meat was covered with black specks indicating that the salt had been removed from solution by precipitation or adsorption.

Inactivation of iron nitrosylsulfides

We examined factors influencing the inactivation of iron nitrosylsulfide. A homogenate was prepared by blending fresh, defatted uncured ham and water, 1:4 by mass. An aliquot of the homogenate was heated at 70 °C for 20 min and the coagulated meat rehomogenized. The homogenates and INS were mixed in various ratios, shaken frequently, and observed at room temperature. Disappearance of INS was determined visually and verified by loss of inhibition of *C. botulinum* in the assay. With concentration of INS in the solution held constant at 250 mg litre^{-1},

Table 3. Absorption of iron nitrosylsulfide (INS) from solution by fresh ham and ham heated to 70 °C. To each tube was added 125 μg INS and the mass (*m*) of meat indicated. +, black colour in solution; –, no colour.

m(meat)/mg	Colour	
	fresh ham	heated ham
60	–	–
40	–	–
20	+	–
10	+	–
8	+	+
6	+	+
4	+	+
2	+	+
1	+	+

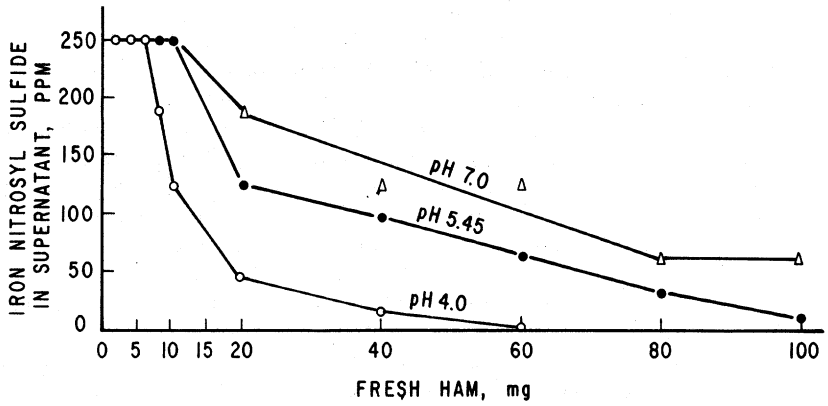

Fig. 1. Effect of pH on the absorption of iron nitrosylsulfides (INS) by meat homogenates. The solution initially contained 500 μg INS. The ordinate refers to equivalent of fresh ham used in preparing the homogenate. The abscissa refers to concentration in mg/litre.

40 mg fresh meat equivalent was required to remove all the black colour from solution, as shown in Table 3. When the meat was denatured by heating to 70 °C before addition to INS solution, only 10 mg of meat was sufficient to absorb the colour. Thus, adsorptive sites were exposed by denaturation.

The effect of pH in the usual range for meat is shown in Figure 1 where 500 μg INS reacted with varying quantities of meat homogenates adjusted to pH 4.0, 5.45, and 7.0 with a buffer of citric acid and KOH. The amount of INS adsorbed was determined by visual comparison with a series of reference solutions of the black INS. More INS was adsorbed by the meat under more acid conditions.

With our present knowledge of the behaviour of the iron nitrosylsulfides, this class of compounds cannot be used to inhibit clostridium in commercial production of canned meat.

Inhibition of clostridium by citric acid

Preliminary studies in test-tubes suggested that some simple organic or inorganic acids could be used to inhibit clostridia, either as a substitute for NO_2^- or in combination with traces of NO_2^-. A series of studies were set up with canned ham inoculated with about 1 000 spores of *C. sporogenes* per can and with citric acid and HCl as the test compounds for this class of substances. The pH of the meat, about 5.5, was reduced to 5.1 by adding the acids at equal molar concentration of acid. But frequently the swelling of the cans proved due to facultative anaerobic bacteria as determined by growth in brewer anaerobic agar. To eliminate these organisms, the effect of heating, as measured at the centre of the can, was assessed by four treatments: salt and sugar only; NO_2^- at 20 mg/kg; citric acid at 1 g/kg; citric acid at 1 g/kg plus NO_2^- at 20 mg/kg. Cans were heated in a waterbath to 68, 80, or 92 °C, taking about 19, 24 and 26 min, respectively, to reach these temperatures. With 68 °C, all cans showed nonspecific swelling.

At 80 °C, two cans of meat out of five with salt and sugar only swelled after 29 days, and were positive for *C. sporogenes*; swelling of the remainder was due to facultative anaerobes. With nitrite and heating to 80 °C, three cans of five were positive within 63 days. With citric acid, four of five cans swelled as a result of *C. sporogenes* in about 93 days; and in meat treated with citric acid and nitrite, three cans swelled in 83 days; the remaining two cans did not swell in 95 days.

At a temperature of 92 °C, all cans with only salt and sugar swelled within 29 days as a result of *C. sporogenes*. With nitrite, 4 positive swellings occurred in 63 days and with citric acid all 5 cans swelled between 93 and 100 days. With nitrite and citric acid, only one can swelled because of *C. sporogenes* and one because of nonspecific micro-organisms. Thus, nonspecific interference can usually be avoided by heating to an internal temperature of 80 °C and cooling immediately. Citric acid increased the shelf life of canned ham inoculated with *C. sporogenes* from one month to three months.

Parallel tests were begun with canned hams inoculated with about 1 000 spores of *C. botulinum* 62A per can. Results were again obscured by the growth of nonspecific bacteria. A different heating schedule eliminated growth of the nonspecific bacteria. The sealed cans were heated in the water bath to 68 °C internal temperature, and were either cooled immediately, as in commercial practice, or

Table 4. Effect of heat treatment in reducing swelling (5 cans per treatment) due to facultatively anaerobic bacteria in canned hams inoculated with *Clostridium botulinum* 62 A. *N*(Cl), number of swollen cans positive for clostridium; *N*(nd), number of cans with swelling due to nondescript bacteria; *t*, time (during which swelling occurred).

Additives	Heat to 68 °C					
	Cool immediately			Hold 30 min		
	N(Cl)	*N*(nd)	*t*/d	*N*(Cl)	*N*(nd)	*t*/d
None	1	4	5–6	5		5–6
$NaNO_2$ 25 mg/kg	2	3	7	4	1	9–12
6 mol/litre		5	22	5		9–26
HCl 6 mol/litre; $NaNO_2$ 25 mg/kg	1	4	22	4	1	13–26
Citric acid 15 g/litre	1	4	28	5		15–21
Citric acid 1.5 g/litre; $NaNO_2$ 25 mg/kg		5	28	4	1	14–27

held at 68 °C for 30 min before storage at 30 °C (Table 4).

Difference was noted in sensitivity and growth pattern between *C. sporogenes* and *C. botulinum*, which grew out more quickly and was more resistant to the effects of NO_2^- and citric acid than *C. sporogenes*. Inasmuch as a number of studies have been reported in the literature in which *C. sporogenes* was used as a test organism, inhibition data from these studies should be translated to *C. botulinum* with caution. Further studies on inhibition of clostridia by organic acids under these conditions are in progress.

Discussion on the session

Experimental conditions

The authors agree on a remark from the audience that in a can other compounds might be produced than under aerobic conditions. They were merely searching for a suitable inhibitor.

Experiments in pasteurized systems

Other micro-organisms than clostridia (e.g. bacilli and enterococci) may survive pasteurization and can grow during incubation thus altering the experimental conditions.

Heat destruction of Roussin salts

During autoclaving, added iron nitrosulfides may be destroyed.

Experiments with different Clostridium species

Dr Ingram emphasized Dr Wasserman's point about the danger of arguing from one *Clostridium* species to another. We know, for example, that *Cl. perfringens* is less resistant than *Cl. botulinum* to salt, but more resistant to nitrite, some strains

much more so. Obviously it would be unwise to assume that concentrations controlling *Cl. botulinum* apply to all clostridia. Moreover *Cl. perfringens* is of special interest as a species capable of causing food poisoning, which is rather common. It would make little sense to prescribe conditions safe for *Cl. botulinum* if they failed also to control *Cl. perfringens* and other practically important species.

Inhibition of clostridia by iron nitrosylsulfides and citric acid in canned ham

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Chemistry of iron nitrosylsulfides

At the last meeting in Zeist, van Roon (1974) and Grever (1973; 1974) described the properties of an iron nitrosylsulfide (INS) and the formation of a clostridial inhibitor in culture media autoclaved with nitrite. In our laboratory, investigation of the Perigo effect yielded some interesting but inexplicable results. Information about the role of iron provided a key. In addition to studies on the formation of the inhibitor in culture medium (Huhtanen & Wasserman 1975; Huhtanen 1975), we concentrated on methods of chemical synthesis of the inhibitor on a large scale. Methods for preparing iron nitrosylsulfides have been described since 1858; however, reaction conditions for these 'Roussin salts' have not been well characterized and succeeding investigators developed their own procedures, each postulating a different empirical formula (Rosenburg 1879; Pavel 1882; Manchot & Linckh 1926). We have studied the preparation of the potassium iron nitrosylsulfide salt under controlled conditions at pH 7–7.5 and 9.0–10.0, as well as the ammonium salt at pH 9. The reaction was in air with equimolar amounts of KNO_3 and of potassium hydrosulfide or ammonium sulfide. An almost equal molar concentration of ferrous sulfate heptahydrate was added to the boiled mixture of the nitrate and sulfide solutions, maintaining the desired pH constantly. Shiny black crystals, recrystallized twice from water, had the proximate analysis shown in Table 1.

The empirical formulae are as shown – salts of pentanitrosyl dithiotriferrate rather than the various formulae shown by others. Potassium salts prepared at

Table 1. Proximate analysis of iron nitrosylsulfide salts prepared at several pH values. *M*, relative molecular mass (molecular weight); *w*, mass fraction (content).

Sample	pH	*M*		*w*(N)/%		*w*(S)/%		*w*(total Fe)/%	
		calc.	found	calc.	found	calc.	found	calc.	found
$[Fe_3(NO)_5S_2]K$	7.0–7.5	420.8	423	16.63	16.39	15.23	15.42	39.81	39.56
$[Fe_3(NO)_5S_2]K$	9.0–10.0	420.8	396	16.63	16.79	15.23	15.06	39.81	39.46
$[Fe_3(NO)_5S_2]NH_4$	∿9.0	399.7	–	21.01	20.78	16.04	16.36	41.92	41.90

neutral or alkaline pH had the same composition, exhibited identical infrared spectra, and were soluble in water, acetone, and ether. The salt produced at pH 9.0–10.0, however, was less stable. On exposure to air and daylight, it began to turn gray after two weeks, and on exposure to direct sunlight, it started to turn gray after a few days. By contrast, the neutral salt remained unchanged for more than two months under the first set of conditions and for longer than two weeks in direct sunlight. Iron in the molecule occurred in divalent and trivalent forms; the latter predominates through oxidation on storage. In our NH_4^+ salt, almost all the iron was as Fe(III).

Inhibition of clostridium by iron nitrosylsulfides

The black, iron nitrosylsulfide had a minimum inhibitory concentration of 0.16 mg/litre as compared to 80 mg/litre for $NaNO_2$ against *Clostridium botulinum* in a tryptone-yeast extract medium. However, when meat or a crude meat extract was added to the medium, inhibition was prevented. This confirms the observations of Grever (1973; 1974). We found that autoclaving INS with the medium inactivated it, indicating that the Perigo factor, formed on autoclaving nitrite in the medium, is probably different from INS. Tests were also carried out with cans of ground fresh uncured ham inoculated with spores of *C. sporogenes* or *C. botulinum*. The 208 x 107 flip-top aluminium cans contained 70 g meat ground twice through a 3/8-inch (1-cm) plate, mixed with aqueous solutions of salt at 38 g/litre $^{-1}$, sugar at 20 g/litre nitrite or inhibitor. A spore suspension was added drop-by-drop to the meat and mixed to give about 1 000 spores/can. The cans were flushed with nitrogen, sealed under vacuum, and heated to 68 °C internal temperature. They were then cooled in water, stored at 30 °C, and observed daily for swelling. A presumptive test for clostridia was carried out by inoculating some material from the centre of swollen cans into Brewer Anaerobic Agar and incubating for 3 days at 35 °C. Gas formation and anaerobic colonies suggested the presence of clostridia; swelling of the cans was due to nonspecific facultatively anaerobic bacteria that did not produce gas in the agar shake tubes. Unfortunately confirmatory toxin tests could not be carried out. A representative comparison of inhibition by nitrite and the iron nitrosylsulfide salt, against *C. sporogenes*, is shown in Table 2.

Thus, in the presence of meat, the nitrosyl salt was not active. The surface of the

Table 2. Inhibition of swelling by nitrite and iron nitrosylsulfide in cans (10 per treatment) of ground ham inoculated with spores of *Clostridium sporogenes*. *t*, time (until swelling); INS, iron nitrosylsulfide.

Treatment	Number of cans swollen	*t*/days
None	7	21– 48
Inoculated	10	15– 25
Inoculated; NO_2^-, 150 mg/kg	9	53–100
Inoculated; INS, 100 mg/kg	10	12– 19