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Relation of sulfhydryl groups to the fading of cured meat

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RELATION OF SULFHYDRYL GROUPS TO THE FADING OF CURED MEAT

by

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I. INTRODUCTION

Fading of cured meat pigments represents an important economic loss to the meat industry. Recognition is given this fact by the recent advent of large scale use of vacuum packaging. Although vacuum packaging represents a practical solution to the fading problem, it involves added expense to the processor and, ultimately, to the consumer. An understanding of the reactions involved in cured meat fading might lead to other solutions to the problem.

Considering the economic importance of cured meat fading, the amount of published information pertaining directly to this phenomenon is surprisingly small. Almost without exception, the work that has been done on fading has been directed toward investigations of the pigment alone. Although the action of light and oxygen on cured meat results in the change of the characteristic pink color to a brown color, there is little reason to assume that the only reaction in such a complex chemical system as meat is between light and oxygen and the pigment. Possible interactions between pigment and other constituents of meat have been neglected.

The present study was undertaken with the objective of gaining some understanding of interactions between the pigment, fat, and sulfhydryl components of meat and their relation to the fading of cured meat pigment.

II. REVIEW OF LITERATURE

A. The Pigments of Meat

The major pigments of meat are myoglobin, hemoglobin and their derivatives. In the study of the nitric oxide derivatives of these pigments, hemoglobin rather than myoglobin often was used. This was mostly a matter of convenience and, in early work, a matter of necessity since myoglobin was not isolated and purified until 1932 (Theorell, 1932). Shenk et al. (1934) found only 10 percent of the residual pigment in beef to be hemoglobin. In spite of the differences between hemoglobin and myoglobin, Urbain and Jensen (1940) considered the properties of hemoglobin and its derivatives sufficiently like those of myoglobin to allow the use of hemoglobin in studies of meat pigments.

The concentration of myoglobin in meats is dependent upon the age and activity of the animal (Millikan, 1939; Poel, 1949; Lawrie, 1950, 1953). Certain muscles appear to contain more myoglobin than do others. The myoglobin concentration was found to be higher in skeletal muscles than in cardiac muscles of dogs, horses (Drabkin, 1950), cattle and pigs (Watson, 1935; Lawrie, 1950). The average myoglobin concentration of beef muscle is 3.7 mg. per gram while pork muscle averages 0.79 mg. per gram of meat for light muscle and 1.44 mg. per gram for dark muscle (Ginger et al., 1954).

B. The Nature of Cured Meat Pigment

1. The action of nitrites on hematin compounds

The effect of nitric oxide on hematin compounds was discovered by Hermann (1865) who observed that nitric oxide changed the color and spectral characteristics of hemoglobin in the absence of oxygen. Gamgee (1868) observed that hemoglobin which had reacted with nitrite would not combine with oxygen and he considered that there were changes in hemoglobin due to combination of nitrite with oxidized hemoglobin.

The reaction between hemoglobin and nitrite was found by several workers to yield a mixture of nitric oxide hemoglobin and methemoglobin (Haldane *et al.*, 1897; Hartridge, 1920; Haurowitz, 1924; Meier, 1925; Brooks, 1937; Marshall and Marshall, 1945). According to Greenberg *et al.* (1943), the reaction of nitrite with hemoglobin *in vitro* is represented by the equation

$$NO_2^- + 2\ HbO_2 \longrightarrow NO_3^- + HbO + O_2$$

where HbO represents methemoglobin. Although temperature, nitrite concentration, and pH had no influence on the ratio of nitrite utilized to methemoglobin formed, the reaction was much more rapid in acid than in neutral or alkaline medium.

In addition to the above reaction, nitric oxide has been observed to combine with methemoglobin (Hartridge, 1920; Anson and Mirsky, 1925). Keilin and Hartree (1937) demonstrated that nitric oxide methemoglobin was unstable and easily reversible to nitric oxide hemoglobin. Barnard (1937) suggested that nitrite combined with the globin of hemoglobin as

well as with the iron prosthetic group; however, Gibson and Roughton (1957) showed that nitric oxide combined only with the heme groups of hemoglobin.

The formation of methemoglobin in the reaction of nitrite with hemoglobin occurs only in the presence of oxygen. Haldane et al. (1897) demonstrated that the amount of methemoglobin formed by nitrite in the absence of oxygen was very low. Brooks (1937) prepared nitric oxide hemoglobin with reduced hemoglobin in the absence of oxygen and failed to find any initial formation of methemoglobin. In the presence of a reducing agent, one mole of nitrite changed one equivalent of reduced hemoglobin to nitric oxide hemoglobin at pH 5.15 to 6.63. The reaction rate decreased with increasing pH and was very slow above pH 7.16. In the absence of reducing agent and oxygen, one mole of nitrite reacted with two equivalents of reduced hemoglobin to form nitric oxide hemoglobin and methemoglobin. The structure of nitric oxide hemoglobin was confirmed as a ferroheme compound by Coryell et al. (1939) through the study of its magnetic properties.

Having studied the effect of nitrites on blood, Haldane (1901) discovered that the spectrum of the red pigment of fresh meat treated with saltpeter was identical to that of nitric oxide hemoglobin. The pigment became insoluble when the meat was boiled in water, but could be extracted with alcohol. The solubility of the denatured pigments of cured meat in acetone was observed by Anderton and Locke (1955) and a method for the determination of cured meat pigment on the basis of its solubility in acetone was developed by Hornsey (1956). Hoagland (1914) attributed

the color of cooked cured meat to nitric oxide hemochromogen produced by the reduction of the color of salted meat during cooking. Urbain and Jensen (1940) reported the pigments of cured meat to be mainly nitric oxide myohemoglobin and nitric oxide myohemochromogen.

2. Effect of pH

Both the development of cured meat color and its destruction are influenced by pH. Gibbons and Rose (1950) showed that the pH of the tissues of animals rested and fed before slaughter was about 5.3 compared to a pH of 6.0 to 6.6 in tissues of animals slaughtered while fatigued. Meat having a low pH had less residual nitrite after curing and retained its color better than did meat having a high pH. Brooks (1938) observed that the reaction between nitrite and hemoglobin in the presence of a reducing agent was slow over the pH range of 7.2 to 7.8 but rapid in the range of 5.2 to 6.6. Conditions of pH and salt concentration present in muscle during curing were found to be adequate for a rapid reaction. According to Duisberg and Miller (1943), the effect of pH is related to the inability of the pigment to take up nitric oxide below pH 4.0 rather than to the instability of nitric oxide hemoglobin at low pH values. These workers considered the optimum pH for curing to be about 5.2. Hornsey (1959) found that maximum conversion of pigment to the nitroso derivative occurred in meat of low pH.

The rate of oxidation of nitric oxide myoglobin is a function of pH and nitrite concentration. Walsh and Rose (1956) found that in the absence of nitrite, nitric oxide myoglobin was stable in the dark in a pH range of 5.0 to 7.5. In the presence of 200 PPM of sodium nitrite, the

rate of oxidation of nitric oxide myoglobin was markedly accelerated below pH 6.3. Urbain and Jensen (1940) had previously found that the oxidation of nitric oxide myoglobin was inhibited by an increase in pH. On the basis of pH effect, Walsh and Rose (1956) concluded that nitric oxide myoglobin was oxidized by nitrous acid below pH 6.3 in the presence of nitrite.

3. Effect of reducing agents

Earlier work on the reaction of nitrite with blood (Haldane et al., 1897; Brooks, 1937; Keilin and Hartree, 1937) showed that reducing agents were required to maintain the pigment in the ferrous or reduced form and to reduce nitrite to nitric oxide. Reducing agents serve a similar purpose in the production of cured meat pigment.

Reducing substances are normally present in muscle tissue. Brooks (1936, 1938) observed that muscle tissue maintained an oxidation potential of - 0.2 volts in the absence of oxygen. The oxygen uptake of pork muscle was about the same as beef muscle. Bacon, however, had a somewhat lower oxygen consumption (Brooks, 1936). Among the components of fresh tissue, Bender et al. (1958) found 2.1 percent reducing sugars (as glucose) on a dry weight basis. Sulfhydryl groups released from protein during heat processing are a source of reducing substances in meat. Watts et al. (1955) observed that the development of cured meat color paralleled the appearance of free sulfhydryl groups. Erdman and Watts (1957) found that cured meat maintained both color and sulfhydryl groups during low temperature storage. Prolonged heat treatment can destroy sulfhydryl groups

in meat. Fraczak and Padjdowski (1955) indicated that 80°C. was the critical temperature for the decomposition of sulfhydryl groups in meat.

Another source of reducing agents in meat is additives introduced during processing. Greenwood et al. (1940) found that sugars improved the color of cured meat by establishing reducing conditions and preventing the oxidation of nitric oxide hemoglobin to methemoglobin in the presence of microorganisms. Ascorbic acid and related compounds have been widely used in recent years to improve the color of cured meats. Watts and Lehman (1952a) found that 0.1 percent ascorbic acid added to meats caused better color development when the meat was heated at 70°C. or frozen at -17°C. These workers (1952b) observed that hemoglobin did not react with ascorbic acid in the absence of oxygen. Ascorbic acid reduced methemoglobin and promoted the reduction of nitrite to nitric oxide. In the presence of oxygen, an undesirable side reaction occurred in which the green pigment choleglobin was formed. According to Hollenbeck and Monahan (1953), the beneficial effect of ascorbic acid in curing meat is due to the reduction of nitrogen dioxide to nitric oxide. Kelley and Watts (1957) observed that cysteine, ascorbic acid and glutathione were capable of promoting the formation of nitric oxide hemoglobin, regenerating this pigment on surfaces of faded meat and protecting surfaces of cured meat from fading when exposed to light.

The reduction of methemoglobin to hemoglobin by ascorbic acid was demonstrated by Gibson (1943) and was found to be catalyzed by iron and copper salts. Ivanova (1950) reported that both ascorbic acid and glutathione reduced methemoglobin to hemoglobin in vitro.

Since nitrite and nitrate are oxidizing agents in acid solution while ascorbic acid is a reducing agent, the compatability of these compounds in a curing mixture is of some concern. Henrickson et al. (1956) reported ascorbic acid protected cured meat color from fading but was not completely stable with nitrite. Hollenbeck and Monahan (1955) concluded that moisture and temperature were important in controlling the reaction between ascorbate and nitrite in dry curing mixtures. In solutions, pH and temperature determined the rate of reaction. A very slow rate of reaction was observed at a pH of 6.5 to 7.0 in meat brines having high salt concentration.

When Siedler and Schweigert (1959) studied the effect of reducing agents on the production of denatured globin nitric oxide myohemochrome in model systems, they observed that ascorbic acid caused a significant loss of metmyoglobin at 60° and 70° C. Nitrite protected the heme from destruction by ascorbic acid and cysteine, but was less effective in the presence of the latter. Dithionite was the only reductant capable of forming the cured meat pigment at 60° C. while all reductants formed the pigment at 70° C. The yields of pigment at 70° C. were dependent on nitrite concentration when cysteine was the reductant, but not when ascorbic acid was the reductant.

4. Effect of metallic ions

Acceleration of the reduction of methemoglobin by ascorbic acid in the presence of cupric or ferrous ions was observed by Gibson (1943). Addition of 8-hydroxyquinoline, which complexes copper, or α,α-dipyridyl,

which complexes ferrous ion, reduced the rate of reduction of methemoglobin by ascorbic acid by as much as 50 percent.

Weiss et al. (1953) found metallic ions increased the formation of nitric oxide hemoglobin in the order Cu> Fe> Zn. The metal chelating agent, ethylenediaminetetraacetic acid, inhibited nitric oxide hemoglobin formation. Cupric and ferrous ions accelerated fading in the absence of ascorbic acid, but protected the color of solutions of nitric oxide hemoglobin in the presence of ascorbic acid. Metallic ions had no effect in solutions of hemoglobin reduced with sodium dithionite. Siedler and Schweigert (1959) noted that ferrous ions had a nitrite sparing effect under certain conditions. The yields of cured meat pigment obtained with cysteine and a low nitrite level were increased by the addition of low levels of ferrous salts.

C. Factors Causing Loss of Color by Cured Meats

1. Color loss by dehydration

A reversible relationship between moisture loss and color change in the absence of air was reported by Winkler (1939). Color change was explained on the basis of pigment concentration. According to Urbain and Ramsbottom (1948), discolorations due to dehydration can be controlled by proper packaging.

2. Color loss by autoxidation

Urbain and Jensen (1940) observed that nitric oxide hemoglobin was susceptible to oxidation in the presence of oxygen. A combination of

high pH (8.5) and low temperature (0 C.) prevented oxidation of nitric oxide hemoglobin for 13 days. Temperature had little effect on oxidation of nitric oxide hemoglobin at higher hydrogen ion concentrations.

Walsh and Rose (1956) described one of the mechanisms of oxidation of nitric oxide myoglobin as a slow autoxidation in air. They visualized the following reaction as occurring:

$$MbNO + 1/2\ O_2 \longrightarrow metMb + NO_2^-$$

While the autoxidation was independent of pH, oxidation of nitric oxide myoglobin by nitrous acid was markedly influenced by pH.

3. Color loss by photooxidation

The requirement for both light and oxygen to produce cured meat fading has been shown by Urbain and Ramsbottom (1948), Allen (1949) and Hornsey (1957). Cured meat remained stable toward light if packaged under vacuum in gas impermeable films.

The reversibility of fading was first observed by Urbain and Ramsbottom (1948). These workers noted that cured meat vacuum packaged in impermeable films decreased in color the first day of storage; thereafter, the color increased to the initial level. Color was reformed by the reducing activity of the meat and the curing agents present. Watts et al. (1955) reported that faded cured meat which had residual free sulfhydryl groups and excess nitrite would recover some of the faded color upon being stored in the dark.

The effect of light on fading depends on the time and intensity of illumination (Hockman, 1946; Allen, 1949; Archer and Bandefield, 1950; Taylor and Pracejus, 1950; Kraft and Ayres, 1954; Hornsey, 1957). There

is some controversy in the literature regarding the effect of various spectral regions of light on fading. Allen (1949) reported that different portions of the visible spectrum adjusted to the same intensity were equally effective in fading cured meats. Although Hockman (1946) considered intensity to be a more important factor than wave length, he suggested a yellow or amber light for cured meat display. On the other hand, Taylor and Pracejus (1950) observed that various types of light sources differed in their fading power, and Archer and Bandefield (1950) reported that shorter wave lengths of visible light were more active in causing fading than were the longer wave lengths. The work of Taylor and Pracejus (1950), Kraft and Ayres (1954) and Hashimoto and Yasui (1956) indicated that ultraviolet light as well as visible light was effective in fading cured meat. Kampschmidt (1955) reported the wave lengths effective in fading of cured meat were those which were absorbed by nitrosomyoglobin, or approximately 350 to 600 mu. This confirmed the finding of Urbain and Ramsbottom (1948) that wrapping cured meat in a red film to filter out light below 600 mu protected cured meat from fading.

Three separate mechanisms for the oxidation of nitric oxide myoglobin were proposed by Walsh and Rose (1956). These are, 1) the oxidation of pigment by nitrous acid at low pH, 2) autoxidation in air, and 3) photooxidation which increased in rate with increasing light intensity. These workers failed to observe a mixture of oxymyoglobin and metmyoglobin forming in the photooxidation of nitric oxide myoglobin and therefore rejected a dissociation mechanism such as was advocated by Urbain and Jensen (1940) and Kampschmidt (1955). They proposed as a possible

mechanism of photooxidation an activated nitric oxide myoglobin which could either deactivate to form nitric oxide myoglobin or lose an electron to oxygen and become oxidized to metmyoglobin and nitrite. Inasmuch as Walsh and Rose studied the native pigment rather than the denatured pigment found in cooked meat, their proposal may not relate to the mechanism of cured meat fading.

Draudt and Deatherage (1956) observed in manometric studies that a portion of the nitric oxide of denatured globin nitric oxide myohemochrome was oxidized to nitrite and nitrate. Carbon dioxide was also found as one of the oxidation products. The increase in oxygen uptake was proportional to the decrease in color of the pigment. In a study of meat components which could cause loss of color, oleic acid and rancid fat were found to affect color of cured meat pigment while methyl laurate and methyl oleate had no effect on color.

D. Relation of Sulfhydryl Groups to Oxidative Processes

Sulfhydryl-containing compounds have been involved in a wide variety of free radical reactions. Waters (1948, p. 73) states:

> The fact that thiols can act as chain carriers in autoxidation, and can add on to olefines by the peroxide-catalyzed radical mechanism, are further indications of the transient existence of neutral thiol radicals.

In relation to enzyme reactions, Waters concluded (1948, p. 283):

> In view of the considerable amount of evidence which proves that free thiol radical, R-S·, can be formed either by the dissociation of disulfides, or by the one-electron oxidation of thiols by ferric and cupric salts, it is not unlikely that these enzymes catalyze chain reactions by way of the oxidation and reduction of thiol groups to free thiol radicals in the

> same way as thiophenol can act as a chain carrier in autoxidation, and amyl disulfide as an autoxidation catalyst.
>
> A sulfur-containing protein could become an active catalyst if a minute proportion only of its disulfide links could momentarily dissociate to the radical form and thereupon dehydrogenate a vicinal (i.e. and absorbed) metabolite and start a reaction chain in biological systems, just as the alkyl disulfides can initiate the dehydrogenation of tetralin.

Tobolsky and Mesrobian (1954, p. 22) proposed that organic hydroperoxides might be formed by the dehydrogenation of a sulfhydryl group, yielding a free radical thiol. Free radical thiols have been used to catalyze the decarbonylation of aldehydes (Barrett and Waters, 1953) and free radical exchanges (Cohen and Wang, 1955). Free radical chain transfer reactions catalyzed by thiols are important in the manufacture of synthetic high polymers such as synthetic rubber and plastics (Walling, 1957, p. 152).

1. Sulfhydryl groups and fat oxidation

The effect of sulfhydryl groups on the oxidation of fats was first observed by Meyerhof (1923) who found that frog muscle in the presence of thioglycollic acid absorbed large amounts of oxygen. When the muscle was extracted with hot alcohol or ether, oxygen absorption ceased. The same reaction with lecithin was traced to the linolenic acid component of lecithin. Meyerhof attributed this reaction to a transfer of oxygen by sulfhydryl groups to the unsaturated fatty acids. The inhibition of the lecithin-sulfhydryl system by cyanide was regarded as evidence of trace metal activation. Copper accelerated the oxidation of lecithin by thioglycollic acid; iron and manganese had no effect on lecithin in the presence of cysteine.

Szent-Györgyi (1924a) was unable to find a fatty acid peroxide as a product in the oxidation of linolenic acid by thioglycollic acid. He proposed (1924b) that sulfhydryl groups bound molecular oxygen in the form of an active peroxide.

In attempting to discover the metabolic significance of glutathione, Hopkins (1925) studied the reaction between this sulfhydryl-containing compound and fats. In systems of glutathione and linoleic or linolenic acid at pH 3-4, sulfhydryl concentration remained constant while oxygen in excess of that required to oxidize glutathione to its disulfide was taken up and a corresponding amount of fatty acid oxidized. Hopkins agreed with the Meyerhof postulate of a peroxide formed by two thiol groups. During decomposition by the thiol peroxide, oxygen was purportedly transferred to the fatty acid and reduced sulfhydryl groups were regenerated. At neutral pH the reaction was altered and sulfhydryl groups were oxidized. When glycerides were used, an induction period of from one to three hours was observed. This interval could be reduced by increasing the glutathione concentration. Hopkins suggested that an autocatalyst in very low concentrations developed slowly in the reaction mixture. He recognized the role of metallic ions in the system and considered that they might be part of the autocatalyst. According to Hopkins, thiols acted as "pseudo" catalysts by assisting in the formation of the autocatalyst.

Allott (1926) considered that some of Hopkins' results could be attributed to autoxidation of the fatty acids or glycerides. He found the activity of glycerides could be increased by aeration and some samples

showed definite uptake of oxygen in the absence of glutathione. Tait and King (1936) reported the oxygen uptake at acid reactions by lecithin and reduced glutathione was greater than that of neutral fat or fatty acids, and also greater than that obtained from lecithin after hydrolysis.

Scarborough and Watts (1949) reported the prooxidant effect of ascorbic acid and cysteine in aqueous fat systems in the absence of phenolic antioxidants. These workers inferred that the prooxidant activity of ascorbic acid and cysteine was related to their property of being reversibly oxidized. Watts and Wong (1951) found ascorbic acid could catalyze the oxidation of linoleic and linolenic acids, but not of oleic acid. The prooxidant activity of ascorbic acid was inhibited by ethylenediaminetetraacetic acid. Holtz (1936) had previously reported the oxidation of linoleic and linolenic acids catalyzed by ascorbic acid was inhibited by cyanide. Deutsch et al. (1941) reported the oxidation of phospholipids catalyzed by ascorbic acid at pH 4 was inhibited by hydroquinone. Phospholipid oxidation catalyzed by ascorbic acid was increased by iron but was not inhibited by cyanide (Elliot and Libet, 1944). Ottolenghi (1959) studied the activity of ascorbic acid and metals on mitochondrial lipids and concluded that a co-oxidation of ascorbic acid and unsaturated fat was mediated by a metallic ion. The rate of the reaction was governed by the concentrations of ascorbic acid, metal and lipid. The peroxidation of lipids in isolated mitochondria was also reported by Tappel and Zalkin (1959a) who concluded that the oxidation was due to hematin catalysis. Sulfhydryl groups lost during oxidation accounted for only 20 percent of the observed oxygen consumption. Low

concentrations of antioxidants inhibited fat peroxidation in mitochondria (Tappel and Zalkin, 1959b).

The decomposition of lipid peroxides by protein sulfhydryl groups was studied by Dubouloz and Fondarai (1953). Free sulfhydryl groups in proteins were rapidly destroyed when peroxide was present in excess. Three to five atoms of peroxide oxygen disappeared for each sulfhydryl group originally present. A similar reaction was observed in liver tissue (Dubouloz et al., 1954).

2. Metallic-sulfhydryl oxidations

Work previously cited in relating sulfhydryl groups to fat oxidation has shown that trace metals are important in sulfhydryl and ascorbic acid catalyzed oxidations. These reports indicated that a reaction occurs between metals and reducing agents.

Mathews and Walker (1909) reported that iron at a level of 6×10^{-6} molar could double the speed of oxidation of cysteine. They believed that the mechanism of this reaction involved the formation of an intermediate compound of a ferric salt with cysteine. Dixon and Tunnicliffe (1923) found the optimum pH for the autoxidation of glutathione, cystine and thioglycollic acid was 7.4 while the reaction was inhibited at pH 4.0. The presence of the disulfide form of the sulfhydryl compound accelerated the reaction. Warburg and Sakuma (1923) demonstrated that the so-called autoxidation of cysteine was caused by trace metal impurities. By careful purification of cysteine, autoxidation was reduced to a low level. Addition of one microgram of iron to 20 mg. of pure cysteine resulted in a 10-fold increase in the amount of oxidation. The oxidation

was inhibited by cyanide or pyrophosphate. Ferric and ferrous iron were equally effective in promoting the oxidation of cysteine (Harrison, 1924). Harrison believed that sulfhydryl compounds were unable to combine directly with molecular oxygen unless they had been activated by iron. Meldrum and Dixon (1930) believed that the autoxidation of glutathione was not accelerated by the addition of iron alone but depended on the presence of some substance able to form catalytically active complexes with the metal.

The mechanism of the metal catalyzed oxidation of cysteine was described by Michaelis (1929). Ferrous salts reacted with cysteine at pH 7 to 8 to give ferritricysteine. This underwent an internal oxidation-reduction to form ferrocysteine plus cystine which, in turn, reacted with two molecules of cysteine to yield free cystine and ferrotricysteine. A cycle was established which ended in the oxidation of all the cysteine to cystine. Because of the oxidation-reduction nature of the iron-cysteine complex, Michaelis and Barron (1929) suggested that an active chemical system could be produced with cysteine, a metallic ion and an oxidant.

In the catalytic oxidation of glutathione by selenite, selenium diglutathione was an active intermediate (Tsen and Tappel, 1958). The work of Dixon and Tunnicliffe (1923) and Meldrum and Dixon (1930) also indicated that a metal complex with the disulfide form of a sulfhydryl compound was the active catalyst.

Albert (1950, 1952) reported that cysteine-metallic ion complexes were more stable than other amino acid-metallic ion complexes. Amino

acids combined most strongly with cupric ion followed in decreasing order by nicklous, zinc, cobaltous, ferrous and manganous ions. Hughes (1950) reported that the mercury-sulfur bond is stronger than the bond between mercury and any other group found in proteins.

Lyman and Barron (1937) found that glutathione was readily oxidized in the presence of a catalyst such as copper, hemin or hemochromogen, but was not oxidized by atmospheric oxygen when dissolved in metal-free buffer. Cyanide inhibited the oxidation of glutathione by copper, but the hemin catalyzed oxidation of glutathione was inhibited only at high concentrations of cyanide.

3. Photochemical oxidations involving sulfhydryl groups

The basic requirement for photochemical reactions is the absorption of radiant energy. Since most compounds which contain a free sulfhydryl group do not absorb visible light (Anslow and Foster, 1932), photochemical reactions involving sulfhydryl groups must be activated either by ultraviolet light or visible light in the presence of a sensitizer which can transfer the photo energy to the sulfhydryl group. Weiss and Fishgold (1936) exposed various sulfhydryl compounds to ultraviolet radiation and detected the production of hydrogen corresponding to the amount of the disulfide formed. Sulfhydryl compounds were also found to react with fluorescent dyes in the dark; fluorescence was quenched by the sulfhydryl groups.

Certain amino acids can be oxidized by visible light in the presence of a sensitizing agent. Weil et al. (1951) reported tyrosine, tryptophane, histidine, methionine and cystine were photooxidized in the

presence of methylene blue. In the photooxidation of blood protein hydrolyzates that had been sensitized by methylene blue, riboflavin, eosin, or protoporphyrin, only the aromatic, heterocyclic and sulfur-containing amino acids were photooxidized (Vodrazka and Sponar, 1957). Brin and Krasnovskii (1957) described the chlorophyll sensitized oxidation of ascorbic acid and cysteine. They postulated a mechanism of reaction involving the photoreduction of the sensitizer in the light, followed by reaction of the photoreduced pigment with oxygen.

Repke (1956) observed that ultraviolet radiation and daylight (in the presence of photosensitizers) inhibited the activity of hydrogen transporting enzymes in the skin. Inactivation reportedly resulted from oxidation of enzyme sulfhydryl groups. The oxidation of sulfhydryl groups by ultraviolet radiation was reported by Kofman (1957). The amount of free sulfhydryl groups in solutions of native egg albumin exposed to ultraviolet light decreased 30 percent in two to three hours following irradiation.

Ginsburg et al. (1957) reported peroxides were formed in animal tissues subjected to X-irradiation. The reactivity of sulfhydryl groups in the enzyme systems of animals subjected to ionizing radiations was increased while the sulfhydryl concentration remained unchanged. Some modification of the sulfhydryl groups was believed to have occurred. Barron (1954) reviewed literature regarding the production of free radicals from sulfhydryl compounds subjected to ionizing radiations in the presence of oxygen. A high yield of oxidizing compounds can be formed in this manner.

Fox et al. (1958) reported the production of a green pigment, identified as sulfmyoglobin, during the irradiation of meat extracts. The pigment was formed in maximum concentration at pH 5.3 with addition of cysteine to the meat extracts. A two-step reaction was believed to have occurred: (1) The production of hydrosulfide ion from the thiol-containing compound followed by (2) a reaction between hydrosulfide ion, myoglobin and an oxidant. Hydrogen peroxide was not considered to be the active oxidant since only cholemyoglobin was obtained by the use of hydrogen peroxide in the reaction mixture.

E. Fat Oxidation

Morris (1954) and Holman (1954) indicated in their reviews that while the exact mechanism of fat oxidation depends on the nature of the fat and the degree of unsaturation, fat autoxidation generally involves the attack of oxygen upon an ∝-methylene radical adjacent to a double bond. A chain reaction is established by the extraction of hydrogen from the ∝-methylene group thereby forming a free radical.

1. Effect of light on fat oxidation

According to Lea (1931a), the light catalyzed autoxidation of fat is autocatalytic and once oxidation has begun, removal of light does not stop the reaction. Even light of low intensity was found to have appreciable influence on the rate of oxidation.

Light has little effect on fat in the absence of oxygen. Holm et al. (1927) and Kilgore (1933) demonstrated that unsaturated fats exposed to light in the absence of air did not increase in peroxide value during

prolonged storage. Goldschmidt and Freudenberg (1934) exposed pure linolenic acid to ultraviolet light in the absence of air. The iodine number of the fatty acid did not change over a period of five days.

A theory for the photochemical acceleration of fat oxidation was proposed by Coe (1938) based on photosensitization of chlorophyll in vegetable oils or hematin compounds in animal fats. According to Coe's theory, peroxides are formed when nascent hydrogen released by the photosensitized pigment combines with molecular oxygen. The induction period for fat oxidation was attributed to the time required for development of active substances.

Lea (1939, p. 144) studied the effect of different spectral regions on fat oxidation and found that fats absorb strongly in the ultraviolet below 260 mu. Coe (1941) reported the wavelengths below 490 mu promoted oxidation while the spectral region from 540 to 740 mu was not effective. Greenbank and Holm (1941) concluded from their studies that the effectiveness of light in promoting fat oxidation increased as the wave length decreased. The blue end of the visible spectrum was most effective in promoting oxidation.

The photooxidation of unsaturated fatty esters in the presence of chlorophyll was reported by Khan et al. (1954). These workers proposed that light energy absorbed by chlorophyll was transferred to the unsaturated fat in some manner, supplying the activation energy for the oxidation. Bateman and Gee (1949a, b) determined that the photooxidation of unconjugated olefins initiated by the photolysis of olefin hydroperoxides proceeds by a chain reaction.

2. Metal catalyzed oxidation

Metals act as prooxidants in fat oxidation by decomposing fat peroxides to form free radicals which can initiate additional chain reactions (Holman, 1954). Lea (1939, p. 160) indicated metallic prooxidants catalyzed the decomposition of natural inhibitors in fat. Metals were effective in reducing the induction period, but thereafter had little effect on the rate of fat oxidation. Walling (1957, p. 427) stated that metals must have two oxidation states to act as oxidation catalysts. Metal sequestering agents such as ethylenediaminetetraacetic acid (EDTA) act either by precipitating the metal or by altering its oxidation-reduction potential. Belcher et al. (1955) reported that EDTA markedly lowered the oxidation potential of ferric-ferrous and cupric-cuprous systems by strongly complexing ions in the higher state of oxidation. This had the effect of making the lower oxidation state a more powerful reductant.

Uri (1956) investigated the aerobic oxidation of unsaturated fatty acids in the presence of metal stearates, phthalocyanines, and octamethyltetraazoporphin. The latter two types of compounds were very much more active than the stearates, showing appreciable activity at a molar concentration of 5×10^{-8}. Cupric ion did not catalyze the production of linoleate hydroperoxide but was active in its decomposition. The most active metal catalysts in the oxidation of linoleic acid were those which are normally oxidized by a one electron transfer (Uri, 1958); metallic ions which are normally reduced by a one electron transfer, such as cupric ion, have little activity. Cupric ion is active in linoleate

oxidation if peroxide is initially present or if reducing agents are present. In this case, free radical intermediates are formed during the reduction of cupric to cuprous ion.

3. Hematin catalyzed oxidation

Haurowitz et al. (1941) and Tappel (1953a) showed that the destruction of hemin and hemoglobin was coupled with the oxidation of unsaturated fatty acids. Iron released from the hematin compounds during their oxidation had little catalytic activity.

The peroxidation and carotene bleaching effect of bacon extracts reported by Reiser (1949) were attributed to an enzyme. Oxidation and bleaching were reduced or eliminated when the bacon extracts were heated. Tappel (1952, 1953a), on the other hand, was unable to demonstrate the presence of lipoxidase in pork, beef, chicken, turkey or fish. Boyd and Adams (1955) could find no evidence of enzymic fat oxidation in beef, pork, uncured bacon, and cured, unsmoked bacon. Tappel (1952) attributed Reiser's results to the hematin compounds in bacon extracts.

The catalytic properties of blood pigments was first demonstrated by Robinson (1924). She established that the oxidative catalysts in blood were the iron porphyrin compounds. These compounds were much more active than inorganic iron and were not inhibited by cyanide as was inorganic iron. Barron and Lyman (1938) reported hemochromogens were better catalysts for unsaturated fat oxidation than hemin, but were inhibited by cyanide while hemin was not. Chain reaction inhibitors prevented the hemin catalyzed oxidation of linseed oil and oleic acid.

The mechanism of oxidation of colloidal linoleate by hemin, hemoglobin, myoglobin, and cytochrome c was established as a free radical chain mechanism by Tappel (1953b, 1955). He considered hematin compounds served as initiators of free radicals by reacting with fatty acid hydroperoxides. The iron porphyrin group of heme proteins and peroxides were considered the essential reactants (Tappel, 1955). Free radical inhibitors such as nordihydroguaiaretic acid, propyl gallate, butylated hydroxyanisole, tocopherol, methylene blue, tryptophane, and sodium diethyldithiocarbamate were effective inhibitors of hematin catalyzed oxidations (Chang and Watts, 1949; Tappel, 1953c, 1954; Lew and Tappel, 1956).

The activation energy of the hematin catalyzed oxidation of linoleate was reported to be 3.3 kilocalories per mole (Tappel, 1953b). This figure was later revised to 5 kilocalories per mole (Maier and Tappel, 1959). In contrast, the autoxidation of linoleate requires an activation energy of over 15 kilocalories per mole (Tappel, 1953b).

4. Effect of curing on fat oxidation

According to Lea (1939, p. 213), curing increases the susceptibility of fat to oxidation. Bacon fat was found to contain an appreciable level of peroxide oxygen (Lea, 1931b). Fat adjacent to lean meat was especially susceptible to oxidation. This suggests the occurrence of hematin catalyzed oxidation at the protein-fat interface.

Although nitrite is a strong oxidizing agent in acid solution, Lea (1939, p. 215) believed that the concentration of nitrite in cured meats was too low and the acidity insufficient for nitrite to be a factor in

the increased susceptibility to oxidation of cured meats. Metallic prooxidants in brines were likewise present in too low a concentration to have any appreciable effect, according to Lea (1939, p. 215). Watts (1954) reported that sodium chloride has been associated with rancidity and discoloration in cured meat. The prooxidant effect of sodium chloride apparently depends on the presence of curing salts, methemoglobin formation being accelerated in the absence of nitrite.

5. Effect of antioxidants

Smoking of cured meat delays the onset of rancidity (Watts, 1954). The antioxidant activity of smoke is presumed to derive from the phenolic substances in smoke. Watts and Faulkner (1954) studied the antioxidant activity of liquid smokes. After six months storage at -17°C., frozen ground pork treated with 0.04 percent liquid smoke had no peroxide value. Pork treated with 0.01 percent butylated hydroxyanisole had a peroxide value of 72 after the same storage period. White (1944) determined that smoked bacon could be stored for two months at -1°C. to -18°C. without becoming rancid; unsmoked bacon was usually rancid after one month. Smith et al. (1945) found that smoking retarded peroxide development in bacon while exposure to light promoted oxidation. Antioxidants retarded the development of peroxides and smoking plus antioxidant treatment was considered to maintain bacon in a suitable condition at least five times as long as untreated bacon. Hanley et al. (1953) treated bacon with hydroquinone, butylated hydroxyanisole, propyl gallate and citric acid, individually and in combination. While rancidity evaluation by a trained taste panel did not correlate with chemical criteria, coatings (lard)

containing 5 to 10 percent butylated hydroxyanisole markedly increased the fat stability.

Antioxidants have had variable effects on the color of cured meats. Watts (1957) reported cured hams treated with polyphosphates had a greater color stability and required a longer time to develop rancidity than paired, untreated hams. Polyphosphates were reported (Watts, 1950; Lehman and Watts, 1951) to have good antioxidant properties in aqueous fat systems. Kraft and Wanderstock (1950) applied the antioxidants nordihydroguaiaretic acid and butylated hydroxyanisole dissolved in a coconut oil base to the surfaces of smoked and precooked smoked hams; they did not observe any increase in color retention in the treated samples.

This review has treated the nature of the pigment of cured meat and the factors which relate to its production and degradation. The nature of reactions by sulfhydryl groups and fat oxidation have received major attention because these relate directly to the present study. The peroxidative effect of sulfhydryl compounds (Meyerhof, 1923; Hopkins, 1925; Wheeldon, 1958) together with the reports of Haurowitz et al. (1941) and Tappel (1955) on the co-oxidation of unsaturated fats and hematin compounds represent a possible fading mechanism. The experimental verification of this mechanism was the major objective of this study.

III. EXPERIMENTAL METHODS AND MATERIALS

A. Materials

1. Meat

The cured meats used in this study included big bologna, picnic ham and "Spam" type canned luncheon meat. All meat was procured from a local market. A Hobart Slicing Machine, Model 411, with the thickness control set at 10 was used to cut meat slices to a thickness of approximately 0.09 inch. A Hobart KitchenAid Model 4 mixer equipped with a grinding attachment containing a number 10 plate with 3/16 inch holes was used for grinding bologna. Meat was ground five times and wrapped in a foil-polyethylene laminate and stored in the refrigerator until used. Ground samples that were to be kept for more than one day were stored in the freezer. Preparation of samples was carried out in a darkened room. Hornsey (1957) observed that even a small amount of illumination during sample preparation could result in fading when cured meat was subsequently placed in the dark.

2. Pigments

a. Myoglobin For the preparation of myoglobin, a combination of the methods of Theorell (1932), Morgan (1936), Ginger et al. (1954) and Draudt and Deatherage (1956) was used with some additional modification. Beef round (17 pounds) was ground in a Hobart Food Cutter, Model 8, equipped with a silent cutter and a grinding attachment. The meat was comminuted in the silent cutter and then ground twice through a number 12-3 plate with 3/16 inch holes. An equal weight of distilled water was

added to the meat and the mixture was allowed to stand overnight at a temperature of 3-5°C. The infusion was filtered through cheesecloth and the filtrate was treated with basic lead acetate until no further protein was precipitated. Completion of protein removal was determined by centrifuging an aliquot of the mixture and adding a few drops of basic lead acetate solution to the supernatant. Appearance of a precipitate indicated incomplete removal of proteins. The pigment solution containing precipitated proteins was centrifuged at 6600 X G in a Model SS-1A Servall Superspeed angle centrifuge.

An approximately 10-fold concentration was obtained by passing the pigment solution through a column containing the free acid form of carboxymethyl cellulose (Peterson and Sober, 1956). The pigment was eluted with 0.1 N phosphate buffer at pH 7.5. The eluted pigment was collected in tubes by means of a Packard automatic fraction collector. The contents of the tubes containing the greatest portion of the pigment were pooled and the solution was brought to a phosphate concentration of 3.0 M at a pH of 6.5 to 7.0. The solution was centrifuged and the filtrate was dialyzed against distilled water at 4°C. to remove phosphate. The filtrate containing the pigment was dialyzed against a saturated ammonium sulfate solution adjusted to pH 7 with addition of ammonium hydroxide. When the chromoprotein had been precipitated, the mixture was centrifuged and the filtrate was discarded. The precipitate was extracted with aliquots of 82 percent saturated ammonium sulfate adjusted to pH 7 until no further color was extracted. The remaining precipitate was discarded. The combined extracts were dialyzed against saturated ammonium

sulfate until the chromoprotein was again precipitated. The precipitate was obtained by centrifugation, dissolved in a minimal amount of distilled water and dialyzed against distilled water until free of sulfate. The pigment solution was standardized as cyanmetmyoglobin according to the method of Drabkin and Austin (1935).

b. Hemoglobin Fresh blood was obtained from a calf at the time of slaughter. The blood was defibrinated by shaking with glass beads and filtering through cheesecloth. The defibrinated blood was centrifuged and the red blood cells washed with 0.85 percent saline. Blood cells were diluted with distilled water until microscopic examination showed only a few intact cells. The hemoglobin solution was standardized by the method of Drabkin and Austin (1935).

c. Sulfhemoglobin Sulfhemoglobin and sulfmyoglobin are produced by the action of hydrogen sulfide on the pigment in the presence of oxygen (Lemberg and Legge, 1949, p. 490). For the present study, sulfhemoglobin was produced by bubbling hydrogen sulfide and air through a hemoglobin solution. The exact mechanism for the conversion of hemoglobin to sulfhemoglobin was not known; however, Lemberg and Legge (1949, p. 491) state that complete conversion of hemoglobin to sulfhemoglobin has never been achieved.

d. Denatured globin nitric oxide hemochrome The pigment of cured meat (DGNOHb) was prepared by heating a mixture of 1.0 ml. hemoglobin, 2.0 ml. of 0.2 percent sodium nitrite, 1.0 ml. of 0.5 molar buffer at pH 5.4 and a trace of sodium dithionite in 12 ml. glass centrifuge tubes at 85°C. for 15 minutes. The denatured pigment was

centrifuged, washed and used in experiments at a level equivalent to a hemoglobin concentration of 3×10^{-4} molar.

3. Chemicals

a. Safflowerseed oil A commercial grade of oil was used to prepare emulsions containing 2.5 ml. safflowerseed oil, 5.0 ml. of 0.02 molar phthalate buffer at pH 6.2 and two drops of Tween 80 (polyoxyethylene sorbitan monooleate). The mixture was emulsified in a hand homogenizer and used at a level of 0.5 ml. per manometer flask.

b. Methyl linoleate The method described by Parker et al. (1955) was used to prepare methyl linoleate. The prepared material had a refractive index of $n_D^{25} = 1.4589$. The predicted purity was 92 to 95 percent methyl linoleate and five to eight percent methyl esters of oleic and saturated acids. Methyl linoleate was stored in a glass stoppered flask at a temperature of $-17^{\circ}C$. Working solutions were prepared by weighing a suitable aliquot into a 10 ml. volumetric flask and adding 0.1 ml. of Tween 40. Deionized water was used to bring the flask contents to volume.

c. Solvents Technical grade hexane (Skelly B) was purified by passing the solvent through columns of activated silica gel. Solvents treated in this manner had zero absorption at 232 mu when compared to a distilled water blank.

Ethyl alcohol was used to aid in breaking the emulsions formed between methyl linoleate, Tween 40 (polyoxyethylene sorbitan monopalmitate) and the aqueous phase in the reaction mixtures. Ethyl alcohol was purified by distilling over potassium hydroxide and metallic zinc. Distilled alcohol had zero absorption at 232 mu compared to distilled water.

d. Antioxidants Butylated hydroxyanisole, butylated hydroxytoluene and propyl gallate were obtained from Eastman Chemical Products, Inc. A sample of nordihydroguaiaretic acid was obtained from Dr. Hammond of the Department of Dairy and Food Industry. Antioxidants were dissolved in ethyl alcohol and used at a level of 0.01 percent.

e. Gases Oxygen and nitrogen were obtained from commercial cylinders and used without further treatment.

4. Packaging films

300 MSAT-80 and 300 LSD cellophane were used in this study because of their high gas permeability and transparency.

B. Methods

1. Determination of cured meat color

a. Optical methods The reflectance ratio method for the determination of cured meat color described by Ramsbottom et al. (1951) and Erdman and Watts (1957) was used in initial studies of cured meat fading. This method employs the ratio of reflectance at 650 mu to that at 570 mu as an index of color in cured meat. A block of U.S.P. magnesium carbonate served as the standard. Measurements were made on a Beckman DU spectrophotometer equipped with a diffuse reflectance attachment.

During the course of this work, a Gardner Automatic Color Difference Meter, Model AC-1, became available. This instrument is a tristimulus colorimeter measuring color relative to a standard. A medium red standard (R_d = 33.8; a = 34.4; b = 12.9) obtained from the manufacturer was used as a reference. A Model 150S Sorensen AC voltage regulator was used with

the color difference meter. Measurements were made on the R_d scale of the instrument and only the "a" values (red-greenness) were recorded.

b. Chemical method The destruction of cured meat pigments was determined by the method of Hornsey (1956). In experiments with bologna, two gram samples of bologna were ground with a mortar and pestle. The ground meat was extracted with 8.6 ml. of a mixture containing 40 ml. of acetone and 3 ml. of distilled water. The absorbancy of the extract was determined at 540 mu. In experiments with denatured globin nitric oxide hemochrome, 1 ml. aliquots of a reaction mixture were centrifuged in 12 ml. glass centrifuge tubes. The precipitate was extracted twice with 2 ml. portions of the acetone solution described above. The color extracts containing acetone nitroso-heme were pooled and the absorbancy was determined at 540 mu.

2. Manometric procedures

A Model WB-4 GME Warburg respirometer was used for the measurement of oxidation in mixtures of pigment, lipids and cysteine. The temperature of the Warburg bath was maintained at 20°C. by circulating tap water through a cooling coil immersed in the bath. All reactants, except the heme pigments which were placed in the side arms, were added to the main compartment of the manometer flasks. The center wells contained 10 percent potassium hydroxide and a small filter paper fan to absorb any carbon dioxide produced. Flasks were equilibrated by shaking for 15 minutes with the manometer stopcocks open. At zero time, the manometer fluid level was adjusted to the reference point, the stopcocks were closed and the sidearm contents tipped in. Readings were corrected for

thermobarometer changes and the oxygen consumption calculated by the use of previously determined flask constants.

3. Treatment of meat slices with reagents

Slices of meat measuring approximately two inches in width and 3.5 inches in length, for measurements on the color difference meter, or one inch in width and two inches in length for reflectance ratio measurements were immersed in solutions of reagents in large beakers. The beakers were agitated frequently to insure good contact of the reagent with all surfaces. After treatment, slices were removed from the solutions and blotted between paper towels. Meat slices were then packaged and their color determined before exposure to light.

4. Exposure of samples to light

Samples were exposed to light in a refrigerated display case at a temperature of 15°C. The display case was illuminated by two 48 inch General Electric 40 watt fluorescent tubes (deluxe cool white) giving a light intensity of 50 to 60 footcandles at the surface of the samples.

a. Slices in film Bologna slices were sealed in cellophane with a Vertrod, Model 8A, electronic heat sealing machine. Samples were placed in the display case in such a manner that each received approximately the same level of illumination.

b. Ground meat in film Ten gram samples of finely ground bologna were placed between sheets of cellophane and pressed into a thin layer between glass plates. The film was sealed on four sides by means of the

Vertrod electronic sealer. Samples were exposed to light as described above.

c. Pigment suspensions in gelatin Hemoglobin at a concentration of 3×10^{-4} molar was converted to denatured globin nitric oxide hemochrome and suspended in a 5 percent gelatin solution. Cysteine, methyl linoleate and ferric salts were added to the gelatin suspension of pigment as required. The suspensions, at a temperature of 40°C. to improve fluidity, were poured into 42 mm. glass petri dishes. Plates were refrigerated until the suspensions were solid before being exposed to light. Color was measured using the Gardner Color Difference Meter.

5. Determination of lipid oxidation

a. Linoleate oxidation The spectrophotometric method described by Tappel et al. (1953) was used for the determination of methyl linoleate oxidation. One ml. aliquots of reaction mixtures were extracted with 2 ml. of Skelly B. The absorbancy at 232 mu was determined on 0.1 ml. of the extract diluted to 3.0 ml. with Skelly B.

b. Iodometric determination of peroxides The method described by Stansby (1941) was used to determine peroxide formation in the fat component of cured meat. Ground meat samples (10 grams) were placed in 125 ml. Erlenmeyer flasks and extracted for 10 minutes with 12 ml. of chloroform at a temperature of 40°C. on a rotary shaker. Samples were covered to exclude light during the extraction period. The slurry of meat and chloroform was filtered and 5 ml. of the filtrate was pipetted into a tared beaker for determination of the total fat extracted. A 10 ml. aliquot of the chloroform extract was placed in a clean 125 ml. Erlenmeyer

flask and deaerated by bubbling with nitrogen for 5 minutes. Fifteen ml. of glacial acetic acid, containing 4 ml. of concentrated hydrochloric acid per liter were added to the chloroform and nitrogen was bubbled through the mixture for one minute. One ml. of a saturated solution of potassium iodide was added and the flask was stoppered and placed in the dark for 5 minutes. At that time, 60 ml. of distilled water containing 3 ml. of 0.1 N hydrochloric acid was added to the flask as well as 2.5 to 3.0 ml. of a 1.5 percent solution of soluble starch. The mixture was shaken vigorously to release iodine from the chloroform layer and the flask contents were titrated with 0.002 N sodium thiosulfate solution to a starch-iodine end point. A microburette with a capillary delivery tube which delivered 0.016 ml. per drop was used for the titration. The peroxide value was calculated as follows:

$$\text{peroxide value} = \frac{\text{ml. thiosulfate X 0.002 X 1000}}{\text{2 X grams fat titrated}}$$

6. Sulfhydryl groups

The qualitative nitroprusside test for sulfhydryl groups as adapted for use with cured meats by Watts et al. (1955) was used to determine the minimum treatment with mercuric chloride that would complex the available sulfhydryl groups. The absence of a positive nitroprusside test after mercuric chloride treatment was interpreted as indicating free sulfhydryl groups were no longer present.

7. Oxidation experiments

Cured meat pigment and methyl linoleate oxidation were determined directly in reaction mixtures containing denatured globin nitric oxide

hemochrome, methyl linoleate and cysteine. Flasks containing the reaction mixtures were maintained at a temperature of 20°C. A stream of oxygen served to keep the denatured pigment in suspension. One ml. aliquots of the reaction mixtures were removed for the determination of residual cured meat pigment and methyl linoleate oxidation.

IV. RESULTS AND DISCUSSION

A. Effect of Sulfhydryl Reagents on the Color of Cured Meat

In preliminary work, treating cured meat with reagents which combined with or oxidized free sulfhydryl groups increased the stability of the pigment when the meat was exposed to light. Sulfhydryl groups have been reported to be important in the production of color in cured meat and for protection of this color against light-induced fading (Watts et al., 1955; Erdman and Watts, 1957). These functions of sulfhydryl groups in cured meats are apparently related to their reducing activity (Kelley and Watts, 1957). The present observations indicate that sulfhydryl groups may function in reactions which result in the oxidation of cured meat pigment.

1. Effect of sulfhydryl reagents on the nitroprusside test in cured meat

Immersion of bologna slices in 0.01 molar mercuric chloride for five minutes, or in 0.1 molar mercuric chloride for one minute was sufficient treatment to prevent the development of a positive nitroprusside test for sulfhydryl groups. Treatment of bologna slices with 0.1 molar sodium iodoacetate for 15 minutes did not prevent the development of a positive test. The failure of iodoacetate to combine with sulfhydryl groups in the meat may be explained by steric hindrance factors which prevented protein bound sulfhydryl groups from reacting with the iodoacetate molecule.

2. Effect of sulfhydryl reagents on color of cured meat exposed to light

Slices of bologna, approximately 2 inches by 1 inch, were dipped into 0.1 molar solutions of iodine, potassium iodide, mercuric chloride and sodium iodoacetate for one minute. The slices were sealed in 300 MSAT-80

cellophane and exposed to light at an intensity of 50 to 60 footcandles. The ratio of reflectance at 650 mu to that at 570 mu was determined on each sample at the intervals indicated in Table 1.

The 650/570 reflectance ratio decreases when cured meat fades under the influence of light (Kraft and Ayres, 1954; Erdman and Watts, 1957). This result is also seen in Table 1. Treatment with 0.1 M iodine caused the meat to have a reddish brown coloration. The other treatments did not alter the appearance of the samples.

Visual examination showed that samples treated with mercuric chloride or with potassium iodide could be distinguished from the control after 3.5 hours of illumination by their greater degree of redness. Of the various treatments employed, mercuric chloride best preserved the color of bologna samples exposed to light. In addition, mercuric chloride was the only reagent which prevented development of a positive nitroprusside test for sulfhydryl groups.

The effect on bologna of treatment with potassium iodide may not have been related to a reaction with sulfhydryl groups. Walsh and Rose (1956) reported that potassium iodide inhibited the photooxidation of nitric oxide myoglobin and they attributed this inhibition to potassium iodide's property of deactivating excited states. Privault (1927) reported that excitation energy in fluorescent systems was dissipated as kinetic energy in the presence of potassium iodide.

The results shown in Table 1 do not indicate any considerable difference in reflectance ratios between controls and treated samples at different time intervals. The suitability of the 650/570 reflectance

Table 1. Effect of sulfhydryl reagents on the reflectance ratio of bologna slices exposed to light

Reagent	Reflectance ratio 650mu/570mu Hours of illumination					
	0	0.5	1.0	1.5	2.5	3.5
Control	2.46	2.20	2.14	1.95	1.93	1.91
0.1 M $HgCl_2$	2.41	2.27	2.24	2.06	1.98	1.87
0.1 M KI	2.32	2.35	2.19	2.08	1.93	1.93
0.1 M I_2	2.13	2.06	1.99	1.86	1.90	1.96
0.1 M CH_3COONa	2.50	2.23	2.20	1.91	1.99	1.75

ratio as a measurement of meat color was questioned since treatment of the bologna slices with mercuric chloride noticeable improved the color retention of the samples exposed to light as compared to untreated controls. Reflectance ratios of replicate samples of bologna treated with 0.1 M mercuric chloride for five minutes were compared to a similar number of controls and the results statistically analyzed (Table 2). Treated samples had significantly higher reflectance ratios than controls after two hours of illumination, while controls had higher reflectance ratios initially and after 16 hours of illumination (Table 2). Visual examination, on the other hand, revealed that treated samples had a greater degree of redness at all times after the initial treatment. The

Table 2. Significance of the difference in reflectance ratios between mercuric chloride treated and untreated bologna slices exposed to light

	Reflectance ratio 650/570 mu[a]			
	Hours of illumination			
	0	2	4	16
0.1 M $HgCl_2$	2.49	2.09	1.94	1.50
Control	2.58	2.02	1.91	1.65
$S_{\bar{d}}$	0.040	0.024	0.030	0.014
t	2.315*	2.894**	0.095	10.22**

[a]Means of 21 samples.

*Significant difference P = 0.05 (Snedecor, 1959, p. 46).

**Significant difference P = 0.01 (Snedecor, 1959, p. 46).

reflectance ratio method, therefore, does not accurately describe color differences under the experimental conditions.

The effect of mercuric chloride on the color of cured meat apparently is related, in some manner, to a reaction between the reagent and sulfhydryl groups present in meat. One possible alternative is a reaction between the reagent and the pigment of cured meat in which the pigment loses its photosensitivity. Bologna slices were dipped into 0.01 and 0.1 molar solutions of mercuric chloride for periods of 1 to 10 minutes and exposed to light. After illumination, the pigment concentration was determined (Hornsey, 1956). Table 3 shows only slight variations in pigment concentration occurred regardless of the degree of mercuric chloride

Table 3. Effect of mercuric chloride treatment on the level of extractable pigment in bologna slices exposed to light

$HgCl_2$	Hours of illumination									
	0		2				4			
	Minutes of $HgCl_2$ treatment									
Molarity	0	10	0	1	5	10	0	1	5	10
0.0	2.15[a]	-	1.87	-	-	-	1.72	-	-	-
0.01	-	2.00		1.87	1.96	1.82		1.85	1.79	1.90
0.10	-	2.18		1.91	2.07	1.97		1.82	1.87	1.84

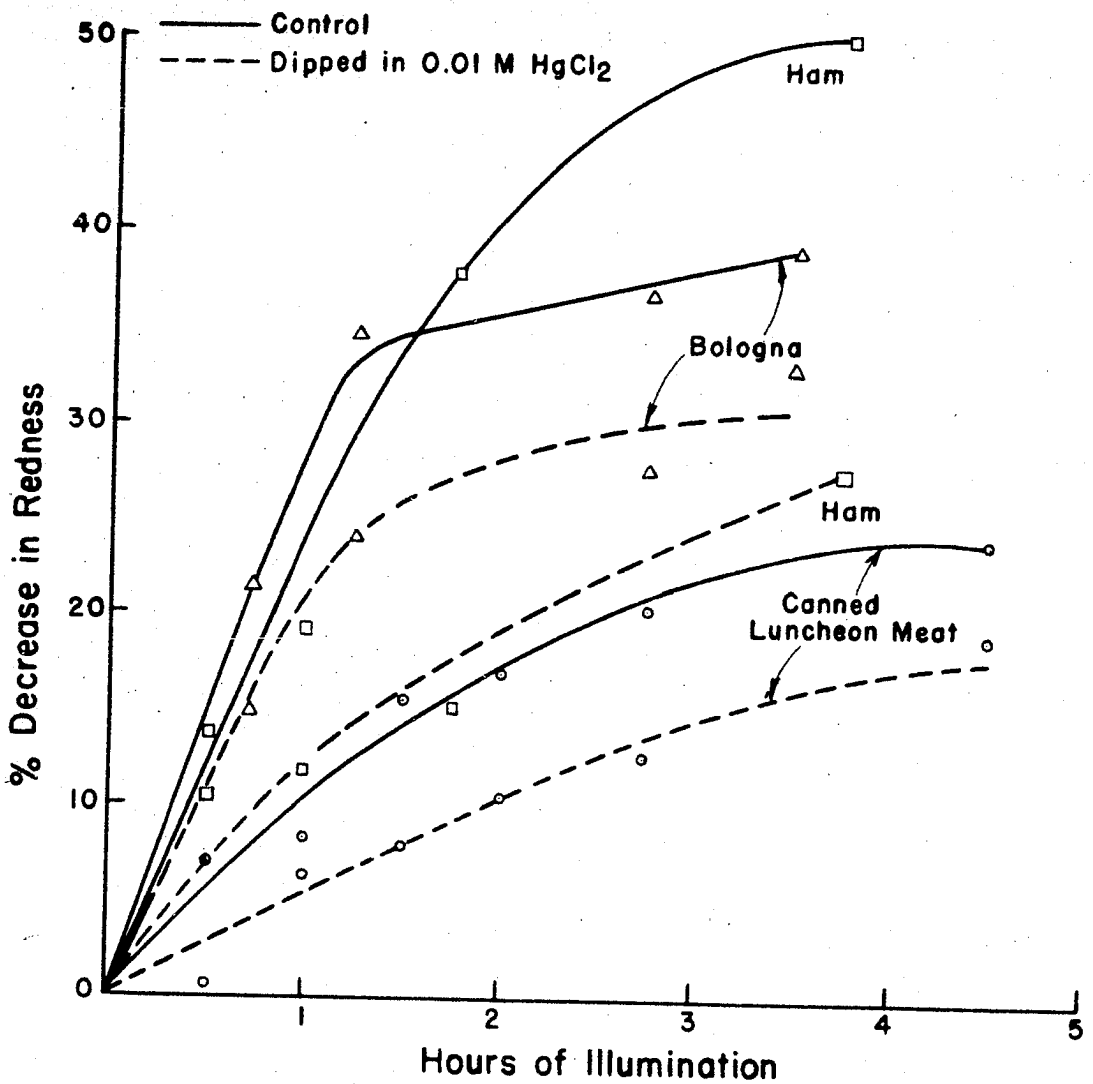
[a]Expressed as mg. of pigment per gram of bologna.

treatment or the time of illumination. Since the extraction procedure has been reported to be specific for denatured globin nitric oxide hemochrome (Hornsey, 1956), presumably treatment with mercuric chloride does not measureably alter the nature of the cured meat pigment.

Fading of cured meats was further studied with the aid of a Gardner Color Difference Meter. Three types of cured meat, sliced canned luncheon meat, sliced picnic ham, and sliced bologna, were treated with 0.01 molar mercuric chloride for five minutes and packaged in 300 MSAT-80 cellophane. Color measurements were made initially and at intervals during exposure to light. Figure 1 shows that cured meats treated with mercuric chloride lost less red color than did untreated controls exposed to light for the same time.

The Gardner Color Difference Meter appears to provide a more satisfactory means of measuring color than does the spectrophotometric

Figure 1. Effect of illumination on fading of cured meats treated with mercuric chloride


Control
Dipped in 0.01 M $HgCl_2$
Ham
Bologna
Ham
Canned
Luncheon Meat
% Decrease in Redness
Hours of Illumination
0
10
20
30
40
50
1
2
3
4
5

reflectance ratio method or the direct extraction method. The reflectance ratio method did not correlate with visual estimations of color in mercuric chloride treated samples versus their controls. In addition, the reflectance ratio is an index of the oxidation state of the pigment and is only indirectly related to color. The error in the extraction method derives from the fact that fading is a surface phenomenon while extraction yields pigments from the interior of the meat which has not been affected by light. Another difficulty of the extraction method is its destructive nature; it is not possible to measure pigment changes in a single sample over a period of time.

B. Hematin Catalyzed Oxidation in the Presence of Cysteine

The observation that destruction of free sulfhydryl groups in cured meat reduces light induced fading suggests that sulfhydryl groups in cured meat might take part in a reaction which could cause the destruction of cured meat pigment. In addition to those reactions involving sulfhydryl groups considered in the Review of Literature, Schales (1938) reported the production of hydrogen peroxide when oxygen was passed through solutions of cysteine, glutathione or thioglycollic acid. Although neither Walsh and Rose (1956) nor Draudt and Deatherage (1956) found evidence of peroxide formation during fading of nitric oxide myoglobin or denatured globin nitric oxide myohemochrome, these workers investigated the action of light on pigment alone. In studies of reaction mixtures containing unsaturated fats, sulfhydryl groups and hematin compounds, Tappel and Zalkin (1959a) attributed the peroxidation of mitochondrial lipids to hematin catalysis. Wheeldon (1958), however, held that lipid peroxidation

in a heart muscle preparation was caused by a metal catalyzed reaction between lipids and BAL (2,3-dimercaptopropanol).

Hematin compounds may be oxidized while acting as catalysts for the oxidation of unsaturated fats (Haurowitz et al., 1941; Tappel, 1953c; Maier and Tappel, 1959). Watts (1957) reported that color stability of cured hams was related to the degree of oxidation of the fat. Reactions between the various components of cured meat are, therefore, directly related to cured meat color. Manometric experiments were performed on model systems of pigments, fats and sulfhydryl groups in an effort to explain the earlier observation of the effect of mercuric chloride on cured meat exposed to light.

1. Manometric studies with safflowerseed oil

Safflowerseed oil was used initially as a source of unsaturated fat; a saturated fatty acid, capric acid, was also tried. Both hemoglobin and myoglobin were used as hematin catalysts. Cysteine served as the source of sulfhydryl groups. Results of these experiments are shown in Table 4.

Cysteine increased the rate of oxygen consumption in systems containing heme pigments and safflowerseed oil. Since a 5-fold and 10-fold increase respectively in cysteine concentration did not double the rate of oxygen consumption, cysteine oxidation alone was probably not responsible for the increase in oxygen consumption. The requirement for an unsaturated fat in this system was demonstrated by replacement with safflowerseed oil of capric acid. No oxygen consumption occurred in this case (Table 4).

Table 4. Oxygen consumption by systems of hematin compounds, cysteine and lipids

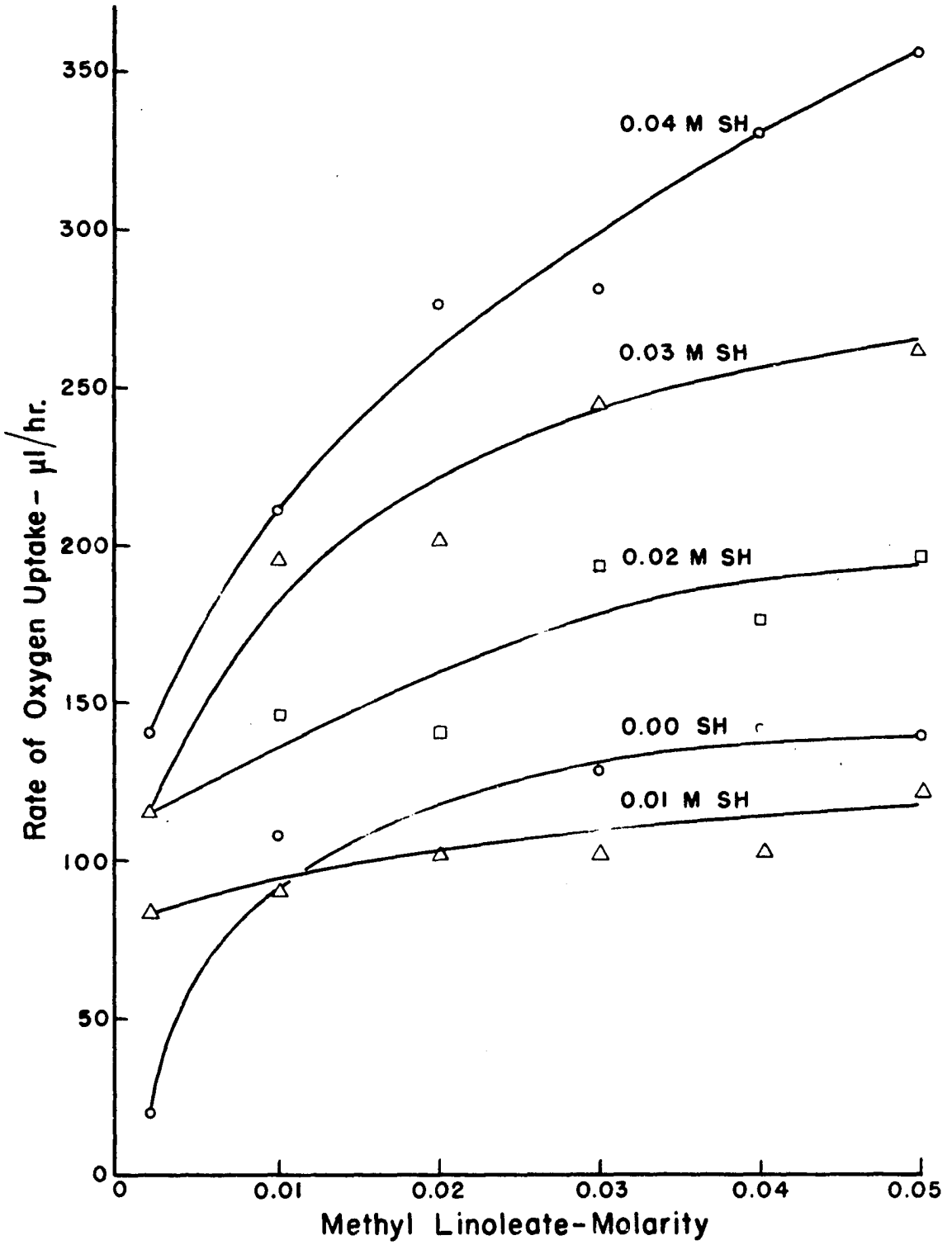
Cysteine uM.	Heme pigment uM.	Lipid	Rate of oxygen consumption ul./hr.
0.0	0.0	Safflowerseed oil	1.5
0.0	1.1 Mb	"	58
2.7	1.1 Mb	"	106
13	1.1 Mb	"	178
27	1.1 Mb	"	198
13	0.9 Hb	"	78
27	0.9 Hb	"	72
13	0.9 Hb	Capric acid	1.4

2. Manometric studies with methyl linoleate

The system of cysteine, heme pigments and methyl linoleate was studied by means of manometric experiments. The effect of methyl linoleate concentration on oxygen consumption by this system at various levels of cysteine is seen in Figure 2. Myoglobin concentration was held constant at 3.7×10^{-5} M. The slopes of the rate curves increase with the cysteine concentration. The rate of oxygen consumption is thus affected both by the methyl linoleate and cysteine concentrations.

Experiments were performed to determine whether the increase in oxygen consumption in the presence of cysteine was caused by the oxidation of cysteine or by a prooxidant effect of cysteine on the oxidation of

Figure 2. Effect of methyl linoleate concentration on oxygen consumption at increasing concentrations of cysteine


Rate of Oxygen Uptake - µl/hr.
350
300
250
200
150
100
50
0
0.04 M SH
0.03 M SH
0.02 M SH
0.00 SH
0.01 M SH
0
0.01
0.02
0.03
0.04
0.05
Methyl Linoleate-Molarity

methyl linoleate. Rates of oxygen consumption were determined in the presence and absence of cysteine at various concentrations of methyl linoleate. A prooxidant effect of cysteine is indicated when the rate of oxygen consumption in the presence of cysteine is greater than the rates of consumption by linoleate and cysteine measured separately. Cysteine concentration was held constant at 0.04 M. and linoleate concentration was varied as shown in Table 5. A prooxidant effect of cysteine is demonstrated at each level of methyl linoleate employed. This indicates that the extra oxygen consumption in this system resulted from the oxidation of unsaturated fats catalyzed in some manner by sulfhydryl groups.

Table 5. Prooxidant effect of cysteine in the oxidation of methyl linoleate

Linoleate M.	Rate of oxygen consumption ul./hr.			
	By cysteine (1)	By linoleate (2)	Theoretical vs. experimental rate Summation (1) + (2)	Cysteine combined with linoleate
0.0	23	-	23	-
0.002	-	89	112	140
0.01	-	89	112	210
0.02	-	115	138	275
0.03	-	130	153	280
0.04	-	138	161	330
0.05	-	139	162	354

The effect of cysteine concentration on oxygen consumption when methyl linoleate concentration was held constant at 0.02 M is shown in Figure 3. Myoglobin was used at a level of 3.7×10^{-5} M. Under the experimental conditions, cysteine did not enhance oxygen consumption at a concentration of 3.3×10^{-3} M. At cysteine concentrations of from 1×10^{-2} to 3.3×10^{-2} M., oxygen consumption was increased by a factor of from 1.5 to 3.0 times the consumption obtained in the absence of cysteine. The effect of cysteine concentration on oxygen consumption in this system appears to be critical.

The relation of cysteine concentration to the degree of prooxidant or catalytic effect on methyl linoleate oxidation was determined as in Table 5. Linoleate concentration was maintained at 0.02 M. and the cysteine concentration was varied as shown in Table 6. A prooxidant effect was obtained only at the highest level (0.033 M.) of cysteine employed. Figure 3 and Table 6 indicate a critical concentration of cysteine is required before a prooxidant effect appears.

3. Study of linoleate oxidation

The direct determination of linoleate oxidation was made in systems containing hemoglobin and cysteine. The low absorbancy of a control containing only methyl linoleate at 232 mu indicates autoxidation of linoleate was not an interfering factor in these experiments (Figure 4). In the presence of cysteine and hemoglobin, linoleate oxidation began after an induction period of about one hour. In the absence of cysteine, linoleate oxidation began immediately at a rapid rate. Cysteine alone was effective as a catalyst in the oxidation of linoleate, though a slower

Figure 3. Effect of varying cysteine concentrations on oxygen consumption by myoglobin and methyl linoleate

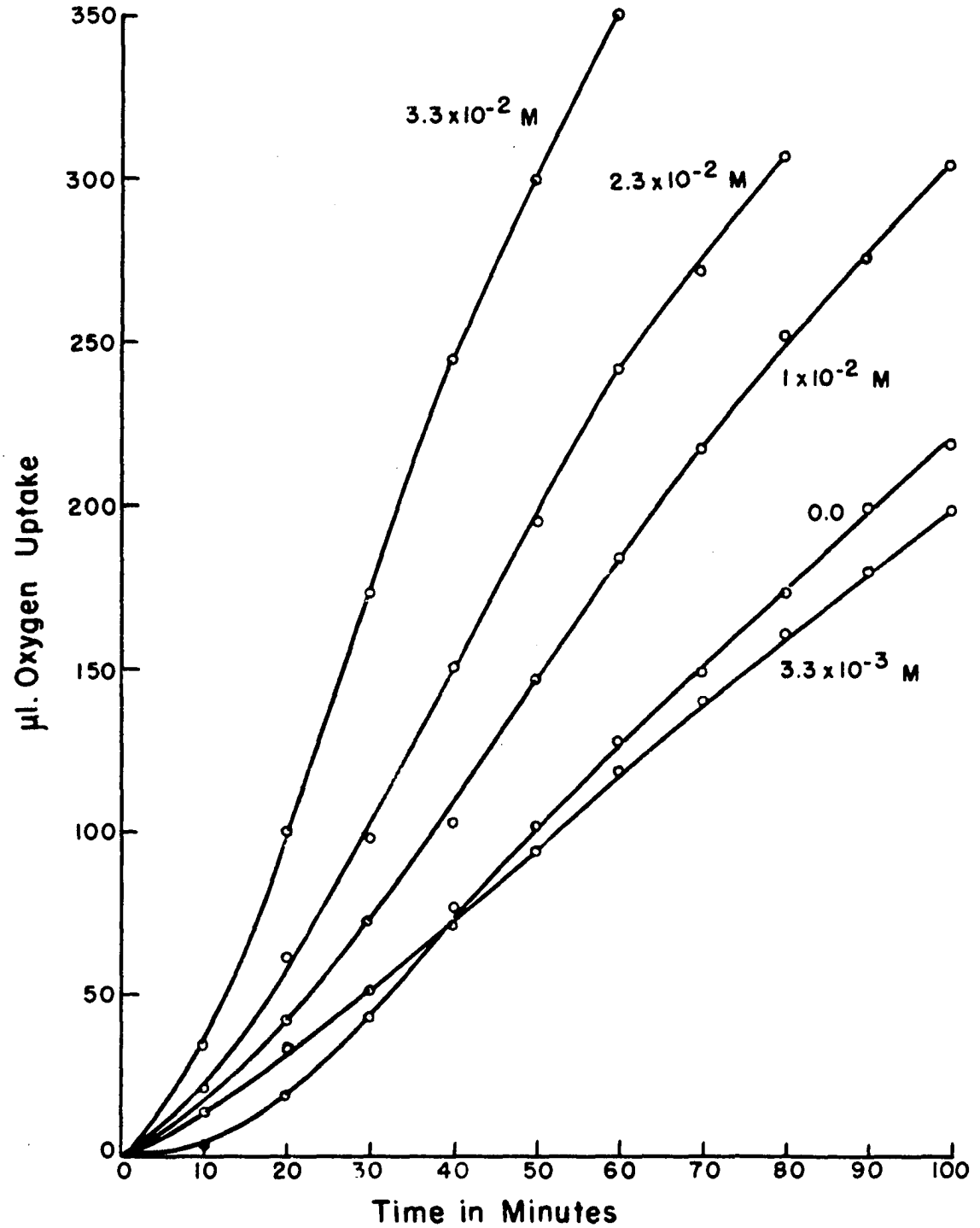

350
300
250
200
150
100
50
0
µl. Oxygen Uptake
3.3 x 10-2 M
2.3 x 10-2 M
1 x 10-2 M
0.0
3.3 x 10-3 M
0
10
20
30
40
50
60
70
80
90
100
Time in Minutes

Table 6. The prooxidant effect of cysteine at various concentrations in the oxidation of methyl linoleate

Cysteine M.	Rate of oxygen consumption ul./hr. By linoleate (1)	By cysteine (2)	Theoretical vs. experimental rate Summation (1) + (2)	Linoleate combined with cysteine
0.0	162	-	162	-
0.0033	-	-29[a]	222	133
0.01		54	268	216
0.023		121	296	283
0.033		235	319	397

[a]The negative rate indicates inhibition of oxygen consumption at this concentration of cysteine.

rate of oxidation was obtained than with hemoglobin alone. After the reaction had proceeded for one hour (Figure 4) linoleate oxidation by cysteine and hemoglobin was about one third that of the oxidation by cysteine alone and one quarter that of hemoglobin. The apparent inhibition of linoleate oxidation in the presence of cysteine and hemoglobin as opposed to the more rapid oxidation by hemoglobin or cysteine alone might be analogous to the inhibition of catalase by cysteine. According to Stern (1932), cysteine, like cyanide, forms an inactive complex with the ferric ion of the heme.

Figure 4. The oxidation of methyl linoleate in the presence of cysteine and hemoglobin

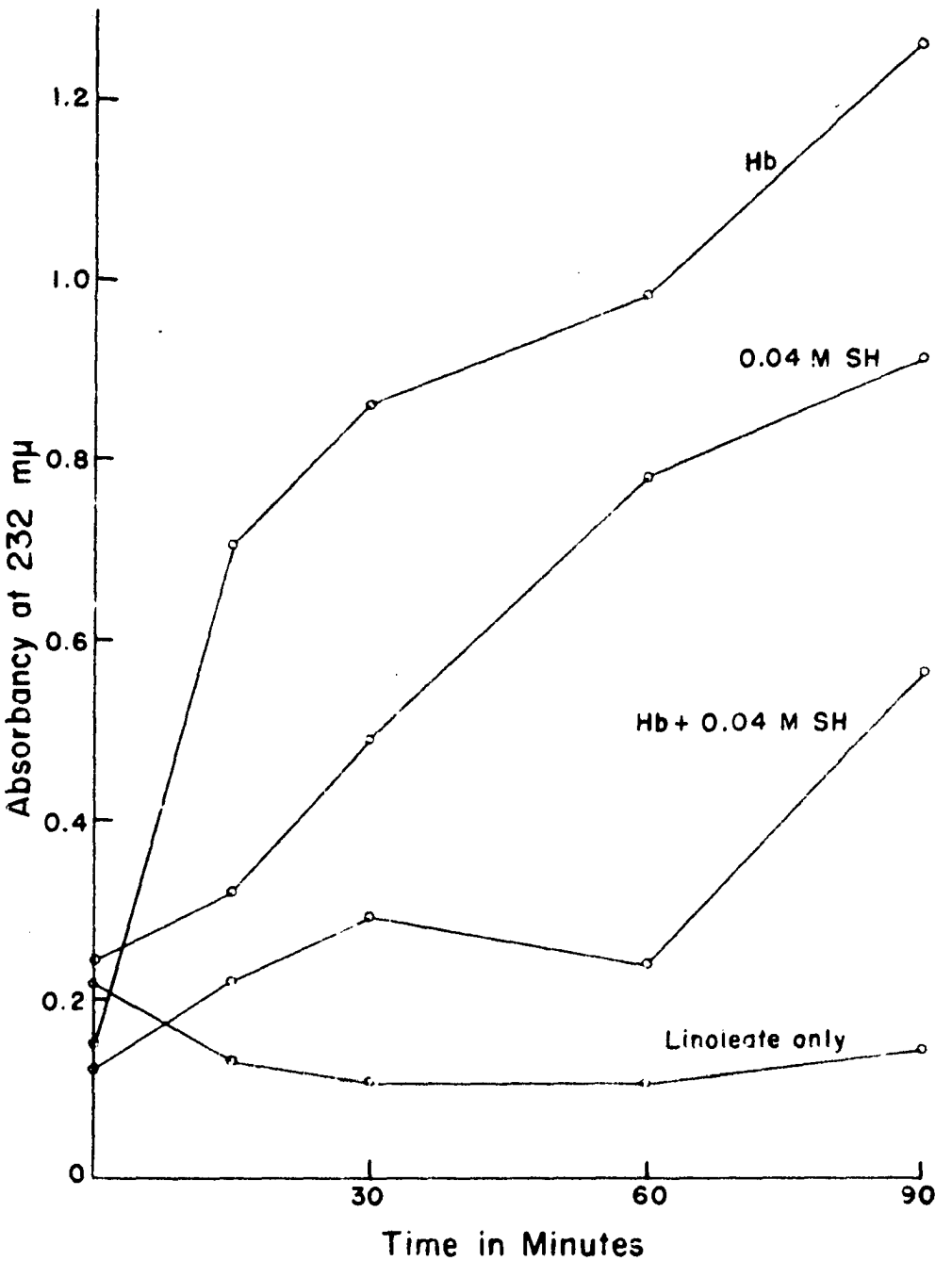
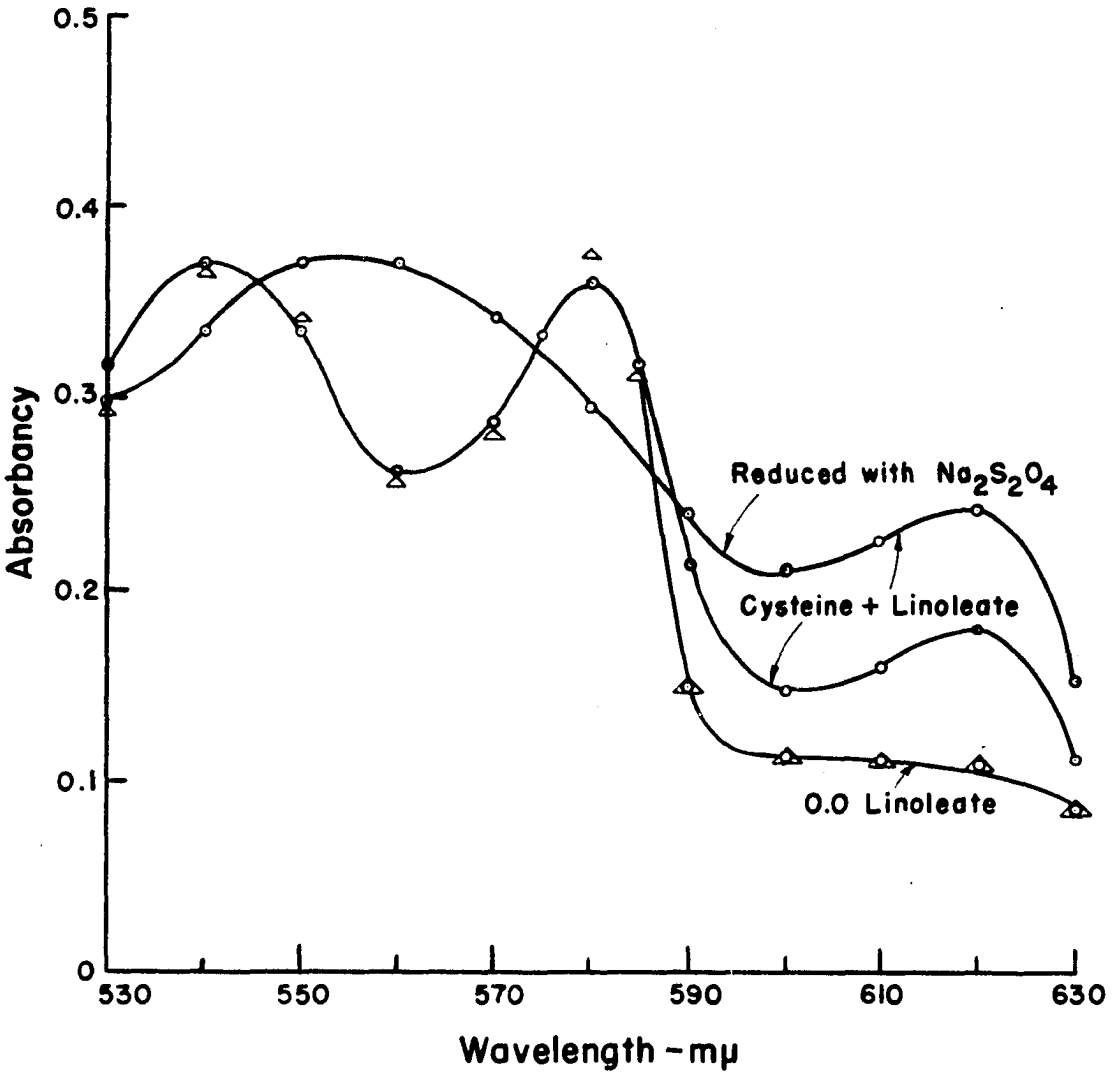

Absorbancy at 232 mμ
1.2
1.0
0.8
0.6
0.4
0.2
0
Hb
0.04 M SH
Hb + 0.04 M SH
Linoleate only
30
60
90
Time in Minutes

Figure 5. The effect of methyl linoleate on the production of sulfmyoglobin in the presence of cysteine


0.5
0.4
0.3
0.2
0.1
0
Absorbancy
530
550
570
590
610
630
Wavelength - mμ
Reduced with $Na_2S_2O_4$
Cysteine + Linoleate
0.0 Linoleate

4. Product of the co-oxidation of cysteine, myoglobin and methyl linoleate

At the completion of manometric experiments, the contents of some flasks were observed to be green in color. The green pigment was identified as sulfmyoglobin by the peak at 620 mu which was intensified when reduced with sodium dithionite (Figure 5). The 620 mu peak did not appear in the absence of linoleate, nor did any green pigment develop.

The production of sulfmyoglobin at cysteine levels which were found to be prooxidant and the absence of sulfmyoglobin when linoleate was not present suggested that the catalytic effect of cysteine might be related to an increased catalytic activity of sulfmyoglobin. The catalytic activity of hemoglobin and sulfhemoglobin were compared in a manometric experiment. The results shown in Table 7 indicate sulfhemoglobin was less active in catalyzing the oxidation of methyl linoleate than was hemoglobin. The prooxidant effect of cysteine, therefore, cannot be attributed to the production of sulfhemoglobin. The production of an inactive complex between the pigment and cysteine may explain the decrease in activity of hemoglobin in the oxidation of linoleate in the presence of cysteine (Figure 5).

Sulfhemoglobin and sulfmyoglobin are produced by the action of hydrogen sulfide on the pigment in the presence of oxygen (Lemberg and Legge, 1949, p. 490). In the present study, there was no evidence for the production of hydrogen sulfide in flasks containing pigment, cysteine and linoleate. Strips of filter paper saturated with lead acetate did not darken in flasks in which sulfmyoglobin was produced during manometric experiments. The failure to form sulfmyoglobin in the absence of

Figure 6. Effect of pH on oxygen consumption by systems containing methyl linoleate, cysteine and myoglobin

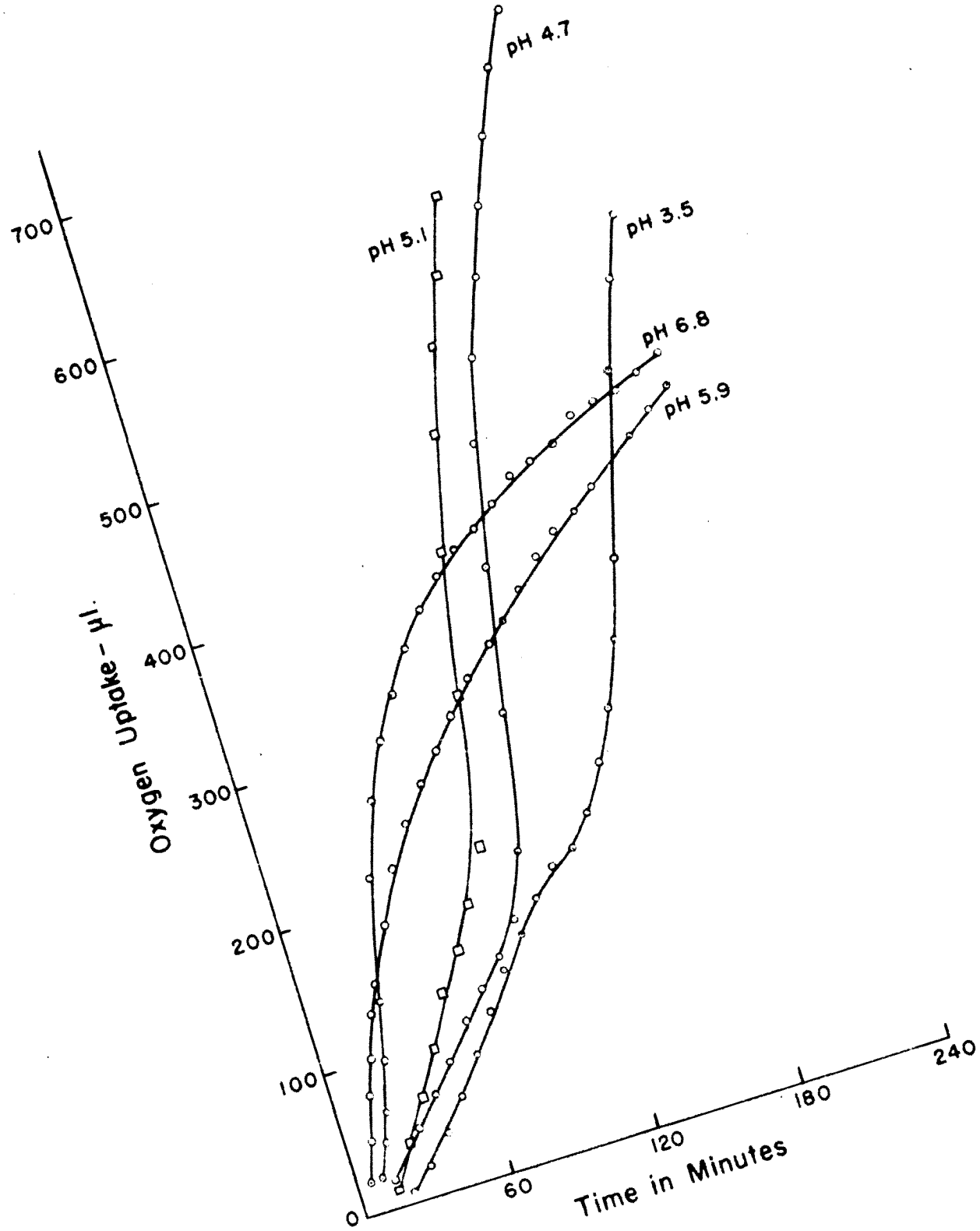

pH 4.7
pH 5.1
pH 3.5
pH 6.8
pH 5.9
700
600
500
400
300
200
100
0
Oxygen Uptake - µl
60
120
180
240
Time in Minutes

Table 7. Effect of hemoglobin and sulfhemoglobin on oxygen consumption by cysteine and 0.04 M. methyl linoleate

Cysteine M.	Rate of oxygen consumption ul./hr.	
	Hemoglobin	Sulfhemoglobin
0.0	38	40
0.03	86	80
0.04	158	56
0.05	63	34

linoleate suggested that the mechanism of formation was similar to that described by Fox et al. (1958) who observed the production of sulfmyoglobin in irradiated meat extracts. Sulfmyoglobin is produced, according to the latter workers, by a reaction between hydrosulfide ion, myoglobin and an oxidant. Hydrosulfide ion was produced by irradiation of thiol-containing compounds.

Possibly ionizing irradiation and hematin catalyzed oxidation of unsaturated fats both provide the conditions required for production of sulfmyoglobin. Barron (1954) stated that the oxidizing entities $H_2O_2^{--}$, OH and HO_2^{--} are formed during the irradiation of water saturated with oxygen. He pointed out that sulfhydryl compounds are readily oxidized by ionizing radiations to the free radical RS· which is a powerful oxidizing agent. According to Tappel (1955), the mechanism of hematin catalyzed oxidations of unsaturated fats involves the production of free radicals during the peroxidatic decomposition of fatty acid hydroperoxides.

Gibson et al. (1958) reported that a free radical, believed to be the OH radical, is produced when metmyoglobin reacts with hydrogen peroxide. These reports suggest certain similarities between ionizing irradiation and hematin catalyzed oxidations with regard to production of free radical intermediates required for the production of sulfmyoglobin.

5. Effect of pH on the prooxidant activity of cysteine

The peroxidation of fats by sulfhydryl compounds has been reported to occur within a pH range of 3 to 5 (Meyerhof, 1923) or 3 to 4 (Hopkins, 1925). Hematin catalyzed oxidation, on the other hand, is reported to be optimum at a higher pH range. Barron and Lyman (1938) found the hemin catalyzed oxidation of linseed oil increased with pH up to pH 6.96, and Tappel (1955) stated the rate of hemoglobin catalyzed linoleate oxidation increased two-fold over the range of pH 7.8 to 9.5.

The effect of pH on the myoglobin catalyzed oxidation of methyl linoleate in the presence of cysteine is shown in Figure 6. Oxygen consumption increased as pH rose from 3.5 to 5.1. At pH 5.9 and above oxygen consumption was diminished. The difference in the curves (Figure 6) obtained in the range of pH 3.5 to 5.1 from those obtained at pH 5.9 and above, probably reflects the change from sulfhydryl catalysis of linoleate oxidation at the lower pH level to hematin catalysis at the higher pH's. The effect of pH is further shown in Table 8. High rates of consumption in the presence of cysteine are correlated with the appearance of sulfmyoglobin within the range of pH 3.5 to 5.1.

The finding of sulfmyoglobin over a pH range of 3.5 to 5.1 with maximum oxygen consumption at the higher pH is in general agreement with

Table 8. Effect of pH on rates of oxygen consumption and sulfmyoglobin production by systems of methyl linoleate and myoglobin in the presence of cysteine

pH	Rate of oxygen consumption ul./hr.	Sulfmyoglobin production
3.2	0	-
3.5	315	+
4.0	515	+
4.7	540	+
5.1	538	+
5.3	162	-
5.9	274	-
6.8	330	-

the finding of Fox et al. (1958) that maximum sulfmyoglobin was produced in irradiated meat extracts at pH 5.2 to 5.3. The latter workers attributed the effect of pH on sulfmyoglobin production to variations in the rates of reaction of hydrosulfide ion, myoglobin and oxidizing agent.

6. Effect of ethylenediaminetetraacetic acid on the prooxidant effect of cysteine

The inhibitory effect of ethylenediaminetetraacetic acid (EDTA) on the prooxidant activity of ascorbic acid and sulfhydryl compounds (Watts and Wong, 1951; Wheeldon, 1958; Ottolenghi, 1959) indicates metallic ions are cofactors for the reaction. The hematin catalyzed oxidation of unsaturated fats, on the other hand, is not affected by EDTA (Watts and

Wong, 1951). The latter result is confirmed by experiments shown in Figure 7 where, in the absence of cysteine, EDTA did not affect the oxygen consumption or absorbancy at 232 mu of systems containing hemoglobin and methyl linoleate. In the presence of cysteine, however, both oxygen consumption and absorbancy at 232 mu was inhibited by EDTA in the presence of heme pigments as well as in their absence.

The effect of EDTA shown in Figure 7 supports the requirement of trace metals for the prooxidant effect of sulfhydryl compounds. In addition, a change in the mechanism of the hematin catalyzed oxidation of linoleate is indicated when cysteine is present. If EDTA inhibits only the sulfhydryl catalyzed oxidation, then the same level of hematin oxidation of linoleate would be expected in the presence of cysteine and EDTA as in their absence.

7. Effect of trace metals on the prooxidant effect of cysteine

Manometric experiments with systems containing ferric, ferrous, cupric and nicklous ions showed that the first two of the above ions had the greatest prooxidant effect both in the presence and absence of hemoglobin (Figure 8). Ferric and ferrous ions were equally active in the presence of hemoglobin. When pigment was omitted from the reaction mixture, ferrous ion yielded the greater oxygen consumption.

Ferrous ion was the only ion tested capable of catalyzing the oxidation of linoleate in the absence of cysteine and hemoglobin (Table 9).

Uri (1958) reported that the most active metal catalysts in the oxidation of linoleic acid were those ions normally oxidized by a single electron transfer, e.g. ferrous ion. Metallic ions that are normally

Figure 7. Effect of EDTA on the hematin and sulfhydryl catalyzed oxidations of methyl linoleate

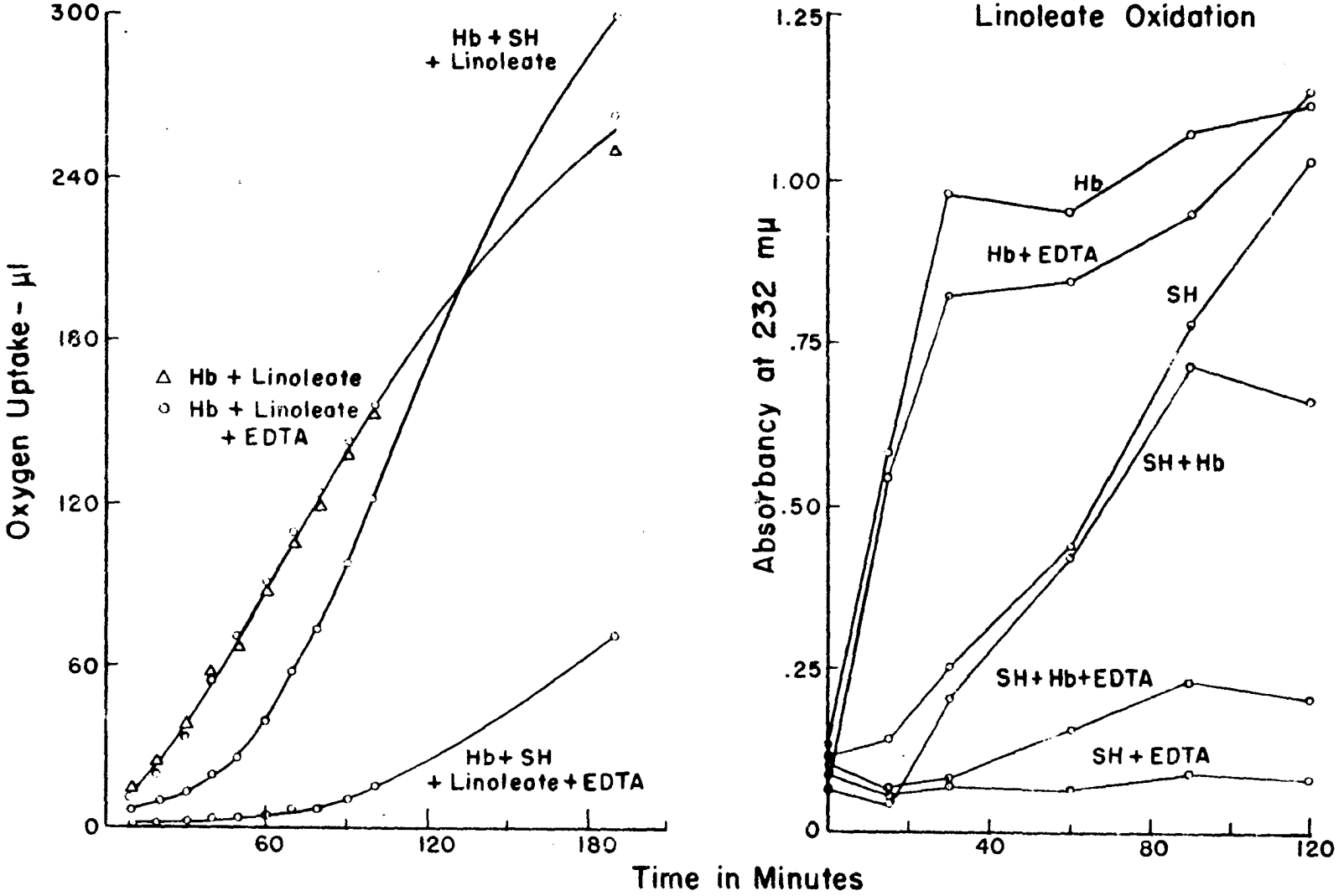

Oxygen Uptake - µl
300
240
180
120
60
0
60
120
180
Hb + SH
+ Linoleate
Δ Hb + Linoleate
o Hb + Linoleate
+ EDTA
Hb+ SH
+ Linoleate + EDTA
Time in Minutes
Linoleate Oxidation
Absorbancy at 232 mµ
1.25
1.00
.75
.50
.25
0
40
80
120
Hb
Hb+ EDTA
SH
SH + Hb
SH+ Hb+EDTA
SH + EDTA

Figure 8. Effect of metallic ions on methly linoleate and cysteine in the presence and absence of hemoglobin

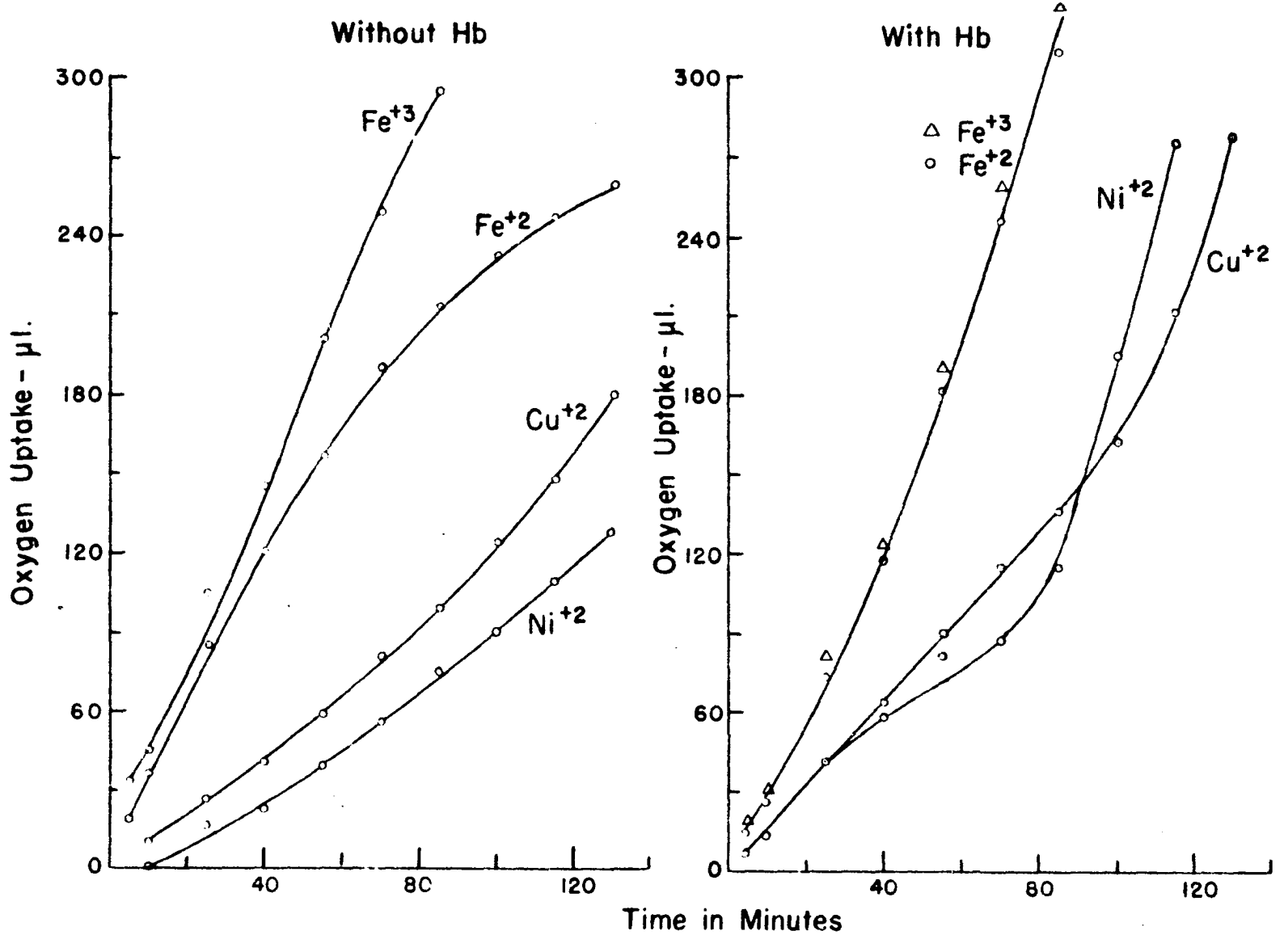

Without Hb
With Hb
Oxygen Uptake - µl.
Time in Minutes
300
240
180
120
60
0
40
80
120
Fe+3
Fe+2
Cu+2
Ni+2

Table 9. Effect of metallic ions on oxygen consumption by methyl linoleate

Metallic ion	Concentration M.	Oxygen consumption ul./hr.
Ferrous	1.3×10^{-3}	107
Ferric	1.3×10^{-3}	5
Cupric	1.3×10^{-3}	0
Nicklous	1.3×10^{-3}	0
Cobaltous	1.3×10^{-3}	0

oxidized by a two electron transfer or reduced by the transfer of a single electron had very little catalytic activity. This explains the catalytic activity of ferrous ion in contrast to the slight activity of cupric, nicklous and cobaltous ions in the oxidation of linoleate (Table 9).

Cupric ion is active in the catalytic decomposition of peroxides (Uri, 1958). Holman (1954) stated that metallic catalysts act by decomposing peroxides to form free radicals. The catalytic activity of cupric ion shown in Figure 8 might be explained by the catalytic decomposition of fat peroxides formed by hematin catalysis. In the absence of hematin compounds, autoxidation of linoleate may have provided the peroxides for cupric ion to act upon. This view is supported by the increase in oxygen consumption after 90 minutes of reaction between cupric ion and linoleate in the absence of hematin compounds (Figure 8).

C. The Oxidation of Cured Meat Pigments by Unsaturated Fats and Cysteine

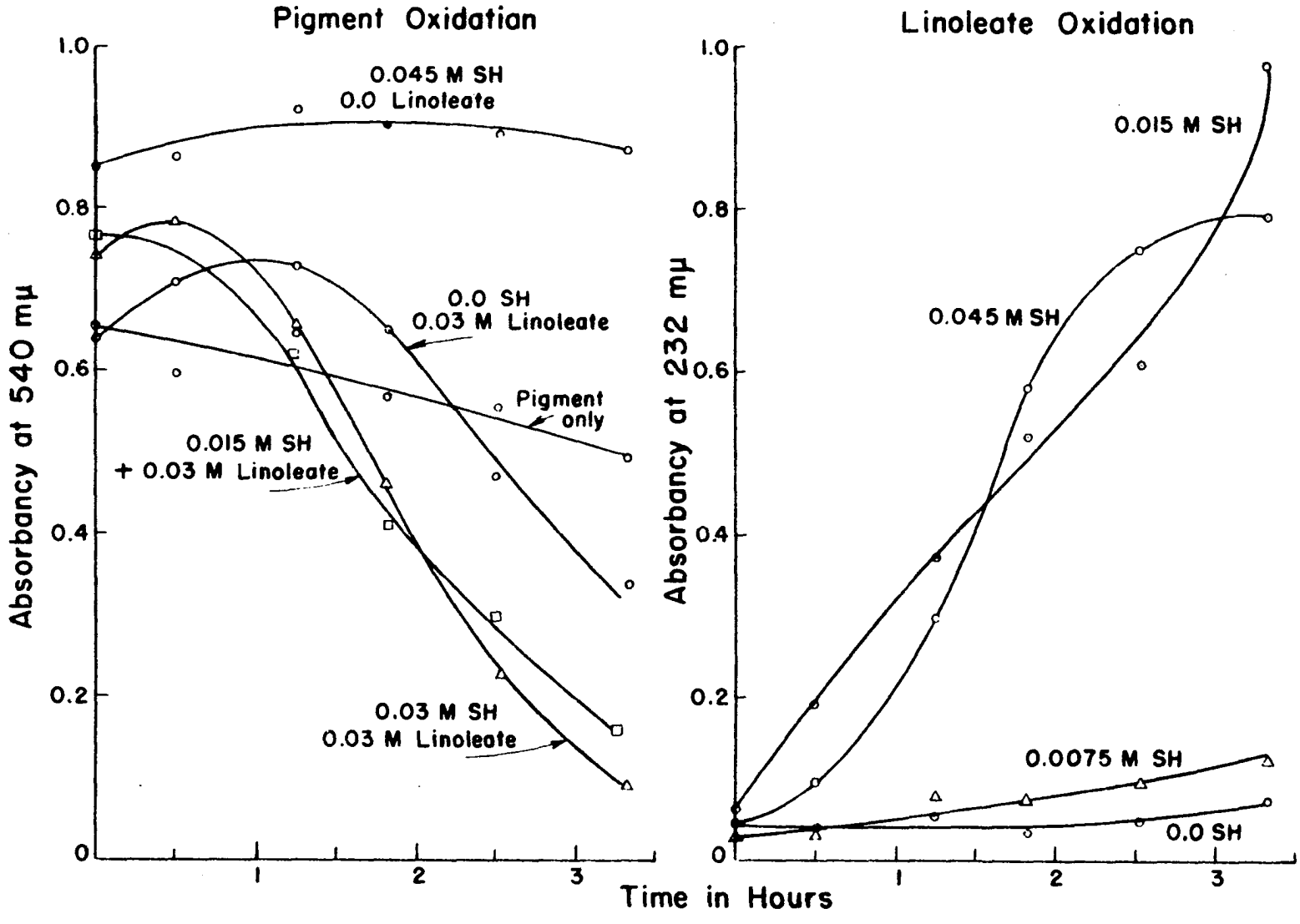
The hematin compounds in cured meats subjected to high processing temperatures are catalytically inactive. To demonstrate loss of catalytic activity, denatured globin nitric oxide myohemochrome (DGNOMb) was used in manometric experiments with 0.04 M. cysteine and 0.03 M. methyl linoleate. No oxygen consumption was observed after four hours reaction in this system.

Although hematin catalysis of unsaturated fats probably does not occur in heat processed cured meats, the catalytic oxidation of these fats by sulfhydryl groups remains possible. The relation between fat oxidation and the destruction of cured meat pigment was studied in systems of DGNOHb (hemoglobin was used to prepare denatured globin nitric oxide hemochrome because the need for large quantities of pigment prevented the use of myoglobin), cysteine and methyl linoleate.

1. Effect of cysteine on the oxidation of denatured globin nitric oxide hemochrome

Cysteine has been found to increase the oxidation of methyl linoleate both in the presence and absence of native heme pigments (Figure 4, 7). Studies with cured meat pigment (DGNOHb) show pigment oxidation is proportional to linoleate oxidation (Figure 9). While cysteine increased the level of linoleate oxidation, the direct relation between cysteine concentration and degree of oxidation observed in manometric experiments (Figure 2, 3; Table 4, 6) was not apparent in systems containing the denatured pigment. The decreased sensitivity to cysteine concentration evident in Figure 9 is attributed to binding of metallic ions by the

Figure 9. Oxidation of cured meat pigment and methyl linoleate in the presence of cysteine


Pigment Oxidation
Linoleate Oxidation
Absorbancy at 540 mμ
Absorbancy at 232 mμ
Time in Hours
0.045 M SH
0.0 Linoleate
0.0 SH
0.03 M Linoleate
Pigment only
0.015 M SH
+ 0.03 M Linoleate
0.03 M SH
0.03 M Linoleate
0.015 M SH
0.045 M SH
0.0075 M SH
0.0 SH
1.0
0.8
0.6
0.4
0.2
0
1
2
3

denatured protein of the pigment. Cysteine protected the pigment against oxidation in the absence of linoleate. This protective effect of cysteine may be related to that observed by Kelley and Watts (1957) concerning cured meat surfaces treated with reducing agents, including cysteine.

2. Effect of EDTA in systems containing DGNOHb

The addition of EDTA to systems of DGNOHb, cysteine and methyl linoleate reduced both lipid and pigment oxidation (Figure 10). Pigment oxidation was further reduced, in the presence of EDTA, when cysteine concentration was reduced. Lowering the concentration of EDTA decreased the induction period for linoleate oxidation and correspondingly increased the level of pigment oxidation. The results shown in Figure 10 as well as those given in Figure 7 are consistent with the idea of a metal-cysteine complex being the active prooxidant.

3. Effect of antioxidants

The data thus far presented have shown that sulfhydryl groups, together with metallic ions, accelerate the oxidation of unsaturated lipid material and that oxidation of cured meat pigment is directly related to this lipid oxidation. Since the oxidation of unsaturated fats is inhibited by antioxidants, it was of interest to determine the effect of antioxidants in these systems.

The antioxidants used in the present study included nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG). Antioxidants were used at a level of 0.01 percent. Figure 11 shows the relationship between

Figure 10. Effect of EDTA on oxidation of cured meat pigment and methyl linoleate in the presence of cysteine

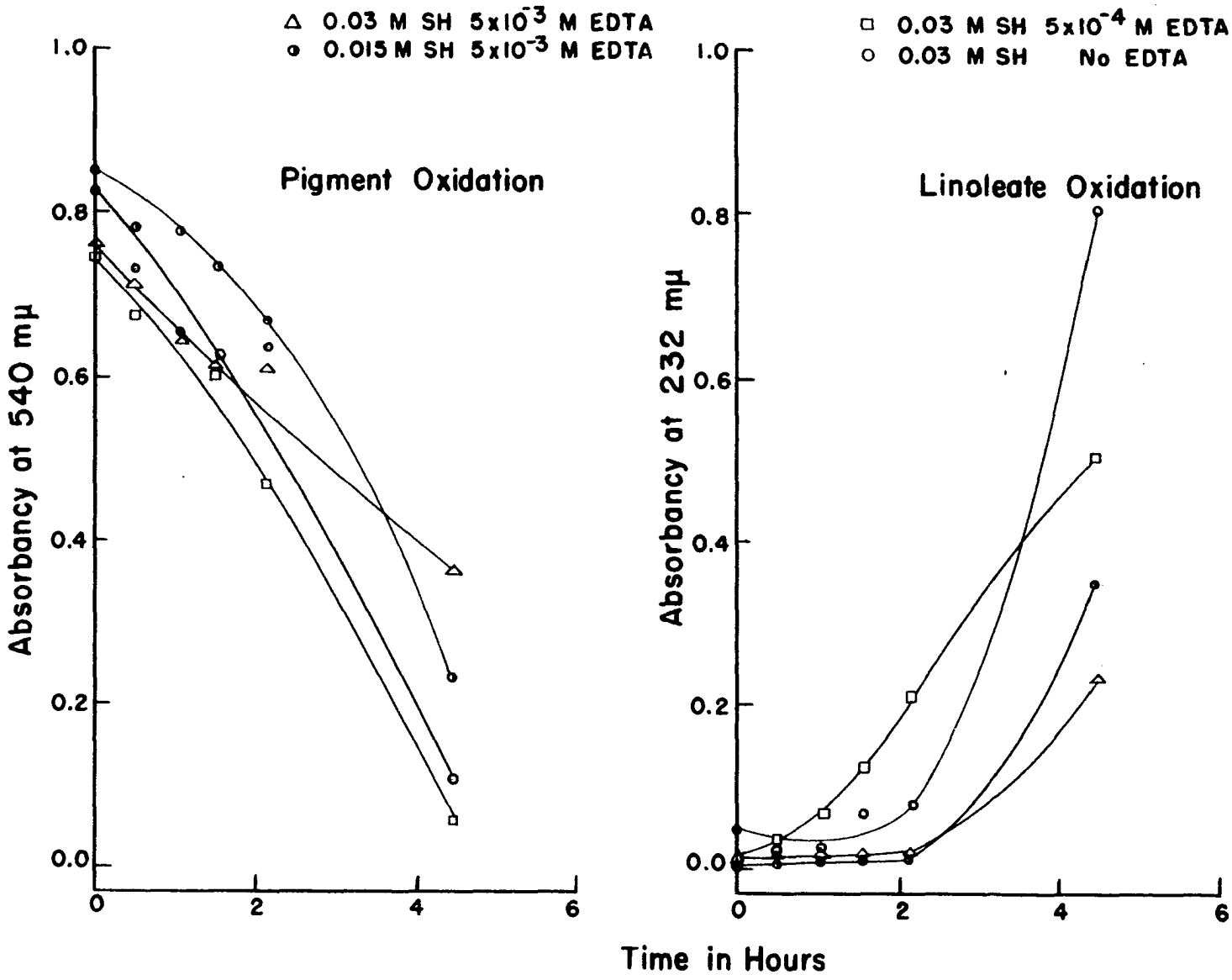
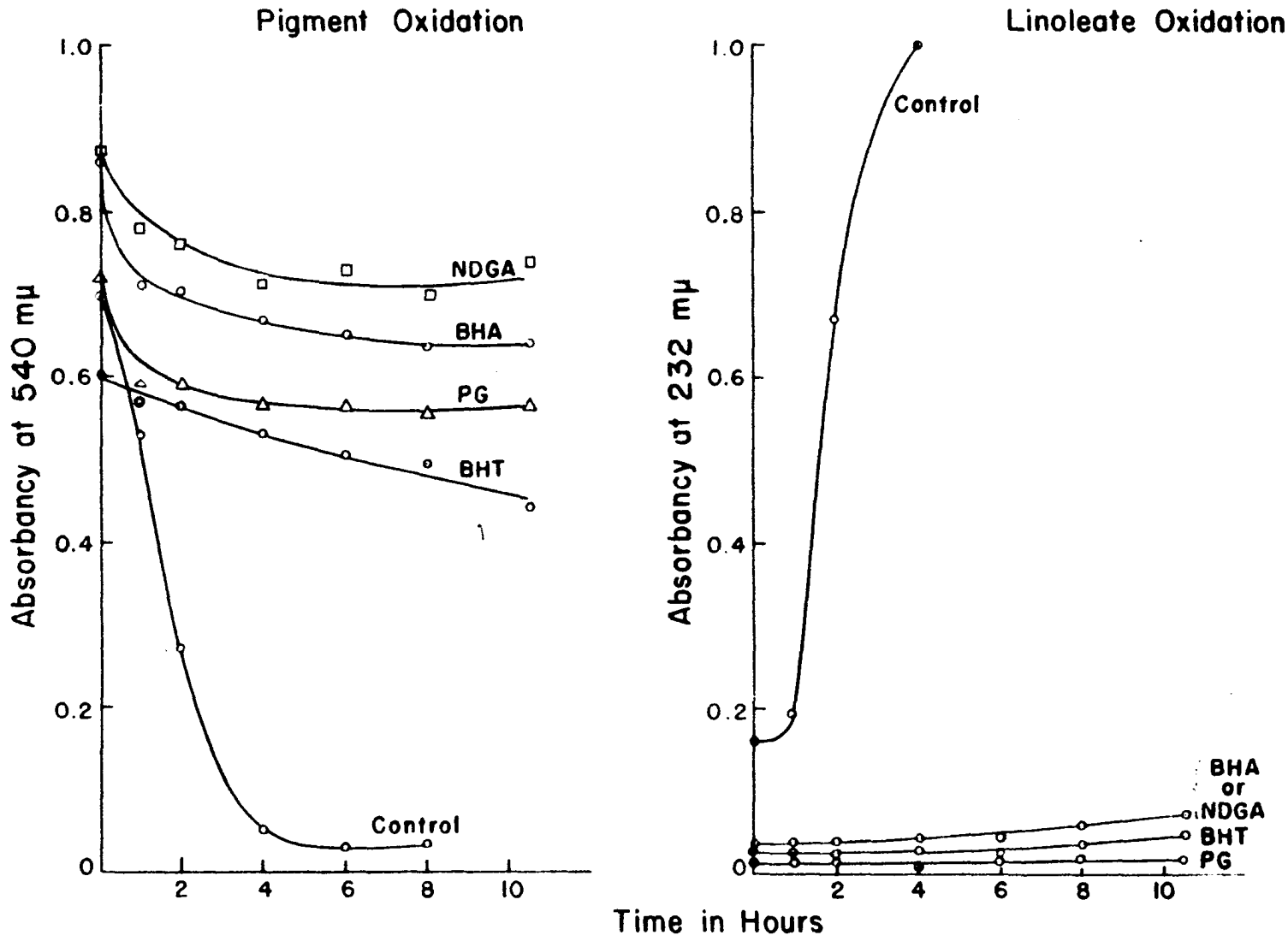

△ 0.03 M SH 5x10⁻³ M EDTA
• 0.015 M SH 5x10⁻³ M EDTA
Pigment Oxidation
Absorbancy at 540 mμ
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
□ 0.03 M SH 5x10⁻⁴ M EDTA
○ 0.03 M SH No EDTA
Linoleate Oxidation
Absorbancy at 232 mμ
Time in Hours

Figure 11. Effect of antioxidants on oxidation of cured meat pigment and methyl linoleate in the presence of cysteine


Pigment Oxidation
Linoleate Oxidation
Absorbancy at 540 mμ
Absorbancy at 232 mμ
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
NDGA
BHA
PG
BHT
Control
Control
BHA
or
NDGA
BHT
PG
Time in Hours

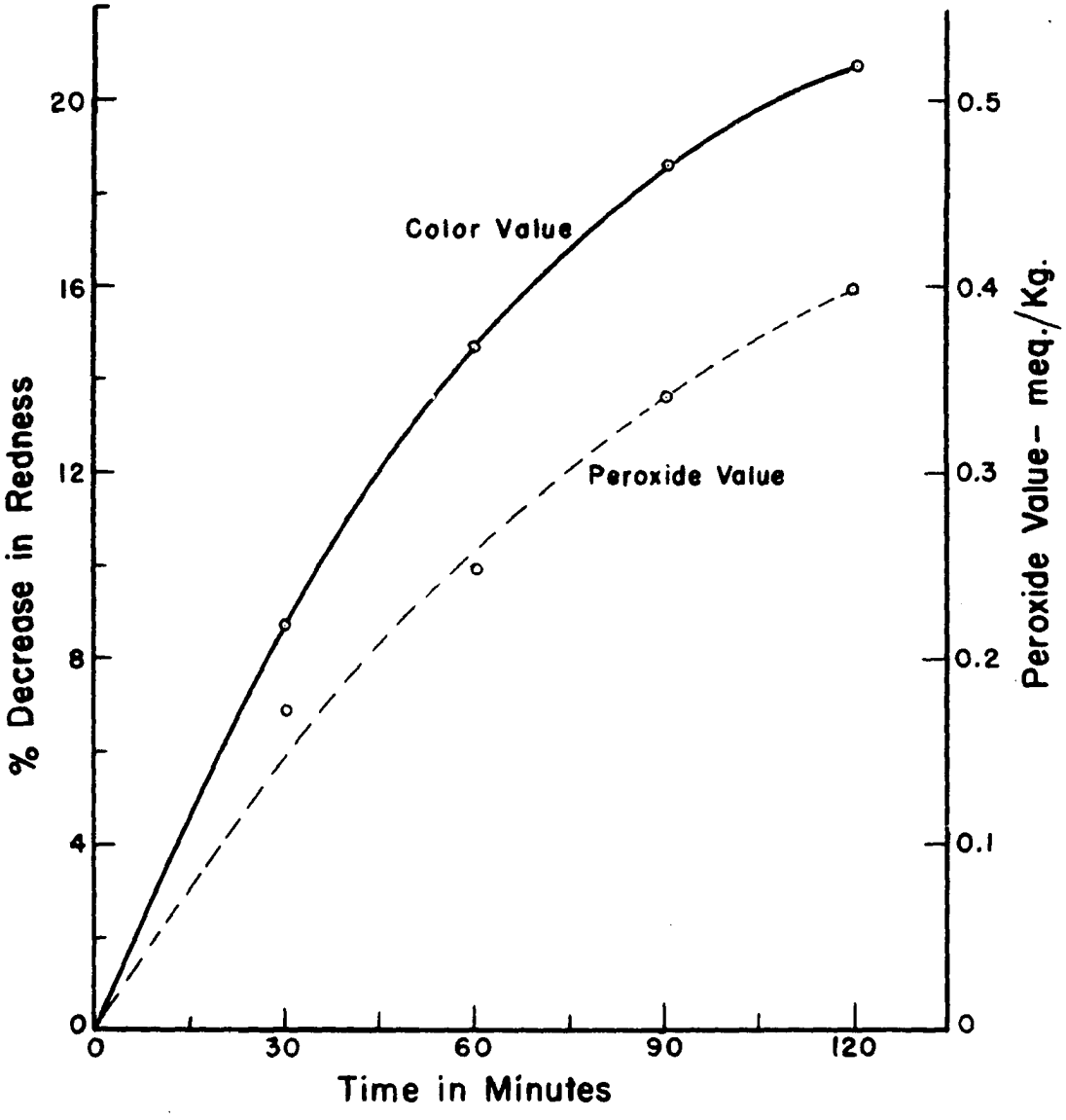
linoleate and pigment oxidation that had previously been observed (Figure 9, 10). All of the antioxidants were effective in the inhibition of linoleate oxidation and, correspondingly, of pigment oxidation. The effect of antioxidants in the system studied supports the theory that pigment destruction in cured meat is related to oxidation of unsaturated fats.

Although antioxidants are shown to protect the pigment from co-oxidation with unsaturated lipid material, the incorporation of antioxidants in cured meats is limited by low water solubility. The treatment of cured meat surfaces with antioxidants dissolved in various carriers has been found to retard development of peroxides (Smith et al., 1945; Hanley et al., 1953); however, antioxidants did not protect the color of cured meat surfaces (Kraft and Wanderstock, 1950).

D. Relation of Fat Oxidation to Fading in Cured Meat

Experiments with systems of cured meat pigment (Figures 9-11) suggested that the pigment was oxidized or faded by a process of co-oxidation with unsaturated fats or, in the present instance, fatty esters. To demonstrate the relationship between fading and fat oxidation in cured meat, thin layers of finely ground bologna were exposed to light. The color of the meat was determined at intervals by means of the Gardner Color Difference Meter. Peroxide value of the extracted fat was also determined at each interval. Figure 12 shows the correlation between the increase in peroxide value and the decrease in redness ("a" value) of the cured meat samples exposed to light.

Figure 12. Effect of illumination on the color and peroxide value of ground bologna


20
16
12
8
4
0
% Decrease in Redness
Color Value
Peroxide Value
0.5
0.4
0.3
0.2
0.1
0
Peroxide Value- meq./Kg.
0
30
60
90
120
Time in Minutes

There is good theoretical agreement between the peroxide value and color loss indicated in Figure 12. An average pigment concentration of 2.0 mg. per 100 grams of bologna (Table 2) may also be expressed as 0.012 millimoles per 100 grams, assuming that all of the pigment in bologna is derived from myoglobin. The error introduced by the hemoglobin which is present in meat would be less than 10 percent since Shenk et al., (1934) reported 10 percent of the residual pigment in beef to be hemoglobin. A peroxide value of 0.4 milliequivalents per killogram of fat is equal to 0.01 milliequivalents per 100 grams of bologna on the basis of a 25 percent fat content. Samples of bologna used in this study ranged from 20 to 24 percent fat. On a theoretical basis the level of peroxide observed is therefore sufficient to account for the oxidation of all the pigment present in the sample.

A simple reaction can be described between DGNOMb and peroxide as follows:

$$\text{DGNOMb} + \text{ROOH} \longrightarrow \text{DGmMb} + \text{ROH} + NO_2^-$$

DGmMb represents the brown ferric hemichrome. Draudt and Deatherage (1956) reported the production of nitrite and nitrate from DGNOMb exposed to light and oxygen. The above reaction is probably an oversimplification since the identity of the actual oxidizing agent is unknown.

E. Effect of Light on Cured Meat Pigment in Suspensions of Gelatin

Except for initial work to demonstrate that sulfhydryl destruction protects the color of cured meat exposed to light, the effect of light in the fading reaction has not been considered in this study. Mixtures of

cured meat pigment, cysteine and methyl linoleate were suspended in gelatin for determination of the effect of light on these systems.

1. Effect of cysteine concentration

Cysteine was added to gelatin suspensions of DGNOHb at the levels indicated in Table 10. The decrease in "a" values using the Gardner Color Difference Meter was a linear function of time for all concentrations of cysteine (Table 10).

While Figure 9 indicates that cysteine protects cured meat pigment from oxidation when in the absence of methyl linoleate, the data presented in Table 10 show that cysteine was able to cause fading of pigment exposed to light. These results may not be contradictory since the two experiments represented different conditions of light, oxygen availability, and temperature. In order to determine whether light or cysteine was causing fading of DGNOHb suspended in gelatin, replicate plates of the suspensions at various cysteine concentrations were exposed to light and an equal number of controls stored in the dark. The sum of the "a" values for all time intervals was obtained for each sample and the analysis of variance was calculated with these values (Table 11). The analysis of variance in Table 11 indicates that cysteine caused the loss of color both in the light and in the dark. Light caused a significant loss of color above and beyond that caused by cysteine.

2. Effect of methyl linoleate, cysteine and ferric ions

To determine the effect of other factors on the photooxidation of cured meat pigment, gelatin suspensions of pigment were prepared with

Table 10. Effect of cysteine concentration on the decrease in redness of pigment suspensions in gelatin exposed to light

Cysteine concentration	Initial "a" value	Percent decrease in "a" after 10 hours	Rate of decrease of "a"
0.0 M.	17.2	9.9	-0.12
0.015	16.9	9.1	-0.10
0.03	16.3	8.3	-0.13
0.042	15.4	10.0	-0.15
0.048	14.7	14.3	-0.21
0.060	13.2	16.7	-0.22

various combinations of cysteine, methyl linoleate and ferric ion. The red component of color ("a" value) was determined initially and after samples were exposed to light for 15 hours. Controls for each treatment were stored in the refrigerator. The results, given in Table 12 as a percentage decrease in redness, indicate each of the treatment combinations had some effect on pigment fading over and above that caused by light. The differences between values obtained in the light and in the dark (Table 12) are only an approximation of the effect of light alone since the treatment effects are not additive.

The effect of cysteine given in Table 12 is in agreement with the results shown in Table 11. That is, cysteine had the greater effect on fading although there was an effect of light in addition to that of cysteine. The combination of cysteine and linoleate yielded the greatest

Table 11. Effect of cysteine at various levels on fading of pigment suspensions in the light and dark

	Sum of "a" values obtained during 18.5 hours Cysteine concentration - M.				
	0	0.015	0.03	0.045	0.06
Exposed to light	190.0	173.1	156.1	119.3	105.8
Dark control	190.7	178.0	162.7	134.6	107.3

Analysis of variance

Source	Degrees of freedom	Sum of squares	Mean square	F
Light	1	7.01	7.01	2.82*
Cysteine	4	795.51	198.88	79.87**
Light X Cysteine	4	5.66	1.42	0.57
Error	10	24.93	2.49	
Total	19	833.02		

*Significance at P = 0.05.

**Significance at P = 0.01.

loss of color both in the light and in the dark. Loss of color in the dark, in the latter case, is attributed to linoleate oxidation catalyzed by cysteine. The addition of ferric ion to the systems shown in Table 12 appears to have had a sparing effect on pigment oxidation; the sparing effect in the dark was larger than that found in the light. This is interpreted as a reduction in autoxidation but not in photooxidation in

Table 12. Effect of cysteine, methyl linoleate and ferric ion on fading of gelatin suspensions of DGNOHb

Treatment	Percentage decrease in "a" after 15 hours	
	Exposed to light	Stored in dark
Pigment only	13.7	0.0
Pigment + Fe[a]	17.0	6.5
Pigment + linoleate[b]	21.0	5.7
Pigment + linoleate + Fe	32.5	4.5
Pigment + cysteine[c]	43.7	2.0
Pigment + cysteine + Fe	26.2	0.0
Pigment + cysteine + linoleate	49.0	32.3
Pigment + cysteine + linoleate + Fe	40.0	14.9

[a]Fe concentration 1.7×10^{-5} M.

[b]Linoleate concentration 0.03 M.

[c]Cysteine concentration 0.03 M.

the systems containing ferric ion. Ferric ion showed the strongest sparing effect when cysteine and linoleate were in combination. The function of metallic ions may have been to alter the nature of the catalytic oxidation of linoleate by cysteine so that instead, a catalytic oxidation of cysteine by ferric ion occurred.

A similar observation was reported by Weiss et al. (1953) who found that trace metals protected cured meat color in the presence of ascorbic acid, but not in the presence of sodium dithionite. The prooxidant effect

of ascorbic acid, as well as of cysteine, in the presence of trace metals has already been pointed out (Scarborough and Watts, 1949; Watts and Wong, 1951; Ottolenghi, 1959).

While there is no doubt that photooxidation alone causes some heme destruction, it is proposed that an important cause of fading is the co-oxidation of pigments with the unsaturated fats present in cured meat. Fat oxidation is accelerated by trace metals which form active fat oxidation catalysts with sulfhydryl groups. It is not clear whether the active sulfhydryl groups in cured meat are protein bound or remain free as in the case of glutathione which occurs naturally in muscle tissue. While the present experimental results have been obtained with the free sulfhydryl groups of cysteine, the experiments of Dubouloz and Fondarai (1953) on reactions between lipid peroxides and protein bound sulfhydryl groups indicate that these groups may also be catalytically active.

This study points out two avenues of research which might lead to a method for controlling fading. Antioxidants may be incorporated in cured meats to inhibit the oxidation of fat. This approach should be especially useful for comminuted cured meat products which are emulsions of fat, protein and water. It may be possible to disperse antioxidants in these emulsions by means of emulsifiers. Non-comminuted cured meat products present a more difficult problem for the dispersion of presently available antioxidants. A second avenue of research is the study of trace metals and their effect on the fading process. Although Weiss, et al. (1953) reported that EDTA inhibited the formation of nitric oxide hemoglobin, Gibson (1943) found that cupric or ferrous ions aided in the reduction of

methemoglobin in the presence of ascorbic acid. Another beneficial effect of trace metals is shown by the sparing action of ferric ion in the presence of cysteine or with cysteine and linoleate (Table 12). EDTA, or other metal sequestering agent, might be used to control the concentration of trace metals in cured meats.

The concept of a photoactivated pigment molecule proposed by Walsh and Rose (1956) is applicable to the role of fat oxidation in pigment fading proposed herein. This view is supported by the report of Khan et al. (1954) that visible light energy absorbed by chlorophyll is transferred to an unsaturated fat in some manner, providing the activation energy for fat oxidation. Conceivably a photoactivated pigment molecule can supply the activation energy for the formation of the sulfhydryl-metal complex which then catalyzes the oxidation of unsaturated fats. An activated pigment molecule may also supply the energy to initiate the autoxidation of unsaturated fats. The failure of mercuric chloride treatment to completely inhibit fading (Figure 1) and the decrease in redness of cured meat pigment exposed to light in the presence of linoleate (Table 12) indicate autoxidation of unsaturated fats may also be a factor in fading.

V. CONCLUSIONS

1. Treatment of cured meat with reagents that react with sulfhydryl groups increases the stability of the meat toward light.

2. The oxidation of unsaturated fats is accelerated by the presence of cysteine. The prooxidant effect is dependent on the concentration of unsaturated fat and cysteine.

3. Methyl linoleate is oxidized by cysteine in the absence of hematin pigments. Trace metals are required and the oxidation is inhibited by the metal complexing agent EDTA.

4. A green pigment identified as sulfmyoglobin is formed when methyl linoleate is oxidized in the presence of myoglobin and cysteine.

5. The prooxidant effect of cysteine on the oxidation of methyl linoleate and the formation of sulfmyoglobin occurs within a pH range of 3.5 to 5.1.

6. Ferric and ferrous ions are more active trace metal prooxidants in the presence of cysteine than are cupric, nicklous or cobaltous ions. In the absence of cysteine and heme pigments, ferrous ion catalyzes the oxidation of linoleate.

7. The destruction of cured meat pigment in mixtures of methyl linoleate and cysteine results from oxidation of the unsaturated fat. Inhibition of fat oxidation by antioxidants protects the color of cured meat pigment.

8. The light-induced fading of bologna is accompanied by an increase in fat peroxide value which is proportional to the decrease in cured meat color.

9. Photooxidation of cured meat pigment occurs in the absence of cysteine or methyl linoleate. The oxidation of pigment exposed to light is increased by cysteine and/or methyl linoleate.

VI. SUMMARY

A study was made of the relation of free sulfhydryl groups to fading of cured meat pigment. Treatment of cured meat with mercuric chloride reagent, which reacts with sulfhydryl groups, increased the stability of cured meats to light.

Manometric experiments on systems containing native pigment, cysteine and an unsaturated fat indicated that sulfhydryl groups were involved in an oxidative reaction. The prooxidant effect of cysteine in this system was demonstrated by separating the oxygen consumption provided by the hematin catalyzed oxidation of methyl linoleate from that caused by sulfhydryl catalysis. The concentration of lipid and cysteine influenced the occurrence and degree of the prooxidant effect by cysteine.

Cysteine catalyzed the oxidation of methyl linoleate in the absence of heme pigments. The metal complexing agent EDTA inhibited the oxidation of linoleate by cysteine alone as well as the hematin catalyzed oxidation of linoleate in the presence of cysteine. EDTA had no effect on the hematin catalyzed oxidation of linoleate in the absence of cysteine.

Ferric and ferrous ions had a greater prooxidant effect in the presence of cysteine than did cupric, cobaltous or nicklous ions. Ferrous ion was the only metallic ion of those tested which actively catalyzed linoleate oxidation in the absence of heme pigments and cysteine.

A green pigment formed when cysteine and linoleate reacted with native myoglobin was identified as sulfmyoglobin. The prooxidant effect of cysteine was not caused by the production of a more highly active hematin catalyst since sulfhemoglobin had less catalytic activity than did

hemoglobin. The prooxidant effect of cysteine and the production of sulfmyoglobin occurred within a pH range of 3.5 to 5.1.

Cured meat pigment concentration in model systems was inversely proportional to linoleate oxidation. In addition, the fading of the red color of cured meat samples exposed to light was directly proportional to the fat peroxide value. EDTA decreased but did not completely inhibit linoleate and cured meat pigment oxidation. The antioxidants NDGA, BHA, BHT and PG inhibited both linoleate and pigment oxidation.

The effect of light was studied in suspensions of cured meat pigment in gelatin. The color of these suspensions decreased in proportion to the increase in cysteine concentration. Most color loss occurred in the presence of cysteine and linoleate. The pigment alone was only slightly altered by light in the absence of cysteine and/or linoleate.

It is proposed that cured meat fades by the co-oxidation of pigment and unsaturated fats. Free sulfhydryl groups and trace metals accelerate this reaction.

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