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Inhibitory effect of some Perigo-type compounds on clostridium spores in pasteurized meat products

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Abstract

In batches of 5–10 cans of cured pasteurized beef and ham products, *S*-nitrosocysteine and nitrite inhibited growth of clostridia but a complex of cysteyl–nitric oxide–ferrate did not. In an experiment in which the cans of beef product were stored at 3 °C immediately after pasteurization, inhibition increased because of nitrite and the increase was related to an increase in the mass fraction of protein-bound nitrite.

The contribution of the rather labile *S*-nitrosothiols to the inhibition in meat products is considered in the light of the results

Introduction

Since the 1973 Symposium on Nitrite in Meat Products (Krol & Tinbergen 1974), little has been published about the Perigo-type inhibition of clostridial growth in meat products but only in culture media. Grever (1975) concluded that the Perigo-type effect could not be induced by addition of cysteine, Fe(II) and nitrite to canned meat products, either pasteurized or sterilized. He observed no inhibition at all. Other authors reported a small contribution of Perigo-type inhibition to bacteriological stability (Ashworth & Spencer 1972; Ashworth et al. 1973; Chang et al. 1974; Chang & Akhtar 1974). This paper considers whether other reaction products of nitrite, besides nitrite itself, may contribute to the bacteriological stability of meat products.

Black Roussin salt was not tested as it seems unlikely that this compound is formed in a pasteurized meat product, little H_2S being produced, and its inhibitory effect is appreciably diminished in meat products (information from C. L. Walters 1974).

Materials and methods

S-Nitrosocysteine was prepared as described by Mirna & Hofmann (1969) and was estimated by ultraviolet spectrometry.

Preparation of the cysteyl-nitric oxide-ferrate. In nitrogen-saturated distilled water, dissolve 11 g cysteine, 5 g $FeSO_4.7H_2O$ and 14 g histidine (buffer). Adjust the

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pH with dilute NaOH to 6.40 and make up to 750 g. Put about 400 g of the solution into a three-necked flask equipped with a gas inlet tube, a dropping funnel, and a slotted tube for the removal of waste gas. The solution was stirred magnetically. Air in the flask is removed with special-grade nitrogen gas, before nitric oxide was introduced. Add other reaction components drop-by-drop through the funnel and allow to react for 30 min at room temperature. Discard the precipitate separated from the solution by nitrogen pressure filtration. Concentration of ferrate in the black solution was estimated by ultraviolet spectrometry (van Roon 1975). An adequate amount was added to the brine used for preparation of the ham product.

Mass fraction of free and protein-bound nitrite in the meat products was estimated by a method of Olsman (1977b).

Spore suspensions of *Clostridium sporogenes* strain 945 (Grever 1975) were kindly supplied by the Netherlands Centre for Meat Technology of the Central Institute for Nutrition and Food Research. Anaerobes were counted on pour plates containing tryptone 15, yeast extract 10 and agar 15 g $litre^{-1}$, pH 7.0 ± 0.1 (range). Aerobes were counted on pour plates with tryptone 15, meat extract 3, dehydrated yeast extract 5, peptonized milk 15, dextrose 1 and agar 15 g $litre^{-1}$, pH 7.0 ± 0.1 (Mossel & Krugers Dagneaux 1959). All the plates were incubated for 3 d at 30 °C.

Preparation of the comminuted ham product. Lean ham, trimmed free from adipose and connective tissue, was minced through an 8-mm plate and mixed. The brine, already containing the inhibitors and *Clostridium sporogenes* spores, was added and mixed with the meat in a bowl chopper. Average mass fractions of additives in the product were NaCl 24 (in first tests 17), commercial phosphate mixture 4, glucose 5, sodium glutamate 0.9 and ascorbic acid 0.4 g kg^{-1}. The spore load was about 10^3/g ham product. The mixture of meat and brine was canned (diam. and height 76 mm x 35 mm or 76 mm x 57 mm) and stored overnight in a chilling room. Next day the cans were heated to a core temperature of 68.9 °C. Cans fitted with thermocouples were used to check the temperature.

Preparation of the comminuted lean beef product. Mass fractions of additives to the minced lean beef product were NaCl 25, caseinate 20, commercial phosphate mixture 5 and starch 40 g kg^{-1}. No ascorbate was used. A product of lower pH was obtained by adding glucono-δ-lactone 5 g kg^{-1}. A suspension of cow-dung was added to give a load of about 10^4 clostridial spores per g beef product. The cans were pasteurized to a core temperature of 80 °C maintained for 10 min.

Incubation test. The cans of ham or beef, 5–10, were incubated at 30 °C. The time it took to produce swells was recorded and was used as a criterion of clostridial growth. Some flat and swollen cans were tested bacteriologically.

Results

Inhibition of clostridia by cysteyl–nitric oxide–ferrate or sodium nitrite in ham product. Figure 1 gives results of the incubation test. The pH of the product

ranged from 5.8 to 6.1. Ferrate made the product look grayish. Inoculated ham from swollen cans contained $> 10^6$ clostridia per g and uninoculated ham from some swollen cans gave counts of $10^4 - 10^5$ per g for aerobic and anaerobic incubation. No spore-forming micro-organisms were detected. The bacteria were not identified or classed.

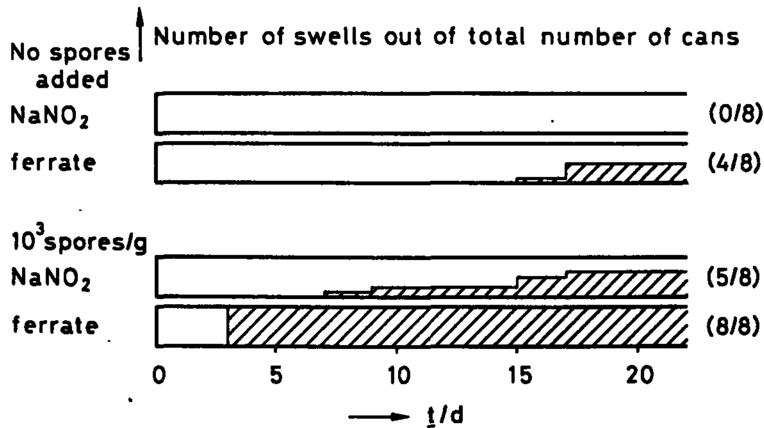

Fig. 1. Inhibition of Clostridia (number of swollen cans, 8 per group) by $NaNO_2$ at a mass fraction of 104 mg kg^{-1} in the pasteurized ham product and by dicysteyl – dinitric oxide – ferrate at a mass fraction of 80 mg kg^{-1} of $NaNO_2$ equivalent in the ham product incubated at 30 °C. 1 mg $NaNO_2$ equivalent = 2.6 mg ferrate. Inoculation with 10^3 spores per g product.

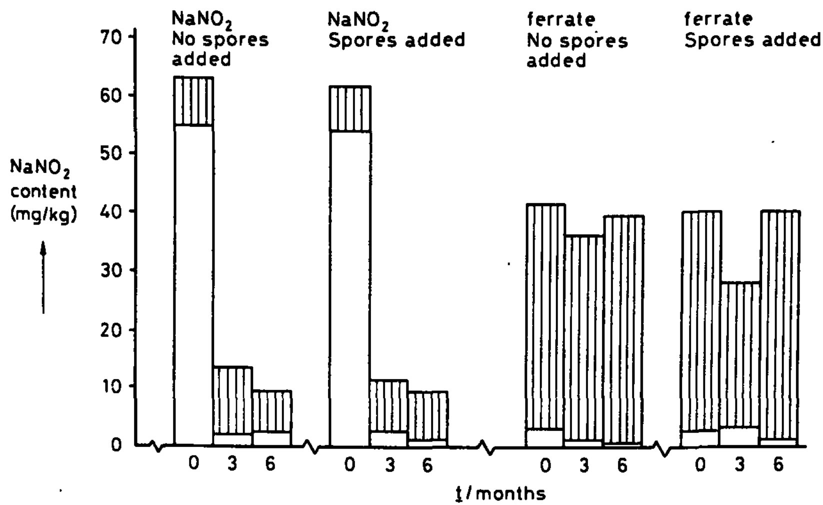

Fig. 2. Effect of storage time (t / months) at 8 °C and addition of $NaNO_2$ or dicysteyl – di nitric oxide – ferrate on mass fractions (mg kg^{-1}) of free (unshaded) and protein-bound (shaded) nitrite (expressed as $NaNO_2$) in a pasteurized ham product. Amounts of additives as in Figure 1.

Thus ferrate was a poorer inhibitor of clostridial growth than nitrite, as was found by Grever (1975).

Some other batches of the ham were stored for up to 6 months at 8 °C. No clostridia grew. The ferrate did not influence the number of viable clostridia. With nitrite, counts of clostridia were about 10^2 per g after 6 months.

Heat treatment reduced the mass fraction of total nitrite considerably (Fig. 2). The added ferrate caused an increase in protein-bound nitrite, which remained almost constant during storage.

Inhibition of clostridia by protein-bound nitrite in beef. Olsman & Krol (1972) and Olsman (1974; this symposium) studied nitrite depletion in a beef product. We tested a possible relation between mass fraction of protein-bound nitrite and clostridial growth in this type of product.

After pasteurization, some batches of cans were immediately put in an incubator at 30 °C; others were first stored at 3 °C in a chilling room (Fig. 3 and 4). Ten cans of each batch were transferred from the chilling room to the incubator and one was used for estimation of free and protein-bound nitrite.

Figures 3 (pH 6.2) and 4 (pH 5.9) indicate a relation between storage time at 3 °C and time at 30 °C before cans containing the same amount of added nitrite swelled. The effect at pH 5.9 seemed greater than at 6.2.

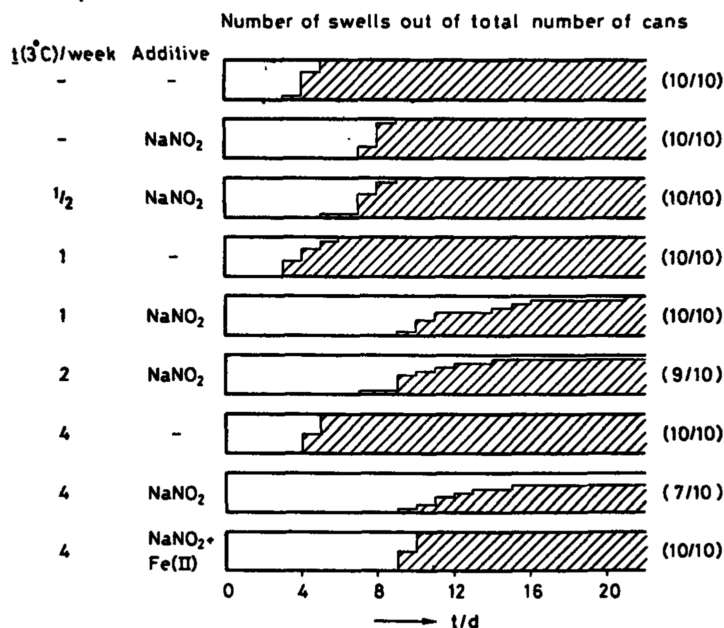

Fig. 3. Effect of storage time (t / week) at 3 °C immediately after pasteurization on the inhibition of Clostridia (number of swollen cans, out of 10) by $NaNO_2$ in a beef product. Inoculation with suspension of cow dung (10^4 spores per g product); pH 6.2. Mass fraction of added $NaNO_2$ 200 mg kg^{-1} and of Fe(II) 50 mg kg^{-1}; subsequent incubation at 30 °C.

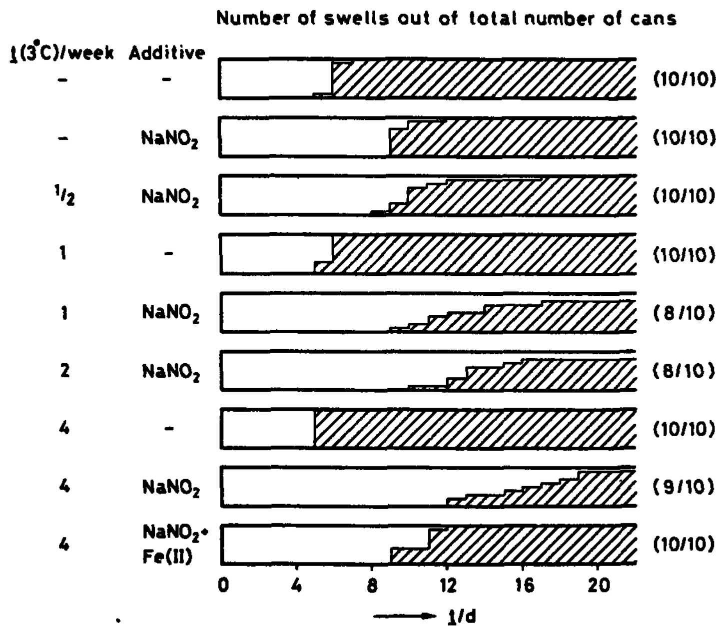

Fig. 4. Effect of storage time (t / week) at 3 °C immediately after pasteurization on the inhibition of Clostridia by $NaNO_2$ in a beef product, pH 5.9. Further conditions as in Figure 3.

During storage at 3 °C, free nitrite fell and protein-bound nitrite increased by a smaller amount (Fig. 5). At pH 5.9, there was more bound nitrite than at 6.2. These results suggest a relation between mass fraction of protein-bound nitrite at the moment of transfer to the incubator and enhancement of the inhibition of clostridia by nitrite.

Addition of a small amount of Fe(II) increased protein-bound nitrite, but reduced inhibition (Fig. 3, 4 and 5).

Inhibition of clostridia by S-nitrosocysteine or sodium nitrite in ham product. A ham product was formulated with several levels of *S*-nitrosocysteine and $NaNO_2$ (Fig. 6 and 7). Meat for some cans was inoculated with the *Clostridium sporogenes* strain 945 (10^3 spores per g product); in uninoculated cans, free and protein-bound nitrite were estimated. After pasteurization, the cans were incubated at 30 °C.

Inoculated cans that swelled all contained 10^6-10^8 *Clostridium sporogenes* per g product. During incubation at 30 °C, all samples developed a flora mainly of enterococci. Counts were sometimes as high as $>10^7$ per g. The enterococci decreased the pH of the product, as 0.55% glucose had been added. The drop in pH in some cans was considerable, from pH 6.5 to 5.8, and could influence results, but probably in a uniform manner. The product with added *S*-nitrosocysteine had the normal pink colour. The two substances inhibited clostridia (blown cans) to a similar extent (Fig. 6 and 7).

Pasteurization decreased free and total nitrite (Fig. 8). Incubation at 30 °C causes free and protein-bound nitrite to decrease rapidly. But even before pasteurization, there was less nitrite than had been added.

S-nitrosocysteine was labile in the brine added to the ham in the bowl chopper. Amounts have been corrected as far as possible, but some nitrosothiol would undoubtedly be decomposed into nitric oxide during mincing and could explain the lower mass fractions of total nitrite in the samples before pasteurization.

Discussion

Nitrite is one of the important factors in the bacteriological stabilization of cured meat products, especially for clostridia. However rather high levels have proved necessary (Ingram 1974; Grever 1974). But free nitrite is depleted in meat products during storage after heat treatment. (Nordin 1969; Olsman & Krol 1972; Olsman 1974 and this symposium; Sebranek 1974). One or more reaction products

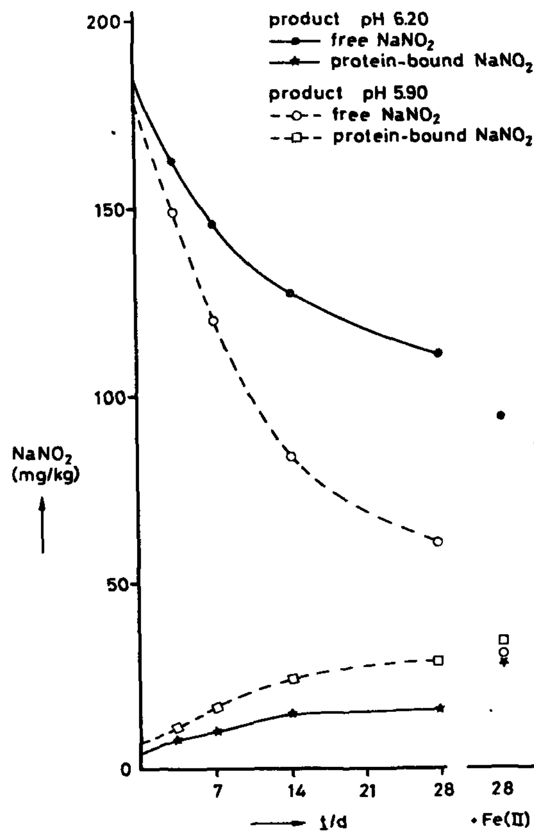

Fig. 5. Levels of free and protein-bound $NaNO_2$ in the beef product as effected by storage at 3 °C.

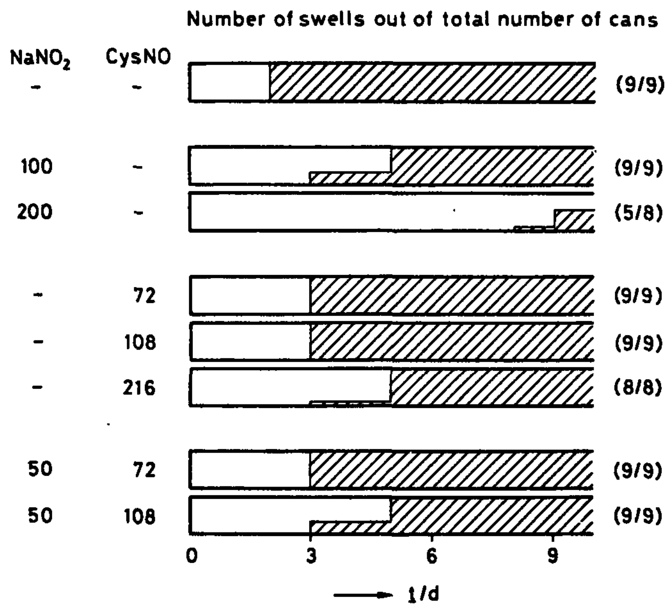

Fig. 6. Inhibition of Clostridia (number of swollen cans, out of 8 or 9) by $NaNO_2$ and *S*-nitrosocysteine in the pasteurized ham product incubated at 30 °C. 1 mg $NaNO_2$ equivalent = 2.2 mg *S*-nitrosocysteine. Inoculation with 10^3 spores per g product.

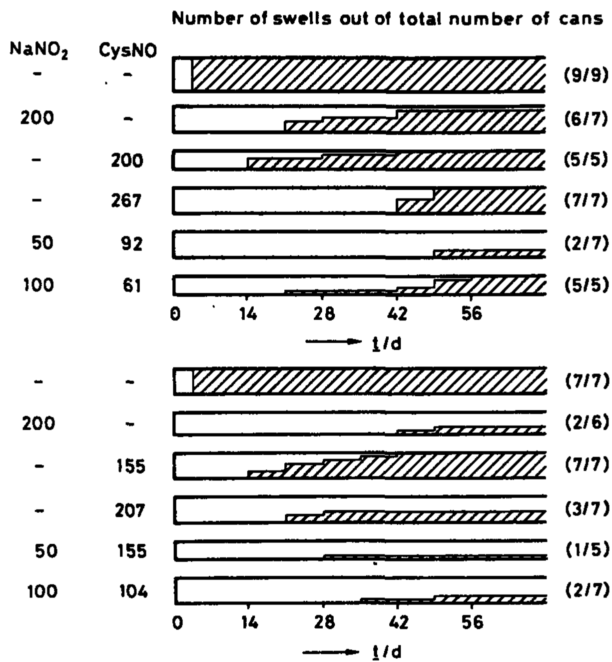

Fig. 7. Inhibition of Clostridia by $NaNO_2$ and *S*-nitrosocysteine in the pasteurized ham product incubated at 30 °C. Except for added mass fractions, the conditions are the same as in Figure 6.

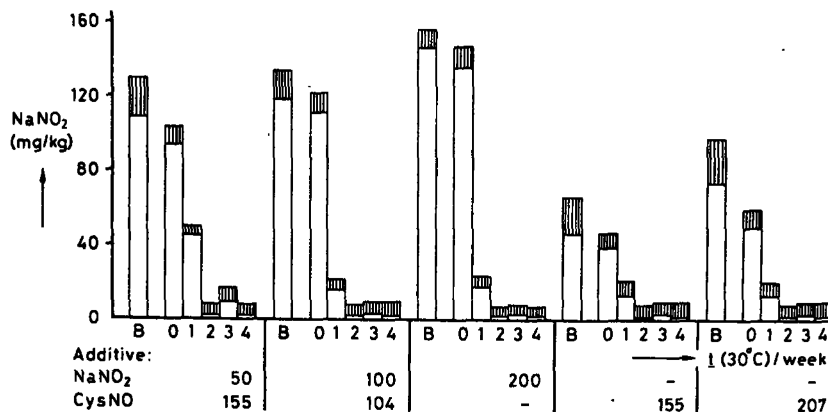

Fig. 8. Mass fractions of free (unshaded) and protein-bound (shaded) nitrite before (B) and after pasteurization (t = 0) and incubation at 30 °C for 1–4 weeks. Additives are expressed as mass fractions of $NaNO_2$ or its equivalent (mg/kg^{-1}).

of nitrite might inhibit growth. A small Perigo-type effect was demonstrated in a meat product depleted of nitrite (Chang & Akhtar 1974; Chang et al. 1974).

Mirna & Hofmann (1969), Olsman & Krol (1972), Olsman (1974 and this symposium), Kubberød et al. (1974) and Fox & Nicholas (1974) have reported formation of a group of reaction products of nitrite: *S*-nitrosothiols. Soluble nitrosothiols have proved inhibitory in culture media (Incze et al. 1974; Moran et al. 1975; Hansen & Levin 1975; Huhtanen 1975). In our tests the soluble *S*-nitrosocysteine inhibited clostridial growth when added at about the same substance content (mol/kg) as nitrite. Total nitrite increased rapidly in the first week of incubation (Fig. 8). Batches with larger amounts of inhibitors could be incubated longer before they blew. There are several possible explanations. Other inhibitory compounds not detected by nitrite analysis may have formed. The majority of the spores may have lost viability, but this aspect was not examined. Growth of enterococci lowered pH in the product, and may retard clostridial growth.

Duration of storage at 3 °C before incubation proved to be related to enhanced inhibition by nitrite. The longer the storage at 3 °C the higher the mass fraction of protein-bound nitrite in the product. But other inhibitors derived from nitrite, might be present. Inhibition of clostridia could be due to insoluble *S*-nitrosothiol, bound through thiol groups of the meat protein. It can hardly be expected that insoluble compounds are inhibitory. Olsman reports in this symposium on the lability of protein-bound nitrite, in particular at higher temperatures like 30 °C. Nitrosothiol decomposes to nitric oxide, which is not likely to be inhibitory (Shank et al. 1962). Presumably nitric oxide reacts with other endogenous substances to enhance inhibition by nitrite.

In contrast to its behaviour in culture media, Fe(II) reduces the inhibitory effect of nitrite (this paper) or removes it (Grever 1975). Fe(II) readily forms coordination complexes with proteins through thiol groups and with nitric oxide (van Roon 1974), so increasing protein-bound nitrite (Fig. 5). Free cysteyl–nitric oxide–ferrate was strongly adsorbed to meat protein (Figure 2), losing its inhibitory proper-

ties. As Fe(II) complexes are more stable than nitrosothiols, small amounts of free nitric oxide would be expected in the products. This might explain the poor inhibitory effect of these compounds in meat products and suggests the utility of nitric oxide in inhibiting bacteria in meat products. Some Perigo-type inhibition might be present in meat products. *S*-Nitrosothiols contribute directly or indirectly to this effect; Fe(II) hinders inhibition.

Discussion on the session

Term Perigo-effect

Dr Ingram commented that the term Perigo effect should *not* be used in relation to meat. It is now quite clear that what Perigo observed is destroyed in the presence of meat. In this context scientists at the Meat Research Institute Bristol always refer to the term 'heated nitrite effect' for meat. There has not yet been any convincing laboratory demonstration with meat of an effect of the magnitude observed by Perigo. Certainly the term ought not to be used of unheated systems.

Safety of meat products

The question was raised which factor is the most important: the added nitrite, the 'protein-bound' nitrite or the 'residual' nitrite in the safety of these products.

The general opinion was that the amount of added nitrite is important but probably during storage some of the 'protein-bound' and 'residual' nitrite will contribute to the stability of the product. 'Protein-bound' nitrite is otherwise poorly defined and depends largely on the circumstances in a particular product.

We should also be careful with the term 'residual' nitrite because low levels of, say, below 5 mg per kg can easily be artifacts originating from nitrite released by unstable nitrosated compounds decomposing during the treatment of the sample before analysis.

Dr Cassens commented that the variation in 'residual' nitrite could also be influenced by the actual properties of the muscle. More specifically red and white muscles differ markedly in many characteristics such as pH, amount of myoglobin, amount and characteristics of lipids, type of predominant metabolism, amount of various minerals present and characteristics of the proteins. These differences are also present at the cellular level where red and white myofibres are easily discerned. The animal geneticist may be successful in minimizing the variation in biological properties of the muscle; neural control of muscle properties offers another approach to the problem. In any case, if the biological variation in muscle were minimized, the variation in 'residual' nitrite could consequently be lowered.

Background of the inhibition by nitrite

Our knowledge of the physiology of the inhibition of micro-organisms by nitrite is still very poor.