Role of Bacteria in the Oxidation of Myoglobin\textsuperscript{1,2}

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**Abstract**

ROBACH, D. L. (Michigan State University, East Lansing), and R. N. COSTILOW. Role of bacteria in the oxidation of myoglobin. Appl. Microbiol. 9:529–533, 1961.—The addition to steaks of cell suspensions of a number of aerobic bacteria and of *Saccharomyces cerevisiae* greatly increased the rate of discoloration. Low inocula resulted in the more rapid appearance of the brown color of metmyoglobin, whereas high cell populations quickly produced the purple color of myoglobin. Sonically treated suspensions of *Pseudomonas geniculata* produced similar changes in surface color but less rapidly. No such effect was observed with *Lactobacillus plantarum*.

The visible changes in color were found to be associated with the oxygen demand of the surface tissue including, of course, the demand of any contaminating microorganisms. Inhibitors of respiratory activity inhibited the rate of discoloration under normal atmospheric conditions. However, when the oxygen level in the atmosphere was reduced, the inhibitors had no significant effect. In an oxygen-free atmosphere, the steak surfaces were the purple color of myoglobin; at 10 mm oxygen pressure, the pigment was oxidized to metmyoglobin and the surface was brown in color. No bacterial activity was necessary for pigment oxidation under low oxygen pressures.

Addition of dilute solutions of glucose oxidase resulted in rapid oxidation of the meat pigment to metmyoglobin both in extracts and on steak surfaces. More concentrated solutions resulted in further oxidation as evidenced by the appearance of a green color. Horseradish extract with a high peroxidase activity added with H\textsubscript{2}O\textsubscript{2} resulted in rapid oxidation of the pigment but neither were very effective alone, although H\textsubscript{2}O\textsubscript{2} did result in a browning reaction in aged steaks.

It is concluded that the primary role of the bacteria in meat discoloration is in the reduction of the oxygen tension in the surface tissue. The implications of the data are discussed and a possible mechanism of myoglobin oxidation is proposed.

Discoloration of fresh prepackaged meat or “loss of bloom,” as it is called by the trade, means that meat loses its bright red appearance. Meat color is due to myoglobin, an iron porphyrin pigment very similar to hemoglobin. The chemistry of meat pigments was reviewed by Schweigert (1956). The heme prosthetic group is attached to a globin protein fraction by the amino acid, histidine. The heme so attached to the protein fraction can form a dissociable compound with oxygen. In the reduced form, the iron of the heme is in the ferrous state and the pigment is purple. Upon exposure to excess molecular oxygen the iron remains in the ferrous state but the pigment becomes oxygenated. This pigment called oxymyoglobin is bright red. The heme portion of myoglobin may be oxidized by various means to metmyoglobin, a brown pigment. The iron of this compound is in the ferric state. The problem of discoloration involves the loss of oxygen to form reduced myoglobin (purple) and oxidation to metmyoglobin (brown).

The fact that bacterial activity is a major factor in pigment changes in fresh prepackaged meat has been well established (Butler, Bratzer, and Mallman, 1953; Costilow et al., 1955). Neill (1925) observed that pneumococci, cell-free extracts of pneumococci, and sterile animal tissue extracts caused the reduction of methemoglobin to hemoglobin when molecular oxygen was excluded. On introduction of small amounts of molecular oxygen, the reverse reaction was demonstrated with intact bacterial cells, cell extracts, and sterile extracts from potatoes. It is believed that the pneumococci bring about oxidation by the production of peroxides or similar compounds (Neill and Hastings, 1925). However, the actual role of bacteria in causing changes in the pigment in meat surface tissue has not been clearly demonstrated. This was the purpose of the investigation reported here.

**Materials and Methods**

Steaks used in this study were from the longissimus dorsi muscle of U. S. Good grade beef ribs purchased locally. The muscle was removed from the ribs and cut into \(\frac{3}{4}\)-in. thick steaks.

The wrapping material employed was du Pont cellophane 300 MSAT-80.\textsuperscript{4} Cells of all the bacteria used except *Lactobacillus*

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\textsuperscript{4} E. I. du Pont de Nemours & Co., Wilmington, Del.
plantarum were grown in nutrient broth shake cultures or on nutrient agar slants. The L. plantarum culture was grown in a Trypticase soy broth (BBL) and the Saccharomyces in dextrose broth (Difco). All cells were washed in saline before being inoculated on steak surfaces by the use of sterile atomizers. Washed cells were also used to prepare cell-free extracts. Cells were broken in a Raytheon, 50-w, 9-kc 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 to be described 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 slice approximately 3 mm thick from the surface of the steak with a Hobart 50 slicing machine. The machine was cleaned and sanitized before each cutting by washing thoroughly with hot detergent and then rinsing with distilled water, hypochlorite (500 ppm), and finally with sterile distilled water. The rotating blade was allowed to spin dry. Platings made of swabblings from the machine demonstrated that the portion which came in contact with the meat slice was essentially free from bacteria.

The thin slice from the surface was 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 sec, a sample of the homogenate was plated with Tryptone glucose extract agar (Difco) (TGE) and observed for bacterial growth. The remainder 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 1 week and at 20 °C for 3 days. For the sake of convenience, the shorter time and higher temperature of incubation were used for all bacterial counts.

A sample of the homogenate was prepared for pigment determination by centrifuging in a Servall SPX centrifuge at full speed for 10 min, and filtering through Whatman no. 40 filter paper. Seven milliliters of filtrate were diluted with 3 ml of distilled water for spectrophotometric analysis with a Beckman DU spectrophotometer. In studies conducted in optically matched Thunberg tubes, a Bausch and Lomb Spectronic 20 colorimeter was used and no dilution of the filtrate was necessary.

Two methods of pigment analysis were used. The first method was essentially that of Butler et al. (1953). The second method of analysis was that of Broumand, Ball, and Stier (1958). Both were found to give comparable results with respect to percentage of metmyoglobin. The latter method has the advantage of making it possible to estimate the relative percentage of each form of the pigment.

The discoloration of steak surfaces was estimated by the Munsell spinning disc method of Nickerson (1946) and an index of fading calculated on the basis of the standards proposed by Butler et al. (1953). Visual observations were also made since the fading index does not reflect the type of pigment change which occurs, because the index values are increased on either deoxygenation or oxidation of the muscle pigment.

Steaks to be incubated in atmospheres of various oxygen pressures were placed unwrapped in vacuum desiccators. The bottom of each desiccator was covered with water to maintain a high relative humidity. The air was replaced by reducing the pressure to less than 50 mm Hg and refilling with nitrogen that had been passed over hot copper shavings to remove contaminating oxygen. This was repeated three times and the desired amount of oxygen added along with the nitrogen on the last fill. The total pressure was adjusted to 750 mm with nitrogen.

RESULTS

Pure cultures of Pseudomonas fluorescens, Pseudomonas aeruginosa, two strains of Pseudomonas geniculata, three Pseudomonas sp., Achromobacter liquefaciens, Flavobacterium rhenuanum, L. plantarum, and Saccharomyces cerevisiae were tested for their effect on the surface color of wrapped beef steaks. At room temperature, the yeast and all the bacteria tested except L. plantarum caused rapid color changes. At 4 ± 1 °C, the yeast had little effect on color but the aerobic bacteria tested were all active. All gave essentially the same results, causing the pigment to change from red to brown and finally to purple. However, when large numbers of cells were added to steak

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8 Baltimore Biological Laboratory, Inc., Baltimore, Md.
9 Difco Laboratories, Inc., Detroit, Mich.
10 Raytheon Corporation, Waltham, Mass.
9Bausch and Lomb Optical Company, Rochester, N. Y.
surfaces, the purple color of myoglobin was noted within a few hours and no further change was observed. *L. plantarum*, an organism which does not utilize oxygen to any appreciable extent, did not bring about the discoloration of beef at either room temperature or at 4 C.

The index of fading value for steak surfaces treated with cell-free preparations (sonically treated) of *P. geniculata* increased more rapidly than that for appropriate controls, demonstrating that the enzymes of *P. geniculata* were active in the discoloration. Steaks inoculated with intact cells discolored more rapidly than those treated with cell extracts. This was expected since the respiratory activity of the intact cells was greater than that of the sonically treated preparations.

Further evidence of the importance of the respiratory activity of the bacteria was obtained on comparing control steaks with inoculated steaks, and inoculated steaks treated with inhibitors demonstrated to greatly reduce the oxygen uptake rate of the test organism using meat as a substrate. As may be noted in Fig. 1, the untreated inoculated steaks had a very significant oxygen demand initially and this was correlated with a high bacterial population and a high index of fading. The steaks were the purple color of myoglobin and no significant amount of metmyoglobin was detected. The inoculated steaks treated with iodoacetate had a high bacterial load but a low oxygen demand and a low fading index initially. These steaks never developed as high a bacterial population or oxygen uptake rate as the control group. However, the metmyoglobin levels increased slightly faster and the change in fading index was comparable with the control. Treatment with a 0.1 M solution of sodium malonate yielded results similar to iodoacetate.

The effect of various oxygen levels on the color of beef steaks is illustrated in Fig. 2. During the first few days of refrigeration, the microbial activity was minor. However, the steaks held under 10 mm oxygen pressure quickly developed a brown color and those under 75 mm oxygen pressure became brown at a slower rate. After the first 5 or 6 days, the microbial activity, undoubt-edly, brought about the color changes noted in the steaks held in a normal atmosphere and in one of oxygen. Inoculated steaks that were run for comparison in this experiment all became purple during the first 6 days and remained this color for the duration of the experiment.

The initial changes occurring under the various oxygen pressures were not altered by the atomizing of

**FIg. 1. Comparison of changes in fading index, metmyoglobin concentration, oxygen demand, and bacterial count of inoculated steaks treated with a 0.1 M solution of iodoacetate with both inoculated and uninoculated control steaks. Groups of steaks with each treatment were prepared and one of each sampled at the times indicated. The steaks were held at 4 ± 1 C during the experiment.**

**FIG. 2. Relative color changes observed on steak surfaces held in various atmospheres. The atmospheres were adjusted in vacuum desiccators by use of oxygen-free nitrogen. The total pressure of each atmosphere was adjusted to 750 mm. The experiment was conducted at 4 ± 1 C. The color notations used and plotted on the graph from top to bottom were: (1) very bright red, (2) bright red, (3) red, (4) dark red, (5) brown-red, (6) brown, (7) brown-purple, (8) purple-brown, (9) purple.**
steaks with solutions of iodoacetate (0.1 M), potassium cyanide (0.01 M), or chlortetracycline (100 ppm). In fact, there was no significant effect of these treatments on the color changes occurring in an atmosphere with 10 mm oxygen pressure throughout the test period (Fig. 3). The cyanide-treated steaks appeared somewhat more red in most instances which probably resulted from reaction with the myoglobin. In higher atmospheres where bacterial activity became an important factor, the inhibitors delayed the appearance of color changes. In the normal atmosphere, chlortetracycline had the greatest delaying action, followed by KCN and then iodoacetate (Fig. 3). The differences are probably due primarily to differences in their effectiveness as bacterial inhibitors. However, KCN may stabilize the pigment to a certain extent by reacting with it.

Atabrine, a specific inhibitor of flavoprotein enzymes, inhibited oxygen uptake by meat tissue; but attempts to test its effect on pigment oxidation under reduced oxygen pressures were unsuccessful. The yellow color of atabrine interfered with both visual observations and spectrophotometric analysis of the meat pigment.

The addition of dilute solutions (0.05%) of a commercial preparation of “pure” glucose oxidase to meat surfaces resulted in rapid browning. At room temperature, the surface color was a definite brown within 15 min after addition. However, if more concentrated solutions of glucose oxidase were used, the oxidation apparently continued because a green color, presumably due to choleglobin, was observed on the surface.

Hydrogen peroxide (0.3%) had no obvious effect on the color of fresh steaks but some browning was observed on its addition to steaks held at 4 ± 1°C for several days.

The oxidation occurring in the presence of glucose oxidase was also demonstrated in meat extracts at room temperature. Within 30 min after addition of the enzyme solution, 86% of the pigment was in the oxidized state and the remainder was in the myoglobin form (Table 1). There was apparently some nonenzymatic activity since enzyme solutions which had been boiled 5 min effected some change in pigment. It will be noted, however, that this change was no greater than the change occurring on addition of both heated and unheated horseradish extracts and only slightly more than that effected by H₂O₂. The horseradish extract had a high peroxidase activity and, when added to a steak surface with 0.3% H₂O₂, caused rapid browning.

**Discussion**

There can be little doubt that the primary role of bacteria in the color changes of fresh meat is in the reduction of the oxygen level in the surface tissue. This is supported by a number of facts, viz., (i) 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; (ii) the oxygen level in the storage atmosphere greatly affects the rate of pigment changes of both inoculated and uninoculated steaks; (iii) oxygen uptake rate of the surface tissue of meat is correlated with microbial activity and with color change; (iv) at intermediate levels of oxygen demand of surface tissue, oxidation to metmyoglobin occurs, whereas, with higher respiration rates, reduction to myoglobin occurs, and this is correlated with similar changes.

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**Table 1. Effect of various additives on the oxidation of myoglobin (Mb) to metmyoglobin (MMb) in meat extracts**

<table>
<thead>
<tr>
<th>Additive</th>
<th>Incubation time (min)*</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>MMb</td>
<td>Mb</td>
<td>MMb</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>H₂O₂ (0.3%)</td>
<td></td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Glucose oxidase (0.001%)</td>
<td></td>
<td>86</td>
<td>11</td>
</tr>
<tr>
<td>Heated glucose oxidase (0.001%)</td>
<td></td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Horseradish extract†</td>
<td></td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>Heated horseradish extract‡</td>
<td></td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

* Incubated in tubes at room temperature (22 ± 1°C).
† Percentages refer to per cent of total pigment.
‡ Added 0.1 ml filtered extract pressed from commercial horseradish to 9.9 ml meat extract.
under controlled oxygen atmospheres; and (v) agents inhibiting the development of high oxygen uptake rates in surface tissue will result in color preservation under normal atmospheric conditions but are ineffective under low oxygen pressures.

This being true, then one must look to the meat tissue per se for the mechanism of pigment changes. Grant (1955) demonstrated that the blocking of succinic dehydrogenase with malonate protected the interior of frozen ground meat from discoloration. This treatment must have eliminated practically all oxygen uptake, since the pigment remained in the oxygenated state. However, malonate did not prevent the oxidation of myoglobin in surface tissue of steaks held at refrigeration temperatures; although it did greatly reduce oxygen uptake of contaminating bacteria. Iodoacetate, an inhibitor of glyceraldehydephosphate dehydrogenase (a glycolytic enzyme) and of other sulfhydryl-containing enzymes, was more effective than malonate on steak surfaces, but was without any measurable effect when the oxygen tension in the atmosphere was reduced to 10 mm. Since chlorotetacycline and KCN had similar effects, it is believed that their primary action on meat surfaces is to inhibit the development of aerobic bacteria.

The production of \( \text{H}_2\text{O}_2 \) by pneumococci leads to methemoglobin formation (Neill, 1925), and results of this study demonstrate that \( \text{H}_2\text{O}_2 \) in combination with peroxidase causes rapid oxidation of myoglobin. Although \( \text{H}_2\text{O}_2 \) production by meat tissue was not demonstrated, the fact that atabrine inhibits oxygen uptake is indirect evidence of flavoprotein enzyme activity. Such activity would yield a certain amount of \( \text{H}_2\text{O}_2 \). Furthermore, glucose oxidase, which produces \( \text{H}_2\text{O}_2 \) as a product of glucose oxidation, results in extensive oxidation of the pigment either in surface tissue or in extracts. Keilin and Hartree (1945) demonstrated that the addition of glucose oxidase and glucose to either washed horse erythrocytes or to laked corpuscles resulted in the oxidation of 100% of the hemoglobin to methemoglobin within a few minutes. Catalase was not essential for this reaction since a solution of recrystallized hemoglobin, free from catalase, was oxidized. In fact, the catalase may have offered some protection because a portion of the partially purified pigment underwent oxidative destruction accompanied by protein denaturation and formation of the green verdohemochromogen. Bingold (1933) demonstrated that catalase did protect oxyhemoglobin from oxidation by high concentration of \( \text{H}_2\text{O}_2 \).

Brooks (1935) found the rate of oxidation of hemoglobin to methemoglobin in buffered, laked, defibrinated blood at different oxygen pressures to be proportional to the concentration of reduced hemoglobin. The decreasing rate, which occurs as oxygen tension is increased, results from the increasing concentration of the oxygenated pigment that is not apparently involved in the reaction. Keilin and Hartree (1945) speculate that this fact explains the protective action of catalase toward hemoglobin when \( \text{H}_2\text{O}_2 \) concentrations are high. There would be considerable oxygen released under such conditions and the pigment would probably be oxygenated and, thus, not susceptible to oxidation. This would not be the case, however, with metabolic \( \text{H}_2\text{O}_2 \) since there would be a net loss of dissolved oxygen in the over-all reaction. Data collected in this work support this idea, since high peroxide levels had no effect when added alone to freshly sliced steaks, but did result in pigment oxidation in steaks held 3 or 4 days at \( 4 \pm 1 \) C. The latter steaks would be expected to have a relatively high oxygen demand and percentage of myoglobin, thus, providing the proper state for oxidation to occur.

Based on the above, it is believed that the reduction of oxygen tension in meat tissue, either by microbial growth or by physical means, results in a great increase in the reduced myoglobin, which, in turn, is oxidized by metabolic \( \text{H}_2\text{O}_2 \) produced either by the meat tissue or by the bacteria. If the oxygen tension is reduced to low enough a level, little or no \( \text{H}_2\text{O}_2 \) can be formed and no appreciable oxidation occurs. Any oxidized myoglobin present is rapidly reduced under such conditions.

**LITERATURE CITED**


