Detection of Glucose Oxidation Products in Chilled Fresh Beef Undergoing Spoilage

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The fate of nutrients during storage of longissimus dorsi muscle at 4°C was examined. Glucose concentrations in meat were shown to decrease concomitantly with an approximately fourfold increase in the activity of glucose dehydrogenase. Gluconate concentrations in meat were determined by an enzyme assay and shown to increase from 2.1 to 40.6 μg/g upon storage of the meat from day 0 to day 6. At day 12, gluconate concentrations had decreased to 5.8 μg/g. Dark firm dry meat, which contains little or no glucose, did not exhibit the same rise and fall in gluconate concentration. Thin-layer chromatographic analysis confirmed the presence of 2-ketogluconate in 6- and 12-day-old longissimus dorsi muscle that had been stored at 4°C. Gluconate concentrations in irradiated sterile meat inoculated with Pseudomonas fluorescens increased from 4.2 to 77.8 μg/g during the first 6 days of storage at 4°C. Therefore, glucose in meat stored at 4°C appeared to be converted to gluconate, 2-ketogluconate, or both extracellularly by one of the main meat spoilage organisms, most likely the pseudomonads.

The aerobic spoilage of chilled fresh meats is largely a surface phenomenon (11). Microorganisms most commonly involved in this spoilage process include species of Pseudomonas, Moraxella, Acinetobacter, and Enterobacter, as well as Brochothrix thermosphacta (8), Pseudomonas spp. in most instances dominating in the spoilage flora. One important factor contributing to the domination by Pseudomonas spp. is ability to grow faster at low temperatures than the other common meat spoilage organisms (7). The primary carbon source for the growth of the Pseudomonas spp., Enterobacter spp., and B. thermosphacta is glucose, whereas amino acids are used by species of Acinetobacter and Moraxella as the carbon source (7).

Lynch and Franklin (15) found that at high temperatures (30°C), the major portion of available glucose is taken directly into Pseudomonas fluorescens and phosphorylated, whereas at low temperatures, the glucose is oxidized extracellularly to gluconate, 2-ketogluconate, or both. These workers stated that the oxidative metabolic pathway may at low temperatures confer a competitive advantage to P. fluorescens over other organisms.

The present study was thus undertaken to determine whether there is a significant conversion of glucose to gluconate, 2-ketogluconate, or both during the storage of meat at chill temperatures.

MATERIALS AND METHODS

Bovine longissimus dorsi muscle (2 to 4 days post-mortem) was obtained from a local abattoir. The meat was trimmed of visible fat and connective tissue with sterile instruments under a laminar flow unit. The muscle was then cut into slices of 10 or 14 g (length, 6.35 or 7.6 cm; width, 2.54 or 3.2 cm, respectively), which were then wrapped in oxygen-permeable polyvinyl chloride film and stored at 4°C. For the analyses, either the entire 10-g slices or 2- or 1-g portions (length, 3.0 or 2.5 cm; width, 1.5 or 1 cm, respectively) cut from the 10- and 14-g slices were used.

Glucose dehydrogenase activity during storage of longissimus dorsi muscle at 4°C. Longissimus dorsi muscle (10 g) was stored for various periods at 4°C in a polyvinyl chloride wrap. At each sampling, two 10-g samples were removed from storage, and each was put into a 50-ml flask containing 20 ml of distilled water and glass beads. Each flask was shaken vigorously by hand for 1 min, and the liquid portion was decanted and centrifuged (17,300 × g; 15 min). The residue was washed twice with distilled water (17,300 × g; 15 min), and the residue containing microbial cells was disrupted by sonication (80 W; 3 min; Heat Systems; Ultrasonics Inc., Plainview, N.Y.). The sonicated material was centrifuged (29,000 × g; 30 min), and glucose dehydrogenase activity in the supernatant extract was assayed by the method of Ng and Dawes (18).

Protein determination. A 2-ml portion of the supernatant extract was added to 2 ml of 2 N NaOH, and the tubes were stored at 4°C. The samples in the tubes were subsequently boiled for 2 min, and protein was estimated by the method of Lowry et al. (14), with bovine serum albumin as the standard.
Glucose and lactic acid analyses. Glucose and lactic acid levels in longissimus dorsi muscle stored for various periods at 4°C were determined by the method of Gill (6).

For the initial sampling, duplicate 1.0-g portions were cut from 10 g of meat; the remainder of the sample was rewrapped in film and put back into storage at 4°C for further sampling on days 6 and 12. Each 1.0-g portion was extensively ground in 6 ml of 6% (wt/vol) perchloric acid. The liquid percolic acid fraction was decanted and centrifuged (12,100 x g; 15 min).

The supernatant fraction was placed on ice. The pH was adjusted to 6.5 with 20% (wt/vol) KOH, and the precipitated KCIO₄ was removed by centrifugation (3,020 x g; 10 min). The glucose oxidase-peroxidase method was used to determine glucose in the supernatant fraction, and the lactic acid dehydrogenase method was used to determine lactic acid (enzyme kits from Sigma Chemical Co., St. Louis, Mo.).

Gluculate analysis. (i) Normal muscle. For the initial sampling, duplicate 2.0-g portions were cut from 14 g of meat; the remainder of the sample was rewrapped in film and put back in storage at 4°C for further sampling on days 6 and 12. Each 2.0-g portion was treated with perchloric acid and ground, as was done for the glucose and lactic acid analyses. The liquid percolic acid fraction was decanted and centrifuged (12,000 x g; 15 min).

The supernatant fraction was placed on ice. The pH was adjusted to 10.0 with 20% (wt/vol) KOH, and the precipitated KClO₄ was removed by centrifugation (3,020 x g; 10 min).

The amount of gluconate in the supernatant fraction was determined by the gluconate kinase–6-phosphogluconate dehydrogenase method (Boehringer Mannheim Corp., 1979. Methods of Enzymatic Food Analysis). All reagents were from Sigma.

(ii) Irradiated inoculated muscle. P. fluorescens, P. putida, a nonfluorescent pseudomonad, Enterobacter agglomerans, and B. thermosphacta were isolated from meat that had been allowed to spoil in air at 4°C. These organisms were maintained on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) at 4°C.

Longissimus dorsi muscle was cut up into thin (3.0-cm length; 1.5-cm width) 2-g slices. The pieces were sterilized with 10 kGy of gamma radiation (Gammacell 220; Atomic Energy of Canada Ltd.), and then the surface of each piece was evenly inoculated with 25 μl of a different bacterial suspension. We prepared the inocula by transferring isolated colonies from APT agar (Difco; grown for 48 h at 25°C) into 0.1% (wt/vol) peptone-water dilution blanks. The optical density of the pepton-water was adjusted (model 240 spectrophotometer; Gilford Instrument Laboratories, Inc., Oberlin, Ohio), and serial dilutions were made to give a final concentration on the meat surface of approximately 10⁷ organisms per g. The meat was stored aerobically at 4°C for 12 days in containers lined with wet blotting paper. Gluconate levels were determined on days 0, 6 and 12 as outlined above. At the same time, microorganisms were enumerated on APT agar plates incubated at 25°C.

Thin-layer chromatography for detection of 2-keto-gluconate in meat undergoing spoilage at 4°C. At each sampling, we group each of five 10-g samples of longissimus dorsi muscle with 15 ml of distilled water, using a mortar and pestle. The homogenate was dehydrated and centrifuged (17,300 x g; 10 min). The supernatant fraction was freeze-dried (Thermomac Industries Corp., Copiague, N.Y.) and reconstituted to 0.5 ml with distilled water, and various amounts (1 to 8 μl) were spotted onto cellulose thin-layer chromatography glass plates (model MN 300; Brinkman Instruments Inc., Rexdale, Ontario). The plates were developed in ethyl acetate-water-glacial acetic acid-formic acid (18:4:3) three times and were carefully dried in a cold air flow between runs (10). To detect 2-ketogluconate, we sprayed the developed plates with a solution containing O-phenylendiamine dihydrochloride, dried the plates for 2 to 3 min at 100°C, and viewed them immediately and after 1 h under both visible and UV light.

RESULTS AND DISCUSSION

Storage of meat at 4°C resulted in decreased levels of both glucose and lactic acid. The level of lactic acid in longissimus dorsi muscle decreased from an initial concentration of 1.3% to 0.23% after 12 days of storage at 4°C (Table 1). Gill (6) has also observed a decrease in the lactic acid concentration of diluted meat juice medium that had been inoculated with a Pseudomonas sp. In another series of experiments, he ob-

### Table 1. Effect of storage at 4°C on glucose dehydrogenase activity and glucose, lactic acid, and gluconate levels in bovine longissimus dorsi muscle

<table>
<thead>
<tr>
<th>Storage (days)</th>
<th>Glucose (%)</th>
<th>Glucose dehydrogenase (nmol of glucose oxidized/min per mg of protein)</th>
<th>Lactic acid (%)</th>
<th>Gluconate (μg/g)</th>
<th>Gluconate concn (μg/g) in DFD meat*&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0615 ± 0.005</td>
<td>2.9 ± 1.86</td>
<td>1.3 ± 0.29</td>
<td>2.1 ± 1.3</td>
<td>1.8 ± 0.14</td>
</tr>
<tr>
<td>6</td>
<td>0.024 ± 0.004</td>
<td>2.9 ± 1.86</td>
<td>0.99 ± 0.01</td>
<td>40.6 ± 5.3</td>
<td>0.40 ± 0.07</td>
</tr>
<tr>
<td>12</td>
<td>0.002 ± 0</td>
<td>11.2 ± 4.16</td>
<td>0.23 ± 0.02</td>
<td>5.8 ± 2.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

*<sup>+</sup> Standard deviations (two duplicate experiments).
*<sup>+</sup> Standard deviations (three duplicate experiments).
*<sup>+</sup> ND, Not detected.

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served that mutton stored until it spoiled still contains a significant amount of lactic acid. Glucose decreased from 0.06% at day 0 to 0.002% at day 12 (Table 1). The levels obtained initially (day 0) were greater than those reported by Newton and Gill (17) but similar to those observed by other workers (4, 5) at the beginning of experiments as well as during storage of meat at chill temperatures.

Gill (6) has further observed that glucose is used preferentially by all meat spoilage organisms except Acinetobacter spp. in meat juice medium stored at 30°C. Gill has also shown that the level of lactic acid does not decrease until the glucose supply has been exhausted. In the present study with 10-g slices of meat stored at 4°C, the glucose and lactic acid levels decreased concurrently (Table 1).

Concomitant with this decrease was an increase in glucose dehydrogenase activity, especially from day 6 to day 12 of storage. Lynch et al. (16) have shown that maximum enzyme activity in P. fluorescens grown at 5°C occurs when the cells approach the stationary phase of growth.

Since the pseudomonads predominate in the microflora of meats undergoing aerobic spoilage at chill temperatures (1, 13), the observations of Lynch et al. (16) may be used to explain the results obtained in our studies. The pseudomonads were the predominant species after 6 to 12 days of storage, and their stationary phase of growth had been reached (total colony-forming units per gram: day 0, 5.0 \times 10^3; day 6, 2.0 \times 10^6; day 12, 1.6 \times 10^{10}); therefore, the substantial increase in glucose dehydrogenase activity can be readily appreciated.

Because glucose dehydrogenase activity increases during the storage of meats, the possibility exists that glucose may not be metabolized entirely via intracellular phosphorylation to glucose-6-phosphate but may be metabolized partially or wholly via extracellular conversion to gluconate, 2-ketogluconate, or both before uptake by the cells.

Indeed, the gluconate concentration increased approximately 20-fold during the first 6 days of storage and subsequently decreased. In addition, 2-ketogluconate was not detected by thin-layer chromatography at day 0 but was detected on days 6 and 12 (data not shown). In dark firm dry meat, which is characterized by a high ultimate pH and a low or absent glucose content (17), little or no gluconate was formed during storage, as was expected (Table 1).

The gluconate concentrations in meat slices that were irradiated and inoculated with P. fluorescens, P. putida, or a nonfluorescent pseudomonad respectively increased approximately 18-, 7-, and 3-fold during the first 6 days of storage and subsequently decreased. No such increases were seen in irradiated meat inoculated with either B. thermosphacta or an Enterobacter sp. (Table 2). Acinetobacter sp. and Moraxella sp. were not tested because our isolates were found not to utilize glucose oxidatively as a carbon source for growth. By day 6, all three pseudomonads had grown on meat to numbers higher than those to which either of the two other species grew (Table 2). These findings agree with those of other workers (7, 9), who have reported that pseudomonads grow faster than other meat spoilage organisms at temperatures of 15°C and lower.

Irradiated uninoculated meat samples were found to contain gluconate concentrations of 4.8, 5.3, and 7.1 \mu g/g on days 0, 6, and 12 of storage at 4°C, respectively. Thus, endogenous meat enzymes were not responsible either for the observed increases in gluconate concentrations in meat during the first 6 days of storage at 4°C or for the subsequent decrease.

Gill (6) found no glucose on the surface of meats when the cell density reaches approximately 3 \times 10^8 colony-forming units per cm². Our results, however, indicate that at 4°C, not only was glucose still present in stored meat samples when the microbial population reached 2.1 \times 10^9 organisms per g, but also that during the storage period, the glucose was converted to
its oxidation products, namely gluconate, 2-ketogluconate, or both. The latter products were subsequently metabolized by the microbial flora.

Gill and Newton (7) have stated that for both fluorescent and nonfluorescent pseudomonads, glucose, amino acids, and lactic acid (in that order) are the initial sources of carbon. The preferential sources of carbon for the enterobacters appear to be similar, with the exception that glucose-6-phosphate is used in preference to amino acids and lactic acid. For B. thermosphacta, glucose and then glutamate are the principal carbon sources.

Our results raise the possibility that once glucose is reduced to levels low enough for catabolite repression to be removed, the microbial flora utilize the gluconate and 2-ketogluconate as carbon sources; the disappearance of amino acids at this time may reflect their utilization as sources of nitrogen as well as carbon. Once the gluconate and 2-ketogluconate are depleted, the amino acids could, of course, satisfy both the carbon and nitrogen needs of the system.

Because of the conversion of glucose to gluconate, 2-ketogluconate, or both, a definite selective advantage could be conferred on the pseudomonads during their competition with other meat spoilage organisms for carbon sources. Moraxella spp. (2; unpublished data) and most of the Acinetobacter spp. studied (3, 7, 12; unpublished data) cannot use glucose as a carbon source. The majority of species of these two genera would also be disadvantaged by their inability to grow at the low pH (5.5 to 5.7) prevailing in fresh chilled meats (7, 9; unpublished data).

Glucose, glucose-6-phosphate, lactic acid, and amino acids have been shown to be metabolized in meat during storage (7). This report shows for the first time the presence, conversion, and utilization of glucose oxidation products in muscle by meat spoilage organisms.

LITERATURE CITED