COLOR STABILITY OF FRESH BEEF AS AFFECTED BY IONIZING RADIATION DOSE RATE AND ADDED INORGANIC PHOSPHATE

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WILLIAM WALLACE BALLANTYNE

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This is to certify that the

thesis entitled

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AND ADDED INORGANIC PHOSPHATE

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William Wallace Ballantyne

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Walter M. Urbain

Major professor

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ABSTRACT

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A method that employs a pasteurizing dose of ionizing radiation has been developed for the centralized cutting of consumer-sized fresh beef cuts to provide a twenty-one day distribution and shelf life. A critical factor in this process is the development of a bright red color in meat at the time of retail sale. The color of fresh meat is primarily dependent upon the chemical form of the heme-containing protein pigment, myoglobin. Purple, reduced myoglobin can be oxygenated to bright red oxymyoglobin or can be oxidized to brown metmyoglobin which is incapable of oxygenation.

Factors that accelerate or decrease the rate of oxidation of the fresh meat pigment to metmyoglobin gain special importance with centralized cutting. Two such factors are the effect of irradiation dose rate, and the influence of added inorganic phosphate.

Reflectance spectrophotometric methods were used to study pigment form conversions resulting from electron and gamma irradiation. The conversions, characterized by a darkening of meat samples, were from the bright red oxymyoglobin pigment form to either brown metmyoglobin or purple myoglobin. The pigment form was a factor of the atmospheric conditions at the time of irradiation. When oxygen was available during the irradiation period, oxidation was dose-rate dependent. Except at the highest electron dose rates studied, the effect did not seem to alter the ability of the vacuum-held beef slices to oxygenate after twenty-one days of storage.

A ten percent solution of sodium tripolyphosphate was used before packaging and irradiation as a dip treatment to reduce exudation or "drip" from fresh beef slices. Treated slices had less oxidized pigment after twenty-one days of vacuum storage than control slices. Spectrophotometric methods were used to compare the effects of treatment with other alkaline compounds with treatment with various phosphate preparations. Oxidation was slowed by phosphate in both fresh beef slices and purified oxymyoglobin solutions under specific conditions of pH and temperature. Dialysis experiments with radioactive phosphate indicated the existance of an association between phosphate and myoglobin.

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INTRODUCTION

The work reported in this study in concerned with the description and the solution of some of the problems encountered during the development of a process for the centralized cutting of fresh beef. Centralized cutting refers to the breaking down of the beef carcass into individually packaged consumer-sized cuts at a central location, and the distribution of desired cuts to the retail outlet. Advantages of this procedure include the removal from retail outlets of many inefficiently used specialized facilities and skilled employees. Savings would also come from the distribution of less bone and meat trimmings and the provision of each outlet with only those meat cuts in accord with market preference. In this way the retail outlet would not be involved with the final step of fresh beef fabrication. This final step would be performed more efficiently and with improved control in centralized facilities.

Present methods of distribution yield only a few days of retail saleable life for the consumer-sized fresh meat cut. Vacuum packaging combined with good refrigeration and sanitation provides a distribution time of 10 to

14 days for wholesale-sized portions. Vacuum packaged consumer-sized cuts are expensive, and because of the reducing conditions created, are an undesirable purple color at the time of retail sale. As an alternative to freezing, which could prolong the time indefinitely for cuts of any size, a recently developed method employing ionizing radiation can provide a 21 day distribution and shelf life for consumer-sized fresh beef cuts (Urbain, et al., 1969). Microbial spoilage is delayed with a pasturization dose of about 100 krad. Liquid exudation or "drip" can be reduced by treatment with certain inorganic phosphates, and color control provided by a two-step packaging system. Consumer-sized portions, packaged in air-permeable film, are irradiated and bulk vacuum packaged, and then removed from vacuum at the retail level within 21 days. Normal red color is obtained by oxygenation of the pigment through the permeable film. The meat is held at 5°C or lower.

The purchase decision regarding a particular meat cut is influenced considerably by the degree of bright red coloration present at the time of sale. Meat color is dependent on the chemical form of the pigment myoglobin. The reduced pigment form, myoglobin, is a purple color and is capable of oxygenation to bright red, oxymyoglobin. The oxidized form of the pigment, brown metmyoglobin, is incapable of oxygenation. Factors that

accelerate or decrease the rate of oxidation of the fresh meat pigment to metmyoglobin gain special importance with centralized cutting. For the process referred to above, two such factors are the effect of irradiation dose rate and the influence of added inorganic phosphate.

Ionizing radiation can be provided by either gamma or electron sources. Although not observed with gamma intensities in the range of 0.05 krad/sec, a darkening of meat samples has occurred under some irradiation conditions at higher electron dose rates. Measurements using a non-destructive reflectance spectrophotometric method were made to identify pigment characteristics following specific irradiation conditions and meat storage.

A gradual oxidation took place after 10 to 14 days in irradiated, vacuum packaged, fresh beef slices held in refrigerated storage at 3°C. However, if the samples were treated with phosphate, the oxidation rate was slowed and generally a superior color resulted when the packages were removed from vacuum and allowed to oxygenate. Studies were conducted with beef slices, various inorganic phosphates, and some other alkaline compounds to determine whether there might be some association between phosphate and myoglobin that would cause decreased oxidation rates. The effect of phosphate on the oxidation rates of myoglobin preparations was also studied.

Radioactive phosphate was used in attempts to quantify an association between phosphate and myoglobin.

REVIEW OF LITERATURE

Myoglobin in Beef Muscle

The major red pigment of fresh meat is the protein myoglobin. This pigment, which acts to transport and store oxygen within the cell, gives the muscle its usual red color.

Myoglobin is a water-soluble sarcoplasmic protein of muscle tissue with a molecular weight of about 17,000 (Lawrie, 1965). It predominates in "red muscles," those characterized by repetitive, slow action over long periods of time, and by greater concentrations of mitochondria and respiratory enzymes than the "white muscles." Muscular activity and age cause increases in myoglobin concentration in an individual animal. Nutrition, breed, sex, and altitude are also determinants.

While myoglobin is considered the predominant pigment of fresh meat color (Fleming et al., 1960), hemoglobin is estimated to constitute from 20 to 35 percent of the total red pigment (Rickansrud and Hendrickson, 1967).

Structure of Myoglobin

Myoglobin is formed by the combination of a single prosthetic heme group and a basic globin polypeptide (Antonini, 1965). The heme group has been shown to be a

stable coordinated compound of iron with protoporphyrin. Four pyrrole rings linked by four methene bridges to form a planar ring system provide the basis for the porphyrin structure (Lemberg and Legge, 1949). Protoporphyrin IX, the porphyrin of hemoglobin and myoglobin, is characterized by eight side chains consisting of 4 methyl, 2 vinyl, and 2 propionic groups.

The iron atom of the heme, at the center of the porphyrin ring, has four of its six coordination sites occupied by bonds to the porphyrin. The heme may be diagrammed as in Figure 1. The fifth and sixth positions, one on either side of the porphyrin plane, are available for combination with other ligands. In myoglobin, the fifth position of the iron interacts with a histidine residue of the globin polypeptide (Kendrew, et al., 1960). The sixth position may be variously bound to ligands such as oxygen, water, carbon monoxide, and nitric oxide.

Kendrew et al. (1960) used X-ray diffraction to study the structure of crystallized sperm whale metmyoglobin. They found that the molecule was approximately spherical with the heme group near the surface. Resolution of 2 Å showed that the globin contained a high degree of right-handed α-helix segments. Increasing resolution to 1.4 Å permitted the determination of each amino acid and its side chain (Kendrew et al., 1961). Of the 1953 amino acid residues found, 118 were involved

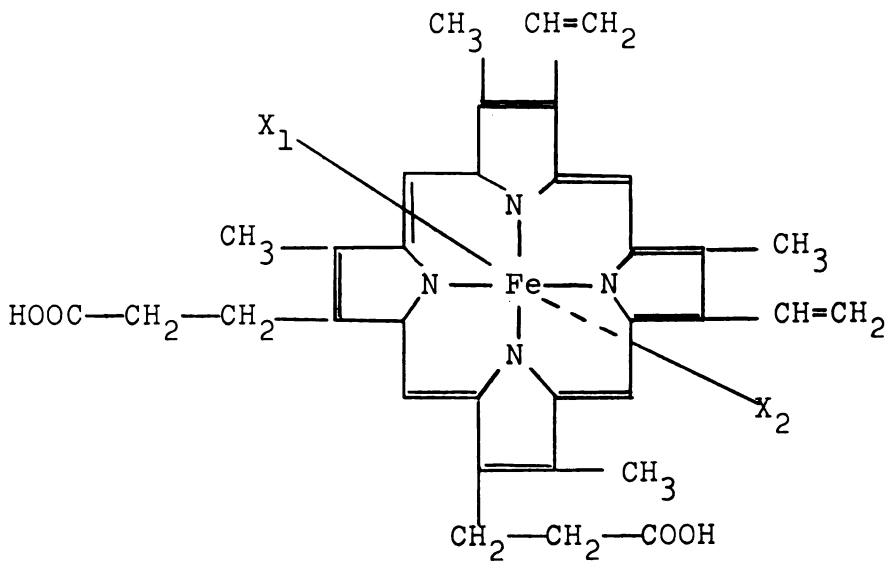


Figure 1. Heme structure.

in the eight α-helix sections. Amino acid sequence studies of sperm whale metmyoglobin by Edmundson and Hirs (1961) and Edmundson (1965) were correlated with these X-ray data to obtain an accurate picture of the molecule. The myoglobin molecule contained only five molecules of water, with non-polar groups located primarily in the interior and polar chains at the surface (Kendrew et al., 1961). Much of the stability of the structure appeared to be due to van der Waals forces from non-polar interactions among the side chains and between the side chains and the heme. The vinyl groups of the heme were directed towards the interior of the molecule and the propionic acid groups were near the surface.

Kendrew et al. (1961) postulated that the interaction of the heme with the globin occurs via three imidazole nitrogen groups of histidine residues. There is a coordination bond direction from the iron to the nearest histidine, F 8, and an apparent indirect linkage to the iron through a water molecule to the "distal histidine", E 7. In addition, there is a probable hydrogen bond between another histidine and a heme propionic acid side chain.

Stryer et al. (1964), using difference Fourier synthesis, were able to demonstrate the binding of an azide ion to the sixth coordination position of the iron. The replacement of water by azide resulted in a shift in the X-ray pattern. A positive peak was thought to

represent the loss of the sulfate ion which was hydrogen bonded to the distal histidine when water bridged it to the sixth position of the iron.

X-ray diffraction has also been used to study the structural differences between crystallized sperm whale myoglobin and metmyoglobin (Nobbs et al., 1966). The structure of myoglobin was very similar to that of metmyoglobin with no evidence of conformational changes or of heme group movement. This was in contrast to the oxygenation of hemoglobin, which is accompanied by gross changes in the relative position of the four subunits (Muirhead, 1963). However, two significant differences were observed (Figure 2). When metmyoglobin was reduced to myoglobin, a peak shift occurred representing the loss of a negative ion from the distal histidine. The other change in the metmyoglobin Fourier synthesis was interpreted as the removal of a water molecule from the sixth coordinated position. There was no water in the myoglobin converted from metmyoglobin, and thus no hydrogen bonding to the distal histidine. The distal histidine, in turn, seemed to lose a sulfate ion upon reduction of the heme iron. The differences would be as diagrammed in Figure 2. The lack of a water molecule confirmed the explanation of paramagnetism proposed by Pauling (1949). Muirhead et al., (1967) have also found that hemoglobin undergoes a reversible transition between two structures, which seems to

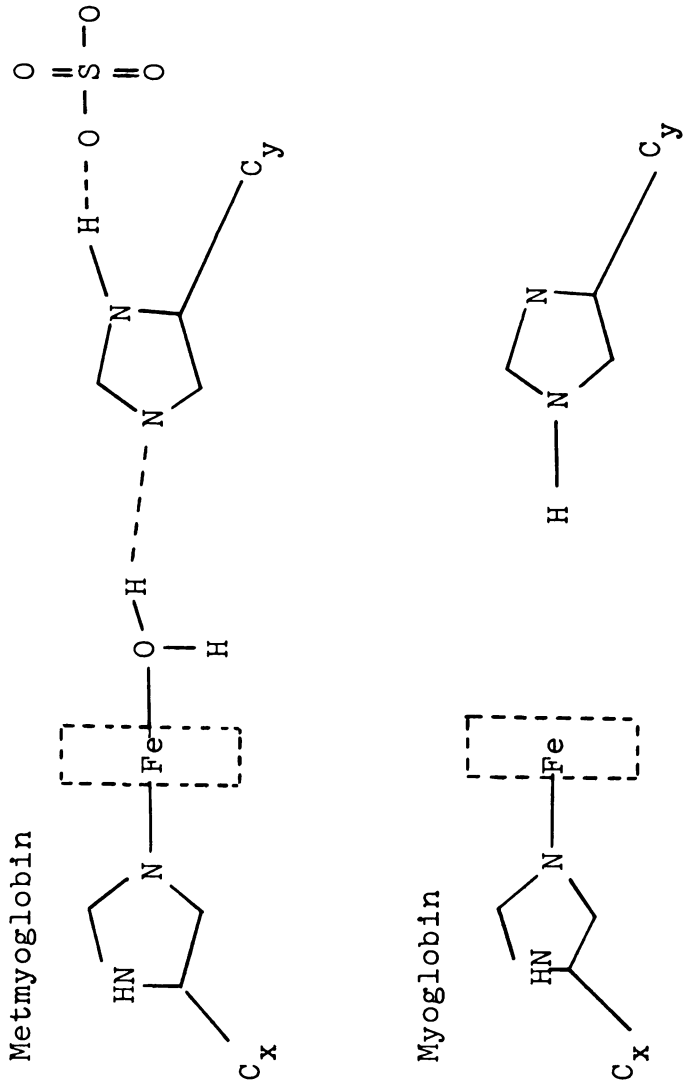


Figure 2. Structural differences between metmyoglobin and myoglobin.

correlate with the presence or absence of a water molecule at the sixth coordination position.

Function of Myoglobin

The most significant feature of the heme-globin combination is the ability of the ferrous iron to combine reversibly with the molecular oxygen, forming an oxygenated derivative stable to oxidation (Antonini, 1965). Ferrous heme can also combine reversibly with ligands such as CO and R-CN in a one to one ratio, but when exposed to oxygen, the constituent iron oxidizes rapidly to the ferric state. Myoglobin is the simplest form of a heme-protein capable of reversible oxygenation. The equilibrium between myoglobin and oxygen can be expressed as:

$$\underset{\text{myoglobin}}{Mb^{+2}} + O_2 \underset{k}{\overset{k'}{\rightleftharpoons}} \underset{\text{oxymyoglobin}}{Mb^{+2}O_2} \qquad K = \frac{k'}{k}$$

The equilibrium constant, K, equals $\frac{[MbO_2]}{[Mb][O_2]}$, which may be expressed in terms of the partial pressure of oxygen as $K = \frac{[MbO_2]}{[Mb]ppO_2}$ (Lemberg and Legge, 1949). If the value for the fractional saturation of O_2, $Y = \frac{[MbO_2]}{[MbO_2+Mb]}$, is plotted against ppO_2, a rectangular hyperbola results (Rossi-Fanelli and Antonini, 1958). The dissociation curve for hemoglobin, as described by the Hill plot, is sigmoidal. The greater affinity of myoglobin than of

hemoglobin for oxygen is significant physiologically in the transfer of oxygen from oxyhemoglobin to the muscle cell.

The mechanism by which binding of oxygen occurs without causing a rapid oxidation is unclear (Antonini, 1965). The binding is likely to be only indirect, with other protein interactions playing a major role. The "distal histidine", the histidine in the direction opposite to the iron-binding imidazole, has been considered to be important (Muirhead and Perutz, 1963). That abnormal hemoglobins without this histidine were characterized by rapid autoxidation would suggest that the iron-oxygen complex was stabilized by hydrogen bonds between the oxygen and this residue. Cann (1965) has implied that nearby aromatic residues were also instrumental in the stabilization of heme interactions. The hydrophobic environment that exists may prevent electron transfer from the oxygen to the ferrous iron.

Color in Meat

The highly conjugated double bond system of the protoporphyrin results in a great degree of resonance and subsequently, in combination with the iron, provides the basis for a very deep color (Lemberg and Legge, 1949). All porphyrins absorb light in the region of 400 to 450 nm, the Soret band. Hemes, when combined with globin

and another ligand, will have a characteristic absorption spectrum in the visible region (Bowen, 1949). Oxymyoglobin has maximum absorption values at 582 and 544 nm and a minimum at 564 nm. Myoglobin absorbs in a broad band around 555 nm. Metmyoglobin has absorption peaks at 510 and 635 nm within a pH range of 5.6 to 7.0.

Reduced myoglobin, Mb, gives a deep purple color to muscle. The oxygenated form of the pigment, oxymyoglobin, MbO_2, has a bright red color. For aesthetic reasons, this pigment form is the desired fresh meat color at the point of retail sale (Rickansrud and Hendrickson, 1967). Conversion of the reduced pigment to the oxidized form results in brown, highly undesirable metmyoglobin, MetMb.

In living tissues, the action of the reducing enzyme system keeps the iron largely in the ferrous form (Ramsey, 1949). Studies by Grant (1955), Stewart et al. (1965b), and others have shown that fresh meat initially also possesses a viable reducing enzyme system with the potential for conversion of metmyoglobin to the reduced form.

Autoxidation

Observations by Brooks (1935) with hemoglobin led to the conclusion that methemoglobin formed first beneath the surface, and at a maximum rate where the partial pressure of O_2 was approximately 20 mm of Hg at 30°C. George and Stratmann (1952a,b) found that autoxidation of

crystallized horse heart myoglobin was similar to that observed with hemoglobin. They reported that under constant conditions of pH, temperature, and phosphate concentration, the autoxidation of myoglobin was first order in air with a mean rate constant of 0.325 ± 0.015 hr^{-1}. The amount of oxygen absorbed during autoxidation was 2.5 moles per mole of MbO_2. In a later report, George and Stratmann (1954) indicated that the autoxidation was first order over the range of 0.3 to 760 mm of oxygen pressure. The rate constant increased with increasing oxygen pressure to a value of 1.0 to 1.4 mm partial pressure of oxygen, and then decreased to a constant value above 30 mm. They also found that the partial pressure for half-saturation was 1.0 to 1.3 mm, and thus the rate of autoxidation was greatest at half-saturation.

George and Stratmann (1952b) proposed a free radical mechanism for autoxidation which would involve the participation of an auxiliary electron-accepting group on the protein molecule. This acted as a catalyst in another reaction regenerating the unoxidized heme. This would protect the heme from autoxidation; however, the mechanism did not account for the additional consumption of oxygen above that required for oxidizing the heme group. They also reported that oxidation of myoglobin to metmyoglobin by oxygen was inhibited by carbon monoxide. The rate of autoxidation increased with hydrogen ion concentration at

both low and high oxygen pressures in the pH range of 5.34 to 6.64 at 30°C. They claimed the change to be only minor and to be due, possibly, to ionization of heme-linked groups on the myoglobin molecule.

Snyder and Ayres (1961) found that autoxidation of crystallized beef myoglobin was similar to that reported for horse myoglobin. They followed autoxidation by measuring the decrease in light absorbance at 580 nm with a Beckman DU spectrophotometer. A high temperature dependency was noted with a Q_{10} value of 4.8 for 0°C to 10°C. The autoxidation rate was first order with respect to unoxidized myoglobin, with a rate constant of $0.22 hr^{-1}$. With similar conditions (0.6 M phosphate buffer at pH 5.7 and 30°C), they found the rate constants to vary depending upon the amount of $Na_2S_2O_4$ added to reduce the MetMb. The rate of autoxidation of myoglobin was affected by concentrations of $Na_2S_2O_4$ greater than about 0.05 percent. When the quantity of $Na_2S_2O_4$ added to reduce the metmyoglobin was limited, the variability of the rate constants fell within a narrower range than previously obtained.

Brown and Dolev (1962) studied the rates of autoxidation of beef and tuna oxymyoglobins at pH 5.9 in phosphate buffers from 0.1 to 0.6 M and at temperatures from 0 to 40°C. The autoxidation was first order with respect to unoxidized myoglobin under all conditions

studied. The rate of oxidation of beef oxymyoglobin decreased with increasing buffer concentration, whereas the rate of oxidation of tuna oxymyoglobin was independent of buffer concentration. Tuna oxymyoglobin was oxidized more slowly than beef at low temperatures at all buffer concentrations, but more rapidly at high temperatures and high buffer concentration. Their experiments with crude beef myoglobin extract resulted in much lower rates of autoxidation than with purified systems. The rate constants reported by Brown and Dolev (1962) were lower than those reported by Snyder and Ayres (1961) with similar experimental conditions, due probably to the use of pH 5.9 rather than pH 5.7.

Brown and Mebine (1969) reported a new mechanism for autoxidation of oxymyoglobin which resulted in a net evolution of 0.75 moles of oxygen per mole of MbO_2 oxidized. By using a specially designed cell and an oxygen electrode, they correlated pigment oxidation with the release (and thus measurement) of oxygen. Their proposal allowed for the known dependence rate on hydrogen ion concentration, and the indirect dependence on oxygen level.

$$Mb^{+2}O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Mb^{+2} + O_2$$

$$H^+ + Mb^{+2} + \tfrac{1}{4}O_2 \xrightarrow{k_3} Mb^{+3} + \tfrac{1}{2}H_2O$$

The experimental results support the proposed use of 0.25 moles of oxygen for the oxidation of oxymyoglobin and the net release of 0.75 moles. The difference between these and previously reported values was attributed to the removal of the reducing agent, sodium hydrosulfite, by ion exchange column. This reducing agent was used in unspecified amounts by George and Stratmann (1952a) with no mention made of its removal. Absolute rates of autoxidation as affected by temperature, pH, and oxygen level were substantially different in systems free of hydrosulfite, but relative effects were the same. Brown and Mebine also found that carboxymethylation of available histidine residues in undenatured sperm whale and tuna myoglobins seemed to have little effect on oxidation rates.

Reducing Systems

That surface color of fresh meat was largely a balance between autoxidation of myoglobin and enzymatic reduction of metmyoglobin was noted by Stewart *et al.* (1965b). They observed that the metmyoglobin-reducing activity (MRA) showed great variability among different animals. The MRA increased with pH from pH 5.1 to pH 7.1 and with temperature from 3°C to 35°C in a given sample. Watts *et al.* (1966) observed that all reductive activity in meat could be stopped by oxidation of

reduced nicotinamide adenine dinucleotide (NADH) and accelerated by oxidized nicotinamide adenine dinucleotide (NAD), and that metmyoglobin reduction occurred after oxygen disappeared from the meat (i.e. under anaerobic conditions). They concluded that both oxygen utilization and metmyoglobin reduction were normally mediated through NAD. It appeared that a loss of substrate rather than a loss of enzyme occurred to limit the reducing capacity.

Saleh and Watts (1968) tested various substrates which donated electrons to NAD for their ability to increase the reduction of metmyoglobin. They believed the primary pathway of electron transport from NADH to metmyoglobin to be through DT diaphorase and quinones. The DT diaphorase catalyzed the reduction of various quinones to quinol, which could reduce ferric heme compounds (Bodwell et al., 1965). A metmyoglobin reductase has not yet been isolated or characterized (Brown and Snyder, 1969).

Brown and Snyder (1969) reported the non-enzymatic reduction of metmyoglobin by NADH and by flavins. This reduction, in the presence of ethylenediaminetetraacetate (EDTA), occurred at rates greater than most of those reported for enzymatic reduction. The system was pH dependent, having greater activity at pH 6.4 than at pH 7.4.

Irradiation Preservation of Meat

The potential for irradiation preservation of fresh meat items has been reviewed by Urbain (1966) and Rhodes and Shepherd (1966). As determined by Yankelovich (1968), a three week extension of shelf life would probably be necessary for completely centralized cutting. Urbain et al. (1968, 1969) and Giddings (1969) have detailed a procedure using ionizing irradiation to permit the extension of fresh meat storage and shelf life. Pasteurization doses of irradiation (50 to 500 krads) which would delay normal microbial spoilage could be used in the centralized cutting of fresh meat. Belo (1968) investigated the usefulness of some inorganic phosphate compounds for slowing drip in consumer-sized fresh meat cuts, and suggested the existence of a lipid oxidation problem upon reoxygenation.

In the proposed process, phosphate-treated irradiated consumer-sized meat cuts, individually packaged in air-permeable film, are bulk packaged in air-impermeable film. After evacuation, they are distributed to retail outlets. Bulk shipments are then opened in the retail outlet as necessary and the reduced pigment permitted to oxygenate.

Effects of Irradiation on Myoglobin

Hannan (1954) stated that discoloration of meat occurred when oxygen was present during irradiation.

Ginger et al. (1955) noted that the susceptibility of myoglobin extracts to radiation damage from cobalt-60 increased with increased purity of the extract. Both oxidation and reduction reactions were obtained by the irradiation of myoglobin in crude extracts. In addition, ground meat packaged and stored in oxygen-impermeable Saran showed little change in myoglobin immediately after irradiation or storage. They reported the apparent conversion of metmyoglobin to myoglobin with irradiation doses of 50 krep to 3000 krep. Spectra were determined on extracted myoglobin. When air-permeable film, Visking, was used, discoloration increased with irradiation dosage and storage.

Laser (1955) exposed solutions of oxyhemoglobin and methemoglobin in air or nitrogen to X-ray doses of 0.8 to 75 krad. He found that the primary spectrophotometric changes in the pigment depended on the initial state of the pigment, and claimed that the changes were independent of oxygen pressure. Oxyhemoglobin oxidation was similar in air and nitrogen; however, methemoglobin reduction was greater in nitrogen than air. He also stated that the amount of oxidation or reduction increased with increasing dose of X-rays, and that, because of secondary reactions, neither was directly proportional to dose.

Tappel (1956) found that a myoglobin extract from pork irradiated in nitrogen atmosphere with doses from 1 to 4 megarads gave an absorption spectrum for oxymyoglobin. In an atmosphere of excess oxygen, the extract developed the brown discoloration of metmyoglobin.

Bernofsky et al. (1959) noted that irradiation of aqueous extracts of beef oxymyoglobin caused a series of reactions in which oxymyoglobin was converted to metmyoglobin, followed by a change to an unidentified red compound stable to further irradiation.

Ambe et al. (1961) found a transformation to insoluble protein aggregates in irradiated hemoglobins and a loss of biological redox or oxygen binding properties. The aggregates did not seem to be formed by simple cross-linking or denaturation. The methionine and histidine were the most radiolabile amino acids.

Brown and Akoyunoglou (1964) reported the effects of gamma radiation on highly purified solutions of sperm whale and tuna myoglobins. Doses of 0 to 500 krad in a nitrogen atmosphere caused precipitation of irradiated myoglobins as a function of dose and concentration. Absorbance at 280 nm increased considerably following irradiation, and changes in the visible region suggested that there was a partial oxymyoglobin and a complete metmyoglobin conversion to substances with spectra similar to oxymyoglobin.

Tarladgis (1965), using reflectance spectrophotometry, studied the pigment changes that occurred in precooked meats during gamma irradiation at doses of 100 to 5000 krad. He found both the spin and the valence state of the iron to be affected by ionizing radiations. The high-spin ferric-porphrin compound of cooked meat was gradually converted by radiation to the low-spin ferrous form. The reduction did not seem to be quantitative with irradiation dose, but at doses above 5×10^2 krad, the dissociation of both ligands of the iron increased and the iron was reduced. He also reported a 25 percent destruction of the pigment with 500 krad of gamma irradiation.

Ho (1967) gamma irradiated sperm whale and tuna metmyoglobin solutions at doses of 500 krad. She found changes in the visible and ultraviolet spectra, with an increase at 280, 540 and 580 nm and a decrease in the Soret region. She reported little change in amino acid composition, no significant change in binding of myoglobin with malonaldehyde, and an increased rate of autoxidation of oxymyoglobin.

The literature indicates considerable awareness of changes associated with irradiation dose; however, none of the research reviewed reported effects of irradiation dose rate on meat pigments.

Limited studies have been conducted on foods with very high dose rates. At high dose rates, there was a decreased ionization of the food because of the increased recombination of free radicals, therefore decreased chemical effects (Pikaev, 1967). Hansen et al. (1965) found a substantial gain in flavor quality in radiation sterilized protein foods when the dose rate was increased from 10^2 to above 10^7 or 10^8 krad/sec. They reasoned that the improvement resulted from a very high energy input which ionized water molecules to an extent that the free radicals created began to recombine rather than react with the solute. Hansen et al. (1965) referred to a study by Taimuty (1957) in which he compared gamma rays of about 100 rep sec^{-1} and electrons at 5×10^8 rep sec^{-1} in foods and vitamins, and observed an effect only in aqueous solutions of ascorbic acid and vitamin A acetate, where the higher dose rate caused less irradiation damage.

Phosphates and Myoglobin

As discussed by Urbain et al. (1968, 1969), a limited number of inorganic phosphate compounds can be considered beneficial for the prevention of drip from fresh meat cuts. The exact mechanism by which they function is not clearly understood. Hellendorn (1960) discussed the action of phosphates on water binding. The effect could be due to the ability of phosphate to split

actomyocin into its component proteins, resulting in the uptake of water. Another theory concerned the possible binding of calcium to the meat, forming bridges between protein fibers. Sherman (1962) stated that meat treated with alkaline polyphosphates exhibited a pH dependent preferential absorption of cations. He theorized that the cations reduced the internal forces of attraction in the protein structure, allowing water retention in proportion to cation concentration.

Yasui et al. (1964a,b) clarified some of the previous studies with their work on rabbit muscle. They classified the specific interactions of inorganic polyphosphates into two types. The first class consisted of inorganic polyphosphates of relatively low molecular weight such as pyrophosphate and tripolyphosphate which had a marked increase in their affinity for myosin in the presence of univalent cations. They also showed increased reactivity with the protein through the formation of divalent metal-phosphate complexes. Among the polyphosphates examined, only pyrophosphate could cause changes in the size and shape of myosin. Tripolyphosphate first had to be hydrolysed to pyrophosphate. Over long periods of time, this occurred by the action of a myosin tripolyphosphatase. Pyrophosphates gradually became ineffective through hydrolysis by pyrophosphatases in meat.

The second type were highly polymerized inorganic phosphates such as hexametaphosphate. Their effect was probably limited to enhancing solubility and extractability of myosin by increasing ionic strength in the meat. They had almost no effect on the viscosity of myosin solutions, and were inhibited in their binding to the protein by high concentrations of univalent and divalent cations.

Belo (1968) has noted that certain inorganic phosphates can improve the reoxygenation ability of fresh beef slices held for three weeks in vacuum storage.

Studies in recent years have shown that various organic and inorganic forms of phosphate apparently interact with hemoglobin and perhaps with myoglobin. Benesch and Benesch (1967) reported the influence of organic phosphates on the oxygenation of hemoglobin. Among the organic phosphate esters present in the human erythrocyte, 2,3-diphosphoglycerate (DPG) was found to be the most abundant and to have the greatest effect on cooperative ligand binding and the overall oxygen affinity of human hemoglobin. They suggested that DPG and related organic phosphates may function as the naturally occurring regulators of oxygen transport by hemoglobin.

Chanutin and Curnish (1967) studied the effect of added organic and inorganic phosphates on the oxygen

equilibrium of human erythrocytes. They found that ATP, GTP, and DPG were particularly effective in decreasing the oxygen affinity of hemoglobin, and were responsible for small changes in heme-heme interaction. Hexametaphosphates caused marked changes in both oxygen affinity and the oxygen equilibrium curve, while tripolyphosphates, tetraphosphates, and pyrophosphates were less effective.

Chanutin and Hermann (1969) used equilibrium dialysis to study the interaction of human deoxy, oxy, and methemoglobin with 2,3-diphosphoglycerate, adenine tri, di, and monophosphates, ribose-5-phosphate, pyrophosphate, tripolyphosphate and tetra and hexametaphosphate. AMP and ribose-5-phosphate were bound in negligible amounts. They determined the number of binding sites and association constants for the rest of the compounds. The results obtained in dilute cacodylic acid buffer at pH 6.5 demonstrated that DPG and other organic and inorganic phosphate compounds bound deoxy, oxy, and methemoglobin.

Only limited information exists concerning the interaction of phosphates with myoglobin. Gillespie et al. (1966) studied the interaction of sperm whale metmyoglobin with phosphates. They compared the rates of denaturation of metmyoglobin components by cupric ions and the consequent decrease in absorbance at the Soret peak at 409 nm. The separated metmyoglobin fractions were

tested after dialysis for one or two days and after deionization on a mixed bed ion exchange column. The denaturation time was much lower for the metmyoglobin that had been deionized, indicating that binding by some ion was affecting denaturation. The additions of phosphate showed a definite effect on the denaturation rate of metmyoglobin by the cupric chloride.

Similar experiments in which phosphate was replaced by sulfate, chromate, arsenate, or borate showed no effect by these ions on the denaturation rate.

Hartzell et al. (1968) compared the reactivity to cupric ions of harbor seal, porpoise, and sperm whale myoglobins. The main point of contrast between the sperm whale myoglobin and the other two was the rate of reaction with cupric ion and the sensitivity of that rate to the presence of phosphate. They were again following denaturation of metmyoglobin by the change in absorbance at 409 nm. They could determine no simple reaction order. The decrease in rate of denaturation with phosphates was more pronounced with sperm whale metmyoglobin.

Brown and Mebine (1969) studied the effect of 2,3-diphosphoglycerate on the oxidation rates of various types of oxymyoglobins. They found the presence of diphosphoglycerate to substantially increase oxidation rates. They reasoned that diphosphoglycerate reduced the oxygen affinity of myoglobin as it does in hemoglobin.

EXPERIMENTAL DESIGN

Work concerning the oxidation of myoglobin will be described as two separate projects. Experiments that demonstrate the influence of irradiation dose rate on meat pigments will be detailed first. The second section will outline procedures and materials used in studying the manner in which inorganic phosphates can affect the oxidation of myoglobin.

Dose Rate Experiments

Urbain et al. (1968, 1969) described a method for the potential use of ionizing radiation in the centralized cutting and distribution of fresh meat cuts. It was noted that when fresh beef slices were electron irradiated under certain conditions, a darkening of surface pigments occurred. As this phenomenon had not been observed previously with gamma irradiation, further studies were initiated to determine its significance. Preliminary tests indicated a general correlation between pigment darkening and dose rate of irradiation.

The beef used for the experiments was either of U.S. Commercial or U.S. Good grade purchased from the Food Stores at Michigan State University. The

semitendinosus muscle from the bottom round, commercially known as the "eye of round," was removed and used fresh. Cross-sectional slices were cut approximately 1.5 cm thick and about 7.5 cm square.

Because of the necessity for drip control and color preservation, all meat samples were dipped for 30 seconds in a 10 percent (by weight) solution of sodium tripolyphosphate, $Na_5P_3O_{10}$, purchased from Calgon Corporation.

Beef slices were wrapped in an oxygen permeable film, "Resinite RMF-61," manufactured by the Borden Chemical Company. This simulated the first step in completely centralized cutting of fresh meat (Giddings, 1969). Air impermeable overwrap pouches composed of a Mylar, Saran and polyethylene laminate, "IKD Super All Vak #13," were supplied by International Kenfield Distributing Company. These bags were vacuum sealed with a Kenfield Vacuum Sealer resulting in commercial vacuum conditions, i.e. about 685 cm (27 in) of mercury. Bags which were to be backfilled with oxygen had an 8 cm length of Tygon tubing glued into the closed end of the pouch. Gas exchange could be controlled by a clamp attached to the tube.

Three packaging conditions were used for dose rate studies. Samples designed to simulate commercial usage were phosphate dipped for 60 seconds following cutting and trimming. They were drained for 30 to 60 minutes

to remove excess phosphate solution, wrapped in oxygen permeable film, and then irradiated individually. Under commercial conditions the packages would be collectively vacuum packaged in wholesale sized units of about 22.5 kilograms. However, in order to observe each sample separately, our samples were packaged individually or as pairs in the air impermeable pouches, evacuated, and stored as desired.

With samples to be irradiated under conditions of vacuum, it was desirable to minimize the time required for treatment and packaging. Following the phosphate dip, free moisture was immediately removed from each sample with a paper towel. The samples were then wrapped individually or in pairs in air permeable film, and vacuum sealed in pouches. These samples were irradiated in the vacuum pouches.

Samples to be treated under oxygen pressure greater than 760 mm were phosphate dipped, drained, wrapped in air permeable film, and then held for 10 hours at 3°C to promote maximum formation of oxymyoglobin. Following this, they were packaged in the pouches equipped with tygon tubing inserts, vacuum sealed, and backfilled through the tubing with oxygen in excess of 760 mm before irradiation.

Two radiation sources were used. The gamma source was a 50,000 curie Cobalt-60 research irradiator. The

cobalt strips were raised from a protective water pool to an enclosed shield in a refrigerated room for irradiation. Samples could be arranged around the shield or in its center-well, thus providing a number of distinct dose rates for gamma irradiation. Most of the irradiation was performed at temperatures of 10°C.

The electron source used was a General Electric One MeV resonant transformer electron beam generator. This machine provides a variety of dose rates. Dose rate was controlled by varying the beam current from 0.1 milliamperes to 1.0 milliamperes, or by varying the distance between the source and the sample. Dose calculations have been made for a standard distance of 47 cm. The distance could be increased to 118 cm. Dose rate determinations were performed by ion chamber method (Clifford, 1968). Total doses were determined for the dose rates used by following changes in the UV spectrum of the acrylic plastic "Perspex H-X," manufactured by Imperial Chemical Industries, England. Spectrophotometric readings were converted to dose using the determinations of Adwalpalker (1969).

Samples were irradiated to a total dose of 200 krad. Electron dose rates of 5, 25, and 50 krad/sec were used for samples in all three packaging systems, and in addition, rates of 0.55, 10, and 15 krad/sec were used

for samples under oxygen pressure. Rates of 0.05 and 0.5 krad/sec were obtained from the gamma source. The dose rates used and their sources are tabulated in Table 1.

Vacuum and atmosphere irradiated samples were stored at 3°C for 21 days, removed from the air impermeable laminate bags, and exposed to atmospheric conditions for one day.

TABLE 1.--Dose rates and source of irradiation.

Dose rate krad/sec.	Radiation source, gamma or electron	Beam current milliamps	Time	Distance from source
50	electron	1.0	4 sec.	47 cm
25	electron	0.5	8 sec.	47 cm
15	electron	0.3	13.3 sec.	47 cm
10	electron	0.2	20 sec.	47 cm
5	electron	0.1	40 sec.	47 cm
0.55	electron	0.1	360 sec.	118 cm
0.5	gamma	---	400 sec.	centerwell
0.05	gamma	---	42 min.	46 cm

Color Measurements

Color determinations were quantified by reflectance spectrophotometry. The method followed was similar to that used by Stewart et al. (1965a) and Snyder and Armstrong (1967). It was non-destructive and provided quantitative values for the percentages of pigment in the myoglobin and metmyoglobin forms.

In meat samples sufficiently thick that no change in reflectance occurred upon further increases in thickness, the reflectance or reflectivity, called R_∞ and read as transmittance, was dependent on the ratio of light absorbed by the pigment to that scattered by the meat structure. The ratio of the absorption coefficient per unit of sample thickness (K) to the scattering coefficient per unit of sample thickness (S) varied with the total light reflected. The value of K, the absorption coefficient, was dependent on computation of S, which involved measurements of reflectance of layers of known thickness against backgrounds of known reflectance. This had not been done for meat, but Stewart et al. (1965a) assumed that S would be reasonably constant under a given set of experimental conditions, and thus K/S values would be proportional to the amount of pigment.

The K/S values accounted for the fact that some of the incident light was scattered by the opaque surface, and that the proportion of light absorbed by the pigments to that scattered decreased with increasing R_∞. Judd and Wyszecki (1963) calculated values for K/S derived from the Kubelka-Munk equation, $K/S = \frac{(1-R_\infty)^2}{2R_\infty}$, which can be used for the conversion of R_∞ values to K/S values. Snyder and Armstrong (1967) have shown, by adding known amounts of pigment to a model system of 5 percent non-fat

dried milk in water, that a linear relationship exists between K/S ratios and the proportions of the three pigment forms as measured at the wavelengths specified.

The method essentially consisted of a comparison of reflectance spectrophotometric values at the isobestic points of the three pigment forms. As shown in Figure 3, the reflectance spectrum for any particular sample had an isobestic point at 525 nm. Regardless of the pigment form, i.e. Mb, MbO_2, or MetMb, the reflectance value at this point was the same for any particular sample. Isobestic points existed at 474 nm for MbO_2 and MetMb, and at 571 nm for Mb and MbO_2. Thus, the reflectance value at 474 nm converted to K/S compared to the value at 525 nm converted to K/S was indicative of the relative amount of Mb present. When all the pigment was in the myoglobin form, the ratio of $\frac{K/S \text{ @ } 474 \text{ nm}}{K/S \text{ @ } 525 \text{ nm}}$ provided a number for 100% Mb. When all the pigment was oxygenated and/or oxidized, i.e. MbO_2 + MetMb = 100%, the same ratio gave a value for 0% Mb. These two extremes were used as the 0% and 100% points on a graph comparing ratio values of $\frac{K/S \text{ @ } 474 \text{ nm}}{K/S \text{ @ } 525 \text{ nm}}$ with percent Mb (Figure 4).

The ratio of $\frac{K/S \text{ @ } 571 \text{ nm}}{K/S \text{ @ } 525 \text{ nm}}$ in the same manner provided the 0% and 100% points on a graph for percent MetMb. When all the pigment was in the oxidized form the ratio was the value for 100% MetMb, and conversely,

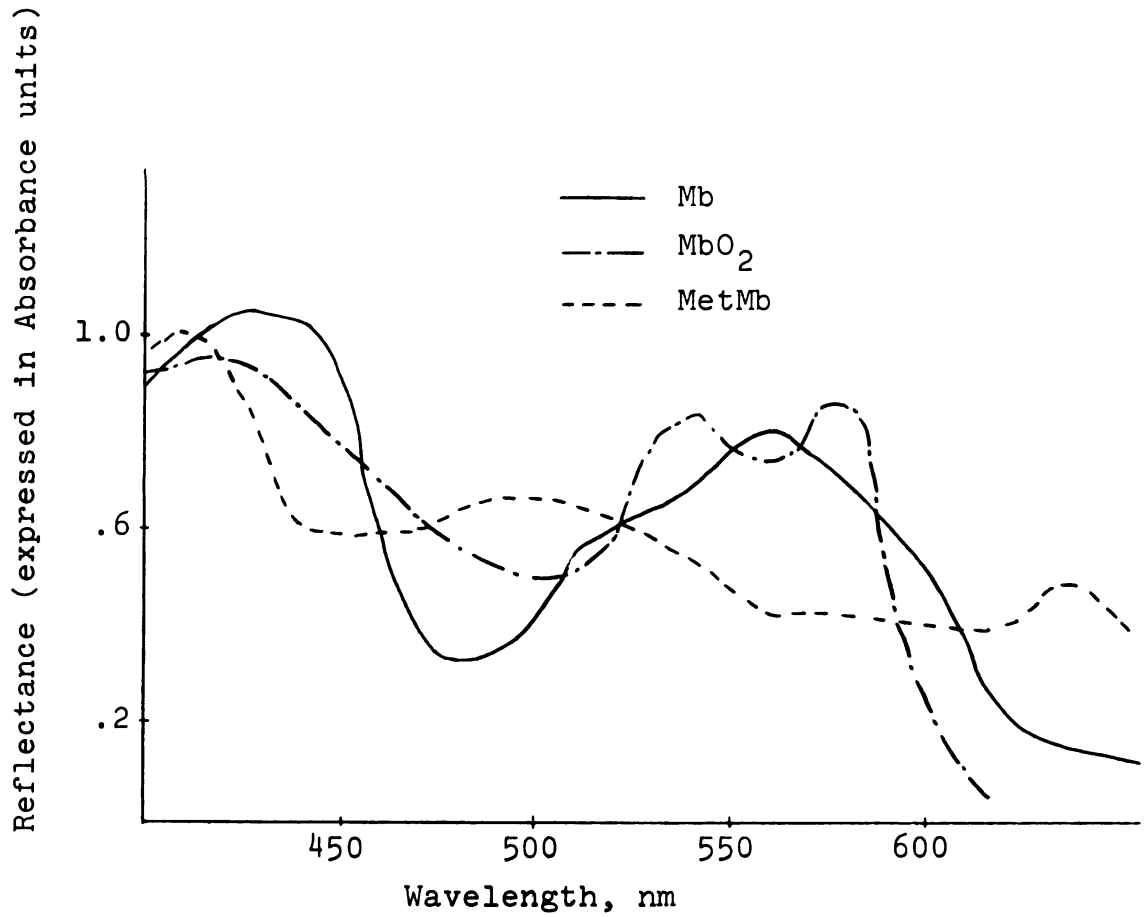


Figure 3. Reflectance spectra of fresh beef samples treated to contain predominantly Mb, MbO_2, and MetMb respectively.

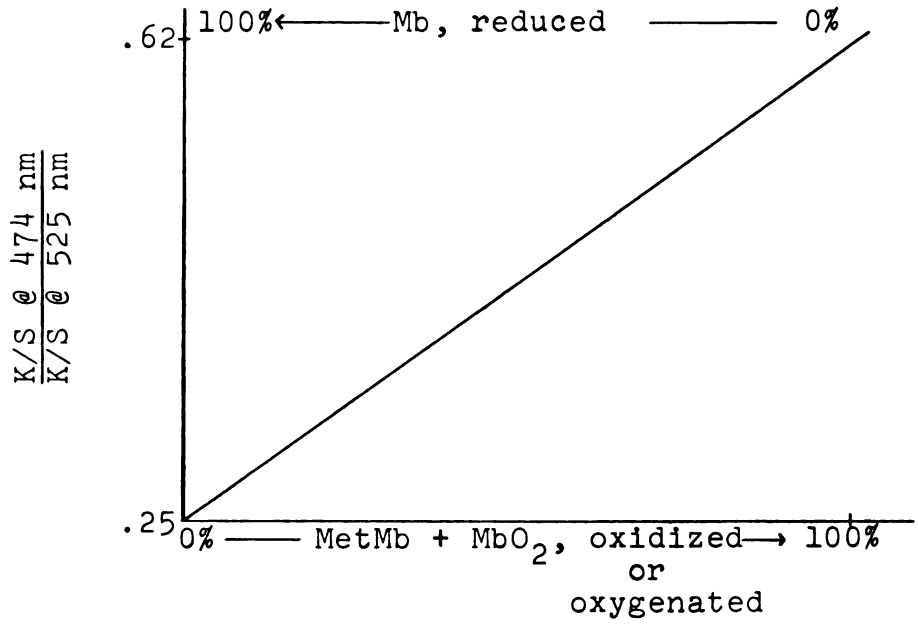


Figure 4. Curve relating the ratio of K/S @ 474 nm to K/S @ 525 nm vs. the percentage of reduced pigment, myoglobin.

when all the pigment was in the reduced form, i.e. Mb + MbO_2 = 100%, the ratio was the value for 0% MetMb (Figure 5).

Once the standard graphs were prepared, relative amounts of Mb and MetMb could be determined for any sample by converting the R_∞ readings to K/S values, determining the respective ratios, and reading the percent Mb and percent MetMb from the graphs. The relative amount of MbO_2 was determined by difference, i.e. $\%MbO_2$ = 100% - (%Mb + %MetMb).

The Standard graphs were prepared by converting all the pigment in a sample to the reduced (Mb) form, all to the oxygenated (MbO_2) form, or all to the oxidized (MetMb) form (Figure 3). To obtain a value for 100% Mb, measurements could be made on a sample immediately following cutting, after evacuation and storage, or on a sample reduced with sodium hydrosulfite, $Na_2S_2O_4$. Exposure of a freshly cut meat surface to the atmosphere or to a pure oxygen atmosphere resulted in the formation of MbO_2. MetMb was obtained by chemical oxidation with potassium ferricyanide.

A Bausch and Lomb Spectrophotometer, Model 505, with reflectance attachment was used to obtain all spectra. Meat slices cut at right angles to the fiber long dimension were placed against a glass slide covering the sample port. The glass, almost optically clear, was

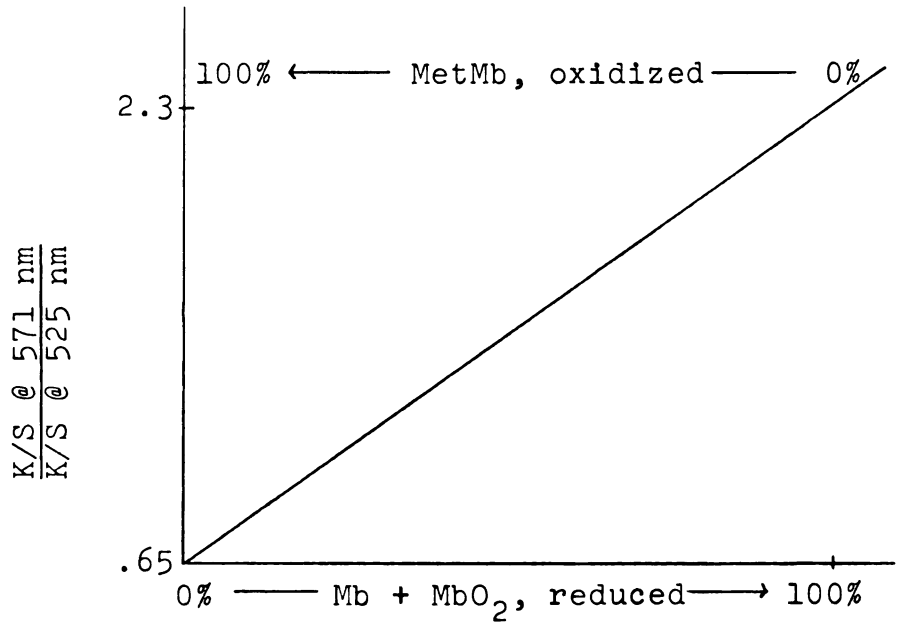


Figure 5. Curve relating the ratio of K/S @ 571 nm to K/S @ 525 nm vs. the percentage of oxidized pigment, metmyoglobin.

necessary to maintain a flat meat surface (Elliot, 1967). A similar glass slide was used to cover the reference port. A magnesium carbonate reference was used to calibrate the instrument. Because the absorbance of the meat sometimes exceeded 1, a flat, gray piece of cardboard was used as a reference, resulting in spectra generally between 0.3 and 0.8 absorbance units (Stewart et al., 1965a). The recording was done as absorbance rather than reflectance in order to exaggerate the spectral peaks. The absorbance readings were converted back to reflectance before conversion into K/S values.

There were a number of problems associated with the reflectance method (Elliot, 1967). The most important problem in storage studies was a variability introduced by changes in water content. Drying of red meat surfaces resulted in a darkening due probably to a concentration of pigments. Repeat measurements were inaccurate unless taken from the same location on any given sample. Well-marbled or fatty pieces of meat gave meaningless results. Samples had to be measured immediately following release of vacuum, as oxygenation began immediately.

Spectrophotometric determinations were taken immediately before and after irradiation, after the vacuum pouch was opened, and after the one day of reoxygenation. Visual observations by a limited number of expert judges were also collected at these times.

Phosphate Studies

The extension of shelf-life and distribution time of centrally cut fresh beef posed the problem of pigment oxidation to brown metmyoglobin. This conversion was delayed to some extent by the effectiveness of the metmyoglobin reducing enzymes and by a system that included vacuum packaging. A noted decrease in oxidation rate occurred when various inorganic phosphates were used as treatments to slow liquid exudation or drip.

Fresh Beef Slices

Experiments were conducted with fresh beef slices in an attempt to quantify some of the effects of phosphates on whole meat. Cross-sectional beef slices were trimmed from U.S. Good grade eye of round muscle. The slices were dipped for 60 seconds into the experimental solutions immediately after cutting, permitted to drain for 30 to 60 minutes, gamma irradiated with 100 krad, and vacuum stored for a 21 day period at 3°C. Reflectance measurements were taken before irradiation, at 21 days, and following a single day of reoxygenation. Surface pH of the fresh meat was determined with a Beckman Expandomatic pH meter. After 22 days of storage, pH measurements were taken both of the surface of the slices and of ground meat samples. Visual observations of color and appearance were made following the reoxygenation.

Preliminary experiments compared the effects of dipping in a solution of sodium tripolyphosphate (NaTPP) with dipping in some other inorganic compounds with similar pH values. The NaTPP was food grade purchased from Calgon Corporation. A 10% solution of it had a pH of about 9.1. Solutions of reagent grade ammonium and sodium hydroxide were prepared with pH values in the range 9.0 to 9.2. Solutions of Tris-HCl and carbonate buffers for pH 9 were prepared according to procedures outlined in "Methods of Enzymology," Vol. I. The test solutions were chilled before use. Color differences were noted visually throughout the refrigerated holding period, and reflectance measurements taken upon termination of storage.

The effect on oxidation rates of solutions with similar phosphate ion concentration but different pH was shown using NaTPP and the three chemical forms of orthophosphate. Solutions of H_3PO_4, NaH_2PO_4, and Na_2HPO_4 were prepared to give phosphate ion concentrations similar to the 10% NaTPP, and the pH of each determined. Before dip and after storage, pH measurements were taken of the meat slices. Visual observations and reflectance data were recorded throughout the tests.

Experiments were also conducted that compared the dibasic form of orthophosphate, Na_2HPO_4, with carbonate and borate buffers, NaTPP, and a commercial blend of

phosphate called "Kena", supplied by Calgon Corporation. Again, visual observations were made throughout the 21 day vacuum holding period, and both visual observations and reflectance recordings taken after removal from vacuum and reoxygenation.

Beef slices from U.S. Commercial and Good grade eye of round were dipped for 60 seconds in 10% solutions of sodium tripolyphosphate, drained, and either allowed to oxygenate or vacuum packaged and held until the purple color indicated a reduced condition. Reflectance spectra were then taken of the oxygenated or reduced meat pigments and compared with controls similarly treated but without the phosphate dip. Shifts in the spectra were noted.

Storage studies were conducted in which the beef slices were dipped in NaTPP following various periods of vacuum storage. All samples were given three weeks of vacuum storage and one day for oxygenation. Tripolyphosphate applications were made at 0, 7, 14, and 21 days of storage. Those slices dipped during the holding period were removed from vacuum, quickly dipped and drained, and returned to vacuum within a 30 minute period. A non-phosphate treated control was compared with the samples by visual and reflectance methods at 21 days and following one day of oxygenation.

Studies Using Unpurified Myoglobin Extracts

Water extracts of myoglobin were obtained from beef slices that had been dipped in a 10% NaTPP solution. The method was a modification of that used by Broumand et al. (1958) and involved grinding the sample, free of trimable fat, in a Kitchen Aid grinder using a 1.9 cm (3/4 in) and then a 0.95 cm (3/8 in) plate, adding one and one-half times the sample weight of deionized distilled water, and shaking in a flask. The mixture was permitted to stand in the refrigerator for an hour and then filtered through a Buchner funnel using Wattman No. 1 filter paper. A control was prepared following similar procedures but using meat not treated with phosphate. Pigment extracts from half of the phosphate treated samples were dialysed in presoaked 25 mm dialysis tubing for three hours against 4 liters of deionized distilled water without changing the water. Oxidative changes were followed spectrophotometrically for four days with a Bausch and Lomb recording spectrophotometer using the analysis method of Broumand et al. (1958).

Preparation of Purified Myoglobin

Purified solutions of oxymyoglobin were prepared. The methods of Yamazake et al. (1964) and Quinn et al. (1964) were modified, resulting in the rapid preparation of stable oxymyoglobin. A U. S. Choice or Good grade

eye was trimmed of external fat and sliced, then visible internal collagenous fibers removed. The slices were ground with a Kitchen Aid grinder through a 1.9 cm (3/4 in) plate, and 700 to 800 gram lots held in 2 liters of cold distilled water per kilogram of muscle for about 30 minutes. A sufficient quantity of 2 N ammonium hydroxide was added to maintain the homogenate above pH 7.5. This was mixed about 10 seconds in a 4 liter Waring blender. The homogenate was then filtered through increasing thicknesses of cheesecloth to remove much of the lipid and fibrous material.

The extract was 70% saturated with ammonium sulfate by adding the solid salt. The pH was adjusted to maintain a pH of 7.5 and the temperature held at less than 4°C. The mixture was centrifuged at 4000 rpm for 15 minutes in a Sorvall Model RC2-B centrifuge. Hemoglobin, which is insoluble in 70% ammonium sulfate (Lewis and Schweigert, 1955), and some fibrious material were precipitated. The supernatant was filtered through cheesecloth to remove remaining membraneous material, saturated with ammonium sulfate, and the pH readjusted to 7.5. About 2 grams of Celite were added and the mixture stirred for 30 minutes. The mixture was filtered through a Buchner funnel and the Celite-myoglobin precipitate collected, scraped from the filter paper, and dissolved

in a small quantity of chilled, deionized distilled water. This mixture was dialysed in presoaked dialysis tubing against several 4 liter batches of deionized distilled water to remove sulfate ion. Final dialysis was against 0.2 M Tris-Maleate-NaOH buffer, pH 7.0. Following dialysis, the Celite-myoglobin mixture was filtered through a Buchner funnel to remove the Celite from the myoglobin. Myoglobin to be stored or used over an extended period of time was then passed through a Millipore filter, Type GS, 0.22μ pore size, to remove bacteria. The myoglobin concentration was usually at least 5×10^{-4} M at this stage of extraction.

Sephadex column chromatography similar to that employed by Quinn (1963) was used to purify the myoglobin. Dry G-75 Sephadex from Pharmacia was placed in an amount of 0.2 M Tris-Maleate-NaOH buffer, pH 7.0, well in excess of that necessary for rehydration. Following equilibrium overnight, the suspension was heated to boiling to remove entrapped air, cooled, and then poured slowly down the wall of a 4.5 x 50 cm column. When a few centimeters of the gel had settled, the screw clamp at the bottom of the column was opened to allow a slow flow of the buffer. When the gel had settled completely, a layer of filter paper was placed on the surface to protect it from disturbance when samples were added.

Samples of myoglobin suspended in the Tris buffer were added to the column in quantities of 10 to 20 ml and eluted with a similar buffer. The gravity flow rate of the column was about 30 ml/hour. The eluant was collected in 5 to 10 ml portions and each read spectrophotometrically at 280 nm and at 525 nm. The absorbance at 280 nm divided by the absorbance at 525 nm gave a measure of myoglobin purity (Quinn, 1963). Those fractions with a ratio of less than 4.5 were retained. The spectrum from 450 nm to 600 nm was also obtained with the Bausch and Lomb 505 recording spectrophotometer to check for the characteristic oxymyoglobin peaks at 544 and 582 nm.

A molar extinction coefficient of 15.1×10^{-3} at 582 nm (Bowen, 1949) was used for estimation of the concentration of myoglobin. Chromatographic purification resulted in a dilution of the myoglobin from concentrations greater than 5×10^{-4} to about 1.5 to 2.0 $\times 10^{-4}$ M. However, there was almost no conversion to metmyoglobin as indicated by the spectrum. Myoglobin could be stored in the purified condition for periods of at least two weeks with minimal autoxidation.

Effect of Dibasic Phosphate on Oxidation Rates of Myoglobin

The influence of inorganic phosphate on oxidation rate was studied. Sephadex-purified oxymyoglobin was dialysed against deionized distilled water to remove the

Tris buffer. Quantities of Na_2HPO_4 were added to one ml fractions of the oxymyoglobin in molar ratios of 1:10, 1:1, and 10:1 phosphate ion to oxymyoglobin. The phosphate-myoglobin solutions were added to 3 ml portions of 0.2 M Tris-HCl buffer to give one mixture of each at pH 5.6, 6.1, 6.5, and 7.0. The samples were incubated in 16 by 125 mm test tubes in a water bath at 30°C ± 0.5°C.

An immediate measurement was taken of each for 0 time, and again at 15, 30, 60, 90, 120, and 150 minutes. No provision was made to keep the temperature at 30°C for the brief time of measurement. The pH value of each sample was determined at the end of a test.

The method of Snyder and Ayres (1961) and Brown and Dolev (1962) was used to determine autoxidation rate. Changes in the peak at 582 nm, characteristic of oxymyoglobin, could be followed with a Bausch and Lomb recording spectrophotometer. The initial absorbance reading from a particular sample was assumed to be representative of 100% oxymyoglobin. With time, oxidation of the pigment resulted in a drop in the height of the peak at 582 nm. After the 150 minute test, a few milligrams of potassium ferricyanide were added, and the resulting absorbance value taken as representative of 100% oxidized pigment. The amount of oxidized pigment as a ratio of the total possible could then be determined for each test.

Association of Radioactive Phosphate and Myoglobin

The possibility for association of phosphate ions and myoglobin was investigated by taking 10 ml of Sephadex-purified myoglobin and adding to it one ml of labeled phosphate. The $Na_2H^{32}PO_4$ had an activity of 5 µc/ml, and a phosphate concentration of 2×10^{-4} mM. The concentration was considerably lower than the 2×10^{-1} mM concentration of the myoglobin. Following a 24 hour refrigerated holding, the mixture was added to a Sephadex column, the visible fraction collected, and a 0.1 ml aliquot of the diluted myoglobin counted on a Nuclear Chicago planchette counter.

An association of phosphate with myoglobin was also investigated by adding one ml of 100 µc/ml $Na_2H^{32}PO_4$ to 10 ml of myoglobin which had been dialysed to remove the Tris buffer. The mixture was held at 3°C overnight and dialysed against distilled water at 3°C. Progress of the dialysis was checked by counting 0.1 ml aliquots from the water changes, and then samples of myoglobin were counted to determine the amounts of radioactive phosphate remaining.

Both of the preceeding tests were qualitative. Further attempts were made to quantify the amount of phosphate that would apparently associate with each mole of myoglobin. Sephadex-purified myoglobin was dialysed to

remove the Tris buffer. The concentration of myoglobin was determined, and samples of $Na_2H^{32}PO_4$ prepared to provide molar ratios of 1:10, 1:2, 1:1, 2:1, 5:1, and 10:1 phosphate to myoglobin. In one series of experiments the radioactive isotope was used as the only source of phosphate, and thus the dialysed myoglobin had to be diluted for the correct combinations. In a later experiment, the labeled phosphate was used as a tracer and the myoglobin was not diluted from the 2×10^{-4} M concentration.

The phosphate solutions with known radioactivity levels were added to myoglobin in the desired molar ratios, held overnight at 3°C, and dialysed in presoaked 25 mm dialysis tubing against deionized distilled water for 24 hours, Aliquots from the dialysis water were counted at 2, 4, 6, 8, and 24 hours. Controls were used containing no myoglobin, but with similar amounts of labeled phosphate as the test samples. Samples of 0.2 ml were counted on a Nuclear Chicago planchette counter with consideration given to machine counting efficiency and the 14.3 day half-life of radioactive phosphorus.

RESULTS AND DISCUSSION

Dose Rate Studies

Preliminary experiments with electron irradiation resulted in obvious visible differences in the color of beef slices irradiated in air. These particular beef slices had been packaged in an air permeable film, Resinite, and were irradiated at room temperatures. The total dose was 200 krad, delivered at two rates, 5 and 50 krad/sec.

Table 2 indicates that the immediate irradiation effect on this meat with the pigment in the oxygenated form, MbO_2, was a conversion to both the purple colored reduced myoglobin, Mb, and to the brown metmyoglobin form, MetMb. This conversion appeared to be directly related to dose rate, with more myoglobin and metmyoglobin produced at 50 krad/sec than at 5 krad/sec.

After 21 days storage and before reoxygenation, the reflectance measurements showed higher metmyoglobin levels in the irradiated samples than in the control samples. There was more metmyoglobin in the samples irradiated at higher dose rates than in those irradiated at lower rates. After reoxygenation as well, the higher dose rate samples had more metmyoglobin than the lower

TABLE 2.--Electron irradiation of beef slices exposed to air; dose rate vs. % of each pigment form.

Dose rate krad/sec.	Pigment form	Before irradiation %	After irradiation %	Following 21 days storage, %	After one day of oxygenation, %
50	Mb	.2	35	36	12
	MbO_2	95	13	1	39
	MetMb	3	52	63	49
5	Mb	4.5	25	49	19
	MbO_2	93	56.5	0	41.5
	MetMb	2.5	18.5	51	39.5
0	Mb	4	4	64	9
	MbO_2	93.5	96	13	74
	MetMb	2.5	0	23	17

dose rate samples, which in turn had more metmyoglobin than the control samples. Neither of the irradiated groups of samples formed as much oxymyoglobin upon oxygenation as did the control samples. These results indicate that dose rate might affect the amount or the rate of oxidized pigment formation in the irradiation process for central cutting of meat. However, because a phosphate dip had not been used, further testing was necessary to establish the effects of dose rate for the actual conditions envisioned in the process.

In other experiments reported by Urbain et al. (1969), absorption spectrophotometric tests of juice expressed from beef slices irradiated at different dose rates gave indications that higher dose rates caused greater metmyoglobin production. Irradiation of beef pigment extracts caused almost total conversion of pigment to metmyoglobin, but there was still some indication of a dose rate effect. In these tests, a considerable decrease in metmyoglobin formation was noted in beef slices irradiated in vacuum as compared to those irradiated in air. However, changes of the pigment form during extraction plus use of unpurified myoglobin might have produced results different from those that would be obtained with beef slices.

Results of more extensive tests with beef phosphate treated and irradiated while exposed to atmospheric conditions are given in Table 3. Figure 6 shows the net

TABLE 3.--Irradiation of beef slices exposed to air; dose rate vs. % of each pigment form.

Dose rate krad/sec Radiation source	Pigment form	Before irradiation, %	After irradiation, %	Following 21 days storage, %	After one day of oxygenation, %
50 electron	Mb	22	33.5	51	21
	MbO_2	55	22	6	58.5
	MetMb	23	44.5	43	20.5
25 electron	Mb	22	17	48.5	23
	MbO_2	56	42	10.5	58
	MetMb	22	41	41	19
5 electron	Mb	19.5	26.5	41	19.5
	MbO_2	60	37	15	67
	MetMb	20.5	36.5	44	13.5
0.5 gamma	Mb	25	41.5	46	12
	MbO_2	50.5	32	14.5	71.5
	MetMb	24.5	26.5	39.5	17.5
0.05 gamma	Mb	21	16.5	41.5	19.5
	MbO_2	59.5	56.5	20.5	61.5
	MetMb	19.5	27	38	19
0 none	Mb	12	2	76.5	22
	MbO_2	65	74	4.5	67.5
	MetMb	23	24	19	10.5

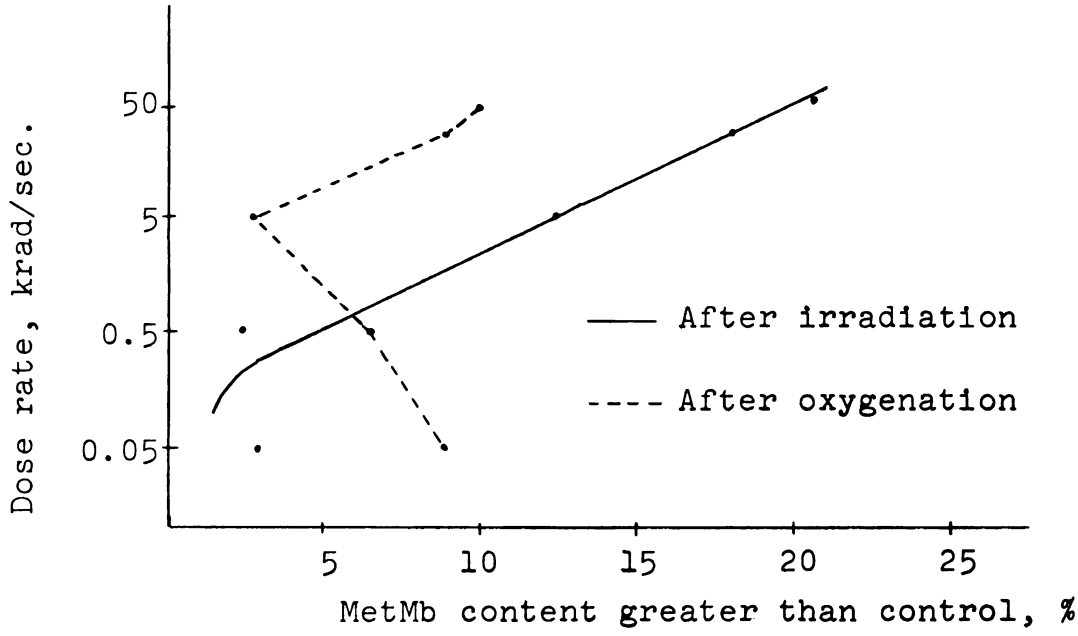


Figure 6. Atmospheric irradiation conditions; dose rate vs. the amount of metmyoglobin greater than in the control.

increase in oxidized pigment (metmyoglobin) in the samples above that in the control immediately following irradiation. As shown previously with the samples in the preliminary study, there was an increase in the amount of oxidized metmyoglobin formed at higher dose rates. The darkening was due to conversion from oxymyoglobin to both myoglobin and metmyoglobin. Visual observations generally indicate significant differences among samples irradiated at the three electron rates and the control, with the highest dose rate producing the darkest sample. The higher gamma rate also caused some darkening, but the lower rate generally resulted in a color that could not be distinguished from the control.

Following the three week storage period, reflectance measurements showed that all the irradiated samples had a similar amount of metmyoglobin, about twice as much as the control. After one day of reoxygenation, spectrophotometric results indicated minor differences among the samples (Figure 6). These differences did not seem dependent on dose rate. Although there was some drop in the metmyoglobin concentration with a corresponding decrease in electron dose rate, both gamma rates resulted in higher ultimate metmyoglobin concentrations than did the lowest electron rate. All the samples had more metmyoglobin than the controls, but spectrophotometrically there was little difference in the values.

However, visual observations after reoxygenation indicate that the highest dose rates, i.e. 25 and 50 krad/sec, may result in metmyoglobin levels too high for acceptibility. Samples irradiated with the lowest electrol rate and the higher gamma rate were generally acceptable. Those irradiated at the lower gamma rate were almost always acceptable. Thus, following reoxygenation after three weeks vacuum storage, the reflectance spectrophotometric results may not parallel visual observations.

Results obtained from irradiation of vacuum samples are given in Table 4. Reflectance spectrophotometry indicates that there was a slightly greater conversion to metmyoglobin with the highest dose rate (Figure 7). Although all the samples had about 50% oxymyoglobin before irradiation, there was almost a complete conversion from this form to myoglobin following irradiation at any dose rate. Visual changes were slight, and darkening was generally noted only with the highest electron dose rate. This would indicate that meat in which about half the pigment was already in the reduced form would not appear to become more purple when the myoglobin concentration increased. The darkening at the highest dose rate was a brown hue on the purple background.

After three weeks of vacuum storage, the reflectance measurements indicated that the higher electron rates may result in slightly greater amounts of oxidized

TABLE 4.--Irradiation of beef slices under vacuum; dose rate vs. % of each pigment form.

Dose rate krad/sec Radiation source	Pigment form	Before irradiation, %	After irradiation, %	Following 21 days storage, %	After one day of oxygenation, %
50 electron	Mb	44.5	81	73	18
	MbO_2	50.5	1.5	8	55
	MetMb	5	17.5	19	27
25 electron	Mb	47	83	71.5	10
	MbO_2	46	9.5	15.5	86
	MetMb	7	7.5	13	4
5 electron	Mb	49	93	65	10
	MbO_2	51	6	24.5	79.5
	MetMb	0	1	10.5	10.5
0.5 gamma	Mb	53	85	78	15.5
	MbO_2	42.5	4	12.5	78.5
	MetMb	4.5	11	9.5	6
0.05 gamma	Mb	43.5	94	85	10
	MbO_2	53	0	12	80
	MetMb	3.5	6	3	10
0 none	Mb	59	59	83.5	17
	MbO_2	38	38	6	75
	MetMb	3	3	10.5	8

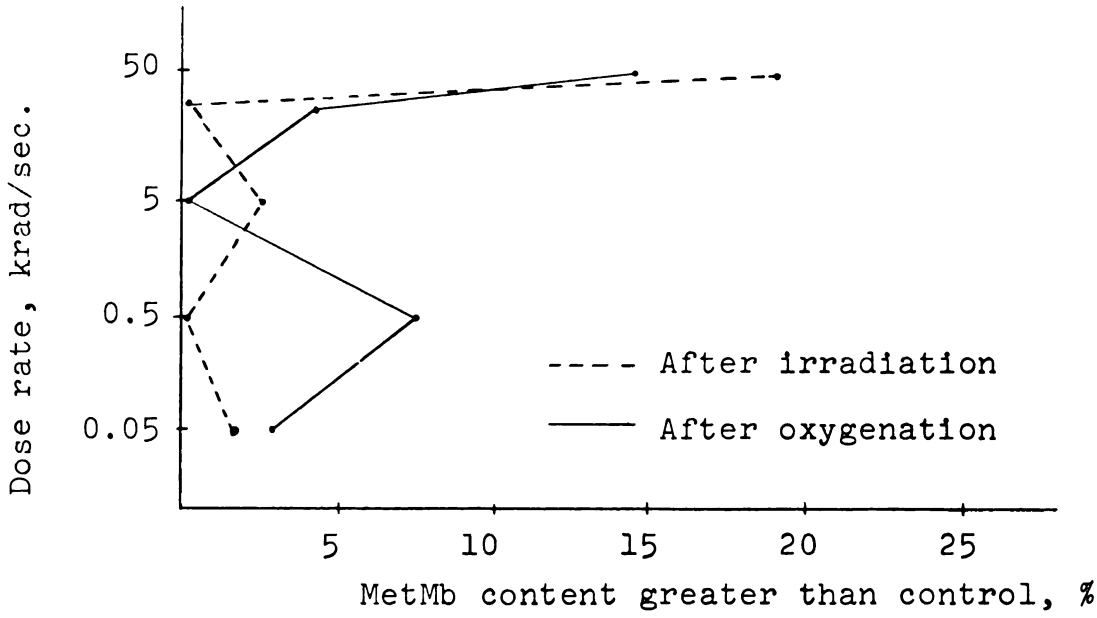


Figure 7. Vacuum irradiation conditions; dose rate vs. the amount of metmyoglobin greater than in the control.

pigment. Following one day of reoxygenation, both the reflectance and the visual observations showed the level of metmyoglobin still to be greatest in the samples treated with the highest dose rate of electron irradiation (Figure 7). Many of these samples were unacceptable. Samples irradiated at the other dose rates were usually an acceptable bright red.

Table 5 contains data from samples irradiated under oxygen pressure of slightly greater than one atmosphere. These samples were not stored. The results exaggerate the effects of the presence of oxygen during irradiation. The number of electron dose rates used for this study was increased. The lowest was almost as low as the higher of the two gamma rates. For the irradiated samples, Figure 8 shows the amount of oxidized pigment greater than that in the control. This differential in oxidized pigment increased as the dose rate increased. The higher gamma rate and the lowest electron rate had similar metmyoglobin concentrations. This would be some verification of the assumption that the effect from gamma rays and electrons is part of a continuum. In contrast to irradiation under atmospheric or vacuum conditions, irradiation under oxygen pressure resulted in definite oxidation at all dose rates. However, there was little difference in the amount of metmyoglobin produced at the gamma rates of 0.05 and 0.5 and the electron

TABLE 5.--Irradiation of beef slices in oxygen conditions; dose rate vs. % of each pigment form.

Dose rate krad/sec Radiation source	Before irradiation			After irradiation		
	%Mb	$\%MbO_2$	%MetMb	%Mb	$\%MbO_2$	%MetMb
50 electron	22	72	6	31	18	51
25 electron	22	73	5	33	18	49
15 electron	10	85	5	18	45	37
10 electron	17	78	5	25	45	30
5 electron	19	75	6	33	43	24
0.55 electron	21	75	4	28	52	20
0.5 gamma	12	86	2	33	50	17
0.05 gamma	11	85	4	16	69	15
0 none	14	82	4	17	83	0

rates of 0.55 and 5.0 krad/sec. At dose rates greater than 5.0, increases in the dose rate resulted in increases in metmyoglobin production.

Visible darkening of the sample surface was observed at all dose rates used, with increased darkening at higher rates. It was impossible to distinguish between

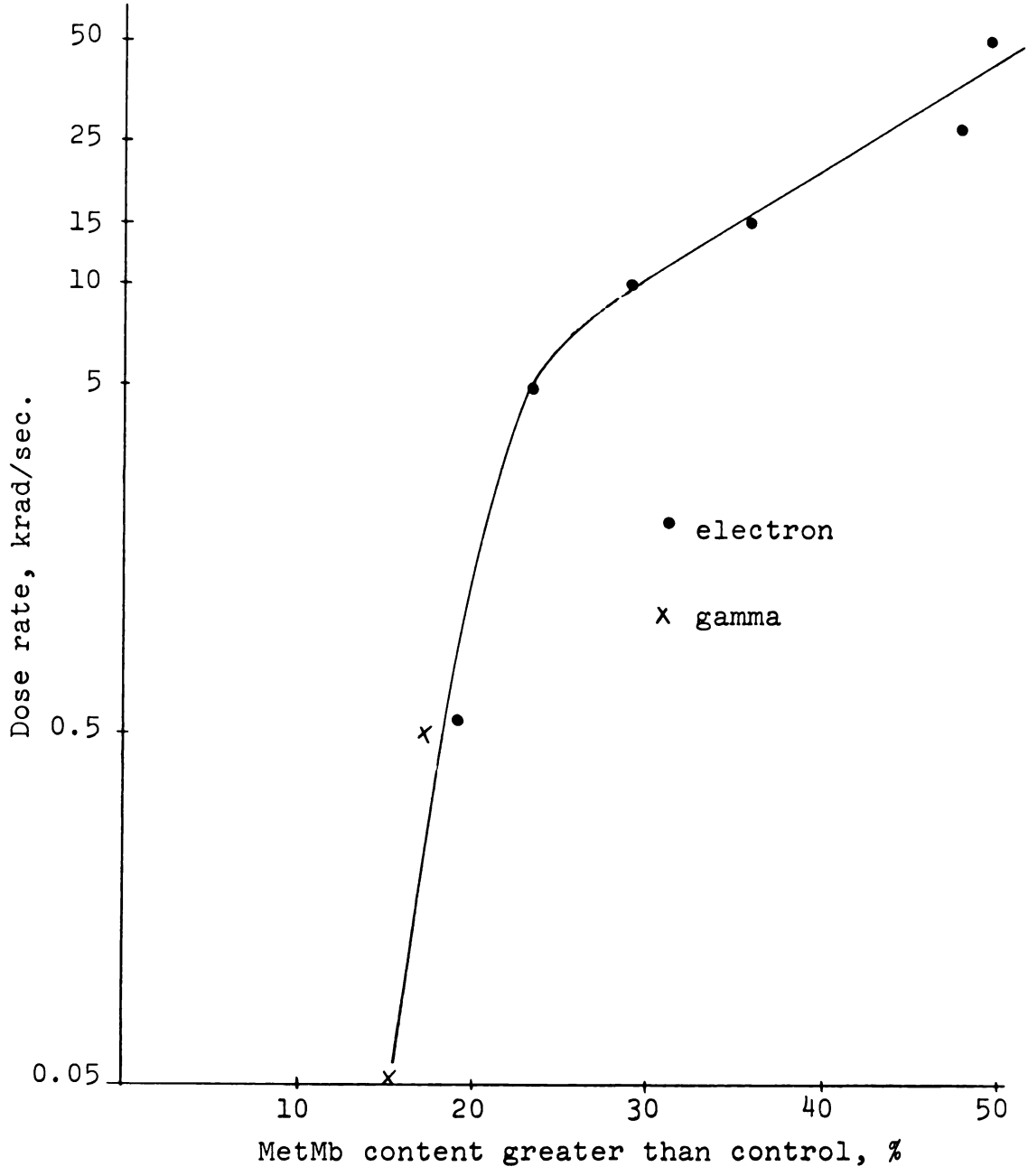


Figure 8. Oxygen irradiation conditions; dose rate vs. the amount of metmyoglobin greater than in the control.

the samples irradiated with the two lowest electron rates and the gamma rates. The irradiation appeared to cause the conversion of oxymyoglobin to both myoglobin and metmyoglobin, but the conversion to myoglobin was not as great as with the two other irradiation conditions. The amount of oxymyoglobin which remained increased almost directly with a decrease in dose rate.

In summary, a meat surface darkening could be observed under certain conditions of pasteurizing doses of irradiation. The darkening, as determined by reflectance spectrophotometry, was due to the conversion of bright red oxymyoglobin pigment to either purple myoglobin or brown metmyoglobin. The conversion, especially to the oxidized pigment form, was very dependent on the conditions at the time of irradiation. The effect was most noticeable with irradiation under oxygen pressure, and least noticeable when conditions of vacuum existed during irradiation. Oxidized pigment formation also appeared to be dependent on the dose rate used. Based on visual observations, this effect was minimal when irradiation was carried out with vacuum packaged beef, was generally noticeable at higher dose rates with meat having access to air, and was pronounced when a pure oxygen atmosphere was present.

The range of dose rates studied was 0.05 krad/sec with gamma rays to 50 krad/sec with electrons. The two

extremes could probably be considered as normal operational dose rates for the respective sources. Only at the highest dose rate, 50 krad/sec, was there any significant oxidation of pigment with vacuum conditions. Electron radiation at the rates studied resulted in oxidation under atmospheric conditions, but gamma radiation at lower dose rates did not cause any changes. At all the dose rates studied, both types of radiation caused pigment oxidation when oxygen conditions existed. The amount of reduced pigment formed by the irradiation did not seem to follow any pattern. There was a large increase in myoglobin at all dose rates when vacuum conditions were used. At some dose rates with atmospheric conditions there was no net increase in myoglobin. With oxygen conditions, increases always occurred.

In the storage studies, only vacuum and atmospheric irradiation conditions were compared. After 21 days of vacuum storage, the amount of reduced pigment was a significant determinant of the oxygenation ability of the meat. The amount of reduced pigment present after storage was generally much greater when vacuum irradiation conditions were used. The amount of metmyoglobin after reoxygenation was similar for all dose rates under atmospheric irradiation conditions, but under vacuum irradiation conditions, the 50 krad/sec rate resulted in slightly greater amounts than did the other rates.

Although the reflectance measurements after reoxygenation of all atmospheric irradiation samples were similar, visual observations indicated that the samples treated with higher dose rates were often unacceptable. Only the highest dose rate with vacuum irradiated samples had enough metmyoglobin to be often judged unacceptable.

There appeared to be little difference in surface effects between gamma and electron irradiation at the dose rates used except for dose rate differences. During storage, the initial visible difference disappeared and a fairly uniform color resulted. This generally occurred within a week after irradiation.

The processes which occur during the irradiation of fresh meat might be thought of in terms of a biological Fricke dosimeter. As described by Taimuty (1962), the Fricke dosimeter is an aqueous solution used to measure the dose from an irradiation source. It is a chemical method based on the radiation-induced oxidation of ferrous ion (Fe^{+2}) in a 0.4 M sulfuric acid solution. Ionizing radiation causes the oxidation of the divalent iron to the trivalent form by a process highly dependent upon the presence of oxygen (Pikaev, 1967). The ferrous iron is primarily oxidized by the hydroxyl radical and hydrogen peroxide produced by the ionization of water:

$$Fe^{+2} + OH \longrightarrow Fe^{+3} + OH^-$$

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH + OH^-$$

When oxygen is present in the solution, the conversion to Fe^{+3} increases due to the reaction:

$$H + O_2 \longrightarrow HO_2$$

which, at low pH values, can oxidize Fe^{+2}:

$$Fe^{+2} + HO_2 + H^+ \longrightarrow Fe^{+3} + H_2O_2$$

The dose range in which air-saturated ferrous sulfate solutions can be used with precision extends from 4 to 40 krad with the upper limit set by oxygen depletion (Taimuty, 1962). The yield of the Fricke dosimeter is independent of dose rate up to about 2×10^3 krad/sec (Schuler and Allen, 1956). Above this rate, care is needed to avoid depletion of oxygen and ferrous ion.

Similar mechanisms could be involved in the irradiation of fresh meat. Fresh meat is about 70% water and provides an adequate source of the initial irradiation radicals, such as H and OH. There exists a single ferrous iron per molecule of reduced myoglobin and oxygen atoms are available either from oxymyoglobin or from atmospheric oxygen present in the package system. The medium, however, is not likely to be as acidic as the Fricke dosimetry system. Meat dipped in sodium tripolyphosphate would have a surface pH of up to 6.2 units.

The oxygenation or appearance of bright red color in meat is dependent upon the equilibrium established between myoglobin and oxymyoglobin:

$$Mb^{+2}O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Mb^{+2} + O_2$$

As proposed by Brown and Mebine (1969), oxidized metmyoglobin is formed from the reduced, unoxygenated myoglobin:

$$Mb^{+2} + O_2 \xrightarrow{k_3} Mb^{+3}$$

George and Stratman (1952b) found that the rate of oxidation is greatest when the partial pressure of oxygen is 1.0 to 1.4 mm of Hg at 30°C. Since the partial pressure for half-saturation is also 1.0 to 1.3 mm of Hg at 30°C, the formation of Mb^{+3} is most rapid when the amounts of Mb^{+2} and $Mb^{+2}O_2$ are equal.

The radiolysis of water creates a number of products, including hydrogen atoms and hydroxyl radicals:

$$H_2O \longrightarrow H + OH$$

Within the meat system, the reducing hydrogen atom can combine with oxygen from oxymyoglobin, if present, creating the hydroperoxyl radical:

$$H + O_2 \longrightarrow HO_2$$

This causes the oxygenation reaction to proceed to the right, as written above. Because of the reduction in the partial pressure of oxygen, the oxidation of myoglobin

to metmyoglobin is promoted. The hydroxyl radical, available oxygen, and perhaps the hydroperoxyl radical all can act as oxidants in this reaction. As the partial pressure of oxygen decreases below 1.0 mm Hg, the indirect dependence of metmyoglobin formation on oxymyoglobin is eliminated and the oxidation of myoglobin proceeds primarily by the OH radical and H_2O_2.

Oxidation would be counteracted or balanced by reductions promoted by the hydrogen atom and possibly the hydrated electron, e^-_{aq}, both major secondary products of the radiolysis of water (Pikaev, 1967):

$$Fe^{+3} + H \longrightarrow Fe^{+2} + H^+$$

At the pH of the meat system there is also the possibility that the hydroperoxyl radical will cause a reduction of Mb^{+3} (Siu, 1957a):

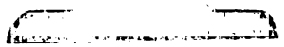
$$HO_2 + Fe^{+3} \longrightarrow Fe^{+2} + O_2 + H^+$$

In addition to the effects of radiolysis products on the ferric ion, the metmyoglobin reducing enzyme system also acts to reduce the metmyoglobin formed (Stewart, et al., 1965b). At the relatively low dose used in this research, the enzyme system is not likely to be inactivated significantly by the radiation (Siu, 1957b).

Within any one of the three irradiation conditions we used, identical factors that might promote a change in the pigment form existed. The amount of ferrous ion and

oxygen and the total dose or energy applied were similar at the beginning of irradiation. The difference was the length of time the dose was applied. An explanation for the immediate dose rate effect in fresh meat could be based on the diffusion of both radiation-produced radicals and oxygen combined with the activity of the metmyoglobin reducing enzymes of the system. As the dose rate increased, the amount of time available for enzyme action or for intermediate substrates to be formed was decreased. In most cases, the visual effects of the differences in dose rate did not seem to be permanent.

In vacuum irradiation conditions, available oxygen was a limiting factor in the oxidation of myoglobin to metmyoglobin. Oxymyoglobin and entrapped pockets of air were the chief sources of oxygen in vacuum packaged fresh meats. The formation of the hydroperoxyl radical would deplete this available oxygen and, in combination with the oxidation of myoglobin to metmyoglobin, would shift the equilibrium of the oxygenation reaction towards greater formation of myoglobin and oxygen. When all the oxygen was used, the oxygen-independent oxidation would be negated by reductions due to reducing radicals and the reducing enzyme system. The net result would be the conversion of oxymyoglobin to myoglobin, some of which would be oxidized to metmyoglobin and then subsequently reduced back to myoglobin. Our results indicated a



small net increase in the amount of metmyoglobin at the highest dose rate.

As with vacuum irradiation conditions, the effects with atmospheric and oxygen irradiation conditions were a balance between oxidation induced by the oxidizing radiation products and reduction by reducing products plus the reducing enzymes present in the meat. However, there was no limitation on oxygen availability except that due to the diffusion rate of oxygen into the meat structure. The dose rate effect, as noted by the decrease in oxygenated pigment and the increase in oxidized pigment, could be a localized effect due to inadequate time for oxygen diffusion. With very high dose rates, there would be a rapid shift in the oxygenation reaction towards $Mt.^{+2}$ and O_2 formation as the hydrogen atoms removed the oxygen molecules from the system. Diffusion of oxygen to that particular location would be insufficient to maintain the initial concentration. The drop in partial pressure of oxygen combined with the high concentration of oxidizing radicals would promote the formation of metmyoglobin. As the length of time for the dose application increased, the diffusion of oxygen would result in a gradual increase in the partial pressure of oxygen and shift the equilibrium towards oxymyoglobin.

The differences observed between the atmospheric and oxygen conditions during irradiation could be

explained by the differences in the quantity of oxygen available for diffusion in either case. Hannan (1954) similarly found that when the oxygen availability increased within a given set of irradiation conditions, the net amount of metmyoglobin increased. Oxygen irradiation conditions resulted in more net metmyoglobin than did atmospheric conditions at a similar dose rate.

Our results indicate that conversion to metmyoglobin was greater if the pressure of oxygen exceeded 760 mm, as in the oxygen irradiation conditions, than if it was about 150 mm, as in the atmospheric conditions. However, Laser (1955) found that following irradiation with X-ray doses ranging from 0.8 to 75 krad and with air and nitrogen atmospheres, solutions of oxyhemoglobin were oxidized to methemoglobin independent of oxygen pressure.

The activity of the metmyoglobin reducing enzymes appeared to be the most significant factor in the pigment conversions following 21 days of vacuum storage. In nearly all the situations studied, there was little visual carry-over of the dose rate effect. However, the highest dose rate applied to the vacuum irradiated samples resulted visually and spectrophotometrically in more metmyoglobin after reoxygenation, and when applied to atmospheric irradiated samples, resulted visually but not spectrophotometrically in more metmyoglobin after

reoxygenation. These observations imply that greater damage was done to the myoglobin molecule or to the reducing enzyme system at higher dose rates. However, no definite explanation can be given for the decreased ability of the meat to reoxygenate after 21 days storage following irradiation at the higher dose rates studied. No literature source could be found that discussed a related situation.

Hansen et al. (1965) and others have studied the effects of very high irradiation intensities on high-protein foods. The radicals produced by ionizing radiation reacted with the solute or recombined with each other, depending on their relative concentrations. If the energy input was fast enough, the electron spurs or tracks along which ionization took place began to overlap, and radicals could recombine. As referred to by Hansen et al. (1965), Taimuty calculated that such dose rate effects were unlikely to be produced in meat at rates less than 10^7 or 10^8 krad/sec. Neither study was, however, concerned with pigment oxidation.

In contrast to the work of Hansen et al. (1965) and others who reported decreased flavor changes at higher dose rates, i.e. 10^7 krad/sec as compared to 10^2 krad/sec, we found an increase in oxidation of meat pigment at higher dose rates, i.e. 5 x 10 as compared to 5 x 10^{-2} krad/sec. There appears to be very little

relationship between the dose rate effect on pigment found in this study and the effects of very high dose rates on flavor reported in the literature. In the dose range of 5×10^{-2} krad/sec to 5 x 10 krad/sec, no previous report of the effect of dose rate on foods has been found.

A possible reason for the immediate, short term dose rate effect observed in meat pigments was outlined. However, the apparent permanent increase in the percentage of oxidized beef pigment with higher dose rates remains unexplained.

Effect of Phosphates

It has been demonstrated that a phosphate dip designed to reduce watery exudation from the vacuum-held, irradiated beef samples was also beneficial to the formation of a bright red color on reoxygenation. Irradiated fresh beef slices not treated with sodium tripolyphosphate or hexametaphosphate generally began to brown after 14 days of vacuum storage (Urbain et al., 1968). It could be assumed that the high pH of the phosphate solutions used increased the pH of the meat sufficiently to result in a pH nearer the optimum of the metmyoglobin reducing enzymes (Stewart et al., 1965b). However, Saleh et al. (1967) stated that substrate availability, not the enzyme, was probably the critical factor. Enzyme action may not, however, be the

entire explanation for the observed effects. Reports of previous studies of the effects of phosphate on fresh meat color which investigate other possibilities have not been found.

In preliminary experiments, fresh beef slices were dipped in phosphate solutions with a phosphate concentration approximately equal to that of a 10% solution of sodium tripolyphosphate. A comparison of the resulting amounts of each pigment form, as measured spectrophotometrically, is given in Table 6. Both ground sample and meat surface pH measurements taken for each sample were similar. The samples dipped in dibasic sodium phosphate were bright red following reoxygenation. Those treated with sodium tripolyphosphate were a faded red, and the remainder were brown in appearance and unacceptable. This experiment indicated the necessity for a phosphate dip of relatively high pH.

Results of experiments designed to compare the usefulness of some chemicals and buffer systems of high pH are listed in Table 7. All solutions were from pH 9.0 to 9.2, but only the dip solutions containing phosphate resulted in favorable reoxygenation color. Although by spectrophotometric determination, the other solutions resulted in less metmyoglobin formation than the control, they were not visibly superior. Initially, the carbonate and Tris-HCl treated samples were as

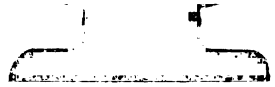


TABLE 6.--Dip solutions with phosphate ion concentrations similar to a 10% sodium tripolyphosphate solution.

Treatment	Solution pH	Pigment form			Final pH of meat	
		%Mb	$\%MbO_2$	%MetMb	ground	surface
Control	5.6 (beef)	22	11	67	5.4	5.5
NaTPP	9.1	6	68	26	5.85	5.9
H_3PO_4	1.5	13	11	76	4.6	4.7
NaH_2PO_4	4.5	2	17	81	5.5	5.5
Na_2HPO_4	9.2	0	80	20	6.2	6.1

TABLE 7.--Comparison of dip solutions with pH values from 9.0 to 9.2; pigment form and meat pH after oxygenation.

Treatment	Pigment form			Final pH of meat	
	%Mb	$\%MbO_2$	%MetMb	ground	surface
Control	20	30	50	5.6	5.6
NaTPP	19	70	11	5.9	5.85
NaOH	20	47	33	5.65	5.65
NH_4OH	23	33	44	5.65	5.65
Carbonate	22	45	33	5.7	5.75
Tris-HCl	24.5	44	31.5	5.75	5.75
Na_2HPO_4	26.5	73.5	0	6.15	6.1

reduced in appearance as the phosphate treated samples, but they began to show increased oxidation after about 2 weeks of vacuum storage. The dibasic sodium phosphate treated samples often had a "dark cutter" appearance after a day of reoxygenation. The control was the most oxidized sample.

Another test, as reported in Table 8, compared borate and carbonate buffers with dibasic sodium phosphate, sodium tripolyphosphate, and Kena, a commercial blend of phosphates. Samples containing phosphate were again superior in color after reoxygenation. The borate buffer dip was superior to the carbonate buffer and the control, but did not give as bright red a color on reoxygenation as phosphate dip. The results obtained with dibasic sodium phosphate were inconsistent. Visually the samples dipped in dibasic sodium phosphate often appeared too purple after reoxygenation, similar to the "dark cutter" appearance of high pH beef. In a few cases, the beneficial effect of the dibasic sodium phosphate seemed to disappear before 21 days and the vacuum-packaged samples would begin to oxidize. The dibasic sodium phosphate seemed to be faster-acting than the sodium tripolyphosphate, which probably had to be hydrolysed by tripolyphosphatases before it could affect the color. This would agree with Yasui et al. (1964a,b) who reported that

TABLE 8.--Comparison of dip solutions with pH values from 9.0 to 9.2; pigment form after oxygenation.

Treatment	Pigment form		
	%Mb	$\%MbO_2$	%MetMb
Control	29	49	22
NaTPP	5	87	8
Carbonate	23	49	28
Borate	5	72	23
Na_2HPO_4	16	84	0
"Kena"	19	81	0

tripolyphosphate must be hydrolysed to a simpler form to be effective in water binding.

Reflectance spectra of beef samples treated with phosphate were compared with spectra of non-treated controls. Spectral peaks at 544 and 582 nm for oxymyoglobin and at 555 nm for myoglobin were similar in both cases. No other shifts were observed in the visible spectrum. These results suggest that it is unlikely that any association of phosphate and myoglobin is sufficiently permanent to result in changes in the visible spectrum.

A series of experiments was conducted to investigate the mechanism of phosphate action. Beef samples were treated with sodium tripolyphosphate following various times of vacuum storage. Beef slices were removed from

vacuum, dipped and drained of excess phosphate, and when necessary, repackaged to be opened 21 days after cutting. The spectrophotometric results shown in Table 9 generally correlated well with visual observations. The control was brown and would not reoxygenate sufficiently, while the samples dipped immediately in phosphate developed a bright red color after exposure to oxygen. Samples treated with phosphate at both one and two weeks also reoxygenated to an acceptable red color. The samples which were dipped in phosphate after two weeks storage had begun to brown, but at 21 days before reoxygenation, they showed an increase in purple color, indicating a reduction of met-myoglobin due to the phosphate treatment. The samples dipped after three weeks vacuum storage would not reoxygenate, and were similar to the control. The results could mean that the phosphate was acting as a substrate or an intermediate for the reducing enzyme system. This system appears to require vacuum conditions, as the treatment at 21 days without a subsequent vacuum storage did not improve the color beyond that of the control.

Unpurified myoglobin extracts were obtained from beef slices dipped in sodium tripolyphosphate and compared with extracts from non-treated controls. Some samples from phosphate dipped beef were dialysed for limited periods of time against deionized distilled water. Results of tests using these crude myoglobin preparations

TABLE 9.--Comparison of sodium tripolyphosphate application times; pigment forms after oxygenation.

Treatment	Pigment form		
	%Mb	$\%MbO_2$	%MetMb
Control	13	52	35
NaTPP at 0 day	8	82	10
NaTPP at 7 days	13	75	12
NaTPP at 14 days	25	75	0
NaTPP at 21 days	15	50	35

were generally not conclusive (Table 10). The time required for the oxidation of the crude extracts was greater than that required for purified myoglobin (Table 11) as was shown by Brown and Dolev (1962). The control initially had a greater amount of oxidized pigment than the treated or treated-dialysed samples. However, after one day the control had a decrease in metmyoglobin concentration while the other two had an increase, with the result that the control had less metmyoglobin after the four day storage period. The treated and treated-dialysed samples were similar in autoxidation characteristics. This could indicate that if the phosphate was the reason for the changes in oxidation rate, some association might exist between phosphate and myoglobin that was not altered significantly by dialysis.

TABLE 10.--Unpurified myoglobin preparations; pigment oxidation with time.

Time	Percentage of Metmyoglobin		
	Control	NaTPP treated	treated, dialysed
1 hour	34	22	22
2 hour	33	24	--
5 hour	--	--	26
8 hour	30	34	25
1 day	11	25	--
2 day	10	26	22
4 day	20	33	30

TABLE 11.--Effect of pH and phosphate on the oxidation rate of myoglobin; time vs. % oxidized pigment.

pH	Time (minutes)	Percentage of oxidized pigment			
		Control	Molar ratio of phosphate to myoglobin		
			1:10	1:1	10:1
5.6	15	25.5	9.5	9.0	8.5
	60	46	30	28.5	31
	120	74.5	52.5	52.5	55
	150	86	63	63	65
6.1	15	17	9.5	8	9.5
	60	23.5	20.5	19.5	20
	120	40.5	37	35.5	34.5
	150	47	44	43	44
6.5	15	6.5	9	8	8
	60	15.5	19	17.5	18.5
	120	24.5	27.5	27	27
	150	29	32.5	31.5	31.5
7.0	15	4	4	5	2.5
	60	8	10	11	8.5
	120	15.5	16	16	13.5
	150	17	18	18.5	15.5

The effects of phosphate and pH on oxidation rates of purified oxymyoglobin solutions are shown in Table 11. These solutions had not been treated with the reducing agent, sodium hydrosulfite. The results are similar to previous findings, and indicate that as the pH was lowered, oxidation rates increased (Brown and Mebine, 1969; Yamazaki et al., 1964; George and Stratmann, 1952b). The comparison of oxidation rates between the control and phosphate treated samples indicated that a definite decrease occurred at pH 5.6 and a minor, but noticeable decrease at pH 6.1. However, at pH 6.4 and 7.0 the oxidation rates of the control and the treated samples were similar.

At pH values of 5.6 to 5.9, Brown and Mebine (1969) found the presence of the organic phosphate, 2,3-diphosphoglycerate, to substantially increase oxidation rates. Benesch and Benesch (1967) and Chanutin and Curnish (1967) demonstrated that 2,3-diphosphoglycerate, other organic phosphates, and some inorganic phosphates, including sodium tripolyphosphate, reduced the oxygen affinity of hemoglobin. Brown and Mebine explained their findings with the fact that the 2,3-diphosphoglycerate increases oxidation by influencing k_1 and k_2 in their proposed mechanism for oxidation:

$$Mb^{+2}O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Mb^{+2} + O_2$$

$$H^+ + Mb^{+2} + \tfrac{1}{4}O_2 \longrightarrow Mb^{+3} + \tfrac{1}{2}H_2O$$

The influence of phosphates on oxidation as reported in Table 11 can be explained by the influence of the phosphate on k_3 using the same mechanism. The oxidation of Mb^{+2} by oxygen might be slowed by phosphate. This could be due to an association of phosphate with myoglobin.

Gillespie et al. (1966) speculated that phosphate could conceivably associate with the distal histidine of myoglobin. Stryer et al. (1966) found by X-ray diffraction studies what they assumed to be a sulfate group attached to the distal histidine. However, they observed this in metmyoglobin and emphasized that crystals of metmyoglobin may have different activities than metmyoglobin in solution. If the phosphate combines with the distal histidine, the heme should be directly influenced by any phosphate addition up to one phosphate per myoglobin. As shown in Table 11, phosphate ratios of 1:10, 1:1, and 10:1 did not result in differences in the oxidation rates. Brown and Mebine (1969) compared ratios of 2:1, 10:1, and 20:1 diphosphoglycerate to myoglobin. They found increased oxidation rates at higher ratios, which would be difficult to explain on the basis of a phosphate interaction with only the distal histidine.

The organic phosphate 2,3-diphosphoglycerate is not, of course, similar in structure to a phosphate ion, and might interact quite differently with the myoglobin molecule.

That some association does exist between inorganic phosphates and myoglobin can be shown with radioactive phosphate (Table 12). Small quantities of $Na_2H^{32}PO_4$ were added to myoglobin solutions, held overnight at 3°C, and then applied to a Sephadex G-75 column. As shown by the table, some radioactive phosphate was eluted from the column with the myoglobin fraction. If it was not bound, because of its small size, the phosphate ion should have been held back on the column and separated from the heavier myoglobin. This experiment was a qualitative indication of association, and could not be used to determine the interaction ratio or its specificity.

A dialysis method also gave indications that an association existed (Table 13). Again, the experiments were qualitative and did not attempt to show any interaction ratio. The molarity of the phosphate was very low, and the number of molecules of phosphate was much lower than the number of molecules of myoglobin. Figure 9 shows that near-equilibrium was reached within two days of dialysis. The amount of radioactive phosphate remaining with the myoglobin was a fairly small percentage of the total after one day of dialysis and decreased very little in an extra day. The count given by the

TABLE 12.--Separation of radioactive phosphate from myoglobin on a Sephadex column; fraction location vs. the count of $Na_2H^{32}PO_4$.

Fraction location		Count/minute (0.1 ml aliquot)
First red color out of the column	5 ml	30
	5 ml	32
	5 ml	36
bright red color	5 ml	39
	5 ml	43
	5 ml	43
buffer after Mb	300 ml	28

TABLE 13.--Separation of radioactive phosphate from myoglobin by dialysis; time vs. count of $Na_2H^{32}PO_4$.

Time (hours after beginning of dialysis)	Count/minute (0.1 ml aliquot)
16	50
20	31
21	36
22	33
28	24
41	13
46	10
52	9
52 (with myoglobin)	1.14×10^4 (2% of added phosphate)

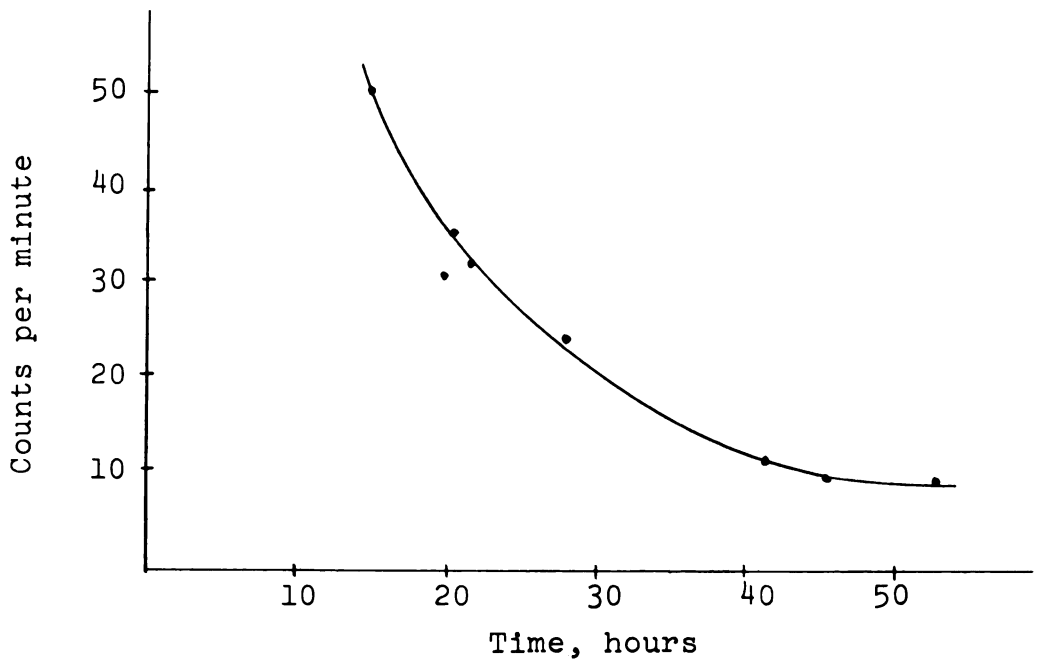


Figure 9. The dialysis to equilibrium of radioactive phosphate from a myoglobin solution.

radioactive phosphate in the myoglobin at the time dialysis was stopped indicated strong association.

Attempts to quantify the possible ratio of association were not successful (Table 14). If, as theorized, there was a 1:1 association, no radioactive phosphate should have been dialysed from the samples with 1:10, 1:2, and 1:1 ratios of phosphate to myoglobin. The counts for 2:1, 5:1, and 10:1 should have been proportional to the amount of phosphate in excess of myoglobin, i.e. equivalent to 4, 8, and 9 ml of added phosphate respectively. However, as shown in Table 14, radioactive phosphate was dialyzed from all the samples, and thus, under the conditions of the test, it would seem unlikely that a 1:1 association existed that could withstand the forces exerted by dialysis. The test sample counts were almost linear with quantity of radioactive phosphate added, and indicated a definite retention of phosphate when myoglobin was present. This suggested, as did the results from the previous tests, that some form of association occurred between phosphate and myoglobin.

Gillespie et al. (1966) studied the effect of cupric ions on metmyoglobin stability to denaturation. They found that incompletely deionized preparations containing phosphate were far more stable than completely deionized preparations. They reported that an apparent mole ratio of 0.5 to 1.5 phosphate to protein was retained in

TABLE 14.--Association of radioactive phosphate with myoglobin; count of radioisotope in dialysis water vs. molar ratios of phosphate to myoglobin.

Condition	Molar ratio of phosphate to myoglobin					
	1:10	1:2	1:1	2:1	5:1	10:1
Quantities of added $^{32}PO_4$, ml	1	4	5	8	10	10
Quantities of Mb added, ml	10	8	5	4	2	1
Average cpm after 24 hour dialysis	455	1870	2315	3450	4605	4495
Count/minute from control (no Mb present), 24 hour dialysis	675	--	2460	--	--	5295
Theoretical count for the control (100% dialysis)	630	--	3150	--	--	6300

Phosphate buffer, pH 6.4,
Mb, 2×10^{-4} M
Radioactive phosphate tracer, 1 in 250
Sample aliquots, 0.2 ml

dialysis experiments. Limited results indicated that increasing the molar ratio of phosphate to myoglobin to 10:1 also caused an increase in the time required for cupric ion to denature metmyoglobin. Our experiment did not show a quantitative association between phosphate and oxymyoglobin; however, it differed from the study of Gillespie in that oxidation of pigment, not denaturation, was studied.

CONCLUSIONS

Both visual and spectrophotometric results indicate that immediate discoloration of meat surface pigment takes place during certain conditions of irradiation. The effect is dose-rate dependent, and results from the conversion of oxymyoglobin to metmyoglobin and in some cases to myoglobin. Except at the highest electron intensities studied, the dose rate effect does not seem to alter the ability of vacuum held beef slices to reoxygenate after 21 days storage.

The results of the studies with phosphate indicate that some association of phosphate and myoglobin seems to exist. Oxidation is slowed by phosphate in both whole beef slices and purified myoglobin solutions under specific conditions. Some form of interaction or association between phosphate and myoglobin can be shown by using the radioactive isotope $^{32}PO_4$; however, this association does not seem to be 1:1 for the conditions used in this study.

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