THE ROLE OF MICROORGANISMS IN THE REDUCTION AND OXIDATION OF MEAT PIGMENTS

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THE ROLE OF PHOSPHATIDES IN THE REDUCTION AND OXIDATION OF MEAT PIGMENTS

By

Donald Lewis Hobach

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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ABSTRACT

Meat color is due to myoglobin, an iron porphyrin pigment. In the reduced form, the iron of the heme is in the ferrous state, and the color is purple. Upon exposure to oxygen the pigment becomes oxygenated, but the iron remains in the ferrous state and the color is bright red. Under certain conditions myoglobin may be oxidized to metmyoglobin, a brown pigment. The iron portion of this compound is in the ferric state. The problem of discoloration then involves the loss of oxygen to form reduced myoglobin (purple) and oxidation to form metmyoglobin (brown).

Various investigators have shown that bacteria are instrumental in the discoloration of fresh beef; however, the exact method or methods involved are not known.

This investigation was an attempt to elucidate the actual role of microorganisms in pigment changes.

Initial studies of a number of species of bacteria and of a yeast showed that only those organisms which metabolize aerobically (possess the Kreb cycle enzymes) would bring about the oxidation of myoglobin to metmyoglobin. Further, only those aerobic organisms which were able to metabolize at low temperatures were shown to cause this reaction at refrigeration temperatures.

Plate counts and manometric studies with homogenates of tissue slices from the surface of steaks showed a direct correlation between numbers of microorganisms, oxygen uptake rate, and color changes. Various bacteriostatic agents applied to steak inhibited bacterial growth and resulted in lower oxygen uptake rates and prolonged color retention; however, the same correlation between the three variables was again noted upon extended storage.

Cell-free extracts prepared by sonic oscillation of heavy cell suspensions of Pseudomonas aeruginosa and Pseudomonas fluorescens were found to bring about the oxidation of myoglobin to metmyoglobin on meat surfaces, and any substance inhibiting oxygen uptake by these enzymes also inhibited pigment oxidation.

Tissue removed from beef muscle and placed in reduced oxygen atmospheres using sterile techniques underwent pigment oxidation at oxygen tensions from 6 mm to 20 mm (measured by a mercury manometer) without the presence of bacteria. In an all N_2 atmosphere only reduction occurred. At higher O_2 tensions, oxidation occurred only after bacterial contamination was evident. These data along with experiments involving glucose oxidase, peroxidase, and specific enzyme inhibitors led to the conclusion that the role of bacteria in meat pigment changes is simply that of lowering the dissolved oxygen level in the surface tissue. The level may be reduced

to the point where natural changes in the meat cause pigment oxidation, and further limitation results in pigment reduction.

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INTRODUCTION

The discoloration of fresh prepackaged beef items is of great economic importance. Voegeli (1952) has shown that almost 26% of the prepackaged meat was removed from self-service cases due to discoloration. This results in financial losses due to the labor of reworking the product which terminates in cheaper cuts as well as weight losses. Ball *et el.* (1957) stated that shelf life of prepackaged fresh meat is only slightly over 48 hours and that the product seldom has a satisfactory appearance for as long as 72 hours. To lengthen the period of marketability, color retention must be prolonged.

Discoloration is called "loss of bloom" by the trade. This means that the meat loses its bright red appearance. Meat color is due to myoglobin which is a pigment resembling hemoglobin in that it is an iron porphyrin, and the heme prothetic group is attached to a globin protein fraction. In the reduced form, the iron of the heme is in the ferrous state and its color is purple. Upon exposure to unlimited oxygen the iron remains in the ferrous state but the pigment is oxygenated. This pigment called oxymyoglobin is bright red. The heme portion of myoglobin may be oxidized by various methods to metmyoglobin, a brown pigment. The iron portion of this compound is in the ferric state. The problem of discoloration or "loss of bloom" involves the loss of oxygen to form reduced

myoglobin (purple) and oxidation to metmyoglobin (brown).

There are three factors to consider when one thinks of discoloration of meats. The factors are physical, chemical, and biological. Physical factors include oxygen tension, temperature, and humidity.

The maximum rate of reduction of myoglobin occurs in the absence of oxygen, but Brooks (1933) found that maximum oxidation occurs at an oxygen pressure of about 4 mm Hg. at 0°C. He further found that as the temperature increased, the rate of the above reactions also increased. Landrock and Wallace (1955) have observed that packaging materials which were coated to prevent excessive loss of moisture resulted in color preservation.

Chemical factors affecting pigment changes include hydrogen ion concentration, antioxidants (ascorbic acid), reducing agents, and oxidizing agents. At a pH of 5.5 and above the surface pigment of meat becomes darkened while at a pH between 4.5 and 5.4 the pigment is a lighter red color. Various authors have observed that ascorbic acid in dilute solutions may preserve "bloom," but in high concentrations it brings about oxidation and discoloration. It is known that reducing agents such as sodium dithionate ($Na_2S_2O_4$) reduces all myoglobin derivatives to reduced myoglobin, and oxidizing agents, such as potassium ferricyanide ($K_3Fe(CN)_6$) oxidize all myoglobin derivatives to metmyoglobin.

A third group of factors which must be considered is the biological. This includes the active meat enzymes and the effects of bacteria and their enzymes. From the results obtained by various authors it appears that in fresh meat the full complement of glycolytic enzymes are present in active form as well as the Kreb cycle enzymes. There is little doubt that at least some of these enzymes are important in color changes. Several investigators have suspected that bacteria and bacterial enzymes influence the color of fresh meats; however, the mechanism(s) involved is not understood. Organisms belonging to the Pseudomonas-Achromobacter group have been associated with fresh beef discoloration and Butler et al. (1953) indicated that these organisms may bring about discoloration of fresh prepackaged beef by lowering the available oxygen to a critical pressure where the formation of metmyoglobin is optimum. The metabolic formation of H_2O_2 by organisms without immediate destruction has also been postulated as causing metmyoglobin formation. It was thought that respiring organisms cause discoloration of fresh prepackaged beef by lowering the oxygen tension; however, other systems may also be active.

The present study was undertaken to determine the actual role of bacteria in the discoloration of fresh prepackaged beef.

REVIEW OF LITERATURE

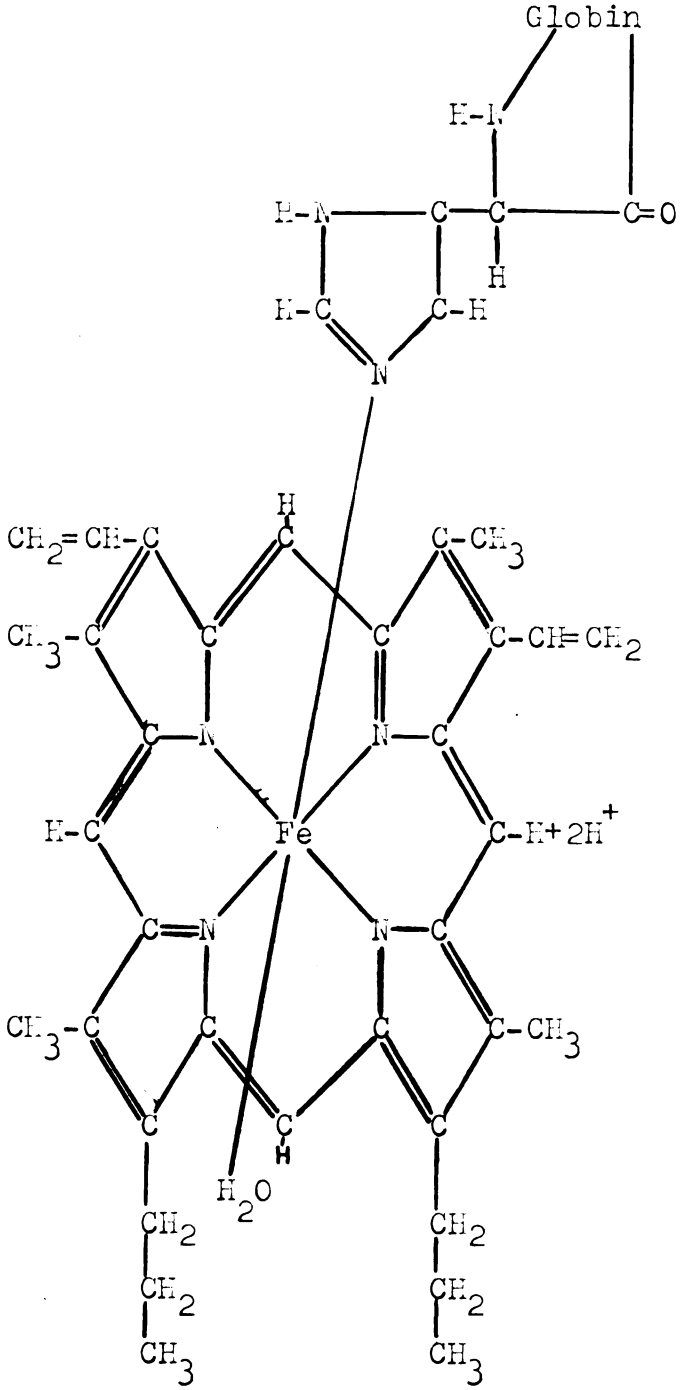
A knowledge of the chemistry of the muscle pigment myoglobin is essential to understand the effects of various factors on the color of meat. The color of prepackaged beef is due to the chemical state of pigment myoglobin. Many interesting theories as to the true character of this pigment have been set forth. The first theory was that the red color of meat was due to blood and could be washed away (Boerhave, 1739). Bichart (1803) agreed, but believed that the muscle obtained its color from the blood which was deposited in the tissue rather than from circulating blood. In the latter part of the nineteenth century much work was done, but it was not until the early part of the twentieth century that Gunther (1921) suggested the term myoglobin for muscle pigments rather than hemoglobin. He was thoroughly convinced that muscle pigment was not identical with the blood pigment hemoglobin.

Whipple (1926) employing many extraction techniques, estimated the myoglobin content of various muscle tissue. More comprehensive analysis of the occurrence of myoglobin and its chemical characteristics occurred during the next few years. It was the contributions of Theorell (1932, 1934) which gave the researchers new grounds for continued investigation, for he succeeded in crystallizing myoglobin. Schenk *et al.* (1934) working with beef rib eye muscle found no parallelism between the myoglobin content and hemoglobin

content. In more recent years, research has made available a more clear understanding of the chemical nature of this pigment and the reactions it undergoes in the muscle.

Chemistry of Myoglobin

Myoglobin, the principal pigment of red meats, is a chromoprotein belonging to the group of hemo-proteins. The prosthetic group is protoporphrin, and the protein fraction is globin. The prophyrin family includes two other very important members, chlorophyll and hemoglobin, both essential for life. Lemberg and Legge (1949) wrote a book dealing with this family of compounds and cited 3,182 references. The prosthetic group of myoglobin, protophorphrin, consists of four pyrrole rings which are coupled to a central iron atom through the nitrogen atoms. This then is an iron porphyrin, and it is the iron atom which makes possible its function as a respiratory pigment; i.e., it can form a dissociable compound with oxygen, the iron remaining in the ferrous state. According to Kendrew (1949), the four valences of the iron atom are connected with the nitrogen of each of the pyrrole rings, and the other two seem to be bound to the protein component. However, according to Schwiegert (1956), the fifth valence of the iron molecule is attached to the imidazole ring of histidine (Figure 1). This ring is bound to the other four pyrrole rings through the iron molecule. The protein



Heme Structure of Myoglobin
Figure
Schweigert, B.S., 1956. Proc. of the Eighth Research Conference, Univ. of Chicago, Chicago, Illinois, P. 61-64

fraction is attached to histidine and not to the iron molecule. The sixth valence of the iron molecule is satisfied by a water molecule, and this seems to be the active site of the heme structures.

Hemoglobin, the blood oxygen carrier of mammals, has a molecular weight of 66,000 - 68,000 according to Haurowitz (1950), and consists of four "units" of the proto-heme-globin complex. He observed myoglobin to have a molecular weight of 17,000 and consists essentially of one of the "units" of hemoglobin, except that the globin or protein fractions differ.

Myoglobin was isolated, crystallized, and characterized by Theorell (1932), and he offered absolute proof that hemoglobin and myoglobin are two different pigments. This proved that Kennedy and Whipple (1926) were wrong when they indicated the pigments were identical compounds. Theorell (1932, 1934, and 1947) elaborated the chemistry of myoglobin showing among other things that the iron content of myoglobin and hemoglobin was identical, 0.345%.

The spectrophotometric studies of Hill (1933) demonstrated that the sharpest absorption band for myoglobin was 5800 Å while hemoglobin was 5700 Å. Under more precise conditions Hill (1936) found the absorption bands for hemoglobin were sharpest at 5770 Å and 5420 Å, compared to 5815 Å and 5446 Å for myoglobin, and that myoglobin had a greater affinity for oxygen than did hemoglobin. He also noted that myoglobin

gave a hyperbolic oxygen dissociation curve compared to a sigmoid curve for hemoglobin. Millikan (1939) substantiated the oxygen dissociation curves of the two pigments as found by Hill, and related the hyperbolic shape of the curve to the physiological oxygen storing function of myoglobin.

Shenk, Hall, and King (1934) noted that myoglobin gave maximum absorption at 582 mμ. This was substaniated by Bowen (1949) and others. Bowen (1949) also observed that myoglobin gave a second absorption peak at 544 mμ. The work of Bowen appeared to be the best of all the literature reviewed on spectrophotometric analysis of myoglobin, so his data for extinction coefficients are used in this study for determinations of percent myoglobin and percent metmyoglobin. Austin and Drabkin (1935) published similar information for hemoglobin. Mangel (1951) in her study of myoglobin used the procedures of Austin and Drabkin.

The advantages of using metmyoglobin cyanide as a reference standard for spectrophotometric analysis in preference to gasometric techniques was demonstrated by Drabkin and Austin (1935). They gave evidence that the reagents used for conversion of the pigments to the cyanide derivative had no significant effect on the absorption spectra of the derivatives at the critical wave lengths.

Generally, there is some blood residue in meats, but Shenk et al. (1934) found that 90% of the meat pigments is usually myoglobin, and hemoglobin was not usually over 10%. They also set up a method for estimating the percentage of these two pigments in a muscle extract by use of the ratio of the optical density measured at wave lengths of 577 mμ and 582 mμ.

Drabkin et al. (1950) studying the distribution of myoglobin concluded that the concentration of myoglobin is not correlated with body size but is more concentrated in the muscles of animals that run fast or work hard such as the dog or the horse. Lawrie (1950) found that a high concentration of myoglobin is usually found in muscles of high physiological activity. Using the figures of Drabkin et al. (1950), Butler et al (1953) calculated the concentration of myoglobin in the skeletal muscles of the heifer to be about 0.40 to 0.47%.

A physical structure for myoglobin was proposed by Kendrew (1949). He states that it consists of two disks, 9 Å thick and 57 Å diameter, parallel to each other and perpendicular to their axis, separated by a layer of liquid of crystallization 6.6 Å thick. Each of the disks is formed by one polypeptide chain, folded on itself in four equal sections. The prosthetic group is perpendicular to the plane of each disk and extends above and below it. The thickness at either point is about 15 Å greater than the disk

itself. He obtained a sedimentation constant for myoglobin of 2.0×10^{-13} in Svedberg's ultra centrifuge.

Myoglobin was first found to have a molecular weight of 3200 by Theorell (1932). However, other workers have obtained different molecular weights for myoglobin. Bowen (1948) in his review states that workers have found the molecular weight of myoglobin to be from 16,850 by osmotic pressure to 17,600 by the rule of simple multiples. He found the molecular weight of myoglobin to be 17,300 and have an average iron content of 0.323%. Rossi-Fanelli (1950) states that the iron content is 0.34%. The isoelectric point of myoglobin was found to be at pH 6.99 by both authors.

The composition of the globin fraction of myoglobin of different species of animals has been analyzed by Rossi-Fanelli (1940, 1941, 1942, 1947, 1948, 1954, 1955 and 1956). He noted that myoglobin has isoleucine but no cystine as contrasted with hemoglobin which contains cystine but no isoleucine. Comparing human myoglobin and hemoglobin, (1955), he noted that myoglobin is richer in glutamic acid, lysine, glycine, and methionine; poorer in threonine, alanine, valine, and arginine. In general, the data showed that the proteins are formed by a significantly different mixture of amino acids.

It was observed by Rossi-Fanelli (1950) that myoglobin forms a hyperbolic dissociation curve which is only slightly affected by pH. The pigment has a large affinity for oxygen and is very easily oxidized to metmyoglobin, and it demonstrates a large resistance to denaturation by alkali. He

also states that hemoglobin has an isoelectric point of 6.78, a molecular weight of 68,000, forms a sigmoid dissociation curve which is moderately affected by pH, has a moderate affinity for oxygen, less easily oxidized to the "met" form than is myoglobin, and has only a small resistance to denaturation by alkali. Thus, the physiochemical constants of the two pigments differ also.

Physiological Role of Myoglobin

The basic facts as to the physiological function of myoglobin were presented by Theorell (1934) and Hill (1933, 1936). Millikan (1936, 1939) and Biorick (1949) have elaborated these basic facts. Summarizing their findings, it appears that myoglobin becomes oxygenated at the expense of the oxyhemoglobin in the peripheral capillaries. The myoglobin as oxymyoglobin transports the oxygen to the cells where it is used in oxidative enzymatic activity. Thus myoglobin acts as an intermediate between the circulatory system and the actively metabolizing cells. The reduced myoglobin may then be reoxygenated to complete the cycle.

Reactions of Myoglobin

Since the reactions of hemoglobin and myoglobin have been found to be similar, the reactions of both will be discussed.

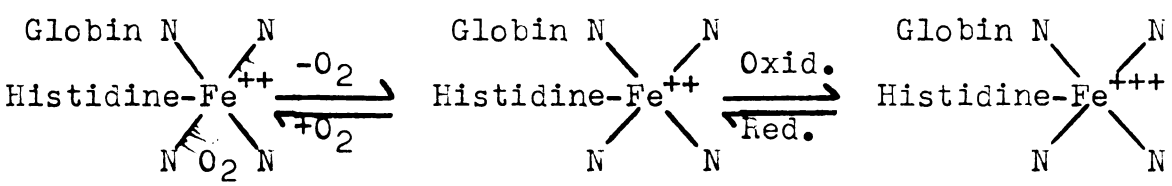
According to Haurowitz *et al.* (1950) hemoglobin is an "aqui-compound" and forms a coordinate bond with water

and not oxygen. He also indicates that oxyhemoglobin does not dissociate into hemoglobin and oxygen in the absence of water and postulates that the following equation demonstrates the true pathway.

$$Hb.(H_2O) + O_2 \longrightarrow HbO_2 + H_2O$$

This may be postulated for myoglobin also.

Much of the early work on the changes of pigments in meat was performed by Brooks (1929, 1931, 1933, 1935, 1936, 1938, 1948, and 1955). He demonstrated that myoglobin was oxidized by oxygen, for in an all nitrogen atmosphere (essentially free of oxygen) the oxidation of myoglobin to metmyoglobin did not occur to any appreciable extent. He postulated the reaction to proceed in the following manner:



Oxymyoglobin (MbO_2) (bright red) Myoglobin (Mb) (purple) Metmyoglobin (Mmb) (brown)

The four nitrogen atoms represent the pyrrole rings which form porphyrin. The protein fraction is connected through histidine to the iron molecule. This reaction is essentially as given by Brooks with formula modifications by Schweigert (1956).

By potentiometric studies, Conant (1923) showed that hemoglobin followed the same pathway. This pathway has met with some disagreement, but the work of George and Stratmann (1952 a,b) tends to validate it. The reduction

of oxymyoglobin to myoglobin or the reverse reaction involves no electron transfer, but the oxidation of myoglobin to metmyoglobin or the opposite reaction involves electron transfer. The iron in myoglobin is in the ferrous state, but the iron in metmyoglobin is in the ferric state.

The Color of Fresh Meat

As stated previously some of the early workers (Boerhave (1739), Bichart (1803)) believed that the red color of meat was derived from the blocd which remained in the tissue. However, since the work of Gunther (1921) it is generally accepted that the color of fresh meat is due to myoglobin. The meat of animals which have been properly bled contains myoglobin (purple), oxymyoglobin (bright red), and metmyoglobin (brown). The color of the meat depends upon the relative amount of each of these three compounds present, which depends upon the storage conditions of the meat (Rickert, 1957 a).

Brooks (1933) and Winkler (1936 b) have observed that the discoloration of fresh meat was caused in general by two factors; viz., desiccation and oxidation of myoglobin to metmyoglobin. The darkening effect due to drying was caused primarily by a concentration of pigments at the surface, with the optical properties of the desiccated tissue perhaps being altered. If the meat is prepackaged and moisture loss is minimized, the reaction which would account for the primary changes in the meat color would then be myoglobin oxidation.

The desired color of fresh prepackaged meat is the bright red of oxygenated myoglobin (MbO_2). Freshly cut meat when first exposed to air is a shade of purple, and the myoglobin is primarily in the reduced state (Mb). Upon exposure to air, oxygenation occurs, and as the myoglobin is changed to oxymyoglobin, the meat becomes a bright red. Mackintosh and Hall (1936), Allen (1948) and Bratzler (1955) pointed out that oxygenation or "blooming" of meat occurs very rapidly within the first 30 minutes after cutting and is accelerated by reducing the temperature.

Factors Influencing the Color of Fresh Prepackaged Meat

Oxygen tension and temperature: The papers by Brooks (1929, 1931, 1933, 1935, 1938) go to great lengths in an effort to discover and explain the factors which influence the reactions of myoglobin. He noted that both the oxygen tension and temperature are very important factors. Temperature is of importance because as it is increased the reaction rate is increased and also the oxygen becomes less soluble, and consequently the partial pressure of oxygen in the meat decreases. To cite a specific example, Brooks (1931) noted the rate constant for Hb $\longrightarrow$ MHb to be about four times as great at 25°C. as at 15°C. Brooks (1933) demonstrated that at 30°F. oxyhemoglobin dissociates rapidly to hemoglobin when the oxygen pressure drops below 80 mm of mercury. However, at this reduced oxygen tension

the hemoglobin is soon oxidized to methemoglobin. He also observed that metmyoglobin formation proceeds more rapidly at low oxygen tensions. Neill and Hastings (1925 a,b) have obtained similar results using hemoglobin solutions. They observed maximum oxidation to take place at an oxygen tension of about 20 mm of mercury. Brooks (1938) discovered that the maximum rate of oxidation of Hb occurred at an oxygen pressure of about 4 mm Hg. at 0°C. This estimate by Brooks was made by noting the rate of color change in a tissue section by use of a microspectroscope.

George and Stratmann (1952 b) employing a more refined gasometric technique observed that the maximum rate of metmyoglobin formation occurred between 1.0 and 1.4 mm oxygen pressure at 30°C. The fact that the rate of oxidation of myoglobin was 4.25 times faster than that of hemoglobin was also noted by these authors. Brooks (1931) suggested that at a constant oxygen pressure the rate of oxyhemoglobin formation is monomolecular with respect to hemoglobin concentration. Neill (1925 c) noted that no oxidizing agent that he used could oxidize hemoglobin to methemoglobin in the absence of molecular oxygen. Both Brooks (1931) and Neill (1925 d) noted that hemoglobin was not oxidized to methemoglobin in the absence of molecular oxygen. From the data of Brooks (1929, 1931) it is evident that at a temperature of 30°C. and an oxygen pressure of 20 mm Hg, the oxygen concentration is low enough to permit

the dissociation of oxyhemoglobin into hemoglobin and yet high enough for oxidation of hemoglobin to methemoglobin. Neill (1925 d) demonstrated further proof of the effect of oxygen tension on hemoglobin when he found that in a pure oxygen atmosphere the concentration of oxyhemoglobin increased and therefore the formation of methemoglobin was retarded. Mangel (1951) stored frozen meat samples under atmospheres of nitrogen, oxygen, and carbon dioxide, with air as a control. She found no significant difference. However, the metmyoglobin formation tended to be slower when the samples were stored under oxygen.

Brooks (1938) found that muscle retains a residue of respiratory enzymes long after slaughter of the animal, and when exposed to air a steady state is reached where the depth to which the oxygen penetrates is determined by the relative rates of its diffusion and uptake.

The following formula for determining the oxygen penetration has been given by Brooks (1938):

$$d = \sqrt{\frac{2Co\ D}{A}}$$

where d = depth of oxygen penetration
Co = pressure of oxygen at the surface of tissue
D = coefficient of diffusion of oxygen through tissues
A = oxygen consumption

The value of A for the muscle of fresh beef as reported by Brooks (1929, 1936) is roughly 10^{-4} cc per gram per minute at 0°C.

Brooks (1935) found the depth of oxygen penetration increased slowly with time, and it decreases with increasing temperature. He found that the depth of oxygen penetration varied from 2 to 5 mm. He also showed that discoloration was confined to the layers where oxygen was present, and metmyoglobin was formed rapidly in the inner surface of the oxymyoglobin layer where oxygen pressure was lowest. The myoglobin in the interior portion of the muscle remained in the reduced form, unchanged. It was estimated by Brooks (1938) that the surface color was affected by about a 2 mm layer of tissue, for no light was reflected from layers deeper than this.

The effect of temperature and vacuum on fresh meat was tested by Rickert et al. (1957 c). They found that meat stored under a vacuum of 20 inches or more underwent an initial loss of redness and returned to redness more rapidly than meat stored under a vacuum of less than 20 inches. Increasing the vacuum under constant storage temperature or increasing the storage temperature while maintaining a constant vacuum increased the rate of initial darkening and shortened the time for the subsequent return to redness. They state that vacuum appears to be necessary for the return to redness, but not for the initial darkening. A lower vacuum resulted in the formation of a gray color which turned red upon increased vacuum.

Rickert et al. (1958) working with atmospheres containing various amounts of O_2 and N_2, noted that increased N_2 caused an increased rate of discoloration of fresh beef. However, the return to redness was faster than at lower N_2 tensions and higher O_2 tensions. Further, they noted that low pressures of CO_2 proved to be more effective in maintaining good color, from both the standpoint of retarding initial discoloration and from the standpoint of holding color thereafter.

Packaging Material: Various wrapping materials were tested by Lavers (1948). He found that as oxygen permeability of the material decreased, metmyoglobin formation increased. He noted that MSAT cellophane, which is oxygen impermeable, caused rapid browning of the meat. MSADT cellophane, which is oxygen permeable, caused less discoloration. The rate of discoloration could be lessened even more by moistening the MSADT cellophane which increases oxygen permeability. He hypothesized that the development of an oxygen permeable transparent sheet which was moisture proof would solve the problem. Landrock and Wallace (1955) performed a detailed study on the relationship of the oxygen permeability of films and discoloration of fresh red meat. They state that cellophanes used for packing fresh meat are unique in that they are coated with a moisture-proof coating on the side to be kept away from the meat. The inner side

then becomes wetted by the meat juice and consequently increases oxygen permeability sufficiently to maintain bloom. The coating prevents excessive loss of moisture, thus, minimizing desiccation. The great oxygen permeability of MSAT 80 cellophane paralleled the increased bright red color of the meat as compared to other less permeable wrapping material. They postulated that the minimum oxygen permeability requirement is about 5000 ml O_2/sq. met./24 hr/atm. at 75°F.

Vinylidene copolymer (cry-o-vac) and MSAT 80 cellophane (coated side out) were found to be superior to all other wraps tested in preserving the flavor and organoleptic quality of fresh meat by Clauss et al. (1957). However, no color observations were recorded.

Reflectance spectrophotometry was employed by Pirko and Ayres (1957) to study the influence of different packaging materials on the discoloration of fresh meat. They observed that films with highest gas permeability (300 MSAT 80-cellophane, polyethylene 0.0015 inches and 80 FM-1 pliofilm) resulted in maximum metmyoglobin formation on the 6th day of storage. As permeability decreased, the rate of metmyoglobin formation increased; however, after one day's storage, the total amount of metmyoglobin was reduced. They consider films of high oxygen permeability to be inferior to films of very low oxygen permeability. It appears that they wish to sacrifice the bright red color of oxymyoglobin for

the purple color of myoglobin to obtain an increased sales life.

Vinylidene copolymer (cry-o-vac) was found to be the best of the various packaging materials used for maintaining good color in lean ground beef by Rickert et al. (1957 a). They determined redness by the Hunter color and color-difference meter. The author doubts if these authors made a distinction between the bright redness of oxymyoglobin and the purple-redness of myoglobin.

Rickert et al. (1958) substantiated the results of Rickert et al. (1957 a). They explain that MSAT 80 cellophane and cellulose acetate both of which have high gas permeability resulted in high redness of the packaged meat for 2 days, then discoloration followed. Steaks wrapped in cellophane-pliofilm laminate film which has low oxygen transmission properties were higher in redness than steaks wrapped in the highly permeable films except for the first few days.

Rickert et al. (1957 a, 1958) and Pirko and Ayres (1957) believe that good color of fresh beef could be maintained by packing it in a completely impermeable film in the absence of oxygen.

Chemicals: The chemistry of the red meat color change having been fairly well characterized, many workers began trying to influence the change. Winkler (1939 b) studied the effects of lowering and raising the pH of meat. He

found that a pH above 5.5 caused darkening and a pH between 4.5 - 5.5 caused lighter colors. This agrees with the findings of Hall, Latscher, and Mackintosh (1944) working with dark cutting beef. However, they believe that the main reason for dark cutters is that the oxygen demand is greater than can be supplied by normal transfer into the tissues, resulting in oxymyoglobin being reduced to myoglobin. The fact that muscle tissue darkens with increased pH was noted by Hill (1928) and Bate-Smith (1948 a,b).

Some chemicals have been shown to effect the oxidation of myoglobin to metmyoglobin. Stilles and Foster (1922), Brooks (1930, 1931, 1936) were among the first to observe that salt increased the rate of oxidation of oxymyoglobin and oxyhemoglobin, and/or myoglobin and hemoglobin to the brown metmyoglobin and methemoglobin respectively.

Ascorbic acid has been shown to bring about the rapid oxidation of hemoglobin solutions (Chang and Watts, 1949) and reduce methemoglobin to hemoglobin (Gibson, 1943). However, Watts and Lehman (1952 a) state that ascorbic acid protects hemoglobin solutions when it is added in low concentrations and at a low temperature. But at high concentrations and high temperatures, it brings about oxidation and discoloration. In another paper (1952 b) they state that ascorbic acid may be useful in preserving the red color at the surface of refrigerated prepackaged meat.

These results have been confirmed by Costilow et al. (1955). Studying the degradation of hemoglobin in living tissue, Garner, Mills and Christman (1951) concluded that ascorbic acid catalyzes the degradation of hemoglobin to choleglobin. This has been substantiated by Lemberg and Legge (1949), Chang and Watts (1949), and Watts and Lehmann (1952). Nicotinic acid has been reported by Coleman (1951) to transform the interior color of meat from purple to a bright red.

Studying the effects of ascorbic acid on meat color, Clauss et al. (1957) noted that when ascorbic acid crystals were added to beef samples they caused an immediate darkening which remained throughout the storage period. The addition of 0.005% solution of ascorbic acid had better color preserving properties than did a 0.01% solution. These results were substantiated by Rickert et al. (1957 b). They also noted that NDGA (Nordehydroguaiaretic acid - 0.05%) had no beneficial effect on surface meat color.

Broumand et al. (1958) in their study of myoglobin reported that sodium dithionite ($Na_2S_2O_4$) reduces all myoglobin derivatives to myoglobin, and potassium ferricyanide ($K_3Fe(CN)_6$) oxidizes all myoglobin derivatives present in meat extracts to metmyoglobin.

Effect of bacteria and their enzymes: The influence of bacteria on the color of fresh meats has been demonstrated by several investigators, but their role in

discoloration has not been clearly defined. Neill (1925 a) and Neill and Hastings (1925) have observed that washed Pneumococcus cells do not have the ability to reduce methemoglobin to hemoglobin unless other substances such as glucose or meat infusion are added to the system, but the cells have oxidizing ability without the addition of these substances. They also noted that cell-free extracts of Pneumococcus had both the ability to oxidize and reduce hemoglobin solutions. Another series of experiments performed by Neill (1925 c) demonstrated that anaerobic bacilli brought about the oxygen dissociation of oxyhemoglobin. Urbain and Greenwood (1940) compared dilute hemoglobin solutions treated with toluene to inhibit bacterial growth to untreated controls. Both sets of tubes held at 10°C were shaken daily for maximum aeration. They noted after 60 days' storage the bacteria-free tubes contained no methemoglobin, but contaminated (control) tubes showed reduced oxygen capacity after two weeks, which was further reduced to about 50% at 60 days. The loss of oxygen capacity coupled with color change of the solution (turned brownish) was proof of methemoglobin formation. The authors believe microorganisms have similar effects on the sensitive heme pigments of meat.

Jensen (1945) was of the opinion that microorganisms both living and dead, as well as their enzymes may oxidize myoglobin to metmyoglobin. He further stated that desiccation intensified this reaction. Allen (1948) stated that

there is a type of meat discoloration where a gray brown color develops when the coloring matter is destroyed by bacteria. Further work by Allen (1949) showed that bacterial growth on prepackaged boneless round steaks held at 34° - 40°F. increased rapidly until the ninth day, after which it remained essentially unchanged.

Further evidence of the oxidation-reduction capabilities of bacteria was presented by Eddy et al. (1952) when they demonstrated that Escherichia coli had the ability to reduce dehydro-ascorbic acid to ascorbic acid. This substantiated the work of Hewitt (1950). Hewitt noted that generally the cultures of bacteria caused reducing conditions which were greatest during the logarithmic growth phase. He noted the lowest O/R potentials when metabolic activity was the greatest. He further explained that once growth is established in a culture, oxygen donators and hydrogen acceptors are taken up by the cells as they carry on their normal metabolic activities. After the log growth phase, metabolic activity decreases and as the oxygen from the air diffuses back into the culture, the O/R potential begins to rise.

Kraft and Ayres (1952) noted that meat samples held at 40°F. generally discolored before any other evidence of spoilage became apparent. They employed surface swabbing techniques and demonstrated that when the count reached about 2×10^6 organisms per square cm. the first detectable

off odor appeared. They concluded that discoloration could not be correlated with organoleptic or microbiological evaluations as a means of determining storage end points.

Initial contamination as well as the temperature of the self-service case have a great influence on storage life of prepackaged meat (Ayres, 1951 a,b). Short steaks could be stored four days longer when the bacterial count was very low. Initial counts on meat cuts prepared in accordance with good sanitary practices had far lower bacterial counts than similar items purchased from local retail outlets. He urged that studies which not only determine the number of organisms present, but also their contributions to its ultimate spoilage, be made.

Achromobacter-Pseudomonas types of organisms were associated with spoilage of fresh beef by Ayres (1951 a). He stated that since the revision of Bergey's Manual, some of the strains previously reported as Achromobacter would be classified as members of the genus Pseudomonas. The majority of strains isolated from spoiled ground beef belonged to this group. At spoilage time in one test, more than 98% of the flora belonged to the Achromobacter-Pseudomonas group. He used off-odors as the principal criterion for spoilage detection, and observed it correlated with slime production and increase in CO_2 production.

The Achromobacter-Pseudomonas type accounted for about 85% of the population during the early storage of meat (first 2 weeks) at 34-38° and 40-50°F. (Halleck et al 1958). During the latter part of storage Pseudomonas fluorescens type organisms constitute approximately 80% of the total count. During the entire storage period lactobacilli accounted for about 5% of the total population.

A slippery condition on poultry due to spore-forming capsulated bacilli closely resembling Bacillus mesentericus was described by Mallman (1932). Sulzbacher (1952 a) compared the generation time for Pseudomonas-type organisms in ground beef and ground pork held at 7°C. A rather heavy inoculum was added to the meat before grinding. He noted the generation time in ground beef to be 4.5 hours and 4.81 and 5.99 hours in ground pork. Kirsch et al. (1952) working with commercial hamburger found initial counts varied from 1.4×10^6 to 9.5×10^6 per gram. Upon subsequent storage (0-2°C.) the organisms multiplied rapidly so that after 6 days' storage 5×10^8 or more organisms were present, and after 8 to 12 days off odors were apparent. Maximum counts ranged from 5×10^8 to 1×10^{10} per gram. In general the off odor was nonputrefactive but typically stale and sour. A taxonomic study of the isolates demonstrated that the great majority belong to the Pseudomonas genus.

It has been reported by Butler et al. (1953) that bacteria commonly found on meat cuts caused discoloration due to increased rate of metmyoglobin formation and caused the production of off odors and slime formation. They state that the changes usually happen in the order mentioned. Shelf life of prepackaged meat was significantly prolonged by lower initial bacterial contamination, and by reducing the storage temperature. They indicated that the organisms (Pseudomonas sp.) may bring about discoloration by lowering the oxygen to a critical pressure where metmyoglobin formation is ideal. The metabolizing organisms lowered the oxygen tension still further which resulted in reducing conditions and the metmyoglobin was reduced to myoglobin.

Myoglobin solutions held at 45°F. were studied by Broumand et al (1958). They noted that nonsterile water extracts contained 100% metmyoglobin within 8 hours while the sterile extract did not become 100% metmyoglobin until about 28 hours. All solutions were in hermetically sealed cuvettes. After 28 hours storage at 48°F. the cuvettes were stored at 95°F. for an additional 148 hours. Within 60 hours the nonsterile cuvette contained all myoglobin, but it took 120 hours for the sterile extract to become entirely reduced. They used spectrophotometric analysis to calculate the amount of various pigments. Their conclusion was similar to that proposed by Butler et al.

(1953); i.e., microorganisms increased the rate of formation of metmyoglobin and upon subsequent holding increase the rate of myoglobin formation.

MEAT ENZYMES

Lemberg and Legge (1949) state that the metabolic formation of H_2O_2 without immediate destruction, leads to metmyoglobin formation. However, Brooks (1938), Lavers (1948) and Urbain (1951) believed that the mere lowering of oxygen tension, without peroxide formation will lead to the same results.

Employing manometric techniques, Schneider and Potter (1943) demonstrated the presence of cytochrome oxidase in beef muscle. They also proved the presence of succinic dehydrogenase by methylene blue reduction. According to Bate-Smith (1942) beef contains about 0.005% cytochrome-C which is about 0.4 micromoles per 100 grams. Grant (1955 a) substantiated the results of Schneider and Potter (1943). He found high alpha-glycerophosphate dehydrogenase activity in steer muscle, but it was absent in cow muscle. Using various enzyme inhibitors in conjunction with frozen ground beef, he noted that only malonic acid of the enzyme inhibitors used provided a bright red interior color. This indicates that succinic dehydrogenase is active in beef. He further found by the use of inhibitors that the following enzymes may show

a limited activity, phosphoglucomutase, phosphatase, enolase, lactic dehydrogenase, malic dehydrogenase, Co-A system and enzymes converting citrate to oxalosuccinate, citrate to malate, alpha-ketoglutarate to succinyl Co-A, and succinyl Co-A to succinate. He presumed L-glutamic acid oxidase, catalase, phosphorylase, and glucose, fructose and formate dehydrogenases to be inactive in frozen ground beef.

In another study Grant (1955 b) inspected the stability of succinoxidase (succinic dehydrogenase plus cytochrome oxidase) in beef tissue. He found that the enzyme is better preserved if the tissue is frozen, is more stable in ground as compared to unground muscle, and is readily destroyed by heat.

Andrews et al. (1952) demonstrated that adenosinetriphosphate, succinic dehydrogenase and glycolytic system enzymes were active in beef muscle, (Longissimus dorsi and Semitendinous). The enzymes within the meat remained active after 4 weeks' storage at 2°C. and only aldolase was found to lose part of its activity. After four weeks its activity was but half that of the original. The aldolase system was shown, therefore, not to be the limiting enzyme in the glycolytic system, since the stabilities varied independently. They concluded that the lack of available substrates in the intact muscle tissue

is the limiting factor in excised muscle tissue metabolism, rather than the instability of specific enzyme systems.

The enzymatic activities of cytochrome oxidase and succinic-oxidase in various excised muscle tissues from a variety of animals were shown by Lawrie (1953). He also found correlation between the percentage of myoglobin in the muscle and the activity of the corresponding enzyme preparation and both were related to the extent of muscle exertion.

PROCEDURE

Steaks used in this study were from the Longissimus dorsi muscle of U. S. Good grade beef ribs. The muscle was removed from the ribs and cut into 1/2 inch thick steaks.

The wrapping material employed was DuPont cellophane 300 MSAT-80 which is for fresh meat packaging.

Cells of Ps. geniculata were grown in nutrient broth shake cultures or on nutrient agar slants. All cells were washed in saline before being inoculated on steak surfaces by the use of sterile atomizers. Washed cells were used for intracellular enzyme preparations also. Cells were broken in a Raytheon, 50 watt, 90 kilocycle sonic oscillator type R-22-3. The suspension was centrifuged to remove cellular debris, and the supernatant was applied to steak surfaces by means of a small brush.

The various enzyme and bacterial inhibitors which were used on the steak surface were applied by sterile atomizers.

Most myoglobin solutions were prepared from Longissimus dorsi muscle; however, some solutions were prepared from beef trimmings. The myoglobin solutions were prepared exactly as the pigment solutions used for spectrophotometric analysis. This procedure which consists of making a water extract of the meat tissue will be discussed in detail later.

Initial studies determining bacterial growth on steak surfaces demonstrated that a surface slicing technique gave more reproducible results than did the conventional swabbing technique. The surface slicing technique consisted of cutting a 3 mm slice from the surface of the experimental steak with a Hobart 50 slicing machine. The machine was cleaned and sterilized before each cutting as follows: washed thoroughly with hot detergent, rinsed with distilled H_2O, followed by hypochlorite (KOCL, 500 ppm), finally rinsed with sterile distilled water, and the rotating blade was allowed to spin dry. Swabbing the machine at various times demonstrated that the portion which came in contact with the meat slice was essentially free from bacteria.

The thin slice from the surface was placed on MSAT 80 wrapping paper, weighed and placed in a sterile, chilled Waring Blender. Refrigerated sterile distilled water was added to give a 1:5 dilution. After blending for 30 seconds, a sample of the homogenate was plated out in tryptone glucose extract agar (TGE) (Difco) and observed for bacterial growth. The rest of the homogenate was used for determining oxygen uptake by the Warburg method and for determining the pigments present by spectrophotometric analysis.

Two incubation temperatures for the TGE agar plates were tested, and it was noted that similar results were

obtained from platings held at 4° C. for one week and at 20° C. for three days. For the sake of convenience the shorter time, higher temperature of incubation was used for all bacterial counts.

Refrigerated meat used in this study was stored in a walk-in cooler at 4° C. (± 1° C). Shelf life was noted to be similar to that obtained in a household type refrigerator.

A sample of the homogenate was prepared for pigment determinations as follows:

a. The suspension was centrifuged in a Servall SPX centrifuge at full speed for 10 minutes.

b. The supernatant was filtered through Whatman No. 40 filter paper.

c. The filtrate was diluted using 7 ml. of filtrate to 3 ml of distilled water.

d. The diluted material was then subjected to spectrophotometric analysis. A Beckman DU with blue filter and a slit width of 0.1 mm was employed.

For some spectrophotometric analysis a Bausch and Lomb spectronic 20 colorimeter was used employing optically matched Thunburg tubes rather than cuvettes. For this procedure the filtrate obtained in b. above was not further diluted.

Two methods of analysis have been tried based on the Lambert-Beers Law which follows:

$$\text{Log } \frac{Io}{I} = A = a\ b\ c \qquad (1)$$

where Io = intensity of the incident light

I = intensity of the emergent light

A = absorbancy or optical density from the spectrophotometer

a = molar extinction coefficient

b = length of light path through the solution in cm.

c = molar concentration

$$c = \frac{A}{a} \text{ when } b = 1.0 \text{ cm} \qquad (2)$$

The first method was essentially that of Butler *et al.* (1953). The optical density was measured in a Beckman Model DU spectrophotometer at wave lengths of 544 and 582 millimicrons. The total pigment concentration was obtained by conversion of the pigments to the CN derivative by adding a drop of 4% solution of potassium ferricyanide and a drop of 1% potassium cyanide to each cuvette after readings were obtained. The resultant metmyoglobin cyanide derivative was measured to 544 millimicrons and the millimolar extinction coefficient of 11.3 given by Bowen (1949) for metmyoglobin cyanide was used to calculate the concentration by the following formula:

$$c = \frac{A}{11.3 \times 10^3} \qquad (3)$$

To estimate the percent metmyoglobin, the difference between the extinction coefficients of oxymyoglobin and

metmyoglobin at 544 and 582 millimicrons was taken as the maximum possible change at these wave lengths. These figures were 9.6 at 544 mμ and 12.1 at 582 mμ as given by Bowen (1949). At each wave length, the change due to metmyoglobin was equal to the molar extinction coefficient (a) of oxymyoglobin minus that of the sample. By dividing the figure obtained by the total change in (A) from oxymyoglobin and metmyoglobin and multiplying by 100, an estimation of the percent myoglobin was obtained. The estimations of the percent metmyoglobin at 544 mμ and 582 mμ were averaged to obtain the final estimate of the percent metmyoglobin.

The second method of analysis used was that of Broumand (1956) which is as follows. According to Bowen (1949) the millimolar extinction coefficient for oxymyoglobin and myoglobin are the same (5.6) at a wave length of 507 mμ while that of metmyoglobin is 9.9 at this wave length. At a wave length of 573 mμ both oxymyoglobin and myoglobin have a millimolar extinction coefficient of 10.6 while that for metmyoglobin at this wave length is 3.0. Theoretically, at these two wave lengths, if the percent metmyoglobin remains constant, so does the millimolar extinction coefficient of the solution; that is, at these wave lengths, the millimolar extinction coefficients of the extract are independent of the relative percent of

myoglobin and oxymyoglobin as long as their combined percentage is constant. Therefore, at 507 mμ, if the millimolar extinction coefficient (E mm) is 9.9, then the extract is 100% metmyoglobin and 0% oxymyoglobin and myoglobin or if at 507 mμ the E mm is 5.6, then the extract is 0% metmyoglobin and 100% oxymyoglobin and myoglobin. If E mm at 573 mμ is 3.0, then the extract contains 100% metmyoglobin and 0% oxymyoglobin and myoglobin or if the E mm is 10.6, then the extract is 0% metmyoglobin and 100% oxymyoglobin and myoglobin. If E mm is greater than 9.9 or less than 5.6, another pigment must be present, but if E mm is equal or greater than 5.6 and equal to or less than 9.9, then the extract contains X% metmyoglobin and 100-X% oxymyoglobin and myoglobin.

A graph for the determination of % metmyoglobin and the % of the combined pigments, oxymyoglobin and myoglobin, can be constructed. Value of $\frac{\text{(a) at 507 mμ}}{\text{(a) at 573 mμ}}$ of a solution containing 100% myoglobin and oxymyoglobin and 0% metmyoglobin is

$$\frac{(100 \times 5.6) + (0 \times 9.9)}{(100 \times 10.6) + (0 \times 3)} = \frac{5.6}{10.6} = .528$$

A solution containing 75% (oxymyoglobin and myoglobin) and 25% metmyoglobin would equal:

$$\frac{\text{(a) at 507 mμ}}{\text{(a) at 573 mμ}} = \frac{(75 \times 5.6) + (25 \times 9.9)}{(75 \times 10.6) + (25 \times 3)} = \frac{6.675}{8.70} = .767$$

50% (oxymyoglobin and myoglobin) and 50% metmyoglobin

$$\frac{(50 \times 5.6) + (50 \times 9.9)}{(50 \times 10.6) + (50 \times 3)} = \frac{7.75}{6.68} = 1.140$$

25% (oxymyoglobin and myoglobin) 75% metmyoglobin

$$\frac{(25 \times 5.6) + (75 \times 9.9)}{(25 \times 10.6) + (75 \times 3.0)} = \frac{8.825}{4.9} = 1.801$$

0% (oxymyoglobin and myoglobin) 100% metmyoglobin

$$\frac{(0 \times 5.6) + (100 \times 9.9) = 9.9}{(0 \times 10.6) + (100 \times 3.0) = 3.0} = 3.3$$

Results of the above calculations for all percentages of metmyoglobin from 0 to 100 can be put into graph form in which $\frac{(a) \text{ at } 507 \text{ m}\mu}{(a) \text{ at } 573 \text{ m}\mu}$ is plotted versus relative concentrations of metmyoglobin and oxymyoglobin plus myoglobin. Thus, by finding the ratio of absorbancy (O.D.) at 507 mμ and 573 mμ $\frac{(\text{O.D. at } 507 \text{ m}\mu)}{(\text{O.D. at } 573 \text{ m}\mu)}$ and referring to the graph, the relative percent of metmyoglobin can be obtained.

Bowen (1949) found that the E mm for metmyoglobin and oxymyoglobin are both 7.6 at a wave length of 473 mμ and the E mm of myoglobin is 4.4; also, the E mm of metmyoglobin and oxymyoglobin are both 3.0 at a wave length of 597 mμ and that of myoglobin is 5.1. By following the procedure as outlined just previously, a second graph can be constructed in which $\frac{(a) \text{ at } 473 \text{ m}\mu}{(a) \text{ at } 597 \text{ m}\mu}$ is plotted versus relative concentrations of myoglobin and metmyoglobin plus oxymyoglobin.

From these two graphs the relative percentages of metmyoglobin and myoglobin can be determined. Presuming not more than 3 pigments are present, the percent of oxymyoglobin can be determined by adding the percent myoglobin and percent metmyoglobin and substracting from 100.

Voegeli (1952) has given detailed instructions for the measurement of the surface color of meats by the use of Munsell spinning disks. The same procedure and similar equipment was used in this study.

The standard color employed for calculations of the index of fading (I) by the formula of Nickerson (1946), as applied by Voegeli (1952) was 7.0 red in hue, 4.0 in value, and 8.0 in chroma. The standard selected for the plotting of color readings of the fresh prepackaged meat was the color of steaks which had been oxygenated under an oxygen pressure of 30 pounds per square inch for one hour. This color was believed by Butler (1953) to approximate closely the color of fully oxygenated myoglobin. It was then possible to find the position of subsequent sample readings in relation to the standard by using the Nickerson (1946) formula. Thus, as the steaks discolor, less pigment is in the oxygenated form, and the index of fading (I) increases in numerical value. Lower values for the index of fading indicates that the steaks have a color more close to the standard steak which was presumed to have its surface pigment fully oxygenated.

RESULTS

The Effects of Microorganisms and Cell-Free Extracts on Meat Pigments

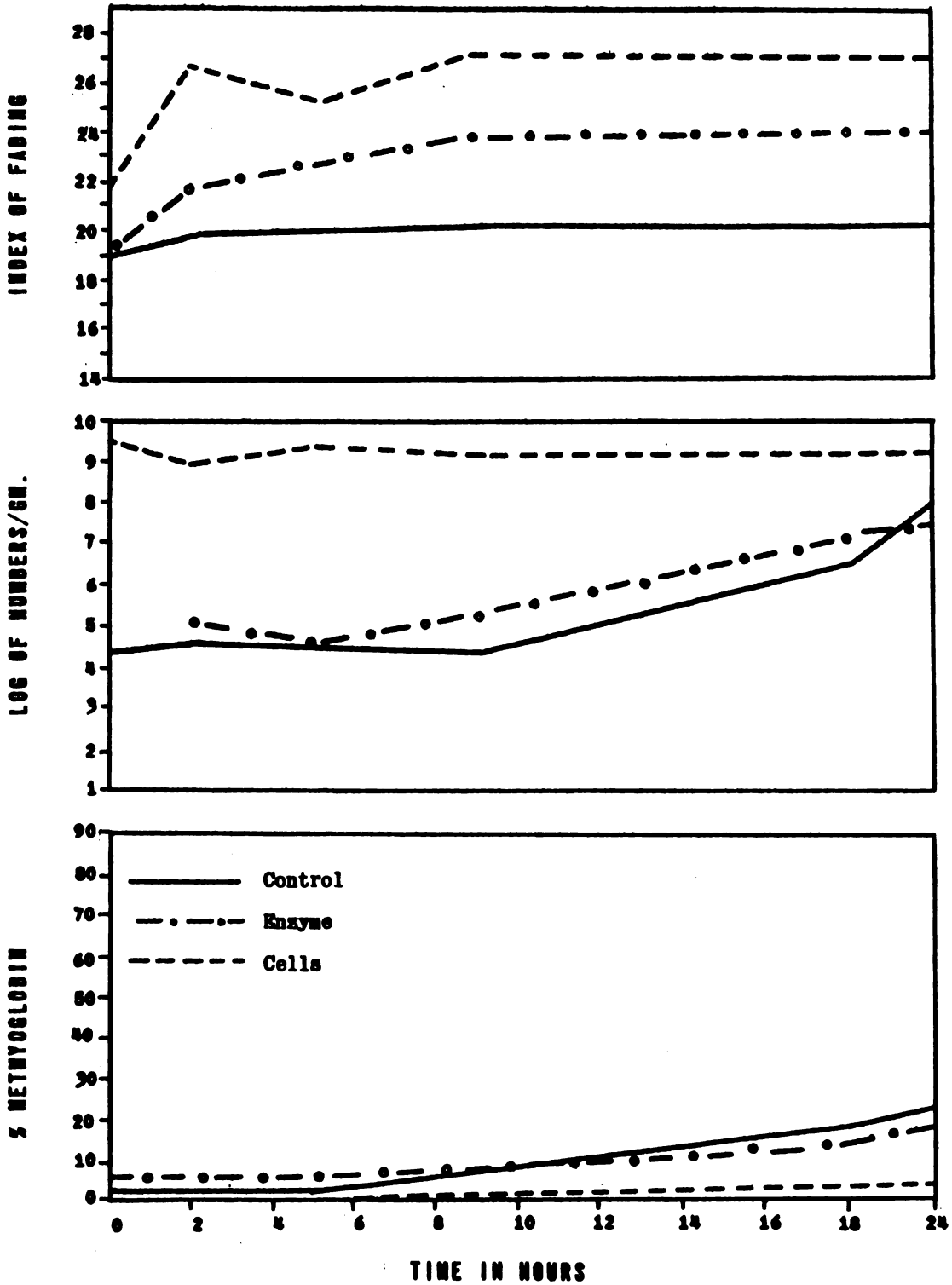
Steak Surface

Pure cultures of *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Archromobacter liquefacians*, *Flavobacterium rhenanus*, *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and two strains of *Pseudomonas geniculata* were tested for their effect on the surface color of wrapped beef steaks at room temperature. All the aerobic strains of bacteria tested, including three untyped strains isolated from discolored meat, caused rapid discoloration as did the one strain of yeast. These bacteria are active in meat discoloration at refrigerated temperatures also. *L. plantarum*, a strain which is microaerophilic and does not contain the Kreb cycle enzyme system, caused no discoloration.

In all subsequent experiments *Ps. geniculata* was used since it was shown to cause discoloration of fresh prepackaged meat by Butler *et al.* (1953). Various levels of inoculum of *Ps. geniculata* were tested for effects on prepackaged meat at 4° C. Data demonstrated that as the bacteria level increased, so did the rate of discoloration. However, the steaks which discolored most rapidly became the purple color of myoglobin with only little evidence of metmyoglobin formation. As the initial level of organisms decreased, so did the rate of discoloration, but these

steaks became brown before turning purple. In all cases the initially bright red steaks became dark red before turning purple or brown.

The effects of intra- and extracellular enzymes of a strain of Ps. geniculata on the pigment of beef steaks were studied. At refrigeration temperature up to nine days there was little or no difference between control steaks and steaks treated with an extracellular enzyme preparation, but those treated with an intracellular enzyme preparation darkened slightly. Initially, the steaks treated with the intracellular enzymes and cells were slightly darker, but at the end of two days only the inoculated steak had discolored completely. A preliminary experiment using the above four treatments conducted at room temperature (23-24°C.) demonstrated that after 15 minutes the inoculated steaks were visually darker, and within one hour those treated with intracellular enzymes were also darker. Both the inoculated steaks and the steaks treated with the intracellular enzyme preparation turned a definite purple after 2 hours. At 24 hours the control steaks and steaks treated with extracellular enzymes began to turn brown. The inoculated steaks remained purple, but the steaks treated with intracellular enzymes were purple-brown at the 24-hour interval. A repeat of this gave similar results. (Fig. 2) Visual observations of steaks held at



EFFECTS OF INTRACELLULAR ENZYMES AND CELLS OF Ps. GENICULATA ON PREPACKAGED BEEF HELD AT ROOM TEMPERATURE

FIGURE 2

30°C. indicated that the changes were similar to those obtained at room temperature.

Cell-free extracts and cells of Ps. aeruginosa also have the ability to cause the discoloration of fresh prepackaged steaks held at room temperature. From the data in Table 1 it can be seen that cells of Ps. aeruginosa cause discoloration at about the same rate as do cells of Ps. geniculata. Cell-free extracts of both organisms bring about discoloration at equal rates but slower than the intact cells.

Attempts were made to determine if Ps. geniculata or a crude preparation of intracellular enzymes of this organism could cause discoloration of unwrapped steaks at room temperature. No color differences were noted after 1 hour. Even after 2 hours, no clear differentiation could be made. However, at 3 hours the inoculated steak was slightly darker than the control or enzyme treated steaks, but darkening due to desiccation was quite advanced and only a slight differentiation could be made. Wrapped steaks which had been inoculated with cells or treated with the same enzyme preparation discolored within 15 to 30 minutes.

Meat Homogenates

Four hundred ml of sterile distilled water was added to 80 grams of meat in a sterile Waring blendor and blended for 1 minute. One hundred ml. of this homogenate

Table I

The effects of Ps. geniculata, Ps. aeruginosa, and a cell-free preparation of the intracellular enzymes of these organisms on the color of fresh prepackaged beef*.

Time in Hours	Control	Ps. geniculata	Ps. aeruginosa	Enzymes of Ps. geniculata	Enzymes of Ps. aeruginosa
0	bright red	bright red	bright red	bright red	bright red
1	bright red	bright red	dark red	slight dark red	slight dark red
2	red	dark red	dark red	dark red	dark red
12	red	purple-brown	purple-red	dark red	dark red
18	red	purple	purple-brown	dark red brown areas	dark red brown areas
24	1/3 red 2/3 brown	purple	purple-brown	3/4 brown 1/4 purple	2/3 brown 1/3 purple

* Experiment was performed at room temperature.

was placed in each of 4 - 500 ml Erlenmeyer flasks and plugged with cotton. Three flasks--inoculated, untreated, and treated with aureomycin (final concentration of 100 ppm)--were placed on a rotary shaker, and the fourth was held as the untreated, unstirred control. Ten ml samples were taken at different intervals, spun down, filtered, and the percent metmyoglobin and percent myoglobin determined spectrophotometrically. At zero time, all samples contained some metmyoglobin, and at 6 hours, samples from the 3 shaken flasks had increased in percent metmyoglobin. The untreated sample which was not shaken was essentially unchanged. No myoglobin could be determined. The percent metmyoglobin in the three flasks which had been shaken increased from about 35% at 6 hours to about 50% at 9 hours. After 2 days' shaking, the control had 15% metmyoglobin and 1% myoglobin, and the inoculated had 24% metmyoglobin and 18% myoglobin, but the sample with aureomycin added contained 90% metmyoglobin and no myoglobin. The standing untreated sample contained 27% metmyoglobin and no myoglobin. Upon further shaking the inoculated sample and control developed a green color typical of choleglobin while the aureomycin-treated sample turned straw color and had developed mold growth. The unstirred untreated meat homogenate developed high populations of bacteria and after 4 days contained 17% metmyoglobin and 34% myoglobin but no choleglobin.

Table 2

Effects of Ps. geniculata and aureomycin on meat homogenates held at 30°C.

Time in Hours	Shaken						Standing	
	Control		Inoculated		Aureomycin		Control	
	% met.	% myo.	% met.	% myo.	% met.	% myo.	% met.	% myo.
0	14	1	15	1	19	4	19	2
6	35	0	35	0	40	0	16	0
9	46	0	51	0	56	0	41	0
48	15	1	24	18	90	0	27	0
Visual observations at 48 hours	green		green		straw colored		red	

From this data (Table 2) it would appear that bacteria did not play an important role in the change of pigments of shaken meat homogenates until after 2 days at which time they began to increase the myoglobin content. Aureomycin seemed to make conditions ideal for the formation of metmyoglobin.

Attempts to measure the oxidation-reduction potentials of inoculated and uninoculated meat homogenates were made. The extracts were placed in cotton plugged flasks into which a platinum electrode and salt bridge were inserted. The salt bridge extended into a beaker containing saturated KCl and the reference electrode. Variance in potential was measured by a galvanometer to which both electrodes were connected. No significant difference between the inoculated and uninoculated extracts was observed because the differences between replications were quite large. However, the Eh of all extracts decrease rather rapidly.

Muscle Pigment Extract

The addition of Ps. geniculata to a solution of oxymyoglobin held at room temperature caused an initial formation of metmyoglobin. However, within five minutes the percent myoglobin was twice that of metmyoglobin, and this ratio existed until all the pigment was a mixture of these two forms. At the end of 3 hours the ratio remained unchanged.

Cell-free enzyme preparations of Ps. geniculata and Ps. aeruginosa were tested for their effects on oxymyoglobin solutions at normal atmosphere and at room temperature (Table 3). It appeared that both enzyme preparations caused an initial increase in metmyoglobin formation; however, it is quite probable that the enzyme preparation absorbed in the range used for measuring metmyoglobin as indicated by the 0-time reading giving false results. The cells probably affected both the range for measuring metmyoglobin and the range for measuring myoglobin for at 0-time an increase in both metmyoglobin and myoglobin was observed. Assuming that after the initial absorption the enzymes and cell no longer interfere, the following observations were made: first, the enzymes of Ps. geniculata showed some action up to 4 hours while the enzymes of Ps. aeruginosa caused a steady increase in both metmyoglobin and myoglobin, and second, the cells of Ps. geniculata reduced oxymyoglobin to myoglobin at a faster rate than did Ps. aeruginosa but demonstrated less ability to oxidize oxymyoglobin to metmyoglobin in a 4-hour interval.

Correlation of O_2 Demand of the Surface Tissue of Meat and Pigment Changes

These studies were undertaken to see if there was a correlation between the activity of the respiratory enzymes present and discoloration. Manometric studies

Table 3

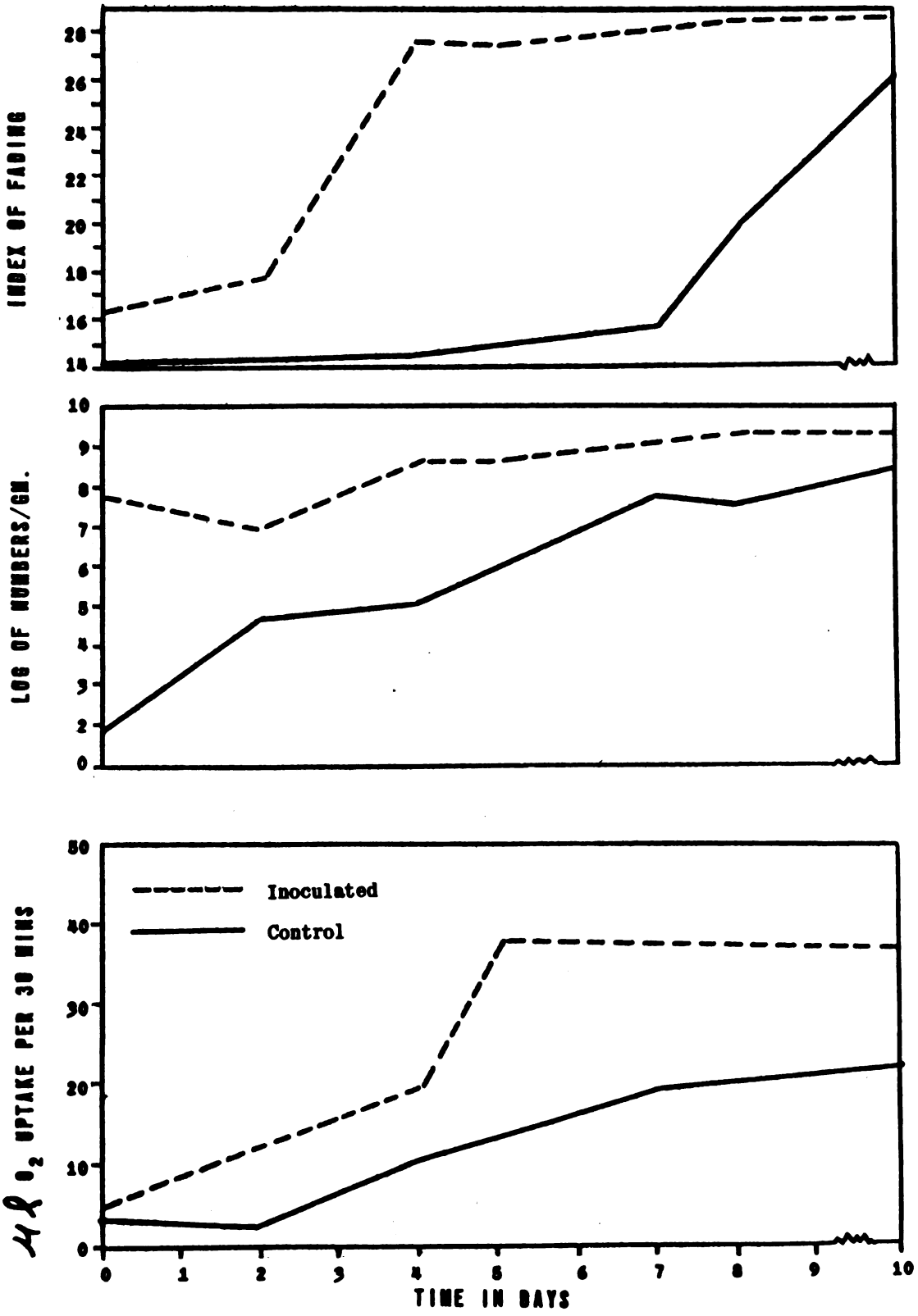
The effects of the intracellular enzymes and cells of Ps. geniculata and Ps. aeruginosa on the rate of pigment change of oxymyoglobin extracts.*

Time in	Control		Ps. geniculata		Ps. aeruginosa		Enzymes of Ps. geniculata		Enzymes of Ps. aeruginosa	
minutes	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.
Before	2	0	0	0	0	0	0	0	1	0
0	0	0	22	25	24	24	16	0	19	0
5	0	0	20	28	24	28	15	0	22	11
10	0	0	21	28	27	26	12	0	22	12
15	0	0	28	64	31	46	14	1	24	12
30	0	0	36	60	39	50	11	0	27	7
45	5	0	35	63	41	49	13	0	25	14
60	3	0	35	65	44	55	13	4	29	17
120	9	0	35	65	44	52	19	7	38	19
180	12	0	35	65	42	51	28	10	41	16

*Oxymyoglobin solutions were held at room temperature

were employed in addition to visual observations, index of fading, and bacterial counts. Various lots of steaks inoculated and uninoculated were tested over a period of 11 days. After visual observations and Munsell disk notations were made, a 3 mm. slice from the surface of each steak was placed in a sterile Waring blendor, diluted 1 to 5 with sterile distilled water and ground for 30 seconds. Two and eight tenths ml. of this homogenate was used to determine µl of O_2 uptake by the conventional Warburg manometric method. Results (Fig. 3) demonstrated that the number of bacteria of the inoculated steaks did not increase greatly, but as the steaks discolored (fading index increased) the µl of O_2 uptake increased. There was a lag in the oxygen uptake by the bacterial cells since even though the counts were high initially, the µl of O_2 uptake was no more than that of the uninoculated control. However, as the steaks discolored, the oxygen uptake rate increased.

A good correlation between the number of bacteria present, rate of discoloration, and oxygen consumption was obtained from the uninoculated group of steaks, i.e. as the bacteria increased in numbers and the oxygen uptake increased, steaks discolored. From these data, it appears that there is good correlation between respiratory activity measured by oxygen uptake and steak discoloration.



EFFECTS OF Ps. GENICULATA ON PREPACKAGED BEEF HELD AT 4°C

FIGURE 3

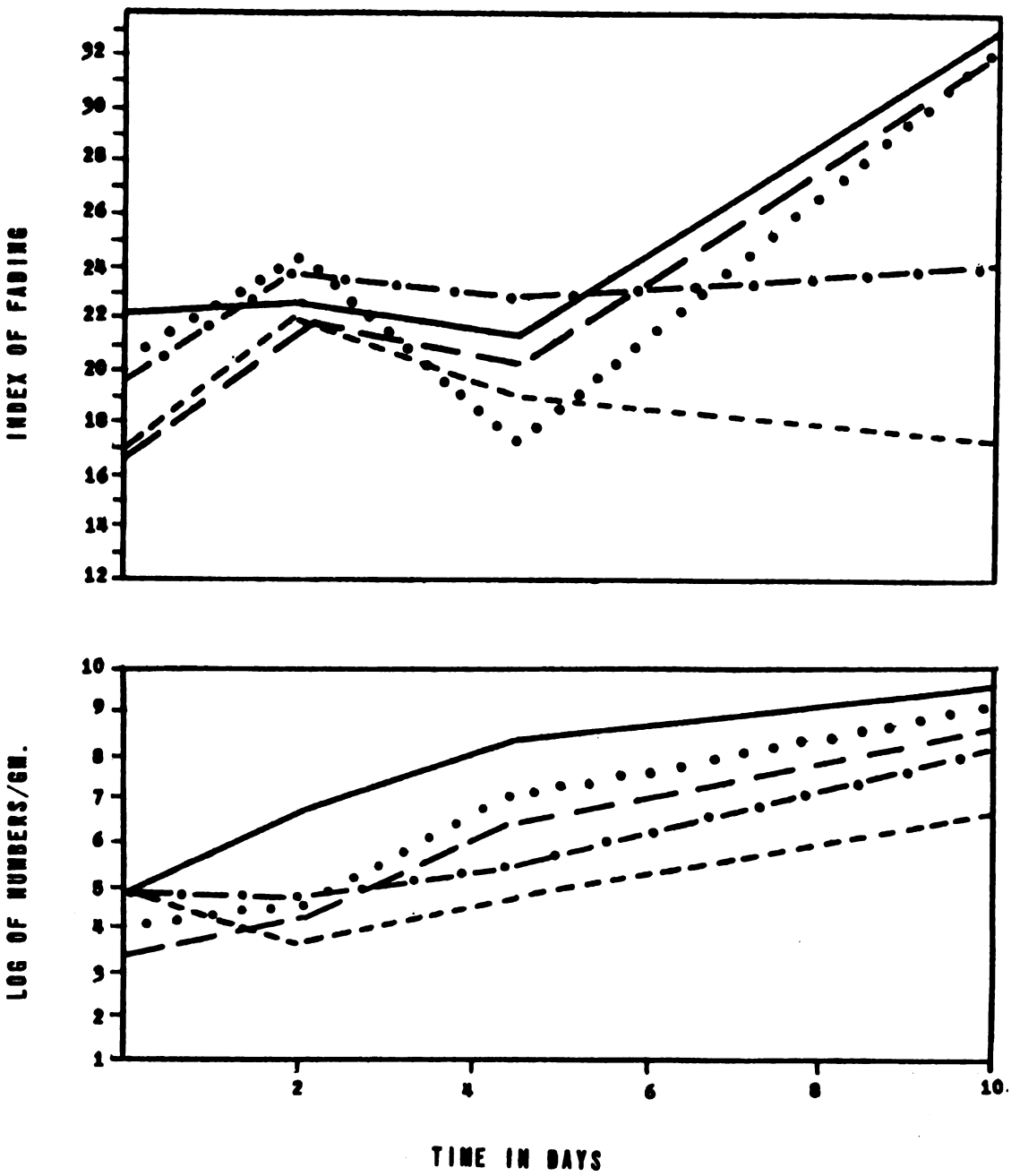
Influence of Respiratory Enzyme Inhibitors on Pigment Changes and Respiratory Activity

Steaks Held at Refrigerated Temperatures

To study the fundamental principles of discoloration of fresh meat by bacteria, various inhibitors were applied to the surface of beef steaks. Various concentrations of aureomycin ranging from 5 to 100 ppm were sprayed on the surfaces of the steaks. From Figure 4 it can be seen that as the concentrations were increased, the bacterial growth was controlled more completely, and the bright red color of oxymyoglobin was retained longer. Further experimentation using aureomycin at 100 ppm demonstrated good control of bacteria as compared to the control and resulted in preservation of a desirable color 3 or 4 days longer than the control (Fig. 5).

One percent solutions of both sorbic acid and sodium sorbate were sprayed on the surface of the beef steaks. Neither form had any effect on color, nor did they control the bacteria. However, preliminary studies using cellophane impregnated with sorbic acid as a wrapping material showed that color retention could be extended somewhat.

Sodium malonate is a competitive inhibitor of succinic dehydrogenase. To test the effect of this respiratory enzyme inhibitor on color retention, a group of steaks was treated with sodium malonate and compared to an



EFFECTS OF VARIOUS LEVELS OF AUREOMYCIN APPLIED TO THE SURFACE OF PREPACKAGED BEEF HELD AT 4°C

——— Control

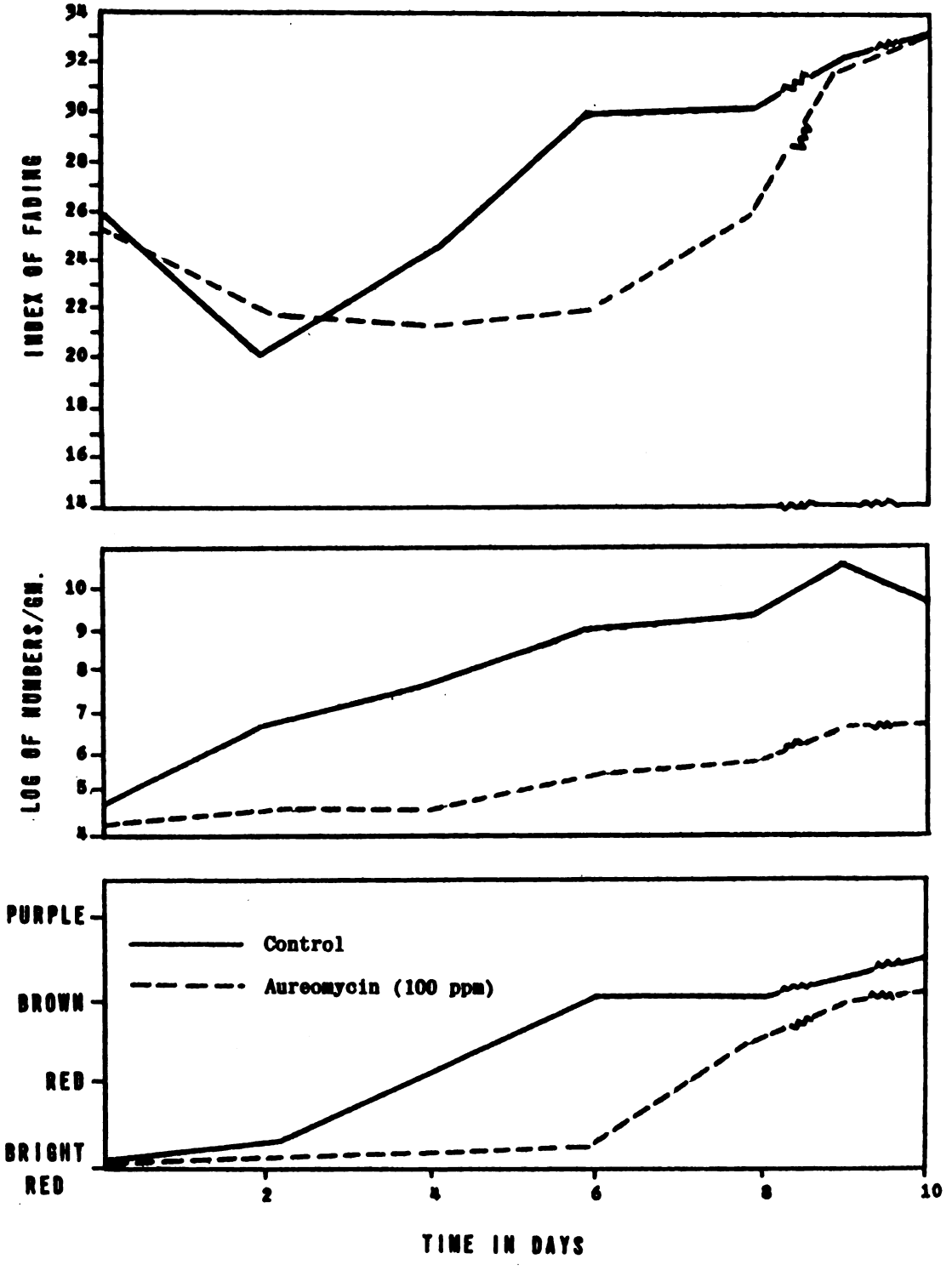
– – – – – – 100 ppm Aureomycin

•—•—•— 50 ppm Aueromycin

— — — 25 ppm Aureomycin

• • • • • • • 5 ppm Aureomycin

FIGURE 4



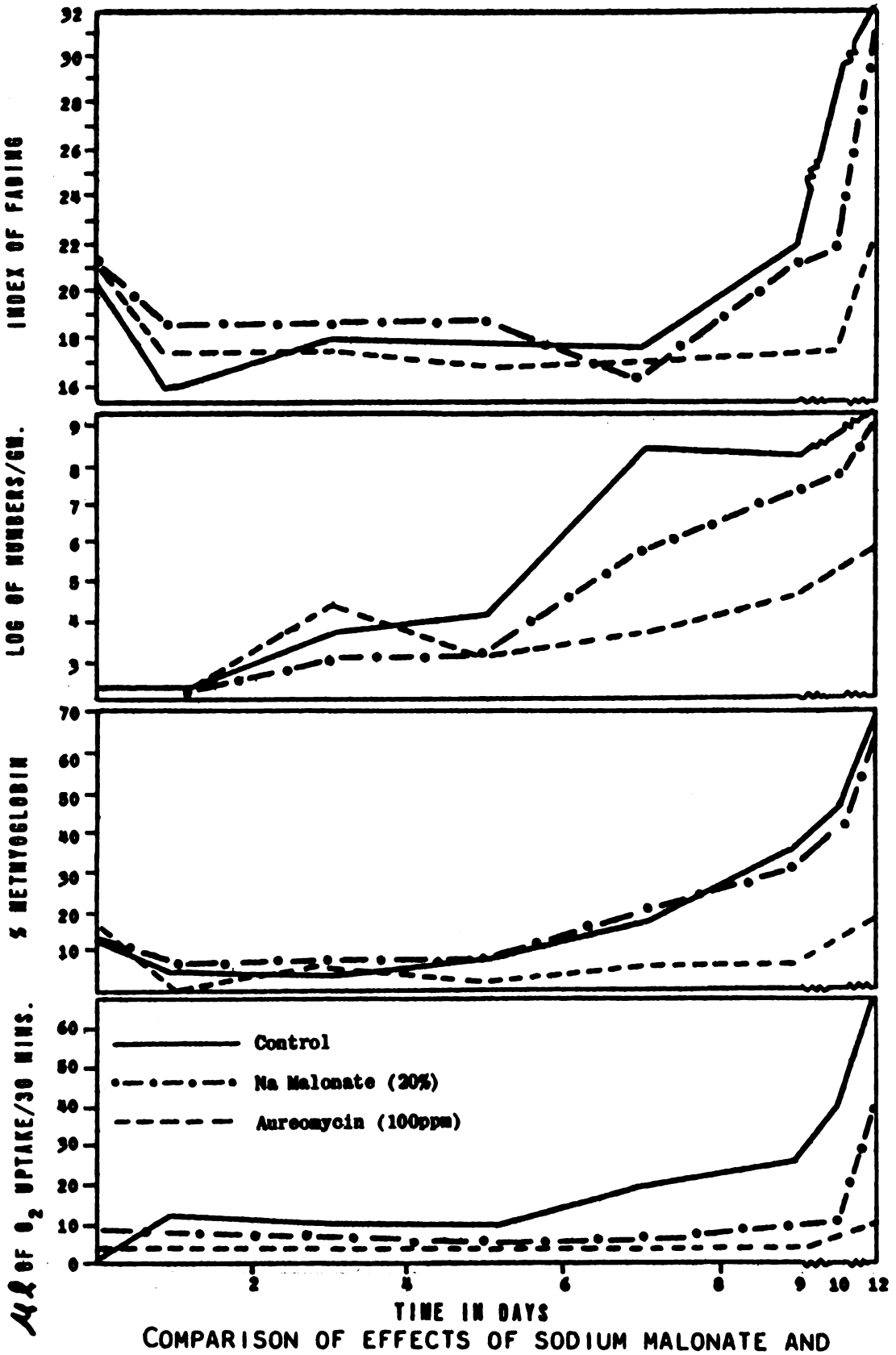
EFFECTS OF AUREOMYCIN ON FRESH PREPACKAGED BEEF HELD AT 4°C

FIGURE 5

aureomycin (100 ppm) treated group and a control group. The steaks treated with aureomycin had low bacteria counts, low metmyoglobin formation, low O_2 uptake, and low index of fading. Sodium malonate demonstrated some bactericidal or bacteriostatic activity, and the index of fading and oxygen uptake were less than the control. However, the percent metmyoglobin formed was practically the same. (Fig. 6). The results illustrated that sodium malonate had some effect on the microflora and color retention of prepackaged beef, but its action was inferior to aureomycin.

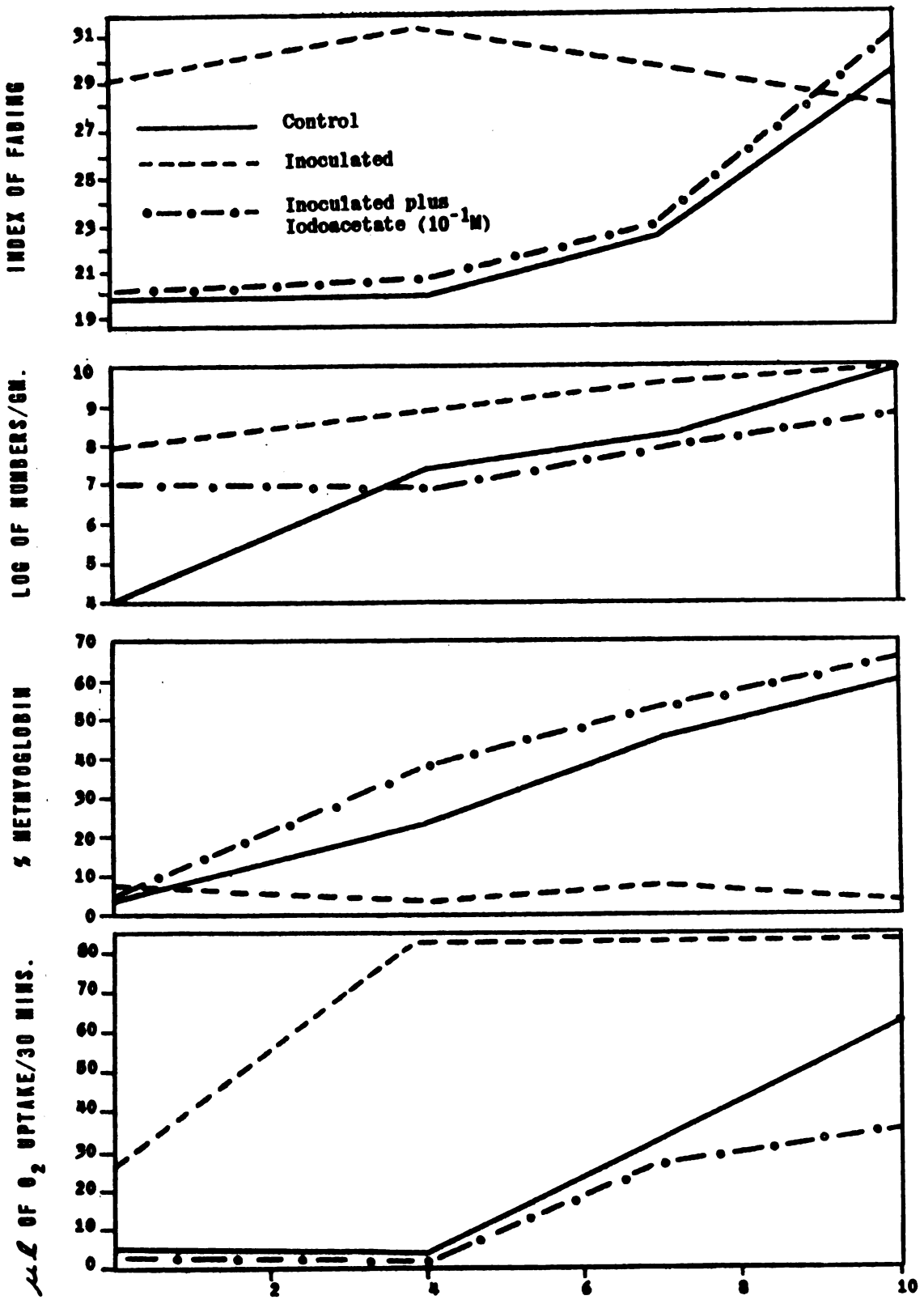
Iodoacetate (0.1M) sprayed on steak surfaces proved much more effective than the 20% malonate. The iodoacetate was applied to inoculated steaks and compared to inoculated untreated and to uninoculated steaks. The inoculated steaks having high bacterial counts and high respiratory activity discolored within 2 days. (Fig. 7). The inoculated steaks sprayed with iodoacetate had a low bacterial population, low respiratory activity, and prolonged color retention. In fact, the action was so pronounced that the inoculated steaks treated with iodoacetate gave counts, respiratory activity, and color notations very comparable to the uninoculated control.

Warburg data demonstrated that cells treated with NaN_3 and KCN had far lower respiratory activity on meat



COMPARISON OF EFFECTS OF SODIUM MALONATE AND AUREOMYCIN APPLIED TO PREPACKAGED BEEF HELD AT 4°C

FIGURE 6



EFFECTS OF IODOACETATE ON INOCULATED PREPACKAGED BEEF HELD AT 4°C

FIGURE 7

substrate than did untreated cells of Ps. geniculata. Cells treated with NaN_3 (0.01 M and KCN (0.1 M) spun out washed, and resuspended in sterile saline, showed about 73% inhibition. NaN3 and KCN added to the meat substrate before the addition of bacteria resulted in complete inhibition of the respiratory enzymes. Final concentration of NaN_3 was 1×10^{-3} molar and KCN was 1×10^{-2} molar. (3.3×10^{-3}M KCN gave similar results.)

Cells treated as above were sprayed on the surface of steaks and these compared to steaks treated with the inhibitors and inoculated, treated but uninoculated, and to a control lot of untreated steaks. All steaks were then packaged and held at 4°C. for visual observations. Results presented in Table 4 indicate that cells pretreated with KCN or NaN_3 as well as cells added in conjunction with these inhibitors caused no discoloration. This demonstrates that respiratory enzymes play an important role in the discoloration of fresh prepackaged beef. It was observed, however, that NaN_3 *per se* caused discoloration of the meat. This might be expected for NaN_3 reacts chemically with porphyrins and the prosthetic group of myoglobin is a porphyrin. The exact reaction involved was not studied in this investigation. When KCN was added to steaks without inoculation, a bright red color was still present after 10 days.

Table 4

Effects of KCN*, NaN_3** and Ps. geniculata inactivated with KCN and NaN_3 on fresh prepackaged beef held at 4°C.

Time in Days	Control	KCN	NaN_3	Ps. geniculata plus KCN	Ps. geniculata Plus NaN_3	Ps. geniculata KCN inactivated	Ps. geniculata NaN_3 inactivated
0	bright red	bright red	bright red	bright red	bright red	bright red	bright red
1	bright red	bright red	dark red	bright red	dark red	bright red	bright red
2	red	bright red	purple red	bright red	purple red	bright red	bright red
4	dark red	bright red	purple brown	bright red	purple brown	dark red	dark red
6	dark red	bright red	purple brown	bright red	purple brown	1/2 dark red 1/2 brown	brown
10	brown	bright red	purple brown	red brown edge	purple brown	1/2 brown 1/2 purple	1/3 brown 2/3 purple

*0.1 molar KCN
**0.01 molar NaN_3

Further studies were completed comparing the activity of treated and untreated cells of fresh prepackaged meat at various oxygen tensions held at 4° C. The various oxygen tensions were obtained by drawing a vacuum in dessicator jars and readmitting N_2 and O_2. Before admitting the final gas mixture the jars were flushed twice with nitrogen gas. Steaks of the various treatments described above plus inoculated untreated steaks were placed in the dessicators at the various oxygen tensions. In an atmosphere of nitrogen all the steaks became purple within an hour. At oxygen tensions between 20 mm and 40 mm O_2 all steaks turned brown between 3 and 6 days. The surface pigment of steaks inoculated with dilute suspensions of actively metabolizing cells was oxidized at a slightly faster rate than that of uninoculated steaks; however, the metmyoglobin of the inoculated steaks was reduced to myoglobin within 1 day after formation. The surface metmyoglobin of the uninoculated steaks was more stable, remaining at least 3 days before being reduced. Steaks treated with aureomycin, iodoacetate, and cells inactivated with KCN gave results similar to the untreated, uninoculated controls. In a normal atmosphere, no metmyoglobin formation could be observed during the first 7 days except for the steak inoculated with active cells. This steak began to discolor after 2 days and within 5 days had become purple with only a slight evidence of browning. The results

indicate that under reduced O_2 tensions surface pigment oxidation is accelerated, and the reaction is not inhibited by either aureomycin or iodoacetate. Also dilute suspensions of Ps. geniculata increase the rate of pigment change only if their respiratory enzymes are active.

Steaks Held at Room Temperature

Early work demonstrated that at room temperature crude intracellular enzyme preparations of Ps. geniculata caused surface discoloration of wrapped beef steaks while extracellular preparations did not. The action of the intracellular enzymes, however, was less pronounced than that demonstrated by intact cells. Preliminary work using crude enzyme extract in conjunction with sodium fluoride (0.01 molar) applied to steak surfaces gave no significant information. That is, at room temperature this enzyme inhibitor had little or no effect on the enzymes causing discoloration. The data indicate one of two things: (1) the concentration of the fluoride was too low to be effective, or (2) the enzymes which fluoride inhibits are not involved in meat discoloration.

In order to obtain further knowledge as to the mechanisms of discoloration of beef at room temperatures, four steaks were treated as follows: (1) untreated control; (2) inoculated, but no further treatment; (3) inoculated and sprayed with 0.1 molar iodoacetate; and (4) inoculated and sprayed with 0.01 molar sodium azide.

All steaks were wrapped with M.S.A.T. 80 cellophane. Results (Fig. 8-A) indicated that both enzyme inhibitors slowed down discoloration up to 45 minutes. At this time, the steak treated with sodium azide turned a dark purple and remained such for the rest of the experiment. At 2 hours, based on final index of fading values, iodoacetate resulted in about a 50% color preservation. That is, the index of fading value lay midway between the index of fading for the untreated control and the index of fading for the inoculated control. Upon refrigeration, all the steaks turned bright red except for the steak treated with sodium azide. This would indicate that sodium azide forms a stable complex with the myoglobin which seems very probable since sodium azide reacts with metalloproteins of which myoglobin is one.

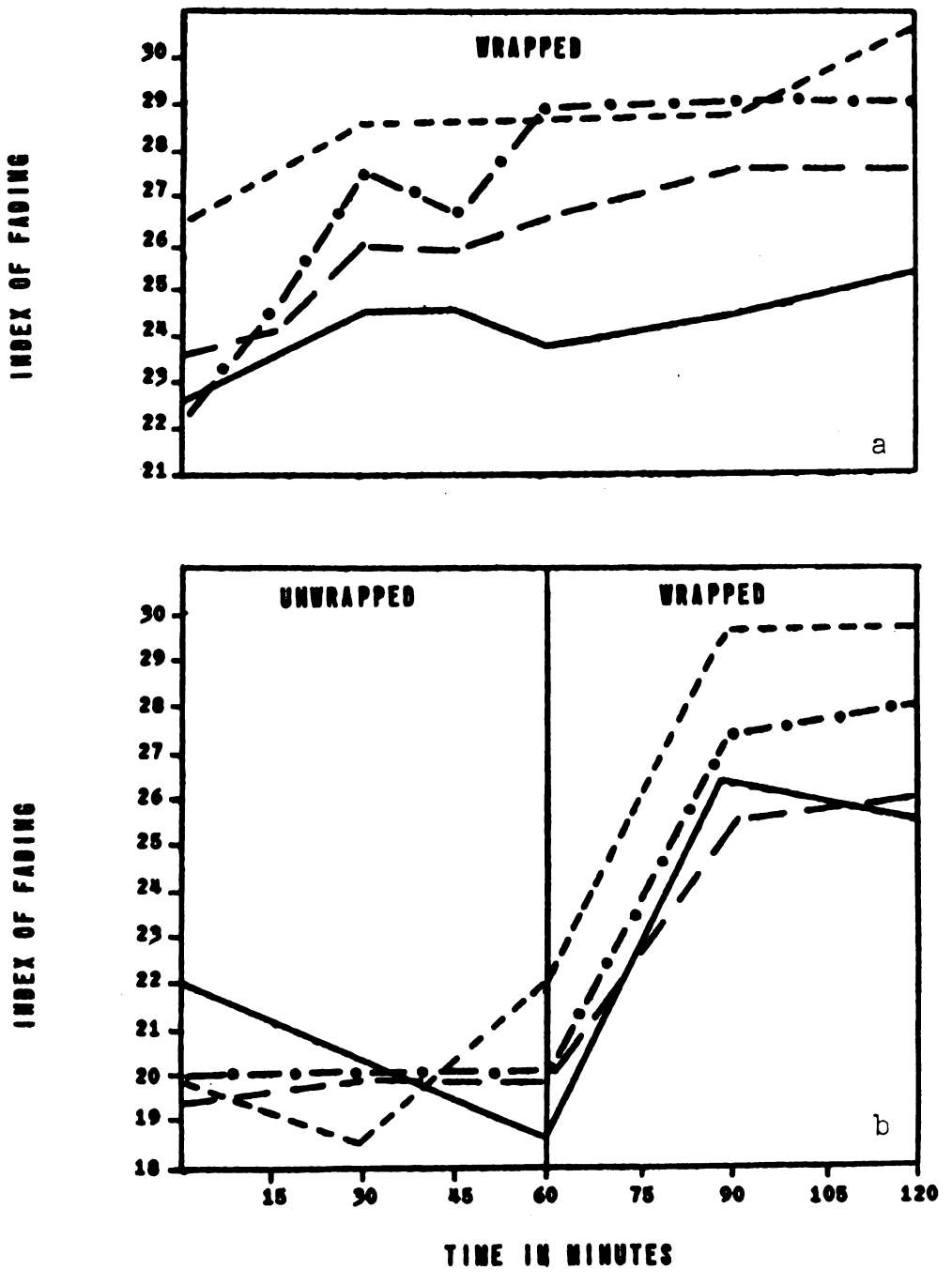
Steaks treated as above, but not wrapped until one hour after treatment, discolored only slightly for the first hour. Only the inoculated control had begun to darken sufficiently to be observed by index of fading values. The index of fading of the uninoculated control was only slightly less than those of the steaks treated with sodium azide and iodoacetate at one hour. After wrapping, all steaks discolored (became purple) rapidly as indicated by visual observations and index of fading values. The inoculated control steaks discolored most rapidly. Sodium azide slowed down the discoloration only very slightly before forming the stable dark purple pigment.

Iodoacetate seemed to be very effective in that it reduced the rate of discoloration to that of the uninoculated controls. (Fig. 8-L).

Muscle Pigment Extracts Held at Room Temperature

Cells of Ps. geniculata have shown the ability of both reduce and oxidize myoglobin solutions. These results were obtained by spectrophotometric analysis of the solutions containing the organisms using a water suspension of the organisms as a blank. Studies comparing treated cells (KCN, NaN_3 and heated) with untreated cells by the above method gave false results, for treating the cells gave them different optical properties which could not be compensated for by a cell suspension blank. The results indicated that cells which were inactivated, as demonstrated by Warburg studies, had the ability to oxidize and reduce the pigment.

A subsequent study was undertaken to note the effects of cells inactivated with KCN. In this study a sample was taken and centrifuged before spectrophotometric analysis. Visual observations of the solutions were also taken. After 30 minutes the myoglobin solution containing the active cells was a definite purple while the solutions containing cells inactivated by heat (35 minutes at 75° C.) and KCN (10^{-1} M) remained red; however, spectrophotometric analysis demonstrated essentially no difference (Table 5).



EFFECTS OF IODOACETATE AND Na AZIDE ON INOCULATED STEAK HELD AT ROOM TEMPERATURE

——— Control

- - - - - Inoculated

— — — Inoculated + Iodoacetate (10^{-1}M)

•—•—•- Inoculated + Na Azide (10^{-1}M)

FIGURE 8

Table 5

Effects of active and inactivated cells of Ps. coniculata on myoglobin pigment extracts held at room temperature

Time in Min.	Control		Active Cells		Cells Inactivated by Heat		Cells Inactivated with KCN	
	% Met.	% Nyo.	% Met.	% Nyo.	% Met.	% Nyo.	% Met.	% Nyo.
0	13	0	13	0	13	0	13	0
10	10	0	13	0	12	0	10	0
20	14	0	16	0	13	0	10	0
30	11	0	16	0	9	0	10	0
45	14	0	16	0	14	0	11	0
60	14	0	23	10	17	2	16	8
240*	19	0	25	63	15	7	17	15
300*	25	0	22	64	19	9	19	2

* Centrifuge tubes were stoppered in order to keep the pigment solutions from coming in contact with air.

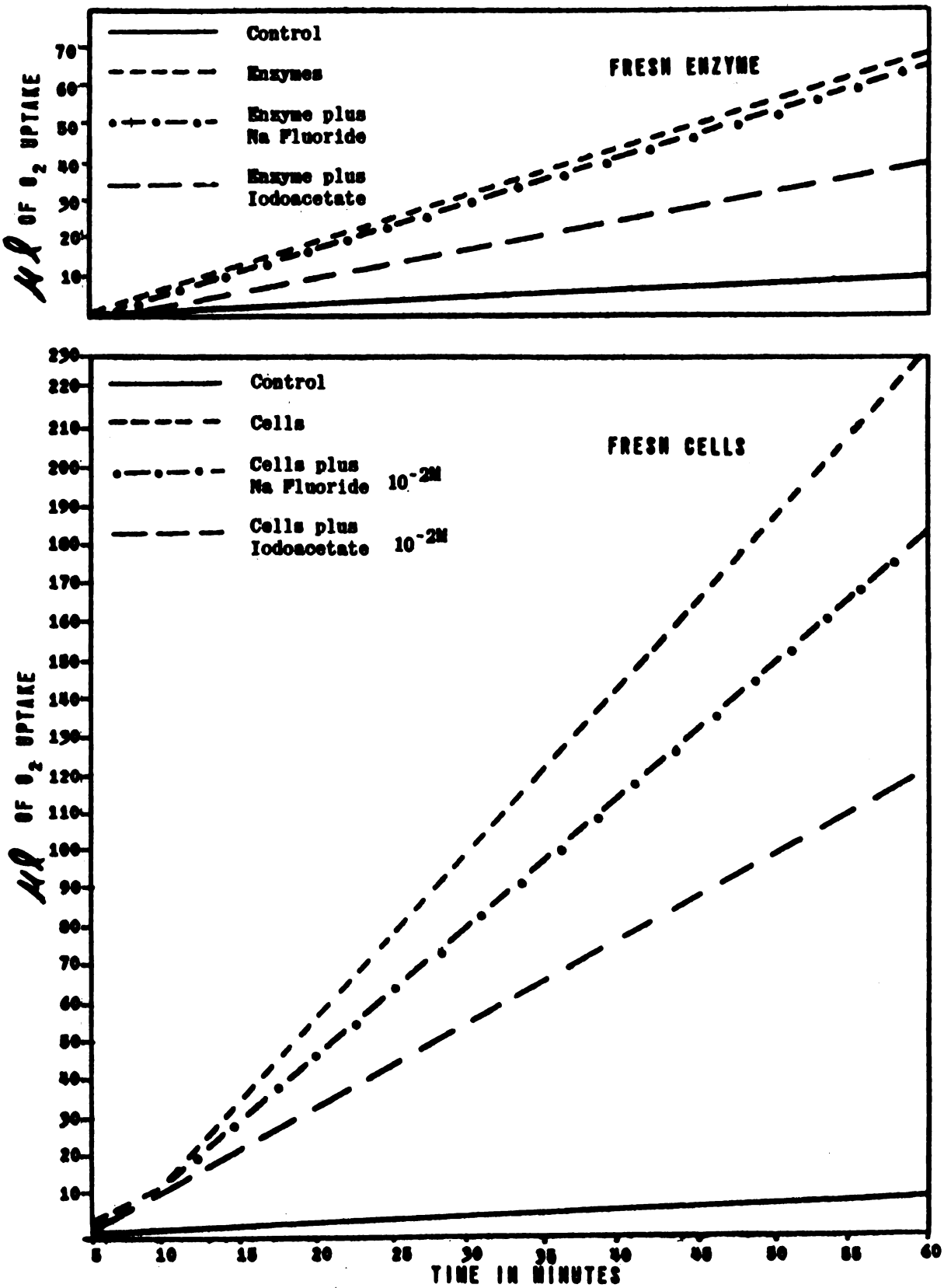
At one hour a centrifuged sample of the inoculated myoglobin solution showed a slight increase in metmyoglobin and myoglobin content, but not nearly as much as visual observations would indicate. At 4 hours samples of the various solutions were placed in centrifuge tubes and rubber stoppers were inserted. The tubes were filled so as to overflow when the stoppers were inserted so that no free air was present in the tube. After centrifugation the solutions were placed in the colorimeter tubes carefully, and spectrophotometric determinations were made. The data demonstrated that active cells had increased the percent metmyoglobin slightly, but primarily the action was reducing the pigment to myoglobin. Treated cells were essentially inactive.

From these results it is evident that one must be careful in his work with myoglobin solutions, especially if spectrophotometric analyses are to be made. The data demonstrate that cells of Ps. geniculata which have cyanide inactivated cytochrome systems are unable to reduce a solution of oxymyoglobin to myoglobin. This is in agreement with the results observed in previous work with myoglobin solutions in which the cells were left in the solution used for spectrophotometric analysis.

Meat Homogenates

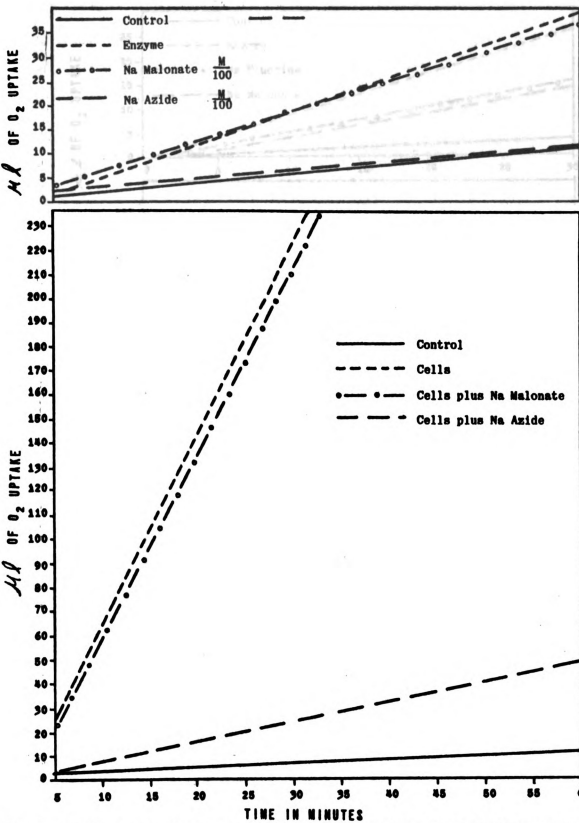
Meat homogenates were used as a substrate in Warburg experiments testing the effects of various enzyme inhibitors on cells and cell-free preparations of Ps. geniculata. Sodium fluoride at a concentration of 0.01 M had no inhibitory effect on the enzyme preparation and only a slight inhibitory effect (18%) on the cells (Fig. 9). However, from the data in Fig. 10, it is evident that 0.1 molar sodium fluoride inhibited the respiratory activity of both whole cells and enzyme preparations. This inhibitor brought about a 50% inhibition of the enzymes and 38% inhibition of the cells. A 0.01 molar solution of sodium malonate was also found to be inactive (Fig. 10), but 0.1 M sodium malonate caused a 50% inhibition of the respiratory activity of the enzyme preparation and the intact cells (Fig. 11). Iodoacetate (0.01 M) reduced the respiratory activity of the cells and cell-free intracellular enzyme preparations by about 50%, and sodium azide (0.01 M) completely inhibited the oxygen uptake by the enzyme preparations and caused virtually complete inhibition of the cells of Ps. geniculata (Fig. 11). One-tenth molar KCN gave results similar to those obtained with sodium azide.

In order to find out if cells of Ps. geniculata utilized an alternate aerobic metabolic pathway at reduced oxygen tensions, the following Warburg experiment



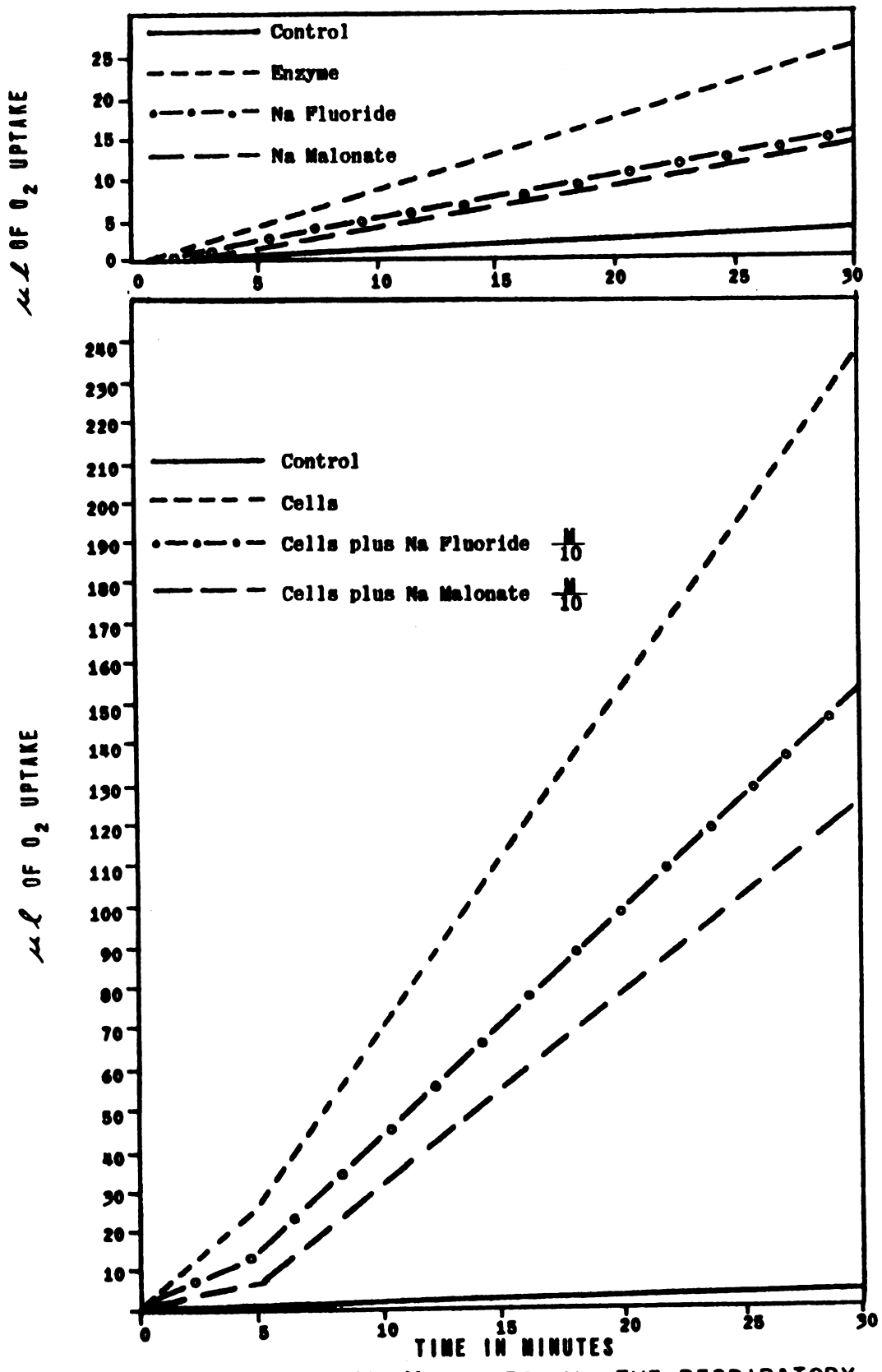
EFFECTS OF Na FLUORIDE AND IODOACETATE ON THE RESPIRATORY ACTIVITY OF CELLS AND INTRACELLULAR ENZYMES OF Ps. GENICULATA

FIGURE 9



EFFECT OF Na MALONATE AND Na AZIDE ON THE RESPIRATORY ACTIVITY CELLS AND INTRACELLULAR ENZYMES OF Ps. GENICULATA

FIGURE 10



EFFECTS OF Na FLUORIDE AND Na MALONATE ON THE RESPIRATORY ACTIVITY OF CELLS AND INTRACELLULAR ENZYMES OF Ps. GENICULATA

FIGURE 11

was undertaken: cells treated with KCN were compared to untreated cells at various reduced oxygen tensions which were obtained by allowing mixture of O_2 and N_2 to flow through the system prior to starting the Warburg analysis. The atmospheres used were: (a) all N_2, (b) 1% O_2 (99% N_2), (c) 5% O_2 (95% N_2), and (d) 10% O_2 (90% N_2). These were compared to normal atmospheres. The μl O_2 uptake was determined for a 30-minute interval (Table 6). The respiratory enzymes of cells treated with KCN were essentially inactive at all atmospheres. The activity of respiratory enzymes of the untreated cells increased as the oxygen tensions increased from 0 to 10%. The enzymatic activity at 5 and 10% oxygen was similar to that at normal atmospheres.

Beef Tissue Slices

Beef tissue homogenates had very low oxygen uptake activity; therefore, tissue slices were tested for respiratory activity. Both myoglobin solution and Ringer solution were used as suspending media in the Warburg flasks. Both gave similar results, so Ringer solution was used for its constituents are defined and controlled.

The tissue slices showed active metabolism as measured by oxygen uptake, and gave essentially the same activity at reduced oxygen tensions as at a normal tension. It was noted that KCN and NaN_3 at final

Table 6

The effect of KCN* on the O_2 uptake by cells of Ps. geniculata at various oxygen tensions

Time in min.	Normal		N_2		1%O_2 + 99%N_2		5%O_2 + 95%N_2		10%O_2 + 90%N_2	
	Cells	Cells + KCN	Cells	Cells + KCN	Cells	Cells + KCN	Cells	Cells + KCN	Cells	Cells + KCN
5	3.42**	1.46	1.4	1.54	0.0	0.0	4.47	0.0	6.1	0.0
10	16.19	5.84	4.2	3.08	1.59	0.0	22.37	3.08	24.28	0.0
15	21.05	5.84	5.6	4.62	1.59	0.0	25.35	3.08	31.88	0.0
20	30.77	7.2	8.4	6.16	4.77	0.0	40.25	3.08	47.08	0.0
25	37.25	7.2	12.6	7.6	6.36	0.0	47.7	4.62	59.23	0.0
30	43.73	7.2	14.0	7.6	6.36	0.0	56.65	4.62	69.83	0.0

* Final concentration of KCN was 6.6×10^{-3} molar
** µl of O_2 uptake are recorded

concentration of 10^{-2} M greatly inhibited the respiration action of the slices both at normal atmospheres and at an atmosphere of reduced oxygen content. The inhibitory action was essentially the same under both conditions (Table 7).

Effects of H_2O_2, Peroxidase and Glucose Oxidase

H_2O_2 (0.3%) added to the surface of fresh steaks held at room temperature or at refrigerated temperature, wrapped or unwrapped, resulted in no metmyoglobin formation. Bubbling occurred indicating catalase activity, but no evidence of pigment oxidation was visible. However, a steak which had been held at a reduced oxygen tension (80 mm O_2) became brown when peroxidase was added to its surface at room temperature under normal atmospheric conditions. In fact, the oxymyoglobin on the surface of most aged steaks was oxidized to metmyoglobin on addition of H_2O_2.

Both fresh and aged solutions of myoglobin pigments were partially oxidized by the addition of H_2O_2. However, from the results in Table 8, it can be seen that the aged solutions of myoglobin were oxidized much more readily than fresh preparations.

Meat extracts gave a positive test for peroxidase using orthophenylene diamine (O.P.D.A.) indicator. To further verify the evidence of peroxidase, KCN, which is known to inhibit this enzyme, was added to the extract

Table 7

Effects of sodium azide and potassium cyanide on the respiratory activity of beef tissue

	Normal Atmosphere			1% Oxygen and 99% Nitrogen		
	Control	NaN_3	KCN	Control	NaN_3	KCN
$\mu l\ O_2$/hr./0.1 gm.	23.8	6.36	5.87	24.6	3.53	4.45

and compared to an untreated control. It was noted that as the concentration of the KCN increased, the peroxidase activity decreased. When compared to a heat-inactivated control, KCN completely inhibited the heat sensitive activity. Potassium iodide plus starch, benzidine, and gum guaiac all failed to give a positive reaction for the presence of H_2O_2 in or on steaks whose pigments were undergoing oxidation. Thus the presence of peroxidase has been shown, but no free H_2O_2 could be detected. However, any H_2O_2 produced would probably be very fleeting due to the presence of catalase in high concentration. Therefore, it is not likely that the tests used are sensitive enough to detect the level of H_2O_2 which might be present.

Effect of Glucose Oxidase

Glucose oxidase when applied to the surface of steaks wrapped or unwrapped, caused the formation of metmyoglobin within five minutes at room temperature. However, the discoloration reaction did not stop at this point but proceeded to the choleglobin stage. Refrigeration did not stop this reaction though it was somewhat slower than at room temperature.

A second trial using dilute solutions of glucose oxidase proved that this enzyme preparation could oxidize the surface pigment of steaks to metmyoglobin without the formation of choleglobin. The results in Table 9 also demonstrate that the heat-treated glucose oxidase was inactive. This would indicate that oxidation caused by

Table 8

Effects of hydrogen peroxide on fresh and aged myoglobin extracts held at room temperature

Time in Min.	Fresh Preparation		Fresh Preparation+H_2O_2		Aged Preparation		Aged Preparation+H_2O_2	
	% met.	% myo.	% met.	% myo.	% met.	% myo.	% met.	% myo.
before addition	3	0	3	0	6	0	5	0
0	9	0	12	0	21	0	50	0
15	10	0	19	0	9	0	39	0
30	7	0	22	0	7	0	42	6
60	9	0	19	0	12	0	40	0

glucose oxidase was enzymatic. H_2O_2 had no effect on the surface pigment of steaks when added alone or in conjunction with glucose oxidase inactivated by heat. Comparing the effects of H_2O_2, glucose oxidase and peroxidase on oxymyoglobin solutions (Table 10), it was observed that H_2O_2 caused some oxidation but was far less active than dilute glucose oxidase. The fact that heated glucose oxidase was active would suggest that some of the activity of the unheated sample was nonenzymatic, but the difference in activity was great enough to show the enzyme system did cause oxidation of oxymyoglobin. Glucose oxidase when more concentrated caused the formation of choleglobin.

Effect of Peroxidase

Peroxidase was obtained by filtering a commercial unpasteurized horseradish preparation. Peroxidase added to the surface of fresh steaks caused no discoloration, nor did 0.3% solution of H_2O_2 at room temperature. However, when added together the steak surface turned brown within 10 minutes; and after holding at 4°C. overnight, the brown pigment became a green-brown color. H_2O_2 added to steaks previously treated with peroxidase turned brown almost immediately.

It was noted that some nonenzymatic oxidation takes place on steaks. Peroxidase solutions inactivated by heat still caused some surface oxidation when used in conjunction with H_2O_2, but the reaction was slower than that of

Table 9

The effects of hydrogen peroxide, glucose oxidase, and a combination of both on the surface color of unwrapped steaks held at room temperature

Time in Min.	Hydrogen Peroxide	Glucose Oxidase	Glucose Oxidase + Hydrogen Peroxide	Heat-Inactivated Glucose Oxidase	Heat-Inactivated Glucose Oxidase + H_2O_2
before addition	red	red	red	red	red
0	red	dark red	dark red	red	red
15	red	red brown	brown	red	red
30	red	dark brown	dark brown	red	red
60	red	dark brown	dark brown	red	red
120	red	dark brown	dark brown	red	red

Table 10

Effects of active and heat-inactivated glucose oxidase and peroxidase on myoglobin extracts held at room temperature

Time in	Control		H_2O_2		Glucose Oxidase		Heated Glucose Oxidase		Peroxidase		Heated Peroxidase	
Mins.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.
0	0	0	3	0	0	0	0	0	2	0	0	0
30	5	0	19	0	86	11	19	0	26	0	25	0
60	9	0	23	3	83	14	34	0	38	0	40	0

the active preparation. The nonenzymatic reaction was shown to interfere far more greatly in myoglobin solutions, causing rapid oxidation (Table 10).

Effects of O_2 Tensions

Steak Surface Pigments

A series of experiments was designed to show the influence of different oxygen levels on the color changes of both inoculated and uninoculated steaks. In the first experiment atmospheres used were oxygen, nitrogen, and air. The wrapped steaks were placed in desiccating jars, a vacuum drawn with a water aspirator, and the jars flushed with the gas to be used. This process was repeated three times.

The results of this study are presented in Tables 11 and 12. The steaks held under an oxygen atmosphere developed a brighter color (lower index of fading values) and maintained it longer than the steaks held in air; however, inoculation with Ps. geniculata greatly reduced the time necessary for a color change to occur in both air and oxygen. The steaks in the nitrogen atmosphere never returned to a bright red, but remained dark red until discoloring further. The uninoculated group held in the N_2 atmosphere developed a brown color and high metmyoglobin percentage. This may have been the result of a low residual oxygen level in this atmosphere. The failure to observe a brown color or a high metmyoglobin percentage in the inoculated group under nitrogen was

Table 11

Effects of nitrogen and oxygen atmospheres on uninoculated prepackaged beef held at 4°C.

Atmospheres and observations	Time in days				
	0	2	4	6	11
NORMAL					
Visual observation	bright red	bright red	bright red	red	2/3 brown 1/3 purple
Index of fading	19.5	17.3	18.0	20.3	29.1
% Metmyoglobin	21.3	5.4	32.2	32.5	35.6
Log No. bacteria	5.3	3.0	4.5	7.6	9.7
OXYGEN					
Visual observation	very bright red	very bright red	very bright red	bright red	1/3 brown 1/2 purple 1/6 red
Index of fading	---	16.2	15.9	15.9	30.9
% Metmyoglobin	---	20.5	19.4	24.7	32.2
Log No. bacteria	---	3.0	4.9	6.7	10.4
NITROGEN					
Visual observation	dark red	purple	brown-purple	brown	purple-brown
Index of fading	---	25.6	27.9	26.8	25.4
% Metmyoglobin	---	26.8	28.2	50.3	16.8
Log No. bacteria	---	3.0	4.5	7.3	8.5

Table 12

Effects of nitrogen and oxygen atmospheres on inoculated* prepackaged beef held at 4°C.

Atmospheres and observations	Time in days				
	0	2	4	6	11
NORMAL					
Visual observations	bright red	bright red	5/6 brown 1/6 dark red	brown-purple	purple
Index of fading	17.3	17.4	25.2	31.6	29.1
% Metmyoglobin	22.8	10.9	31.6	25.6	3.6
Log No. bacteria	6.5	7.4	9.2	9.6	10.1
OXYGEN					
Visual observations	very bright red	very bright red	red	brown-purple	purple
Index of fading	---	14.3	18.8	28.5	31.8
% Metmyoglobin	---	19.3	17.9	20.3	7.4
Log No. bacteria	---	7.4	9.3	9.9	10.3
NITROGEN					
Visual observations	dark red	purple	purple	purple	purple
Index of fading	---	24.2	28.8	27.4	23.3
% Metmyoglobin	---	24.9	20.8	14.3	7.9
Log No. bacteria	---	7.2	7.2	8.9	9.2

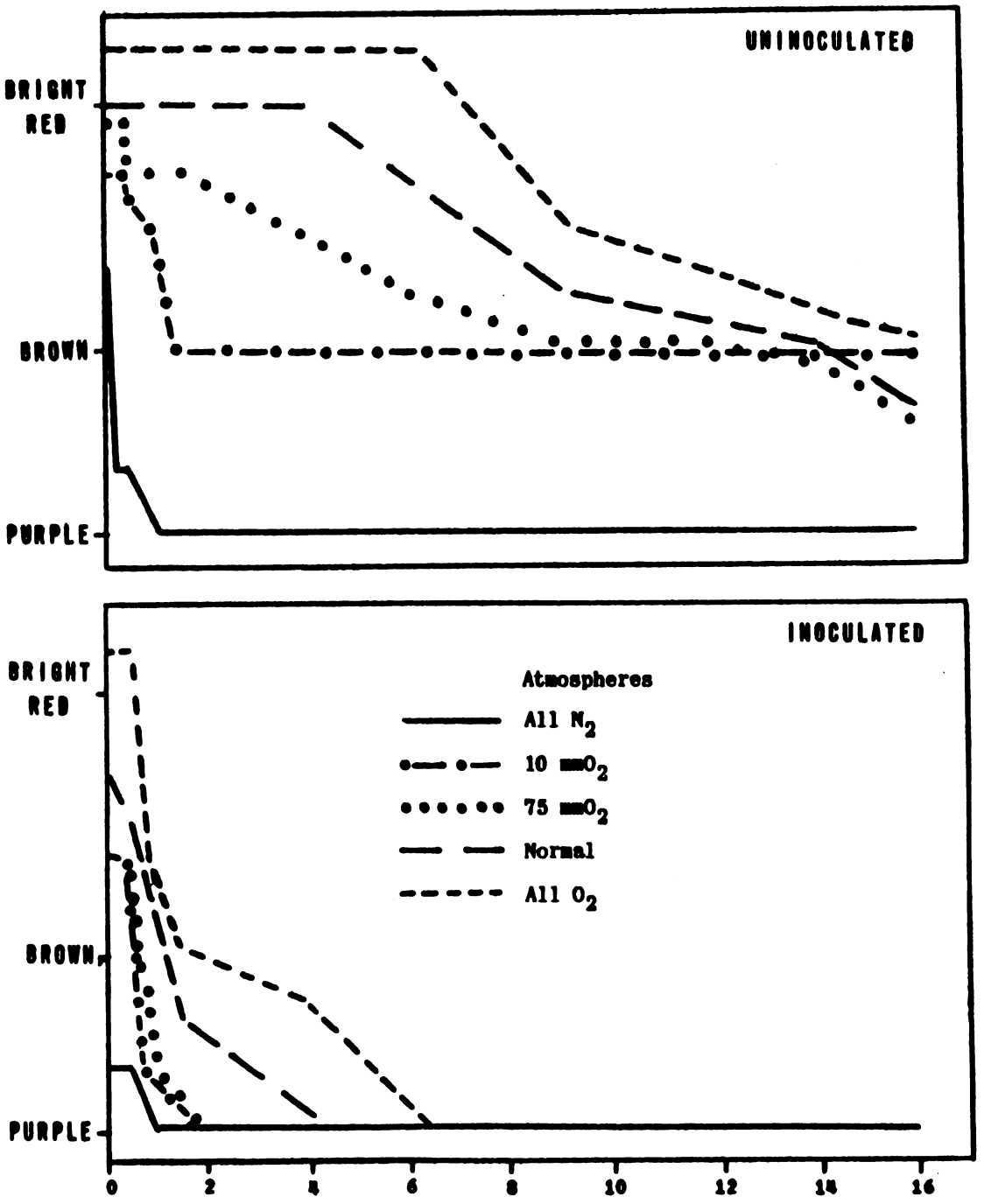
*Inoculated with Pseudomonas geniculata

probably due to the rapid utilization of residual oxygen by the bacteria.

To get further information on the influence of various oxygen levels on fresh meat color, a more comprehensive experiment was designed. This involved storing prepackaged meat at various oxygen tensions. Control steaks inoculated steaks, and steaks treated with cell-free extracts of Ps. geniculata were stored under each atmosphere. The atmospheres were obtained in vacuum desiccator jars by pulling a vacuum with a "hi-vac" pump, measuring in the desired oxygen pressure by the use of a mercury manometer, and bringing the internal pressure near atmospheric pressure with nitrogen. Visual observations of color were made during the 12-days' storage under refrigeration. Observations of the control and inoculated groups of steaks are shown in Figure 12.

All steaks held in the absence of O_2 turned purple within 1-1/2 hours and remained purple throughout the experiment.

The steaks held at 10 mm of O_2 demonstrated some differences as to treatments. The uninoculated steaks began to darken within 12 hours, and at 36 hours became a definite brown and remained brown. The steaks which were inoculated turned dark red immediately, and after 24 hours became purple without the formation of a brown pigment. The steaks treated with the intracellular enzymes gave results similar to the inoculated steaks,



THE EFFECTS OF VARIOUS OXYGEN TENSIONS ON THE COLOR OF PREPACKAGED BEEF HELD AT 4°C

FIGURE 12

but before turning purple, browning was observed.

The inoculated steaks and those treated with the enzyme preparation held at 75 mm O_2 gave results similar to those obtained at 10 mm O_2; however, discoloration caused by the enzymes was less pronounced. The uninoculated steak was a bright red up to 2 hours and then became a dark red turning brown within 6 days and finally began to turn purple after 16 days. It should be noted that up to this point only the uninoculated steak held at 75 mm O_2 was bright red initially.

Inoculated steaks held at normal atmosphere began to darken within one hour and within 24 hours turned purple. The steak treated with enzymes demonstrated a slight darkening for the first 2 hours, but then returned to a bright red. Subsequent discoloration of the steaks treated with enzymes was probably due to bacteria and not to the enzyme preparation per se. Results of the enzyme-treated steak were similar to the results of the uninoculated steak except for the initial darkening.

All the steaks in the oxygen atmosphere were very bright red for the first few hours. The inoculated steak was first to discolor, having a brown periphery and a dark red center by 24 hours. At 4 days the steak was partially purple, and after 6 days it was all purple. The steak treated with enzymes and the uninoculated steak began to discolor after 6 days, probably due to bacterial action.

Two experiments were completed in desiccator jars testing the effects of iodoacetate, aureomycin, and KCN. In the first test, steaks wrapped in KCAT 80 cellophane were used. In the nitrogen atmosphere, all steaks turned purple after 2 days and remained as such until the experiment terminated. All the steaks which were exposed to an atmosphere containing 10 mm O_2, except that treated with KCN, began to turn brown at 3 days and remained brown to the tenth day at which time the steaks became a mixed brown-purple color. The steak treated with KCN began to turn purple after 3 days. The surface pigment of the steak treated with KCN was completely reduced after 5 days, but at no time was the brown color of metmyoglobin observed. Steaks exposed to 75 mm O_2 became red upon refrigeration, but not the bright red that was observed on steaks held at normal or complete oxygen atmospheres. The control steak was first to discolor. Iodoacetate afforded about 1 day of color preservation and the aureomycin about 4 days as compared to the control. The KCN-treated steak retained its red color for about a day longer than the aureomycin-treated steak and then became a dark red and finally purple. Again, all the steaks except that treated with KCN became brown before turning purple.

The color changes observed with steaks held at normal atmosphere were similar to those held at 75 mm O_2 except that the initial color was bright red. Steaks

held in an atmosphere of oxygen retained the bright red color 3 days longer than at normal atmosphere. It was also noted that these steaks turned a purple color with only little evidence of metmyoglobin formation.

The various bacterial inhibitors did show some color retention, but only at atmospheres containing high amounts of oxygen. In an atmosphere of all N_2 and 10 mm O_2, they were inactive. It should be noted that at no time was the formation of metmyoglobin clearly defined on those steaks treated with KCN except in an atmosphere of all oxygen.

The above experiment was repeated using steaks which were not wrapped. The KCN treatment was omitted. The results were similar to those of the previous experiment.

From this information, it appears that the oxygen present at the steak surface plays an important role in the rate of discoloration. At lower oxygen tensions (10 mm O_2), metmyoglobin is formed within 3 days while at higher oxygen tensions (normal) metmyoglobin is not produced until the fifth day. It would appear that at the lower oxygen tensions bacteria play only a small part, if any, for the addition of bacterial inhibitors (excluding KCN) does not slow down the metmyoglobin formation. At higher oxygen tensions, however, the opposite is true, that is, bacteria do play an important role in metmyoglobin formation for the addition of bacterial inhibitors, especially

aureomycin, slows down metmyoglobin formation considerably.

To check the effect of temperature on these color changes, steaks wrapped and unwrapped, with and without the addition of iodoacetate were placed in atmospheres of all nitrogen, nitrogen plus 10 mm O_2, nitrogen plus 75 mm O_2, normal, and all oxygen. Storage was at room temperature.

Steaks in the nitrogen atmosphere became purple immediately and remained so through the experiment. In the other atmospheres browning occurred within 2 days. The steaks held in the normal and all-oxygen atmospheres developed a large amount of metmyoglobin as did the unwrapped steaks and the steaks treated with iodoacetate held at 10 mm O_2. The other steaks turned purple without becoming a definite brown. Thus, at room temperature the formation of metmyoglobin was accelerated, but the reduction to myoglobin was also accelerated. In the all oxygen atmosphere, steaks were all brown at 2 days, while at refrigerated temperature no definite browning could be observed. Iodoacetate did not decrease metmyoglobin formation, but in all cases tended to increase its formation.

The second experiment in this series was the same as the first except that various oxygen levels were obtained by drawing partial vacuums in desiccating jars.

Considering that air contains approximately 20% oxygen, and knowing the barometric pressure, a vacuum can be pulled to the point where the gas left in the desiccator jar would contain the amount of oxygen desired. (For example, if the barometric pressure is 740 mm of mercury and a vacuum of 640 mm of mercury is drawn, 100 mm of gas would be left in the jar of which 20% or 20 mm is oxygen.) Results indicate that at room temperature metmyoglobin formation proceeds at a maximum rate near an oxygen tension of 20 mm O_2. Again, steaks treated with iodoacetate tended to form metmyoglobin more completely except in an atmosphere containing 20 mm O_2 where all steaks became brown about the same time.

The third experiment in this series was undertaken to test the effect of Ps. geniculata on steaks held in partial vacuum with various oxygen levels. Again various oxygen levels were obtained by pulling partial vacuums in desiccator jars. All the inoculated steaks at the various oxygen levels turned purple within one-half hour and remained purple. The uninoculated steaks exposed to oxygen levels of 20 mm and 40 mm demonstrated some browning on the second and third day, but the rest of the steaks turned purple without browning. Ps. geniculata hastened the formation of the purple color at 20 mm O_2, 40 mm O_2, and 60 mm O_2 and kept the pigment of steaks in the reduced form. The pigment of the

uninoculated steaks was partially oxidized to metmyoglobin before being entirely reduced to myoglobin.

It has been shown that at reduced atmospheres especially at atmospheres containing 10 mm O_2 the formation of metmyoglobin from oxymyoglobin on steaks is accelerated. This occurs even when the bacterial inhibitors are added, but it was not known if all the bacteria had been inhibited. The following experiment was conducted to see if metmyoglobin could be produced on pieces of meat without bacterial growth by reducing the amount of available oxygen. To accomplish this, pieces of meat were extracted by sterile technique, placed in sterile petri dishes, and in turn placed in a sterile desiccator jar at which time petri dish covers were removed. Each jar contained three meat samples. The various oxygen tensions were obtained by adding mixtures of N_2 and O_2 to the jars after they had been evacuated. All gases were run through a sterile glass wool filter to keep conditions within the desiccator jars free of microbial growth. All jars were refrigerated. Results (Table 13) show that at reduced oxygen tensions the pigment of muscle tissue can be oxidized to metmyoglobin within 36 hours without the presence of bacteria. Spectrophotometric analysis as well as visual observations bears out this fact. The rate of metmyoglobin formation was essentially the same at oxygen

Table 13

The effects of reduced oxygen atmospheres on sterile excised muscle tissue held at 4°C.

Time in Hours	8 mm. O_2			10 mm. O_2			15 mm. O_2			20 mm. O_2		
Before	bright red			bright red			bright red			bright red		
0	dark red			dark red			dark red			dark red		
12	dark red			dark red			dark red			dark red		
24	brown-red			brown-red			brown-red			brown-red		
36	brown			brown			brown			brown		
48	brown			brown			brown			brown		
	% met.	% myo.	No. bact.	% met.	% myo.	No. bact.	% met.	% myo.	No. bact.	% met.	% myo.	No. bact.
	45	0	0	44	0	0	44	0	0	64	0	0

tensions of 8 mm, 10 mm, 15 mm, and 20 mm. Bacterial counts made by plating with tryptone glucose extract agar (Difco) demonstrated no significant growth.

A second investigation (Table 14) was performed using a wider range of O_2 tensions comparing excised tissue to aseptically handled beef steaks. Results showed that steaks did not react to the various atmospheres as readily as the excised tissue. At 10 mm O_2 and 25 mm O_2 the excised tissue turned brown within 48 hours, and the steaks did not become entirely brown until 96 hours at 10 mm O_2 and 120 hours at 25 mm O_2. The important thing is, however, that the steaks did become brown without the presence of a significant number of bacteria. At 50 mm O_2 the steak turned brown after 144 hours while the excised tissue began to turn brown at 48 hours. Counts indicated that the tissue was without bacterial growth, and the steak had insignificant numbers of bacteria present at the time of browning. However, the steak held under a normal atmosphere and at an atmosphere containing 75 mm O_2 became brown after 12 days' storage, and counts revealed high numbers of bacteria (1×10^8). This would indicate that the discoloration was caused by the bacteria. The excised tissue became brown, but after only 6 days.

Table 14

The effects of reduced oxygen atmospheres on sterile excised muscle tissue compared to aseptically handled steaks held at 4°C.

Time in	10 mm. O_2		25 mm. O_2		50 mm. O_2		75 mm. O_2		Normal	
Hours	Steak	Tissue	Steak	Tissue	Steak	Tissue	Steak	Tissue	Steak	Tissue
before	bright red	bright red	bright red	bright red	bright red	bright red	bright red	bright red	bright red	bright red
0	dark red	dark red	dark red	dark red	red	dark red	bright red	dark red	bright red	dark red
4	dark red	dark red	bright red	dark red	bright red	red	bright red	red	bright red	red
24	dark red	red brown	red	red brown	bright red	dark red	bright red	red	bright red	red
48	red brown	brown	red	brown	red	red brown	bright red	red	bright red	red
72	brown red	brown	dark red	brown	red	brown red	bright red	red	bright red	red
96	brown	brown	red brown	brown	red	brown	red	dark red	bright red	dark red

Muscle Pigment Extracts

The importance of oxygen levels and bacteria on discoloration of fresh prepackaged and unpackaged beef steaks having been demonstrated, it was thought that further information could be obtained by subjecting oxymyoglobin extracts to various oxygen levels with the addition of bacterial suspensions and various bacterial inhibitors. The oxymyoglobin solutions were placed in Thunburg tubes, and the bacterial suspension or bacterial inhibitor was placed in the side arm of the top of the tubes. The top having been put in place, a vacuum was pulled with a vacuum pump. Various oxygen levels were obtained by pulling partial vacuums or by pulling a vacuum and readmitting various oxygen and nitrogen mixtures. Before removing the Thunburg tube from the vacuum system, the top was turned so as to close the internal gas system from the external air. Change in pigment was determined spectrophotometrically. All of the Thunburg tubes used were of the same optical nature, i.e., at a given wave length the tubes filled with water gave similar optical density values. Also, before this method of pigment measurement was used, its accuracy was tested using myoglobin solutions treated with sodium hydrosulfite and potassium ferricyanide. Sodium hydrosulfite reduces the pigment to the myoglobin form, and potassium ferricyanide oxidizes the pigment to the metmyoglobin form. Excellent

results were obtained in both cases.

Oxygen tension was found to have only a slight effect on inoculated pigment solutions; that is, oxygen tensions from near zero to 150 mm gave comparable results.

Baker's yeast under atmospheric conditions caused little or no change of the pigment oxymyoglobin, but at a partial pressure of 10 mm O_2 the yeast apparently had changed about 80% of the oxymyoglobin pigment after 2 hours. The pigment mixture was found to contain the following relative percentages: 27% metmyoglobin, 54% myoglobin, and 19% oxymyoglobin. Glucose (0.1%) was added to the pigment solution to enhance the metabolism of the yeast cells.

Oxygen tension had no effect on pigment solutions inoculated with Ps. geniculata. Therefore, an attempt was made to see if reduced oxygen atmospheres had any effect on "sterile" pigment solutions. Aureomycin was added to the oxymyoglobin solutions in an attempt to render them bacteria free. The solutions held at various oxygen tensions from 10 mm O_2 to normal atmosphere demonstrated no essential difference after 3 hours. Each solution was considered bacteria free for no bacterial growth was observed when 1 ml of each solution was plated out in TGE agar and incubated for 1 week.

These data would tend to minimize the importance of oxygen tension in the reaction of oxymyoglobin being

either reduced to myoglobin or oxidized to metmyoglobin with or without the aid of bacteria. However, an equilibrium between the gas phase and liquid phase within the Thunburg tubes may not have been reached. This difficulty was partially alleviated by placing the tubes wrapped in cheesecloth on a shaker between the readings. An oxymyoglobin solution containing cells of Ps. geniculata was compared to oxymyoglobin solution containing aureomycin (10 ppm) to inhibit microbial growth. The solutions were placed at oxygen levels of 0, 5, 10, and 20 mm O_2. Results in Table 15 indicate that as the oxygen levels are lowered, metmyoglobin and myoglobin formation is increased in both lot 1 (aureomycin treated) and lot 2 (inoculated). In an atmosphere containing no oxygen at 40 minutes, both lots contained 80% myoglobin and metmyoglobin at a ratio of 2:1. In the reduced oxygen atmospheres (5, 10, and 20 mm O_2) there is essentially no difference in metmyoglobin formation between lot 1 and 2; however, the organisms in lot 2 increased the myoglobin content substantially. This would indicate that the bacteria caused an increase in the reduction of oxymyoglobin but had no significant oxidative ability under these conditions. Further data at O_2 tensions between 20 mm O_2 and normal demonstrated results similar to those obtained at normal O_2 tensions.

Pigment changes of oxymyoglobin solutions caused by reduced oxygen tension and bacteria are slowed down

by refrigeration (4°C.), but are not inhibited by it. At 4°C. Ps. geniculata demonstrated greater pigment reducing and oxidizing ability in a partial vacuum than at normal atmospheric conditions compared to uninoculated pigment controls which were subjected to the same conditions. A day was required for the cells of Ps. geniculata to change 80% or more of the pigment from oxymyoglobin to a mixture of myoglobin and metmyoglobin. Once more the ratio was about 1 part metmyoglobin to 2 parts myoglobin. The two pigment solutions treated with aureomycin demonstrated no myoglobin formation after 12 days, yet the sample held in a vacuum contained 81% metmyoglobin, and that held at normal atmosphere contained 66% metmyoglobin. At 3 weeks the measurements indicated 90% and 71% metmyoglobin respectively. No myoglobin was observed. Plating in TGE agar demonstrated no growth. This would indicate that metmyoglobin can be produced in extracts without bacterial activity. However, the role of aureomycin in the reaction (s) is unknown.

Effect of Antioxidants

Three samples of antioxidants from the Griffith Laboratories were used: G-4 containing vegetable oil, lecithin, propylgallate, and citric acid; G-15 containing vegetable oil, lecithin, butylated hydroxytoluene, propylgallate, citric acid, and butylated hydroxyanisole; and G-16 containing vegetable oil, monoglycerides,

Table 15

Effects of various oxygen tensions* on oxymyoglobin extracts with and without the presence of bacteria.

Shaking Time in Minutes	Lot No.**	Oxygen Levels									
		0 mm O_2		5 mm O_2		10 mm O_2		20 mm O_2		Normal	
		% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.
Before	Lot 1	1	0	2	0	3	0	2	0	3	0
	Lot 2	3	0	2	0	2	0	1	0	3	0
0	Lot 1	9	1	4	0	3	0	3	0	3	0
	Lot 2	7	0	7	0	4	0	7	0	3	0
20	Lot 1	25	60	16	13	11	10	13	3	9	0
	Lot 2	21	60	16	35	14	13	13	10	9	0
40	Lot 1	30	62	19	12	14	4	15	4	13	0
	Lot 2	23	67	21	46	16	29	16	22	14	0

* Oxygen tensions were obtained by pulling partial vacuums in Thunburg tubes. Equilibrium was hastened by shaking the tubes between readings.

** Lot 1 contained 10 ppm aureomycin to inhibit bacterial activity.
Lot 2 was inoculated with Ps. geniculata.

butylated hydroxytoluene, propylgallate, citric acid, and butylated hydroxyanisole. All were in liquid form.

Three samples from the Eastman Chemical Products, Inc. were also used. Tenox II, a liquid, contained butylated hydroxyanisole, propylgallate, citric acid, and propylene glycol; Tenox BHA, crystals of butylated hydroxyanisole; and Tenox PG which was propylgallate crystals.

The three samples from the Griffith Laboratories were dark solutions, and initially slightly masked the red color of the meat. All 3 gave similar results in that they caused uninoculated fresh prepackaged beef steaks to discolor about 3 days before control steaks. The treated steaks became brown (methyoglobin) and remained brown for about 2 weeks before turning purple (myoglobin). The control steak turned brown (methyoglobin) 3 days later than the treated steaks, yet it remained brown only a day before becoming partially purple; within 2 days the surface pigment of control steaks was completely reduced.

Tenox II gave results similar to the Griffith products, but when the solution was first added to the meat, a milk-like precipitate formed.

Tenox PG and Tenox BHA did not fully dissolve, and, therefore, a portion remained as white crystals on the meat surface. Tenox PG gave similar results to the Griffith

products, i. e., browning after 3 days and preservation of the brown color for about 2 weeks. Tenox BHA gave slightly different results. The steak treated with Tenox BHA did not turn brown until after the control steak, but it retained the brown color for a longer period of time than the control steak. However, the pigment of the steak turned purple about a week before the other antioxidant-treated steaks.

All of the antioxidants used slowed down the initial rate of discoloration of steaks inoculated with Ps. geniculata; however, the steaks treated with the antioxidants retained the brown color of metmyoglobin for a longer period of time than did the inoculated control steak.

In general, the antioxidants used did not delay the formation of metmyoglobin, but after its formation did tend to keep it from being reduced to myoglobin.

A 1% solution of sodium isoascorbate was compared to a 1% solution of ascorbic acid as applied to the surfaces of inoculated and uninoculated steaks. Data observed indicated that neither solution had any apparent effect on the bacteria present nor on the % metmyoglobin formed. The ascorbic acid solution did slow down discoloration of both the inoculated and uninoculated steaks somewhat, but sodium isoascorbate was ineffective.

Comparing the results obtained on uninoculated steaks treated with 1% solution of isoascorbic acid and a 1% solution of ascorbic acid, it was observed that

there was no significant difference in regard to bacteria counts and % metmyoglobin formed. Each demonstrated some color preservation. Steaks which were inoculated and treated with the two antioxidants gave similar results, but the color preservation was less pronounced.

The application of 10% sodium ascorbate, 10% sodium isoascorbate, or a mixture containing 5% sodium ascorbic and 5% ascorbic acid applied to steak surfaces resulted in no final color preservation and initially caused a slight discoloration which remained for about 2 days. Ten percent ascorbic acid when applied to a steak surface also caused this initial discoloration (slight dark red) which remained about 2 days but resulted in color preservation of 4 days beyond that of the control.

Effect of Ascorbic Acid and of a Mixture of Ascorbic Acid and Sodium Nicotinate on the Color of Prepackaged Steaks

A series of experiments were run comparing the effect of 1% ascorbic acid (A.A.) solution and a solution containing 8% sodium nicotinate (N.N.) and 2% A.A. on the surface pigment of meat. Visual observation, index of fading, and bacterial counts were performed on the treated steaks and on untreated steaks after various storage intervals.

The results of these studies are presented in Tables 16 and 17. It was obvious that the N.N. plus A.A. mixture resulted in a brighter red color initially and maintained this color for a much longer period than that

of either the control group or the A.A. treated group of steaks. This was true even when the steaks were inoculated with a suspension of Ps. geniculata (Fig. 13). No effect of the treatments on the bacterial growth was evident.

Table 16

Effects of ascorbic acid and ascorbic acid plus sodium nicotinate treatments on the rate of discoloration and bacterial growth on steaks.*

Treatment and observations	Time in days					
	0	2	4	6	8	10
CONTROL						
Visual observation	red	bright red	red	red, brown edge	1/2 brown 1/2 purple	3/4 brown 1/4 purple
Index of fading	22.3	20.0	21.3	19.8	30.4	31.2
Log. No. bacteria	3.3	3.9	6.3	7.6	7.6	7.7
ASCORBIC ACID TREATED						
Visual observation	red	bright red	bright red	dark red	2/3 brown 1/3 purple	brown
Index of fading	22.9	17.6	17.9	23.3	29.6	33.8
Log. No. bacteria	4.2	4.0	6.1	6.7	8.7	7.8
ASCORBIC ACID PLUS Na NICOTINATE TREATED						
Visual observations	red	bright red	very bright red	very bright red	bright red	1/5 brown 4/5 red
Index of fading	20.7	17.4	10.4	15.7	19.6	25.2
Log. no. bacteria	3.8	3.5	6.6	7.3	8.4	8.5

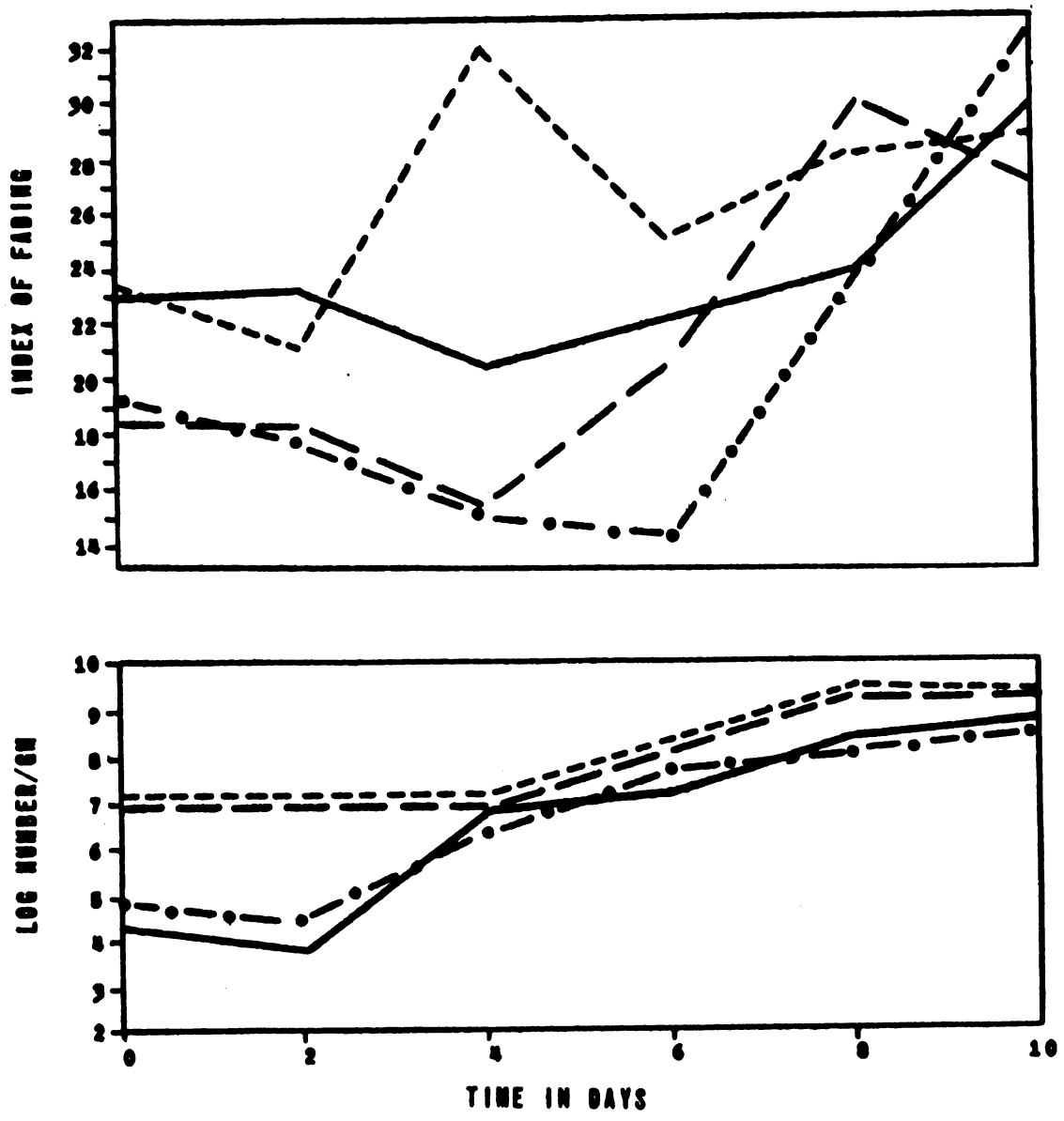
*Experiment conducted in a household type refrigerator.

Table 17

Effects of ascorbic acid and ascorlic acid plus sodium nicotinate treatments on the rate of discoloration and bacterial growth on steaks*.

Treatment and observations	Time in days 0	2	4	6	8	10
CONTROL						
Visual observations	bright red	very bright red	very bright red	red, brown tip	red, brown tip	2/3 brown 1/3 purple
Index of fading	17.5	14.6	15.5	22.3	19.4	27.8
Log. No. bacteria	5.0	4.5	7.1	8.2	7.9	9.1
ASCORBIC ACID TREATED						
Visual observations	bright red	very bright red	red	2/3 brown 1/3 red	5/6 brown 1/6 purple	brown
Index of fading	20.6	16.5	20.2	28.0	33.1	31.9
Log. No. bacteria	3.7	5.0	7.1	8.7	9.1	9.3
ASCORBIC ACID PLUS Na NICOTINATE TREATED						
Visual observations	bright red	very bright red	very bright red	very bright red	red, brown tip	2/3 brown 1/3 red-purple
Index of fading	19.4	15.0	15.5	15.0	18.3	29.4
Log. No. bacteria	3.8	3.5	6.6	7.3	8.4	8.5

*Experiment conducted in a self-service meat case.



COMPARISON OF THE EFFECTS OF A MIXTURE OF ASCORBIC ACID AND SODIUM NICOTINATE ON INOCULATED AND UNINOCULATED PREPACKAGED BEEF HELD AT 4°C

——— Control

- - - - - Inoculated

•—•—•— Mixture

— — — Inoculated + Mixture

FIGURE 13

DISCUSSION

The fact that of the various organisms tested only those organisms which have the ability to respire aerobically caused meat discoloration would indicate the importance of this system in pigment oxidation and reduction. Further, only those aerobic organisms which grow readily at low temperatures were found to cause discoloration of refrigerated prepackaged beef. These findings are in agreement with Ayres (1951a), Butler et al. (1953), and Mallech et al. (1958). These authors attributed meat discoloration to psychrophilic organisms belonging to the Achromobacter-Pseudomonas group. The assumption that the respiratory enzymes of the bacteria are active in causing pigment change is substantiated by two findings. First, the extracellular enzymes of Ps. penicillata and Ps. aeruginosa were found to be inactive, but the intracellular enzymes of these organisms brought about the discoloration of fresh meat at room temperature. The intracellular enzymes of both organisms also hastened the change of oxymyoglobin solutions. In both cases the action of these enzymes was primarily reduction, and only slight oxidative action was noted.

The second finding which helps to prove this assumption is that good correlation between steak discoloration and respiratory activity of the organisms present

on the steak surface was observed. As the number of organisms and their aerobic respiration increased, the steaks lost the bright red color of oxymyoglobin in favor of a dark red color. Upon further increase in respiration the meat pigment became brown. At this point the myoglobin pigment was in the oxidized form, but as the respiratory activity continued to increase, the oxidized pigment, metmyoglobin, was soon reduced to myoglobin. Pigment changes in this order have also been reported by Butler et al. (1953) and Halleck et al. (1958). Butler and his coworkers further observed correlation between surface discoloration of fresh prepackaged beef and the number of bacteria present, but they did not attempt to measure respiratory activity.

Various known enzyme inhibitors were employed in order to obtain better knowledge of which specific enzyme systems of Ps. <u>perniculata</u> are active in the reduction and oxidation of oxymyoglobin. The inability of 0.01 M sodium fluoride to inhibit the respiration of Ps. <u>perniculata</u> as measured by the Warburg was expected since at low concentrations sodium fluoride inhibits enolase only. This enzyme is not critical in respiration by this organism since it follows the pentose-shunt pathway. The inability to inhibit respiration was correlated with its inability to inhibit the action of Ps. <u>perniculata</u> on meat color. Sodium fluoride at a concentration of 0.1 M demonstrated CO_2 respiratory

inhibition and also decreased the rate of discoloration of beef steaks as caused by bacteria. Since phospholipases are inactivated by sodium fluoride at higher concentration, this was as predicted. Sodium malonate, iodoacetate, sodium azide, and potassium cyanide caused inhibition of the respiratory enzymes of Ps. fluorescens. The degree of of inhibition by each agent was well correlated with the ability of the inhibitor to preserve the bright red color of meat with the exception of sodium azide. Though sodium azide completely inhibited the oxygen uptake of both cells and cell-free extracts of Ps. fluorescens, it afforded no protection of oxymyoglobin. However, the pigment change which occurs in the presence of sodium azide is not due to bacteria, but is due to a chemical reaction between the globin pigment and this agent. This statement is made in light of the fact that bacteria treated with sodium azide and subsequently washed were unable to either reduce or oxidize oxymyoglobin.

The ability of sodium malonate ($5C_4$) to partially inhibit the respiratory enzymes of Ps. fluorescens was well correlated with its ability to preserve the brightness of prepackaged meat. Grant (1955a) noted that of the various inhibitors he tested, only malonic acid provided a bright red interior color of frozen ground beef. Since he neglected to make observations for bacterial activity and in light of the above observations, it is possible he

was observing the inhibition of bacterial enzymes as well as meat enzymes.

From the results obtained by the use of various enzyme inhibitors it is apparent that the enzymes involved in the aerobic respiration of Ps. aeruginosa are also involved in meat discoloration. The rate of microbial respiration which depends on the number of organisms under a given circumstance was found to be important not only in determining the rate of meat discoloration, but also in determining the type of discoloration reaction. That is, large populations of actively respiring organisms caused rapid color change on steak surfaces, but the change was primarily the reduction of oxymyoglobin to myoglobin. Smaller populations of the same organism caused discoloration at a slower rate but resulted not only in reduction but also in the oxidation. Thus, as respiration increased, the oxygen available at the surface of the prepackaged steaks became limited, and the steaks discolored. It appeared that the limitation of oxygen was not only responsible for pigment change, but also the type of change which occurred; i.e., oxidation or reduction. Results of various tests indicated that maximum pigment reduction occurred at storage oxygen pressure near 10 mm O_2 at 5°C. The pigment of meat tissue and small steaks handled aseptically was oxidized at oxygen tensions of 10 mm to 50 mm without the presence of bacteria. Due to the observation that the presence of bacteria did not hasten the oxidation process, it was

believed that they were inactive in this reaction. As the temperature increased, the maximum rate of oxidation was found to occur at higher oxygen tensions. These results agree with those of Brooks (1931, 1933, and 1938).

Having shown that oxidation occurred at lower oxygen tensions, two possible mechanisms were postulated. Both were based on the theory that bacteria cause pigment changes by lowering oxygen tensions. It is possible that at a critical point oxidation occurs, but on further aerobic metabolism the available O_2 is lowered still further and results in a strictly physiochemical pigment reduction. In short, this simply involves either a physical or biological limitation of O_2 which results in nonenzymatic pigment oxidation or reduction depending upon the degree of limitation. The second possibility is that the pigment is enzymatically oxidized at the reduced O_2 tension. At reduced oxygen atmospheres the activity of the cytochrome system of both the bacterial cells and the meat tissue would be greatly reduced while the action of any flavin system present would not be so greatly reduced. Thus, one might expect an accumulation of H_2O_2 which in the presence of peroxidase would oxidize the pigment.

Sodium azide and potassium cyanide poison the cytochrome system but have no inhibitory effects on flavin systems. Thus, if there was appreciable flavin enzyme activity in either cells or meat tissue slices at low oxygen tension, either NaN_3 or KCN would not inhibit respiration

to the same extent as at the higher oxygen levels. Results of such studies demonstrated that this was not the case with either cells of Ps. <u>graveolens</u> or with meat tissue slices. This, plus the fact that KCN treated cells would not cause either oxidation or reduction of prepackaged steaks held in normal or reduced oxygen atmospheres, casts considerable doubt on the validity of the enzymatic theory proposed.

Since catalase also acts as a peroxidase, it is possible that at reduced oxygen tensions catalase loses its ability to break down H_2O_2 to molecular oxygen and water, yet the peroxidase activity remains active. The peroxidase could then decompose H_2O_2 to water and atomic oxygen utilizing myoglobin as an electron donor. This action would result in the formation of metmyoglobin. However, as the oxygen is further limited, no H_2O_2 would be formed, and no further oxidation would then occur. Also, the oxidized pigment would be reduced in the absence of oxygen. Heat extracts were shown to have peroxidase activity, but all efforts to demonstrate the presence of H_2O_2 in meat undergoing oxidation were unsuccessful. However, H_2O_2 may be produced and subsequently broken down without being detected.

Glucose oxidase in dilute solutions brought about the oxidation of the surface pigment of steaks. However, in more concentrated solutions the pigment was changed to

choleglobin. This enzyme system produces H_2O_2 from the oxidation of glucose and has an active catalase present to break it down. Hydrogen peroxide (0.3%) was inactive on fresh steaks, but the pigment at the surface of aged steaks was readily oxidized to metmyoglobin. Dilute solutions of peroxidase caused the oxidation of meat pigments. This reaction could be hastened by the addition of H_2O_2; however, inactivated peroxidase solutions were also active. The rate difference was large enough to show that the enzyme was active in the oxidation reaction. More concentrated solutions of peroxidase plus H_2O_2 caused choleglobin formation. These results demonstrate the possibility of a peroxidase system causing pigment oxidation, but the inability to show the presence of free H_2O_2 in tissues where the pigment was undergoing oxidation or of enhanced flavin enzyme activity at low oxygen tensions made it impossible to conclusively prove that this mechanism was active. The study of this reaction was not pursued further since the purpose of this investigation was to study the role of bacteria in pigment oxidation and reduction, and this reaction was not directly caused by the bacteria.

There can be little doubt that the primary role of the bacteria in the color changes of fresh meat tissue is in the reduction of the oxygen level in the surface tissue. This is supported by a number of facts; viz., (a) pigment oxidation

and reduction can be controlled by physical adjustment of the oxygen level in the storage atmosphere in the absence of a significant number of bacteria, (b) the oxygen level in the storage atmosphere greatly affects the rate of pigment changes on both inoculated and uninoculated steaks, (c) oxygen uptake rate of the surface tissue of meat is directly correlated with color change; thus, the higher the numbers of aerobic organisms on a given piece of meat, the greater the oxygen demand and the more rapid the color change, (d) at intermediate levels of oxygen demand of surface tissue, oxidation to metmyoglobin occurs while with higher respiration rates, reduction to myoglobin occurs; and this is correlated with similar changes under controlled oxygen atmospheres, and (e) any agent inhibiting respiration will result in color preservation providing it does not chemically react with the pigment itself.

SUMMARY

All the aerobic organisms tested which included strains of Pseudomonas, Flavobacterium, Achromobacter, and one yeast, Saccharomyces cerevisiae had the ability to reduce oxymyoglobin at room temperature; however, only those organisms which grow readily at low temperatures were found to cause discoloration of refrigerated prepackaged beef. At room temperature, the intracellular enzymes of Ps. reticulata and Ps. aeruginosa caused discoloration of fresh meat, but the extracellular enzymes were inactive.

Good correlation between steak discoloration, number of bacteria present on the steak surface, and respiratory activity of these organisms was observed. The ability of various enzyme inhibitors to inhibit the respiratory activity of Ps. reticulata as measured by conventional Warburg techniques was correlated with the ability of these agents to preserve the "bloom" of both inoculated and control fresh prepackaged beef steaks. Sodium azide did not inhibit changes of oxymyoglobin even though it completely inhibited the respiratory enzymes of Ps. reticulata. This pigment change was considered to be due to a chemical reaction between oxymyoglobin and sodium azide and not due to bacteria.

The results show conclusively that bacteria lower the oxygen tension at the surface of steaks, and the lowering of oxygen tensions results in the discoloration of the steaks. The type of pigment change which can be observed depends upon the rate of free oxygen removal from the surface area; i.e., slow reduction of oxygen tensions resulted in pigment oxidation, and fast reduction resulted in pigment reduction. The pigment of meat tissue was found to be oxidized at reduced oxygen tension (10 mm to 50 mm Hg.) without the presence of bacteria. Therefore, it appears that the main function of the bacteria is to lower the oxygen level, and the pigment is then oxidized or reduced either by non-enzymatic or enzymatic reactions which may occur in the absence of microorganisms.

A study of the possible mechanisms involved in pigment oxidation demonstrated that there was not a high activity of flavin enzyme system at low oxygen tensions resulting in the formation of H_2O_2 either with cells of Ps. geniculata, beef tissue slices or homogenates. Catalase and peroxidase activity was shown to exist in meat and meat homogenates. Hydrogen peroxide (0.3%) was essentially unable to oxidize the pigment at the surface of fresh beef steaks, or oxymyoglobin solutions obtained from fresh meat; however, when added to aged steaks or oxymyoglobin solutions from aged steaks, oxidation was apparent. Dilute glucose oxidase had

the ability to oxidize oxyhemoglobin to methemoglobin, but more concentrated glucose oxidase caused the formation of cholehemoglobin, a green pigment. Heat inactivated glucose oxidase brought about pigment oxidation, but the reaction was much slower than with the active enzyme. Dilute solutions of peroxidase caused the oxidation of meat pigments and this reaction was hastened by the addition of H_2O_2. Inactivated peroxidase solutions were also found to have some activity. As applied to steel surfaces the rate difference was large enough to show that the enzyme was active in the oxidation reaction. More concentrated solutions of peroxidase plus 0.3% H_2O_2 cause cholehemoglobin formation. These results indicate the possibility that peroxidase may be important in pigment oxidation. Further work is indicated on this phase of the problem.

APPENDIX

Appendix

Table 1

The Effects of Various Organisms on the Pigment of Prepackaged Beef Held at Room Temperature

Time in Hours	0	1/2	1	2	3
Organisms					
Pseudomonas aeruginosa	bright red	dark red-purple	purple-brown	brown	brown-purple
fluorescens	bright red	red purple	purple-brown	purple	purple
geniculata	bright red	dark red purple	purple-brown	purple	purple
species	bright red	dark red purple	brown	purple	purple
Achromobacter liquefaciens	bright red	dark red-purple	purple brown	purple	purple
Flavobacterium rhenanus	bright red	dark red	dark red	dark red	purple-brown
Lactobacillus plantarum	bright red	bright red	red	red	red
Saccharomyces cerevisiae	bright red	dark red-purple	brown-purple	purple	purple
Control	bright red	red	red	red	red

Appendix

Table 2

The Effects of Various Organisms on the Pigment of Prepackaged Beef Held at 4°C

Time in Days	0	1/2	1	2	3	4
Organisms						
Pseudomonas aeruginosa	brown-purple	dark red	brown	brown-purple	purple	purple
fluorescens	purple	dark red	red-purple	red-purple	purple-brown	purple
geniculata	purple	dark red	red-purple	brown-purple	purple	purple
species	purple	dark red	red-purple	brown-purple	purple	purple
Achromobacter liquefaciens	purple	dark red	dark red	dark red	purple-brown	purple
Flavobacterium rhenanus	dark red	red	red	dark red	red-purple	purple
Lactobacillus plantarum	red	red	bright red	bright red	bright red	bright red
Saccharomyces cerevisiae	brown-purple	red-purple	red-purple	red-purple	dark red	red
Control	red	red	bright red	bright red	red	red

Appendix

Table 3

Effect of Various Levels of Ps. geniculata on Prepackaged Beef Held at 4°C.

Time in Days/Hrs.	Inoculation levels[1]				
	1	0.5	0.25	0.12	Control
0	slightly dark red	red	bright red	bright red	bright red
1/2 hr.	dark red	red	bright red	bright red	bright red
1 hr.	dark red	dark red	bright red	bright red	bright red
4 hr.	dark red	dark red	bright red	bright red	bright red
12 hr.	dark red-purple-brown	dark red-purple-brown	bright red	bright red	bright red
1 day	purple-brown	purple-brown	red	bright red	bright red
2 days	purple-brown	purple-brown	dark-red brownish	dark-red brownish	bright red
3 days	purple	purple-brown	brown	brown	bright red
4 days	purple	purple	3/4 purple 1/4 brown	1/2 brown 1/2 purple	bright red
5 days	purple	purple	purple	1/4 brown 3/4 purple	red
6 days	purple	purple	purple	purple	dark red
7 days	purple	purple	purple	purple	dark red-brown

[1] A cell suspension was diluted before inoculation to give the relative levels noted.

Appendix

Table 4

Effects of Cells and Intracellular Enzymes of Ps. fenículata on Prepackaged Beef Held at Room Temperature

Time in Hours	Treatment / Observations	Control				Inoculated				Intracellular Enzyme			
		Visual	Log No. Bact.	F.I.	% Met.	Visual	Log No. Bact.	F.I.	% Met.	Visual	Log No. Bact.	F.I.	% Met.
0		bright red	4.5	19.5	1.0	sl. dark red	9.7	22.3	1.63	bright red	--	19.6	2.5
2		bright red	4.7	19.9	2.3	dark red-purple	9.2	27.16	1.0	sl. dark red	9.2	21.5	3.3
5		bright red	4.5	20.0	1.96	dark red-purple	9.4	26.3	1.16	dark red sl. purple	4.5	23.85	5.63
9		red	4.6	20.72	5.13	purple	9.3	28.5	0.0	dark red purple	9.4	24.8	7.85
16		red brown-edges	6.4	20.45	20.55	purple	9.3	28.1	0.0	purple sl. brown	7.4	25.46	19.9
24		red brown-edges	7.9	20.08	28.23	purple	9.6	28.31	2.15	purple sl. brown	7.7	25.56	19.45

Appendix

Table 5

The Effects of Ps. geniculata, Ps. aeruginosa, and a Cell-free Preparation of the Intracellular Enzymes of These Organisms on the Color of Fresh Prepackaged Beef*.

Time in Hours	Control	Ps. geniculata	Ps. aeruginosa	Enzymes of Ps. geniculata	Enzymes of Ps. aeruginosa
0	bright red	bright red	bright red	bright red	bright red
1	bright red	bright red	dark red	slight dark red	slight dark red
2	red	dark red	dark red	dark red	dark red
12	red	purple-brown	purple-red	dark red	dark red
18	red	purple	purple-brown	dark red brown areas	dark red brown areas
24	1/3 red 2/3 brown	purple	purple-brown	3/4 brown 1/4 purple	2/3 brown 1/3 purple

* Experiment was performed at room temperature.

Appendix

Table 6

Effects of Ps. geniculata and Aureomycin on Meat Homogenates Held at 30°C.

Time in Hours	Shaken						Standing	
	Control		Inoculated		Aureomycin		Control	
	% met.	% myo.	% met.	% myo.	% met.	% myo.	% met.	% myo.
0	14	1	15	1	19	4	19	2
6	35	0	35	0	40	0	16	0
9	46	0	51	0	56	0	41	0
48	15	1	24	18	90	0	27	0
Visual observations at 48 hours	green		green		straw colored		red	

Appendix

Table 7

The Effects of the Intracellular Enzymes and Cells of Ps. geniculata and Ps. aeruginosa on the Rate of Pigment Change of Oxymyoglobin Extracts.*

Time in	Control		Ps. geniculata		Ps. aeruginosa		Enzymes of Ps. geniculata		Enzymes of Ps. aeruginosa	
minutes	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.
before	2	0	0	0	0	0	0	0	1	0
0	0	0	22	25	24	24	16	0	19	0
5	0	0	20	28	24	28	15	0	22	11
10	0	0	21	28	27	26	12	0	22	12
15	0	0	28	64	31	46	14	1	24	12
30	0	0	36	60	39	50	11	0	27	7
45	5	0	35	63	41	49	13	0	25	14
60	3	0	35	65	44	55	13	4	29	17
120	9	0	35	65	44	52	19	7	38	19
180	12	0	35	65	42	51	28	10	41	16

*Oxymyoglobin solutions were held at room temperature.

Appendix

Table 8

Effects of *Ps. geniculata* on Prepackaged Beef Held at 4° C.

Time in Days	Control				Inoculated			
	Visual Observation	Log No. Bacteria	Fading Index	ul O_2 Uptake per 30 Mins.	Visual Observation	Log No. Bacteria	Fading Index	ul O_2 Uptake per 30 Mins.
0	bright red	<1	14.1	3.63	bright red	7.6	16.5	2.62
2	bright red	4.7	14.8	2.42	bright red brown edge	6.9	17.5	11.79
4	bright red	5.2	14.4	9.68	brown-purple	8.5	27.75	19.65
5	--	--	--	--	brown	8.7	27.50	38.72
7	bright red brown edges	7.8	15.9	19.36	--	--	--	--
8	bright red	7.3	19.5	18.1	1/3 brown 2/3 purple	9.42	29.5	--
11	brown-purple	8.5	26.3	20.57	purple	9.4	28.9	35.38

Appendix

Table 9

The Effects of Various Levels of Aureomycin Applied to the Surface of Prepackaged Beef Held at 4°C.

Time in Days	Aureomycin 100 ppm Visual P.I.	Aureomycin 100 ppm Log no. Bact.	Aureomycin 50 ppm Visual P.I.	Aureomycin 50 ppm Log no. Bact.	Aureomycin 25 ppm Visual P.I.	Aureomycin 25 ppm Log no. Bact.	Aureomycin 5 ppm Visual P.I.	Aureomycin 5 ppm Log no. Bact.	Control Visual P.I.	Control Log no. Bact.
0	bright red 17.3	4.75	bright red 19.0	4.77	bright red 17.3	3.6	bright red 20.3	3.95	bright red 22.1	4.71
2	bright red 22.9	3.7	bright red 25.7	4.52	bright red 23.1	4.11	bright red 24.3	4.98	bright red 23.3	6.34
5	bright red 19.0	4.48	bright red 22.3	5.94	bright red 19.0	6.46	bright red 16.7	6.99	bright red 21.1	8.19
10	bright red brown tip 15.7	6.64	2/3 red 1/3 brown 24.2	8.14	brown 33.3	8.71	brown 31.9	8.96	brown 33.1	9.43

Appendix

Table 10

Effect of Aureomycin (100 ppm) on Fresh Prepackaged Beef Held at 4°C.

Time in Days	Aureomycin			Control		
	Visual	F.I.	Log T.C. bacteria	Visual	F.I.	Log T.C. bacteria
0	red	25.6	4.2	red	26.2	4.56
2	bright red	22.1	4.71	bright red	19.8	6.64
4	bright red	21.3	4.48	sl. dark red	24.2	7.58
6	bright red	22.5	5.51	dark red-brown	30.4	9.05
8	1/3 dark red 2/3 brown	26.33	5.61	brown	30.7	9.26
12	brown	32.2	6.65	brown	32.05	10.31
15	brown	32.2	6.65	brown	31.65	9.76

Appendix

Table 11

Effect of 1% solution of sorbic acid and sodium sorbate on fresh prepackaged beef held at 4°C.

Time in Days	Sorbic Acid			Sodium Sorbate			Control		
	Visual	F.I.	Log No. bacteria	Visual	F.I.	Log No. bacteria	Visual	F.I.	Log No. bacteria
0	red	24.2	5.13	red	24.2	5.26	bright red	21.7	4.71
2	red	23.5	7.18	red	25.2	6.26	bright red	21.3	6.34
5	brown	30.26	8.35	red	22.7	7.94	bright red	21.1	8.18
10	brown	34.6	9.51	brown	33.1	9.34	brown	33.1	9.43

Appendix

Table 12

Comparison of Effect of Aureomycin (100 ppm) and Na Malonate (20%)* Applied to Prepackaged Beef Held at 4°C.

Time in Days	Treatments / Observations	Control				Na malonate				Aureomycin			
		Log no. /ct.	M.I.	% Met.	µl O_2 Utile	Log no. /ct.	M.I.	% Met.	µl O_2 Utile	Log no. /ct.	M.I.	% Met.	µl O_2 Utile
0		<1	1.9	10.6	--	<1	20.74	11.6	6.55	<1	20.6	12.8	1.65
1		<1	15.8	2.4	9.08	<1	18.75	5.1	6.60	<1	17.3	0.42	2.62
3		3.70	17.76	3.1	4.84	3.0	19.1	4.7	3.2	4.2	17.6	3.3	2.62
5		4.04	17.6	4.1	7.22	3.17	19.1	5.0	3.95	3.17	16.7	1.7	2.62
7		7.89	17.38	16.5	19.30	5.70	16.1	17.2	0.00	3.70	16.54	7.1	0.00
9		7.89	21.9	34.0	25.41	7.39	21.0	32.5	7.85	4.48	17.4	6.9	0.00
10		8.42	20.4	43.9	36.72	7.70	21.3	40.3	9.90	5.35	17.38	10.7	5.24
12		9.26	31.55	69.9	68.97	9.0	30.9	67.3	39.60	5.88	21.88	16.9	2.62

* Both Aureomycin and Na malonate were buffered at pH 5.8 with 0.1 M phosphate buffer

Appendix

Table 13

Effect of Iodoacetate (1/10) on Inoculated Prepackaged Beef Steaks Held at 4°C.

Time in Days	Control					Inoculated					Inoculated + Iodoacetate				
	Visual	F.I.	% Met.	µl O_2 Uptake per 30 Min.	Log no. Bact.	Visual	F.I.	% Met.	µl O_2 Uptake per 30 Min.	Log no. Bact.	Visual	F.I.	% Met.	µl O_2 Uptake per 30 Min.	Log no. Bact.
0	bright red	19.9	4	5.32	1.15	dark red	29.9	8	26.1	6.0	bright red	20.4	6	1.46	7.18
4	bright red sl. dark edges	19.9	23	2.96	7.31	purple	31.5	4	6.5	6.88	dark red	22.0	38	1.46	6.95
7	dark red brown edge	22.4	45	31.2	8.13	purple	27.6	8	14[illegible].6	9.11	dark red brown edge	23.1	55	16.7	8.0
10	brown	29.05	58	62	9.05	purple	28.4	3	113.1	9.9	brown	31.3	65	34.8	8.19

Appendix

Table 14

Effects of KCN*, NaN_3** and Ps. penicillata Inactivated with KCN and NaN_3 on Fresh Prepackaged Beef Held at 4°C.

Time in Days	Control	KCN	NaN_3	Ps. penicillata plus KCN	Ps. penicillata plus NaN_3	Ps. penicillata KCN inactivated	Ps. penicillata NaN_3 inactivated
0	bright red	bright red	bright red	bright red	bright red	bright red	bright red
1	bright red	bright red	dark red	bright red	dark red	bright red	bright red
2	red	bright red	purple red	bright red	purple red	bright red	bright red
4	dark red	bright red	purple brown	bright red	purple brown	dark red	dark red
6	dark red	bright red	purple brown	bright red	purple brown	1/2 dark red 1/2 brown	brown
10	brown	bright red	purple brown	red brown edge	purple brown	1/2 brown 1/2 purple	1/3 brown 2/3 purple

* 0.1 molar KCN
** 0.01 molar NaN_3

Appendix

Table 15

The Effects of Cells of Ps. graniculata Treated with TCA on the Color of Prepackaged Beef at Various Oxygen Tensions Held at 4° C.

Time in Days	10 mm O_2			20 mm O_2			Normal		
	Cells	Cells + TCA	Control	Cells	Cells + TCA	Control	Cells	Cells + TCA	Normal
before	bright red	bright red	bright red	bright red	bright red	bright red	bright red	bright red	bright red
0	dark red	red	red	red	red	red	bright red	bright red	bright red
1	red-purple	red-purple	red-purple	red	red	red	bright red	bright red	bright red
2	purple-brown	purple-brown	purple brown	dark red	dark red	dark red	dark red	bright red	bright red
3	purple	purple	purple	red-purple	red-purple	red-purple	red-brown	bright red	bright red
5	purple	purple-brown	purple-brown	purple	purple-brown	purple-brown	red-brown	bright red	bright red
6	purple	purple-brown	brown	purple	purple-brown	brown	purple	bright red	bright red

Appendix

Table 16

The Effects of Iodoacetate and Na Azide on Inoculated Prepackaged Steaks Held at Room Temperature

Time in Min.	Index of Fading			
	Control	Inoculated	Inoculated plus Azide	Inoculated plus Iodoacetate
0	22.6	26.6	22.6	23.7
15	23.9	27.7	24.4	24.2
30	24.4	28.6	27.6	25.2
45	24.4	28.6	26.6	25.9
60	23.6	28.9	27.6	26.6
90	24.4	27.8	26.9	27.5
120	25.4	30.6	28.9	27.5

Appendix

Table 17

The Effects of Iodoacetate and Na Azide on Inoculated Steaks Held at Room Temperature

Time in Min.	Index of Fading			
	Control	Inoculated	Inoculated plus Azide	Inoculated plus Iodoacetate
unwrapped				
0	22.0	19.9	20.1	19.8
30	20.1	18.5	20.1	20.1
60	16.7	22.1	19.9	19.9
wrapped				
90	20.4	29.4	27.3	25.4
120	25.2	29.4	29.0	26.0

Appendix

Table 18

Effects of Active and Inactivated Cells of Ps. reniculata on Myoglobin Pigment Extracts Held at Room Temperature

Time in Min.	Control		Active Cells		Cells Inactivated by Heat		Cells Inactivated with KCN	
	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.
0	13	0	13	0	13	0	13	0
10	10	0	13	0	12	0	10	0
20	14	0	16	0	13	0	10	0
30	11	0	16	0	9	0	10	0
45	14	0	16	0	14	0	11	0
60	14	0	23	10	17	2	16	0
240*	19	0	25	63	15	7	17	15
300*	25	0	22	64	19	9	19	2

* Centrifuge tubes were stoppered in order to keep the pigment solutions from coming in contact with air.

Appendix

Table 19

The Effects of Sodium Fluoride* and Iodoacetate**
on the Respiratory Activity of Cells and Intracellular Enzymes of Is. periculata

Time in Min.	Accumulative Microliters of O_2 Uptake						
	Control	Enzyme	Cells	Enzyme Plus NaF	Cells Plus NaF	Enzyme Plus Iodoacetate	Cells Plus Iodoacetate
5	0.0	2.84	7.85	4.72	7.4	---	4.8
10	0.0	8.52	17.27	4.72	16.28	1.51	15.24
15	1.56	15.62	36.07	12.57	31.08	4.53	24.12
20	1.56	22.72	54.91	20.42	46.83	10.57	37.42
25	3.12	29.82	75.3	26.76	63.63	15.1	49.22
30	4.68	35.5	97.3	29.64	87.3	19.6	58.1
35	6.42	42.6	119.3	37.69	97.7	22.65	69.9
40	6.24	46.86	141.3	42.41	114.0	25.67	78.78
45	6.24	52.54	166.4	46.69	131.7	30.02	90.58
50	6.24	56.8	191.5	53.41	149.4	31.61	99.46
55	7.80	62.48	213.5	61.26	165.7	36.14	109.9
60	9.36	66.74	241.1	67.54	187.2	40.67	120.3

* Sodium fluoride was 10^{-3} molar
** Iodoacetate was 10^{-3} molar

Appendix

Table 20

The Effect of Sodium Malonate* and Sodium Azide** on the Respiratory Activity of Cells and Intracellular Enzymes of Ps. aeruginosa

Time in Min.	Cumulative Microliters of O_2 Uptake						
	Control	Enzyme	Cells	Enzyme Plus Malonate	Cells Plus Malonate	Enzyme Plus Azide	Cells Plus Azide
5	1.56	1.42	23.6	3.14	19.2	1.51	1.48
10	1.56	5.68	45.6	7.85	57.6	4.53	2.99
15	3.12	9.94	103.7	9.42	105.6	4.53	7.40
20	3.12	11.36	141.1	10.99	130.2	4.53	10.36
25	4.68	14.2	180.6	15.7	176.0	4.53	16.28
30	4.68	18.46	241.8	17.27	233.7	5.04	22.2
35	4.68	21.30	277.9	21.18	256.9	7.59	25.16
40	7.8	25.56	336.0	23.55	300.3	7.06	31.08
45	9.36	29.82	392.6	26.69	385.5	10.57	35.52
50	9.36	32.66	444.3	31.40	412.9	10.57	39.96
55	9.36	34.08	496.1	32.97	460.3	10.57	42.92
60	10.92	36.34	559.7	34.11	504.7	10.57	47.36

* Sodium malonate was 10^{-2} molar
** Sodium azide was 10^{-2} molar

Appendix

Table 21

The Effect of Sodium Malonate* and Sodium Fluoride** on the Respiratory Activity of Cells and Intracellular Enzymes of *H. pulcherrima*

Time in Min.	Accumulative Microliters of O_2 Uptake						
	Control	Enzyme	Cells	Enzyme Plus Na Fluoride	Cells Plus Na Fluoride	Enzyme Plus Na Malonate	Cells Plus Na Malonate
5	0	2.84	25.1	1.91	12.1	3.14	8.88
10	0	8.32	67.5	4.53	34.7	6.28	20.63
15	1.56	12.78	109.9	6.04	69.4	7.28	47.33
20	1.56	18.46	155.4	9.14	101.1	7.85	78.43
25	3.12	22.72	191.5	13.16	122.3	10.99	99.13
30	3.12	25.50	244.9	15.18	154.0	14.13	131.7

* Sodium Malonate was 10^{-1} Molar
** Sodium Fluoride was 10^{-1} Molar

Appendix

Table 22

The Effect of KCN* and NaN_3** on the Respiratory Activity of Cells of Ps. penicillata

Time in Min.	Accumulative Microliters of O_2 Uptake			
	Endogenous	Cells	Cells plus NaN_3	Cells plus KCN
5	1.62	59	0.0	0.0
10	4.86	97.8	3.08	0.0
15	8.10	150.4	6.16	1.46
20	12.96	210.9	10.78	5.84
25	16.2	250.9	12.32	7.30
30	17.82	289.9	13.4	8.76
35	21.06	342.6	16.94	11.68
40	24.3	394.2	20.02	14.60
45	25.92	441.8	23.1	16.06
50	29.16	494.4	26.18	17.52
55	30.78	547.0	29.26	18.98
60	33.9	599.6	33.9	23.6

* KCN was 10^{-2} Molar
** NaN_3 was 10^{-3} Molar

Appendix

Table 23

The Effect of KCN* on the O_2 Uptake by Cells of Ps. geniculata at Various Oxygen Tensions

Time in min.	Normal		N_2		1%O_2 + 99%N_2		5%O_2 + 95%N_2		10%O_2 + 90%N_2	
	Cells	Cells + KCN	Cells	Cells + KCN	Cells	Cells + KCN	Cells	Cells + KCN	Cells	Cells + KCN
5	3.42**	1.46	1.4	1.54	0.0	0.0	4.47	0.0	6.1	0.0
10	16.19	5.84	4.2	3.08	1.59	0.0	22.37	3.08	24.28	0.0
15	21.05	5.84	5.6	4.62	1.59	0.0	25.35	3.08	31.88	0.0
20	30.77	7.2	8.4	6.16	4.77	0.0	40.25	3.08	47.08	0.0
25	37.25	7.2	12.6	7.6	6.36	0.0	47.7	4.62	59.23	0.0
30	43.73	7.2	14.0	7.6	6.36	0.0	56.65	4.62	69.83	0.0

* Final concentration of KCN was 6.6×10^{-3} molar

** µl of O_2 uptake are recorded

Appendix

Table 24

Effects of Sodium Azide and Potassium Cyanide on the Respiratory Activity of Beef Tissue

	Normal Atmosphere			1% Oxygen and 99% Nitrogen		
	Control	NaN_3	KCN	Control	NaN_3	KCN
$\mu l\ O_2$/hr./0.1 gm.	23.8	6.36	5.87	24.6	3.53	4.45

Appendix

Table 25

Effects of Hydrogen Peroxide on Fresh and Aged Myoglobin Extracts Held at Room Temperature

Time in Min.	Fresh Preparation		Fresh Preparation+H_2O_2		Aged Preparation		Aged Preparation+H_2O_2	
	% met.	% myo.	% met.	% myo.	% met.	% myo.	% met.	% myo.
before addition	3	0	3	0	6	0	5	0
0	9	0	12	0	21	0	50	0
15	10	0	19	0	9	0	39	0
30	7	0	22	0	7	0	42	6
60	9	0	19	0	12	0	40	0

Appendix

Table 26

The Effects of Hydrogen Peroxide, Glucose Oxidase, and a Combination of Both on the Surface Color of Unwrapped Steaks Held at Room Temperature

Time in Min.	Hydrogen Peroxide	Glucose Oxidase	Glucose Oxidase + Hydrogen Peroxide	Heat-Inactivated Glucose Oxidase	H_2O_2+ Heat-Inactivated Glucose Oxidase
before addition	red	red	red	red	red
0	red	dark red	dark red	red	red
15	red	red brown	brown	red	red
30	red	dark brown	dark brown	red	red
60	red	dark brown	dark brown	red	red
120	red	dark brown	dark brown	red	red

Appendix

Table 27

Effects of Active and Heat-Inactivated Glucose Oxidase and Peroxidase on Myoglobin Extracts Held at Room Temperature

Time in	Control		H_2O_2		Glucose Oxidase		Heated Glucose Oxidase		Peroxidase		Heated Peroxidase	
Mins.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.
0	0	0	3	0	0	0	0	0	2	0	0	0
30	5	0	19	0	66	11	19	0	26	0	25	0
60	9	0	23	3	83	14	34	0	38	0	40	0

Appendix

Table 28

Effects of Nitrogen and Oxygen Atmospheres on Uninoculated Prepackaged Beef Held at 4°C.

Atmospheres and observations	Time in days				
	0	2	4	6	11
NORMAL					
Visual observation	bright red	bright red	bright red	red	2/3 brown 1/3 purple
Index of fading	19.5	17.3	18.0	20.3	29.1
% Metmyoglobin	21.3	5.4	32.2	32.5	35.6
Log No. bacteria	5.3	3.0	4.5	7.6	9.7
OXYGEN					
Visual observation	very bright red	very bright red	very bright red	bright red	1/3 brown 1/2 purple 1/6 red
Index of fading	---	16.2	15.9	15.9	30.9
% Metmyoglobin	---	20.5	19.4	24.7	32.2
Log No. bacteria	---	3.0	4.9	6.7	10.4
NITROGEN					
Visual observation	dark red	purple	brown-purple	brown	purple-brown
Index of fading	---	25.6	27.9	26.8	25.4
% Metmyoglobin	---	26.8	28.2	50.3	16.8
Log No. bacteria	---	3.0	4.5	7.3	8.5

Appendix

Table 29

Effects of Nitrogen and Oxygen Atmospheres on Inoculated* Prepackaged Beef Held at 4°C.

Atmospheres and observations	Time in days				
	0	2	4	6	11
NORMAL					
Visual observations	bright red	bright red	5/6 brown 1/6 dark red	brown-purple	purple
Index of fading	17.3	17.4	25.2	31.6	29.1
% Metmyoglobin	22.8	10.9	31.6	25.6	3.6
Log No. bacteria	6.5	7.4	9.2	9.6	10.1
OXYGEN					
Visual observations	very bright red	very bright red	red	brown-purple	purple
Index of fading	---	14.3	18.8	28.5	31.8
% Metmyoglobin	---	19.3	17.9	20.3	7.4
Log No. bacteria	---	7.4	9.3	9.9	10.3
NITROGEN					
Visual observations	dark red	purple	purple	purple	purple
Index of fading	---	24.2	28.8	27.4	23.3
% Metmyoglobin	---	24.9	20.8	14.3	7.9
Log No. bacteria	---	7.2	7.2	8.9	9.2

*Inoculated with Pseudomonas geniculata

Appendix

Table 30

Effect of Various Oxygen Atmospheres on the Color of Prepackaged Beef Steaks Held at 4°C.

Time in hours (days)		Atmospheres				
		No Oxygen	10 % O_2	75 % O_2	Normal	All O_2
0		dark red	red	bright red	bright red	very bright red
1/2		dark red-purple	red	bright red	bright red	very bright red
1		purple-dark red	red	bright red	bright red	very bright red
1-1/2		purple	red	bright red	bright red	very bright red
12	(1/2)	purple	dark red	red	bright red	very bright red
24	(1)	purple	dark red	red	bright red	very bright red
36	(1-1/2)	purple	brown	red	bright red	very bright red
96	(4)	purple	brown	dark red	bright red	very bright red
144	(6)	purple	brown	1/2 dark red 1/2 brown	red	very bright red
216	(9)	purple	brown	brown	1/2 dark red 1/2 brown	dark red brown edge
264	(11)	purple	brown	brown	1/6 red, 1/6 brown 1/6 purple	3/6 dark brown 1/6 brown
336	(14)	purple	brown	brown	brown	1/3 dark red 2/3 brown
384	(16)	purple	brown	1/3 brown 1/3 purple	1/2 brown 1/2 purple	brown purple edge

Appendix

Table 31

Effect of Various Oxygen Atmospheres on the Color of Inoculated* Prepackaged Beef Steaks Held at 4°C.

Time in Hours (Days)	Atmospheres				
	No O_2	25% O_2	75% O_2	Normal	All O_2
0	dark red	dark red	dark red	bright red	very bright red
1/2	purple-dark red	dark red	dark red	red	very bright red
1	purple-dark red	dark red	dark red	dark red	very bright red
1-1/2	purple	dark red	dark red	dark red	very bright red
12 (1/2)	purple	dark red	dark red	dark red	very bright red
24 (1)	purple	purple-dark red	purple-dark red	purple-dark red	very bright red
36 (1-1/2)	purple	purple	purple	purple-brown	dark red sl. brown edge
96 (4)	purple	purple	purple	purple	purple-dark red
144 (6)	purple	purple	purple	purple	purple
216 (9)	purple	purple	purple	purple	purple
264 (11)	purple	purple	purple	purple	purple
336 (14)	purple	purple	purple	purple	purple
384 (16)	purple	purple	purple	purple	purple

* Inoculated with Pseudomonas putrefaciens

Appendix

Table 32

Effect of Nitrogen and Oxygen Atmospheres on Uninoculated Prepackaged Beef Steaks Treated with Various Bacterial Inhibitors and Held at 4°C.

Time in Days	Treatments	Atmospheres				
		All N_2	10 mm O_2	75 mm O_2	Normal	All O_2
0	NOR	dark red	dark red	dark red	red	bright red
	Aureomycin	dark red	dark red	dark red	red	bright red
	Iodoacetate	dark red	dark red	dark red	red	bright red
	Control	dark red	dark red	dark red	red	bright red
1/2	NOR	dark red-purple	dark red	red	bright red	bright red
	Aureomycin	dark red-purple	dark red	red	bright red	bright red
	Iodoacetate	dark red-purple	dark red	red	bright red	bright red
	Control	dark red-purple	dark red	red	bright red	bright red
1	NOR	dark red-purple	dark red-purple	red	bright red	bright red
	Aureomycin	dark red-purple	dark red-purple	red	bright red	bright red
	Iodoacetate	dark red-purple	dark red-purple	red	bright red	bright red
	Control	dark red-purple	dark red-purple	red	bright red	bright red
3	NOR	purple	dark red-purple	red	bright red	bright red
	Aureomycin	purple	purple-brown	red	bright red	bright red
	Iodoacetate	purple	purple-brown	red	bright red	bright red
	Control	purple	purple-brown	red	bright red	bright red
5	NOR	purple	purple-red	red	red	bright red
	Aureomycin	purple	brown-purple	red	red	bright red
	Iodoacetate	purple	brown-purple	red	red	bright red
	Control	purple	brown-purple	red (brown edge)	2/3 red 1/3 brown	bright red

Table 32 (Continued)

Time in Days	Treatments	Atmospheres				
		All N_2	10 mm O_2	75 mm O_2	normal	All O_2
7	N.C.	purple	purple-red	red	dark red	bright red
	Aureomycin	purple	brown	red	bright red	bright red
	Iodoacetate	purple	brown	brown-red	brown-dark red	bright red
	Control	purple	brown	brown-purple	brown	red (purple edge)
10	N.C.	purple	purple-red	red	red-brownish areas	red
	Aureomycin	purple	brown	red	red	red
	Iodoacetate	purple	brown	1/3 red 2/3 brown	purple	red
	Control	purple	(purple edge)	purple	purple	purple
12	N.C.	-	purple-red	dark red	purple	dark red
	Aureomycin	-	brown-purple	brown	red-brown tip	red
	Iodoacetate	-	brown-purple	2/3 brown 1/3 purple	purple	purple
	Control	-	brown-purple	purple	purple	purple
14	N.C.	-	purple	dark red	purple	dark red-purple tip
	Aureomycin	-	purple-brown	brown	1/2 brown 1/2 purple	red
	Iodoacetate	-	purple-brown	purple	purple	purple
	Control	-	purple	purple	purple	purple-brown tip
17	N.C.	-	purple	purple red	purple	1/4 red 3/4 purple
	Aureomycin	-	purple-brown	brown	1/2 brown 1/2 purple	red-brown spots
	Iodoacetate	-	purple-brown	purple	purple	purple
	Control	-	purple	purple	purple	purple

Appendix

Table 33

Effect of Nitrogen and Oxygen Atmospheres on Uninoculated, Unpackaged Beef Steaks Treated with Various Bacterial Inhibitors and Held at 4°C.

Time in Days	Treatments	Atmospheres				
		All N_2	10 mm O_2	75 mm O_2	Normal	All O_2
0	Aureomycin	purple	dark red	red	bright red	bright red
	Iodoacetate	purple	dark red	red	bright red	bright red
	Control	purple	dark red	red	bright red	bright red
1/2	Aureomycin	purple	dark red	red	bright red	bright red
	Iodoacetate	purple	dark red	red	bright red	bright red
	Control	purple	dark red	red	bright red	bright red
1	Aureomycin	purple	red	bright red	bright red	bright red
	Iodoacetate	purple	red	bright red	bright red	bright red
	Control	purple	red	bright red	bright red	bright red
3	Aureomycin	dark purple	dark red	bright red	bright red	bright red
	Iodoacetate	dark purple	dark red-brownish	bright red	bright red	bright red
	Control	dark purple	dark red	bright red	bright red	bright red
5	Aureomycin	dark purple	red-brown	bright red	bright red	bright red
	Iodoacetate	dark purple	brown	red	bright red	bright red
	Control	dark purple	brown	red	bright red	bright red
7	Aureomycin	dark purple	brown	bright red	bright red	bright red
	Iodoacetate	dark purple	brown	dark red	red	bright red
	Control	dark purple	brown	dark red	dark red (brown edge)	bright red

Appendix

Table 33 (Continued)

Time in Days	Treatments	Atmospheres				
		All N_2	10 mm O_2	75 mm O_2	Normal	All O_2
8	Aureomycin	dark purple brown		bright red	bright red	bright red
	Iodoacetate	dark purple brown		dark red	dark red	red
	Control	dark purple brown		dark red-brown spot	dark red	red
9	Aureomycin	dark purple brown		red	bright red	bright red
	Iodoacetate	dark purple brown-purple edge		dark red	dark red	red
	Control	dark purple brown-purple edge		1/3 red-1/3 purple 1/3 brown	2/3 dark red 1/3 purple	dark red
10	Aureomycin	dark purple brown		red	bright red	bright red
	Iodoacetate	dark purple brown-purple edge		dark red-brownish	dark red	dark red
	Control	dark purple brown-purple edge		1/2 purple 1/2 brown	1/2 dark red 1/2 purple	dark red
12	Aureomycin	dark purple brown		red	dark red	bright red
	Iodoacetate	dark purple brown-purple edge		purple-brown	dark red	dark red
	Control	dark purple brown-purple edge		purple-brown	1/2 dark red 1/2 purple	dark red-brown edge
14	Aureomycin	dark purple brown		brown red	dark red	red
	Iodoacetate	dark purple brown-purple edge		brown-purple edge	red-brown purple edge	purple-brown
	Control	dark purple brown-purple edge		purple	dark purple	dark purple

Appendix

Table 34

Effect of Nitrogen and Oxygen Atmospheres on Packaged and Unpackaged Fresh Beef Steaks with and Without the Addition of Iodoacetate at Room Temperature

Time in Hours	Treatments	Atmospheres				
		All N_2	10 mm O_2	75 mm O_2	Normal	All O_2
0	wrapped	purple	dark red	dark red	bright red	bright red
	unwrapped	purple	dark red	dark red	bright red	bright red
	iodoacetate + wrapped	purple	dark red	dark red	bright red	bright red
	iodoacetate + unwrapped	purple	dark red	dark red	bright red	bright red
1	wrapped	dark purple	dark red	red	bright red	bright red
	unwrapped	dark purple	dark red	red	bright red	bright red
	iodoacetate + wrapped	dark purple	dark red	red	bright red	bright red
	iodoacetate + unwrapped	dark purple	dark red	red	bright red	bright red
2	wrapped	dark purple	red-purple	dark red	red	bright red
	unwrapped	dark purple	red-purple	dark red	red	bright red
	iodoacetate + wrapped	dark purple	red-purple	dark red	red	bright red
	iodoacetate + unwrapped	dark purple	red-purple	dark red	red	bright red
3	wrapped	dark purple	purple-red	dark red	red	bright red
	unwrapped	dark purple	purple-red	dark red	red	bright red
	iodoacetate + wrapped	dark purple	purple-red	dark red	red	bright red
	iodoacetate + unwrapped	dark purple	purple-red	dark red	red	bright red
5	wrapped	dark purple	purple	purple	red	red
	unwrapped	dark purple	purple-red	dark red	red	red
	iodoacetate + wrapped	dark purple	purple-red	purple	red	red
	iodoacetate + unwrapped	dark purple	purple-red	dark red	red	red

Appendix

Table 34 (Continued)

Time in Hours	Treatments	Atmospheres				
		All N_2	10 mm O_2	75 mm O_2	Normal	All O_2
7	wrapped	dark purple	purple	purple	red	red
	unwrapped	dark purple	purple-red	dark red	red	red
	I.A. wrapped	dark purple	purple-brown	purple-brown	dark red	red
	I.A. unwrapped	dark purple	dark-purple	dark purple	dark red	red
9	wrapped	dark purple	purple	purple	red	red
	unwrapped	dark purple	purple-red	dark red	red	red
	I.A. wrapped	dark purple	purple-brown	brown-purple	dark red	dark red
	I.A. unwrapped	dark purple	dark purple	dark purple	dark red	dark red
24	wrapped	dark purple	purple	purple	dark red	dark red
	unwrapped	dark purple	purple-red	purple-red	dark red	dark red
	I.A. wrapped	dark purple	purple-brown	brown-purple	purple-brown	purple-brown
	I.A. unwrapped	dark purple	dark purple-brown	brown-purple	dark red-brown	dark red-brown
30	wrapped	dark purple	purple	-	purple	dark red
	unwrapped	dark purple	purple-red	-	purple	dark red
	I.A. wrapped	dark purple	purple-brown	-	brown	purple-brown
	I.A. unwrapped	dark purple	dark purple-brown	-	brown	purple-brown
48	wrapped	-	purple-brown	-	-	brown
	unwrapped	-	brown-purple edge	-	-	brown
	I.A. wrapped	-	brown-purple	-	-	brown
	I.A. unwrapped	-	brown	-	-	brown

Appendix

Table 35

Effect of Various Oxygen Levels on Inoculated* and Uninoculated Prepackaged Beef Steaks Held at Room Temperature

Time in Hours	Treatment	Oxygen Levels			
		10 mm O_2	20 mm O_2	40 mm O_2	60 mm O_2
0	inoculated	dark red	dark red	dark red	red
	uninoculated	dark red	dark red	dark red	red
1/2	inoculated	purple	purple	red-purple	purple
	uninoculated	purple	purple	red-purple	dark red
1	inoculated	purple	purple	purple	purple
	uninoculated	purple	purple	purple	purple
48	inoculated	purple	purple	purple	purple
	uninoculated	purple	purple	purple-brown	purple
72	inoculated	purple	purple	purple	purple
	uninoculated	purple	purple-brown tip	1/2 brown 1/2 purple	purple
96	inoculated	purple	purple	purple	purple
	uninoculated	purple	purple	purple	purple
144	inoculated	purple	purple	purple	purple
	uninoculated	purple	purple	purple	purple

* Inoculated with Pseudomonas graciliata

Appendix

Table 36

The Effects of Reduced Oxygen Atmospheres on Sterile Excised Muscle Tissue Held at 4°C.

Time in Hours	8 mm. O_2			10 mm. O_2			15 mm. O_2			20 mm. O_2		
before	bright red			bright red			bright red			bright red		
0	dark red			dark red			dark red			dark red		
12	dark red			dark red			dark red			dark red		
24	brown-red			brown-red			brown-red			brown-red		
36	brown			brown			brown			brown		
48	brown			brown			brown			brown		
	% met.	% myo.	No. bact.	% met.	% myo.	No. bact.	% met.	% myo.	No. bact.	% met.	% myo.	No. bact.
	45	0	0	44	0	0	44	0	0	64	0	0

Appendix

Table 37

The Effects of Reduced Oxygen Atmospheres on Sterile Excised Muscle Tissue Compared to Aseptically Handled Steaks Held at 4°C.

Time in	10 mm. O_2		25 mm. O_2		50 mm. O_2		75 mm. O_2		Normal	
Hours	Steak	Tissue	Steak	Tissue	Steak	Tissue	Steak	Tissue	Steak	Tissue
Before	bright red	bright red	bright red	bright red	bright red	bright red	bright red	bright red	bright red	bright red
0	dark red	dark red	dark red	dark red	red	dark red	bright red	dark red	bright red	dark red
4	dark red	dark red	bright red	dark red	bright red	red	bright red	red	bright red	red
24	dark red	red brown	red	red brown	bright red	dark red	bright red	red	bright red	red
48	red brown	brown	red	brown	red	red brown	bright red	red	bright red	red
72	brown red	brown	dark red	brown	red	brown red	bright red	red	bright red	red
96	brown	brown	red brown	brown	red	brown	red	dark red	bright red	dark red

Appendix

Table 38

Effects of Various Oxygen Tensions* on Oxymyoglobin Extracts with and Without the Presence of Bacteria.

Shaking Time in Minutes	Lot No.**	Oxygen Levels									
		0 mm O_2		5 mm O_2		10 mm O_2		20 mm O_2		Normal	
		% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.	% Met.	% Myo.
Before	Lot 1	1	0	2	0	3	0	2	0	3	0
	Lot 2	3	0	2	0	2	0	1	0	3	0
0	Lot 1	9	1	4	0	3	0	3	0	3	0
	Lot 2	7	0	7	0	4	0	7	0	3	0
20	Lot 1	25	60	16	13	11	10	13	3	9	0
	Lot 2	21	60	16	35	14	13	13	10	9	0
40	Lot 1	30	62	19	12	14	4	15	4	13	0
	Lot 2	23	67	21	46	16	29	16	22	14	0

* Oxygen tensions were obtained by pulling partial vacuums in Thunburg tubes. Equilibrium was hastened by shaking the tubes between readings.

** Lot 1 contained 10 ppm aureomycin to inhibit bacterial activity.
Lot 2 was inoculated with Ps. geniculata.

Appendix

Table 39

Effects of Ascorbic Acid and Ascorbic Acid Plus Sodium Nicotinate Treatments on the Rate of Discoloration and Bacterial Growth on Steaks.*

Treatment and observations	Time in days					
	0	2	4	6	8	10
CONTROL						
Visual observation	red	bright red	red	red, brown edge	1/2 brown 1/2 purple	3/4 brown 1/4 purple
Index of fading	22.3	20.0	21.3	19.8	30.4	31.2
Log. No. bacteria	3.3	3.9	6.3	7.6	7.6	7.7
ASCORBIC ACID TREATED						
Visual observation	red	bright red	bright red	dark red	2/3 brown 1/3 purple	brown
Index of fading	22.9	17.6	17.9	23.3	25.6	33.8
Log. No. bacteria	4.2	4.0	6.1	6.7	8.7	7.8
ASCORBIC ACID PLUS Na NICOTINATE TREATED						
Visual observations	red	bright red	very bright red	very bright red	bright red	1/5 brown 4/5 red
Index of fading	20.7	17.4	10.4	15.7	19.6	25.2
Log. No. bacteria	3.8	3.5	6.6	7.3	8.4	8.5

*Experiment conducted in a household type refrigerator.

Appendix

Table 40

Effects of Ascorbic Acid and Ascorbic Acid Plus Sodium Nicotinate Treatments on the Rate of Discoloration and Bacterial Growth on Steaks*.

Treatment and observations	Time in days					
	0	2	4	6	8	10
CONTROL						
Visual observations	bright red	very bright red	very bright red	red, brown tip	red, brown tip	2/3 brown 1/3 purple
Index of fading	17.5	14.6	15.5	22.3	19.4	27.8
Log. No. bacteria	5.0	4.5	7.1	8.2	7.9	9.1
ASCORBIC ACID TREATED						
Visual observations	bright red	very bright red	red	2/3 brown 1/3 red	5/6 brown 1/6 purple	brown
Index of fading	20.6	16.5	20.2	28.0	33.1	31.9
Log. No. bacteria	3.7	5.0	7.1	8.7	9.1	9.3
ASCORBIC ACID PLUS NaNICOTINATE TREATED						
Visual observations	bright red	very bright red	very bright red	very bright red	red, brown tip	2/3 brown 1/3 red-purple
Index of fading	19.4	15.0	15.5	15.0	18.3	29.4
Log. No. bacteria	3.8	3.5	6.6	7.3	8.4	8.5

*Experiment conducted in a self-service meat case.

Appendix

Table 41

Effect of Ascorbic Acid Plus Sodium Nicotinate on the Rate of Discoloration and Bacterial Growth on Inoculated Steaks

Treatments and Observations	0	2	4	6	8	9
UNINOCULATED CONTROL						
Visual observation	bright red	bright red	bright red	red	1/3 brn. 2/3 dk. red	7/8 brn. 1/8 pur.
Fading index	21.7	18.9	20.6	22.2	24.0	30.0
Log. no. bacteria	4.2	3.8	6.7	7.3	8.3	8.6
UNINOCULATED WITH A. A. AND N.A.						
Visual observation	bright red	bright red	very bright red	very bright red	7/8 red 1/8 brn.	7/8 brn. 1/8 pur.
Fading index	18.8	17.3	15.2	14.7	24.0	32.7
Log no. bacteria	4.8	4.4	6.4	7.6	8.3	8.5
INOCULATED CONTROL						
Visual observation	red	red	3/4 brn. 1/4 red	1/2 brn. 1/2 red-pur.	brn.	brn.
Fading index	23.2	21.0	32.1	25.3	26.9	28.9
Log. no. bacteria	7.2	7.2	7.1	8.3	9.5	9.4
INOCULATED WITH A. A. AND N. A.						
Visual observation	bright red	bright red	bright red	red	brn.	pur.-brn.
Fading index	18.4	17.7	19.5	20.7	30.0	27.0
Log. no. bacteria	6.9	7.0	6.8	8.1	9.2	9.2

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