of lipid oxidation.

Oxidation of Aqueous, Buffered Dispersions of Phosphatidyl Ethanolamine

The studies discussed in the previous section showed that myoglobin failed to accelerate lipid oxidation in cooked beef or pork muscle, even though catalysis of lipid oxidation by heme compounds is a generally accepted phenomenon. To explore the effect of myoglobin on lipid oxidation in a less complex and variable environment than meat, model systems containing purified phospholipids were devised. Younathan and Watts (1960) have shown that phospholipids are the class of lipids most rapidly oxidized in cooked meat. Thus, phospholipid (phosphatidyl ethanolamine) was used in the model systems. The ability of non-heme iron and myoglobin to accelerate the oxidation of phospholipids in aqueous, buffered dispersions was measured by a variety of methods.

Oxygen Uptake by Aqueous, Buffered PE Dispersions

As shown in Table 5, increasing the PE concentration in the buffered dispersions resulted in an increase in oxygen consumption. At all except the lowest PE concentration studied, the moles of oxygen consumed per mole of PE were relatively constant. In subsequent experiments, a PE concentration of either 2.5 or 3.0 x 10^{-4} M was used, since the rate of oxygen uptake was readily measured and reproducible at these levels.

Table 5. The effect of PE concentration on oxygen consumption during 10 hr oxidation at 38°C

PE Concentration[a]	Moles O_2 Consumed[b]	Moles O_2/Mole PE
1.23 x 10^{-4} M	0.7	1.2
2.46 x 10^{-4} M	2.0	1.6
4.00 x 10^{-4} M	2.9	1.5
5.0 x 10^{-4} M	3.7	1.7

[a] PE was dispersed in 0.05 M borate buffer, pH 7.0, containing 0.25% Tween 20.

[b] Oxygen uptake was measured in a Differential Respirometer (Model GR14, Gilson Medical Electronics, Middleton, Wisconsin).

The PE dispersions were buffered at pH 5.5 and 7.0 with 0.05 M tris-maleate or borate buffer. A pH value of 5.5 is in the pH range where non-heme iron exerts maximum prooxidant activity (Wills, 1965). Undenatured meat would normally have a pH value close to 5.5; however, heating causes a slight increase in the pH of muscle (Bendall and Wismir-Pedersen, 1962). While the pH of meat would not normally be as high as 7.0, PE has been reported to oxidize most rapidly at a pH between 7.0 and 8.0 (Corliss, 1968). Myoglobin has been reported to be an active catalyst of lipid oxidation at both acid and slightly alkaline pH values (O'Brien, 1969; Liu, 1970).

The rate of oxygen consumption by PE dispersions over a 24 hr period is shown in Figure 5. PE underwent immediate and rapid oxidation at pH 7.0. At pH 5.5 the initial rate of oxygen consumption was much slower.

The effect of ferrous iron on the oxidation of an aqueous, buffered PE dispersion is shown in Figure 6. At pH 5.5, 1.5×10^{-4} M Fe^{+2} increased the rate of oxygen uptake. When the same concentration of Fe^{+2} was added to a PE dispersion buffered at pH 7.0, a slightly inhibitory effect on oxygen consumption was observed.

Figure 7 shows that metmyoglobin at a concentration of 5.0×10^{-6} M also increased the rate of oxidation of a PE dispersion at pH 5.5. However, at pH 7.0 the same concentration of metmyoglobin was observed to have a slightly depressing effect on the oxygen consumption of PE dispersions. Lower metmyoglobin concentrations

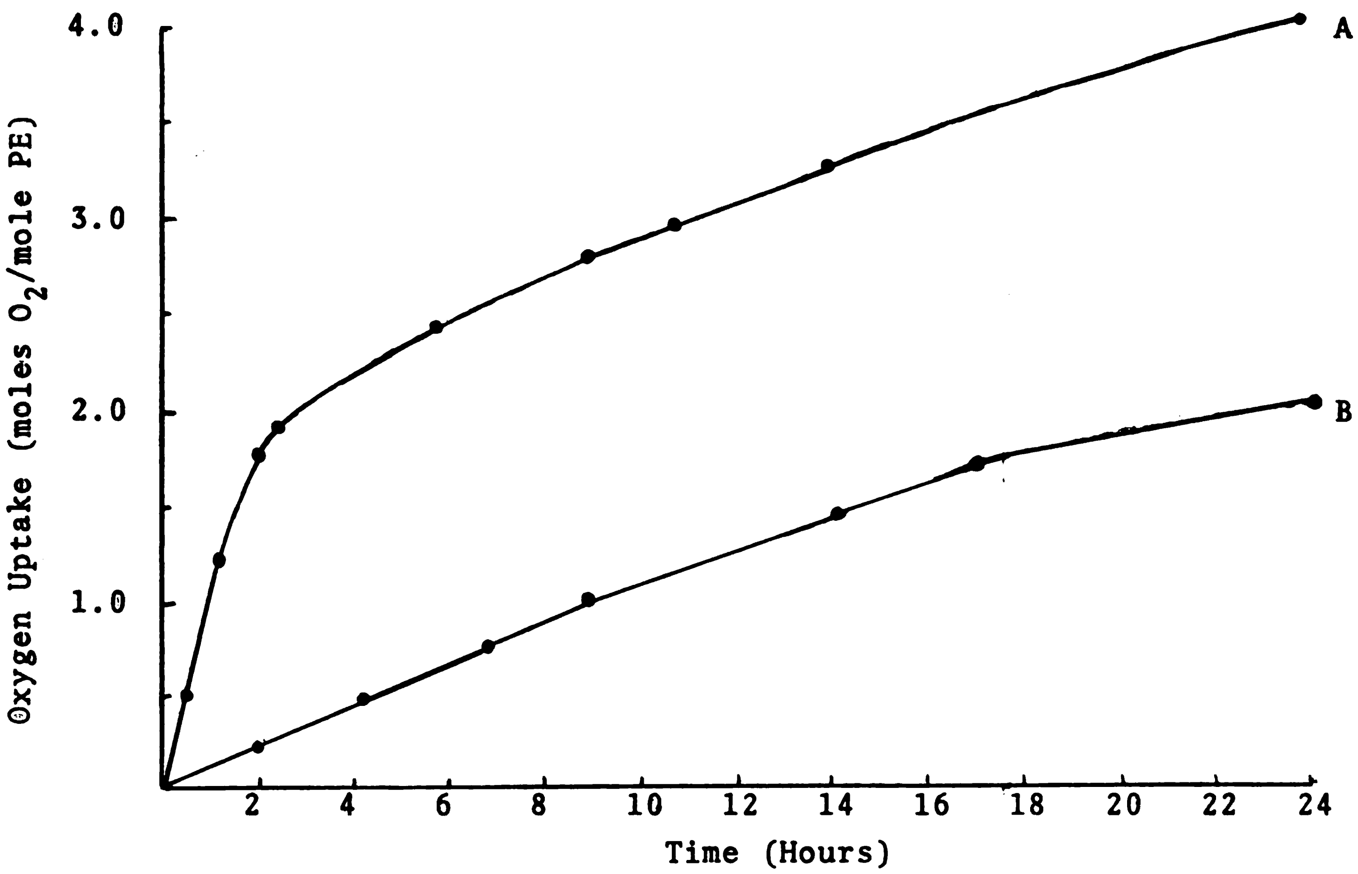


Figure 5. Oxygen uptake of an oxidizing PE dispersion at 38°C. A = 3.0 x 10^{-6} M PE dispersed in 0.05 M tris-maleate buffer, pH 7.0, containing 0.25% Tween 20; B = 3.0 x 10^{-6} M PE dispersed in 0.05 M tris-maleate buffer, pH 5.5, containing 0.25% Tween 20. Oxygen uptake measured in a Differential Respirometer (Model GR14, Gilson Medical Electronics, Middleton, Wisconsin).

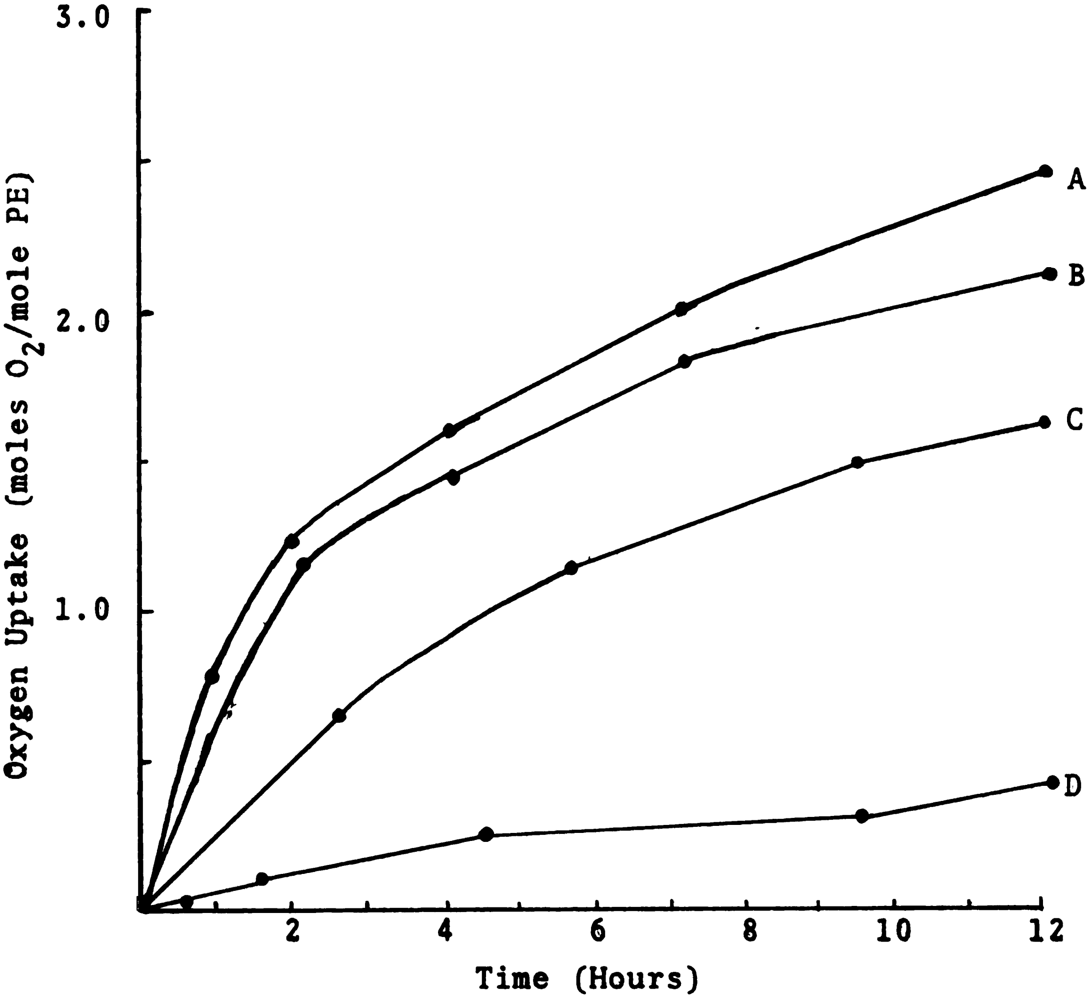


Figure 6. Oxygen uptake of an oxidizing PE dispersion containing Fe^{+2} at 38°C. A = 2.5×10^{-4} M PE dispersed in 0.05 M tris-maleate buffer containing 0.25% Tween 20 at pH 7.0; B = conditions same as A, 1.5×10^{-4} M Fe^{+2} added; C = 2.5×10^{-4} M PE and 1.5×10^{-4} M Fe^{+2} dispersed in 0.05 M tris-maleate buffer, pH 5.5, containing 0.25% Tween 20; D = conditions same as C, no Fe^{+2}.

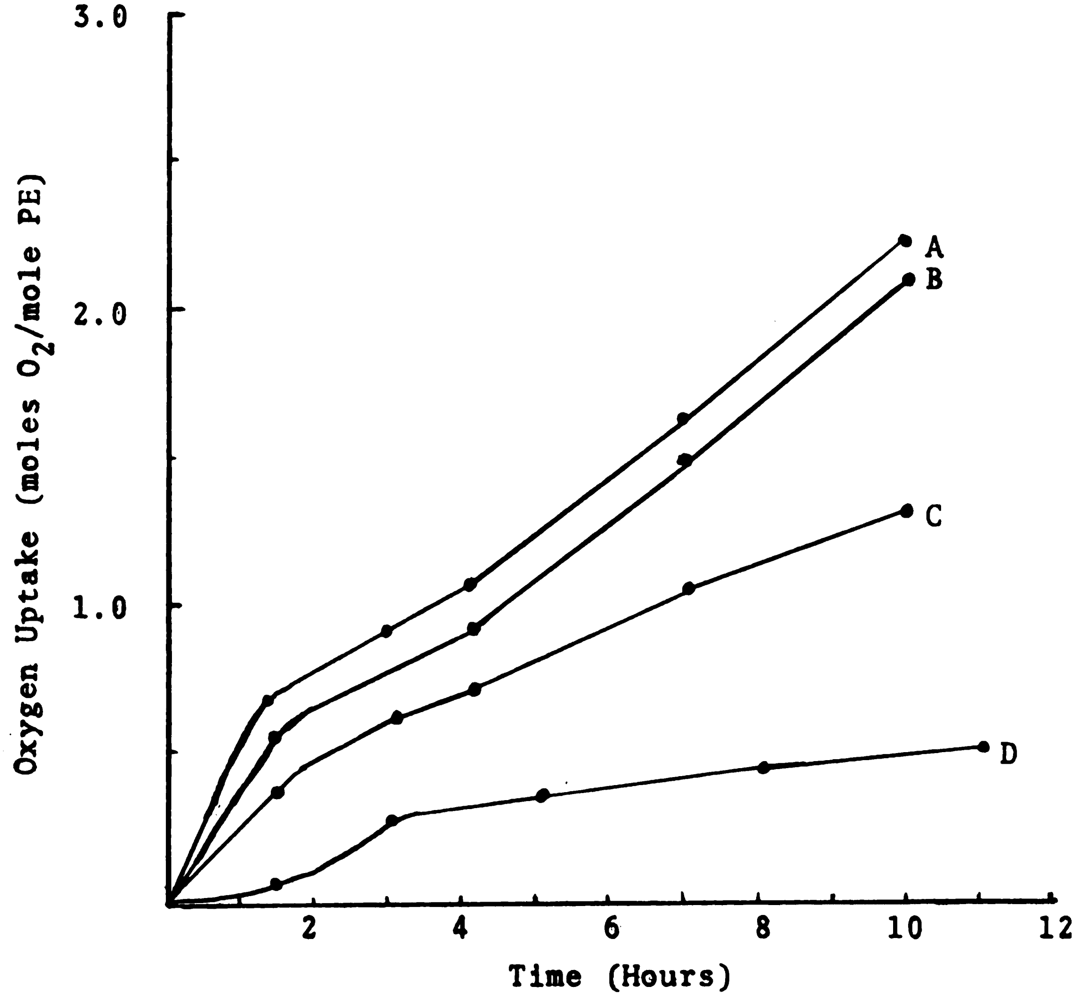


Figure 7. Oxygen uptake of an oxidizing PE dispersion containing metmyoglobin (MMb) at 38°C. A = 2.5 x 10^{-4} M PE in 0.05 M tris-maleate buffer, pH 7.0, containing 0.25% Tween 20; B = same conditions as A + 5.0 x 10^{-6} M MMb; C = 2.5 x 10^{-4} M PE and 5.0 x 10^{-6} M MMb in 0.05 M tris-maleate buffer, pH 5.5, containing 0.25% Tween 20; D = same conditions as C, except no MMb.

(2.5×10^{-6} M, 1.0×10^{-6} M or 4.0×10^{-7} M) did not accelerate oxygen uptake at pH 7.0.

Apparently both metmyoglobin and ferrous iron are capable of accelerating oxygen uptake by a dispersion of PE buffered at pH 5.5. At pH 7.0, neither metmyoglobin nor ferrous iron increased the rate of oxygen uptake. Ferrous iron has been reported to have maximum catalytic activity at pH 5.0 to 5.5 (Wills, 1965) and to cease functioning as a prooxidant above pH 6.4 (Liu, 1970). Myoglobin catalysis has been reported to be less pH sensitive than non-heme iron catalysis (Wills, 1965). The metmyoglobin catalysis at pH 5.5 noted in the present study may be due to some unfolding of the myoglobin structure. This would result in greater exposure of the heme group, thus accelerating oxidation. At pH 7.0, the heme group may be less available for interaction with the phospholipid fatty acids.

TBA Values of Oxidizing Aqueous Dispersions of PE

Table 6 shows the absorbance due to TBA reactive material measured after 24 hr of oxidation at 38°C. Higher absorbance values were observed for the PE dispersions buffered at pH 7.0 than for those at pH 5.5. Ferrous iron accelerated production of TBA reactants at pH 5.5. The TBA values for the samples with Fe^{+2} were 1.4 times greater than control values. At pH 7.0, the incorporation of Fe^{+2} in PE dispersions resulted in a 1.2 fold increase in TBA values. Ascorbic acid plus Fe^{+2} increased TBA values to

Table 6. The effect of Fe^{+2}, metmyoglobin and ascorbic acid on the production of TBA reactants in PE dispersions held at 38°C for 24 hr

Sample	A (538 nm)	
	pH 5.5	pH 7.0
Control--PE alone[a]	0.37	0.46
PE + Fe^{+2}[b]	0.53	0.54
PE + Fe^{+2} and ascorbic acid[c]	0.74	0.72
PE + metmyoglobin[d]	0.45	0.48

[a] 5.0×10^{-4} M PE dispersed in 0.05 M borate buffer.

[b] Fe^{+2}--1.5×10^{-4} M.

[c] Ascorbic acid--1.0×10^{-4} M.

[d] Metmyoglobin--1.0×10^{-6} M.

twice the control level at pH 5.5. At pH 7.0, the addition of Fe^{+2} plus ascorbic acid resulted in a 1.6 fold increase in the production of TBA reactants. Myoglobin increased TBA values slightly at pH 5.5; however, at pH 7.0, no increase in TBA absorbance was noted due to the addition of myoglobin.

With one exception, the TBA values reported in Table 6 are in agreement with oxygen uptake data presented in the previous section. Ferrous iron did not accelerate oxygen uptake at pH 7.0 (Figure 6); however, an increase in TBA absorbance was noted for the PE samples containing Fe^{+2} and Fe^{+2} plus ascorbic acid (Table 6). Non-heme iron and ascorbic acid have been reported to cause high TBA values, which do not reflect the true extent of oxidation occurring in a sample (Wills, 1964; Castell *et al.*, 1966; Castell and Spears, 1968). Thus, the TBA values measured in the samples containing iron and ascorbic acid may reflect interference in the TBA test.

Fluorescence of Oxidized Dispersions of PE

Aqueous dispersions of PE developed a yellow-brown discoloration after several hours of oxygen uptake. The extent of browning appeared to be related to the amount of oxygen consumed by the dispersed PE. Lea (1957) and Corliss (1968) have noted the rapid development of brown discolorations in oxidizing PE.

The concentration of free amino groups has also been shown to decrease during PE oxidation (Lea, 1957; Corliss, 1968). Mono- and dicarbonyls, which are secondary degradation products of peroxides produced by lipid oxidation, are known to condense with primary amines to yield conjugated chromophoric Schiff-base systems (Fletcher and Tappel, 1970). The fluorescence spectra of Schiff-base compounds formed by condensation of carbonyls and amino groups have been reported by Fletcher and Tappel (1970).

Measurement of fluorescent products could provide another method for evaluating the extent of oxidation in PE dispersions. Consequently, the fluorescence of oxidizing PE dispersions was measured.

In the present investigation, the oxidizing aqueous dispersions of PE contained a fluorescent substance, which could be extracted with chloroform. The excitation and emission maxima for the chloroform extract of oxidized PE are shown in Figure 8. The wavelength for maximum excitation was 360 nm. Maximum fluorescent emission was at 430 nm. The fluorescing compounds were not further characterized; however, their fluorescent excitation and emission maxima correspond to those reported for Schiff-base compounds formed by carbonyl-amine condensation (Fletcher and Tappel, 1970).

Figure 9 illustrates the development of fluorescent material in oxidizing PE dispersions. At pH 7.0, fluorescent intensity increased slowly during the first 6 hr of oxidation. As shown in Figure 5, a very rapid rate of oxygen uptake was noted during this time period. From 6-12 hours, the fluorescent intensity of oxidizing PE dispersions increased rapidly (Figure 9). Apparently, the maximum fluorescent intensity was reached after 12 hours, and by 24 hours the relative intensity had decreased. At pH 5.5, where oxygen uptake was less rapid, less fluorescent material was formed. The production of fluorescent material at pH 5.5 increased rapidly between 6 and 12 hr. However, the relative intensity after 24 hr of oxidation at pH 5.5 was slightly greater than the value at 12 hr.

Table 7 shows that incorporation of 1.5×10^{-4} M Fe^{+2} into the PE dispersions increased the production of fluorescent material at pH 5.5. At pH 7.0, however, a

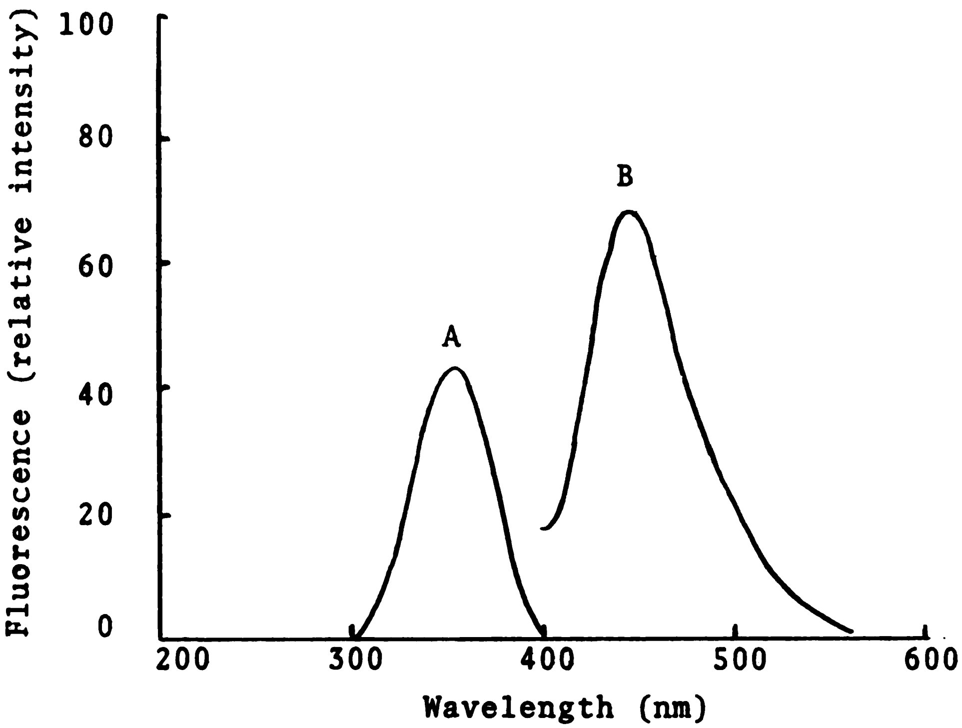


Figure 8. Excitation and emission curves for the chloroform extract of an oxidizing PE dispersion. A = excitation spectrum, B = emission spectrum.

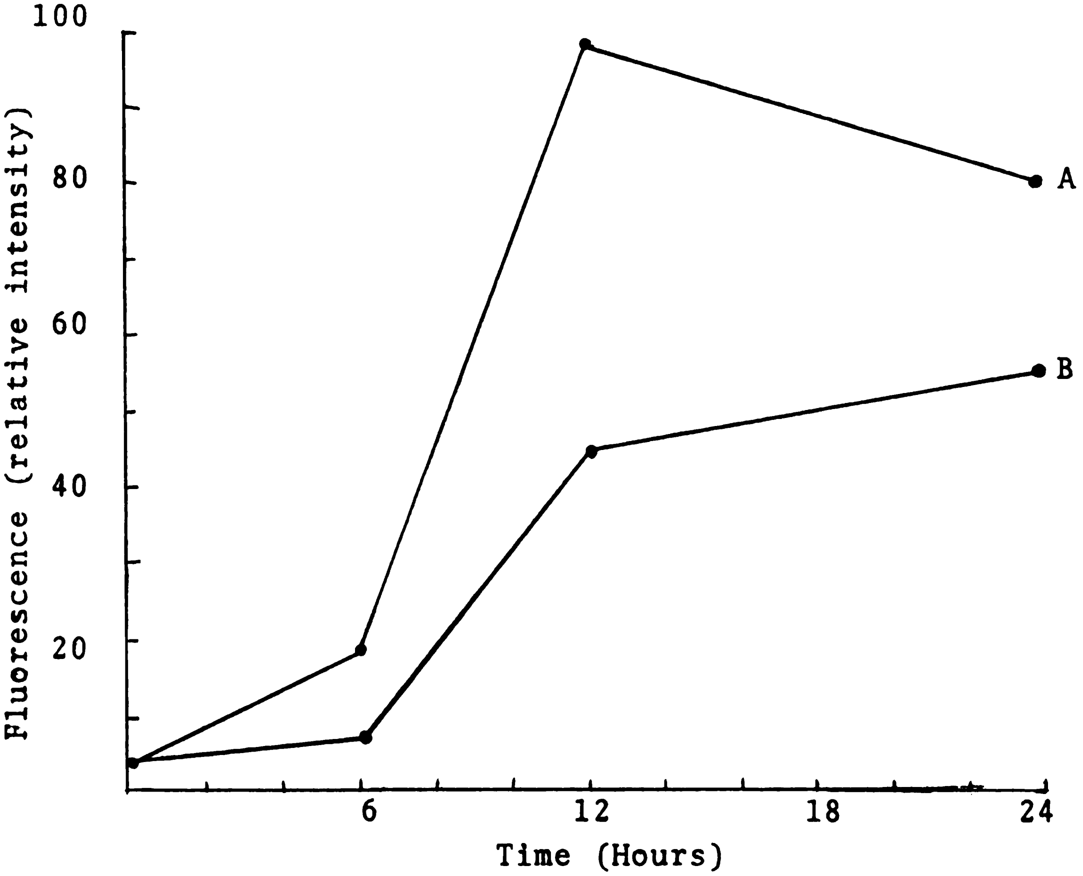


Figure 9. Fluorescent intensity in a PE dispersion as a function of hours of oxidation at 38°C. A = PE dispersed in 0.05 M tris-maleate buffer, pH 7.0, containing 0.25% Tween 20; B = Same conditions as A, except pH of 5.5.

decrease in the fluorescent intensity was noted when Fe^{+2} was added to the oxidizing PE dispersions.

Table 7. The change in fluorescence of an oxidizing PE dispersion due to the addition of Fe^{+2} or metmyoglobin

Sample	Increase over PE alone pH 5.5	 pH 7.0
PE + Fe^{+2}[a]	2.7x	0.73x
PE + metmyoglobin[b]	2.8x	1.9x

[a] 3.0×10^{-4} M PE and 1.5×10^{-4} M Fe^{+2} dispersed in 0.05 M tris-maleate buffer containing 0.25% Tween 20.

[b] 3.0×10^{-4} M PE and 2.5×10^{-6} M metmyoglobin dispersed in 0.05 M tris-maleate buffer containing 0.25% Tween 20.

These results reflect the effect of Fe^{+2} on oxygen consumption in the PE dispersions. At pH 5.5, Fe^{+2} increased oxygen uptake (Figure 6), apparently resulting in a greater concentration of carbonyl reactants, as shown by the increase in fluorescence (Table 7). Ferrous iron decreased oxygen consumption at pH 7.0 (Figure 6) and a corresponding decrease in fluorescent materials was noted (Table 7).

Metmyoglobin increased the production of fluorescent material at pH 5.5 (Table 7). Again, this presumably reflects the greater avilability of carbonyl reactants due to the prooxidant activity of myoglobin. At pH 7.0, addition of metmyoglobin to PE dispersions also resulted in an increase in the production of fluorescent materials, even though metmyoglobin failed to increase oxygen uptake at pH 7.0 (Figure 7).

No fluorescence was present in blanks containing only buffer and metmyoglobin or ferrous iron. The aqueous phase remaining after the PE dispersion had been extracted with chloroform also failed to fluoresce. It was also noted that the myoglobin which precipitated during the chloroform extraction and centrifugation was bright red in color at pH 7.0 and grayish-brown at pH 5.5. The visible spectra of PE dispersions containing metmyoglobin after 24 hr of oxidation at pH 7.0 were very similar to the spectra of freshly prepared samples or of buffer and metmyoglobin which had been incubated for 24 hr at 38°C. After 24 hr of oxidation at 38°C, the PE dispersions containing myoglobin at pH 5.5 failed to show the peaks which are characteristic of metmyoglobin. The spectra of control samples containing pH 5.5 buffer and metmyoglobin were not changed by 24 hr of incubation at 38°C.

Apparently the heme moiety of metmyoglobin is capable of catalyzing phospholipid oxidation at pH 5.5 and is degraded as a result of lipid oxidation. At pH 7.0, lipid oxidation occurs rapidly in PE dispersions, even though metmyoglobin is not capable of accelerating lipid oxidation at this pH. The configuration of the apomyoglobin at pH 7.0 apparently shields the heme group from destruction by lipid oxidation.

Kendrick and Watts (1969) and Nakamura and Nishida (1971) have reported that the spectra of hemoproteins are altered when inhibiting concentrations of hemoproteins are in contact with unsaturated fatty acids. They have reported that peaks at 535 and 560-563 nm are characteristic of the inhibitory complexes. This spectral shift was not noted in the current study in any of the PE dispersions containing metmyoglobin. However, the spectral shift was noted when metmyoglobin was dissolved in a buffer containing 0.002 M sodium lauryl sulfate. A corresponding change from brown to red was apparent in the metmyoglobin solution. The ability of these solutions to accelerate or inhibit lipid oxidation was not evaluated.

Fatty Acid Composition of Fresh and Oxidized PE

The fatty acid composition of freshly prepared egg yolk phosphatidyl ethanolamine is shown in Table 8. Values reported by Hornstein *et al.* (1961) for beef and pork phospholipids are also given for comparison. The egg yolk PE contained 18.4% arachidonic acid, which is similar to the level reported for muscle phospholipids. While egg yolk PE contained less linoleic acid (18:2) than muscle phospholipids, it also contained 11.3% of long chain fatty acids, tentatively identified as 22:3 and 22:6 fatty acids. It should be noted that these fatty acids were extremely labile and care in extracting and isolating PE was necessary in order to obtain the reported levels.

Table 8. The fatty acid composition of PE from egg yolk and pork and beef muscle phospholipids

	% Composition		
Fatty acid	Egg yolk PE[a]	Beef phospholipids[b]	Pork phospholipids[b]
16:0	15.2	13.2	20.0
16:1	Trace	2.2	2.3
18:0	25.4	15.6	11.0
18:1	19.4	21.2	16.2
18:2	10.1	20.2	27.9
18:3	Trace	1.8	1.0
20:4	18.4	19.2	16.3
22:3 + 22:6	11.3	-	-

[a] Peak area percent.

[b] Hornstein *et al.* (1961).

While the fatty acid composition of the purified egg yolk PE and muscle PE are not identical, the values reported in Table 8 indicate that both contain large quantities of fatty acids which would be susceptible to oxidation. Thus, purified muscle PE might be expected to behave similarly to isolated egg yolk PE in model systems.

Table 9 shows the loss in unsaturated fatty acids in PE dispersions after 24 hr of oxidation at 38°C. Since loss of unsaturated fatty acids results in increased percentages of the remaining fatty acids, results are expressed as the ratio of unsaturated fatty acids to saturated fatty acids. Thus, the lower the ratio, the greater the degree of oxidation occurring in the sample.

Table 9. Ratio of unsaturated fatty acids to saturated fatty acids in fresh PE and PE held for 24 hr at 38°C

Sample	Ratio of unsaturated fatty acids to saturated fatty acids	
	pH 5.5	pH 7.0
Freshly prepared PE[a]--no storage	1.60	1.60
PE held 24 hr at 38°C[a]	0.80	0.65
PE + Fe^{+2} held 24 hr at 38°C[b]	0.32	0.50
PE + Mb held 24 hr at 38°C[c]	0.72	0.69

[a] 3.0×10^{-4} M PE dispersed in 0.05 M tris-maleate buffer.

[b] 3.0×10^{-4} M PE and 1.5×10^{-4} M Fe^{+2} dispersed in 0.05 M tris-maleate buffer.

[c] 3.0×10^{-4} M PE and 2.5×10^{-6} M metmyoglobin dispersed in 0.05 M tris-maleate buffer.

The destruction of unsaturated fatty acids was more rapid at pH 7.0 than at pH 5.5 (Table 9). The most highly unsaturated fatty acids in PE were oxidized most rapidly. The $C_{22:6}$, $C_{22:3}$ and $C_{20:4}$ fatty acids were completely degraded during 24 hr of oxidation in the samples containing Fe^{+2}. Although extensive degradation of $C_{18:2}$ occurred, residual levels of this fatty acid were observed in all samples.

The addition of Fe^{+2} resulted in extensive fatty acid degradation at pH 5.5 (Table 9). At pH 7.0, however, the prooxidant effect of Fe^{+2} was less pronounced. When metmyoglobin was added to the PE dispersion at pH 5.5, the loss of unsaturated fatty acids was also accelerated. Metmyoglobin did not increase the degradation of unsaturated fatty acids at pH 7.0.

Volatiles Produced by Oxidizing Phospholipid Dispersions

During the previous experiments, a characteristic odor was noted in the oxidized PE dispersions. In an attempt to isolate and identify some of the odorous components, the volatiles from the headspace of an aqueous dispersion of phospholipids were analyzed by gas-liquid chromatography. A typical chromatogram of the headspace volatiles from oxidized phospholipids is shown in Figure 10. One of the major peaks (peak 5) had a retention time identical to n-hexanal. The odor of the column effluent from peak 5 was the same as that of standard n-hexanal. Gaddis *et al.* (1961) have reported that n-hexanal is a product of the autoxidation of linoleate, and Evans (1961) has proposed a mechanism to explain its formation. Peak 7 had the same retention time as heptaldehyde. The retention time of peak 9 was identical to a nonanal standard.

Volatiles Produced by Oxidized Meat

The odors of the volatiles in the headspace above the cooked meat samples stored for 72 hr at 4°C was noted.

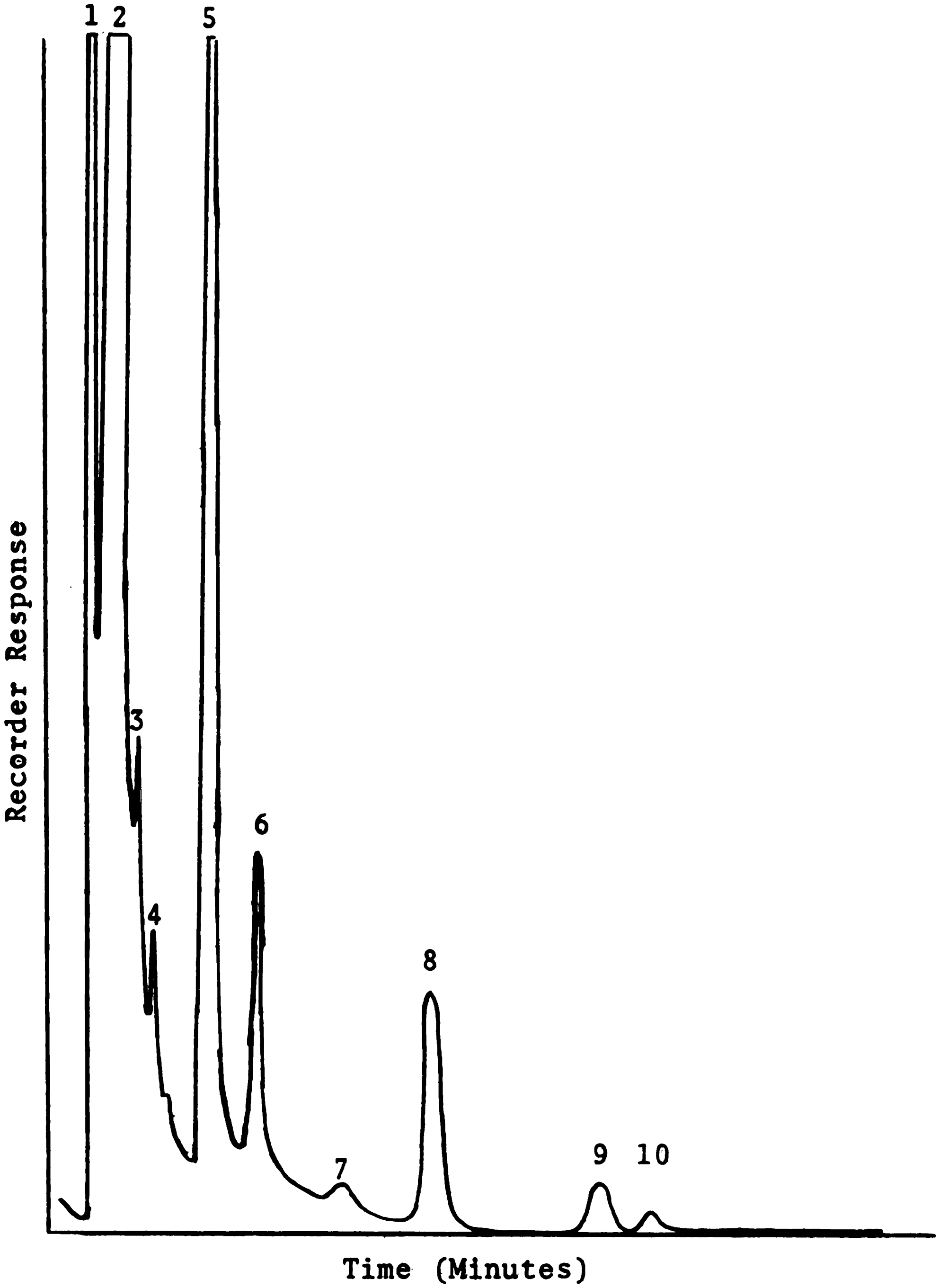


Figure 10. Gas-liquid chromatogram of headspace volatiles from oxidized phospholipid dispersions.

The control sample and the sample containing tripolyphosphate had a pleasant, "boiled-meat" aroma, whereas those containing 5 ppm Fe^{+2} were distinctly rancid. Samples of the headspace vapors above these samples were analyzed by gas-liquid chromatography. A typical chromatogram of the volatiles produced in meat samples containing Fe^{+2} is shown in Figure 11. Peak 4 had a retention time identical to that of n-hexanal. The odor of the column effluent from this peak was the same as the hexanal standard. Peak 5 and peak 7 had retention times identical to those of heptaldehyde and nonanal. The identity of these peaks was not confirmed by other means. However, all of these compounds have been reported to occur in heated beef (Herz and Chang, 1970). Several of the unidentified peaks had characteristic odors; for example, peaks 1 and 2 had an odor like sulfur-containing compounds.

Chromatograms of the headspace vapors above control meat samples and the samples containing tripolyphosphate were similar to the chromatogram shown in Figure 11. Differences were noted in the relative areas of many of the peaks. This was especially noticeable in the case of the n-hexanal peak, which was apparently related to the degree of oxidation occurring in the samples. The hexanal peak was smallest in the samples containing tripolyphosphate. The control sample, which did not contain either anti- or prooxidants, had a hexanal peak twice the size of that in the tripolyphosphate sample. The addition of 5 ppm Fe^{+2} resulted in a hexanal peak that was 4.5 times that of the

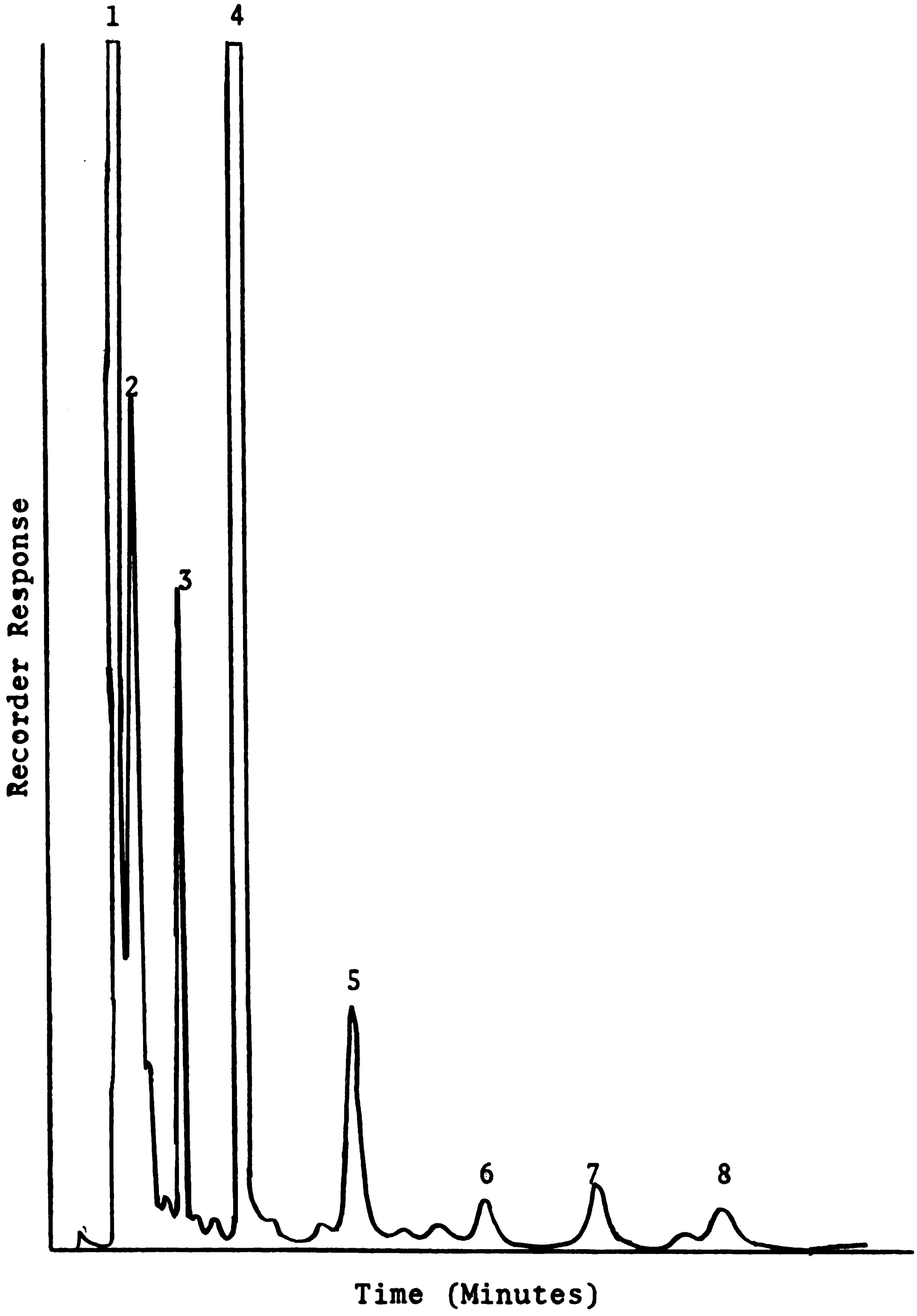


Figure 11. Gas-liquid chromatogram of headspace volatiles from oxidized meat containing 5 ppm Fe^{+2}.

sample with tripolyphosphate. The area of several other peaks, including the peaks with an odor like sulfur-containing compounds, increased somewhat in the sample with Fe^{+2}. A variety of these compounds undoubtedly contribute to the characteristic stale flavors of cooked, stored meat.

A comparison of the chromatograms in Figures 10 and 11 indicated that many of the same volatile compounds were present in the headspace above oxidizing PE dispersions and above oxidized meat. Hexanal appeared to increase as a result of lipid oxidation in both PE and cooked meat. El-Gharbawi and Dugan (1965) reported that n-hexanal increased greatly during the storage of freeze-dried beef. Cross and Ziegler (1965) also found that hexanal was present in greater quantities in uncured than cured meat, presumably due to greater lipid oxidation in the uncured meat.

Hexanal is obviously only one of the components associated with the characteristic odor of stored, cooked meat products. The quantities of hexanal present indicate that it may make a significant contribution to stale flavors in cooked meat. Further isolation and identification of the volatiles produced by oxidized meat is needed for a better understanding of the formation of undesirable odors in cooked, stored meat products.

SUMMARY AND CONCLUSIONS

Studies with heated, water-extracted beef and pork muscle demonstrated that prooxidant substances can be extracted from muscle tissue with water. Much of the prooxidant activity was removed from the aqueous muscle extract by dialysis. These observations indicated that low-molecular weight substances play a major role in acceleration of lipid oxidation in cooked meat.

It was hypothesized that non-heme iron is a major prooxidant in the muscle extract. This hypothesis was tested by measuring the ability of ferrous iron to accelerate lipid oxidation in water-extracted, cooked beef and pork muscle. Levels of Fe^{+2} as low as 1 ppm were effective in accelerating lipid oxidation. Ascorbic acid enhanced the prooxidant activity of Fe^{+2}. However, myoglobin added to the water-extracted muscle at levels ranging from 1-10 mg/g failed to exhibit prooxidant activity. It was concluded that non-heme iron is a major prooxidant in cooked meat, while myoglobin does not accelerate lipid oxidation in denatured muscle.

The effect of metmyoglobin and non-heme iron on the oxidation of phosphatidyl ethanolamine dispersions was evaluated by a variety of methods. Both metmyoglobin and ferrous iron were effective in accelerating the oxygen uptake in PE dispersions buffered at pH 5.5. The accelerating effect of metmyoglobin and Fe^{+2} on oxygen uptake in PE dispersions at pH 5.5 was confirmed by higher TBA values, greater production of fluorescent material and increased losses of unsaturated fatty acids. The data clearly indicate that both metmyoglobin and Fe^{+2} are capable of catalyzing the oxidation of phospholipids at pH 5.5.

At pH 7.0, neither metmyoglobin nor Fe^{+2} accelerated oxygen uptake in PE dispersions. Metmyoglobin did increase the production of fluorescent materials at pH 7.0, but failed to increase TBA values or enhance fatty acid destruction. Ferrous iron increased TBA values at pH 7.0, but did not accelerate the production of fluorescent materials. The results suggested that metmyoglobin and Fe^{+2} failed to accelerate phospholipid oxidation at pH 7.0. Spectral studies indicated that metmyoglobin catalysis at pH 5.5 was accompanied by degradation of the heme groups. At pH 7.0, however, the heme groups were not destroyed.

Gas-liquid chromatography was used to separate the volatile compounds produced by oxidizing PE dispersions and cooked meat. The volatiles found in the headspace above oxidizing phospholipid dispersions were similar to the headspace volatiles in a sample of oxidized, cooked meat. Hexanal was a major component of the volatiles from both oxidized phospholipids and rancid cooked meat. The concentration of hexanal was apparently related to the degree of off-odors in the meat.

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