Hydrogen Sulfide Production by Bacteria and Sulfmyoglobin Formation in Prepacked Chilled Beef

D. J. NICOL, M. K. SHAW, AND D. A. LEDWARD

Meat Research Laboratory, C.S.I.R.O. Division of Food Preservation, Cannon Hill, Queensland, 4170, Australia

Received for publication 6 March 1970

Meat stored at 1 to 2°C under low oxygen tensions, either in gas-impermeable packs or in controlled atmospheres, occasionally exhibited an undesirable green exudate. The green pigment was identified spectrophotometrically as sulfmyoglobin. The conversion of myoglobin to sulfmyoglobin resulted from the production of H₂S by bacteria tentatively identified as Pseudomonas mephitica. This organism produced H₂S only when the oxygen tension was about 1% and the pH of the meat was 6.0 and above.

During the storage of fresh meat, green discolorations have been observed, which are normally attributed to sulfmyoglobin, cholemyglobin, or verdoheme formation (5). Little definitive information is available, however, regarding the conditions necessary for the formation of any of these green pigments on fresh meat.

During experiments on packaging of fresh beef, a bright green exudate was occasionally noticed when conditions of low O₂ tension (~1%) were established in the atmosphere surrounding muscles of high ultimate pH (above 6.0). This paper reports the identification of this green pigment as sulfmyoglobin and identifies the H₂S-producing organism. The conditions necessary for H₂S production by this organism were also determined.

MATERIALS AND METHODS

Packaging and storage of meat. Ox muscles (Longissimus dorsi) were wrapped in a gas-permeable film (MSADT 80) and stored at 2°C in a gas-tight container filled with a mixture of 0.5% O₂, 10% CO₂, and the balance nitrogen. These are referred to as the experimental packages. The commercial packages consisted of L. dorsi vacuum-packed in gas-impermeable Cryovac S (polyvinylidene chloride copolymer, W. R. Grace Co.) and stored at 1 to 2°C.

Identification of the pigment. Immediately after opening the experimental or commercial packages, the green exudate was washed from the wraps with distilled water and centrifuged at 10,000 × g for 10 min at 2°C. The spectrum of the clear supernatant was recorded (Shimadzu Seisakusho Ltd., Kyoto, Japan, model MPS-50L) against an appropriate blank after dilution with water; treatment with sodium dithionite (2%) for 5 min; treatment with alkali (5 N NaOH) for 1.5 hr and dithionite (2%) for 5 min; treatment with ferricyanide [0.1% K₃Fe(CN)₆] for 5 min; exposure to air over a large surface area for 3 hr and again after treatment of this solution with dithionite for 5 min.

In all but the case in which the supernatant was exposed to air, 2 ml of reagent was added to 10 ml of solution. Cuvettes used had a path length 1 cm, and the range scanned was 550 to 700 nm.

Measurement of gas concentration. Gas samples (0.1 ml) were withdrawn from the gas-tight container or through the packaging film of the commercial packages with a gas-tight hypodermic syringe. The composition of the gas phase was determined by using a 25V gas partitioner (Fisher Scientific Co., Pittsburg, Pa.).

Differential enumeration of bacterial flora. Excised surface meat samples (1 cm²) were blended with 0.1% peptone water and surface-plated onto tryptone-phytone-yeast extract-agar (TPY) and lead acetate-agar containing 0.001% cysteine (LAC). TPY plates were used to enumerate the total bacterial flora. LAC plates were used to differentiate the H₂S-producing bacteria, when incubated in an atmosphere of N₂ and 0.4% O₂ for 21 days at 2°C or 2 days at 20°C. Organisms from colonies exhibiting darkening on LAC plates were isolated and identified.

Identification of H₂S-producing isolates. Identification of the H₂S-producing organisms was made according to the Manual of Microbiological Methods (10) and to Skerman (9). In addition, the following biochemical tests were performed: gelatin liquefaction (7), conversion of ethyl alcohol to acetic acid (11), and pigment production (4).

Conditions necessary for H₂S production. The effects of oxygen tension, temperature, and pH on the production of H₂S by the above isolates were determined by stab inoculation into slanted and butted tubes of LAC, adjusted to pH values from...
RESULTS AND DISCUSSION

Identification of the pigment. The spectra of the exudate from the commercial packages, after the treatments described, are shown in Fig. 1. The spectral analysis of the exudate from the experimental packages yielded similar results to those obtained from the commercial packages.

The peak given by the original solution at 617 nm is characteristic of sulffmyoglobin (6), although ferrocholeglobin and ferrocholemyoglobin also exhibit absorption maxima in this region at about 628 and 635 nm, respectively. The disappearance of this band upon treatment with alkali and dithionite eliminated the possibility that the pigment was either of the choleglobins. The band is merely shifted to shorter wavelengths (about 620 nm) with the choleglobins (6) whereas sulf-myglobin, upon denaturation, forms the protomyochrome with a strong absorption peak at 557 to 558 nm (6). The other reactions confirm the pigment to be sulfmyoglobin. Thus, oxidation with ferricyanide or molecular oxygen yielded the red met sulfmyoglobin, and the absorption peak at 617 nm disappeared (8). Treatment with dithionite led to reduction back to the green sulfmyoglobin and reappearance of the peak at 617 nm.

The peak at 580 nm observed in the original solution was due to the presence of unreacted oxy myoglobin, which upon reduction with dithionite yielded reduced myoglobin, with a peak at 560 nm, whereas oxidation by ferricyanide yielded metmyoglobin with a slight absorption peak at 630 nm (6). Comparison of the extinction coefficients, at 580 and 617 nm, of oxy myoglobin and sulfmyoglobin (6, 8) indicated that the pigments were present in approximately equal amounts in the original exudate. The absorption due to the nonsulfur-containing pigments, at wavelengths greater than 600 nm, is only about one-tenth of that due to sulfmyoglobin (8) and thus would not complicate the spectral analysis, at these wavelengths, of the sulfmyoglobin in the exudate.

Isolation and identification of the \( \text{H}_2\text{S} \)-producing bacteria. Approximately one-tenth of the \( \text{H}_2\text{S} \)-producing organisms found on the LAC plates were screened, giving a total of 40 isolates. All of these isolates were pseudomonads.

Chai et al. (2) isolated an organism from fish that was capable of producing \( \text{H}_2\text{S} \) at 1 C. They identified this organism as \textit{Pseudomonas putrefaciens}. All of the \( \text{H}_2\text{S} \)-producing organisms isolated from the experimental packages, and 90% of those isolated from the commercial packages, exhibited reactions differing from \textit{P. putrefaciens} in litmus milk reaction and in rapidity of gelatin liquefaction. Ayres (1) reported the isolation of \textit{P. putrefaciens} and \textit{P. mephitica} from meat and regards these two organisms as closely related. Our isolates fitted the description of \textit{P. mephitica} (Bergey's Manual, 7th ed.), except that in the original isolation of this organism Claydon and Hammer (3) reported no \( \text{H}_2\text{S} \) production.

The remaining 10% of the \( \text{H}_2\text{S} \)-producing isolates from the commercial packages differed from \textit{P. mephitica} also in litmus milk reaction and oxidation of glucose to acid.

One of the isolates fitting the description of \textit{P. mephitica} but producing \( \text{H}_2\text{S} \) was used for further study.

Conditions necessary for \( \text{H}_2\text{S} \) production. \( \text{H}_2\text{S} \) production was observed in the LAC tubes, at \( \text{pH} \) 6.0 and above, after 2 days at 20 C and 14 days at 2 C. No \( \text{H}_2\text{S} \) production was observed at \( \text{pH} \) 5.9 or below under these conditions. The \( \text{H}_2\text{S} \) production occurred only between the deeper butt and the slant-butt junction, i.e., only where limited \( \text{O}_2 \) was available.

Thus, when meat with a \( \text{pH} \) of 6.0 and above is held under low \( \text{O}_2 \) tension, there is likelihood of a
green discoloration due to the formation of sulfmyoglobin. This has been confirmed in the study of commercial packages and by experiments in which meat was inoculated with the isolated P. mephitica. The relevant data for four commercial packages are shown in Table 1, which demonstrates the unique conditions necessary for sulfmyoglobin formation.

Thus in packs B and C, in which the O2 tension was low (1 to 2%), and the pH above 6.0, sulfmyoglobin was formed even though the proportion of H2S producers was only 2 to 5% of the total population. In pack D, although the proportion of H2S producers was 40% of the total population and the pH above 6.0, sulfmyoglobin was not formed because the O2 tension was high (18%). In pack A, with 4% H2S producers and low O2 tension (2%), there was no evidence of sulfmyoglobin because the pH was below 6.0 (5.7). For all other packages showing sulfmyoglobin formation, the combination of high muscle pH (above 6.0) and low O2 tension (1 to 3%) was found.

Thus, sulfmyoglobin formation is observed only on meat having a pH above 6.0, since at lower pH values the bacteria are unable to produce H2S.

In reinoculation experiments, a culture of P. mephitica was inoculated onto meat of pH 6.4 and stored under low O2 tension (~1%) for 21 days at 2 C. The green exudate of sulfmyoglobin was observed after 14 days, and the color became more intense on further storage.

Low O2 tensions have two effects on the formation of sulfmyoglobin. (i) They allow the bacteria to produce H2S from sulfur-containing amino acids (9), and (ii) they allow the formation of the green reduced sulfmyoglobin, as at higher O2 tensions oxidation to the red metsulfmyoglobin occurs (8).

To avoid this green discoloration under low O2 tensions (in gas-impermeable packages or controlled atmospheres), meat of a high ultimate pH should not be used, since these conditions may allow bacterial H2S production and subsequent sulfmyoglobin formation.

**TABLE 1. Effects of internal atmosphere and meat pH on sulfmyoglobin formation in commercial packages after storage at 2 C for 21 days**

| Sulfmyoglobin formationa | pH of meat | O2 in package | Total count/cm² (TPYb Agar) | No. of H2S-producing organisms/cm² (LACc agar) |
|--------------------------|------------|---------------|-----------------------------|---------------------------------------------|
| (A) No                   | 5.70       | 2.1           | 4.5 x 10⁷                   | 1.9 x 10⁴                                  |
| (B) Yes                  | 6.55       | 2.3           | 1.7 x 10⁶                   | 8.8 x 10⁴                                  |
| (C) Yes                  | 6.05       | 1.2           | 2.9 x 10⁶                   | 5.8 x 10⁴                                  |
| (D) No                   | 6.25       | 18.0          | 8.5 x 10⁷                   | 3.8 x 10⁷                                  |

a Letters A to D indicate four commercial packages.

b Tryptone-phytane-yeast extract.
c Lead acetate-agar containing 0.001% cysteine.

**LITERATURE CITED**

1. Ayres, J. C. 1960. Relationship of organisms of the genus *Pseudomonas* to the spoilage of meat, poultry and eggs. J. Appl. Bacteriol. 24:471-486.
2. Chai, T., C. Chen, A. Rosen, and R. E. Levin. 1968. Detection and incidence of specific species of spoilage bacteria on fish. J. Appl. Bacteriol. 24:1738-1741.
3. Claydon, T. J., and B. W. Hammer. 1939. A skunk-like odor of bacterial origin in butter. J. Bacteriol. 37:251-258.
4. King, E. O., M. K. Ward, and D. E. Rancey. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.
5. Lawrie, R. A. 1966. Meat science. Pergamon Press, Oxford.
6. Lemberg, R., and J. W. Legge. 1949. Hematin compounds and bile pigments. Interscience Publishers Inc., New York.
7. Levin, R. E. 1968. Detection and incidence of specific species of spoilage bacteria on fish. J. Appl. Bacteriol. 24:1734-1737.
8. Nicholls, P. 1961. The formation and properties of sulphmyoglobin and sulphatase. Biochem. J. 81:374-383.
9. Skerman, V. B. D. 1959. A guide to the identification of the genera of bacteria, 1st ed. Williams & Wilkins Co., Baltimore.
10. Society of American Bacteriologists. 1957. Manual of microbiological methods. McGraw-Hill Book Co., Inc., New York.
11. Stanier, R. Y. 1947. Acetic acid production from ethanol by fluorescent pseudomonads. J. Bacteriol. 54:191-194.