

Evaluation of Inosine Monophosphate and Hypoxanthine as Indicators of Bacterial Growth in Stored Red Meat

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(Received for publication August 12, 1982)

ABSTRACT

Newly developed high performance liquid chromatography (HPLC) methods demonstrated that changes in inosine monophosphate (IMP) and hypoxanthine occurred during storage of beef held at 5°C. The inability of a spoilage organism (*Pseudomonas fluorescens*) to metabolize nucleotides in broth culture suggested that there was not a causal relationship between bacterial growth and these changes. Experiments designed to selectively inactivate (a) the bacteria flora of meat but not its enzymes (treatment with γ -irradiation or ethylene oxide), (b) meat enzymes (inoculating autoclaved meat with pure cultures of bacteria) or (c) both bacteria and meat enzymes (uninoculated autoclaved meat) demonstrated that changes in IMP and hypoxanthine levels were the result of activity of endogenous meat enzymes.

Inosine monophosphate (IMP) and hypoxanthine have been suggested as chemical indicators of quality in stored meat (5,6,9), but it has never been shown whether these chemical changes result from microbial activity or enzymes endogenous to the meat itself. Studies of microbial spoilage of meat have shown that most of the important biochemical changes occur in the meat's aqueous phase (1,2,4,7,8). The aqueous phase of meat contains nonvolatile compounds, such as amino acids, nucleotides, oligopeptides and carbohydrates, all of which can serve as ready substrates for bacterial growth. Column, thin-layer and paper chromatographic methods for the identification of these compounds in complex matrices are severely limited (5,6,9). Recent advances in high performance liquid chromatography (HPLC) offer improved quantitative methods for characterizing biochemical changes of these nonvolatile components found in the aqueous phase of meat.

In this study, HPLC methods have been developed to follow changes in IMP and hypoxanthine levels in meat stored at 5°C, and to determine if these changes are the result of microbial activity or endogenous meat enzymes.

MATERIALS AND METHODS

Reagents

l-Amino acids, creatine, creatinine, adenosine, guanosine, inosine monophosphate and hypoxanthine (gold label) were obtained from Calbiochem (La Jolla, CA). Glucose (reagent grade) was obtained from J. T. Baker (Phillipsburg, NJ).

Cultures and culture conditions

Bacteria used in this study were short, gram-negative rods previously isolated from spoiled ground beef. They were identified as *Pseudomonas fluorescens*, *P. fluorescens* (biotype II) and the indigenous microflora of the experimental meat samples.

Viable bacteria were counted by surface plating appropriate serial dilutions (made in 0.1% peptone [Difco] water) onto nutrient agar (Difco) with a Spiral plater (Spiral Systems, Bethesda, MD). Plates were counted after 48 h of aerobic incubation at 20°C. For the meat samples, 50 g of meat were placed in a Stomacher bag, 200 ml of sterile distilled water added, and the mixture was massaged for 3 min. The meat slurry (5 ml) was then added to 5 ml of peptone water and appropriate serial dilutions were surface plated as above.

Meat preparation

For preliminary experiments, the outside portion of a beef-bottom round (~2.5 cm) was cut away using a sterile knife and the lower bacterial count meat was removed from the center. Both portions were ground twice in a sterilized grinder through a 3/16-in. plate and allowed to spoil normally at 5°C. The meat was used to follow changes in IMP and hypoxanthine with bacterial growth. To determine if meat enzymes were responsible for the changes in IMP and hypoxanthine in meat, a 6.8-kg (15-lb) piece of beef-bottom round was used. This meat was obtained from an animal that had been slaughtered less than 48 h before the start of the experiment and had a viable count per g (VC/g) less than 1.4×10^2 . The outside portion was removed as described above. A portion of the center (1500 g) was sterilized with ethylene oxide for 7 h at 40°C in a Cryotherm sterilizer and then twice ground through a sterile grinder (3/16-in. plate). Two other 1500-g center portions were twice ground as above. One was used as an untreated control and the other was vacuum-packaged, packed in ice and γ -irradiated using a cesium-137 irradiator for 1 h. The total radiation dose was 1 Mrad. The outer portion (1500 g) was twice ground as above and autoclaved as a layer in a covered pan for 20 min at 121°C. The control, irradiated, autoclaved or ethylene oxide-treated meat was split into three portions containing 500 g each. For each treatment, the first portion served as the uninoculated control, the second portion was inoculated with the spoilage isolate, *P. fluorescens*. The third portion was inoculated with a reference strain of *P. fluorescens*. These 12 samples were incubated at 5°C. Plate counts were done by periodically removing 25-g samples from each portion and placing each in a Stomacher

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bag. Sterile distilled water (100 ml) was added to each 25-g sample, massaged for 2 min in a Stomacher 400 lab blender and sampled for bacteriological analysis as described above. The aqueous mixture (3°C) was centrifuged for 10 min at 10,000 RPM. Lipids were removed by passing the supernatant through glass wool. Proteins were removed by passing through Sephadex G-10 or coagulated using a method described by Gill (3). The solution, free of lipid and protein was diluted to 500 ml with water for chromatographic analysis.

Chromatography

Chromatographic separations were done on a Waters Associates (Milford, MA) system which included the following components: Model 6000A solvent delivery system, Model U6K injector, 30 cm × 4 mm i.d. μ Bondapak C₁₈ column, and Model 450 variable wavelength detector equipped with a stop-flow auto-scanning device. The UV detector head was continuously purged with dry nitrogen to permit stable operation at 200 nm. Samples (25 μ l) of the protein-free diluted meat and basal medium were injected on the column. When not in use, the columns were purged with several column volumes of aqueous methanol before storage to prevent bacterial growth and to facilitate column re-equilibration.

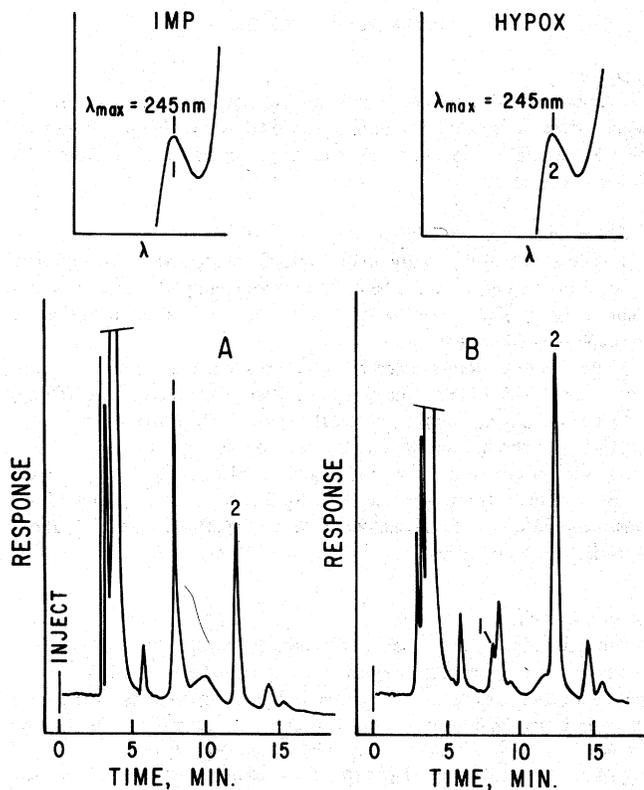


Figure 1. HPLC profiles of meat stored from zero (A) to 10 days (B) at 5°C. Peaks 1 and 2 inosine monophosphate (IMP) and hypoxanthine, respectively. (Reversed-phase chromatography column μ -Bondapak C₁₈; mobile phase, 0.01 M KH₂PO₄, pH = 5.00; UV detector 200 nm).

RESULTS AND DISCUSSION

Examination of reversed-phase high performance liquid chromatographic (RP-HPLC) profiles indicated that, during the preliminary experiments with cow knuckle meat, biochemical changes had taken place in the aqueous phase of beef which had been stored at 5°C for 10 d (Fig. 1). Since the VC/g increased from 3×10^4 to 3×10^8 , it ap-

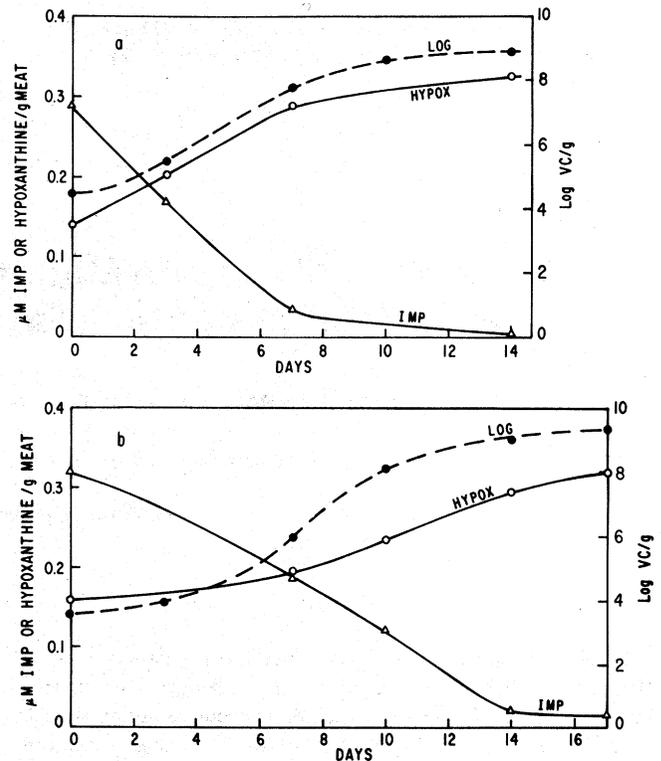


Figure 2. Changes in IMP (Δ) and hypoxanthine (\circ) levels vs. the viable count per g (\bullet) for cow knuckle stored at 5°C: (a) outside portion of cow knuckle and (b) low bacterial count center portion of cow knuckle.

peared that these changes were related to microbial growth. The concentration of inosine monophosphate (IMP) 1 decreased dramatically as hypoxanthine 2 increased (Fig. 1). These compounds were easily identified by retention times and stop-flow UV-scanning from 320 nm to 220 nm after they were partitioned on the chromatographic column and trapped in the detector cell. Both compounds had a $\lambda_{max} = 245$ nm in the mobile phase for which the separation was done (Fig. 1, insets). RP-HPLC was used to follow concentrations of IMP and hypoxanthine for cow knuckle in which the outside portion, containing higher numbers of bacteria, was aseptically separated from the center portion. The accuracy of this analysis was determined by spiking three different samples with IMP and hypoxanthine before deproteinization. Recovery was within 2% of the theoretical value IMP and within 4% for hypoxanthine. Concentrations of IMP decreased more rapidly for the outside portion of ground cow knuckle which had the highest VC/g at day 0 (Fig. 2a). The point at which the concentrations of IMP began to level-off coincided with bacteria entering the stationary phase of growth, which varied between 7 and 14 d for the two samples. Hypoxanthine levels, which have been reported to have potential use for describing organoleptic changes in meat during low temperature storage (3), agreed well with VC/g for the outside portion of ground cow knuckle (Fig. 2a). However, for the aseptically excised center portion, correlation was not as good since the concentration of hypoxanthine did not increase as rapidly as bacterial growth (Fig. 2b).

TABLE 1. Experimental design for selective inactivation of meat enzymes and bacteria.

Inoculum	Treatment			
	Control	Autoclave	Ethylene oxide	γ -Irradiation
Endogenous flora	a	b	c	d
<i>P. fluorescens</i>	e	f	g	h
<i>P. fluorescens</i> (biotype II)	i	j	k	l

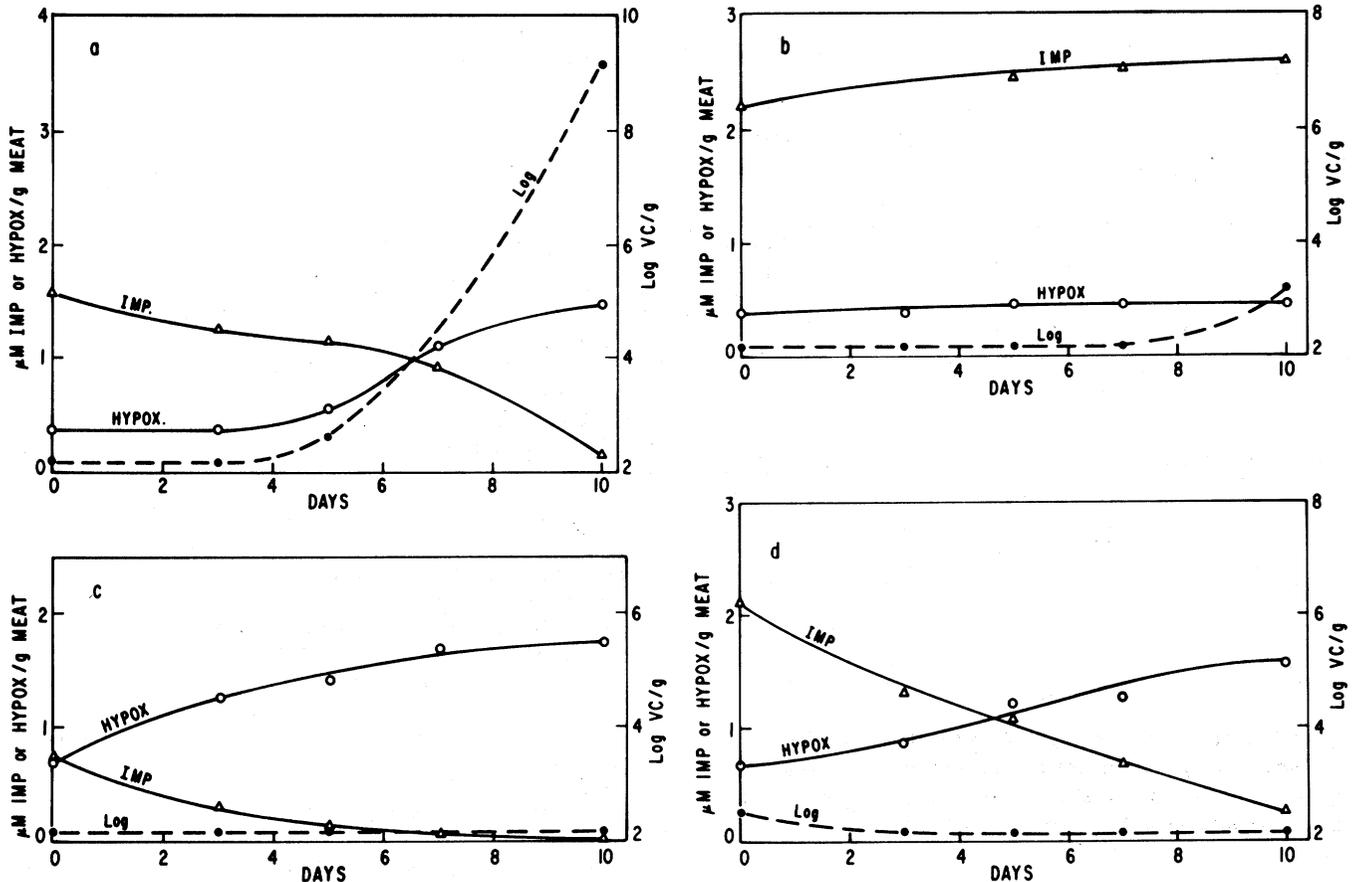


Figure 3. Changes in IMP (Δ) and hypoxanthine (\circ) levels vs. the viable count per g (\bullet) for cow bottom round stored at 5°C: (a) control, uninoculated, (b) Autoclave, uninoculated, (c) ethylene oxide, uninoculated and (d) γ -irradiation, uninoculated.

To determine if changes in IMP which occurred in the beef resulted from bacterial activity, a broth culture system was developed. It consisted of the basal medium, containing glucose, amino acids and nucleotides, and was inoculated with the meat spoilage *P. fluorescens*. In all cases, amino acids were utilized at 5°C. Nucleotides were not utilized as indicated by HPLC measurements and the absence of turbidity. Also included were experiments in which nucleotides were the sole source of carbon and nitrogen. These suggested that the decrease of IMP in meat resulted from the activity of muscle enzymes. In addition to nucleotides, creatine and creatinine levels, also determined by HPLC, were unchanged in the presence of *P. fluorescens* incubated at 5°C.

Since the nucleotides were not metabolized by *P. fluorescens* in broth culture, the utilization of IMP and subsequent formation of hypoxanthine in meat was investigated. The bacteria in the meat were selectively inactivated

by treatment with either γ -irradiation or ethylene oxide, and both the meat enzymes and bacteria were destroyed in the autoclave (Table 1). Three sets of meat were investigated. The first contained the endogenous microflora and sets 2 and 3 were inoculated with the spoilage isolate of *P. fluorescens* and the reference strain of *P. fluorescens*, respectively, to determine the effects of a large bacterial inoculum on IMP and hypoxanthine levels in meat.

In the cases where the meat enzymes were not destroyed (Fig. 3a, c, d) hypoxanthine increased and IMP decreased independent of bacterial growth. Because similar results were obtained with uninoculated meat and meat inoculated with either *P. fluorescens* strain, only representative results are shown. For the autoclaved sample in which the meat enzymes were destroyed, concentrations of IMP and hypoxanthine were unchanged. Comparison of the irradiated samples (Fig. 3d and 4a,b) demonstrated that a large inoculum of either strain of *P. fluorescens* did not

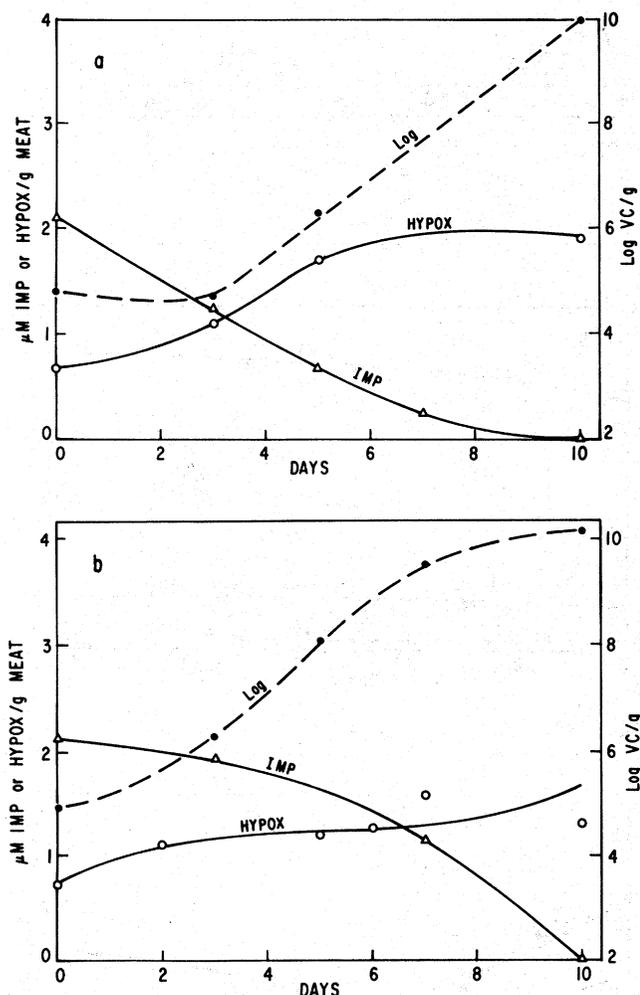


Figure 4. Changes in IMP (Δ) and hypoxanthine (\circ) levels vs. the viable count per g (\bullet) for cow bottom round stored at 5°C: (a) γ -irradiation, inoculated with *P. fluorescens* and (b) γ -irradiation, inoculated with *P. fluorescens* (biotype II).

alter the pattern of changes in the meat. If the lag phase bacteria played an important role in the conversion, the reaction would have been more rapid in the presence of the large inoculated sample than with the endogenous flora. This was not observed. Therefore, the conversion of IMP to hypoxanthine must be due to the endogenous meat enzymes.

These data conclusively demonstrate that changes in the concentrations of IMP and hypoxanthine in meat are the result of meat enzymes and not microbial activity. Attempts by previous authors (5,6,9) to use these two compounds as indicators of organoleptic changes in meat were partially

successful because the chemical changes indicative of spoilage resulted from the activity of endogenous meat enzymes. Although IMP and hypoxanthine cannot be used as indicators of microbial spoilage, they may be useful as an index of the freshness of stored meat, particularly in the case of perishable foods which have been exposed to low-level irradiation to extend their shelf-life. Such foods would be susceptible to atypical spoilage due to residual meat enzyme activity rather than bacterial growth. While traditional bacterial indices of spoilage would not apply, a chemical index of spoilage would be useful.

ACKNOWLEDGMENTS

We thank Drs. J. J. Shieh, T. A. Foglia and J. L. Smith for their helpful suggestions and Miss J. Bowers for carrying out most of the HPLC determinations. We also thank Dr. S. Doores (Penn State University) for identification of the meat spoilage organism and for supplying the culture of *P. fluorescens* biotype II.

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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