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Proceedings of the Second International Symposium on Nitrite in Meat Products

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Editors: B.J. Tinbergen and B. Krol

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Preface

Early in 1974 the proceedings of the first international symposium on nitrite in meat products was published. Besides more than 20 contributions and the discussion on them, they included the conclusions and resolutions agreed there. Being a topical problem, they enjoyed a wide interest from research institutes, industry and government institutions, as we have observed from reactions in the written and spoken word. A second symposium, again at Zeist, was organized for September 1976. Between the two meetings, scientists of diverse disciplines throughout the world have been engaged in examining the usefulness and the risks of the additive nitrite. For a variety of reasons, the number of contributions was doubled.

Again, the value was shown of bringing together experts in an international meeting with ample opportunity for open discussion. It became clear that the additive nitrite is still practically irreplaceable as a potent inhibitor of micro-organisms in meat products. The contribution from meat products to a person's total intake of nitrite in food and to the hazards of nitrosamine formation are relatively small.

We are indebted to the staff of Pudoc for their well known competence in preparing these large second proceedings for publication.

B. J. Tinbergen
B. Krol

Word of welcome

H. de Boer

Research Group for Meat and Meat Products TNO, Zeist

It is a pleasure for me to make a start by saying some words of welcome to you on the occasion of this second Symposium on Nitrite in Meat Products. After the first Symposium, there has been forethought on the most suitable next meeting place. Together with Professor Cassens, we decided to hold it in Zeist again, so we meet together now in this nice place of retreat. Our Research Group for Meat and Meat Products TNO has gladly given support to this follow-up of the former Symposium of September 1973. The same holds true for the Dutch authorities sponsoring the activity. Again we highly appreciate the financial aid given by the Commodity Board of Livestock and Meat, by the Ministry of Public Health and Environmental Hygiene, and by the Organization for Nutrition and Food Research TNO.

We feel happy that Pudoc (Centre for Agricultural Publishing and Documentation) will again give its competent aid in publishing the proceedings.

The marrow of this Symposium is its technical program. In its conception and organization, the efforts of our colleagues of the Netherlands Centre of Meat Technology TNO, in particular Professor Krol and Dr Tinbergen, were most important, as was your response in program development and scientific contributions. We also appreciate the readiness of invited experts to act as discussion leaders of individual sessions.

I hope this second Symposium will contribute both to broader understanding and to adequate policy on nitrite in meat products. The problem forms part of a much wider problem dealing with modern food technology and control of food quality and human nutrition in order to avoid health hazards. Here, in general, understanding of principles and scientific objectivity are essential for finding sound solutions. The complexity of the problems of this kind requires co-operation of scientists of different disciplines, which is also a dominant feature of these Symposia.

The first Symposium could be characterized primarily as surveying and weighing up the different aspects of the complicated problem, particularly the toxicological and microbiological implications. The expert views from different angles and the open discussion have undoubtedly contributed to a more balanced approach in new research as well as in food policy. Even so, we see that national regulations differ considerably in implementation. It will be of interest to evaluate the effectiveness of different approaches on this occasion.

Primarily, however, this Symposium is a scientific follow-up of the former one, focusing in particular on questions of formation and occurrence of nitrosamines and of their analysis. These are key-points in the complex of questions on nitrite as an active compound in meat processing. I hope the co-operation in this international nucleus of experts of different disciplines will result in good progress, which may also benefit others involved in the problem elsewhere. I hope the discussions will result in valuable conclusions and recommendations, making the Symposium and its proceedings most informative to all interested parties.

Microbiological session

Reporters: J.H. Houben, S.J. Mulder

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Role of nitrite and ascorbate in the microbiological safety of vacuum-packed sliced bacon

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Abstract

The role of nitrite and ascorbate in the microbiological safety of vacuum-packed sliced bacon was examined in a series of curing and inoculation tests over 9 months. Mildly salted pork (containing about 4% (w/w) NaCl on water, that is mass of salt divided by mass of salt and water) and no nitrite or nitrate readily supported growth of *Clostridium botulinum*. In bacon with sodium nitrite at an intended mass fraction of 100mg/kg (observed mean 71 mg/kg) and potassium nitrate at 250 mg/kg (observed mean 193mg/kg), growth of *C. botulinum* was significantly reduced. Protection was greater if the intended mass fraction of nitrite was increased to 200 mg/kg (observed mean 196 mg/kg). Sodium ascorbate at a mass fraction of up to 2 000 mg/kg (observed, about 1 600 mg/kg) did not reduce the protection afforded by nitrite against *C. botulinum*. Medium-salted pork and bacon (containing about 5.5% NaCl on water) did not readily support growth of *C. botulinum*. *Staphylococcus aureus* grew at 15–25 °C in bacon (with about 5.5% NaCl on water), irrespective of the presence of nitrite, nitrate or ascorbate, but enterotoxin development was prevented by vacuum-packing. Shelf life of vacuum-packed bacon was increased by nitrite and nitrate, but quality deteriorated with ascorbate on prolonged storage.

Introduction

Nitrite is used to give colour, flavour and microbiological stability to bacon, and may combine with certain amines during cooking to form traces of nitrosamines. Among suggestions to reduce nitrosamine levels formed in cooked bacon are reducing the amount of nitrite used in curing and adding ascorbates to react with the nitrite during subsequent storage and cooking of the bacon. Addition of ascorbates has been extensively studied and, though reductions in amounts of nitrosamine found on cooking have been reported (Mottram et al. 1975), there are no comprehensive data on the microbiological safety and keeping of bacons containing sufficient ascorbate to reduce nitrosamine formation.

The effect of sodium ascorbate at mass fractions of about 1 000 and 2 000 mg/kg in bacon and pork on toxin production by *Clostridium botulinum* and *Staphylococcus aureus* that had been purposely inoculated into commercially manufactured vacuum-packed sliced bacons.

Materials and methods

Bacon cures

The bacons were typical British back bacons not heavily cured and cold-smoked.

Series A bacons consisted of 11 cures manufactured over 5 weeks, and Series B of 14 cures manufactured over 4 weeks; a total of about 2 200 packs (120 g each) were produced. In each series, bacons were prepared with either about 4.0% sodium chloride on water (hereafter called 'mildly salted bacons') or about 5.5% (hereafter called 'medium-salted bacons'). The compositions of the curing brines were adjusted to give the mass fractions of nitrite, nitrate and ascorbate in the bacon at the time of inoculation with *C. botulinum* or *S. aureus*, as shown in Table 1.

The following curing procedure was used in all the tests. Back blocks were selected for medium lean colour (in order to produce bacon in the pH range 6.0–6.2) and fat of medium hardness. Blocks were injected with brine, which contained polyphosphate, to a pick-up of 13 ± 1% by weight and cover-brined. Blocks were cold smoked, tempered and sliced. The rashers were packed at random 3 per pouch and were vacuum-packed.

Chemical analysis

Nitrite, nitrate, ascorbate, moisture and pH were assayed by official methods (GB-SAC 1973, p. 153-158) with minor modifications for nitrite in the presence of ascorbate. Sodium chloride was estimated either by the method of Halliday & Wood (1966) or by Volhard titration.

Storage trials of bacons inoculated with S. aureus

Spores from 21 strains of *C. botulinum*, including proteolytic types A, B and F and non-proteolytic types B, E and F, were mixed in equal numbers, washed free of toxin, resuspended in distilled water at a number concentration of 10^5 or 10^8 spores/ml, and heat-shocked at 60 °C for 30 min. Each bacon pack (Series A and B) was opened and 0.01 ml of a spore suspension (i.e. 10^3 or 10^6 spores) was injected with a Hamilton microsyringe into and between the rashers in each pack; packs were re-sealed under vacuum. Inoculated packs were stored for 21 or 28 days at 25 °C or 30 °C. After storage, each pack of bacon was homogenized with phosphate buffer (pH 6.5) containing gelatin (mass concn 2g/litre), and the homogenate tested in mice for botulinum toxin as described by Baird-Parker (1969a). Five or ten replicates were always tested.

Storage trials of bacons inoculated with Staphylococcus aureus

Three strains of *S. aureus* producing enterotoxin A were used to prepare an inoculum (0.1 ml) containing about 10^5 cells of each strain. Packs of Series A bacon were opened and inoculated between rashers with the *S. aureus* suspension. Half the packs were re-sealed under vacuum and the remainder left open. Open and evacuated packs were stored at 15 °C to represent poor chill conditions and at

25 °C to represent storage at ambient temperature. Inoculated bacon was examined for *S. aureus* and enterotoxin (detection limit 10^2 cells/g and 0.005 μg enterotoxin/g bacon) after 14, 21 and 28 days storage. *S. aureus* was counted on the medium of Baird-Parker (1969b) and the presence of enterotoxin A in concentrated bacon extracts was estimated by the Crowle Micro double-diffusion agar slide technique (Holbrook & Baird-Parker 1975).

Results

Chemical analysis

Salt levels in the bacons at the time of inoculation with *C. botulinum* or *S. aureus* generally agreed with the designed levels, but showed typical batch-to-batch variation (Table 1). Observed mass fractions of nitrite and ascorbate were always lower than intended, owing to interactions between them during curing. Hereafter the bacons are designated by intended levels of curing salts (Table 1, Cols 3, 5, 7). The observed pH range of 6.0–6.2 was consistent with the selection of blocks of pork of medium colour.

Table 1. Analyses of bacons at time of inoculation with *Clostridium botulinum* (Series B cures). Values of means of 5 assays. w = mass fraction = (mass of component)/(mass of bacon).

NaCl on water (%)		w (Ascorbate)/mg · kg^{-1}		w (Nitrite)/mg · kg^{-1}		w (Nitrate)/mg · kg^{-1}	
intended	observed	intended	observed	intended	observed	intended	observed
5.5	4.7	2000	1250	200	126	250	195
(medium	5.1	1000	450	200	103	250	246
salted	5.2	0	0	200	143	250	231
bacons)	6.3	2000	1640	100	47	250	289
	6.6	1000	550	100	67	250	171
	4.9	0	0	100	88	250	187
Control[1]	5.0	0	0	0	0	0	0
4	4.8	2000	1610	200	102	250	222
(mildly	4.1	1000	790	200	135	250	143
salted	4.9	0	0	200	196	250	170
bacons)	3.6	2000	1500	100	66	250	203
	4.7	1000	940	100	75	250	217
	2.9	0	0	100	71	250	193
Control[1]	4.3	0	0	0	0	0	0

1. Control containing sodium chloride alone.

Fate of C. botulinum in ascorbate free bacon

The proportion of inoculated packs that developed botulinum toxin during storage is shown in Tables 2 and 3. The botulinum toxins detected were Type A or Type B or mixtures of the two.

Mildly salted pork containing about 4% NaCl on water but with no added nitrite or nitrate, readily supported growth of *C. botulinum* and toxin production from an inoculum of 10^3 spores per pack during storage in evacuated packs at 25 °C (Table 2). However vacuum-packed mildly salted bacons of similar salt content but containing nitrite at an intended mass fraction of 100 mg/kg (observed mean 71 mg/kg) and nitrate at 250 mg/kg (observed mean 193 mg/kg) developed toxin more slowly and fewer packs became toxic during storage. When the intended nitrite level was increased to 200 mg/kg (observed mean 196 mg/kg) none of the packs became toxic after storage at 25 °C for up to 28 days. When the inoculum was increased to 10^6 spores per pack, all salted pork without nitrite or nitrate became toxic; nitrite at intended mass fractions of 100 and 200 mg/kg still depressed *C. botulinum* in bacon stored at 25 °C but its effect was negligible at 30 °C.

Medium-salted pork containing about 5.5% NaCl on water and no added nitrite or nitrate did not readily support toxin production from an inoculum of 10^3 spores of *C. botulinum* per pack during storage at 25 °C (Table 3). When the inoculum was

Table 2. Number of packs with botulinum toxin/number of packs tested of mildly salted bacons containing about 4% salt on water (Series B cures), and inoculated with 10^3 or 10^6 spores per pack ('low'; 'high') after storage at a temperature of 25 or 30 °C for 21 or 28 days. w = intended mass fraction (see also Table 1).

w/mg · kg^{-1}		Packs toxic/packs tested:					
nitrite	ascorbate	25 °C				30 °C	
		21 days		28 days		28 days	
		low	high	low	high	low	high
0[1]	0	4/5	4/5	10/10	10/10	10/10	10/10
100	0	0/5	6/10	3/10	7/10	6/10	9/10
200	0	0/5	1/5	0/10	3/10	6/10	10/10
100	1000	0/10	0/10	0/10	0/10	2/10	7/10
100	2000	0/10	0/10	0/10	0/10	0/10	9/10
200	1000	0/5	1/5	0/10	1/10	9/10	10/10
200	2000	0/5	0/5	0/10	6/10	6/10	10/10

1. Control containing sodium chloride alone.

Table 3. Number of packs with botulinum toxin/number of packs tested of medium salted bacons containing about 5.5% NaCl on water and nitrate at an intended mass fraction of 250 mg/kg (Series A and B cures), and inoculated with 10^3 ('low') or 10^6 ('high') spores per pack. Storage at 25 or 30 °C for 21 or 28 days. w = intended mass fraction (Table 1).

w/mg · kg^{-1}		Packs toxic/packs tested:				
nitrite	ascorbate	25 °C				30 °C
		21 days		28 days		28 days
		low	high	low	high	high
0[1]	0	0/5	4/10	0/10	2/10	10/10
100	0	0/5	0/10	1/10	0/10	5/10
200	0	0/5	0/10	0/5	3/10	8/10
100	1000	.	0/10	.	1/10	0/10
100	2000	0/5	0/10	0/10	0/10	1/10
200	1000	.	0/10	.	0/10	4/10
200	2000	0/5	1/10	0/10	3/10	10/10

1. Control containing sodium chloride alone.

Table 4. Risk of botulinum toxin production relative to that in medium-salted bacon with nitrite at an intended mass fraction (w) of 200 mg/kg and nitrate at 250 mg/kg.

w (ascorbate)/mg · kg^{-1}	Risks of toxin production					
	mildly salted			medium-salted		
	w (nitrite)/mg · kg^{-1}			w (nitrite)/mg · kg^{-1}		
	0[1]	100	200	0[1]	100	200
0	440***	20***	15***	29*	1.3	1.0
1000	.	1.2	6.1	.	0.08+	0.4
2000	.	0.8	26	.	0.05+	1.7

1. Control containing sodium chloride alone.

* Risk of toxin production significantly greater than that of the reference bacon. (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

\+ Risk of toxin production significantly less than that of the reference bacon. ($P < 0.001$).

increased to 10^6 spores per pack, more packs became toxic. However vacuum-packed bacon of similar salt content containing intended levels of 100 mg/kg nitrite (observed mean 88 mg/kg) and 250 mg/kg nitrate (observed mean 187 mg/kg) and inoculated with 10^6 spores per pack did not become toxic after storage at 25 °C for 28 days; some packs became toxic after storage at 30 °C (Table 3). When the intended mass fraction of nitrite was increased to 200 mg/kg (observed 143 mg/kg), results were erratic but still showed that the presence of nitrite and nitrate delayed the development of botulinum toxin.

Effect of ascorbate on antibotulinum activity of nitrite

Nitrite was still effective against *C. botulinum* when sodium ascorbate was included in either mildly salted or medium salted bacons (Tables 2 and 3). Combinations of nitrite at 100 mg/kg and ascorbate at 1 000 or 2 000 mg/kg (intended mass fractions) interacted to enhance the effectiveness of nitrite.

Risks of botulinum toxin in the bacons

Analysis of the data by computer showed that ascorbate at intended mass fractions of 1 000 or 2 000 mg/kg in medium-salted bacon containing about 5.5% NaCl on water, nitrate at 250 mg/kg and nitrite at 200 mg/kg did not significantly increase the risk of toxin formation (Table 4). Indeed, in bacon nitrite at 100 mg/kg and ascorbate at up to 2 000 mg/kg there was an unexplained but significant decrease in the risk of toxin production.

Under the same storage and inoculation conditions, mildly salted bacon containing about 4.0% NaCl on water and nitrite at up to 200 mg/kg had a greater risk of supporting growth and toxin development by *C. botulinum* than did medium-salted bacon. If nitrite and nitrate were both omitted from cures, the risks of toxin formation were increased 20-fold for medium-salted pork and 440-fold for mildly salted pork.

Fate of Staphylococcus aureus in bacon

Staphylococcus aureus grew well in medium-salted pork or bacon stored at 15 or 25 °C, irrespective of the initial mass fractions of nitrite or ascorbate in the bacon. Growth was faster at 25 °C than at 15 °C, and attained much higher rates and higher numbers in open than evacuated packs. Number content of *S. aureus* in evacuated packs stored at 15°C did not exceed 10^7 g^{-1}. Enterotoxin A was found in all open packs of bacon and counts these exceeded 10^7 g^{-1}; it was not found in any of the evacuated packs of bacon. All toxic packs had definite 'off' odours. Neither nitrite level nor presence of ascorbate had any effect on amounts of enterotoxin produced in open packs.

Discussion

These results indicate that sodium nitrite in the presence of potassium nitrate at 250 mg/kg and two concentrations of sodium chloride delay or inhibit growth and

toxin production by *Clostridium botulinum* in factory-manufactured bacon of pH 6.0–6.2. Previous data indicate that inhibition is mainly, perhaps entirely, due to nitrite and that nitrate probably plays no role in such bacon. However, nitrate may play a role in bacons of higher pH (Roberts 1975). As the nitrite level in bacon increased, fewer packs became toxic. However inhibition by nitrite depends on many factors, such as salt (presence increased inhibition), storage temperature (increase in storage temperature decreased inhibition) and inoculum (increase in number of spores decreased inhibition). Similar results have been reported by Collins-Thompson et al. (1974) and Christiansen et al. (1974).

The variability in level of sodium chloride in the bacons compares favourably with that found in normal commercial practice. Although medium-salted bacons (about 5.5% NaCl on water) did not readily support growth of *Clostrid* ⁻ *botulinum*, mildly salted bacon (about 4% salt on water) did. Our inoculatic
that, if level of salt is marginally low, sodium nitrite is essential to inhibit ue.
ment of *C. botulinum*. The salt-on-water levels of similar vacuum-packed slicec back bacon produced commercially by The Wall's Meat Company Ltd during the tests had a mean of 5.4% with a standard deviation of 0.9 (162 samples tested), and thus about 9% of production bacon would be expected to have a salt level of 4% or less. In a recent survey by Roberts (1975b, pers. commun.) of bacon pigs in the United Kingdom, *C. botulinum* Types A and proteolytic Type B were found in samples of pork from 19 out of 26 pigs. Furthermore, he found *C. botulinum* type A in 34 out of 78 samples of unsliced collar bacon.

Our results provide no evidence that the effectiveness of sodium nitrite is diminished by sodium ascorbate at the pH of our bacons. This conflicts with the claim by Bowen & Deibel (1974) that protection by nitrite is diminished by ascorbate at a mass fraction of 1–2 g/kg. However, because of their longer curing and the hot smoking, their ascorbate-containing bacon contained less nitrite, 0–20 mg/kg, at the time of inoculation than those we used, 47–135 mg/kg, and therefore would not be expected to produce a similar inhibition of *C. botulinum*.

The different levels of curing salts affected the keeping of the bacons: medium-salted bacons kept better than mildly salted bacons. The presence of sodium ascorbate tended to shorten the shelf life of bacons at normal ambient temperature; spoilage studies were at ambient temperature to imitate conditions in the British market. However keeping was not always predictable during our tests. Although these curing tests were not designed to assess the affects of potassium nitrate as such on the keeping of bacons, recent studies have shown that potassium nitrate (at about 300 mg/kg) retarded 'cabbage odour' in vacuum-packed bacon inoculated with *Proteus mirabilis* and stored at ambient temperature, particularly in bacon of higher pH, about pH 6.2 (B. L. Austin, unpublished results).

Discussion on the session

Ascorbic acid and the outgrowth of clostridia

The question was raised whether ascorbic acid affects the outgrowth of clostridia. Dr Baird-Parker answered that they had no data relating to bacon but would have expected (from observations on the growth of clostridia in media containing up to 10% sodium ascorbate) a stimulation rather than an inhibition. Data presented

at the 1st symposium in September 1973 also indicated no effect of ascorbate (at 1 000 mg/kg) in a pork macerate broth.

A suggestion from the audience was made that at very high levels, i.e. about 2 000 mg/kg, significant amounts of dehydroascorbic acid could be formed and this compound might be inhibitory.

Ascorbic acid and nitrite depletion

With ascorbic acid, the nitrite depletion rate is greatly accelerated. It was suggested that during this depletion compounds are produced which possess inhibiting properties on microbial growth. However, in inoculation experiments in nitrite-depleted bacon and ham, Dr Baird-Parker observed no inhibition. Maybe the spores have to be present at the moment that nitrite is added, in other words when the nitrite concentration is high.

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Effect of nitrite on the development of Staphylococcus aureus in fermented sausages

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Abstract

Nitrite suppressed development of *Staphylococcus aureus* in dry sausage, especially with fermentation at 27 °C for two days in brine but also for 3 days in air, according to the usual Dutch manufacturing procedure. A special *Lactobacillus* starter, developed in the Netherlands, was used in all tests. The pH was a minor factor when sufficient nitrite had been added.

In air-fermented sausages, nitrite did not sufficiently suppress growth of *S. aureus* in the surface of the sausages if they were ripened in air, but did so if they were ripened in a nitrogen atmosphere. Nitrate had no influence on *S. aureus* in the Dutch sausage manufacturing process.

Introduction

Many factors in staphylococcal growth coincide in fermented sausages, such as relative activity of water (a_w), pH, (an)aerobic conditions, nitrite, natural and inoculated competing micro-organisms. The significance of sodium chloride for staphylococci has long been recognized (Chapman 1945). The effects of salt content – or a_w – and pH, alone or in combination, have been widely studied, both on growth and enterotoxin production (Genigeorgis et al. 1971; Troller 1972; Troller & Stinson 1975). Aerobic conditions favour development of *S. aureus* (Buchanan & Solberg 1972; Christiansen & Foster 1965; Genigeorgis et al. 1972; Scott 1957) and production of enterotoxins (Barber & Deibel 1972). Moreover, nitrite is very effective under anaerobic conditions, but is less inhibitory against *S. aureus* in aerobic conditions (Buchanan & Solberg 1972; Castellani & Niven 1955; Christiansen & Foster 1965; Lechowich et al. 1956). Nitrate is active against *S. aureus*, but only in combination with nitrite (Jedlicka et al. 1975) or when nitrite has been formed from the nitrate (Lechowich et al. 1956). Some staphylococcal strains even reduce nitrate (Bacus & Deibel 1972). Food micro-organisms may inhibit *S. aureus* under many circumstances (Graves & Frazier 1963), but sodium chloride retards saprophytic organisms and encourages staphylococci (Peterson et al. 1964). Lactic acid bacteria may inhibit *S. aureus* (Graves & Frazier 1963; Haines & Harmon 1973; Kao & Frazier 1966) by depletion or otherwise (Gilliland & Speck 1972; Iandolo et al. 1965). Cocci seem more effective than lactobacilli (Haines & Harmon 1973; Kao & Frazier 1966). However a clear distinction was not always made between the effects

of pH and lactic acid bacteria. Some suggest that pH is not important (Iandolo et al. 1965) or only important early in fermentation (Daly et al. 1973); others found a distinct effect using glucono-δ-lactone (Barber & Deibel 1972).

However the experimental variables – broth cultures and sausages; different compositions of media, and sausage mixtures; starter cultures; and fermentation temperatures – did not allow us to predict the behaviour of *S. aureus* in Dutch dry sausages. Dry sausages from the Dutch meat industry are usually brine-ripened and not air-ripened as in most other countries. They are fermented by means of a special *Lactobacillus* starter. The brine-ripened dry sausage is manufactured by fermentation of a sausage mixture with 2% (by mass) sodium chloride and a suitable amount of nitrite, in casings, in a brine containing 9% sodium chloride and a suitable amount of nitrite with a pH of about 4.6. After brine fermentation for 2–3 days at a temperature of 27 °C, the sausages are smoked for at least 24 h at low temperature and then dried in an atmosphere of about 80% relative humidity and at a temperature of 12 to 15 °C.

Therefore it seemed worth while to examine the development of *S. aureus* in brine-ripened sausages. Since, however, sometimes a significant growth of *S. aureus* had been observed in small diameter air-ripened Dutch sausages, air-ripening was included in this study too.

Material and methods

The dry sausage mixture was prepared from frozen beef (58% by mass), pork fat (29%), cooked rind (10%), sodium chloride (2% for brine-ripened sausages and 3% for air-ripened sausages), and sodium ascorbate (0.05%). Usually dextrose (0.7%) was added and 0.5% of a broth culture of *Lactobacillus* strain 301, a strain used for industrial production of dry sausage, isolated by the Department of Meat Technology in the past years, and very suitable for the production of modern dry sausage. Nitrite was added as nitrite (0.6%) containing sodium chloride. Spices were omitted. In some tests, potassium nitrate was added.

From 10 available strains of *S. aureus*, one of the most rapidly growing strains was selected for inoculation of the sausages: strain 40 from the National Institute of Public Health at Bilthoven, reported to produce Enterotoxin D. The strain was maintained on tryptone soya agar slants; a series of daily transfers were made into brain-heart infusion BNI at 37 °C before use for inoculation. The number content of *S. aureus* introduced into sausage mixture was about 10^3 g^{-1}. The sausage mixture was prepared with a 50-litre laboratory cutter, was stuffed in fibrous cellulose casings, diameter 75 mm, in 1-kg portions, which were clipped. For the brine fermentation, the sausages were immersed (1:2 by mass) in a brine solution containing sodium chloride at a mass concentration of 90g $litre^{-1}$ and a mixture of KH_2PO_4 and K_2HPO_4 at about 10 g $litre^{-1}$ to obtain a pH of 4.6. The mass concentrations of nitrite and nitrate in the brine were always twice those initially in the sausage mixture. The brining was at 27 °C for 48 h.

For air fermentation, sausages were suspended in a small incubator with forced circulation of air at 27 °C. To prevent excessive evaporation under these conditions, the sausages were suspended in air or nitrogen in wide closed impermeable plastic bags for 72 h.

After brining or air-ripening, the sausages were not-smoked, but were immediately transferred to a drying room at a temperature of 12–15 °C and a relative humidity of 80–85%.

Aerobic bacteria were counted on plates of tryptone – dextrose – yeast extract – milk agar (Mossel & Krugers Dagneaux 1959). Inoculated lactic acid bacteria could be recognized on these plates. The number of *S. aureus* cells was estimated on Baird-Parker's agar (ISO-DIS-5551 1976). *S. aureus* was estimated in the core of the sausages and in the outer centimetre after removing the casing. The pH was measured in triplicate with an Electrofact pH-meter. Moisture, sodium chloride and nitrite contents were estimated by routine methods.

Results

The number content of inoculated lactic acid bacteria increased in both air-ripened and brine-ripened sausages from 10^7 g^{-1} after inoculation to 10^8 g^{-1} after

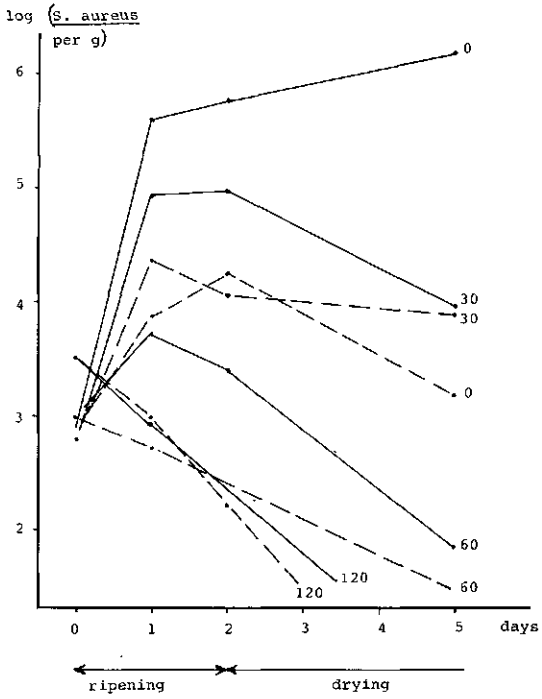

Fig. 1. Effect of nitrite on number content ($\log_{10}$ scale) of *Staphylococcus aureus* in dry sausage during brine fermentation ('ripening') and drying. Solid lines, surface centimetre of sausage; broken lines, core; the cure was with nitrite at mass fractions of 0, 30, 60, and 120 mg/kg (as in the labels of the lines).

24 h and 10^9 g^{-1} after 48 h. The pH decreased from about 5.9 to 5.2 in 24-h brining and to 5.4 in 24-h air-ripening; after 48 h, the pH of both types of sausages was the same, 5.0–5.1, and decreased to about 4.8 during the subsequent drying. Mass fraction of moisture in the brine-ripened sausages decreased from about 51% to 49% during brine fermentation; and of sodium chloride increased from about 2.1 to 3.4%, the corresponding mass fraction in the brine phase of the sausage, $m(NaCl)/m(NaCl + H_2O)$ from about 4.0 to 6.5%.

Since water could not evaporate during ripening of the air-ripened sausages, the mass fractions of moisture and of sodium chloride in the sausage and in the brine phase, respectively about 50, 3.0 and 5.7% did not change considerably. Nitrite, after a considerable decrease during manufacture of the mixture, decreased further during fermentation to 1–3 mg/kg. A strong inhibitory effect was found (Fig. 1) in brine-ripened sausages as mass fraction of added nitrite was increased from 0 to 120 mg/kg. With little nitrite, an initial development of *S. aureus* was followed by a significant decrease. With more nitrite, only a decrease was observed. The different behaviour of *S. aureus* in the core and in the surface layer of air-ripened sausages, already reported by Barber & Deibel (1972), was also observed in brine-

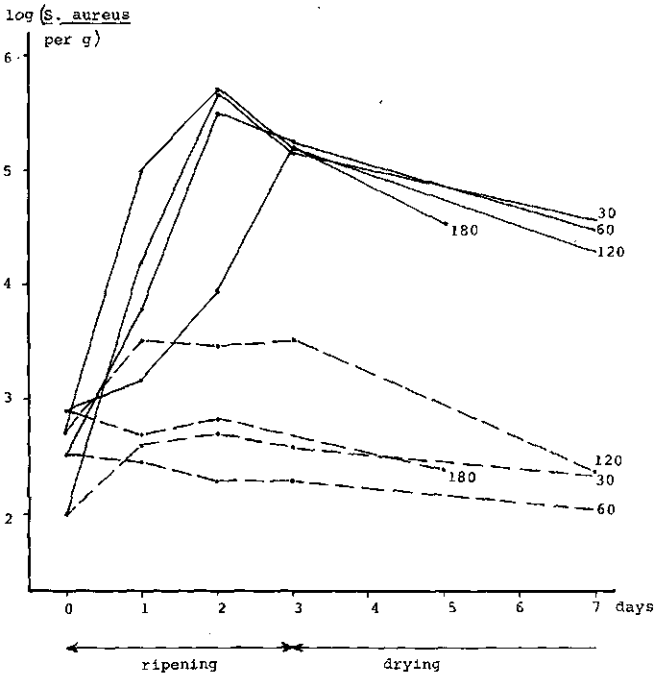

Fig. 2. Effect of nitrite on number content (log_{10} scale) of *Staphylococcus aureus* in dry sausage during air fermentation ('ripening') and drying. Solid lines, surface centimetre of sausage; broken lines, core; the cure was with nitrite at mass fractions of 30, 60, 120 and 180 mg/kg (as in the labels of the lines).

fermented sausages. However, the differences between core and surface disappeared with more nitrite initially. In air-ripened sausages (Fig. 2), however, growth of *S. aureus* in the surface layer was hardly affected by nitrite, even with the largest amount (as observed with broth cultures by Lechowich et al. 1956). Tests where the air in the plastic bags was replaced by nitrogen during ripening showed that the main reason for the increase of *S. aureus* in the surface layer of these sausages was the aerobic condition of the surface (Fig. 3).

Figure 4 shows that a rapid pH decrease to a sufficient low level, though desired for slicing and general microbiological reasons, is not necessary to prevent *S. aureus* from growing as long as sufficient nitrite has been added. With little nitrite or with only nitrate (Daly et al. 1973), a small effect of a rapid pH decrease may be observed.

No effect of nitrate added to nitrite-containing sausage mixtures could be found on growth of *S. aureus*, with either brine cure or air cure. When nitrate was added and nitrite not, *S. aureus* developed as in sausages prepared with sodium chloride only. Probably nitrate was not significantly reduced in the sausage mixtures tested: the ingredients had a relatively low bacterial count and the starter culture could not reduce nitrate.

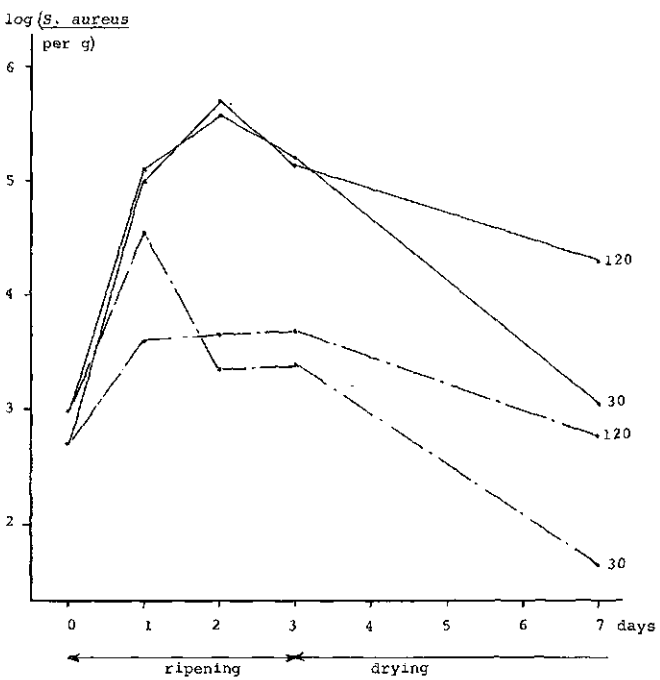

Fig. 3. Effect of ripening in air (solid line) or nitrogen (broken line) on number content ($\log_{10}$ scale) of *Staphylococcus aureus* in the surface of air-ripened sausages prepared with nitrite at mass fractions of 30 and 120 mg/kg (as in the labels of the lines).

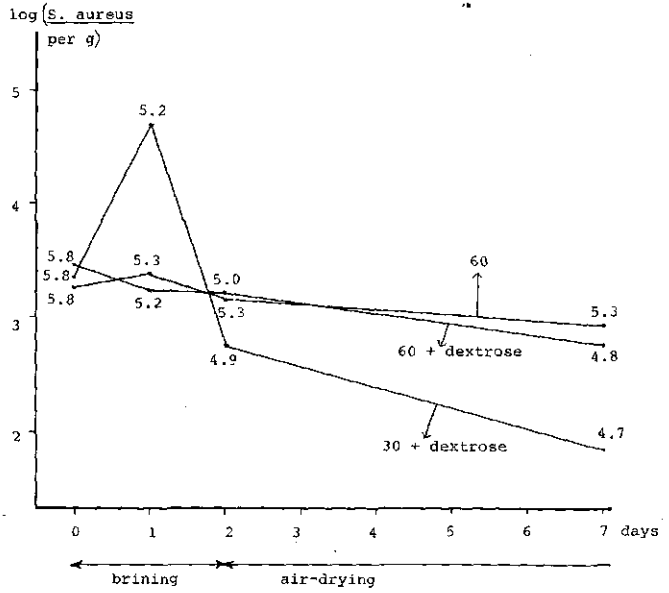

Fig. 4. Effect of dextrose (mass fraction in sausage mixture 7 g/kg) and of mass fraction of nitrite (30 or 60 mg/kg) on number content ($\log_{10}$ scale) of *Staphylococcus aureus* in the surface centimetre of the sausage. Values of pH initially and after 1, 2, and 7 days are marked alongside the respective lines.

Discussion

Nitrite is a very major factor in control of *S. aureus* in fermented sausages, a factor sometimes overlooked in dry sausage experiments. The inhibitory effect of nitrite under anaerobic conditions in dry sausages, first demonstrated by Lechowich et al. (1956) and confirmed by Barber & Deibel (1972), does not prevent high counts of staphylococci in some commercial dry sausages (Baran & Stevenson 1975; Barber & Deibel 1972), probably because the nitrite is sometimes formed slowly by natural or inoculated micro-organisms and because most sausages are air-ripened. However many small-diameter air-ripened dry or semi-dry sausages in the United States (Goodfellow & Brown 1975) and in the Netherlands proved free from *S. aureus*.

This study confirms the views of Lechowich et al. (1956) and Barber & Deibel (1972) and shows that growth of *S. aureus* in the surface layer of brine-fermented sausages is less and is more affected by normal amounts of nitrite than growth in the surface layer of air-fermented sausages. Small amounts of nitrite are very effective in suppressing staphylococcal growth in the core of the sausages, though nitrite was more effective in the core of brine-fermented than in the core of air-fermented sausages.

Brine-fermentation seems to be a more effective procedure to suppress *S. aureus* than air-fermentation. Though use of only nitrate may insufficiently suppress *S. aureus* (Daly et al. 1973) or nitrite burn (Bacus and Deibel, 1972), a mixed starter containing micrococci could suppress *S. aureus* (Niskanen & Nurmi 1976).

Under Dutch circumstances, where a starter not reducing nitrate is used, nitrate is useless, either alone or in combination with nitrite.

Discussion on the session

Growth of Staphylococcus aureus

Starters developed for rapid fermentation of Dutch dry sausages give the best results at a ripening temperature of about 27 °C. This temperature is most likely more stimulatory for the growth of *S. aureus* than lower ripening temperatures. Growth reduction of *S. aureus* was reported in dry sausages inoculated with starter cultures containing micrococci. In milk lactobacillus starters compete for the substrate and sometimes produce metabolic substances which inhibit growth of *S. aureus*.

Sugar in dry sausage may promote growth of *S. aureus* but may inhibit by being metabolized to acids and other compounds. Moreover fermentation of sugar lowers the redox potential also resulting in growth inhibition.

Little is known about growth of *S. aureus* in other types of dry sausages, ripened in other ways.

Enterotoxin production

No toxin determinations were made in the described experiments because the numbers of *S. aureus* were regarded to be to low (maximum of 10^6 cells per gram). A remark from the audience was made that no toxin formation was found either on growth of *S. aureus* in bacon.

In the presence of an inhibitory factor, toxin formation generally stops before the growth comes to an end. An exception to this rule was observed for one specific strain of *S. aureus*.

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Nitrite and nitrate in the control of Clostridium botulinum in cured meats

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Abstract

Clostridium botulinum has been demonstrated in pork in the United Kingdom. Clostridia grew in collar and gammon bacon stored in vacuum packs but rarely in back bacon. Growth in collar and gammon was related more to pH than to the presence of nitrate. In pasteurized cured meat heat treatment, nitrite concentration and incubation (storage) temperature were important factors in determining whether growth of and toxin production by *Cl. botulinum* types A and B occurred. Use of nitrate often led to high residual levels of nitrite unless sodium ascorbate was also present. The frequent production of toxin at 15 °C emphasizes the need for refrigerated storage of pasteurized cured meat products. The many factors shown to be important in controlling *Cl. botulinum* cast doubt on the wisdom of legislating for nitrite alone.

The microbiological reasons for the use of nitrite and nitrate in cured meats were reviewed by Ingram (1974). The triple inhibitory interaction of nitrite, pH and sodium chloride had been clearly shown by Roberts & Ingram (1973) using one strain each of *Clostridium botulinum* types A, B, E and F, and those data were used by Roberts (1974) in developing a measure of the antimicrobial properties of cured meat systems and for developing a model to predict the growth or inhibition of *Cl. botulinum* in those systems.

In a subsequent study on a pasteurized pork slurry, *Cl. botulinum* types A and B grew less with increasing nitrite concentrations and with lower incubation temperatures (Roberts et al. 1976).

This paper presents data on the occurrence of *Cl. botulinum* in pork and bacon in the United Kingdom, on the effect of different concentrations of nitrite and nitrate on the growth of clostridia in vacuum-packed bacon, and on studies, similar to those reported previously (Roberts et al. 1976), on the effects of combinations of curing salts on the growth of spores of *Cl. botulinum* types A and B in pasteurized pork slurry incubated at low temperatures.

Methods

Occurrence of Cl. botulinum

The methods of detecting *Cl. botulinum* in bacon and pork were enrichment of the meat sample and testing for toxin with mice as described in Roberts & Smart (1976a, b).

Production of bacon and assessment after storage

For full details of the production of Wiltshire bacon with different nitrite and nitrate concentrations, and of its microbiological assessment after storage, see Taylor & Shaw (1975). Methods for the study of clostridial growth in this bacon have been published separately (Roberts & Smart, 1976b).

Heated cured meats

For methods of studying growth of *Cl. botulinum* type A and B spores in pasteurized pork slurry, see Roberts et al. (1976). Growth medium: 4 kg trimmed pork + 6 litre water: final mass fraction of water 86–88% and of fat 0.7–2.8%.

Additives

NaCl (salt on water, g/litre) 25, 35; $NaNO_2$ (mg/kg) 0, 100, 200, 300; $NaNO_3$ (mg/kg) 0, 500; sodium ascorbate 0, 1 000 mg/kg and equimolar for nitrite; polyphosphate 0,3 g/litre; pH (not buffered) no added polyphosphate 5.6–5.92, with polyphosphate 5.97–6.29.

Inoculum

Spores of *Cl. botulinum* type A NCTC 3806; NCTC 9837; NCTC 7272; 62; 33; type B NCTC 3807; NCTC 751; 213; CN 1356; Cn 5009. A mixed spore crop containing equal numbers of each of the above ten strains was used at 10 and 10^3 spores per bottle (28 g slurry).
Incubation temperatures: 35, 20, 17.5, 15 °C.
Incubation time: up to 6 months.
Heat treatments: unheated; low heat 80 °C for 7 min (centre of slurry to 70 °C; high heat 80 °C for 7 min and 70 °C for 1 h.
Replicates: unheated, 5 bottles per treatment; low heat, 10 bottles per treatment; high heat, 10 bottles per treatment.

In these tests, all the curing salts and additives were mixed with the pork slurry, and the spores were added before heating, so that the nitrite had been heated with the meat and spores. Growth was assessed visually on a 5-point scale (0 = initial slurry; from 1 = slight gas production to 5 = meat markedly proteolysed and bright pink), and was related to toxin production. Bottles incubated at 35 °C were examined for growth weekly, at 20 °C every 2 weeks, at 17.5 °C every 3 weeks, and at 15 °C every 4 weeks up to a maximum time of 6 months. Production of *Cl. botulinum* toxin was tested by centrifuging the supernatant at a rate of 4 500 min^{-1} for 10 min) to remove meat particles, and injecting 0.5 ml intraperitoneally into mice of liveweight 20–24 g, which were then observed for 72 h for symptoms of botulism (abdominal pinching, respiratory impairment). Representative samples were also neutralized before injection with types A and B monovalent antitoxins (Wellcome Reagents Ltd, Beckenham, Kent) to confirm identity.

Results

Occurrence of Cl. botulinum in pork

If the continued use of nitrite is to be justified by its contribution towards the inhibition of *Cl. botulinum* in cured meat products, that organism must be demonstrated in pork. Surveys in the United States of meat and meat products, discussed fully in Roberts & Smart (1976a), indicated that *Cl. botulinum* was not common, but occasionally a cell was detectable at 1 per 0.6–3 kg of pork. A subsequent survey in the United Kingdom (Roberts & Smart 1976a) of bacon produced experimentally from commercial bacon pigs, showed that of 297 samples from 25 to 100 g, total 16.5 kg, 36 contained *Cl. botulinum* types A (23) or B (13). A more systematic survey of that factory, covering pork from 7 suppliers each sampled on 5 occasions (10 samples of 30 g each from fore-end and gammon-end of back on each occasion) demonstrated *Cl. botulinum* of types A, B and C in 30 of 684 samples (Roberts & Smart 1976b). Our data show about 2 *Cl. botulinum* cells per kilogram pork, a somewhat higher value than that from published surveys. But we used much

Table 1. Growth of Clostridium spp. in different cuts of vacuum-packed bacon stored at 15, 20 and 25°C. In Column 2, '29/76' means that clostridium increased at least 10-fold in 29 of 76 samples. From Roberts & Smart (1976a, Table 8).

Increase over initial count	Number of packs with the stated increase / number of packs sampled				
	collar	gammon	back lean	back fat	total
> 10	29/76	9/40	2/69	3/24	43/209
> 100	23/76	8/40	1/69	1/24	33/209

Table 2. Effect of $NaNO_3$ added at a mass fraction of 500 mg/kg on growth of *Clostridium* spp. in vacuum packs of bacon stored at 15, 20 and 25°C. In Column 2, '12/38' means that clostridium increased at least 10-fold in 12 of 38 samples. From Roberts & Smart (1976a, Table 9).

Increase over initial count		Number of packs with the stated increase / number of packs sampled			
		collar	gammon	back lean	back fat
With $NaNO_3$	10	12/38	2/20	1/35	1/12
	100	8/38	1/20	1/35	1/12
No $NaNO_3$	10	17/38	7/20	1/34	1/12
	100	15/38	7/20	0/34	1/12

larger samples than most investigators. The distribution was erratic: on 3 of 5 visits to the commercial premises, sampling all 7 suppliers, *Cl. botulinum* was not demonstrated in any of 3 x 140 samples of 30 g. On one visit, it was demonstrated in 20 of 138 samples.

Cl. botulinum undoubtedly occurs from time to time in British pork, and this must be clearly understood when changes in technology are proposed.

Unheated cured products

We tested whether a smaller amount of nitrite in Wiltshire bacon would permit growth of clostridia when the bacon was stored in vacuum packs at 15 °C for up to 19 days, 20 °C for 12 days or 25 °C for 9 days, the temperature and times being governed by the shelf-life required. The methods used for counting 'total clostridia' were imperfect (Roberts & Smart 1976a): hence only a difference in count by a factor of 10 or 100 between initial and stored samples indicated growth.

Table 3. Effect of $NaNO_3$ added at a mass fraction of 500 mg/kg on number of packs (out of 80 sampled) where clostridia increased more than 10-fold.

pH	Number of packs where clostridia increased 10-fold	
	with $NaNO_3$	without $NaNO_3$
< 6.09	4	7
> 6.09	15	18
< 6.29	11	16
> 6.29	6	11

Table 4. Effect of $NaNO_3$ added at a mass fraction of 500 mg/kg on increase in clostridia in bacon. Each pair of columns represents 80 packs tested.

pH		Number of packs with stated increase			
		with nitrate		without nitrate	
		> 10	> 100	> 10	> 100
< 6.09		3	1	2	5
> 6.09		4	11	7	11
< 6.29		7	4	6	10
> 6.29		0	6	3	8
	Total	14	22	18	34

At the lowest mass fractions of nitrite (about 100 mg/kg in the bacon), growth of clostridia did not occur in samples of the back lean (pH 5.7–5.9) or back fat, (pH 6.2–6.4) but sometimes occurred in the collar (pH 6.1–6.2) and gammon (pH 6.2–6.3) although salt-on-water values were usually higher in collar and gammon than in back. The number of occasions on which growth of clostridia was detected is summarized in Table 1 which shows that collar and gammon supported clostridial growth more often than back.

The effect of $NaNO_3$ is illustrated in Table 2, where superficial examination suggests that nitrate slightly reduced the growth of clostridia in collar and gammon. If pH be considered and results for less than 10 days at 5 and 15 °C be ignored, where clostridial growth was unlikely, a different conclusion is warranted. Table 3 shows that $NaNO_3$ at 500 mg/kg did not significantly affect the proportion of packs in which multiplication of clostridia occurred, whatever the pH. Moreover, the multiplication factor revealed no restriction by nitrate, whatever the pH (Table 4).

The pH of the bacon clearly had a major influence on incidence of clostridial growth in the packs; despite large variations in composition in other respects. Table 5 shows that clostridia multiplied in only about 5% of the packs at pH below 6.0, but in about 40% at pH above 6.2.

Although *Cl. botulinum* and *Cl. perfringens* both occurred in this bacon, there was no evidence that either grew under any of the storage conditions, even in packs where general growth of clostridia was observed.

Nitrate appeared to retard spoilage somewhat of vacuum-packed bacon of high pH (Table 6). The effect was however not great; and in tests with about 200 mg/kg instead of 500, there was no difference.

Table 5. Influence of pH on number (N) and proportion (δ) of bacon packs in which clostridia increased 10-fold ('growth').

lim pH	Below lim pH			Above lim pH		
	N (total)	with growth		N (total)	with growth	
		N	δ/%		N	δ/%
5.69	3	1	33	157	43	27
5.79	32	1	3	128	43	34
5.89	55	2	4	105	42	40
5.99	68	5	7	92	39	42
6.09	84	11	13	76	33	43
6.19	97	18	18	63	26	41
6.29	120	27	22	40	17	42
6.39	128	30	23	32	14	43
6.49	152	42	28	8	2	25

Heated cured products

We simulated commercial practice by making a slurry of pork containing different combinations of curing salts and other additives, to which were added small numbers of spores of *Cl. botulinum* types A and B. The containers were then closed, heated, cooled to below the final storage temperature, then stored for up to 6 months, and assessed for visible changes ('spoilage') and for toxin production.

Table 6. Effect of nitrate (mass fraction of $NaNO_3$ 500 mg/kg) on number of vacuum packs (out of 7) of Wiltshire bacon with spoilage odour. From Taylor et al. (1976, Tables 2 & 3). Mass fractions of $NaNO_2$ recovered by analysis were 550 – 600 and 25 – 30 mg/kg in samples with and without added nitrate, respectively. t, storage time; T, storage temperature; w, mass fraction.

Cut	$w(NaNO_2$ added)/mg · kg^{-1}	pH	Salt on water/%	t/d	$T = 5$°C		$T = 15$°C	
					$NaNO_3$	none	$NaNO_3$	none
Collar	95	6.2	6.7 – 7.1	5	.	.	0	2
				9	0	0	0	1
				15	0	0	2	3
				20	1	2	.	.
Collar	120 – 150	6.5	7.4 – 7.8	5	.	.	0	0
				9	1	0	1	1
				15	1	1	1	3
				20	1	2	.	.
Back	110 – 120	6.2	6.5 – 7.1	7	.	.	0	0
				12	0	0	0	0
				19	.	.	1	2
				23	0	1	.	.
				35	0	2	.	.

Table 7. Effect of sodium ascorbate (mass fraction 1000 mg/kg) and polyphosphate (concentration 3 g/litre) on growth of *Clostridium botulinum* types A and B spores in pasteurized pork slurry. w = mass fraction added. T = storage temperature. + = growth. 0 = no growth. Slurry 1000 spores per bottle; heated at 80 °C for 7 min, incubated for 6 months.

NaCl on water/g · litre^{-1}	$w(NaNO_2$ added)/mg · kg^{-1}	No ascorbate & polyphosphate				Ascorbate & polyphosphate			
		storage temp./°C				storage temp./°C			
		15	17.5	20	35	15	17.5	20	35
25	0	0	+	+	+	+	+	+	+
25	100	+	+	+	+	0	+	+	+
25	200	0	+	+	+	0	+	+	+
25	300	0	0	0	+	0	+	+	+

Growth assessed visually

Effect of incubation temperature and added nitrite. At 2.5% NaCl on water and $NaNO_2$ at 100 mg/kg and in the absence of sodium ascorbate and polyphosphate, growth of *Cl. botulinum* occurred at all incubation temperatures. Increasing $NaNO_2$ to 300 mg/kg prevented growth at 15, 17.5 and 20 °C, but growth still occurred at 35 °C.

In slurries containing sodium ascorbate at 1 000 mg/kg and polyphosphate at 3 g/litre but no nitrite, growth occurred at all temperatures. Addition of $NaNO_2$ at 100 mg/kg prevented growth (during 6 months at 15 °C) but not at higher temperatures. Addition of $NaNO_2$ at 200 or 300 mg/kg reduced and delayed growth at 17.5, 20 and 35 °C, but did not prevent it (Table 7).

Effect of sodium ascorbate and polyphosphate. In slurries containing 2.5% NaCl and $NaNO_2$, addition of sodium ascorbate and polyphosphate never reduced growth, and sometimes stimulated it, particularly with more nitrite and higher storage temperatures. Polyphosphate increased the pH by about 0.35.

We have used only one commercially available polyphosphate in the knowledge that Dr B. Jarvis (Leatherhead Food Research Association) is continuing his work on polyphosphates. He reports some polyphosphates to be inhibitory, but we do not know the extent of inhibition.

Effect of sodium nitrate (added at 500 mg/kg). In slurries containing 2.5% NaCl on water and $NaNO_2$ at 200 mg/kg, there was slightly more growth at all incubation temperatures when nitrate was present. With 3.5% NaCl on water and $NaNO_2$ at 200 mg/kg, less growth occurred than in its absence at all storage temperatures except 35 °C. If polyphosphate and ascorbate were added as well as 3.5% NaCl on water and $NaNO_2$ at 200 mg/kg, nitrate encouraged growth at 20 °C, and inhibited it at 35 °C.

Table 8. Effect of storage temperature (T) and number of spores inoculated per bottle on proportion (%) of bottles producing toxin with *Clostridium botulinum* types A and B spores in pork slurry. U, unheated; L, heated for 7 min at 80°C; H, 7 min at 80°C and then 1 h at 70°C. Each value under U, L or H represents an aggregate for slurries of 18 compositions. Storage was for 6 months.

T/°C	10 spores per bottle				1000 spores per bottle			
	U	L	H	all	U	L	H	all
35	47	45	29	39.1	90	74	47	66.2
20	33	51	25	37.1	84	72	65	71.7
17.5	24	40	19	28.7	68	72	63	67.7
15	10	25	9	16.0	33	48	39	41.7

Toxin production

We compared proportions of bottles containing toxin after storage for 6 months. Means were calculated across different compositions and treatments.

Effect of incubation temperature and inoculum (Table 8). Fewer bottles inoculated with a small number of spores (10 spores bottle) contained toxin than of those with 1 000 spores. A storage temperature of 17.5 °C instead of 35 °C resulted in slightly fewer bottles containing toxin, but a temperature of 15 °C gave considerably less.

Effect of sodium nitrite and inoculum (Table 9). Again, fewer bottles inoculated with 10 spores contained toxin. With either 10 or 1 000 spores, toxin production was reduced with increasing nitrite.

Effect of heat treatment and inoculum (Table 10). The shorter heat treatment (80 °C for 7 min) raised the centre of the slurry only briefly to 70 °C and the

Table 9. Effect of sodium nitrite and inoculum on proportion (%) of bottles producing toxin with *Clostridium botulinum* in pork slurry. w = mass fraction (of additive). U, unheated; L, heated for 7 min at 80 °C; H, heated for 7 min at 80 °C and then 1 h at 70 °C. Storage was for 6 months.

$w(NaNO_2)$/ mg · kg^{-1}	10 spores per bottle				1000 spores per bottle			
	U	L	H	all	U	L	H	all
0	*62	82	82	78.5	90	95	99	95.5
100	49	73	30	51.3	89	94	66	81.7
200	8	9	4	6.8	66	52	46	50.8
300	11	17	1	9.5	45	34	23	32.0
300(omitting slurry '18')	7	2	2	2.7	40	12	27	23.7)

Table 10. Effect of heat treatment and inoculum level on proportion (%) of bottles producing toxin with *Clostridium botulinum* in pork slurry. Values are aggregated for different compositions of slurry.

Temp. (time)	10 spores per bottle	1000 spores per bottle
unheated	28.6	68.9
80 °C (7 min)	40.4	66.7
80 °C (7 min); 70 °C (1 h)	20.8	53.6

minimum acceptable for pasteurized cured meat products. Holding the centre temperature at 70 °C for 1 h reduced the proportion of bottles containing toxin at both inoculum levels.

Changes in nitrite in the pork

All the pork slurries were monitored for changes in mass fraction of nitrite (expressed as $NaNO_2$) during storage. Generally nitrite fell, and did so fastest at the highest temperature of storage and slowest at the lowest. Added nitrate would provide a potential reservoir of nitrite, perhaps made available by microbial reduction, since spores would survive the modest heating to which these products were subjected. In some treatments more nitrite was detected after 1–2 months than had been added. This only occurred in slurries to which nitrate had also been added and to which ascorbate had not been added, which had been heated at 70 °C for 1 h and which were being stored at 15–20 °C. In them, mass fraction of $NaNO_2$ rose from 100 mg/kg initially to 250–320 mg/kg or from 200 mg/kg initially to 260–360 mg/kg, values sometimes remaining for 2 months above the United Kingdom's permissible limit of 200 mg/kg.

Discussion

The occurrence of *Cl. botulinum* in pork in the United Kingdom has been established at counts of the order of those found by workers in North America (Roberts 1975; Roberts & Smart 1976a, b).

Clostridia grew in collar and gammon bacon stored in vacuum packs, but rarely in back bacon. There was no evidence that *Cl. perfringens* and *Cl. botulinum* grew, although the test (25 °C, for 6 days) was not stringent. Growth of clostridia in collar and gammon was related more to pH than to presence or absence of nitrate in the cure. The microbiological stability of the product was not greatly affected by nitrate (Taylor et al. 1976).

In pasteurized cured meat, the heat treatment may be important. Toxin was more often produced when more spores were present initially, but it is difficult to diminish the number of spores in meat or meat products.

The effects of sodium ascorbate, polyphosphate and sodium nitrate are complex, although no marked inhibition of growth or toxin production was observed. Sodium ascorbate would not be expected to reduce growth, but might promote it. This was not observed regularly, but it seems unwise to insist upon its incorporation in cured meats when so little information is available on bacteriological consequences. Similarly, the one commercially used polyphosphate tested was not inhibitory, although some others are reported to have inhibitory properties (B. Jarvis, pers. commun.); the extent and reproducibility of this inhibition needs study. The effect of sodium nitrate was also inconsistent: it never increased inhibition but occasionally stimulated growth. Besides in all the tests where $NaNO_3$ was added initially at 500 mg/kg, more nitrite was often detected during storgae, sometimes exceeding the limit for $NaNO_2$ of 200 mg/kg at present permitted in the United Kingdom. Hence justification for retention of sodium nitrate in pasteurized cured meat products is considerably weakened.

The content of sodium nitrite and the storage temperature were the two major factors. The frequent production of toxin at 15 °C indicates the need for more data at temperatures approaching the minimum for growth (commonly regarded as 10 °C), and the desirability for refrigerated storage of pasteurized cured meat products. In the United Kingdom storage of pasteurized cured meats below the minimum temperature for growth of *Cl. botulinum* cannot be guaranteed, in distribution where the volume is enormous, in retail where they are often treated like fully processed canned goods, or in the home, since only about half British homes have refrigerators. Exposure at 15 °C or more is almost certain. Pork in the United Kingdom occasionally contains *Cl. botulinum* and so home-produced cured meats presumably also contain *Cl. botulinum* from time to time. Since botulism is unknown from these products, any extravagant reduction in the amount of sodium nitrite seems imprudent, certainly until the various factors inhibiting microbial growth can be expressed quantitatively. Any reduction in amount of nitrite must be counterbalanced by some other factor. The wisdom of legislating for nitrite alone must be questioned.

Discussion on the session

Occurrence of Clostridium botulinum in pork

The samples of muscles investigated were taken from the fore-end and gammon-end. In addition, collar muscles of high pH were examined and *Cl. botulinum* was shown to be present.

Inhibition by polyphosphates

The authors did not observe any inhibiting effect of polyphosphates during the present investigation. Dr Walters, speaking on behalf of Dr Jarvis, reported a marked inhibitory effect of two polyphosphates on toxin production by *Cl. botulinum* up to an incubation temperature of about 25 °C. He wondered whether a similar effect could be obscured in the authors data. Dr Roberts replied that inhibition by the one polyphosphate used was not observed at any incubation temperature.

Experimental conditions

A question was raised about the great number of positives produced in the paper which, of course, does not reflect common commercial practice. The reply was that the data presented are for low concentrations of NaCl and include, for example, some tests with no nitrite and no nitrate. Hence the overall comparisons include a high proportion of conditions supporting the growth of *Cl. botulinum*. In fact, when nitrite is included at 200–300 mg/kg very little growth occurred.

The ultimate aim of this work was to produce a model predicting whether growth of *Cl. botulinum* could occur, hence the need to obtain data at low-salt low-nitrite levels.

Production of canned meat products without nitrite

In the United Kingdom, products like beef stew and poultry products are manufactured without added nitrite. The beef stew is heated to a much higher temperature than normal canned meat products. In the Netherlands, meat balls in natural juice are produced without nitrite.

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Inhibitory effect of nitrite reaction products and of degradation products of food additives

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Abstract

The reaction of nitrite with carbonyl compounds containing an activated methylene group (like some ketones of fat degradation products) may lead to the formation of nitrolic acids, among which ethylnitrolic acid has an especially marked inhibitory effect against a strain of *Micrococcus, Enterobacter liquefaciens, Escherichia coli* and *Staphylococcus aureus* even at a mass fraction of 10 mg/kg. Propylnitrolic acid inhibited these strains at about 100 mg/kg only.

By the reaction of amino sugars with nitrite unstable 2,5-anhydroderivates are formed, which are easily converted into corresponding aldehydes. Heating aqueous acidic solutions of carbohydrates results in the formation of sugar aldehydes also. The growth of *Micrococcus* and *Staph. aureus* was inhibited by 5-hydroxymethylfurfural at 500 mg/kg; *Ent. liquefaciens* showed a certain effect at about 2 000 mg/kg only; *Esch. coli* was not inhibited by these mass fractions of 5-hydroxymethylfurfural.

Among the other compounds tested, only 3,4-dihydroxyphenylalanine was inhibitory against three of the bacterial strains.

Introduction

The antimicrobial activity of nitrite and its reaction products with amino acids and several other constituents of the meat tissue has been frequently discussed (Hansen & Levin 1975; Incze et al. 1974; Mirna & Coretti 1974; Moran et al. 1975; Roberts 1975; van Roon 1973). In the conversion of nitrite to nitrosothiols, for example *S*-nitrosocysteine (CySNO), low-molecular sulfhydryl compounds play a predominant role. The sulfhydryl groups of myosin, the main component of meat proteins, are involved in the loss of nitrite during curing to a rather limited extent (Kubberød et al. 1974). The inhibitory effect of CySNO has proved to be slight, whereas the black Roussin salt, $NH_4\,[FeS_3(NO)_6\,NO_2]\cdot H_2O$, used as model substance, has demonstrated a marked antimicrobial effect particularly in liquid media, strongly reduced by addition of diluted meat juice (Grever 1973; Mirna & Coretti 1974; van Roon 1973). Recent investigations of Asan & Solberg (1976) cast doubt on the activity of CySNO and the black Roussin salt, respectively, to cause the antimicrobial action of heated media as described by Perigo (1967).

Our purpose was to investigate the inhibitory activity of reaction products of nitrite with degradation compounds of carbohydrates and fats as well as the direct action by some of these substances on the growth of a *Micrococcus* strain (isolated

from a starter culture), *Enterobacter liquefaciens*, *Escherichia coli* and *Staphylococcus aureus* in a liquid culture medium.

Materials and methods

Tables 1–3 list the compounds tested. Some of these compounds were also commercially available. Table 1 also contains two reaction products from nitrite with sulfites, potassium nitrilosulfonate, $N(SO_3K)_3 \cdot 2\,H_2O$, and potassium *N*-nitrosohydroxylamine-*N*-sulfonate, $K_2N_2O_2SO_3$, (Pélouze salt), which were included as model substances in these investigations.

The compounds under investigation were tested for their inhibitory action on the growth of a 20-h culture of a *Micrococcus* strain, of *E. liquefaciens*, *E. coli* and *Staph. aureus* (10^5 cells/ml) inoculated in a liquid culture medium (Standard-I-Bouillon, E. Merck) at pH 6.0. To 9 ml of the medium 1 ml of a sterile solution, containing 1 mg of the substance to be investigated, was added. From a 10 ml

Table 1. Reaction products with nitrite.

No	Substance	References/Sources of supply
1	dehydroascorbic acid	Kenyon & Munro 1948/Hofmann - La Roche Inc.
2	3-nitrotyrosine	/E. Merck
3	3,4-dihydroxyphenylalanine,	/E. Merck
4	2,5-anhydro-D-mannose	Bera et al. 1956; Peat 1946
5	ethylnitrolic acid	Schmid et al. 1951
6	propylnitrolic acid	V. Meyer 1875
7	potassium nitrilosulfonate	Sisler & Audrieth 1938
8	potassium *N*-nitrosohydroxylamine-*N*-sulfonate	Moeller 1957; Weitz & Achterberg 1933

Table 2. Condensation and degradation products of ascorbic acid and of carbohydrates.

No	Substance	References/Sources of supply
9	ascorbic acid-cysteine	Meyer-Döring & Perkow 1958
10	ascorbic acid-methionine	Meyer-Döring & Perkow 1958
11	ascorbic acid-dl-alanine	Meyer-Döring & Perkow 1958
12	ascorbic acid-3-hydroxymethyl-indole (ascorbigen)	Kiss & Neukom 1966
13	furfural	/E. Merck
14	4-butyrolactone	/Fluka AG
15	γ-crotonolactone	/Fluka AG
16	2-furoic acid	/Fluka AG
17	furoin	/Fluka AG
18	5-hydroxymethyl-2-furfural	/E. Merck

sample with 100 mg/litre of the substance tested, Standard-I-Bouillon was diluted twice ten-fold to 10 and 1 mg/litre. Besides an inoculated control without test material, a sample containing sodium nitrite at 200 mg/litre served as another control. Hence, of this value 100 mg/litre is the presumed degradation product in test samples.

Results and discussion

The nitrosation of phenols, of compounds with activated methylene groups or of sulfhydryls is in general kinetically favoured over *N*-nitrosation reactions (Gilbert et al. 1975). In the reaction of nitrite with albumin under the conditions in the human stomach 6-hydroxynorleucine, 3-nitrotyrosine and 3,4-dihydroxyphenylalanine (DOPA) and a further unidentified substance could be traced by means of amino acid analysis (Knowles et al. 1974; Woolford et al. 1976a). In the microbiological test with 3-nitrotyrosine and DOPA only the latter showed a faint inhibitory effect against *Micrococcus, E. liquefaciens* and *E. coli* (Table 4). The compound 6-hydroxynorleucine, was not tested because it was not available to us.

Furfural and 5-hydroxymethyl-2-furfural (5-HMF) are formed by deamination of amino sugars, 5-HMF present mainly in connective tissue, tendon and cartilage. D-Glucosamine (chitosamine) reacts with nitrous acid predominantly to 2,5-anhy-

Table 3. Condensation products of cysteine with aldehydes and carbohydrates.

No	Substance	References
19	thiazolindine-4-carboxylic acid	Schubert 1936; Ratner & Clarke 1937
20	cysteine-furfural	Schubert 1936
21	cysteine-glucose	Schubert 1936
22	cysteine-lactose	Schubert 1936

Table 4. Growth inhibition of the strains with different substances. – , no inhibition at a mass fraction of 100 mg/litre; + , faint inhibition at 100 mg/litre; ++ , inhibition at 10 mg/litre.

No	Substance	*Micrococcus*	*E. liquefaciens*	*E. coli*	*Staph. aureus*
3	DOPA	+	+	+	–
4	2,5-anhydro-D-mannose	–	–	–	+
5	ethylnitrolic acid	++	++	++	++
6	propylnitrolic acid	+	+	+	+
9	ascorbic acid cysteine	–	–	–	+
14	4-butyrolactone	–	–	–	+
15	γ-crotonolactone	–	+	–	+
16	2-furoic acid	+	–	–	+
17	furoin	+	–	–	–

dro-D-mannose (chitose); by heating the anhydro-sugar it is readily transformed to 5-HMF (Bera et al. 1956; Defaye 1970; Newth 1951; Peat 1946). By an analogous reaction, D-galactosamine forms 2,5-anhydro-D-talose (Defaye 1970; Shafizadeh 1958); this compound decomposes at room temperature to 5-HMF (Defaye 1964). According to the investigations of Freudenreich (1976), values of glucosamine of about 100 mg/kg glucosamine and galactosamine of 20 mg/kg in meat with large amounts of connective tissue were calculated from the glucosamine and galactosamine values found in fat-free connective tissue. Therefore, the conversion of amino sugars into aldehydes seems of greater interest for nitrite loss rather than displaying a primarily antimicrobial effect.

Riha & Solberg (1971) observed a suppressed growth of *Clostridium perfringens*, which occurred after autoclaving nitrite-free culture media containing glucose and fructose. Such an inhibitory effect of substrates containing carbohydrates (Erickson & Fabian 1942; Tarkow et al. 1942) may be explained by the formation of furfurals (Singh et al. 1948; Wolfrom et al. 1948). Especially the heating of carbohydrates in acid media leads to formation of sugar aldehydes. With degradation of sucrose and fructose to aldehydes, higher yields are obtained than in the degradation of glucose (Singh et al. 1948; Newth 1951). This effect is accelerated in the presence of glycine (Wolfrom et al. 1949). Furthermore the mass fraction of glucose – about 1 000 mg/kg in aged bovine muscle (Hamm et al. 1973) – originated from degradation of glycogen, therefore influencing the formation of 5-HMF. Lee et al. (1976) stated that red muscle contains less glycogen than white, the result is a lower pH in aged white muscle and also a higher loss of nitrite in the cured meat. Amounts of 5-HMF, formed from added carbohydrates and those produced by glycolysis, are comparatively high in contrast to those substances arising from deamination of amino sugars. If aqueous solutions of sucrose or fructose of low acidity are autoclaved at 120–140 °C for 2.5 h, 20 to 30% of the sugar initially present is decomposed into 5-HMF. If a sausage mixture contains sucrose at a mass fraction of 10 mg/kg, then a mass fraction of aldehydes more than 1 000 mg/kg might be present after adequate heat treatment.

In order to prove the inhibitory action of higher amounts of this aldehyde, concentrations of 5-HMF of 200, 500 and 2 000 mg/litre were tested (Table 5). Growth was distinctly inhibited for strains of *Micrococcus* and *Staph. aureus* by more than 500 mg/litre. The activity of *E. liquefaciens* was slightly reduced only at 2,000 mg/litre. *E. coli* was not inhibited at the concentrations tested.

By addition of reducing sulfur-containing substances to acid-hydrolysed carbohydrate mashes, the antimicrobial effect of inhibitors formed during the process is

Table 5. Inhibitory action of 5-hydroxymethylfurfural on growth of bacteria. ρ, mass concentration; – , no inhibition; + , faint inhibition; ++ , distinct inhibition.

ρ(5-HMF)/mg · litre^{-1}	*Micrococcus*	*E. liquefaciens*	*E. coli*	*Staph. aureus*
200	–	–	–	–
500	+	–	–	+
2000	++	+	–	++

eliminated; this may be due to the binding of sugar aldehydes to sulfhydryls (Block 1949). By condensation of aldehydes with cysteine, substituted thiazolindines were formed and can be synthesized in good yields. For example, formaldehyde reacts with cysteine at pH 1.5–12 during formation of the stable thiazolindine-4-carboxylic acid (Schubert 1936; Ratner & Clarke 1937).

Besides the easy nitrosation of phenolic compounds, reactions of nitrous acid with substances bearing an activated methylene group are possible. Ketones as degradation products of fat oxidation (Gilbert et al. 1975) may transform to nitroso ketones which undergo rearrangement according to the following equation:

$$R'-\overset{\overset{O}{\|}}{C}-CH_2-R'' + HONO \xrightarrow{-H_2O} R'-\overset{\overset{O}{\|}}{C}-\underset{\underset{NO}{|}}{CH}-R'' \rightleftharpoons R'-\overset{\overset{O}{\|}}{C}-\overset{\overset{NOH}{\|}}{C}-R'' \quad (1)$$

Recently, Namiki & Kada (1975) identified ethylnitrolic acid by reaction of nitrite with sorbic acid among other substances. As both compounds may be contained in foodstuffs, for instance in meat products as additives, this statement is of special importance.[1] Nitrolic acids are easily formed by reaction of 1-nitroalkanes with nitrous acid according to Equation 2 (Meyer 1875; Schmid et al. 1951):

$$R'-CH_2-NO_2 + HONO \xrightarrow{-H_2O} R'-\underset{\underset{NO}{|}}{CH}-NO_2 \rightleftharpoons R'-\overset{\overset{NOH}{\|}}{C}-NO_2 \quad (2)$$

The aliphatic nitrolic acids of low molecular weight are pale-yellowish crystalline substances, and form red alkali salts. The free acids are rather more stable than the alkali salts. The kinetics of the decay of ethylnitrolic acid, which is catalysed by hydrogen ions, was investigated by Maschka & Mirna (1951). As intermediates, ethyl nitryl oxide, nitrous acid and hydroxylamine were postulated, which react according to the gross Equation 3 to acetic acid and dinitrogen oxide:

$$CH_3-\overset{\overset{NOH}{\|}}{C}-NO_2 \xrightarrow{H^+} CH_3COOH + N_2O \quad (3)$$

The microbial activity of ethylnitrolic acid against a strain of *E. coli* was tested by Namiki & Kada (1975); they found an inhibitory effect at a mass fraction of 25 to 50 mg/kg, whereas sodium nitrite yielded positive results at 1 500–3 000 mg/kg and sorbic acid at 2 000–4 000 mg/kg only. Therefore nitrolic acids were included in our tests. As we have found, ethylnitrolic acid showed an inhibitory effect against the strains tested even at 10 mg/litre.

1. German Food Regulations do not at present permit the addition of sorbic acid in meat products.

Ethylnitrolic acid causes damage to bacterial deoxyribonucleate (Kada 1973; Namiki & Kada 1975). Therefore the possibility of mutagenic action by nitrite reaction products in addition to carcinogenic properties should be studied.

The inhibitory effect of ascorbic acid or of sodium ascorbate or both on microbiological processes in meat products might be explained by the rapid release of nitrogen oxide from nitrite. Further by the effect of the undissociated nitrous acid, whose amount depends on pH, according to Riha & Solberg (1975a), who (1975b) also presumed that the inhibitory effect of *Clostridium perfringens* may be due to reactions of nitrite with constituents of the cell membranes; they assumed a blockage of functional SH-containing enzyme systems. Meat products with added ascorbate as acid or sodium salt have less residual nitrite but distinctly more residual nitrate. Ascorbic acid is rapidly degraded by oxygen, traces of heavy metals, and by enzymes. So the inhibitory effect of such an additive is not necessarily to be expected. In plant and animal tissues, ascorbic acid is also found in bound form (Fürtig & Pohloudek-Fabini 1965). This kind of depot or reserve ascorbic acid is better protected from oxidation than the free form. Moreover ascorbic acid, like glucuronic acid, has been reported, to develop a detoxifying effect in the body. In the literature special attention has been paid to the investigation of ascorbigen, which is formed from *Brassica* spp. by an artefact during isolation from the plant tissue. This compound is formed from the enzymically released 3-hydroxy-methylindole with the ascorbic acid (Kiss & Neukom 1966). So far, there are no exact findings on the binding of ascorbic acid in animal tissues and in body fluids. Presumably binding is via an iron-phosphate complex in the liver and spleen.

Pronounced therapeutical properties are ascribed to compounds of ascorb with amino acids, especially cysteine. These compounds are said to be effective against *Streptococcus haemolyticus* in animal tests, even more so than certain sulfonamides and antibiotics (Meyer-Döring 1957; Meyer-Döring & Perkow 1958). Our investigations have not shown a substantial inhibition of bacterial growth by the compounds synthesized according to the procedures described in the patent of Meyer-Döring & Perkow (1958). Indeed, it is not certain whether the compounds were identical with those tested by those authors.

Drake et al. (1947) claim that dehydroascorbic acid, which forms mainly by reaction of ascorbic acid with nitrite, may form addition compounds with cysteine and with glutathione. So far, we were not successful in isolating one of these compounds.

The reaction of various amino acids with nitrite, with the exception of cysteine, scarcely occurs in weakly acid media (Riha & Solberg 1973). Ågren (1940) found similar results at an earlier date; on the basis of data from Schubert (1939), he succeeded in isolating a condensation product of cysteine-glucose; it is assumed to be a compound with a thiazolindine structure. Weitzel et al. (1957, 1959) have synthesized a number of condensation products by reaction of cysteine with reducing sugars. It has not been proved, however, whether these compounds have, in fact, a thiazolindine structure. According to those authors, we have, therefore, used the same nomenclature for these substances. The reaction of cysteine with ascorbic acid does not lead to only one distinct compound, not even when the method described by Weitzel et al. (1959) is used.

Tatum et al. (1969) heated aqueous solutions of ascorbic acid and found 15

degradation products, mainly furfural. Among the degradation products investigated, only 4-butyrolactone, γ-crotonolactone, 2-furoic acid and furoin inhibited solely one or two of the strains tested.

Acknowledgment

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Discussion on the session

5-Hydroxymethylfurfural

The authors estimate the formation of this compound from amino sugars in a meat product without the addition of sugars to be about 50 mg per kg. The inhibitory level of 5-hydroxymethylfurfural on several non-sporeforming micro-organisms seemed to be 500 mg per kg. With sugars in a meat product, this level might be reached. No data on the inhibition of sporeformers are available.

Furfural

According to the authors, it seems unlikely that furfural will be formed in significant amounts in meat products, as this compound is mainly derived from pentoses. There is some evidence in literature that furfural is less inhibitory towards micro-organisms than 5-hydroxymethylfurfural.

Nitrolic acids

Ethylnitrolic acid has been shown to possess an antimicrobial activity at the 10 mg per kg level. This compound is volatile by steam distillation and rather stable in weak acid media (pH about 6) and its degradation is catalyzed by hydrogen ions. From ethylnitrolic acid, acetic acid and N_2O are formed. The salts are not very stable.

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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

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	+	+

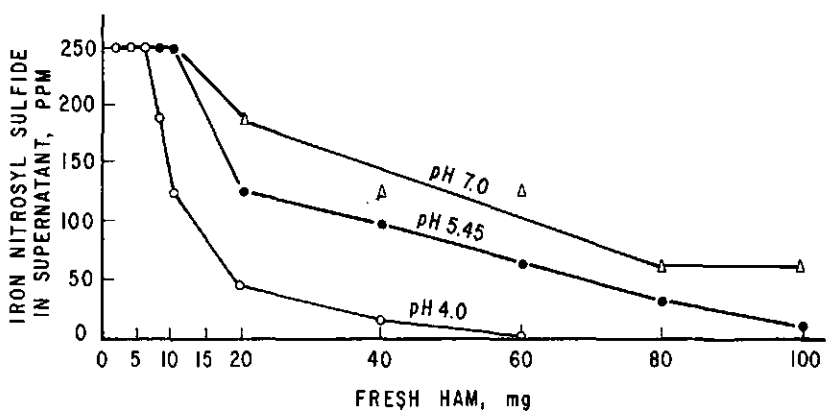

Fig. 1. Effect of pH on the absorption of iron nitrosylsulfides (INS) by meat homogenates. The solution initially contained 500 μg INS. The ordinate refers to equivalent of fresh ham used in preparing the homogenate. The abscissa refers to concentration in mg/litre.

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.

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

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

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.

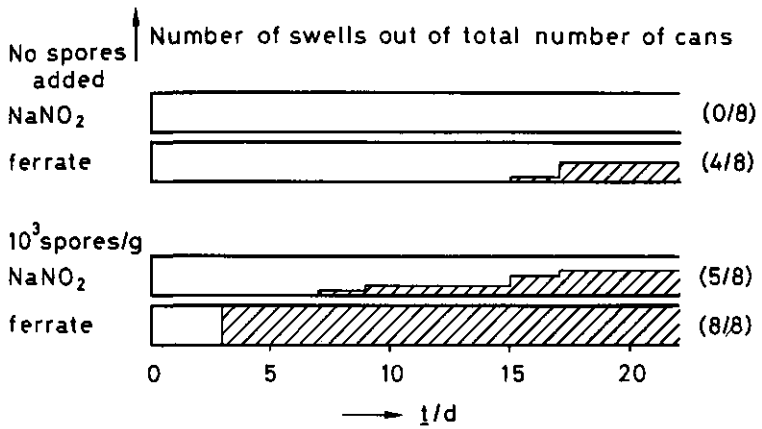

Fig. 1. Inhibition of Clostridia (number of swollen cans, 8 per group) by $NaNO_2$ at a mass fraction of 104 mg kg^{-1} in the pasteurized ham product and by dicysteyl – dinitric oxide – ferrate at a mass fraction of 80 mg kg^{-1} of $NaNO_2$ equivalent in the ham product incubated at 30 °C. 1 mg $NaNO_2$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

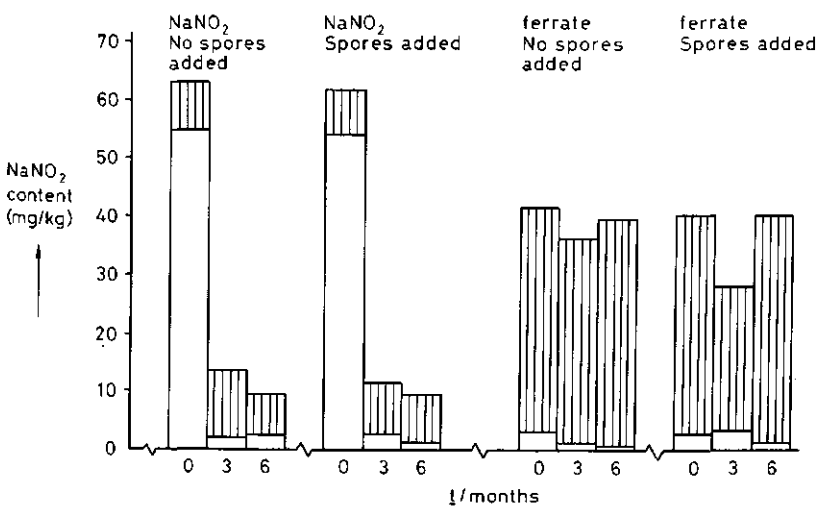

Fig. 2. Effect of storage time (t / months) at 8 °C and addition of $NaNO_2$ or dicysteyl – di nitric oxide – ferrate on mass fractions (mg kg^{-1}) of free (unshaded) and protein-bound (shaded) nitrite (expressed as $NaNO_2$) in a pasteurized ham product. Amounts of additives as in Figure 1.

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.

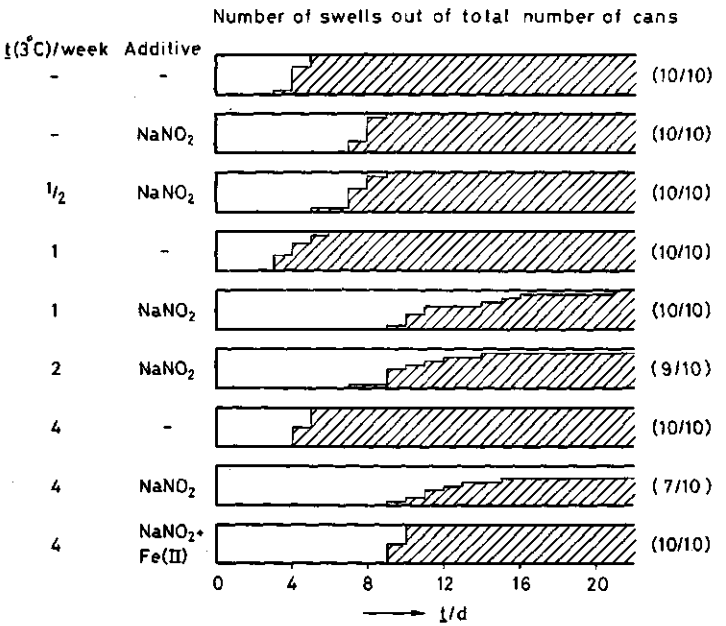

Fig. 3. Effect of storage time (t / week) at 3 °C immediately after pasteurization on the inhibition of Clostridia (number of swollen cans, out of 10) by $NaNO_2$ in a beef product. Inoculation with suspension of cow dung (10^4 spores per g product); pH 6.2. Mass fraction of added $NaNO_2$ 200 mg kg^{-1}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

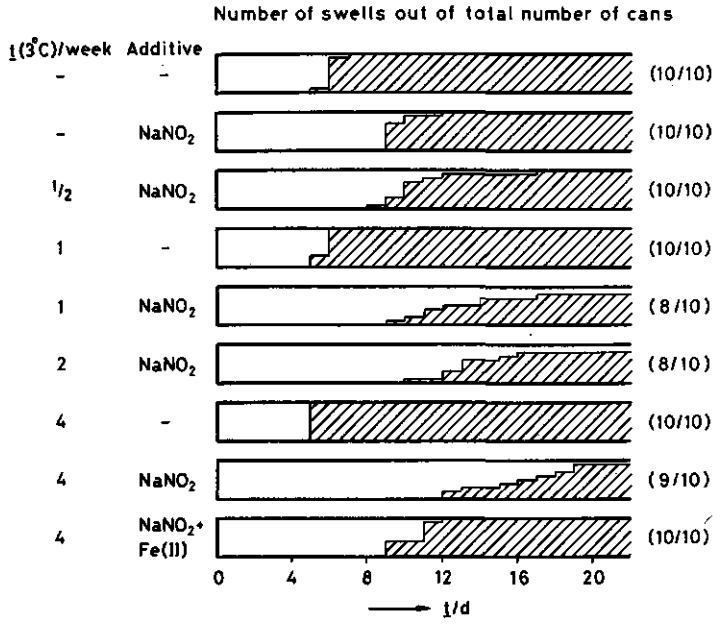

Fig. 4. Effect of storage time (t / week) at 3 °C immediately after pasteurization on the inhibition of Clostridia by $NaNO_2$ in a beef product, pH 5.9. Further conditions as in Figure 3.

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

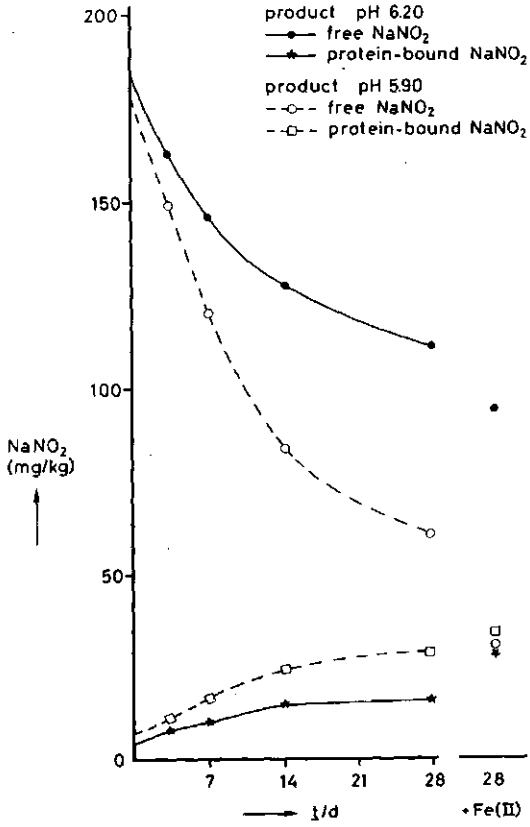

Fig. 5. Levels of free and protein-bound $NaNO_2$ in the beef product as effected by storage at 3 °C.

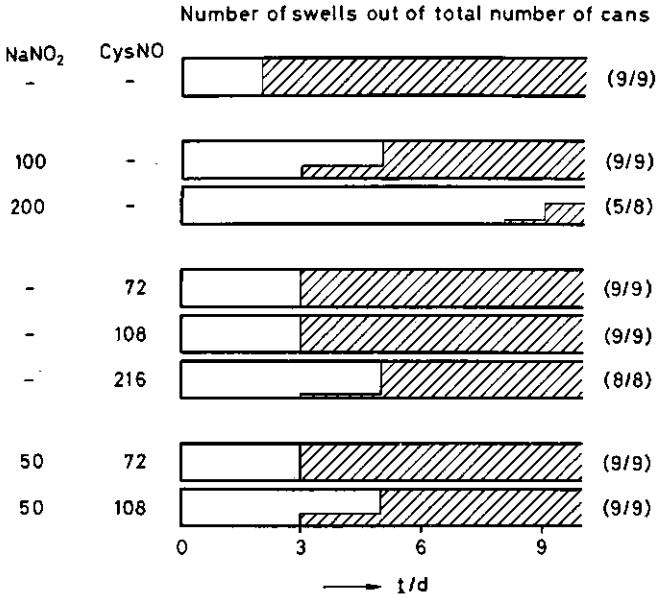

Fig. 6. Inhibition of Clostridia (number of swollen cans, out of 8 or 9) by $NaNO_2$ and *S*-nitrosocysteine in the pasteurized ham product incubated at 30 °C. 1 mg $NaNO_2$ equivalent = 2.2 mg *S*-nitrosocysteine. Inoculation with 10^3 spores per g product.

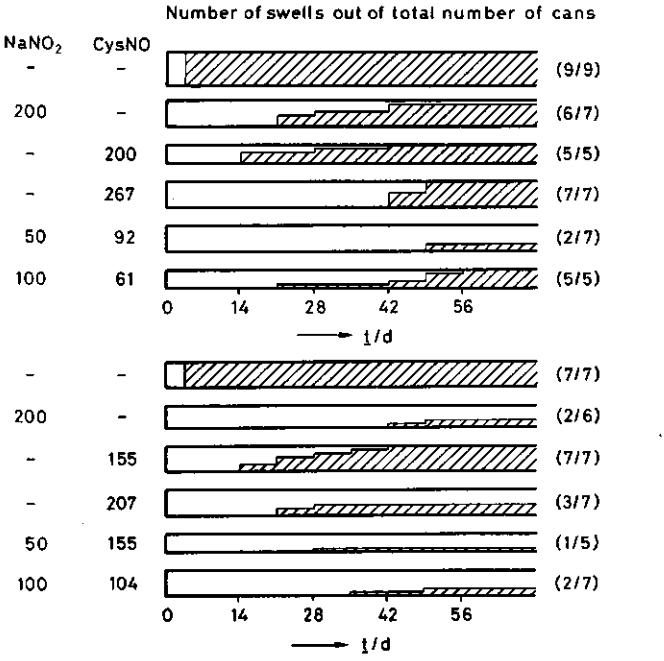

Fig. 7. Inhibition of Clostridia by $NaNO_2$ and *S*-nitrosocysteine in the pasteurized ham product incubated at 30 °C. Except for added mass fractions, the conditions are the same as in Figure 6.

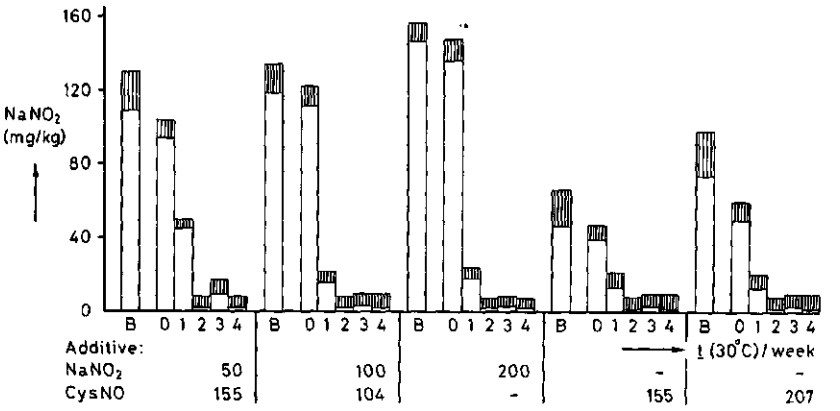

Fig. 8. Mass fractions of free (unshaded) and protein-bound (shaded) nitrite before (B) and after pasteurization (t = 0) and incubation at 30 °C for 1–4 weeks. Additives are expressed as mass fractions of $NaNO_2$ or its equivalent (mg/kg^{-1}).

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.

Conclusions and recommendations of the microbiological session, Tuesday 7 September

1. As emphasized by Perigo, we must seek compounds some 10 times as inhibitory as nitrite, to explain the difference between canning practice and laboratory experiment. Of all the compounds so far surveyed, only the Roussin salts and the nitrolic acids possess this property and it now seems clear that the Roussin salts can be ruled out.

2. Attention should be directed especially to nitrolic acids.

3. The possibility of a synergistic action of hydroxymethylfurfural should be remembered.

Technological session

Reporters: P. C. Moerman, P. S. van Roon

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Processing and sensory evaluation of a primal cut cured with nitrite alone or nitrite-nitrate mixture

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In 1975, the Expert Panel on Nitrosamines appointed by the Secretary of the United States Department of Agriculture proposed eliminating nitrate salts from cured meat products (USDA 1975). The traditional saltpeter used in cures for many centuries has been coupled with nitrite in recent years as information developed indicating nitrate was reduced to nitrite, which is the actual active component. Several kinds of cured meat, however, are still currently processed with nitrate only or mixtures of nitrate and nitrite. Dried beef is one of these products, cured by immersion for as long as 6 weeks in a brine containing nitrate and nitrite. Consultation with several producers of dried beef who had tested an all-nitrite cure revealed dissatisfaction with the process, including concern over the increased occurrence of uncured areas in the pieces of meat. Other users of the nitrate cure insisted that the flavour differed from that with nitrite cure.

In an effort to learn more about the process of curing meat for dried beef, and probably other primal cuts of meat cured in a similar manner, we examined the process and the product.

Materials and methods

Sirloin tips, weighing 7–9 lb (3–4 kg), and known in the trade as beef knuckles, were loaded into plastic vats to a total weight of 1500 lb (781 kg) and covered with 75 gallons (288 litre) of cure brine. The brine contained salt at a mass concentration of 242 g litre^{-1}, sugar at about 30 g litre^{-1} and the appropriate cure ingredients. The conventional brine contained $NaNO_3$ 7750 mg litre^{-1} and $NaNO_2$ at 1670 mg litre^{-1}; the experimental brines were prepared with $NaNO_2$ at 2 200 mg litre^{-1}. Curing was at 5 °C and the meat was turned once every week by transferring to another vat, and covering with the same brine. Single knuckles were removed from the brines after 1, 3, 5 and 6 weeks for analysis. Samples of the brine were also taken for analysis. After 6 weeks, the cured beef knuckles were dried at 65 °C for about 3 days to achieve a yield 60–65% of the original mass of the meat.

Nitrite and nitrate analyses were by the methods of US-AOAC (1975); nitrate concentrations were also estimated with the nitrate-specific electrode. After direct extraction by the method of Fiddler et al. (in preparation), samples of meat were analysed for dimethylnitrosamine (DMN), diethylnitrosamine (DEN), methylethyl-

nitrosamine (MEN), *N*-nitrosopyrrolidine (NPyr), *N*-nitrosopiperidine (NPip), and *N*-nitrosomorpholine (Mor). Chloride was measured with the chloride-specific electrode placed on the surface of the meat.

The dried beef was assessed with groups of about 40 laboratory staff in a consumer-type panel. A paired-comparison test was used to establish difference in flavour between samples, and those panelists noticing a difference were asked to indicate a preference for one of the pair. A 9-point hedonic test was also used to evaluate consumer acceptance of the dried beef. Creamed chipped beef was prepared from a white sauce plus dried beef pieces and evaluated with the hedonic test. Statistical significance was determined by standard methods (Amerine et al. 1965).

The brines were evaluated microbiologically. Total number concentration of aerobes was estimated on regular APT agar and an APT agar with 10% NaCl. Yeasts were detected on potato dextrose agar and Rogosa SL medium was used to identify lactic acid bacteria. Micrococci were detected on mannitol salt agar. All plates were incubated at 25 °C for 4–5 days. All media were obtained from Difco Laboratories[1].

Results and discussion

Microbiologically there was little difference between brines containing NO_2^- alone and those containing $NO_2^- - NO_3^-$. The total number concentrations of viable cells in brine were low, beginning with $10-10^2$ per ml and attaining 10^7 per ml in the $NO_2^- - NO_3^-$ brine and 10^6 per ml in the NO_2^- brine in 5–6 weeks. Yeast predominated, although gram + rods and cocci, both catalase positive and negative, were present in substantial numbers. The composition of the brine did not influence composition of the developing flora.

The pH of the brines initially was 7.0 for the $NO_2^- - NO_3^-$ brine and 6.6 for NO_2^-. After 1 week, the pH of both types had dropped to 5.5–5.6. The experimental NO_2^- brine remained at pH 5.6 through the 6-week curing period, whereas the pH of the regular brine dropped gradually to 5.2.

The content of sodium chloride in the meat was measured, beginning with samples from the third week of cure. Diffusion of salt into the meat was shown by analysing samples from the surface and from the centre of the piece. Variations occurred since different pieces of meat were used at each time interval but, as expected, the content of NaCl was greater at the surface than at the centre after three weeks (Table 1). Maximum salt content in the surface of the meat was attained after about 5 weeks of curing and then declined, possibly through changes in osmotic pressure. The highest mass fraction of salt recorded was 75–88 g/kg. On soaking the meat, salt was removed principally from the surface. The dried product contained much salt: 160–210 g/kg at the surface and about 120 g/kg at the centre.

The fate of the nitrite and nitrate in the brines and the meat is shown in Table 2. The regular brine formula contained a substantial amount of NO_3^- (7 750 mg/litre) and about 35% of it disappeared in the first week, levelling off at a residual 40%

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

after 5 weeks. Little nitrate appeared in the experimental NO_2^- brine. The nitrite concentrations of the regular and experimental brines were 1 670 and 2 200 mg/litre, respectively. The rate of disappearance of NO_2^- from the two brines was about the same, with 52–56% disappearing in 1 week and 90% in 6 weeks. The presence of nitrate in the regular brine had little effect on the concentration of nitrite.

In the meat removed from regular brine, content of nitrate increased with a gradient from the outside of the piece to the centre, ranging from 2 300 to 1 600 mg/kg. Sodium nitrite reached about 300 mg/kg in the outer layers and increased from 0 to almost 100 mg/kg at the centre. Meat cured with nitrite only contained $NaNO_3$ – up to 127 mg/kg at the third week and 103 mg/kg in the dried product.

Table 1. Effect of curing with NO_3^- and NO_2^- or NO_2^- alone on content of NaCl in cured beef. t, time (of curing).

t/week	Site	NaCl content/g kg^{-1}	
		NO_3^- & NO_2^-	NO_2^-
3	surface	53	40
	centre	36	10
5	surface	76	88
	centre	44	64
6	surface	63	64
	centre	49	54
Dried	surface	205	158
	centre	115	117

Table 2. Nitrite and nitrate in brine and beef. t, time (of curing); w, mass fraction (content); ρ, mass concentration; Reg, regular NO_3^- and NO_2^- brine; Exp, NO_2^- brine.

t/week	Brine				Beef				
	$\rho(NO_2^-)$/mg · litre^{-1}		$\rho(NO_2^-)$/mg · litre^{-1}		site	$w(NO_3^-)$mg · kg^{-1}		$w(NO_2^-)$/mg · kg^{-1}	
	Reg	Exp	Reg	Exp		Reg	Exp	Reg	Exp
0	7748	5	1670	2208		—	—	—	—
1	5120	10	814	968	Surface	827	69	68	40
					Centre	152	16	0	0
3	4129	6	570	740	Surface	1813	127	300	391
					Centre	1200	104	48	46
5	3126	5	174	326	Surface	2384	58	248	408
					Centre	1303	74	77	210
6	3000	8	157	276	Surface	2278	112	258	346
					Centre	1600	98	92	190
Dried						3097	103	37	26

Content of $NaNO_2$ in this meat were a little greater than in the meat from the regular cure.

Contents of $NaNO_2$ in both products after drying, smoking and storage were about the same: 26–37 mg/kg. These low values in the final consumer package give no indication of the amounts to which the meat was exposed during immersion nor the danger of nitrosamine formation. None of the nitrosamines tested for were detected in the knuckles cured with the regular brine (Table 3). However, in the nitrite – cured samples, contents of 3–4 μg/kg of dimethylnitrosamine were found after 3 and 5 weeks, respectively, and the final cured dried product contained 14 μg/kg.

Table 3. Dimethylnitrosamine in cured dry beef with regular cure (Reg, nitrite and nitrate) and with the experimental cure (Exp, nitrite only). w, mass fraction (content); t, time (of curing); –, not detected (dimethylnitrosamine was confirmed by mass spectrometry in all samples where it was detected).

t/week	w(dimethylnitrosamine)/μg · kg^{-1}	
	Reg	Exp
0	–	–
1	–	–
3	–	3
5	–	4
6	–	–
Dried beef	–	14

Table 4. Sensory evaluation of dried beef. A = beef cured in regular NO_3^- – NO_2^- brine. B = beef cured in NO_2^- brine. O = dislike extremely; 5 = neither like nor dislike; 9 = like extremely. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; NS, $P \leq 0.05$.

Cure	Difference		Preference of 'Yes'
	No	Yes	
Pooled A vs Pooled B	3	38***	30 – Pooled A**
7A vs 6B	11	27*	18 – 7A^{NS}
7A vs 5B	18	22^{NS}	17 – 7A*
5A vs 2A	10	34***	19 – 2A^{NS}
6A vs 7A	19	24^{NS}	13 – 7A^{NS}
3B vs 4B	21	19^{NS}	11 – 3B^{NS}
1B vs 2B	19	21^{NS}	17 – 1B**

Table 5. Hedonic test. Scores with the same index (a, b, c) are not significantly different.

	Cure	Number of panelists	Score
Dried beef	5B	44	5.72[c]
	6B	45	5.36[c]
	1A	28	6.93[d]
	7A	22	6.67[d]
Cream chipped beef	2A	43	7.02[e]
	6B	34	6.55[e]

No evidence of uncured areas was found in the nitrite-treated knuckles. The apparently random occurrence of these areas, and the fact that they were observed only after the dried product was sliced, made it difficult to determine the cause. Studies are being conducted on the diffusion of cure ingredients into meat in an effort to understand this cure process more fully.

Sensory evaluation of pooled dried beef samples cured in NO_3^- and NO_2^- or NO_2^- only showed that a significant number of panelists could detect a difference between them and that the number of the panelists preferring the dried beef cured in the regular brine was significant. However, there were variations in flavour between knuckles cured in the same brine which might account, in part, for the results obtained (Table 4).

In a hedonic test (Table 5), the beef cured in NO_2^- only was rated an average of 5.5 (neither like nor dislike) whereas the NO_3^-–NO_2^- cured beef scored 6.8 ('like'). The regular-cure product was thus more acceptable. However, there was a general impression that a consumer would accept the NO_2^- cured product if the regular-cure beef were not available for comparison.

Since the extreme saltiness of the dried beef could have affected the panelist responses, it was decided to evaluate the products as creamed chipped beef, which is probably the most familiar preparation of dried beef. A hedonic comparison of one sample each of the regular-cure and experimental-cure beef in this form showed that both preparations were well accepted.

Studies are continuing, not only with beef knuckles cured in NO_2^- brine but also with knuckles injected with pickle containing lower concentrations of nitrite and immersed in brines of various composition, in an effort to produce a product acceptable to the consumer with a minimum of nitrite or nitrate in the curing process.

Acknowledgments

We gratefully acknowledge the participation of J. B. Fox, J. L. Smith, S. A. Palumbo and J. W. Pensabene in some analysis of the brine and beef products. The knuckles were cured and dried beef prepared with the cooperation of the E. W. Knauss Company, Quakertown, Pa.

Discussion on the session

Nitrate-free curing

This paper has some additional points of interest in the general comparison of nitrate-free with traditional cures. In practice, the result of eliminating nitrate is that brines contain more nitrite and that nitrite concentrations in the surface of the meat are correspondingly higher immediately after cure. In the system with nitrate, the critical microbiological question is what decides whether the nitrate is reduced during storage and distribution. Anaerobic conditions favour this; otherwise the critical factors are obscure.

A later paper on methods suggests strongly that most reports of nitrate being produced from nitrite are procedural artefacts. Because of this phenomenon, it is regarded as impossible to legislate for zero nitrate and control embracing nitrate and nitrite on equal terms should be envisaged.

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Consumer evaluation of the flavour of bacon cured with and without sodium nitrite

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The flavor of bacon has been, for the most part, related to the use of nitrite salts in the cure. Mottram & Rhodes (1974) reported at the last meeting that increasing concentrations of nitrite produced more bacon flavour and decreased the level of 'porky' flavor. Herring (1973) demonstrated that the acceptability of bacon made without nitrite decreased with storage time. The data of both workers show, however, that initially, bacon made without nitrite had a certain amount of acceptability. In our earlier studies (Kimoto et al. 1976) with bacon prepared with and without nitrite in the laboratory, panelists rated the flavour of the 'no-nitrite' bacon comparably with that of a popular national brand bacon. An 'Open House' demonstration for the public at our Center presented us with the opportunity of conducting a large-scale consumer test of the flavour of bacon prepared with and without nitrite.

Experimental

Bacon was prepared by a national producer according to his standard procedure. The basic cure solution contained salt, sugar, tripolyphosphate, and ascorbate. Half the bellies were pumped with this solution, the other half received this solution plus $NaNO_2$ at 120 mg/litre. Processing, smoking, and packaging were the same as for the commercial product. The bacons were stored and shipped under refrigerated conditions. Fifteen days after being processed the bacon was fried for 5–6 min at 165 °C, and submitted to our Open House visitors for evaluation. These panelists were given a questionnaire requesting information on sex (male or female); age group (< 21; 21–30; 31–40; > 41), and frequency of eating bacon (at least once a week; at least once a month; less often). They were asked to rate the flavour of the bacon on a 7-point hedonic scale (1 = dislike extremely; 7 = like extremely) and to indicate their preference for the bacon made with $NaNO_2$ or that made without $NaNO_2$. The results were analysed by standard statistical procedures.

Results and Discussion

Of the 969 visitors who participated in the test, the responses of 265 had to be rejected because the questionnaire was filled out incompletely or incorrectly. The

Table 1. Hedonic Evaluation and Preference Selection of Bacon Cured With and Without Nitrite.

Category	Average Rating[1]		
	nitrite cure	no nitrite cure	Average Preference[2]
Female	6.16	6.10	1.50
Male	6.07	5.97	1.50
< 21	6.13	5.95	1.46
< 30	5.90	5.95	1.50
< 40	6.17	6.13	1.50
> 41	6.18	6.09	1.53
Eat once a week	6.20	6.13	1.52
Eat once a month	6.06	6.07	1.50
Eat less frequently	6.04	5.83	1.47
Average	6.10	6.00	

1. Based on 7 point scale: 1 = dislike very much; 7 = like very much.
2. Preference for bacon cured with nitrite = 1; preference for bacon cured without nitrite = 2.

remaining 704 responses came from 350 men and 354 women. The data were analysed for all variables – sex, age, frequency of consumption, and all possible interactions. The results are shown in Table 1. Regardless of the variable, the average for that group differed only slightly from a value of 6.0 ('like moderately') for bacon prepared with nitrite and that prepared without nitrite. Although the preference could have been determined from the ratings in the hedonic test, the panelists were requested to list their preference. For purposes of analysis, preferences for bacon made with nitrite were rated as 1 and those for bacon without nitrite as 2. Table 1 indicates an average value of about 1.5 for each variable, indicating no preference between the flavour of the two bacons.

There was a difference in the colour of the bacons after frying which was not hidden or disguised.

It appears, therefore, that bacon with flavour acceptable to the average consumer can be made by processing with salt and no nitrite. It is possible, however, that smoking the product may provide desirable notes as well. This study dealt only with flavour and did not consider the effects of nitrite on storage stability or the prevention of growth of *Clostridium botulinum*.

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Irradiation as a conceivable way of reducing nitrites and nitrates in cured meats

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Introduction

Nitrite is indispensable as a curing ingredient for producing characteristic colour and flavour of cured meats and for the control of growth and toxin formation by *Clostridium botulinum* in cured unirradiated meats. The main portion of nitrite added to unirradiated cured meats (156 to 200 mg/kg) is for control of *C. botulinum*. Processing of cured meats with sterilizing doses of ionizing radiation (radappertization) obviates the need for that amount of nitrite required for controlling *C. botulinum* in cured unirradiated meats. Our initial work on irradiation-sterilized (radappertized) ham has shown that irradiation allowed reduction of the amount of the added nitrite ($NaNO_2$) from 156 to 25 mg/kg, provided that $NaNO_3$ was supplied at 50 to 100 mg/kg in combination with nitrite (Wierbicki & Heiligman 1974). At the First International Symposium on Nitrite a conclusion was drawn up that 'Irradiation is a conceivable way of reducing the quantities of nitrite in processed meat products' (Krol & Tinbergen 1974, p. 213). In the meantime, further research and development was conducted on radappertized ham and new research was initiated on radappertized corned beef and partly fried (pre-fried) bacon. This paper presents a digest of the most important findings.

Experimental

Details on the technology of the products, quality evaluation and the analytical procedures are given in the following publications: Shults et al. 1976; Wierbicki et al. 1974a, 1974b, 1975a, 1975b and 1976. For nitrosamine analysis, slices of pre-fried bacon were heated for 3 min on each side in an oven at 205 °C. The fried bacon meat was then ground and 25-g random samples digested with methanolic KOH for 5 hours. Isolation and cleanup procedures were carried out as described by Fazio et al. (1971). Bacon drippings were collected and prepared for the nitrosamine analysis according to White et al. (1974). Gas chromatographic separation procedures and confirmation by mass spectrometry were described previously (Pensabene et al. 1974).

Results

Ham

In May 1974, we procured 1 000 kg of fully cooked smoked ham from one meat packer and in February 1975, 26 000 kg from another packer which were then vacuum-packed and irradiated with the sterilizing dose of 3.7 to 4.3 Mrad of gamma-radiation from cobalt–60 at –30 ± 10 °C product temperature. The ham was cured with the common curing ingredients: 2.4 ± 0.3% NaCl; 500 ± 50 mg/kg sodium ascorbate and sodium erythorbate (AE) 1:1 mixture; 0.4 ± 0.1% sodium tripolyphosphate (TPP) and only 25 mg/kg $NaNO_2$ and 100 mg/kg $NaNO_3$. Both irradiated and unirradiated samples were of excellent quality without undercured colour spots throughout the hams, while using industrial injecto-pumping equipment. On the research side, samples of fully cooked smoked ham were processed with the following $NaNO_2$ + $NaNO_3$ combinations: 150 + 0, 25 + 0, 25 + 50, 25 + 100, 50 + 25, 50 + 50, 50 + 75, 75 + 50 mg/kg, respectively. All unirradiated hams were highly acceptable without significant differences in the quality scores (colour, flavour, texture, appearance and general acceptance). Among the irradiated samples, the hams cured with only nitrite (at 150 and 25 mg/kg $NaNO_2$) were unacceptable, particularly on account of the colour; the ham cured with $NaNO_2$ at 25 mg/kg and $NaNO_3$ at 50 mg/kg was acceptable but slightly inferior in colour. The least amount of $NaNO_2$ + $NaNO_3$ for acceptable radappertized ham is a total of 75 mg/kg of which a half to two-thirds (37.5 to 50 mg/kg) shall be $NaNO_2$.

Confirmatory tests are in progress. The residual nitrite in radappertized ham (1 to 4 mg/kg) is within the range of the experimental error for the method used for estimating nitrite. None of the six nitrosamines ($ONNMe_2$, $ONNEt_2$, ONN(Me)Et, ONPip, ONMor, and ONPyr) were detected in the radappertized ham, shortly after irradiation and after 14 months of unrefrigerated storage. Nitrosamines are being estimated in representative samples from the 26 000-kg lot, subdivided into four groups: gamma-irradiated with cobalt-60; electron-irradiated; thermally sterilized to the lethality level in the coldest spot of F = 6; and frozen control as a part of the general studies on wholesomeness of radappertized ham. The nitrosamines will be estimated on three additional industries' procurements of ham representing a total of about 90 000 kg of ham.

Irradiation of ham cured only with $NaNO_3$ (without $NaNO_2$) resulted in a characteristic pink colour of cured meat in the irradiated ham, particularly when irradiation preceded thermal processing. It indicates that irradiation causes a reduction of nitrate to nitrite. Analysis is in progress to prove this effect of irradiation on cured meat pigment, nitrosylmyoglobin.

Corned beef

Beef briskets were cured with NaCl at 1.5 ± 0.2%, AE at 500 ± 50 mg/kg, and the following additions of $NaNO_2$ + $NaNO_3$: 150 + 600, 25 + 100, 150 + 0 and 25 + 0 mg/kg, respectively. After cooking in boiling water, the resulting corned beef was chilled, vacuum-packed and irradiated with the sterilizing dose of ionizing radiation for corned beef of 2.5 to 3.3 Mrad at –30 ± 10 °C product temperature.

Sensory evaluation of the irradiated and unirradiated samples showed acceptable corned beef product for all cures, with slightly lower scores for colour and general acceptance for the corned beef processed with only 25 mg/kg addition of $NaNO_2$ without $NaNO_3$. In contrast to radappertized ham, acceptable radappertized corned beef can be produced with the maximum permitted amount of $NaNO_2$ (156 mg/kg) without $NaNO_3$. However, this addition of $NaNO_2$ can be substantially limited in combination with nitrate. Our target is to produce high-quality radappertized corned beef with a maximum of 75 mg/kg of added mixture of $NaNO_2$ + $NaNO_3$. Tests are in progress on this, including estimation of nitrosamines in irradiated and unirradiated corned beef processed with the reduced additions of nitrite and nitrate.

Pre-fried Bacon

Fresh, chilled (unfrozen) raw bacon bellies, 4 days post mortem, were injecto-pumped (by hand) to 11% weight of the bacon bellies with six different curing solutions containing intended additions of the curing agents to the bacon, smokehouse-processed to internal temperature of 53.3 °C and 100% yield-to-raw

Table 1. Intended additions of curing agents to raw bacon during injecto-pumping. TPP, sodium tripolyphosphate; AE, 1:1 mixture of sodium ascorbate and sodium erythorbate; –, not added.

Lot No	NaCl (%)	sucrose (%)	TPP (%)	$NaNO_2$ (mg/kg)	$NaNO_3$ (mg/kg)	AE (mg/kg)
1	1.5	0.75	0.3	150	500	500
2	1.5	–	–	150	500	500
3	1.5	–	–	25	–	500
4	1.5	–	–	25	100	500
5	1.5	–	–	25	100	1000
6	1.5	0.75	0.3	25	100	1000

Table 2. Analytical composition of cured fresh bacon 3 days after smokehouse processing. T, trace; TPP, sodium tripolyphosphate; ASC, ascorbate; ·, not determined.

Lot No	H_2O (%)	Fat (%)	Protein (%)	NaCl (%)	Sucrose (%)	TPP (%)	pH	Residue, mg/kg		
								$NaNO_2$	$NaNO_3$	Asc
1	30.3	58.7	8.5	1.49	0.82	0.29	6.6	48	487	294
2	34.2	53.4	9.5	1.64	T	.	6.1	21	571	291
3	30.4	58.7	8.7	1.48	.	T	6.2	7	.	347
4	37.4	51.6	9.7	1.57	.	.	6.1	6	55	355
5	31.5	56.6	9.3	1.64	T	T	5.9	7	24	728
6	31.5	55.9	9.2	1.58	0.73	0.31	6.2	8	17	698

Table 3. Chemical composition of partly fried unirradiated (C) and irradiated (Ir) bacon. NPN, non-protein nitrogen (% in total N); FFA, free fatty acids (expressed as % oleic acid in fat; TBA, malonaldehyde; T, trace.

		Lot 1		Lot 2		Lot 3		Lot 4		Lot 5		Lot 6	
Component	Amount	Ir	C	Ir	C	Ir	C	Ir	C	Ir	C	Ir	C
H_2O	(%)	23.4	19.1	21.6	25.6	25.3	23.6	22.0	18.6	24.0	25.3	21.2	22.4
Protein	(%)	20.4	18.8	22.2	24.1	20.3	19.2	23.2	21.0	23.2	19.7	15.9	14.8
Fat	(%)	50.0	56.7	51.0	46.0	49.5	53.0	50.0	55.1	47.0	49.8	57.7	57.6
NaCl	(%)	3.09	3.39	4.13	4.07	3.61	3.26	3.81	4.41	4.48	4.02	2.69	2.46
Ash	(%)	4.31	4.37	4.50	4.78	4.04	3.76	4.40	4.85	4.93	4.23	3.50	3.49
Sugar	(%)	1.31	1.18	0.19	0.17	0.07	0.08	0.16	T	0.16	0.14	1.25	1.12
NPN	(% in N)	10.4	9.0	10.4	10.6	11.1	9.8	10.2	10.4	9.4	10.8	11.0	11.4
P	(%)	0.31	0.31	0.20	0.22	0.17	0.16	0.20	0.18	0.20	0.17	0.25	0.25
FFA	(% in fat)	0.73	0.59	0.70	0.65	0.67	0.58	0.74	0.60	0.70	0.58	0.65	0.52
TBA	(mg/kg)	0.33	0.21	0.34	0.17	0.49	0.58	0.55	0.49	0.48	0.49	0.38	0.30
$NaNO_2$	(mg/kg)	2.7	2.3	2.0	1.8	0.7	1.2	1.2	1.2	1.2	1.0	1.4	0.9
$NaNO_3$	(mg/kg)	877	1157	1114	1281	0	0	223	356	237	192	124	115
pH		6.0	6.1	6.0	6.1	6.0	6.1	5.9	6.0	6.0	6.1	6.0	6.1

(Table 1). Representative samples of the raw processed bacon were analysed 3 days after smokehouse processing (Table 2). Within two weeks, sliced processed (raw) bacon was shipped at refrigeration temperature from the bacon processor to the US Army Natick R & D Command's Radiation Laboratory where it was partly fried (pre-fried) at 205 °C in an electric oven until reduction in weight-to-raw was 60% (1 kg partly fried = 2.5 kg raw bacon). The partly fried bacon was then vacuum-packed into round metal cans, 8.1 cm in diameter and 14.1 cm high (0.5 kg bacon per can) and irradiated with a sterilizing dose of 2.3 to 2.8 Mrad at ambient tempera-

Table 4. Hedonic preference ratings[1] of partly fried irradiated and unirradiated bacon. $\bar{X}$, mean; $s(x)$, standard deviation.

Lot No	Irradiated		Unirradiated	
	$\bar{x}$	$s(x)$	$\bar{x}$	$s(x)$
1	6.2	1.6	7.3	1.1
2	6.4	1.6	6.8	1.5
3	6.8	1.8	7.1	1.1
4	6.4	1.7	7.2	1.3
5	6.6	1.2	6.2	1.6
6	6.4	1.4	6.5	1.4
Least signif. diff.	0.5		0.6	

1. 9-point hedonic scale, Peryam & Pilgrim (1957).

Table 5. Presence of *nitrosopyrrolidine*[1] in partly fried irradiated and unirradiated bacon. –, not detectable, ·, not estimated.

Lot No	Irradiated		Unirradiated	
	fried bacon	fat drips	fried bacon	fat drips
1	–	·	–	·
2	–	–	–	–
2	–	–	·	·
3	–	·	–	·
4	–	–	–	–
4	–	–	·	·
5	–	·	–	·
6	–	·	–	·

1. Determined by the Eastern Regional Research Center Laboratories, US Department of Agriculture, Philadelphia, PA.

ture, then stored without refrigeration until evaluation. The unirradiated control samples were stored frozen at −29 °C; the irradiated bacon samples for estimation of nitrosamines were also stored frozen until analysed. Table 3 gives the analytical composition of the irradiated and unirradiated bacon samples. Characteristic of all the samples is a very low content of residual nitrite ($NaNO_2$ 0.7 to 2.7 mg/kg) and the relatively high nitrate ($NaNO_3$) content, that indicates that the nitrate was mainly present in the lean portion of the bacon. The organoleptic evaluation of the fried bacon for colour, flavour, texture, appearance and hedonic preference showed high ratings, also for the Bacon Lot 3 cured with $NaNO_2$ at only 25 mg/kg without nitrate. Table 4 gives the hedonic preference ratings for the bacon samples as obtained by a 36-member consumer panel. The only inferior ratings for Bacon Lot 3 were obtained for the colour of raw bacon, before frying (which was less pink and faded rapidly when exposed to light) as compared with the colour of the raw bacon of the other five lots.

Nitrosopyrrolidine (ONPyr) was not detected in the fried irradiated and unirradiated bacon (after the partly fried bacon was additionally fried to a crispy state) both in the edible parts of fried-crisp bacon and in the fat drippings in any sample (Table 5). Partly fried (pre-fried) bacon free from ONPyr can be produced from fresh chilled bacon bellies, the cures specified in this paper and the regular smokehouse treatment used for processing of the United States kind of bacon. The partly fried bacon to be preserved by irradiation can be produced with greatly reduced nitrite addition. According to the results presented here, the addition of 25 mg/kg of $NaNO_2$ supplemented with 25 mg/kg $NaNO_3$ should be sufficient to produce partly fried irradiated bacon. The addition of $NaNO_3$ at 25 mg/kg should improve the colour of raw bacon and could serve as additional source of nitrite in the irradiated product. Additional series of bacon with reduced nitrite (and including the addition of $NaNO_2$ and $NaNO_3$, each to 25 mg/kg) will be produced to confirm the findings and conclusions presented in this paper. Irradiation of partly fried bacon to get a shelf-stable product has an advantage of providing a highly acceptable product with reduced salt (NaCl) content and eliminating the dependence on the strict control of the product's water activity, which are major prerequisites for the partly fried (pre-fried) unirradiated bacon to be stored without refrigeration.

Conclusions

1. The main part of the total allowable added nitrite (156 to 200 mg/kg $NaNO_2$) to cured unirradiated meats is used for the control of *C. botulinum* with only a small fraction (about 25 mg/kg) needed for the development of the characteristic colour, flavour and taste of the products.
2. In irradiation-sterilized (radappertized) cured meats, the addition of nitrite can be reduced to the level needed only for the development of the colour, flavour and taste of the products.
3. However, small amounts of nitrate ($NaNO_3$) have to be used along with the reduced additions of nitrite to prevent fading of the cured-meat colour and possibly to supplement the nitrite and to scavenge electrons produced in meats by irradiation.
4. In radappertized ham and corned beef, the total added nitrite and nitrate

should be 75 mg/kg, from which a third to a half should be nitrite, to produce products of good quality as they are known to the consumers. Cooked sausages should be in the same category, subject to experimental proof.

5. In radappertized partly fried (pre-fried) bacon the total addition of 1:1 mixture of nitrite and nitrate in the amount of 50 mg/kg should be sufficient to produce a product of good quality. This addition of nitrite and nitrate to radappertized raw bacon should also be sufficient, subject to experimental confirmation.

6. No nitrosamines were found in fully cooked smoked ham preserved by radappertization. The absence of nitrosamines in other radappertized cured meats (corned beef, cooked sausages, etc.) has to be confirmed experimentally.

7. No nitrosopyrrolidine (ONPyr) was found in radappertized partly fried bacon after additional frying to a crispy state for table serving. However, in a preliminary study on radappertized unfried (raw) bacon, even with the smaller addition on nitrite, ONPyr was detected after frying for serving. More research has to be done to produce radappertized raw bacon free on ONPyr.

8. In the United States, irradiation has been legally defined as a food additive according to the 1958 Food Additive Amendment to the Federal Food, Drug and Cosmetic Act. Consequently, if irradiated foods are to be permitted for unrestricted human consumption, the wholesomeness of irradiated foods must be established and proper clearances obtained from the Food and Drug Administration. This phase of the food irradiation program in the United States has first priority.

9. Once the irradiation processing of foods, including cured meats, is approved by the health authorities, irradiation indeed will be a unique way of reducing the amount of nitrite added to cured meat products.

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Formation of nitrosamines in fermented meat products

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Summary

Nitrosamines may be formed in meat products cured with nitrite or nitrate, especially if raw meat products are left to mature longer, or if other matured foods such as cheese have been added to them and if heated at high temperatures. In a short literature survey, the effects were illustrated of different handling and various additives on the nitrosamine formation.

The effect was investigated of various additives (sodium ascorbate, ascorbic acid, dextrose, glucono-δ-lactone and starter cultures) in raw sausages produced under normal factory conditions. To provoke reactions, diethylamine was added at 10 mg/kg to some of the batches. Gas chromatography coupled with mass spectrometry, with a limit of detection of 1 ng, indicated that the sausages without added diethylamine contained none of the nitrosamines dimethylnitrosamine, diethylnitrosamine, nitrosopiperidine, nitrosopyrrolidine, methylethylnitrosamine and nitrosomorpholine. In some samples to which diethylamine had been added, traces of diethylnitrosamine (at most 3.5 μg/kg) were found. The additives employed under the above conditions showed no effects.

Three ways of restricting formation of nitrosamines in foods or in the human stomach should be considered: ensuring that the diet is low in nitrosating substances; ensuring that foods are low in amines and that use of drugs containing amines is restricted; and avoiding processes that favour reactions in foods between nitrosating substances and nitrosatable amines.

Introduction

Under certain conditions, nitrosamines may be formed in meat products that have been manufactured with curing substances (nitrite, nitrate). This is especially so when raw meat products are left to mature for a longer time or when other matured foods such as cheese have been added to them and when such meat products are heated at high temperatures. That is to say, nitrosamines will only be formed if nitrosating substances as well as nitrosatable amines are present under certain reaction conditions.

Fried bacon is a meat product that often contains nitrosamines, because nitro-

satable amines are formed during the maturing process and because high frying temperatures offer favourable conditions for the formation of nitrosamines. Normally, nitrosopyrrolidine is not found in raw bacon (Fazio et al. 1973; Pate et al. 1971; Patterson et al. 1974; Sen et al. 1973a). The formation of nitrosopyrrolidine begins at 80–100 °C (Gray & Dugan 1974) and reaches its maximum at 185 °C (Pensabene et al. 1974).

Equally important is the time of heating. Under normal heating conditions, no dimethylnitrosamine could be found in frankfurters to which sodium nitrite had been added at 750 mg/kg. However dimethylnitrosamine at 10–11 µg/kg could be traced in samples with an addition of such unusually high $NaNO_2$ contents as 1 500 mg/kg or of the same amount of sodium nitrite combined with $NaNO_3$ at 1 700 or even 17 000 mg/kg. A lengthening of the smoking and heating process from 2 to 4 h under the same conditions doubled the amount of dimethylnitrosamine. (Fiddler et al. 1972).

For bacon, the cooked-out fat always contains more nitrosopyrrolidine than the solid residue remaining after frying (Fazio et al. 1973; Fiddler et al. 1974; Pensabene et al. 1974; Sen et al. 1973). Moreover, by removing the adipose tissue before frying, the formation of nitrosamines in the lean part of the bacon was inhibited or at least diminished (Fiddler et al. 1974). Some investigators suppose that under these conditions fat-soluble nitrosamines volatilize, whereas in fatty products the cooked-out fat holds on to the nitrosamines (Fazio et al. 1973). Others believe that some components in the adipose tissue serve as precursors for nitrosopyrrolidine (Fiddler et al. 1974). Such a theory is supported by experiments in the course of which lean and fat parts of bacon were fried separately (Mottram et al. 1976): Nitrosopyrrolidine was almost exclusively found in the fat samples, and 70–80% of the total amount of nitrosopyrrolidine formed during frying was to be found in the cooking vapours. However about 94% of the nitrosopyrrolidine found in the cooking vapours originated from the fat. For the further investigation on this problem, we suggest that a meat product (e.g. frankfurters) should be produced with different contents of fat. There should be no chance for the fat to cook out and then conditions would be favourable for the formation of nitrosamines.

Within the limit of about 150 mg/kg, which is possible in the use of 'Nitritpökelsalz' (i.e. sodium chloride with 0.5–0.6% (by mass) sodium nitrite), the amount of the added nitrite seems to be less important for the formation of nitrosamines than other factors (Hustad et al. 1973).

Different additives commonly used in the manufacture of meat products showed significant effects on the formation of nitrosamines.

In ready-mixed spices with nitrite, large amounts of nitrosamines were sometimes found, mostly in mixtures containing black pepper or paprika (Sen et al. 1974b). Black pepper seems to favour the formation of nitrosopiperidine; paprika especially the formation of nitrosopyrrolidine (Sen et al. 1973b). In mixtures of spices and nitrate, no nitrosamines have been observed so far. It has yet to be discovered whether the nitrosatable components of the spices are predominantly of a primary nature or connected with certain preparation procedures (e.g. fermentation).

Under certain conditions, reducing agents (e.g. ascorbic acid, sodium ascorbate, sodium erythorbate, ascorbyl palmitate, cysteine, glutathione, and the antioxidants

propyl gallate, hydroquinone and α-tocopherol) show a significantly decreased formation of nitrosamines in cured meat products (Ender & Čeh 1971; Fiddler et al. 1972; Fiddler et al. 1973a; Fiddler et al. 1973b; Gray et al. 1975b; Greenberg 1974; Kotter et al. 1975; Mirvish et al. 1972; Sen et al. 1975). For an effective limitation of nitrosamine formation, there needs to be a certain ratio of reducing agents to nitrite. Low ratios of the above even seem to favour the formation of nitrosamine (Kawabata 1974, Kawabata et al. 1974b). The mass ratio of ascorbic acid to nitrite at least be 2:1 (Ender & Čeh. 1971, Mirvish et al. 1972).

Similarly to ascorbic acid, glucono-δ-lactone (GδL) favours the reduction of nitrite and its addition therefore is recommended as a safety factor (Leistner et al. 1974). On the other hand, meat-free model systems to which dimethylamine had been added showed an increase in dimethylnitrosamine formation in the presence of GδL (Fiddler, et al. 1973a). The investigators suppose an effect caused by the pH. Nevertheless at the same pH, sodium ascorbate and sodium erythorbate inhibit the nitrosating of dimethylamine. Canned meat products with an unusually large addition of nitrite to which GδL was added contained, likewise, higher levels of nitrosamines (van Logten et al. 1972).

In meat-free media containing nitrate and amines, some micro-organisms 'synthesized' nitrosamines (Hawksworth et al. 1971). Nevertheless, in raw sausages, after addition of starter cultures, no nitrosamines were found, although through microbial activity an increased formation of amines can be expected (Palumbo et al. 1974). Obviously some starter cultures inhibit the growth of amine-forming bacteria.

The content of nitrosatable amines responsible for the formation of nitrosamines mainly depends on the maturity of the meat employed for the manufacture of meat products. The content of amines in warm-blooded muscle is regarded as low. During storage and further processing, especially curing and maturing with bacterial fermentation, there is an increase in the content of amines. Only the content of methylamine seems to decrease during storage and maturation. The contents of dimethylamine, trimethylamine, and propylamine increased 2–3 fold. Ethylamine, diethylamine, and *iso*propylamine seem, during maturation, to stay constantly low. (Patterson & Mottram 1974).

Some primary amines found in meat, such as histamine, putrescine, tyramine, tryptamine, colamine, agmatine, and cadaverine, are important precursors for secondary amines (Niewiarowicz 1963). Some of them increased 10-fold during the maturation of raw sausages (Dierick et al. 1974). Putrescine and cadaverine are especially important, because of their possible conversion to pyrrolidine and piperidine during heating (Lijinsky & Epstein 1970).

It would be expected that blood, through its content of amines, induces the formation of nitrosamines (Kawabata 1974; Kawabata et al. 1974; Tozawa & Sato 1974). The observation that haemoglobin has the same effect may depend on the fact that no pure haemoglobin was used, all the more so, as other investigators have observed the opposite (Frouin et al. 1974). The latter is more understandable because both haemoglobin and myoglobin compete with other nitrosatable substances for the available NO.

Initially we studied the effect of ascorbic acid, GδL, and starter cultures in quickly matured raw sausages on the nitrosamine formation (Kotter et al. 1976).

To provoke reactions, we added diethylamine at 10 mg/kg to some of the batches.

The raw sausages were produced under normal factory conditions. The material used for the production (30% prok, 30% beef and 40% pork back fat) was frozen for 36 h at −18 °C, 4 days after slaughter. White pepper 3 g/kg and a pickling salt 30 g/kg containing sodium chloride and sodium nitrite 0.5–0.6% were added to all batches of the raw material. Dextrose, sodium ascorbate or ascorbic acid, and GδL and starter cultures were added in different combinations to the different batches. The sausages were matured for 7 days in a climatic chamber with a temperature decreasing from 24 to 18 °C and a relative humidity from 95 to 75%. Between the 4th and the 6th day, they were slightly smoked. They were ripened and stored at a temperature of 14 °C and a humidity of 75%. The conditions of maturing were kept constant despite the different additives.

The sausages were examined for dimethylnitrosamine, diethylnitrosamine, nitrosopiperidine, and nitrosopyrrolidine, on the 12th or 13th day and particularly on the 29th day. Most samples were also examined for nitrate, nitrite and for their percentage of reddening. For the investigation, 250 g of the sausages were used and were checked by Walters (Kotter et al. 1976) for the presence of nitrosamines by means of gas chromatography coupled with high-resolution mass spectrometry. The limit of detection of the researched compounds was about 1 ng. The recovery of nitrosomethylethylamine added was generally in the range of 50–70%.

In all batches without added diethylamine, none of the nitrosamines were found. The additives employed under the above conditions showed no effects. In some of the batches to which diethylamine was added at 10 mg/kg, traces of diethylnitrosamine (at most 3.5 μg/kg) were found. A relation between the amounts of diethylnitrosamine found and the additives employed was not observed. We could not confirm the assumption that the formation of diethylnitrosamine was greater at low pH values, as had been found with solutions.

On the 25th day, one of our series with an addition of diethylamine was also examined by Hauser and Heiz (Kotter et al. 1976) who used a modified procedure for the detection of the four nitrosamines and, additionally, for methylethylnitrosamine and nitrosomorpholine. With the same limit of detection, none of the nitrosamines were found.

When the usual additives and our common nitrite pickling salt ($NaNO_2$ about 150 mg/kg) were used in the production of fermented sausages, and when neither nitrate nor any undefined curing brine (which might contain high concentrations of nitrosatable amines) were added, there was no evidence of the formation of nitrosamines.

In line with our present knowledge, 3 ways of restricting the possible formation of nitrosamine in foods or in the human stomach should be considered.

1. *Ensuring that the diet is low in nitrosating substances.* This is difficult because a lot of food, especially that of plant origin, contains in natural state large amounts of nitrosating substances, either a priori or because of the special effects of pollution. One must try to diminish the 'natural' contents of nitrosating substances by changing the effects of pollution in the production of those foods. Moreover, one has to prove whether certain foods have to be subjected to a special microbiological fermentation in the presence of ascorbic acid for the decomposition of nitrate and

nitrite. The amounts of nitrosating substances in foodstuffs originating from additives indicated because of hygiene or manufacture are small, compared with the amounts of nitrosating substances which are originally in food or especially in saliva without any relation to the diet. Nevertheless, especially in those countries in which the use of a large amount of nitrite is still allowed in meat products, it must be reconsidered whether the amount of nitrite tolerated up to now is necessary. The possibility to differentiate between added and residual amounts of nitrite to be tolerated with reference to the microbiological necessity and the toxicological acceptability must also be considered.

2. *Ensuring that foods are low in amines and that the use of drugs containing amines is more controlled.* Our information about the amine contents in foods is limited. Probably the kind of feed will have special importance because nitrosamines originating from feed are known to be occasionally present in milk (Juszkiewicz et al. 1974; Möhler et al. 1974). Here the maturation of foods, mainly the bacterial fermentation, needs special attention. Possibly some maturation processes have to be limited. Drugs containing amines, e.g. some out of the groups of antihistamines (Burimamid), analgaesics (*para*-aminophenol derivatives), anticonvulsants (Phenacetin, generally the acetylurea derivatives), should not be taken in together with foodstuffs containing nitrosating substances; at least one should await resorption of the amine-containing drugs.

3. *Excluding methods of handling that favour reactions between nitrosating substances and nitrosatable amines in foods.* The above is especially relevant to foods and food combinations in which larger amounts of nitrosating substances *and* nitrosatable amines are to be expected. This is of particular interest because the total amount of nitrosating substances that are eaten with the food or that are taken in by other ways can hardly be reduced, even after reducing the allowed additives. The same applies to amines. By decreasing the addition of nitrosating substances in food, it is therefore impossible to prevent the danger of nitrosamine formation in the stomach decisively. Our chief concern must therefore be to restrict the favouring of nitrosamine formation in food through other precautions (e.g. exclusion of the high heating of certain foods such as fermented nitrite-containing meat products and nitrite-containing cheese).

So far, of the ways of preventing nitrosamine formation, practically only the reduction of nitrite additions and a total prohibition of nitrate have been discussed. This applies also to countries in which the addition of nitrite has already been rather limited.

Investigations have shown that a certain reduction in nitrite content of our nitrite pickling salt, which still contains 0.5–0.6% (by mass) nitrite, leads to satisfactory results in reddening, stability in colour, and curing aroma, if supporting measures are also taken (Wirth 1973). However, problems will still remain for shelf-life. In certain products, a reduction in the amount of nitrite added involves even an increased hygiene risk. It cannot be disputed that certain amounts of added nitrite must be used in order to have a sufficient bactericidal effect, because of permanent danger of food poisoning. The more so, as in countries where nitrite pickling salt is used, food poisoning possibly increases as a result of the tendency to salt less. To fix a lower input of nitrite and a lower level of residual nitrite for

low-risk products, also as an indication of low added levels, it is not necessary to provide pickling salt with less nitrite in addition to the one in use so far which which guarantees the nitrite content necessary for products with a higher hygiene risk. It is possible, for example, to limit the addition of pickling salt to 2% (by mass) for all meat products. This ensures that low-salted meat products contain enough nitrite. For meat products that are normally salted to a higher extent, you then have to use pure sodium chloride besides 2% pickling salt.

An obligatory use of reducing agents in products to which nitrite is added would surely also help to remove the danger of nitrosamine formation.

It seems most important to limit high-heating of fermented nitrite-containing products. This does not mean that quickly cured products, in which lower contents of amines are to be expected, are absolutely harmless when heated at high temperatures, for instance when being fried. Undoubtedly frying of nitrite-containing meat products together with cheese is also dangerous.

In a further research programme, closer studies have been started on the problems in heating raw fermented meat products. As in our first investigations, different additives will be used. Furthermore, the fresh, quickly cured and more heavily fermented samples will be subject to different heating procedures.

Discussion on the session

Amines and nitrosamines in uncured pork

Methylamine (MA), dimethylamine (DMA), trimethylamine (TMA), ethylamine (EA), diethylamine (DEA), *n*-propylamine (n-PA) and *iso*propylamine (*iso*-PA) were detected in eye-muscle of pork carcasses. During bacon manufacture, DMA, TMA, n-PA and iso-PA increased consistently. In subsequent storage, only DMA and TNA increased (Patterson & Mottram 1974).

Amine formation (MA and DMA + TMA) in *un*cured pork appeared independent of bacterial growth during storage at 20 and 5 °C (Patterson & Edwards 1975).

In an uncured meat product, Groenen (Contrib. 3.4.4) found from 0.1 to about 1 µg per kg of DMNA after frying. Perhaps low 'natural' nitrite and nitrate levels contribute to the formation of this compound. Methods for an accurate determination of nitrite and nitrate at levels of 1 mg per kg and lower are lacking at the moment.

Residual concentration of nitrite

There is uncertainty about the relative importance of input and residual concentrations of nitrite. American investigators emphasized that residual nitrite changes in amount, with age and condition of the product and does not reflect the antimicrobial character of the nitrite in the product. European workers have emphasized residual levels of nitrite.

It has become clear that even in unheated meat systems an important part of the antimicrobial effect in cured meat systems may arise from compounds derived from nitrite. Thus it may be the *difference* between the initial and residual concentrations – i.e. the nitrite which disappears – that is specially significant, even though much of it may be in ineffective forms.

Obviously, the amount of nitrite which reacts with the meat system and there-

fore disappears, is primarily determined by the amount of input nitrite and therefore gives a general measure of the effects.

Influence of glucono-δ-lactone

Dr Kotter remarked on the advantage of glucono-δ-lactone (GδL) to induce rapid disappearance of nitrite and to give low residual concentrations, as if these were obviously desirable. However it seems that this would be likely to increase, in corresponding degree, nitrosation of the food; and it is questionable whether this is an advantage. To decide, we would require information on the relative importance of nitrosation in the food and in the stomach.

Comment of P. L. Schuller

Dr Kotter mentioned that in canned meat products, prepared with an unusually large addition of nitrite and to which also GδL was added, contained, likewise, higher levels of nitrosamines (van Logten et al. 1972). I would like to draw the audience's attention that Professor Kotter draws this conclusion. The experiment was a toxicological study designed to study the possible formation of nitrosamines, using rate as a detector. At that time at the Dutch National Institute of Public Health had no reliable method for the determination of volatile NA at low levels. The Government Chemist (GB) kindly did some chemical analyses. In my opinion, it is not justifiable to draw any conclusion about the influence of GδL on the formation of nitrosamines on the basis of these few figures. The only conclusion drawn by the authors was: that there was no evidence of any pre-neoplastic change or tumour formation that could be attributed to feeding on the canned meat.

General discussion on the Technological Session

Residual nitrite

Microbiologists are not yet ready to accept the suggestion that safety is compatible with zero residual nitrite. There are indications (Ashworth & Spencer 1972; Ashworth et al. 1973) that *residual* nitrite is related to control of Clostridia during long periods of storage. The United State Department of Agriculture (USDA) routinely analyses production samples of bacon for residual nitrite at the time of manufacture. At a recent meeting of the USDA Expert Panel on Nitrites, Dr H. Mussman presented data which showed that about 80% of bacon samples analysed contained residual nitrite at less than 25 mg/kg. Nitrite intake from cured meats means relative high concentrations for shorter periods and nitrite intake from saliva means lower concentrations for longer periods. It is questionable which is the most important combination.

Stability of ascorbic acid during heating

The work of Davidek and coworkers showed that the content of ascorbic acid had dropped immediately after processing. Later on, during storage, ascorbic acid was formed back by reduction of dehydroascorbic acid by the SH groups that became exposed during heating.

Conclusions and recommendations of the technological session, Tuesday 7 September

1. Evidence is available that nitrate is required for curing certain products. Nitrate levels should be as low as possible since the nitrate may act as a reservoir of nitrosating ability.

2. More research is needed on peculiarities of conditions for individual products.

3. A consumer-type panel failed to show preference for bacon cured with nitrite over the same product without nitrite. However both were smoked.

4. Irradiation may substitute for the preserving action of nitrite but, if so, some nitrate must be added to the product.

5. It is desirable to ensure that levels of nitrosating agents and amines are low in food products. Such is difficult to achieve, but experience in and knowledge of processing and handling of these products is crucial to minimize the chance of reaction between nitrite and amines.

6. It was generally agreed that the levels of residual nitrite should be as low as possible, but there was reluctance to support a blanket statement that residual nitrite was completely unsignificant from a microbiological point of view.

7. Some opinion was expressed that the intake of nitrite from food should not exceed those from one's own saliva. This presents a problem, since the nitrite intake from food occurs as a dose superposed on the steady intake from saliva.

8. Concern is expressed about the significance of the analytical figures for residual nitrite.

Chemical session – Reactions with nitrite

Reporters: P. S. van Roon, P. C. Moerman

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Fate of nitrite in meat[1]

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Abstract

The objective was to establish the fate of nitrite in cured meats. Nitrite labelled with ^{15}N was added to model systems and to meat systems to measure the amount of label distributed to various sites or fractions of the product. As a generalization from tests over 5 years, the following proportions of nitrite originally added were recovered: in myoglobin 5–15, nitrate 1–10, nitrite 5–20, gas 1–5, sulfhydryl 5–15, lipid 1–5 and protein 20–30%, representing a total recovery of 38–100% (usually 70–80%).

Introduction

Three years ago, we (Sebranek et al. 1974) reported our results on fate of nitrite to the First International Symposium on Nitrite in Meat Products. We elected to study the fate of nitrite when concern about nitrosamines and residual nitrite surfaced, because it was obvious that nitrite did not merely disappear from a cured meat but was converted to other forms, which may be lost or remain in the product. The rationale for such work is that the form of the reacted nitrite is unknown and must be elucidated before concern over the use of nitrite as a meat-curing agent can be erased. Even though meat is an extremely complex system, and the methods for use of ^{15}N pose some problems, our results (Cassens et al. 1974b; Sebranek *et al.* 1974) and those from other laboratories (Olsman 1974; Fujimaki et al. 1975; Woolford et al. 1976b) clearly indicate that much of the lost nitrite remains in the product in a form other than nitrite. The results described here summarize our continuing efforts to identify the fate of reacted nitrite.

Influence of characteristics of the meat

The properties of a given muscle reflect the proportion of red and white fibre types present (Cooper *et al.* 1969). We have considered the influence of muscle

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2. On leave from Station de Recherches sur la Viande, INRA, France.

type on residual nitrite and the results are shown in Table 1 (Lee *et al.* 1976). The pH of white muscle post mortem is generally lower than that of red muscle and the lower pH results in a lower residual nitrite if the muscle is cured. Olsman & Krol (1972) used a meat system and found nitrite was depleted more rapidly if the pH was lower. The lower pH probably alters rate of reaction of nitrite with some component of meat or makes a site more available for reaction. If the pH difference is removed by selecting muscles with different colour but similar pH, or by chemically altering the pH, then the redder muscle has a lower residual nitrite. This effect is probably due to the higher content of myoglobin in red muscle and the reaction of nitrite with it. Though the results were not unexpected, they illustrate two considerations. First, the selection of raw material for experiment and the sampling procedure should take consideration of the characteristics of the muscle. Second, the processor could influence residual nitrite by selecting the type of muscle used.

Reaction of nitrite with myoglobin

Tarladgis (1962) suggested that the pigment of heated cured muscle is a dinitrosyl-haemochrome. This has led to the hypothesis that the globin portion of the myoglobin molecule is detached by heating and that both of the free coordination positions of iron are occupied by nitric oxide in the presence of nitrite. We (Lee & Cassens 1976) studied the amount of ^{15}N, from labelled nitrite, complexed to both unheated and heated pigment in a model system and found that the heated samples contained about twice as much ^{15}N as unheated samples. Our results support the conclusion of Tarladgis (1962), and indicate that the amount of nitrite reacted to form nitrosylmyoglobin may be up to twice the amount previously thought.

Table 1. Residual nitrite (expressed as $NaNO_2$ by mass), in white and red portions of semitendinosus muscle. Values are means of 3 preparations. Samples were prepared with mass fractions of added water 30 g/kg and nitrite 156 mg/kg. After mixing, samples were stored at 3 °C for 15 h, cooked at 65 °C for 2 h and analysed after 3 days storage. Samples 3 and 4 contained 0.4% disodium phosphate and 0.47% sodium ascorbate by mass. Permission Institute of Food Technologists.

Sample	White		Red	
	pH	nitrite/mg · kg^{-1}	pH	nitrite/mg · kg^{-1}
1	5.8	45	6.1	71
2	5.35	56	5.75	93
3	6.1	81	6.1	54
3	6.4	68	6.4	47
4	6.4	83	6.4	73

Reaction of nitrite with protein

The recent conclusion of Woolford et al. (1976a) is that reaction of nitrite with non-haem proteins represents a major pathway for loss of nitrite. Previous interest in proteins, in regard to meat curing, has centred on reaction of nitrite with sulfhydryl groups but the results from various laboratories have not been consistent or conclusive. Mirna & Hofmann (1969) used ground meat and showed a decrease in both nitrite and sulfhydryl groups by about 30% during two weeks of storage. Woolford (1974) estimated that between 8 and 25% of added nitrite reacted to form nitrosothiols, in an anaerobic system. Kubberød et al. (1974) concluded that formation of nitrosothiols was not important in loss of nitrite, confirming earlier data of Olsman & Krol (1972). Evidence is available from a number of sources that a protein extract, low-molecular peptides or amino acids function in depletion of nitrite (Ando et al. 1971; Fox & Nicholas 1974; Tinbergen 1974; Sebranek et al. 1973) Woolford *et al.* (1976b) studied the problem by incubating protein with nitrite labelled with ^{15}N. Results from a typical test are shown in Figure 1. As nitrite declined, the amount of nitrite nitrogen chemically bound to bovine serum albumin increased. Recovery of labelled nitrogen was poor, presumably through inadequacy of the Kjeldahl conversion. The highest recoveries of ^{15}N were 80–90%.

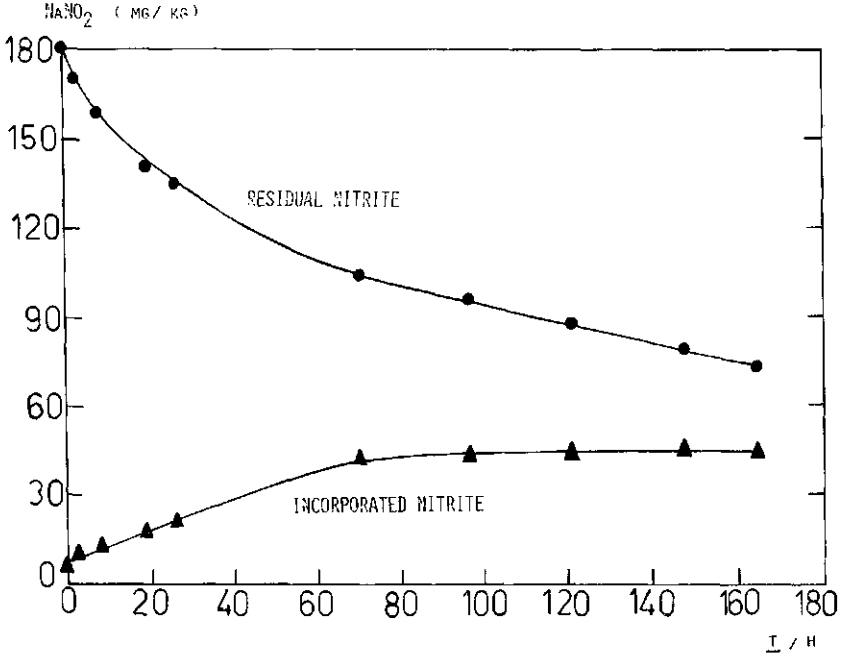

Fig. 1. Mass fractions of nitrite remaining and incorporated on incubation of bovine serum albumin 0.1 kg litre^{-1} and sodium nitrite (0.2 g litre^{-1}) at pH 5.5 and 20 °C in buffer of phosphate (0.1 mol litre^{-1}) and citrate. Dialysed for 36 h to isolate protein. Nitrogen content measured with CuSe and H_2SO_4 only. Permission Institute of Food Technologists.

Nitrite-treated myosin was analysed for amino acids (Fig. 2). The dotted peaks represent compounds not found in the amino acid analysis of unnitrosated myosin.

On the basis of retention time on the ion-exchange column and the chemistry of the possible nitrite reactions, G was identified as 3,4-dihydroxyphenylalanine and H as 3-nitrotyrosine. Commercial samples of the two compounds had identical retention times, when chromatographed singly or added as a spike to hydrolysed protein samples. Peak F, appearing next to alanine, had the same retention time on the ion-exchange column as the reaction product of nitrite with free tryptophan.

More ^{15}N enrichment of the myosin occurred than could be accounted for by the above compounds. The amide linkage of proteins could be nitrosated. Poly-L-lysine, polyalanine and poly-L-glutamic acid are poly-(amino acids) in which nitrosation could occur only at the amide linkage. After incubation of these compounds with ^{15}N labelled nitrite at a mass concentration of 200 mg/litre and at pH 5.0, all were found to contain ^{15}N. The likely products are nitrosamides. Further evidence was obtained by the Eisenbrandt & Preussmann (1970) denitrosation procedure, which liberated nitrite from the nitrosation product of polyalanine.

Reaction of nitrite with fat

Adipose tissue occurs in meat in diverse amounts and is composed not only of lipid but also of connective tissue and other proteins and water. Although it is considered a rather inert tissue, Frouin et al. (1975) offered proof that nitric oxide reacts with unsaturated fatty acids.

Goutefongea et al. (1976) cured backfat and bellyfat with nitrite (156 mg/litre, labelled with ^{15}N) to establish the importance of fat in the fate of nitrite. We found that about 90% of the added nitrite was recovered as free nitrite from the entire tissue. Of the label, 6–9% was recovered in connective tissue extracted from

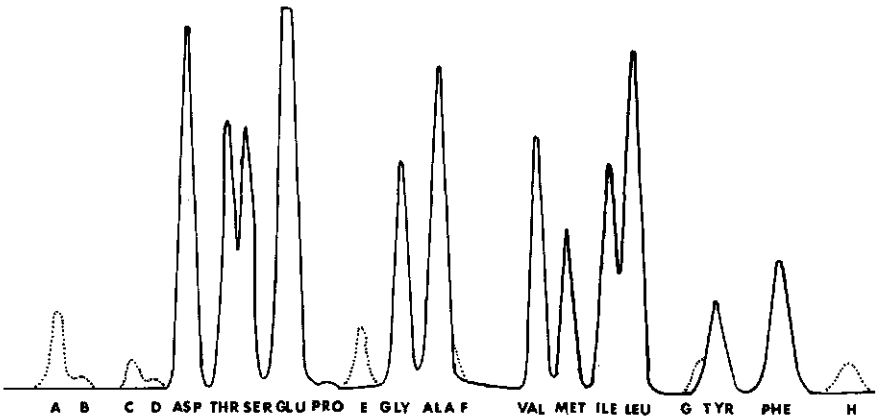

Fig. 2. Amino acid composition of nitrosated myosin. Dotted peaks (magnified) appear on nitrosation. Initial concentration of nitrite 10 g litre^{-1} and myosin (65 g litre^{-1}) in buffer of phosphate 0.1 mol litre^{-1} and citric acid, pH 3.0; KCl 0.1 mol litre^{-1}. Protein precipitated with acetone after 24 h. Permission Institute of Food Technologists.

cured fat, most of which was analysed as free nitrite. If connective tissue was extracted from belly fat before curing and incubated with nitrite (1 g/litre) labelled with ^{15}N, 5–7% of the label was bound (belly fat contains about 3.5% connective tissue by mass). The lipid fraction extracted from cured fat contained 2–5% of the initially added nitrite, at least half of which was bound. When fatty acids and glycerides were treated with nitrite labelled with ^{15}N (1 g/litre) the amount of label incorporated was considerably higher in more unsaturated compounds.

Nitrite in bacon

Using pork bellies cured with sodium nitrite labelled with ^{15}N, with and without sodium ascorbate, we attempted to trace the reaction pathways of nitrite in bacon (Woolford & Cassens 1976c). The depletion of nitrite, initial mass fraction 156 mg/kg was followed during the processing operation. Subsequent tests during the storage of the ^{15}N-labelled sliced vacuum-packed bacon showed further depletion of nitrite in both the lean and adipose tissue portions, the lower mass fractions of nitrite being found in the bacon to which ascorbate had been added. The formation and depletion of nitrate was also noted. Analysis of the protein and lipid portions showed incorporation of ^{15}N into both. Mass spectrometry showed that between 73 and 87% of the added ^{15}N remained in the bacon lean. The adipose portion contained much less ^{15}N, 20–25% of that added. A hot-water extract of both lean and adipose fractions showed the presence of ^{15}N-containing compounds greater than the ^{15}N due to nitrite and nitrate. One effect of the addition of ascorbate was to force ^{15}N into a water-soluble form. Examination of connective tissue isolated from the adipose tissue portion showed incorporation of the ^{15}N, equivalent to a mass fraction of $NaNO_2$ of 6 mg/kg in bacon without ascorbate against 2.5 mg/kg for bacon with added ascorbate.

Data on ^{15}N also showed incorporation of about 25% of the added nitrite into the muscle proteins of both bacons, and incorporation of 10% of the added nitrite into the lipid fraction of the adipose tissue.

Conclusions

A substantial proportion of the nitrite reacts with the non-haem proteins. Estimates for the amount of nitrite bound to sulfhydryl groups span a wide range, but we favour a low value. Certain amino acids are nitrosated and nitrite also reacts with amide linkages. Further study is required not only because of the amount of nitrite reacted with non-haem proteins but also because of the nature of the products formed.

A moderate amount of nitrite reacts with the muscle pigment myoglobin, particularly in red, high-myoglobin muscles.

Ascorbate promotes depletion of nitrite in bacon. When ^{15}N-labelled nitrite is used, the number or amount of water-soluble ^{15}N-containing compounds increases. There is also an increased loss of ^{15}N from the system in the presence of ascorbate.

About 10% of nitrite, a mass fraction (expressed as $NaNO_2$) of 15 mg/kg, seems to have reacted after addition to adipose tissue under conditions relevant to meat

curing. Reaction occurs with connective tissue and unsaturated fatty acids.

The biochemical properties of muscle vary greatly and affect the fate of nitrite.

Discussion on the session

Residual nitrite

Variations are due to muscle properties, e.g. in relation to red and white fibre content. Genetics could be a means to reduce this source of variability. Addition of ascorbate reduces the level of residual nitrite. In particular, the hot-water extract contained more non-nitrite ^{15}N, whereas the bound ^{15}N differed little from the value obtained without added ascorbate.

Unsaturated fatty acids

The reaction products of $Na^{15}NO_2$ and fatty acids or glycerides of various degrees of unsaturation are found, generally, to bind more ^{15}N in compounds having more unsaturation. Reaction kinetics of nitrite depletion is not influenced by highly unsaturated fatty acids. It seems possible that nitrosated compounds, formed in the water phase migrate into the lipid phase.

Bacon

Adipose tissue contains, besides lipid, also connective tissue and water. The latter tissue might be the reactive component. It appears that nitrosopyrrolidine levels in rind are much lower than in the associated fat despite the fact that the highest levels of collagen and residual nitrite are found in the rind. If lean, fat, and rind have been separated and fried separately to produce *N*-nitrosopyrrolidine, the results in Table 2 are obtained.

Differences in NPyr content of lean and fat can be attributed to the different temperatures in the tissues during frying. In the lean, a temperature of 110 °C could be observed, whilst that in the lipid was as high as 150–160 °C.

Table 2. Contents of *N*-nitrosopyrrolidine (NPyr, pg/kg) in separately fried lean, fat and rind, and residual nitrite (mg/kg) before frying. Data from Patterson et al. (1976) and Mottram et al. (1976). –, not detected.

	Number of estimates	Lean		Fat		Rind		Exuded fat
		NPyr	nitrite	NPyr	nitrite	NPyr	nitrite	NPyr
Components fried separately	4	–	76	13	42	1	407	7
Rasher fried whole	6	2	113	3	36	3	437	6

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Chemical behaviour of nitrite in meat products – 1. The stability of proteinbound nitrite during storage

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Abstract

In model meat products of pH 5.35–5.8, more than half the free nitrite lost during the first days of storage was recovered as protein-bound nitrite. Bound nitrite increased to a maximum and then gradually declined during storage with kinetics similar to those for the depletion of free nitrite. However the activation energy for decomposition of bound nitrite was significantly higher than that for disappearance of free nitrite, and was practically independent of pH.

Introduction

The depletion of nitrite during heat processing and storage of meat products is a well-known phenomenon (Nordin 1969; Olsman & Krol 1972; Olsman 1974; 1975; Hill et al. 1973; Fox & Nicholas 1974). The reactive agent was established to be the undissociated nitrous acid rather than the nitrite ion (Olsman 1975). In the pH range prevailing in meat products only a small fraction of the nitrite is present as HNO_2, which may therefore be considered as a leak through which the reservoir of nitrite is emptied. At pH 6.0 or more, the nitrite loss followed first-order kinetics, whereas at lower values the order was between 1 and 2 (Olsman & Krol 1972). Furthermore the depletion rate constant was proportional to the content of lean meat in the meat product.

In tests on the reactivity of various amino acids towards nitrite, cysteine was by far the most effective in decreasing the nitrite content of the model system (Olsman 1975). Saville's studies (1958) suggested that nitrosocysteine was formed, and this was confirmed by Mirna & Hofmann (1969). According to Mirna (1970), the protein-bound cysteine in meat products can also be converted into nitrosothiol, for which he developed an assay method. This method was improved in our department (Olsman 1977c) and has been used to study the fate of nitrite in heat-processed meat products during storage. Besides nitrosothiol, the nitrite bound as NO in coordination complexes of iron with protein-bound thiol groups (like complex II of van Roon 1974) was also estimated by this procedure. The assay value may include still more unknown forms of bound nitrite, as suggested by results of experiments in which the thiol groups had been blocked by alkylation with vinylpyridine (Olsman 1974). Taking these findings into account, we categorized the bound nitrite estimated by our modification of Mirna's method as 'protein-bound nitrite'.

The aim of the present study was to examine the stability of bound nitrite in model meat products at various storage temperatures and different pH values. This information would extend our knowledge of the chemical behaviour of nitrite in meat products, and could have bearing on the microbiological stability of heat-processed meat products (Incze et al. 1974; van Roon & Olsman, *this Symposium*, p. 53).

Materials and methods

Preparation of model products

Three batches were prepared with different amounts of glucono-δ-lactone (GDL) to obtain products of different pH. For the rest, the composition of the three batches was the same (Table 1).

The emulsion was divided into three 1 000-g portions, to which 6, 9 and 12 g of GDL was added, respectively. By thorough comminution in a chopper bowl, this was distributed uniformly over the emulsion mass. After de-aerating in a vacuum mixer, it was put into lacquered 75 mm x 38 mm cans to a net weight of 120 ± 1 g. The cans were heated for 43 min in water of 75 or 90 °C (Table 2).

The low pH values in the final products were chosen deliberately to obtain high levels of bound nitrite (Olsman & Krol 1972) in combination with a relatively high level of added nitrite and a mild heat treatment. Although those conditions deviated from normal processing practice for most meat products, they enabled us to trace levels of protein-bound nitrite with satisfactory accuracy during prolonged storage. All cans were stored at 0 °C until free nitrite in the product had been reduced to a content of $NaNO_2$ of < 20 mg/kg. At that time, all three products

Table 1. Composition of model product, expressed as percentage by mass before addition of glucono-δ-lactone.

Ingredient	%
Beef, fresh and lean	72
Ice-water	19
Caseinate	2
Starch	4
Sodium chloride	2.5
Polyphosphates	0.5
Sodium nitrite	0.04

Table 2. Treatment of the meat products. GDL, glucono-δ-lactone.

Product	GDL added (g per kg model product)	Heating temperature (°C)	pH of final product
A	6	90	5.80
B	9	75	5.60
C	12	75	5.35

were divided in 4 groups with equal numbers of cans and were stored at 0, 10, 20 and 29 °C.

Analytical methods

The procedures for estimating free and bound nitrite will be published later (Olsman 1977b). The procedure for bound nitrite was a modification of Mirna's method (1970), in which the bound nitrite in the protein-rich residue after acetone extraction was released by treatment with mercuric chloride.

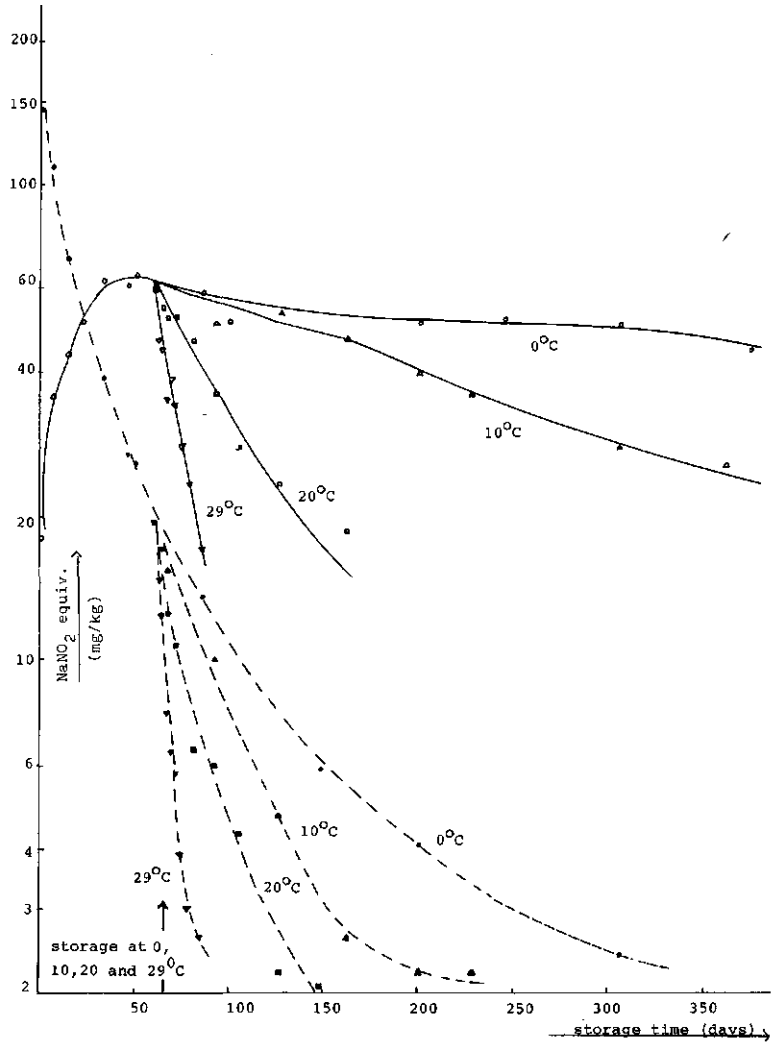

Fig. 1. Changes in levels of free (broken line) and bound nitrite (solid line) in Product A during storage.

Results and discussion

Figures 1, 2 and 3 show the changes in content of free and protein-bound nitrite during storage of the model products A, B and C, respectively. There was a conspicuous increase of the level of bound nitrite at the beginning of the storage period at 0 °C.

As shown in Table 3, 50% or more of the lost free nitrite was recovered as bound nitrite early in storage. This would suggest that initially the reaction

$$HNO_2 + RSH \xrightarrow[I]{k} RS{\cdot}NO + H_2O \qquad (1)$$

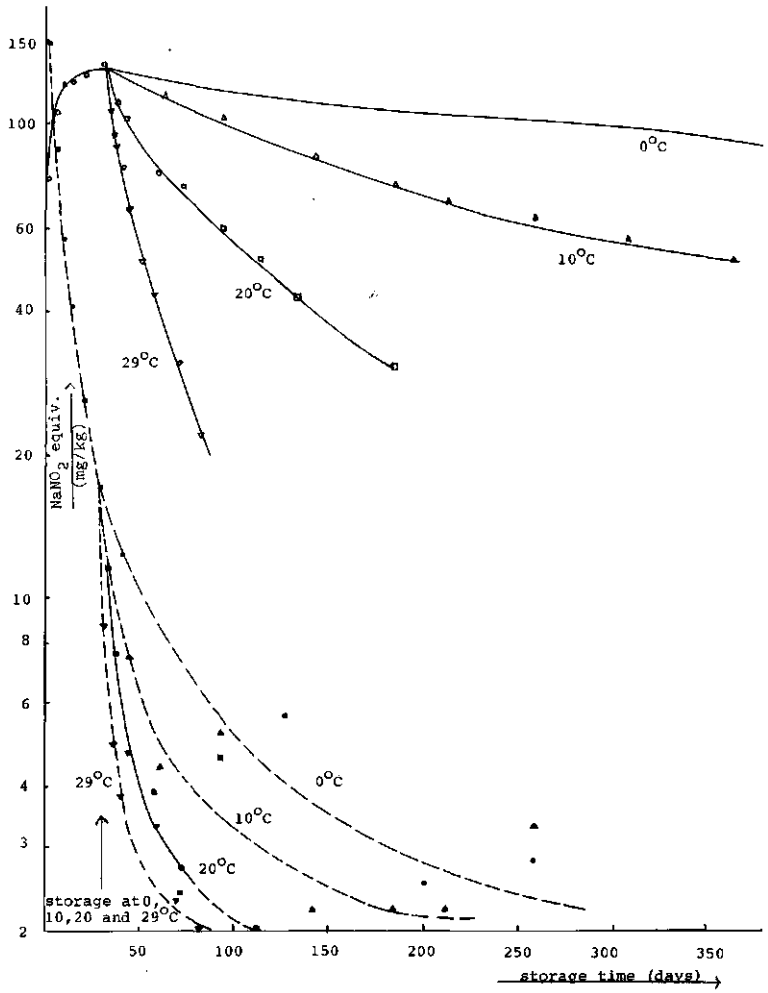

Fig. 2. Changes in levels of free (broken line) and bound (solid line) nitrite in Product B during storage.

is responsible for the disappearance of the greater part of free nitrite. RS·NO represents the protein(thiol)-bound nitrite. This reaction was proposed earlier by Mirna & Hofman (1969). After some time, the bound level reached a maximum and a temperature-dependent decline started. That decrease could be due to the reaction

$$\text{RS·NO} \xrightarrow{k_2} \text{RS} + \text{NO} \qquad (2)$$

(Fox & Ackerman 1968). If Reactions 1 and 2 were the only two to play a role in

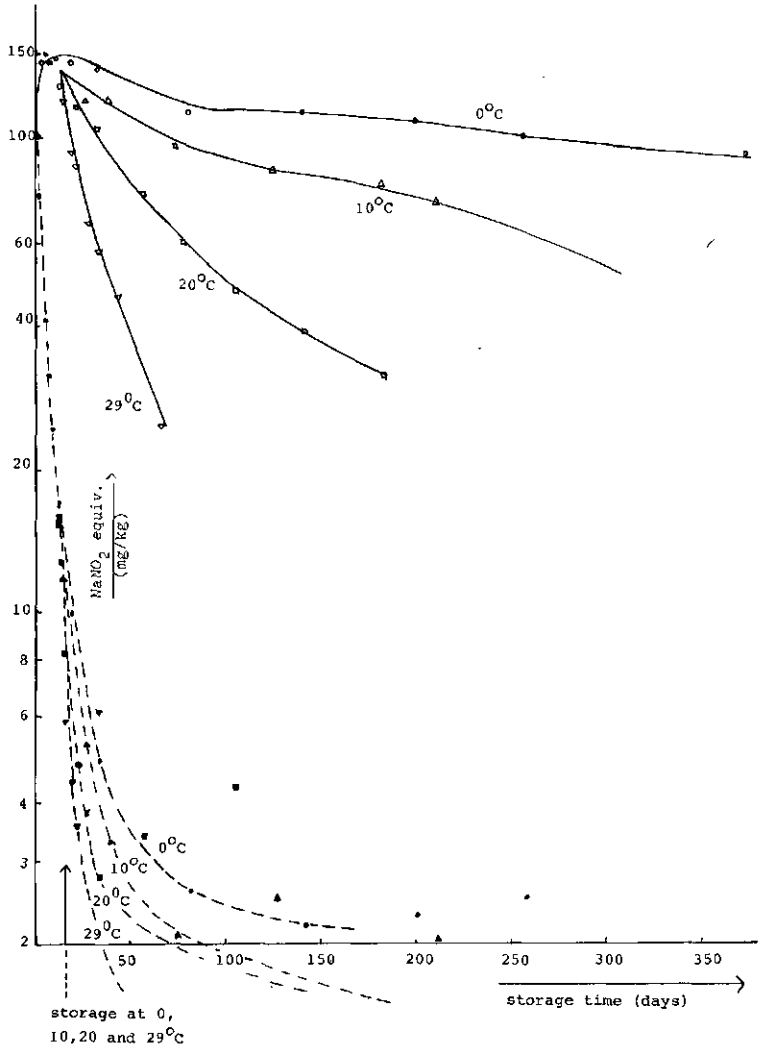

Fig. 3. Changes in levels of free (broken line) and bound nitrite (solid line) in Product C during storage.

Table 3. Changes in free and bound nitrite (expressed as $NaNO_2$, mg/kg) during initial storage, and percentage of lost free nitrite recovered as bound nitrite.

Product	Storage time (days)	$NaNO_2$ (mg/kg)		Change in $NaNO_2$ (mg/kg)		Recovery (%)
		free	bound	free	bound	
A	1	144.3	18.0			
	7	108.6	35.7	−35.7	17.7	49.6
B	1	147.5	74.8			
	5	88.6	106.4	−58.9	31.6	53.7
C	1	100.0	108.7			
	2	75.1	125.5	−24.9	16.8	67.5
	5	40.7	144.3	−34.4	18.8	54.7
	7	31.4	150.8	− 9.3	6.5	69.9

Table 4. Contents of bound and free nitrite and tangents to the second-order regression curves for the depletion of free nitrite at 0 °C at the maximum of bound nitrite, and rate constant of Reaction 2 (k_2 in day^{-1}) calculated from them.

	A	B	C
Max. level of bound nitrite ($NaNO_2$ equiv., mg/kg)	64	132	150
Free nitrite at max. of bound nitrite ($NaNO_2$ equiv., mg/kg)	28	19	24
2nd-order regression eqn. 10^3 lg [$NaNO_2$] =	2125 − 16.71 t + 0.0518 t^2	2198 − 51.7 t + 0.610 t^2	2021 − 7.93 t + 1.202 t^2
d(lg [HNO_2])/dt at max. of bound nitrite	− 0.01194	− 0.0200	− 0.0577
$k_2 \times 10^3$, in day^{-1}	0.0235	0.0216	0.1151

the depletion of free and the formation and decomposition of protein-bound nitrite, the rate of change of the content of bound nitrite would be

$$= k_1 [HNO_2] [RSH] - k_2 [RS{\cdot}NO] \quad (3)$$

and, at the maximum:

$$d(\lg [HNO_2])/dt = k_1 [RSH] = k_2 [RS{\cdot}NO]/[HNO_2] \quad (4)$$

Let me say again that in fact, the analytically estimated protein-bound nitrite covers other forms of bound nitrite than RS·NO alone.

From this equation, the rate constant k_2 at 0 °C can be calculated. Table 4 gives it with the maximum of bound nitrite, the corresponding level of free nitrite and the tangent to the depletion curve at this content of nitrite for the three products A, B and C. The parabolic model was taken for nitrite depletion (Olsman 1974).

Another k_2 value was calculated from the regression between the level of bound nitrite and storage time after the maximum. The decrease in bound nitrite may be mathematically described in the same way as the depletion of free nitrite: by a

linear equation $\lg [RS{\cdot}NO] = a + bt$ or, more precisely by the second-order equation $\lg [RS{\cdot}NO] = a + b_1 t + b_2 t^2$, which fits the data significantly better.

Tables 5 A and B give both equations for all three products at four storage temperatures. The k_2 value or, in 2nd-order equations, the values for $d(\lg [RS{\cdot}NO])/dt$ at storage temperature 0 °C, calculated for the maximum of bound nitrite, exceeded the values of Table 4 by a factor ranging from 5 to 22. Thus the model of consecutive Reactions 1 and 2 fails. From the depletion curves for RS·NO beyond the maximum, the decomposition rate of bound nitrite would be expected to decrease rather than increase with longer storage.

However, the following hypothetical reaction scheme seems to fit in with our results:

$$\begin{array}{l} \qquad\quad RS{\cdot}NO \\ (b)\ \downarrow\ \uparrow + RSH \end{array}$$

$$HNO_2 + X \xrightarrow{(a)} X{\cdot}NO \xrightarrow{(c)} \ldots \qquad (5)$$

X is a compound very easily nitrosated by Reaction 5a and then itself acting as a nitrosating agent. By nitrosation of thiol groups in the reversible nitrosation Reaction 5b, RS·NO is formed. In the first storage period and at the relatively low pH of our products, X-NO is formed more rapidly than meat constituents can be nitrosated by Reaction 5c. A reservoir of nitrosating power builds up. Initially more than half the nitrite is temporarily stored in this way (Table 3). When the level of free nitrite has been reduced to a certain extent, the reverse Reaction 5b becomes predominant and the RS·NO reservoir empties at a gradually increasing rate until finally most of the nitrite has been converted into a variety of stable nitrosated compounds by Reaction 5c. The concept of an intermediary nitrosating agent X·NO fits in with the observation of Ando (1974) that, out of several lean meat fractions, the low-molecular weight dialysable fraction was most active one both in nitrite

Table 5 A. Linear regression equations between the logarithm of the protein-bound nitrite level and storage time t, in days. k_2 = rate constant of RS.NO → RS + NO; r = correlation coefficient, s = standard deviation.

Product	Storage temp., °C	Regression eqn 10^3 1 g [RS.NO] =	r	$k_2 \times 10^3$ in days^{-1} ± s
A	0.0	= 1800 − 0.37 t	− 0.952	0.37 ± 0.07
	10.1	= 1864 − 1.29 t	− 0.988	1.29 ± 0.08
	20.0	= 2105 − 5.71 t	− 0.979	5.71 ± 0.42
	29.1	= 2996 − 20.54 t	− 0.985	20.54 ± 1.38
B	0.0	= 2123 − 0.47 t	− 0.986	0.47 ± 0.04
	10.1	= 2121 − 1.22 t	− 0.985	1.22 ± 0.07
	20.0	= 2187 − 4.01 t	− 0.984	4.01 ± 0.25
	29.1	= 2482 − 14.30 t	− 0.992	14.30 ± 0.63
C	0.0	= 2157 − 0.59 t	− 0.952	0.59 ± 0.07
	10.1	= 2134 − 1.36 t	− 0.981	1.36 ± 0.10
	20.0	= 2159 − 3.98 t	− 0.982	3.98 ± 0.27
	29.1	= 2265 − 13.48 t	− 0.984	13.48 ± 0.93

Table 5 B. Second-order regression equations between the logarithm of the protein-bound nitrite level and storage time t, in days; s = standard deviation.

Product	Storage temp., °C	Regression equation 10^3 1g RS.NO =	$10^3 \times d(1g\ [RS.NO])/dt \pm s$		
			at [RS.NO] = 50 mg $NaNO_2$ equiv./kg	at [RS.NO] = 100 mg $NaNO_2$ equiv./kg	at [RS.NO] max.
A	0	$= 1793 - 0.29\,t - 0.00017\,t^2$	0.38 ± 0.63		0.31 ± 0.42
	10.1	$= 1866 - 1.31\,t + 0.00006\,t^2$	1.29 ± 0.46		
	20.0	$= 2416 - 12.06\,t + 0.0288\,t^2$	7.29 ± 3.43		
	29.1	$= 3411 - 32.07\,t + 0.0788\,t^2$			
B	0.0	$= 2134 - 0.66\,t + 0.00049\,t^2$		0.42 ± 0.19	0.63 ± 0.12
	10.1	$= 2167 - 1.98\,t + 0.00203\,t^2$		1.60 ± 0.16	
	20.0	$= 2281 - 6.59\,t + 0.0128\,t^2$		5.39 ± 0.85	
	29.1	$= 2770 - 26.04\,t + 0.1070\,t^2$		18.67 ± 2.88	
C	0.0	$= 2176 - 10.58\,t + 0.00134\,t^2$		0.42 ± 0.29	1.03 ± 0.18
	10.1	$= 2146 - 16.93\,t + 0.00112\,t^2$		1.48 ± 0.43	
	20.0	$= 2229 - 68.44\,t + 0.0158\,t^2$		5.68 ± 0.26	
	29.1	$= 2142 - 23.46\,t + 0.127\,t^2$		18.47 ± 2.73	

Table 6. Activation energies, ΔE, in kJ/mol, with standard deviations of the decomposition of bound nitrite of the depletion of free nitrite at different pH values.

Product	pH	ΔE in kJ · mol^{-1} of depletion of free nitrite	ΔE in kJ · mol^{-1} of decompn. of bound nitrite	
			k_2	d(lg [RS.NO])/dt
A	5.81	54 ± 3	81 ± 2	103 ± 3
B	5.58	46 ± 1	79 ± 5	87 ± 5
C	5.36	43 ± 2	72 ± 5	88 ± 2

depletion and NOMb formation. The fact that both reactions proceeded even more rapidly in whole sarcoplasma (Ando et al. 1971) can be explained by assuming that the low-molecular dialysable compound X can now pass on NO to the high-molecular compounds of the sarcoplasma. Reaction Scheme 5 implies that the progressive decline of the slope of the depletion curve of free nitrite, d(lg [$NaNO_2$])/dt – which is due to the rapid transformation of HNO_2 into RS·NO in the first part of the storage period and the increasing importance of the back Reaction 5b – is more pronounced the lower the pH of the product. This is in line with our data.

From the increase in k_2 and d(lg [RS·NO])/dt with storage temperature, the activation energy ΔE of the back Reaction 5b was calculated by the Arrhenius equation. Table 6 gives the calculated ΔE together with ΔE for the depletion of free nitrite determined in an earlier study (Olsman 1975). The latter are considerably lower and much more pH-dependent than ΔE calculated from depletion of bound nitrite, which increased rather than decreased with pH of the product. The supposition of Mirna (1974) that bound nitrite was more stable the lower the pH is refuted by this finding. The difference in activation energy between the depletion of free nitrite and the decomposition of bound nitrite implies that at lower storage temperatures bound nitrite should rise to higher levels than at higher temperatures. This has indeed been noticed in earlier experiments at our laboratory.

Discussion on the session

Protein-bound nitrite

The protein-bound nitrite assay method was developed for purely analytical purposes: to estimate the total nitrite content in meat products. $HgCl_2$ is used to release the bound nitrite. The normal level of this fraction in meat products is about 10–15 mg/kg, expressed as $NaNO_2$. The protein-bound nitrite should be considered, just as free nitrite, as a reservoir for nitrosation. The indication that the 'protein-bound nitrite' fraction includes intermediates capable of transferring NO groups is of cardinal importance in relation to the significance of 'residual nitrite'. Since such components might apparently be similarly capable of promoting nitrosamine formation, whether during cooking or in the stomach, their microbiological significance requires investigation also.

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Chemical behaviour of nitrite in meat products. 2. Effect of iron and ethylenediaminetetraacetate on the stability of protein-bound nitrite

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Abstract

Addition of Fe^{2+} at 0.1 or 1 mmol kg^{-1} resulted in higher contents of protein-bound nitrite (PBN) in the stored product, whereas EDTA has the opposite effect. The effect was more pronounced at pH 6.2 than at 5.65, and was completely absent at pH 5.1.

Introduction

Addition of ferrous sulfate to meat products increased the content of protein-bound nitrite during storage of the product (Olsman & Krol 1972). It was supposed that a ferric coordination complex was formed with cysteine residues and NO like those described by van Roon (1974). However our observations were limited to a model product of pH 6.3. This report provides data on the effect of ferrous ions and the metal chelator ethylenediaminetetraacetate (EDTA) on contents of protein-bound nitrite in three model meat products with pH ranging from 5.10 to 6.20.

Table 1. Amounts of additives to 1 kg of the basic emulsion, and treatment of 12 products. GDL, glucono-δ-lactone; EDTA, ethylenediaminetetraacetate.

Product	GDL	$FeSO_4 \cdot (NH_4)_2 SO_4 \cdot 6H_2O$		Na_2EDTA		Heating temp. (°C)	pH of final product
	(g)	(mg)	(mmol)	(g)	(mmol)		
D	–	–	–	–	–	105	6.19
E	7.3	–	–	–	–	85	5.66
F	14.6	–	–	–	–	70	5.11
G	–	39.1	0.1	–	–	105	6.19
H	7.3	39.1	0.1	–	–	85	5.66
J	14.6	39.1	0.1	–	–	70	5.10
K	–	39.1	1.0	–	–	105	6.17
L	7.3	39.1	1.0	–	–	85	5.66
M	14.6	39.1	1.0	–	–	70	5.10
N	–	–	–	1.86	5	105	6.15
P	7.3	–	–	1.86	5	85	5.63
Q	14.6	–	–	1.86	5	70	5.07

Materials and methods

Preparations of model products

Table 1 shows amounts of additives and heat treatments of 12 portions of the basic emulsion of the same composition as in Part 1, Table 1. The additives were distributed uniformly as in Part 1. The ferrous ammonium sulfate and the EDTA were dissolved in part of the water used for the preparation of the emulsion. The EDTA solution was adjusted to pH 6.3 with sodium hydroxide. The 12 batches were canned as in Part 1. Heating temperatures are given in Table 1. All products were stored at 15 °C.

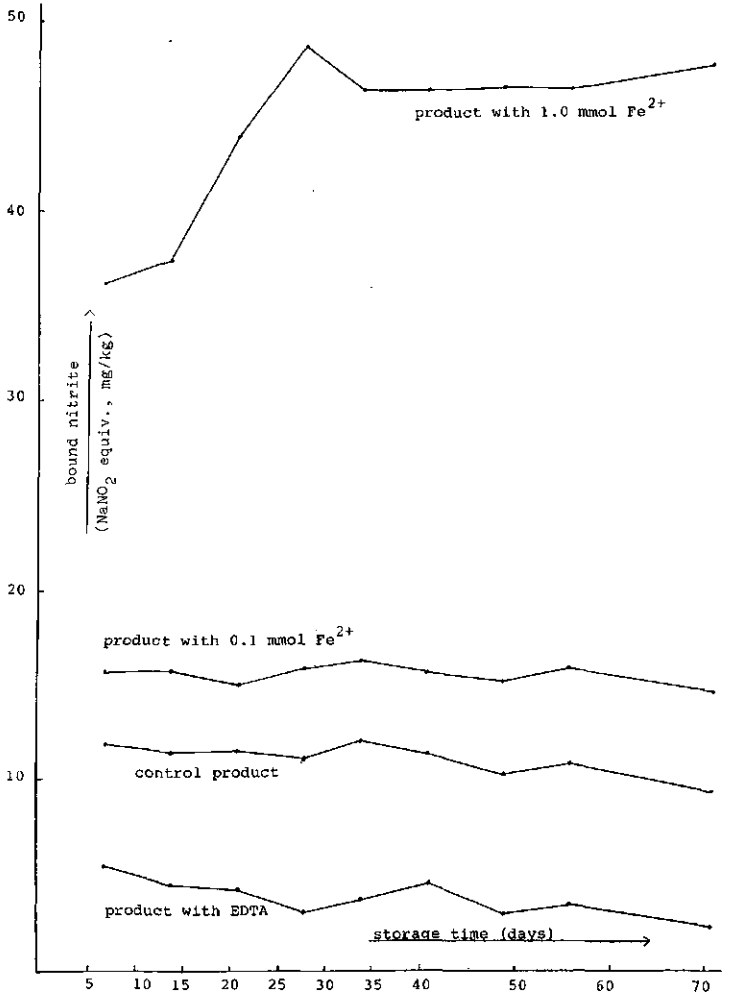

Fig. 1. Changes in bound nitrite level during storage of products of pH ≈ 6.2

Analytical method

Protein-bound nitrite was estimated as in Part 1.

Results and discussion

Figures 1, 2 and 3 show the changes in bound nitrite during storage at 15 °C in the control products with iron at 0.1 and 1 mmol kg^{-1} and with EDTA at pH 6.18, 5.65 and 5.10, respectively. The average levels generally increased markedly with decreasing pH.

At pH 6.18, addition of ferrous ions markedly increased content of bound nitrite (Fig. 1). In the product with ferrous ammonium sulphate at 0.1 mmol kg^{-1}, the content was 30% more than in control product, hardly changing during storage. In the product with 1.0 mmol kg^{-1}, bound nitrite was four times as high, rising considerably during the first 3 or 4 weeks, and then remaining almost constant. Bound nitrite tended to decrease slightly in the control product. With EDTA, endogenous ferrous ions and various other metal ions would be tightly bound in complexes, and the bound nitrite was only 30–40% of that in the control products, declining during storage.

The products of pH 5.65 behaved the same, though less pronouncedly (Fig. 2).

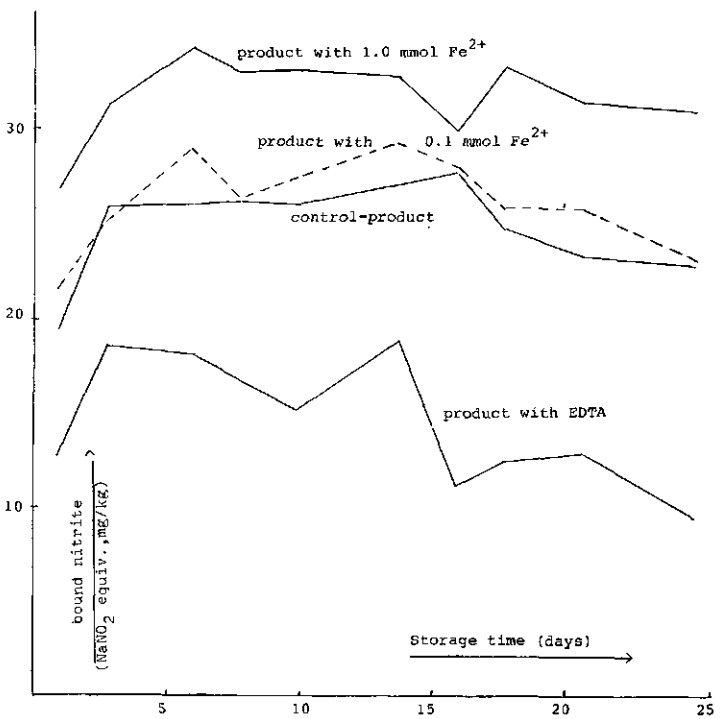

Fig. 2. Changes in bound nitrite level during storage of products of pH ≈ 5.65

With Fe^{2+} 1 mmol kg^{-1}, bound nitrite was only 30% higher than in the control product, and with, EDTA, the content was 50 to 60% of that in the control. There were hardly any differences in content of bound nitrite between the four products of pH 5.10 (Fig. 3). With Fe^{2+} 1 mmol kg^{-1}, the content was even slightly lower than in the control product. Moreover, in all 4 products, contents decreased markedly during storage.

Evidently the stabilizing effect of iron ions on bound nitrite is strongly pH-dependent, and disappears at a pH as low as 5.1.

Depletion rate of free nitrite was not significantly influenced by EDTA or ferrous ions.

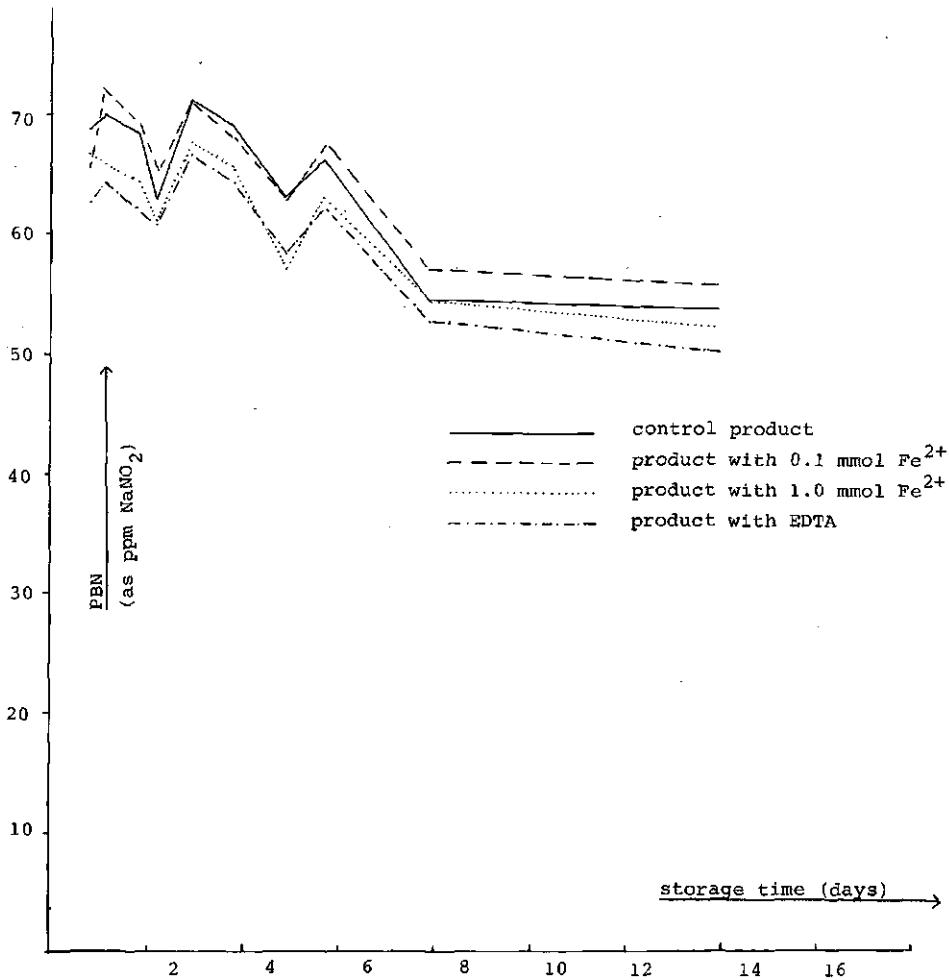

Fig. 3. Changes in PBN level during storage of products of pH ≈ 5.1.

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Nitrates and nitrites: reinterpretation of analytical data by means of bound nitrous oxide[1]

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Abstract

'Nitrate' in 28 samples of dried sausage and 6 samples of cooked ham differed widely by two standard French methods (NF-V-4409, 4410). Another method gave zero readings in accord with predictions from redox potentials that at equilibrium all nitrate should be reduced. Analysis by other techniques and the release of nitrous oxide (NO) from the meat with partial or high vacuum suggested that most was present as bound NO. According to predictions, nitrosamines would be formed only if amounts of nitrite are excessive, if nitrite and amine are in contact with air, or if the meat is roasted.

The estimation of nitrates and nitrites by titration, derived from the methods of Griess and Grau-Mirna, is laid down by French standards (FR-AFNOR-NF-V-4409 & 4410). Until recently, this raised no problems. Like many others, however, we have been trying to reduce the amount of these additives, and have observed the apparent formation of nitrate, in meats known to be reducing. It is difficult to accept oxidation of nitrite at redox potentials below 0.2, and often negative, for pH less than 7, or even 6.5.

So we simultaneously estimated nitrate, by the techniques of Richement (Fe SO_4) and Deniges (diphenylamine sulfate), whose sensitivity thresholds are about 20–30 mg/kg, in the media studied (Table 1). The results show an obvious contradiction between the quantitative analyses and the characterizations: we never confirmed the presence of nitrates in meat products where none had been added, although according to the AFNOR methods they were present in mass fractions often far above the thresholds of detection.

A theoretical study of the equilibrium of nitrates, nitrites and nitric oxide showed that NO_2^- would not be oxidized but would be almost all reduced to the gas NO, which is not very soluble in an aqueous medium. Studies by Sebranek et al. (1973; 1974) and Cassens et al. (1974) have shown that little gas is given off into the atmosphere from nitrite: a maximum of 15%, and more probably less than 5% of the added nitrite. Many authors, with analytical techniques similar to those of

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AFNOR (methods that titrate NO and NO_2^- without distinguishing between them), have shown that there is an appreciable fraction of bound 'nitrite' (or NO). Our own studies have confirmed the presence in meat of numerous compounds capable of fixing NO and of greatly increasing its solubility. Nitric oxide could thus exist in appreciable amounts in a bound form in meat products.

We tried to characterize the presence in meat of this NO, and the ion NO_2^-. The presence of large amounts of NO can easily be shown. The most reliable technique is to modify Zambelli's reactions, eliminating passage through an acid medium. The reagent (sulfanilic acid + phenol) is added directly to an ammoniacal medium. The oxide NO then gives a characteristic spectrum at 400 nm, whereas nitrites do not

Table 1. Mass fractions of 'nitrates' and 'nitrites' (expressed as $NaNO_2$ in mg/kg) in fresh meat products manufactured without KNO_3. Cd, reduced with cadmium and hydrazine sulfate. No nitrate was detected by the methods of Richemont and Deniges.

Product	Amount of $NaNO_2$ added initially	Amount measured in finished product (Griess method)	Amount of nitrite formed on reduction with Cd
Dry	216	0	52
sausage	216	0	31
	216	0	33
	216	6	8
	216	5	40
	216	4	21
	216	4	36
	216	4	74
	216	4	55
	216	2	23
	162	2	11
	108	0	31
	108	2	24
	108	4	21
	54	0	0
	54	0	0
	54	2	9
	27	0	0
	27	3	7
	27	0	24
	13	0	0
	13	0	0
	13	2	22
	13	2	23
	0	2	11
	0	2	10
	0	0	0
	0	2	7
Cooked	96	24	0
hams	96	40	81
	96	13	9
	72	11	10
	48	9	0
	12	22	24

react. This reaction is detectable at mass fractions < 5 mg/kg and shows large amounts of NO in ham and sausages.

Another technique is that of Treadwell: Na_2SO_3 10% (by mass) in an alkaline medium gives characteristic spectra, which enable NO, NO_3^- and NO_2^- to be distinguished. The reaction is detectable at mass fractions < 1 mg/kg for the first two and about 100 mg/kg for NO_2^-. This reaction also characterized the presence of large amounts of NO, or the absence of NO_3^-, if none had been added. It did not characterize NO_2^- with certainty (Fig. 1). We nevertheless consider that the reliability of this reaction is relative, as sulfites are strongly reactive. Although they did not appreciably modify the redoxpotential (0 V), we could not exclude artefacts in a medium as complex as meats.

We also studied distillation at 40 °C in a high vacuum (pressure 27 Pa) of hams and sausages made with pure nitrite. We observed that all the measurable 'nitrite' was evaporable, and sometimes even the evaporated fraction that we condensed contained more 'nitrite' measurable by the AFNOR method than the original product (Table 2). Nitrite, however, would not be volatile, particularly at the experimental pH of 6–7, unlike NO. The 'nitrite' was thus not present in the NO_2^- form.

On the advice of Mr Truffert (whom we would like to thank here), we studied the action of antipyrine (dimethyl-1,5-phenyl-2-pyrazolone) which enables the set NO + NO_2^- to be measured quantitatively. Titration was optimized by mixing equal quantities of the solution to be measured and 0.5% antipyrine in H_2SO_4 5% v/v. Concentrations were measured colorimetrically at a wavelength of 350 nm after 45 min. Nitrates gave no reaction in these conditions either in a pure medium, or added to meat products. This technique gave almost exactly the same total NO + NO_2^- (+ NO_3^- ??) as AFNOR-NF-V-4410, on products made with pure nitrite (without nitrate) (Fig. 2).

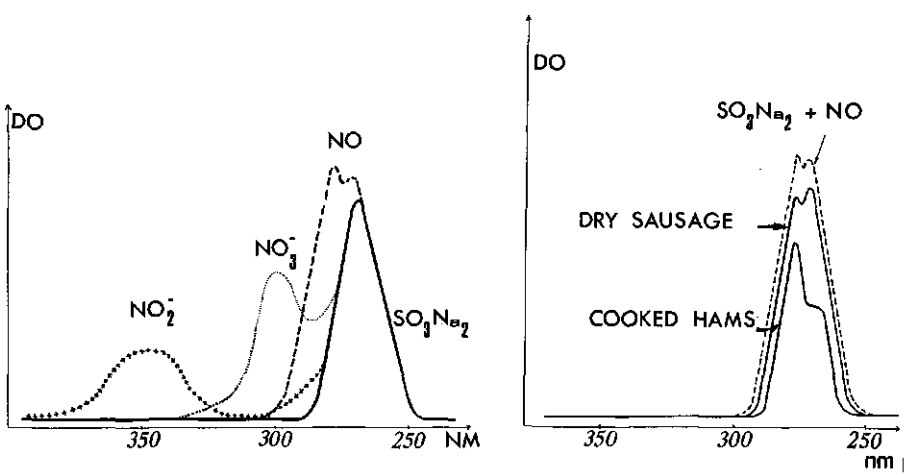

Fig. 1. Estimation of NO, NO_3^- and NO_2^- by reaction with alkaline sulfite and spectrometry. Left. Absorbance (DO) of reference solutions. Right. Absorbance of preparations from cooked hams and dry sausage (pH8) manufactured without nitrite.

The correlation coefficients between these two techniques ranged from 0.993 to 0.998 for dry sausages and hams, and the slopes of regression were close to 1 (0.96–1.06). With these meat products, the technique gave significantly higher values than nitrite titration NF-V-4409. But this does not seem true with products manufactured with added nitrate, even though it has not been possible to show any interference by the nitrate, in spite of numerous studies in which it has been added during titration. The results then seem to be slightly lower than those given by AFNOR-NF-V-4409.

Table 2. Mass fraction of 'nitrates' and 'nitrite' (expressed as $NaNO_2$, mg/kg) after distillation of m products diluted with water (1:10 by mass) under partial or high vacuum. –, not estimated.

Product	Amount of $NaNO_2$ added	Amount measured			Pressure of distillation (kPa)
		in product after Cd reduction (Griess method)	in dry residue of distillation after Cd reduction (Griess method)	in distillate	
Dry sausage	216	62	33	62	6.7
	0	25	3	52	6.7
	20	17	10	–	6.7
	20	17	0	–	0.027
Cooked hams	96	75	13	–	6.7
	96	75	0	–	0.027
	12	25	20	–	6.7
	12	25	0	–	0.027

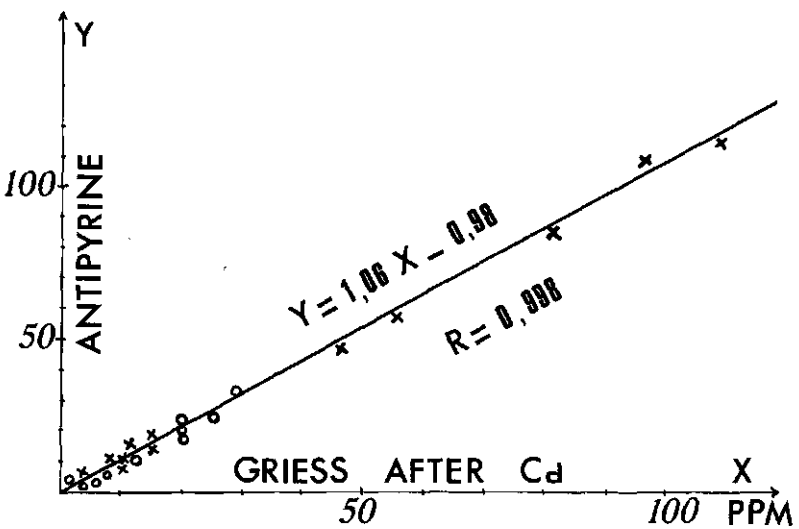

Fig. 2. Correlation between estimates of NO and NO_2^- in dry sausage (X) and cooked hams (o) by the Griess method after reduction with cadmium and hydrazine sulfate (FR-AFNOR-NF-V-4) and with antipyrine.

Conclusion

Undoubtedly the standard methods of measuring nitrates and nitrites do not give accurate results, and analysts should be cautious in interpreting data. This caution is reinforced by certain results in Table 1, the distillation studies, and later tests: in some samples both of ham and sausages, 'nitrite' is formed from the meat itself. This phenomenon has already been observed by Huynh in spoiled meat. The amounts formed do not exceed a few milligram per kilogram meat – but they undoubtedly exist – though we do not know how they are formed.

The problems of nitrates and nitrites are far more complex than we used to think. As has been pointed out by the groups of Cantoni (1973–1975), of Cassens and Sebranek (1973–1974), and by, Fan & Tannenbaum (1973), Frouin et al. (1975; 1976a, b), Goutefongea (1973), Mirna & Hofmann (1969), Mirvish (1970) and others, there are important bonding phenomena of the substances measured under the names of nitrites and nitrates in meats. Hitherto these phenomena were attributed to bound nitrite, i.e. the bonding of NO_2^- . Theoretical calculations forecast the rapid breakdown of NO_2^- into NO in meat products. The specific reactions we used confirm all the theoretical forecasts: we regularly detected NO; the substance measured as nitrite was totally volatile; we never detected NO_2^- as such; in meat products we always found either NO or NO_3^- , never NO_2^- . We had already shown that numerous compounds in meat can fix NO and keep it in solution, so confirming the results.

The following hypothesis seems to us the most plausible in meat products (Fig. 3). All the nitrite is reduced to NO. This oxide is highly reactive, and becomes fixed to some degree to many compounds in meat, such as proteins, thiols, hydroxyls, carboxyls, reducing agents and haematic compounds. There is a complex equilibrium in meats between the various substances that fix NO with variable force. This equilibrium is upset during analysis by the reagents, which release some

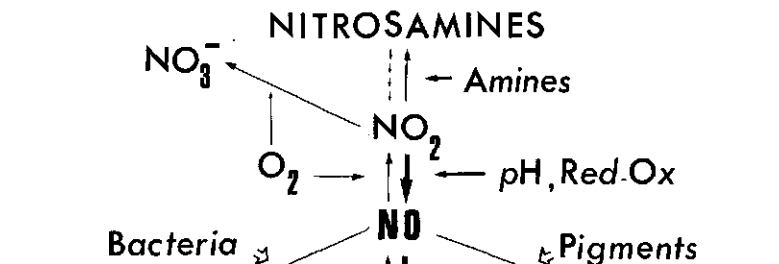

Fig. 3. Probable scheme of reaction of NO_2^- in meat products.

of NO, but the amount measured depends on conditions. The reducing agents (cadmium with hydrazine sulfate) cause more NO to be freed (Table 1). Probably there are also interactions in the defaecation, extraction and filtration (Mirna & Hofmann 1969).

This hypothesis explains why analysis is difficult and why introsamines form only in certain conditions. If large amounts of nitrite are added, the reducing and fixing capacities of the meat are saturated, NO_2^- remains, and nitrosamines are formed.

If the nitrite and amines are in contact in the presence of air, particularly in spice mixtures, the nitrite cannot be reduced to NO, as the redox potential is too high, and nitrosamines are formed.

If the products are roasted, that is heated until the NO is given off, as in our distillation tests, nitrosamines are formed as the air reoxidizes NO. Substances that fix NO, such as sodium ascorbate, reduce the formation of nitrosamine (Fiddler 1973b), and as the hypothesis would lead one to expect.

The hypothesis of bound NO is central in analytical, toxicological and bacteriological problems raised by nitrites in meat products. Further studies are needed to find out more of its implications.

Discussion on the session

Estimation of NO with Griess reagent

According to the author, NO reacts with the Griess reagent giving exactly the same colour. The measurements were in a nitrogen atmosphere. Others believe that under these circumstances no reaction would occur unless an oxidant is present like H_2O_2. Perhaps traces of O_2 were dissolved.

Frying bacon under exclusion of air

This paper indicates that nitrosamines can only be formed in the presence of air because of oxidation of nitric oxide into nitrite. Has anybody experience with fried bacon in absence of air (submerged in fat)? This paper suggests that this would prevent nitrosamine formation.

Conclusions and recommendations of the chemical session, reactions with nitrite, Wednesday 8 September

1. Pathways of nitrite especially with regard to its reaction with connective tissue as well as with fats have been studied.

2. Ascorbate promotes depletion of nitrite, but it increases the amount of reaction products bound to the water-extractable compounds.

3. Study of the behaviour of nitrite bound to proteins showed that this type of nitrite may act as a reservoir of trans-nitrosating power and should be considered to be equally important as free nitrite. Its significance for establishing levels for legal regulations was questioned.

4. Current analytical methods should be re-examined.

5. The differentiation between various nitrosating species would be a helpful mean to study their possible influence on the formation of *N*-nitrosamines.

Chemical session – Formation of nitrosamines

Reporters: P. S. van Roon, P. C. Moerman

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Nitrosation of food amines under stomach conditions[1]

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Abstract

Using foods as sources of nitrosatable amines, studies have been made of the formation of *N*-nitroso compounds under conditions simulating those within the stomach and also in man. A three-fold higher concentration of thiocyanate, a stimulator of nitrosation, was observed in saliva and gastric juice of smokers than of non-smokers. Pentagastrin stimulation of hydrochloric acid secretion reduced concentration of thiocyanate in stomach contents but that of nitrite remained unchanged from those within the fasting stomach. After Ingestion of lettuce containing 800–1400 mg/kg KNO_3 by normal adults, concentration of nitrite in saliva increased rapidly and remained high for a further 4 h. After consumption of a meal containing nitrite, its concentration within the stomach increased, along with pH, for about 45 min before returning towards fasting value. *N*-Nitrosopiperidine (NOPip) and *N*-Nitrosopyrrolidine were the predominant volatile nitrosamines produced when foods were incubated with high levels of nitrite (10 g/kg) atypical of the stomach. At more characteristic concentration of nitrite, nitrosamine formation was reduced and was greatly dependent on thiocyanate level. On occasions, traces of NOPip were detected in the gastric contents of volunteers after ingestion of foods containing nitrite.

Introduction

Greenblatt et al. (1971) advanced various hypotheses to explain the discrepancy between the carcinogenic effect in terms of number of lung adenomas induced by ingestion of nitrite and *N*-methylaniline and that anticipated from the calculated formation of *N*-nitrosomethylaniline *in vivo*. These included extraction of the non-ionized amine ($pK_a = 4.85$) by fats in ingested food even at pH 3–4, removal of the amine by precipitation of insoluble salts and physiological effects of the amine on gastric secretion and function.

In long-term feeding studies of nitrite and morpholine in rats (Shank & Newberne 1976), nitrite in the diet was considered to induce more tumours than did amine; the lowest mass fractions of nitrite and morpholine studied, namely 5 mg/kg for both, proved carcinogenic to a small proportion of the rats. On the other hand, Telling et al. (1976) concluded that the contents of dimethylamine and pyrrolidine added to the diet had a larger influence on the formation of the corresponding

1. Previously published (Walters et al. 1976). Republished by permission International Agency for Research on Cancer, Lyon, France.

nitrosamines in the stomachs of rats than did concentration of nitrite in drinking water.

On reaction with nitrous acid, complex tertiary amines such as the analgesic aminopyrine (Lijinsky & Greenblatt 1972) and the fungicide ziram (Eisenbrand et al. 1974) can give rise to simpler volatile nitrosamines. In order to determine the availability in foods of amine precursors that could form nitrosamines, Walters et al. (1974) treated foods at acid pH with higher amounts of nitrous acid than typical of the food consumer; afterwards the products were fractionated on the basis of their volatility in steam and extractability in dichloromethane. By combined gas chromatography and high-resolution mass spectrometry, the four volatile nitrosamines *N*-nitroso piperidine, pyrrolidine, dimethylamine and diethylamine were shown to arise from nitrosation of milk products.

Nitrate is widely distributed in vegetables and water supplies and can readily be reduced to nitrite by bacterial or plant enzymes. The ingestion of vegetables and extracts containing large amounts of nitrate markedly elevates concentrations of nitrite in saliva (Stephany & Schuller 1975), presumably through reduction by oral bacteria (Tannenbaum et al. 1974; Maruyama et al. 1976). By virtue of its greater reactivity, nitrite itself is not as widely distributed but is useful as a food additive in protecting the consumer against *Clostridium botulinum* and other undesirable micro-organisms.

In kinetic studies, thiocyanate markedly stimulated the nitrosation of an amine, particularly at pH below 3 (Boyland & Walker 1974). The anion occurs in the saliva, particularly of smokers (Densen et al. 1967); higher concentration of thiocyanate in urine of smokers than in that of non-smokers has also been observed. Nitrite also occurs physiologically human saliva usually at a substance concentration of 80–140 μmol/litre (6–10 mg litre^{-1})(Tannenbaum et al. 1974). In some people concentration of nitrite in whole saliva varied little over several months but in other people concentration varied greatly with time; meals containing various amounts of nitrate had little or no effect on the concentration of nitrite in saliva 1 h later.

Table 1. Effect of eating 70 g lettuce on substance concentration of nitrite in saliva. Mean concentration before eating lettuce was 63 μmol · litre^{-1}. *t*, time (after eating lettuce); *c*, substance concentration; *P*, probability. Values are means for 2 groups of 20 people sampled on 4 successive days in a witch-over trial. The lettuce contained 51–91 mg KNO_3 (0.5–0.9 mmol).

t/h	$c(NO_2^-)$/μmol · litre^{-1}		*P*
	no lettuce	with lettuce	
0	49	97	< 0.01
2	26	76	< 0.001
4	33	53	< 0.05
6	26	44	> 0.05

Methods and results

Factors influencing nitrosation

Nitrite in saliva Substance concentration of nitrite in saliva of normal people was estimated, irrespective of meals, at 90 min intervals at 08:30–17:00 h on 2 consecutive days with Merckoquant nitrite test sticks (Hofmann 1976).

Substance concentration of nitrite in the saliva of normal persons fell generally within the range 0–70 mmol litre^{-1} (up to 5 mg/litre, expressed as KNO_2) and no significant trends were discernible in individuals throughout two working days. Two individuals were found to have inexplicably higher concentrations ranging from 140–280 μmol litre^{-1} in the early morning but reverted to 'normal' values during the day. In one person, a mean value of 13 over one day was followed by one of 86 the following day but generally the observed fluctuations were less marked. The group was composed of smokers and non-smokers, but no difference was apparent between the two.

Effect of dietary nitrite on values in saliva To assess the effect of dietary nitrate, 20 people each ate 70 g lettuce containing 51–91 mg KNO_3 and a similar control group did not. The following day, the roles were reversed and so on for four days. Nitrite was estimated in saliva directly before and after ingestion of the lettuce and subsequently after 2, 4 and 6 h.

Table 1 illustrates the effects on nitrite in saliva of eating lettuce. Significant increases in the salivary nitrite level were obtained directly after the ingestion of lettuce and after 2 and 4 but not after 6 h.

Nitrite has been detected in the fasting gastric juice of healthy persons and patients examined by the Gastro-enterology Department of the Central Middlesex Hospital, London N.W. 10. The data has been provided by courtesy of Dr W. S. J. Ruddell and Dr L. M. Blendis. The mean concentration observed was closely asssociated with acidity. When substance concentration of titratable H^+ was <10 mmol litre^{-1}, nitrite values in gastric juice of fasting people ranged up to 36 μmol litre^{-1}, whereas in six patients with pernicious anaemia and complete achlorhydria values were 59–240 μmol litre^{-1}.

Nitrite in diet and gastric contents By the method of Nicholas & Nason (1957), nitrite was estimated in gastric juice either from normal people or after ingestion of a meal containing nitrite. Gastric samples were obtained with nasal Ryles tubes, before and at intervals after the meal for up to 88 min. The meal consisted of a fried egg (40 g), bread (32 g), butter (16 g), cheese (22 g), biscuits (17 g), milk (200 cm^3) and 80 g luncheon meat (produced at BFMIRA without added nitrate), which provided nitrite to a calculated aggregate concentration of 0.83 mmol litre^{-1} (expressed as KNO_2 58 mg litre^{-1}) in the whole meal. Samples of saliva were taken after each time interval and nitrite was estimated with Merckoquant test sticks.

After the meal, a rapid rise in both pH and gastric nitrite ensued, with maxima after about 40 min (Fig. 1). Thereafter the pH fell towards the mean fasting value and the mean concentration of nitrite decreased sharply. Throughout the whole period, the average concentration of nitrite in saliva did not vary markedly, al-

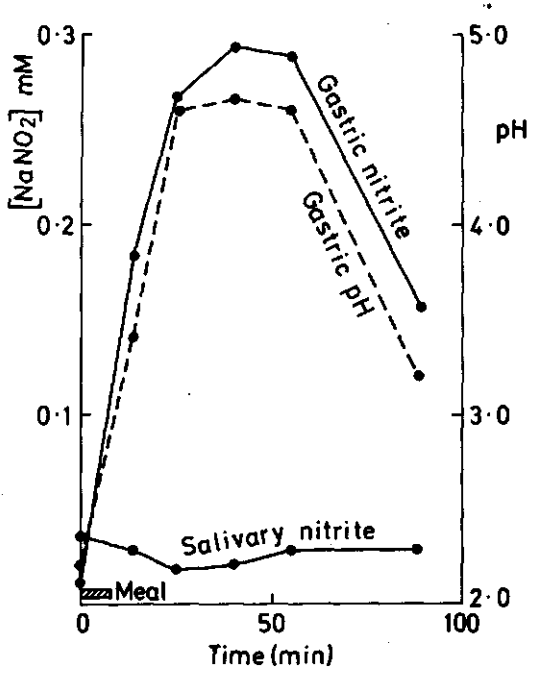

Fig. 1. Effect of ingestion of meal containing at a substance concentration of 0.83 mmol litre^{-1} on gastric pH and gastric and salivary concentrations.

Table 2. Effect of smoking on thiocyanate (SCN^-) in whole saliva and fasting gastric juice. n, number (of people sampled); c, substance concentration; $s(\bar{c})$, standard deviation.

Fluid	Group	n	$\bar{c}$ (SCN^-) ± $s(\bar{c})$/mmol · litre^{-1}
Saliva	non-smoker	15	1.82 ± 0.71
	smoker	15	5.45 ± 0.77
Gastric	non-smoker	20	0.48 ± 0.34
juice	smoker	6	1.49 ± 0.23

though it tended to diminish somewhat after the meal, presumably by 'dilution' with the dietary intake, and then to return towards the initial value.

Thiocyanate The difference between the thiocyanate levels in the saliva of smokers and non-smokers (Densen et al. 1967) was confirmed and extended to the concentrations found in gastric juice (Table 2). Thus, although thiocyanate was present at lower concentrations in the stomach than in the saliva, the concentrations from smokers were about three times those of non-smokers.

Total phenols Phenols were estimated with Folin & Ciocalteu reagent, in 19 observations on the gastric contents of four fasting non-smokers and ranged 1.2–4.3 mmol litre^{-1}, average 2.06. The corresponding range for five observations on one

smoker was 1.5–3.5 mmol litre^{-1}, average 2.64. Thus, no significant difference was observed in the few samples so far examined.

Acid secretion and endogenous nitrite and thiocyanate Pentagastrin stimulation of the fasted human stomach caused a fall in pH (Fig. 2), the pH being maintained throughout the time period despite removal of gastric contents at intervals. Over the same period, thiocyanate fell to mean concentrations less than half those in the fasted stomach, the depression after each time interval being significant with probability ranging from 0.001–0.02. The initial mean substance concentration of thiocyanate in 17 people was 1.0 mmol litre^{-1}; after pentagastrin stimulation, the minimum was 0.32. A similar fall in total phenols (expressed as phenol) was observed during the pentagastrin test, which was significant after all time intervals except 0–15 min. No change was detected in gastric nitrite during pentagastrin stimulation. The mean initial substance concentration of nitrite in gastric contents was 5.5 μmol litre^{-1}; minimum and maximum after pentagastrin stimulation were 5.4 and 7.2, respectively, neither of which was significantly different from initial values.

Nitrosation of food amines in vitro Homogenates of similar meals comprising egg, bread, butter, cheese, biscuits, milk and luncheon meat were prepared in an MSE Atomix with or without added human gastric juice to provide an aggregate sub-

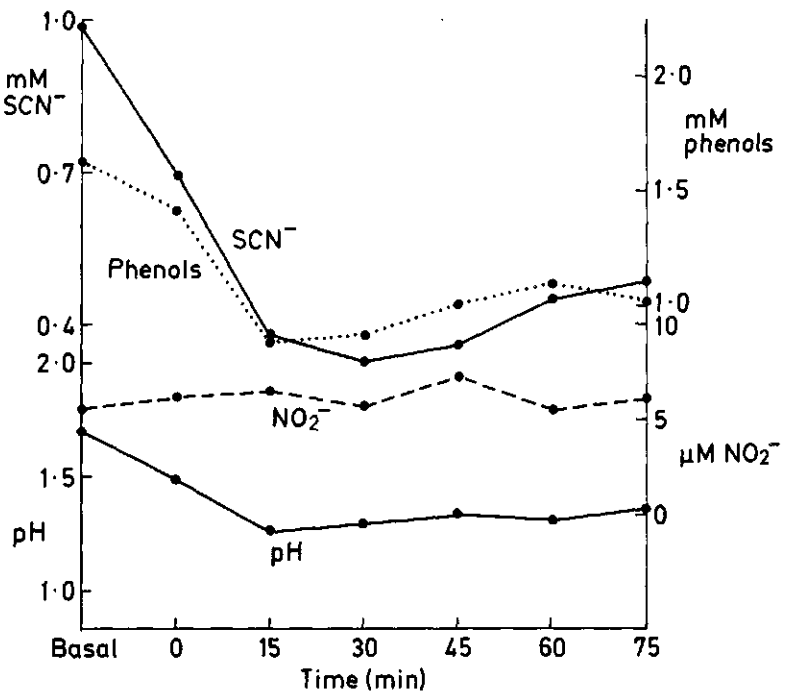

Fig. 2. Gastric pH, nitrite, thiocyanate and total phenols after pentagastrin stimulation and acid secretion. Data after 17 people.

stance concentration of nitrite of about 0.43 mmol litre^{-1} (expressed as KNO_3 30 mg litre^{-1}). After adjustment of the pH, where necessary, to pH 2.0, the homogenates were incubated for 3 h at 37 °C. Any volatile nitrosamines formed were separated by distillation under reduced pressure in the manner of Telling et al. (1976). After extraction from the distillates with dichloromethane and concentration of the extracts and transfer to hexane, volatile nitrosamines were separated detected and estimated by combined gas chromatography and high-resolution Hitachi and Perkin Elmer RMU7L mass spectrometer as detector in the manner of Crathorne et al. (1975).

The predominant volatile nitrosamines arising from the deliberate nitrosation of foods, and particularly those of dairy origin, in acid solution with large concentrations of nitrite (0.15 mol litre^{-1}) atypical of those likely within the human stomach are *N*-nitrosopiperidine (NOPip) and *N*-nitrosopyrrolidine (NOPyr). The maximum mass fractions of each detected by combined gas chromatography and mass spectrometry were 83 and 42 μg/kg, respectively.

During incubation for 3 h at pH 2.0 and 37 °C of egg, bread, butter, cheese, milk and luncheon meat containing nitrite to provide an aggregate substance concentration of 0.46 mmol/litre (expressed as KNO_2 32 mg/litre^{-1}), the production of NOPip was reduced to about a tenth of that under 'drastic' conditions and no NOPyr was detected in the absence of added thiocyanate (Table 3). The formation of NOPip was, however, markedly enhanced by inclusion of thiocyanate at concentrations within the range 0–32 mg litre^{-1}, which also raised NOPyr production above the limit of detection.

Table 3. Effect of thiocyanate (SCN^-) concentration on formation of volatile nitrosamines from food amines. *c*, substance concentration (initially in the homogenated meal); *w*; mass fraction; NOPyr, *N*-nitrosopyrrolidone; NOPip, *N*-nitrosopiperidine.

$c(SCN^-)$/mmol · litre^{-1}	w(NOPyr)/μg · kg^{-1}	w(NOPip)/μg · kg^{-1}
0	0.0	8.0
0.2	2.0	36
0.6	1.0	30
1.2	1.0	34
3.0	1.0	51

Table 4. Formation of volatile nitrosamines from food amines during incubation in the presence of thiocyanate. (c = 1.2 mmol · litre^{-1}) and nitrite (c = 0.43 mmol · litre^{-1}). *t*, time (of incubation); *w*, mass fraction; NOPyr, *N*-nitrosopyrrolidine; NOPip, *N*-nitrosopiperidine.

t/min	w(NOPyr)/μg · kg^{-1}	w(NOPip)/μg · kg^{-1}
15	0.2	6.7
30	0.0	2.1
180	0.3	5.1

The effects of the time of incubation in human gastric juice upon formation of NOPyr and NOPip in the presence of thiocyanate are shown in Table 4. The restricted diet gave rise to less NOPip than the more complete meal. Though variation in the production of NOPip from food amines with little nitrite was such that its change with time was insignificant, the important finding was that it formed so rapidly. Too little NOPyr was formed to assess its variation with time of incubation.

Incubation of foods and nitrosation of amines in the stomach Oral tubes of about 5 mm bore permitted both entry and subsequent recovery of slurries of meals to and from the stomachs of volunteers after requisite time intervals; in this way, we effectively sampled the whole contents of the stomach. From tests with food slurries containing phenol red, which is not absorbed from the stomach, we estimated recoveries of ingested food.

The homegenates of meals, which contained egg, milk and luncheon meat, were of a consistency to permit passage along the oral tubes; the aggregate substance concentration of nitrite was within the range 0.46–0.77 mmol litre^{-1} (expressed as KNO_2 32–53 mg litre^{-1}).

Table 5 shows the formation of *N*-nitrosopiperidine detected as in studies in vitro in stomach contents recovered from a smoker and a non-smoker. Trace amounts of *N*-nitrosopiperidine were detected after 30 min in four persons out of fourteen, three times from the smoker and once from the non-smoker.

In order to incoporate salivary thiocyanate, meals containing nitrite at a concentration of 0.49–0.64 mmol litre^{-1} (34–44 mg litre^{-1}) were eaten whole and as much as possible was recovered. Only 40–50% of intake was recovered in this manner, as compared with a mean of 85% for the slurry and neither *N*-nitrosopiperidine nor *N*-nitrosopyrrolidine was detected on any of six persons after 30 or 60 min in the stomach.

Table 5. Formation of *N*-nitrosopiperidine after recovery of a food slurry from the human stomach at time intervals. *t*, time (in stomach); *c*, substance concentration (initially in slurry); *w*, mass fraction (in stomach contents). Data from 14 persons.

t/min	$c(NO_2^-)$/mmol · litre^{-1}	w(NOPip)/μg · kg^{-1}	
		smoker	non-smoker
15	0.46	0	0
15	0.46	0	0
15	0.46	0	0
30	0.46	0.5	0.4
30	0.46	0	0
30	0.46	0	0
30	0.46	0	0
30	0.55	0.3	0
30	0.49	0	0
30	0.77	0.5	0
60	0.62	0	0

Discussion

Stephany & Schuller (1975) induced much higher concentrations of nitrite in saliva than those reported in this paper after ingestion of vegetables or their extracts rich in nitrate. Their results show that the duration of high concentrations in saliva as well as the magnitude of the rise in concentration depend on intake of nitrate. Since vegetables can contain more than the lettuce samples employed in these studies (Kamm et al. 1965), people living on a diet with a high proportion of vegetables will probably be subject to even higher concentrations of nitrite in the saliva and hence also in the stomach.

The higher concentrations of thiocyanate in the saliva of smokers than of non-smokers are in keeping with the observations of Densen et al. (1967) although the relative mean concentrations in these studies are somewhat less than reported even for light smokers; this is probably because the mean found for the group of non-smokers was inexplicably higher than that previously reported. Furthermore, the fall in gastric thiocyanate after pentagastrin stimulation of acid secretion supports the view that it is of salivary origin. The concentrations of thiocyanate in the gastric juice of fasting smokers and non-smokers fall within the range considered significant for stimulation of the nitrosation of secondary amines at pH 3.0 and below (Boyland & Walker 1974). Certainly, for the production of *N*-nitrosopiperidine from food amines, thiocyanate at 0.2 mmol litre^{-1} and more is effective *in vitro*.

The precursors of *N*-nitrosopiperidine and *N*-nitrosopyrrolidine in foods in contact with nitrite may well not be the bases themselves but derivatives such as piperine, which can occur in peppers; certainly, the formation of these volatile nitrosamines from spices treated with nitrite is well authenticated (Sen et al. 1973; Gough & Goodhead 1975) and putrescine, which can be present in foods, has been recognized as a precursor to *N*-nitrosopyrrolidine (Warthesen 1975). The concentration of *N*-nitrosopyrrolidine formed at low concentrations of nitrite is only a small fraction of that obtained from foods at a much higher concentration of nitrite but that of *N*-nitrosopiperidine over a time representative of the residence time in the stomach was not greatly affected by such variations; this suggests that the mechanisms of formation of the two nitrosamines were different.

Although the production of *N*-nitrosopiperidine from food amines is quite rapid *in vitro*, nitrosation is unlikely to proceed in the stomach until acid secretion has lowered the pH towards the fasting values close to the optimum for formation of *N*-nitroso derivatives from secondary amines. Some time was required to distribute the nitrite present in the food throughout the stomach contents, as evidenced by the duration of the rise in its concentration in samples recovered for analysis. This implies that locally higher concentrations persist initially but presumably with environmental pH values not conducive to nitrosation. The maximum concentrations reached in the stomach were, however, in keeping with the distribution of ingested nitrite throughout a volume of stomach contents of about 1 litre, so that losses by absorption or by passage into the duodenum would not have been great. Though the compound isolated and concentrated from stomach contents conformed both in retention time in gas chromatography and in mass divided by charge in high-resolution mass spectrometry with *N*-nitrosopiperidine, considerable

variations in recoveries would be anticipated at the extremely low mass fractions encountered. No positive responses for *N*-nitrosopiperidine have so far been obtained within a residence time of 30 min in the stomach, in accord with the time interval necessary for acid secretion to lower the pH.

Acknowledgments

This research was supported financially by the Cancer Research Campaign. The practical work was undertaken by Mrs B. E. Wells, Miss C. Dyke and Mr P. J. Colley. Grateful thanks are expressed to Professor J. N. Hunt, Guys Hospital Medical School, London S.E.1, and his colleagues Miss M. Fisher and Mr R. Burn-Murdoch for their invaluable assistance with tests *in vivo*.

Discussion on the session

Effect of thiocyanate on nitrosopiperidine formation

The catalytic effect of this compound is pH-dependent. It appears to be active only at pH values below 3.5. The amount of SCN^- in saliva seems to be subject to rather large variations. It was suggested that this compound is a metabolite of constituents of tobacco smoke.

Use of test sticks for determination of nitrite in saliva

The correlation between quantitative data from sticks and data from a standard colorimetric determination was thought to be poor. The authors experience was that the test sticks are reasonably accurate up to about 10 mg/kg. Much higher nitrite concentrations resulting from higher nitrate intakes would have been noticed if they had occurred.

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Increased N-nitrosamine formation in model fat systems containing sodium ascorbate

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The use of reducing agents such as sodium ascorbate (NaAsc) has been suggested as a practical method of reducing nitrosamine formation in cured meats and the United States Department of Agriculture is proposing the compulsory use of NaAsc or sodium erythorbate in bacon curing (Food Chemistry News 1975). The reaction of NaAsc with nitrite in mildly acidic solution is much more rapid than the nitrosation of secondary amines, and in model aqueous systems the presence of excess NaAsc results in a considerable reduction in amine nitrosation (Mirvish et al. 1972; Mottram et al. 1975). Lower levels of nitrosamines have been found in certain cured meats prepared with NaAsc (Herring 1973; Fiddler et al. 1973).

The major nitrosamine problem in cured meats is the occurrence of NPYR in fried bacon and its formation has been shown to be associated with the fat (Fiddler et al. 1974; Patterson et al. 1976). Adipose tissue contains by mass 85% lipid and we have examined the effect of NaAsc on the nitrosation of dipropylamine and pyrrolidine in a model system resembling this essentially non-polar environment. The amine was dissolved in benzene or corn (maize) oil and shaken at room temperature with a small amount of a buffered aqueous solution containing $NaNO_2$

Table 1. Effect of sodium ascorbate (NaAsc) and ascorbyl palmitate (Asc Pal) on the nitrosation of secondary amines in a model fat system. DPA, dipropylamine; PYR, pyrrolidine; 'increase' is yield of nitrosamine in the presence of reducing agent divided by yield in control; –, not determined.

Amine	Aqueous	Nitrosamine Yield (%)			Increase with	
	pH	control	NaAsc	Asc Pal	NaAsc	Asc Pal
Benzene as non-polar phase						
DPA	5.0	4.45	25.5	3.0	5.7	0.67
DPA	6.0	0.13	1.63	–	12.7	–
PYR	5.0	0.28	5.73	0.38	20.5	1.36
PYR	6.0	0.10	0.72	–	7.2	–
Corn oil as non-polar phase						
DPA	5.0	0.24	5.91	0.19	24.6	0.79
PYR	5.0	0.42	3.48	0.04	8.3	0.10

and NaAsc. The presence of NaAsc in this two-phase system resulted in a considerable increase in the yield of both *N*-nitrosodipropylamine and *N*-nitrosopyrrolidine, quite contrary to the observations in single-phase aqueous solution (Table 1).

In aqueous solution, ascorbate reduces nitrite to nitric oxide, which is oxidized to N_2O_3 and NO_2 and, since in non-polar solvents all three oxides of nitrogen are powerful nitrosating species, rapid nitrosation of the amines occurs in the benzene and corn oil. Such a rapid nitrosation does not occur in aqueous solution because NO_2 and N_2O_3 are readily hydrolysed.

When ascorbyl palmitate was used instead of NaAsc a reduction in nitrosamine yield was observed because the lipophilic ester is insoluble in water and consequently did not reduce the nitrite but remained in the non-polar phase to compete with any nitrosating species produced.

This report has shown that NaAsc increases the nitrosation of secondary amines in a mixed aqueous/non-polar system. Bacon is a similar two-phase system in which *N*-nitrosamine formation is known to be associated with the fat and, although the reactants and conditions will differ from those in model systems, it seems unwise to legislate for the compulsory addition of reducing agents to bacon cures until the mechanism of nitrosamine formation in bacon during frying is fully understood.

Experimental Detail

Dipropylamine (DPA) or pyrrolidine (PYR) (0.125 mmol) dissolved in benzene or corn oil (100 ml) was shaken at room temperature (about 20 °C) for 2 h with a buffered aqueous solution (15 ml) containing NaAsc or solid ascorbyl palmitate (AscPal) (0.5 mmol) and $NaNO_2$ (0.25 mmol). A control mixture contained no reducing agent. After neutralizing, the nitrosamines were extracted by steam distillation and determined using a Coulson electrolytic conductivity detector operating in the N-specific mode. The nitrosamines were confirmed by GC-MS with an LKB 9 000 mass spectrometer.

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Formation of carcinogenic nitrosamines in relation to nitrite in meat products

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Introduction

Treatment of laboratory animals with nitrite concurrently with certain amino compounds has inducted tumors by formation of *N*-nitroso compounds in vivo, as has been reported by many authors. Nitrite, whether occurring naturally or as a food additive, has therefore been widely considered as a risk in the induction of tumors in man. In order to evaluate whether a real hazard is present it is useful to know how much nitrosamines can be expected to be formed from the amounts of the precursors likely to be consumed by man. As a direct estimate of nitrosamine synthesis in vivo has not been possible in man as yet, we tried to get some data by three less direct ways.

In a first series of tests, we investigated how long after ingestion nitrite would be available for reaction with amines in the human stomach. Similarily absorption from the stomach of an easily nitrosable amine – amidopyrine (Ap) – was tested. Then, simulating stomach conditions in vitro, we reacted the nitrite contained in some cured meat products with a commercially available Ap preparation and measured the *N*-nitrosodimethylamine (NDMA) synthesized.

In a second series of tests, we measured in rats the renal excretion of unmetabolized NDMA after oral application of the carcinogen or of the precursors nitrite and Ap. We wished to test, whether, as in dogs given piperazine concurrently with ham (Sander et al. 1973), the estimate of the nitroso compound in the urine can be used to measure its formation *in vivo*. If so, it might be worthwhile to examine the urine of people eating food containing nitrite. Positive results should especially be obtainable from patients known to use Ap.

Further series of tests on the transformation of non-carcinogenic nitroso compounds into carcinogenic ones showed that the use of nitrite as a food additive could result in production of nitroso aminoacids. Decarboxylation of such compounds, especially of *N*-nitrosoproline, has been discussed as a source of carcinogenic nitrosamines. The transformation can occur as a thermal decarboxylation, but also a bacterial decarboxylation was described (Kawabata & Miyakoshi 1975). We therefore tested 12 strains of *Pseudomonas* freshly isolated from patients. The bacteria were incubated under different conditions with nitrosoproline and the reaction mixtures were then analysed for nitrosopyrrolidine.

Materials and methods

Availability of nitrite or amidopyrine in the human stomach

Gastric juice from a volunteer, who had fasted for at least 8 h, was sampled at different times after ingestion of a broth made by boiling 100 g boiled ham in about 100 ml water for 10 min. Portions of gastric content were also recovered by physically induced vomiting 15, 30 or 60 min after ingestion of 100 g ground boiled ham. Nitrite in 10-g samples was estimated by the method of Stoja (1969) after estimation of pH.

Similarily the availability of Ap in the gastric juice was measured by collecting 10-ml samples every 10 min by stomach tube after a volunteer had ingested 100 mg Ap dissolved in 100-ml water. The quantitative estimation of the drug was in dichloromethane extracts by photometry at 273 nm. We checked the stability of Ap in gastric juice, as well as the amounts of Ap metabolites that might influence the photometric estimate.

Reaction of nitrite in cured meat and amidopyrine in vitro; tests with spinach

Boiled ham, raw cured meat, cured boiled pork chops and, in some further tests, frozen spinach was boiled for 10 min and the broth filtered through a sieve. Nitrite was estimated in the ham broth immediately after boiling, but the spinach broth samples were left at room temperature for several hours to permit bacteria to reduce nitrate to nitrite.

Samples of the extracts (170–200 ml) were brought to 37 °C in a water bath. Ap, 100 mg per original sample of 100–150 g, was added in tablet form. During the first 15 of the 25 min, total reaction time the reaction mixtures were gradually acidified to pH 3 with HCl 125 g/litre. The reaction was stopped by adding NaOH 300 g/litre.

In the spinach samples, the time required for bacterial formation of nitrite varied greatly from package to package. To obtain a more complete reaction curve, nitrite was therefore added to several samples. The nitrosation reaction was stopped in the spinach samples by adding sulfamic acid 100 g.

Except in the last tests described (bacterial decarboxylation), NDMA was estimated after steam-distillation and extraction of the NaCl-saturated distillate by GC (Hewlett Packard 5 750 G, 6 ft (1.83 m) glass column, 2.5% OV 17 on Haloport F; column temp. 98 °C; injection port temp. 310 °C, nitrogen specific flame-ionization detector, 400 °C; carrier gas; He).

Renal excretion of nitrosodimethylamine in rats

Rats (SIV_{50} strain, female, bodyweight 260–320 g, groups of 20) were each given 5 ml of NDMA in aqueous solution or Ap immediately followed by sodium nitrite by stomach tube. The rats were kept in metabolic cages and urine was collected in ice-cooled vessels for 4 h. To ensure the collection of all and only the urine produced during the time, the rats were induced to urinate, by applying light pressure on the bladder region, before the administration of the test solutions and

again before removal of the rats from the cages. The urine was extracted thrice with diethyl ether (ether:urine = 2,5:1 by volume) with the aqueous protion ice-cooled between extractions. The extracts were dried with sodium sulfate and concentrated to 0.5 ml. NDMA was estimated by GLC as described above. Known amounts of NDMA, 5–500 μg, extracted from 40-ml urine blanks, gave a procedural recovery of 36%.

Bacterial decarboxylation

From pathological laboratory specimens, 12 strains of *Pseudomonas* species were isolated and were cultured overnight in Mueller-Hinton medium, 500 ml per sample. After centrifuging and washing in physiological sodium chloride solution, the bacterial cells were resuspended in 10 ml sodium chloride solution. For each strain, three flasks containing 100 ml sodium chloride solution were inoculated with 1 ml of that bacterial suspension yielding a number concentration of 10^5 to

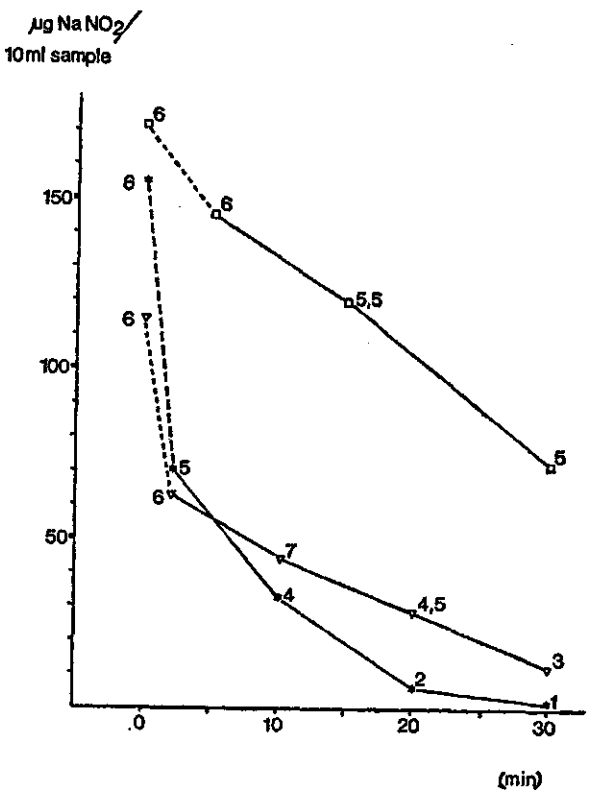

Fig. 1. Decrease of nitrite concentration in 10 ml aliquots of the gastric contents after ingestion of a broth made by boiling 100 g boiled ham in 100 ml water (Experiment 1, △; Experiment 2. *), and after ingestion of 100 g boiled ham (Experiment 3, ▫). Values at t = 0 represent the $NaNO_2$ in the broth, or in experiment 3, the concentration in the meat (10 g) itself, values along the line are pH.

10^6 ml^{-1}. They were incubated at 37 °C for 24 h concurrently with 3 more flasks without bacteria. *N*-Nitrosopyrrolidine at a concentration of 100 µg/litre or *N*-nitrosoproline at a concentration of 500 or 10 µg was added before incubation.

To isolate *N*-nitrosopyrrolidine, 50 ml of potassium carbonate, substance concentration of 0.5 K_2CO_3 3 mol $litre^{-1}$, and 45 g of sodium chloride were added to the flasks after incubation. The samples were then extracted four times with diethyl ether (50 ml, then trice 40 ml). The combined extracts were dried over calcium chloride and concentrated by vacuum distillation to 1 ml. The nitrosopyrrolidine was estimated by GC with a Siemens GC L350 (5% Carbowax 20 M on Chromosorb WAW 60–80 mesh, 2-m column, column temp. 110 °C nitrogen-specific flame ionization detector, 260 °C, carrier gas N). Further tests were with our photolytic method (Sander 1968) to find out whether the nitroso group was split off by the bacteria.

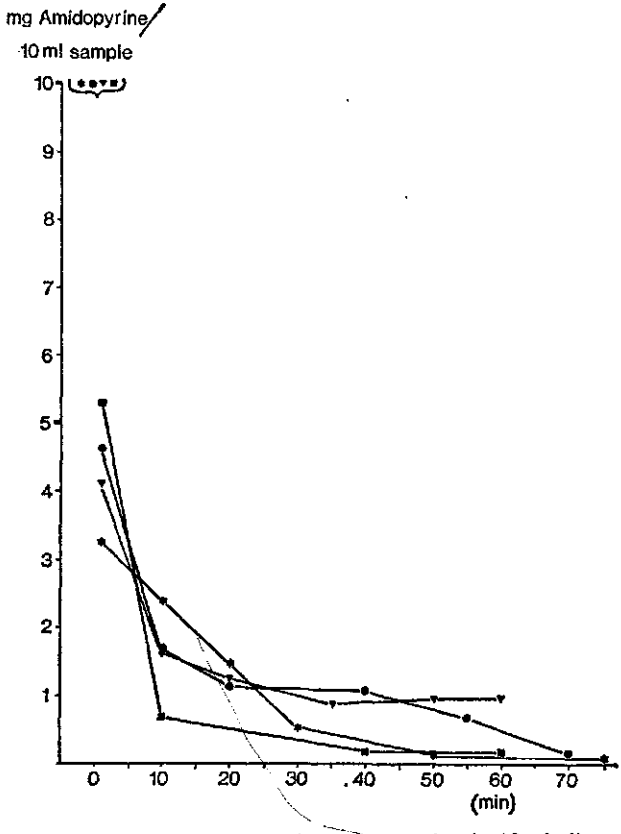

Fig. 2. Decrease of amidopyrine concentration in 10 ml aliquots of the gastric contents after ingestion of 100 mg plus 100 ml water for 4 experiments. Values at t = 0 represent the concentration of the solution ingested. The first measurements were made at t = 1 min.

Results

Tests in the human stomach

According to the acidity of the gastric contents (Fig. 1), resorption and dilution, nitrite in the stomach decreased rapidly when the compound was ingested in broth, amounts of $NaNO_2$ in meat extracts ranging 1.1–5.1 mg for 100 g meat. After 2 min only half the original was found, after 10 min a quarter, and after 20–30 min levels approached those found in gastric juice (corresponding to mass concentration of $NaNO_2$ of 50–500 μg litre^{-1} before ingestion of the test solution (Fig. 1, Tests 1 and 2). When the volunteer consumed boiled ham itself and not a broth, the nitrite remained significantly longer in the stomach (Fig. 1, Test 3).

The recovery of Ap from gastric juice was about 90%. However, through the slight absorbance $A(\lambda = 273\ nm) = 0.05$, produced by the gastric juice itself, the method was intensive at mass concentrations in the sample below 15 mg/litre. Figure 2 shows the results of 4 tests, to find out how long Ap would be available for reaction in the human stomach if ingested at a time when the stomach was otherwise empty. In all tests, only 30–50% of the initial concentration (10 g/litre) was found after

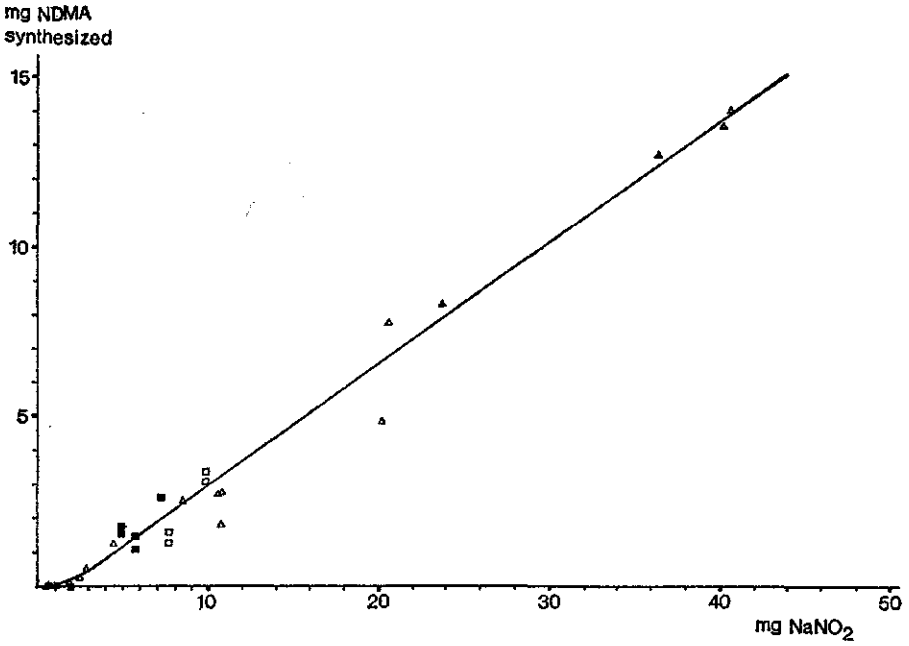

Fig. 3. NDMA synthesized from 100 mg Ap in extracts of 100–150 g boiled ham (□ ■) or spinach (△ ▲). Closed figures: nitrite is already present or naturally occurring in the foodstuffs. Open figures: additional nitrite was added to the extracts. Acidification to pH 3 with 12.5% HCl over a period of 15 min. Total reaction time: 25 min, T = 37 °C. Reaction volumes: 170–200 ml. Two samples containing less than 2 mg nitrite yielded positive (< 15 μg) results for NDMA.

1 min. This concentration decreased rapidly and after 10 min no more than 0.25 g litre^{-1} remained in gastric contents. Since Ap was not degraded in gastric juice, this fall in concentration must be due to transport of the material out of the stomach and to dilution by gastric juice or saliva. The pH was highly variable but had no effect on Ap concentrations.

Reaction in vitro

The recovery of NDMA from ham or spinach reaction mixtures was low (= 8%) but reproducible, and was linear between 62.5 μg and 10 mg. Both alkaline (ham samples) and acidic (spinach samples) distillation yielded the same recoveries. The amounts of NDMA formed during the reaction are shown in Fig. 3. Except for very low amounts of nitrite (2.5 mg in 170–200 ml reaction mixture), the amount of NDMA formed increased rectilinearly with amount of nitrite. Even with only 4–5 mg sodium nitrite in the reaction mixture, several milligrams of NDMA were formed.

Tests in rats

The amount of NDMA recovered in urine from rats during the first 4 h after administration of the compound is illustrated in Fig. 4. Since the amount of nitrosamine excreted was dependent on the volume of urine excreted; all observations were expressed as concentrations in urine. The volumes of urine collected were 11–60 ml for 20 rats.

The lowest oral dose at which NDMA was still detectable in the urine from 20 rats was 12.5 mg, a dosage (on bodyweight basis) of about 2mg/kg. The absolute amounts excreted at that dose were found to be 0.4–2.4 μg, representing a re-

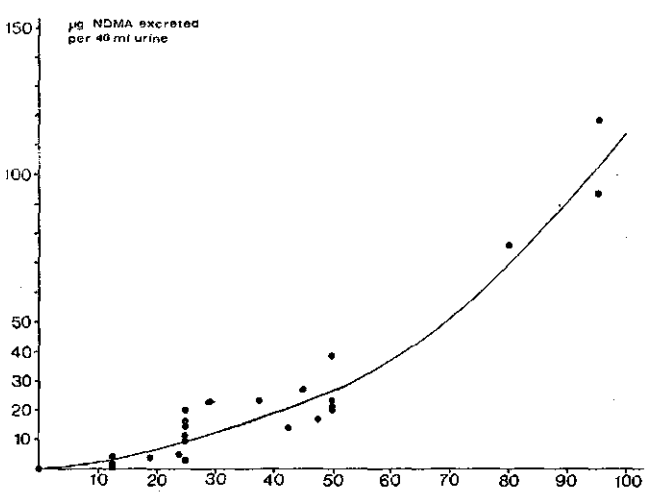

Fig. 4. Excretion of NDMA in the urine of rats collected for 4 h after oral administration of the compound.

covery of only 0 003–0 019%. At higher doses increasingly more nitrosamine was detected in the urine, but even at a dosage of 15 mg/kg the recovery was well below 1%. Although the same rats were always used and the experiments were always conducted at the same time each day, the points were widely scattered, probably because of daily individual differences in absorption, metabolism, and excretion of the compound.

The results of several tests on the synthesis of NDMA in rats are shown in Table 1. If the amounts of NDMA excreted (again highly variable) are averaged, and synthesis calculated from the excretion curve, the extent of reaction can be roughly estimated at 30%. When only one or neither of the precursors was administered, no NDMA was found.

Tests on bacterial decarboxylation

With a detection limit by mass fraction of 2–5 μg/kg, *N*-nitrosopyrrolidine was not found in any of the samples where *N*-nitrosoproline was incubated with *Pseudomonas* species. With the photolytic method, there was no loss of nitroso group by bacterial transformation in any of the test mixtures. Neither was *N*-nitrosopyrrolidine degraded.

Discussion

In our tests in the human stomach, we demonstrated that the availability of the precursors of *N*-nitroso compounds corresponds well to that found in experimental animals (Epstein 1971). Nitrite and amines are rapidly transported from the stomach when ingested in solution. The nitrosamine yield will therefore depend largely upon the time interval between ingestion of the compounds. If a tablet of an easily nitrosable drug is swallowed together with broth or soup containing nitrite, the amount of the nitroso compound formed may well approach the yield in our tests in vitro. It may be much higher if the meat used to prepare the meal contained more nitrite as is usual in Germany. With the relatively low content of residual nitrite in our German products, we found milligram yields of the strongly carcinogenic NDMA when a daily dose of Ap reacted with the nitrite of a normal portion of meat. The use of Ap and some other easily nitrosable drugs like piperazine is widespread. It is also often recommended to take such drugs at mealtimes. For a more solid meal, the yields are probably not much lower. This can also be deducted from tests on dogs with piperazine (Sander et al. 1973). Thus nitrosamines are quite often formed in the stomach in amounts a hundred or a thousand fold those in the nitrite-treated food itself. Therefore nitrite in food has to be reduced as far as possible. Use of other methods to reduce nitrosamine formation, especially from some drugs, is essential.

The problem of transformation of non-carcinogenic nitroso compounds into carcinogenic ones has not yet received due consideration. The tests we have done on the bacterial decarboxylation of nitrosamino acids are only a beginning.

Discussion on the session

NDMA concentration in urine

Considering the low levels of dimethylnitrosamine in urine, one should be aware of metabolic processes in the animal.

Identification of NDMA in the model experiments

The method of identification of NDMA in the model systems with amidopyrine and extracts of cooked, cured ham and spinach was discussed. Sander gave the additional information that the samples had been analyzed for NDMA before and after ultraviolet irradiation. The NDMA peak always disappeared with irradiation, as it should. Still, some doubts were expressed about the certainty of identification. The use of extracts in the model experiments might introduce interfering compounds. For instance, pyrazines, nitriles and halogenated aromatic compounds could interfere during estimation of nitrosamines with a thermionic detector. Telling said that during ultraviolet irradiation some nitramine would be formed which would be easily detectable by electron capture.

Table 1. Nitrosodimethylamine (NDMA) in urine of rats after administration by stomach tube o amidopyrine (Ap) 10 mg per rat immediately followed by $NaNO_2$ 10 mg in aqueous solution per rat t, time (of collection from time of administration); m, mass (or weight); Δm, mass given (or dose) $-\Delta V$, volume lost (by 20 rats); $-\Delta m$, mass lost in urine (assuming 36% recovery by extraction); ρ mass concentration (in urine). Rats were induced to urinate before administration and at the time o collection.

t/h	m(20 rats)/g	$\frac{\Delta m(NaNO_2)}{m(\text{rats}) \cdot 10^{-6}}$	$-\Delta V$(urine)/ml	$-\Delta m$(NDMA)/μg	ρ(NDMA)/μg · litre^{-1}
2	5380	38.1	13.0	1.4	110
4	5320	37.6	24.5	2.7	110
4	5290	37.8	25.0	6.4	258
4	5270	37.9	32.0	3.1	98
4	5230	38.2	32.5	6.2	192
4	5250	38.1	30.5	3.4	112
4	4860[1]	39.1	22.0	4.7	212
4	5410	36.9	36.0	4.7	132
4	6580	30.4	21.0	2.7	130
4	6650	30.1	12.0	1.4	112
6	5250	38.1	43.5	1.28	30
6	5250	38.1	45.0	2.36	52
6	5270	37.9	50.0	2.38	48

1. Group of 19 rats instead of 20.

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Specific detection systems for the chromatographic analysis of nitrosamines

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Introduction

We have previously reported our use of the photohydrolytic production of nitrite from *N*-nitroso compounds as a specific means of detecting this class of compounds by liquid chromatography (Fan & Tannenbaum 1971; Iwaoka & Tannenbaum 1976b). This detection system, in conjunction with high-pressure liquid chromatography (HPLC), has proven especially useful in its ability to specifically reveal *N*-nitroso compounds amidst a complexity of other species. We here report the use of both normal-phase and reverse-phase HPLC, coupled with any of three detectors, to analyse *N*-nitroso species found in both a complex organic reaction mixture and in food extract. We analysed both a nitrosated spermidine, *N*-(3-aminopropyl)-1,4-butanediamine, mixture and an extract of raw bacon, using HPLC in conjunction with the following detectors:

1. an ultraviolet detector (254 nm) (model 440, Waters Associates)
2. a thermal energy analyzer specific to *N*-nitroso compounds (TEA) (Thermo-Electron, Waltham, Mass.)
3. our specific photohydrolysis system, which couples the cleaved nitrite to a Griess reagent, and allows detection of the resultant dye by its absorbance at 546 nm (model 440 detector, Waters). The extract of raw bacon was also analysed using gas chromatography (GC) with the TEA detector.

Experimental and results

Nitrosation of spermidine

The nitrosation was effected by dissolving 1.28 g (5 mmol) spermidine trihydrochloride (Sigma) and 3.10 g (45 mmol) sodium nitrite in 125 ml distilled water. The solution was acidified to pH 3.0–3.5 by slow addition of dilute HCL. The reaction flask was placed in a water bath at 37 °C, where it remained for 3 hours. The reacted mixture was then extracted with 200 ml dichloromethane. The organic extract was shaken with distilled water, and dried over $MgSO_4$. After being dried, the extract was concentrated to a few milliliters by evaporation under nitrogen. The concentrate was finally backwashed with a few milliliters of distilled water. Both the organic and the aqueous phases were examined chromatograpically.

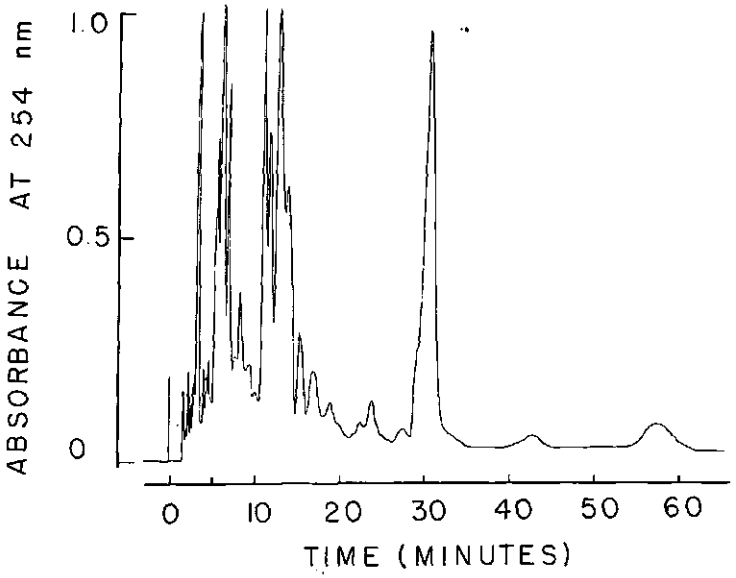

Fig. 1. A 7-µl sample of CH_2Cl_2 extract of nitrosated spermidine separated on the µPorasil column detected at 254 nm. The mobile phase was CH_2Cl_2 and isooctane 3/1 by vol.; flow rate 2 ml/min.

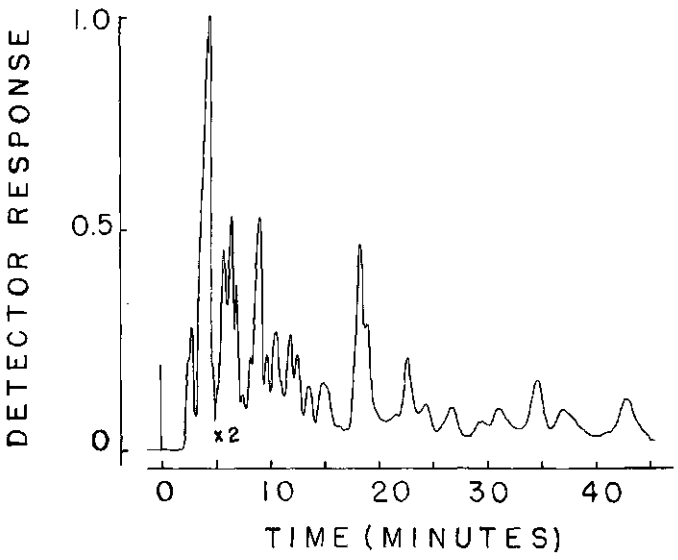

Fig. 2. A 1-µl sample of CH_2Cl_2 extract of nitrosated spermidine, separated on the µPorasil column, detected by TEA. The mobile phase was isooctane and acetone 19/1 by vol.; flow rate 2 ml/min.

Chromatographic examination of the organic phases

Initial examination of the dichloromethane concentrate with a Corasil II (Waters Associates) column 3 ft (0.91 m) long and a 254-nm detector yielded fourteen poorly resolved peaks. Further analysis with a pre-packed μPorasil column (Waters) 1 ft (0.3 m) long revealed some thirty peaks of ultraviolet absorption (Fig. 1). With separations on the μPorasil column and detected by TEA specific for *N*-nitroso, again nearly thirty peaks were revealed (Fig. 2). This large number of fairly well resolved nitroso compounds by HPLC was not matched by our attempts to separate the reaction mixture by GC with a TEA detector. Examination of reference solutions under the same conditions indicated that the biologically important compounds dimethylnitrosamine, diethylnitrosamine, nitrosopyrrolidine and nitrosomorpholine all have elution times similar to the times of peaks in our sample chromatograms. These similarities are useful points of reference, but are no identification of the sample peaks.

Chromatographic examination of the aqueous phase

Examination of the aqueous backwash of the concentrated spermidine reaction mixture on a Partisil ODS reverse-phase column (Reeve Angel) revealed eight or nine species absorbing ultraviolet radiation (Fig. 3). Detection by photohydrolysis

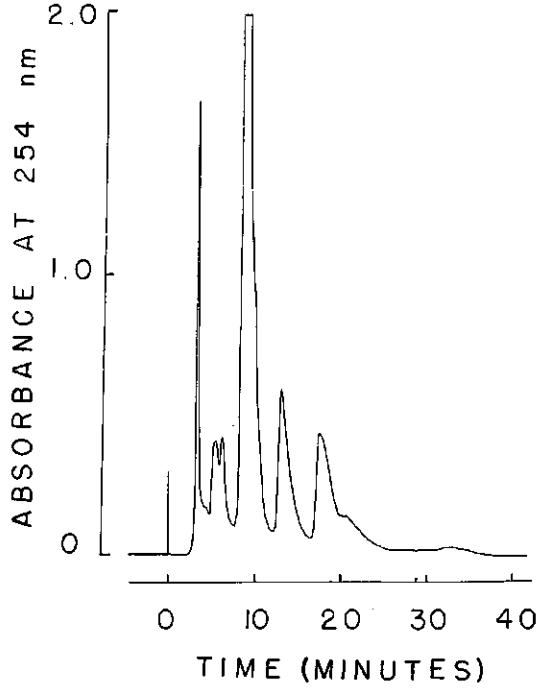

Fig. 3. A 10-μl sample of the aqueous wash of CH_2Cl_2 extract of nitrosated spermidine, separated on the Partisil ODS column, detected at 254 nm. The mobile phase was distilled water; flow rate 1 ml/min.

showed that all of these species, with the possible exception of one, were also *N*-nitroso compounds (Fig. 4).

Analysis for nitrosoamino acids in raw bacon

The bacon was extracted and further treated as described in Appendix 1. The bacon was a commercial sample with nitrosopyrrolidine at a mass fraction of 50 mg/kg after frying for 3 min per side at 170 °C. The final evaporation of the CH_2Cl_2 extract of the raw bacon was stopped when the volume had been reduced to 2 ml. The concentrate was divided into two 1-ml portions, one of which was analysed by GC, the other by HPLC.

GC analysis of bacon extract

The 1-ml CH_2Cl_2 extract was transferred to a reaction vial and evaporated to dryness. The residue was reacted with 1 ml isopropenyloxytrimethylsilane (Applied Science, State College, Pa).

GC conditions: column 6 ft (0.9 m) long, inner diam. 2.2 mm filled with 3% OV-17 on Chromosorb G 80/100
carrier helium; flow rate 30 ml/min
oven temp. 120 °C
injector temp. 175 °C
detector TEA

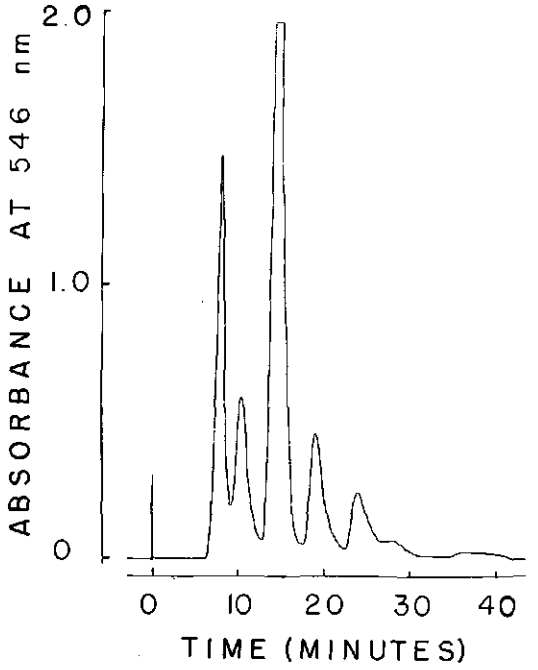

Fig. 4. A 10-µl sample of the aqueous wash of CH_2Cl_2 extract of nitrosated spermidine, separated on the Partisil ODS column, detected with the photohydrolysis system. The mobile phase was distilled water; flow rate 1 ml/min.

The chromatogram (Fig. 5) shows a peak at the same retention time as an authentic sample of trimethylsilylnitrosoproline (TMS-NPro). Other smaller peaks can be seen, corresponding to unknown compounds in the sample, which may be *N*-nitroso compounds.

The amount of NPro in the original bacon sample was estimated by comparing the area of the GC peak to that of a known amount of dibutylnitrosamine. The TEA responds to *N*-nitroso compounds on a molar basis, so the areas of different compounds can be compared if they are corrected for molecular weight. The GC data indicate an original mass fraction of 70 μg/kg of NPro in the raw bacon.

HPLC analysis of bacon extract

The other 1-ml portion of the bacon extract was also evaporated to dryness and then taken up in 0.5 ml water. Analysis of this aqueous sample on a pre-packed μC_{18} Bondapak column (Waters) using a 254 nm detector revealed some eighteen peaks (Fig. 6). Photohydrolytic detection, however, yielded at most four (Fig. 7, top). Comparison with a NPro reference indicated that the first two closely spaced peaks in the sample chromatogram corresponded to NPro, the doublet resulting from the separation of the syn and anti conformers of the molecule (Fig. 7, top; Iwaoka & Tannenbaum 1976a). The final two peaks of the chromatogram were not revealed by GC-TEA analysis, and have yet to be identified.

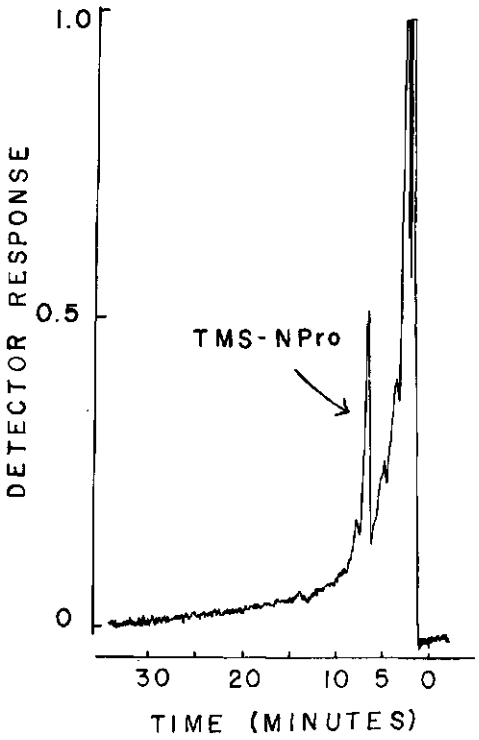

Fig. 5. A 1.7-μl sample of CH_2Cl_2 extract from raw bacon after work-up for NPro analysis and trimethylsilylation. Separated by GC on OV-17, with TEA detector. TMS-NPro, trimethylsilylnitrosoproline.

Comparison of ultraviolet absorbance of the NPro reference and the bacon extract indicated that the raw bacon originally contained a mass fraction of about 80 μg/kg.

Discussion

The selectivity of our photohydrolytic detection system is especially evident in the analysis of raw bacon. Even though the cleanup and extraction procedure is specific for anionic species, HPLC separation followed by ultraviolet detection still reveals quite a few species. Photohydrolysis, however, selectively revealed only the *N*-nitroso compounds, and the many other species present in greater amounts did not interfere. This easy elimination of non-nitroso background made extensive clean-up unnecessary. The photohydrolytic detection system is sensitive as well, capable of detecting *N*-nitroso compounds in amounts of less than 50 ng per injection.

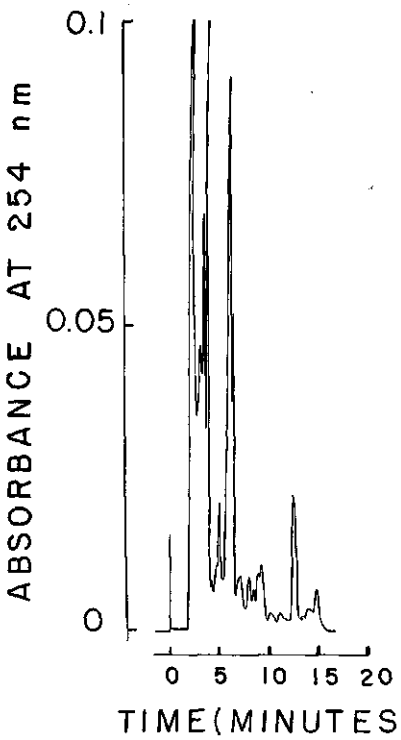

Fig. 6. Raw bacon extract, taken up in water, separated on the C_{18} μBondapak column, detected at 254 nm. The mobile phase was Na_2HPO_4 in distilled water 10 g/litre; flow rate 1.5 ml/min. The injected volume was 30 μl.

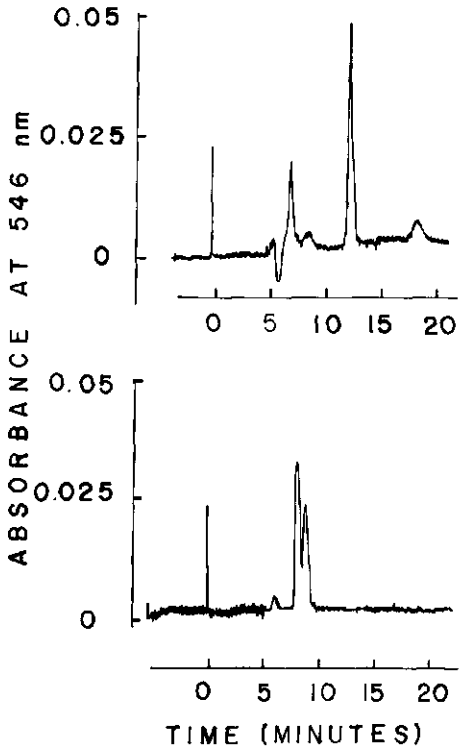

Fig. 7. Separation on C_{18} μBondapak column with mobile phase of Na_2HPO_4 in distilled water 10 g/litre; flow rate 1.5 ml/min. Top: Raw bacon extract taken up in water. The injection size was 30 μl. Bottom: Nitrosoproline reference in water.

The analysis of nitrosated spermidine demonstrates the suitability of HPLC in separating the many *N*-nitroso compounds present in the mixture. Because HPLC is non-destructive, it is especially useful in collecting fractions for further characterization.

A comparison of HPLC separations of the aqueous wash of reacted spermidine as detected by 254 nm and as detected by photohydrolysis (Fig. 3 and 4) shows that the latter detection system, though it involves the addition of considerable dead space between the column and the detector, results in only a small loss in resolution. The chromatograms of the aqueous wash appear to contain far fewer components than those of the CH_2Cl_2 extract, but this is not actually so, as preliminary results using a more efficient reverse-phase column indicate that each of the major peaks can be resolved into several others. This implies that the backwash is similar in complexity to the CH_2Cl_2 extract for *N*-nitroso compounds. It would in fact be expected that all but the least polar of the *N*-nitroso compounds present in the CH_2Cl_2 extract would partition back into water to some extent.

The identity of several of the major *N*-nitroso compounds resulting from the nitrosation of spermidine, notably nitrosopyrrolidine and γ-butenyl-(β-propenyl) nitrosamine, has been previously established (Bills *et. al.* 1973; Hildrum *et. al.* 1975), yet it is evident from our work that many more *N*-nitroso compounds can be resolved by chromatography and could be identified.

Comparison of photohydrolysis and TEA, the two selective detection procedures on the nitrosated spermidine, reveals essentially identical selectivity for *N*-nitroso compounds. It was convenient to use photohydrolysis for an aqueous eluant and TEA for an organic solvent eluant. The TEA is orders of magnitude more sensitive than photohydrolysis, but use of both techniques provides the analyst with greater flexibility in technique and types of chromatographic columns. It would be useful to apply both techniques when the structure of the unknown(s) is uncertain.

The agreement on the mass fraction of nitrosoproline in the raw bacon between the GC and HPLC procedures is excellent (70 and 80 μg/kg). Several other samples of raw bacon produced in the order of 10 μg/kg of nitrosopyrrolidine upon frying, but failed to reveal the presence of nitrosoproline. Thus these results cast doubt on the significance of pre-formed nitrosoproline as a nitrosopyrrolidine precursor.

Acknowledgment

Financial support was provided by Grant 2-PO1-ES00597 and Public Health Service Contract NO1-CP-33315 from the Public Health Service. We thank Dr David Fine and Dr Tsai-yi Fan of the Thermo-Electron Corp., Waltham, M for use of this TEA-HPLC system.

Appendix 1. Procedure for extraction of nitrosoamino acids from bacon

1. Homogenize 100 g of raw bacon in 0.15 litre of distilled water. Make sure a representative sample of the bacon is obtained. Use 50 ml of water to rinse out homogenizer.
2. Centrifuge homogenate at 5 000 rev. per min and 0 °C for 5–10 min or until

the fat solidifies in the centrifuge bottle. Carefully pour off supernatant and store at 4 °C.

3. Repeat homogenization and centrifuging steps twice more with bacon solids and combine 3 supernatant fractions. Discard solids.

4. Filter chilled supernatant fraction with Buchner funnel to remove remaining solidified fat and meat particles.

5. Add filtered supernatant to ion-exchange column at a flow rate of 3–5 ml/min. Column is a glass tube 20–30 in (50–75 mm) long, 1 in (25.4 mm) inner diam, filled with 100 ml of Dowex 2 2 x 8–100 strongly basic anion-exchange resin in the chloride form. Discard column eluate.

6. Wash column with 300 ml of distilled water or until eluate is clear. Flow rate 5–7 ml/min. Discard eluate.

7. Elute nitrosoamino acids from ion-exchange column with 500 ml of NaCl 1 mol/litre adjusted to pH 1 with HCl. Flow rate about 5–7 ml/min.

8. Remove water from column eluant on rotary evaporator connected to an aspirator vacuum. Keep water-bath at 50 °C. Be sure residual salts are absolutely dry. If salts are not completely dried, the nitroamino acids are difficult to extract.

9. Extract nitrosamino acids from salts with ten portions of 50 ml of dichloromethane (CH_2Cl_2).

10. Combine all CH_2Cl_2 extracts and concentrate in a 500 ml Kuderna-Danish evaporator with a graduated test-tube bottom. Use a straight glass chimney instead of a Snyder column on top of the evaporator. Keep water-bath at 60 °C.

11. Concentrate sample in Kuderna-Danish until about 5 ml of CH_2Cl_2 remains. Remove apparatus from water bath and cool the external surface of the evaporator with cold water to condense volatile CH_2Cl_2. Remove graduated test-tube and evaporate CH_2Cl_2 to 2 ml.

Discussion on the session

Nitrosation of spermidine

With the analytical techniques used, specific for *N*-nitroso compounds, about 30 peaks were observed after nitrosation of spermidine. Only a part of these were confirmed to be *N*-nitrosamines. The other peaks have not been identified yet. Spermidine was selected, as it occurs in some foods (e.g. cereals, organs, meat) at fairly high contents.

Precursors of nitrosopyrrolidine in fried bacon

One possible precursor is nitrosoproline formed – not in the raw bacon, but during the frying process – by the nitrosation of proline. Nitrosoproline could rapidly break down to give nitrosopyrrolidine. However, there may be other precursors as well. For instance, collagen can yield nitrosopyrrolidine on pyrolysis in the presence of nitrite.

Nitrosoalkylcyanimides

Dr Tannenbaum also presented some recent results of a study on nitrosoalkylcyanimides. In saliva, primary amines appear to be converted by bacteria into small

amounts of alkylcyanamides. In the stomach nitrosation takes place, resulting in the formation of highly mutagenic and carcinogenic nitrosoalkylcyanamides. Contents up to 10 μg/kg have been detected, despite a half-value time of these compounds smaller than 1 min. They are powerful alkylating agents. Aliphatic as well as aromatic amines can be converted.

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen.

Analysis, formation and occurrence of volatile and non-volatile N-nitroso compounds: recent results

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Abstract

In 17 out of 52 cured meat products, volatile nitrosamines (1–66 μg/kg) were detected. After frying, all of the products tested (18) contained nitrosamines. Contents of *N*-nitrosopyrrolidine and *N*-nitrosopiperidine were substantially higher in most samples by the frying process. A method for the detection of *N*-nitroso-3-hydroxypyrrolidine at levels of μg/kg in foods was developed. In 7 out of 11 different cured meat products, the compound was detected at contents of up to 9 μg/kg after frying. Heat-induced decarboxylation of *N*-nitrososarcosine, *N*-nitrosoproline and *N*-nitroso-3-hydroxyproline was studied in model systems and the relationship between heating temperature and yield of nitrosamines investigated. A method of trace analysis for nitrosamino acids in foods was developed.

Introduction

The occurrence of carcinogenic nitrosamines in several types of foods is well established. They have been found in a wide range of different commodities, such as cured meat products, fish and cheese. *N*-Nitrosopyrrolidine has been found regularly in cooked bacon (Reviews: Fiddler 1975; Scanlan 1975). As yet, there are few reliable data on the occurrence of nitrosamines in German food products (Eisenbrand et al. 1975). We therefore analysed a first series of samples, mainly cured meat products, from the German market. In some of these samples, we analysed for nitrosamines before and after frying. The formation of volatile nitrosamines by heat-induced decarboxylation of nitrosamino acids was studied in silicone oil and by frying of ham. In these model tests *N*-nitroso-3-hydroxypyrrolidine was formed from *N*-nitroso-3-hydroxyproline at a rate similar to that for *N*-nitrosopyrrolidine from N-Nitrosoproline. An analytical procedure was developed for estimation of *N*-nitroso-3-hydroxypyrrolidine and for nitrosamino acids in foods.

Materials and methods

Reagents were analytical grade; solvents were purified and distilled before use (Eisenbrand et al. 1975a). Unlabelled and ^{14}C-labelled nitrosamino acids were prepared according to published procedure (Hansen et al. 1974), with slight modifications. N-Nitroso-3-hydroxypyrrolidine was prepared by a modification of a method described by Krüger & Bertram (1975).

Origin and preparation of samples for estimation of volatile nitrosamines

Food samples were obtained from local stores. Nitrite and nitrate contents were estimated colorimetrically (ISO-3 091 1975). Some samples were fried after analysis in a normal frying procedure in an open pan: the minced sample (100 g) was cooked for 4 min on one side and for 2 min on the other at about 185–200 °C. The frying temperature was controlled by a thermoelement (Heraeus Fus-O-MAT); when necessary (for meat loaf) 5–10 ml of vegetable oil, ready heated to 180 °C, was used to simulate normal frying conditions. After cooling, the samples were analysed for nitrosamines.

The analytical procedure for volatile nitrosamines is outlined in Figure 1. The method consists of distillation from weakly alkaline medium in vacuo, extraction of the acidified distillate with dichloromethane, evaporation to a small volume and detection of nitrosamines by two complementary methods: a) direct with a modified Hall electrolytic conductivity detector (ECD) in pyrolytic mode and b) formation of heptafluorobutyryl derivatives after acid-catalysed denitrosation of nitrosamines and mass fragmentography in a low-resolution GC-MS instrument. As internal references, *N*-nitroso-[^{14}C]dimethylamine (^{14}C-NDMA) and *N*-nitrosodi-*n*-propylamine (NDPA) were added to each sample. The method has been described in detail recently (Eisenbrand et al. 1975a). Apparently, positive results by either method, which were not confirmed by the other, were regarded as negative. Whenever possible, but not always, positive results were confirmed by GC with high resolution MS.

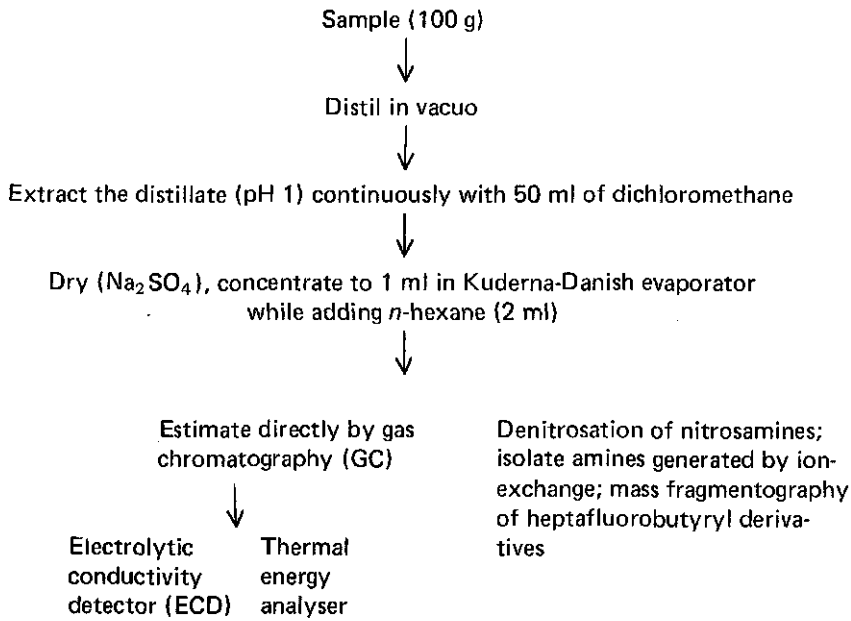

Fig. 1. Isolation and estimation of volatile nitrosamines from foods.

Heat-induced decarboxylation of nitrosamino acids (8.47 μmol in 0.1 ml acetonitril in silicone oil (2 ml) was studied in sealed reaction tubes immersed for 10 min in an oil bath held at specified temperatures (100–250 °C). After heating, the tubes were cooled to −60 °C and the contents transferred into a distiller. Nitrosamines were isolated by distillation in vacuo and extraction of the distillates with dichloromethane (Fig. 1). To correct for losses during working-up, ^{14}C-NDMA was added as an internal reference to the decarboxylation mixture of nitrososarcosine, and *N*-ni-

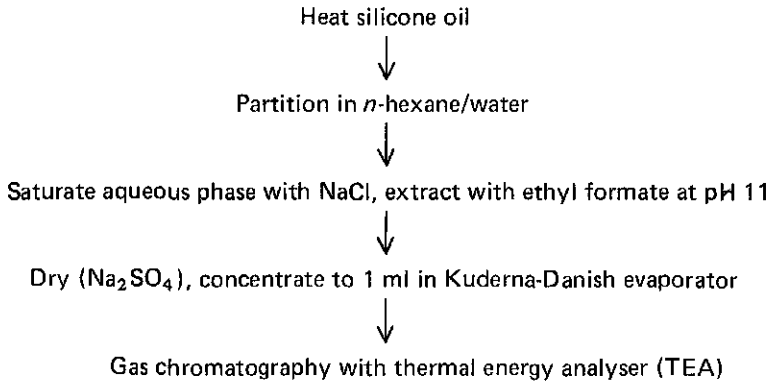

Fig. 2. Isolation and estimation of *N*-nitroso-3-hydroxypyrrolidine from silicone oil. Recovery 76%; 7 tests; standard deviation 3.8.

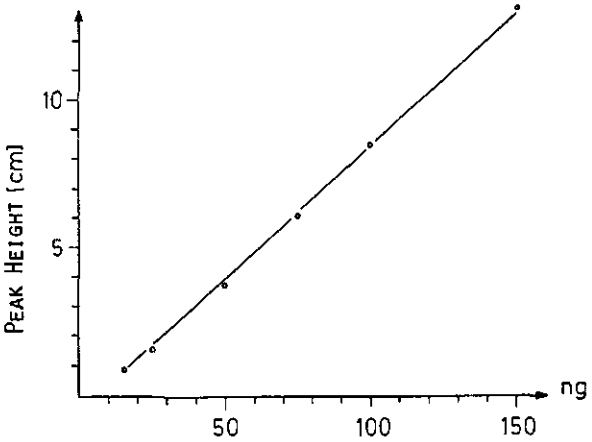

Fig. 3. Calibration graph for *N*-nitroso-3-hydroxypyrrolidine detected by thermal energy analysis. Column 1.8 m long, 2.2 mm inner diam. glass; 10% OV 225 on 80/100 Gas Chrom Q; carrier gas argon; flow rate 45 ml/min. Temp. column 220 °C; injection port 225 °C.

troso-di-*n*-butylamine (NDBA) to that of nitrosoproline. Compounds were detected by ECD.

Decarboxylation of N-nitroso-3-hydroxyproline

Sealed tubes were heated as above. After cooling, the reaction mixture was transferred into *n*-hexane (10 ml) and the organic phase extracted thrice with 1 ml water. The pooled, aqueous phases were saturated with NaCl, adjusted to pH 11 and extracted thrice with 12 ml ethyl formate. *N*-Nitroso-3-hydroxypyrrolidine (NHPyr) in the concentrated organic phase was estimated by gas chromatography with a TEA detector (Fine et al. 1975) by comparison with external references (Fig. 2). A calibration graph for NHPyr was linear in the selected range (15–150 ng; Fig. 3).

Decarboxylation of N-nitrosoproline and N-nitrososarcosine during frying of ham

Samples of comminuted ham (50 g), fortified with nitrosoproline and nitrososarcosine (40 µmol in 1 ml H_2O), were cooked in a frying pan in 3 ml of vegetable oil. The temperature of the oil was controlled by a thermoelement. Temperatures of 180–190 and 210–220 °C were reached after 4 min; the samples were turned and held at these temperatures for another 4 min. After cooling, the samples were transferred into a 1-litre round-bottomed flask, ^{14}C-NDMA and NDBA were added as internal references and the nitrosamines isolated as described (Fig. 1). Two samples of ham, fried without added nitrosamino acids, served as references for calculating nitrosamine yields. At the selected sensitivity, these samples showed no trace of nitrosamine.

Decarboxylation of N-nitroso-3-hydroxyproline during frying of ham

Frying was as above. After cooling, 50 g fried ham was homogenized with 10 g Celite 545 in 100 ml of methanol and water (10/1 by vol.). The homogenate was filtered through a Büchner funnel and the residue was extracted twice more in the same way first with 100 ml and then 150 ml of the solvent. The residue was finally rinsed with another 150 ml of the solvent. The filtrate (500 ml) was concentrated in a rotary evaporator to about 50 ml and further worked up as in Figure 4, except for the column chromatographic step which was not needed in these tests. In the final concentrate, NHPyr was estimated by gas chromatography with a TEA detector as described; chromatograms were free from interference (Fig. 5).

Distribution between fried product and steam of nitrosamines generated from N-nitrosoamino acids

Ham samples, fortified with *N*-nitrosoamino acids (40 µmol in 1 ml H_2O) were cooked in a flat-bottomed flask connected via a distillation bridge (ground joints) to a trap held at −80 °C. After frying, the bridge was rinsed with water and the washings added to the contents of the trap. The fried ham and the contents of the trap were worked up separately and analysed for nitrosamines as described. Unfor-

tified samples served as reference for calculation of nitrosamine yields; they showed no trace of the respective nitrosamines at the selected sensitivity.

Estimation of N-nitroso-3-hydroxypyrrolidine in foods

Food samples (50 g) were worked up as outlined in Figure 4. Sample concentrates (0.2 ml) were analysed by GC-TEA for NHPyr before column chromatography; columes of 1 µl were injected. When the TEA detector indicated the presence of a nitrosamine, the concentrate was put onto a column 5 cm high and 6 mm

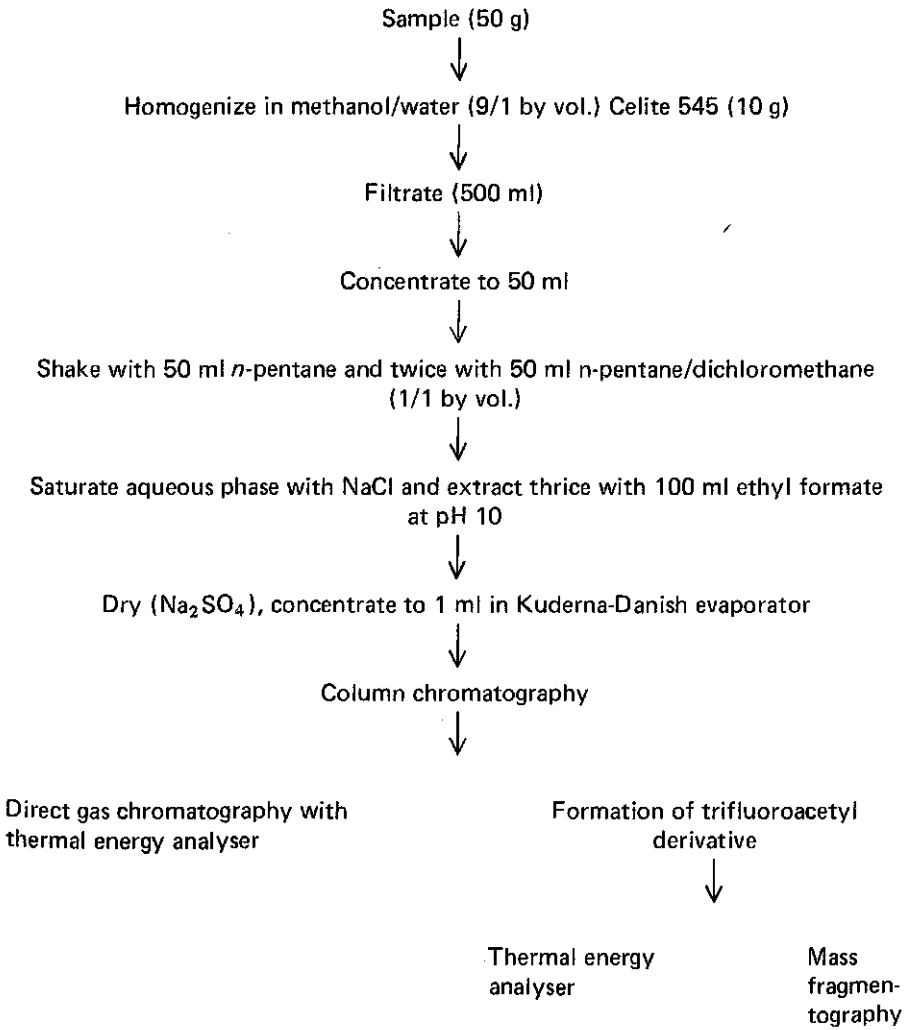

Fig. 4. Isolation and estimation of *N*-nitroso-3-hydroxypyrrolidine from foods. Recovery 55%; 7 tests; standard deviation 3.8.

inner diam. of basic alumina (activity 1, Merck) covered with 2 cm of strongly acidic SE-cellulose (Serva, Heidelberg). The column was eluted with acetone and dichloromethane (1/1 by vol.), the first 20 ml of the eluate contained the nitrosamine. After concentration to 0.2 ml, 4 µl of the eluate were injected and the compound again detected by TEA.

Trifluoroacetylation of N-nitro-3-hydroxypyrrolidine and GC-TEA

Acetonitrile (0.3 ml) was added to the sample of concentrate (0.2 ml in CH_2Cl_2 and acetone), which was concentrated to 0.1 ml. *N*-Methyl-bis-trifluoroacetamide (MBTFA) (0.1 ml) was added and the mixture heated in a sealed vial at 60 °C for 2 h. Aliquots of 4 µl were introduced.

Estimation of N-nitrosoamino acids in foods

Food samples (50 g) were worked up as outlined in Figure 6. The final residues after evaporation of the ion-exchange eluates were reacted with 200 µl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) as previously (Eisenbrand et al. 1975a). The trimethylsilyl were separated by gas chromatography with the TEA detector. N-nitroso-[^{14}C]sarcosine and *N*-nitroso[^{14}C]proline served as internal references.

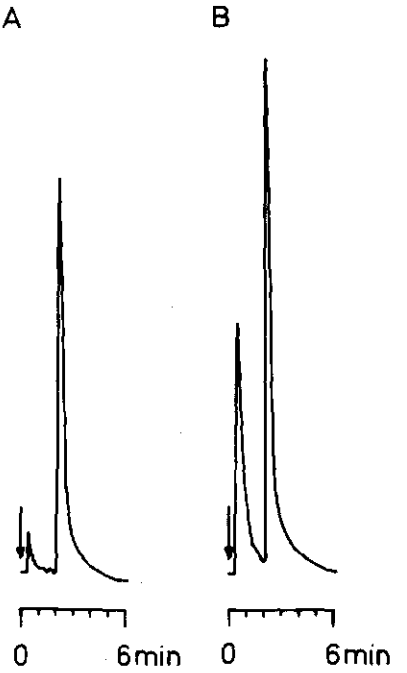

Fig. 5. Decarboxylation of added *N*-nitroso-3-hydroxyproline during frying of raw ham (roher Schinken). Column 1.8 m long, 2mm inner diam. glass; 10% OV 225 on 80/100 Gas Chrom Q; carrier gas argon; flow rate 45 ml/min.

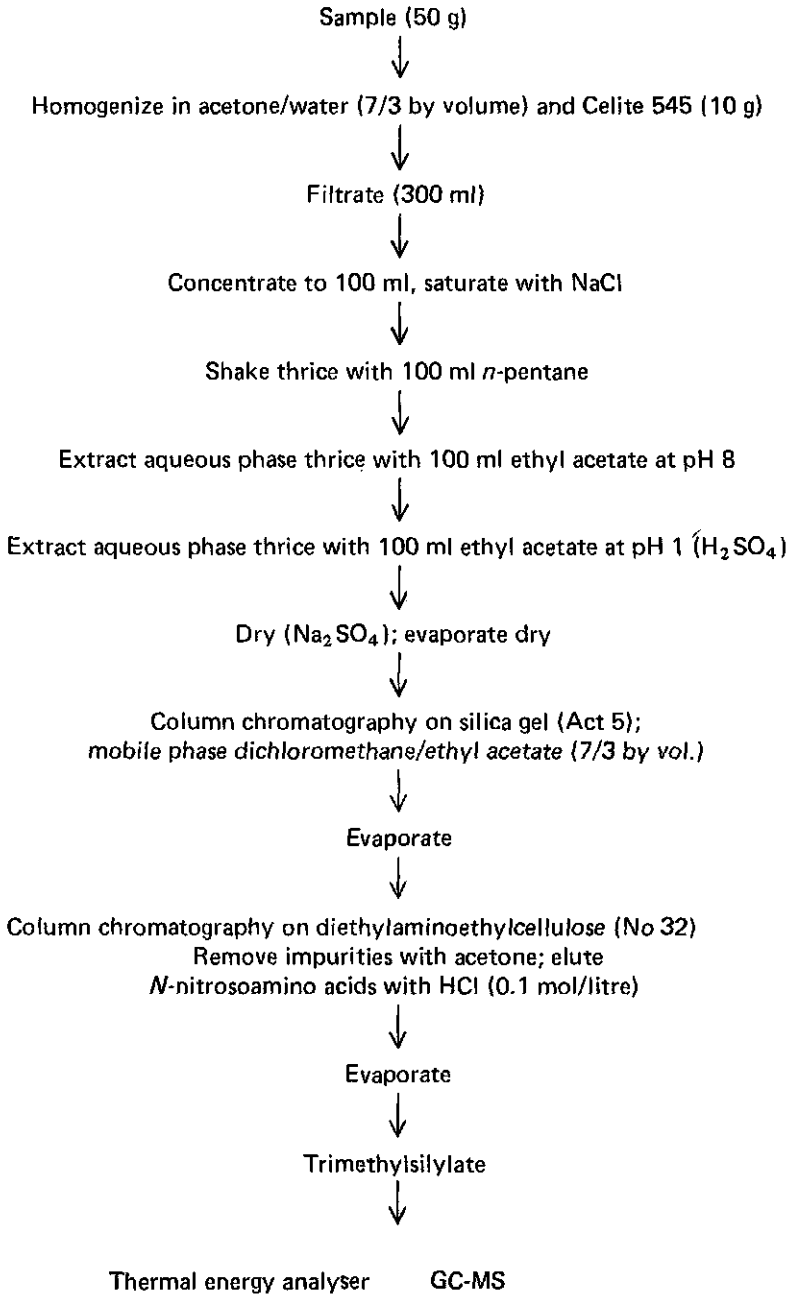

Fig. 6. Isolation and estimation of *N*-nitrosamino acids from foods.

Gas chromatography

Volatile nitrosamines and heptafluorobutyryl derivatives Columns and separation conditions were essentially the same as previously (Eisenbrand et al. 1975a).

N-Nitroso-3-hydroxypyrrolidine. A glass column 1.8 m long and 2mm inner diameter with 10% OV-225 on 80/100 mesh Gaschrom Q was connected via a glass-lined steel capillary (20 cm long; 0.5 mm inner diam.; make SGE) to the pyrolytic furnace of the TEA detector. The gas chromatograph was a VARIAN 1 520 instrument. For decarboxylation experiments, the temperatures were column 220 °C and injection port 225 °C. For analysis of food samples, a temperature programme was used: initial temp. 100 °C, after 10 s programmed at 10 °C/min^{-1} up to 230 °C. Carrier gas argon; flow rate 45 ml/min.

Trifluoroacetyl derivative of N-nitroso-3-hydroxypyrrolidine. A glass column 3 m long and 2 mm inner diameter with 3% QF1 on 80/100 mesh Gaschrom Q was connected to the TEA detector as described. Temp. column 10 s isothermal at 115 °C, then programmed at 4 °C/min^{-1} up to 140 °C; injection port 230 °C. Carrier gas argon; flow rate 40 ml/min.

Trimethylsilyl derivatives of N-nitrosamino acids. A glass column 1.2 m long and 2 mm inner diameter with 6% OV 17 on 80/100 mesh Gaschrom Q was connected to the TEA detector as described. Temp. column 2 min isothermal 115 °C, then programmed at 10 °C/min^{-1} up to 210 °C; injection port 230 °C. Carrier gas argon; flow rate 40 ml/min.

High resolution GC-MS. An AEI-MS 902S mass spectrometer was used at a resolution of 10 000–13 000. Molecular ions of the respective nitrosamines were detected by the peak-matching technique, using appropriate fragments of perfluorokerosene as references. An unsilanized glass column (1.8 m long; 2 mm inner diam.) with 3% DEGS on unsilanized chromosorb W was connected via membrane separator to the ion source. These measurements were done by Dr Fehlhaber (Farbwerke Hoechst).

Table 1. Nitrosamines found in meat products. *w*, mass fraction; NDMA, *N*-nitrosodimethylamine; NPip, *N*-nitrosopiperidine; NPyr, *N*-nitrosopyrrolidine; +, confirmed by mass spectrometry; ·, not estimated.

No	Type	*w*(nitrosamine)/μg · kg^{-1}		*w*(nitrite)/ mg · kg^{-1}	*w*(nitrite)/ mg · kg^{-1}
		uncooked	cooked		
367 356	Liver loaf	NDMA 1	NDMA 1+	4	22
361 351	Meat loaf	NDMA 8+	NPip 2 NPyr 5	4	10
362 352	Meat loaf	NDMA 2+		3	17

365 354	Meat loaf	NDMA 1	NDMA 1 NPip 1 NPyr 6	4	7
301 298	Peppered ham Pfefferschinken	NPip 4+ NPyr 8+	NDMA 1+ NPip 10+ NPyr 27+	2	239
200	Peppered ham Pfefferschinken Outer layer of lean portion	NDMA 54 NPip 250 NPyr 15		84	24
313	Peppered ham Pfefferschinken Whole sample 200	NDMA 3+ NPip 66+ NPyr 15+		84	24
328 329	Peppered ham	NDMA 1	NDMA 6+ NPip 64+ NPyr 46+	16	57
309 312	Type of ham, Smoked Katenschinken	NDMA 6+ NPip 2+	NDMA 3 NPip 2 NPyr 9	16	593
303 304	Type of ham, Smoked Rauchfleisch	NDMA 1	NDMA 2+ NPyr 3	77	262
358 347	Type of bacon Dürrfleisch	NDMA 2+	NDMA 1 NPip 4 NPyr 32	15	28
360 349	Type of bacon Dürrfleisch	NDMA 2	NDMA 4+ NPip 4+ NPyr 40+	164	141
359 348	Type of bacon Schwarzwälder Speck	NDMA 1	NDMA 2+ NPyr 25+	1	890
357 346	Type of bacon Bauchspeck Geräuchert	NDMA 3+ NPyr 17	NDMA 1+ NPip 1 NPyr 10	7	12
299 300	Type of bacon Dürrfleisch	NDMA 1	NDMA 1+ NPip 2+ NPyr 35+	104	167
342 369	Pepper-salami Pfeffersalami	NDMA 2+ NPip 4 NPyr 2	NDMA 12+ NPip 50+ NPyr 11+	1	58
341 371	„ „	–	NDMA 5+ NPip 15+ NPyr 12+	1	13
338 372	„	–	NDMA 3 NPip 18 NPyr 7	–	62
339 373	„	NDMA 1+ NPip 6+	NDMA 3 NPip 25 NPyr 7	1	5
344 374	„	NDMA 10+	NDMA 1 NPip 6 NPyr 9	1	16

Results and discussion

Content of volatile nitrosamines in cured meat products

A total of 52 cured meat products, including different types of bacon, ham and fermented sausages were analysed. In 17 of these products (a third) nitrosamines were detected in contents of 1–66 μg/kg. NDMA (1–10 μg/kg) was found in 16 of the positive samples, NPyr (2–17 μg/kg) in 5 and NPip (2–66 μg/kg) also in 5 of the samples. In 11 samples, only one nitrosamine (NDMA) was found, in 4 two and in 3 all three nitrosamines. All but one of the positive samples and two negatives (pepper salami) were fried under the conditions specified. All of the fried samples (18) contained nitrosamines; in most samples, the content increased or *N*-nitroso compounds not detectable before cooking were found afterwards (15 samples). The content of NDMA decreased in 6 samples but increased in 9, including those, in which the nitrosamine could not be detected before cooking. The content of NPyr decreased in 1 sample, but increased in 14, including 12 in which it was not detectable before. The content of NPip did not decrease after cooking, but increased in 13 samples, including 11 in which it was not detectable before. Results obtained from 4 different types of meat products are listed in Table 1: liver loaf and meat loaf, ham, bacon, and salami sausage.

Table 2. Mass fractions (w) of nitrosamines in different types of meat product before (u) and after (c) cooking. NDMA, *N*-nitrosomethylamine; NPip, *N*-nitrosopiperidine; NPyr, *N*-nitrosopyrrolidine; n, number of samples.

Type	n	$w/\mu g \cdot kg^{-1}$					
		NDMA		NPip		NPyr	
		u	c	u	c	u	c
Bacon	5	1.8	1.8	0	2.4	3.4	28.4
Liver loaf, meat loaf	4	3	1.3	–	0.8	–	2.8
Pepper salami	5	2.8	4.8	2	22.8	0.4	9.2
Ham	4	2	3	1.5	19	2	21.3

Table 3. Decarboxylation of added *N*-itrosamino acids (40 μmol in 50 g) during frying of ham (roher Schinken) at different temperatures n, number of samples; $\bar{x}$, mean; $s(\bar{x})$, standard error of the mean.

N-Nitrosoamino acid added	*N*-Nitrosamine found	n	Yield (% of theoretical)			
			180 – 190 °C		210 – 220 °C	
			$\bar{x}$	$s(\bar{x})$	$\bar{x}$	$s(\bar{x})$
N-Nitrososarcosine	*N*-Nitrosodimethylamine	6	2,3	0,3	2,8	0,2
N-Nitrosoproline	*N*-Nitrosopyrrolidine	6	0,24	0,03	0,42	0,04
N-Nitroso-3-hydroxyproline	*N*-Nitroso-3-hydroxy-pyrrolidine	5	2,6	0,4	2,7	0,3

There was no correlation between contents of nitrosamines and of residual nitrite. This result is in contrast to the findings of Sen et al. (1976c), who concluded from their analytical work on fried bacon that nitrosation reactions by residual nitrite, occurring during cooking of bacon, play a greater role, at least in the formation of NPyr than the decarboxylation of *N*-nitrosoproline. A plausible explanation for this disagreement can be found in the different frying conditions: in the work of Sen et al. (1976c), most of the bacon samples were fried at a much lower temperature (134–142 °C) than in our tests (185–200 °C). Decarboxylation of *N*-nitrosamino acids occurs at a substantial rate only at temperatures above 150 °C.

Mean contents of NDMA either remained constant (bacon) or increased only slightly with cooking (Table 2). However contents of NPip and NPyr were substantially higher after cooking in all 4 types of product; in meat loaf, they were detectable after cooking but not before. In the other groups, contents increased by factors of up to 23.

Table 4. Decarboxylation of added *N*-nitrosamino acids (40 µmol in 50 g) during frying of ham (Roher Schinken) in a closed system; ratio of amount of nitrosamines in ham to that in steam, and proportions (range between samples).

N-Nitrosoamino acid added	*N*-nitrosamine formed	*n*	Ratio		Proportion	
			$\bar{x}$	$s(\bar{x})$	ham	distillate
N-Nitrososarcosine	N-Nitroso-dimethylamine	5	0,28	0,07	17 – 26	74 – 83
N-Nitrosoproline	N-Nitroso-pyrrolidine	5	1,08	0,17	48 – 55	45 – 52
N-Nitrosohydroxy-proline	N-Nitroso-3-hydroxy-pyrrolidine	5	18,7	7,9	92 – 96	4 – 8

Table 5. Mass fractions (w) of *N*-Nitroso-3-hydroxypyrrolidine (*NHPyr*) found in meat products by TEA detector. –, not detected; ·, not estimated.

No	Type of product	w(NHPyr)/µg · kg^{-1}		w(nitrite)/mg · kg^{-1}
		uncooked	cooked	
1	Ham		–	4
5		–	9	68
11		–	5	
3	Boiled ham	< 1	2	33
7		·	–	13
10	Salami	–	< 1	2
9	Pepper salami	–	5	2
2	Meat loaf	·	2	9
6		·	–	5
4	Bologna	·	–	30
8		–	3	9

Heat-induced decarboxylation of N-nitrosamino acids in silicone oil

Formation of nitrosamines by decarboxylation became substantial beyond 150 °C (Fig. 7). Maximum yields of NDMA from *N*-nitrososarcosine were reached at about 230 °C (90% of theory); yields of NPyr from *N*-nitrososarcosine and NHPyr from *N*-nitroso-3-hydroxyproline were much lower: 11 and 9%, respectively. There are two possible explanations: (a) *N*-nitrososarcosine might be decarboxylated easier than the heterocyclic *N*-nitrosamino acid, (b) NPyr and NHPyr are equally rapidly formed but decompose to a greater extent than NDMA. Tests are under way to answer that question. For *N*-nitrosoproline (Fig. 7B), a similar relationship between formation of NPyr and heating temperature in silicone oil was found by Pensabene et al. (1974). Under different conditions (25 ml silicone oil; powdered *N*-nitrosoproline; 100-ml flask) they obtained a lower maximum yield of NPyr (2.4% of theoretical).

Heat-induced decarboxylation of N-nitrosoamino acids during frying of ham

Yields of nitrosamines after frying of ham under the conditions used were much lower than in silicone oil (Table 3), as would be expected since the food matrix would be heated to temperatures sufficiently high to effect decarboxylation only on the outside whereas the inner part would be warmed up to a lesser extent. The water content of the ham would also help to lower the effective temperatures. Moreover, since frying was in a normal open frying pan, a substantial proportion of the nitrosamines formed would volatilize with the cooking steam.

About 80% of NDMA formed from *N* nitrososarcosine and about half the NPyr formed from *N*-nitrosopyrrolidine volatilized with the steam (Table 4). As expected, NHPyr is hardly volatile, 92–96% remaining in the fried product. Distribution values of NDMA and NPyr correspond well with those found by Sen et al. (1976d) after frying of bacon; in similar experiments, Gough et al. (1976) found a somewhat higher volatility for both compounds.

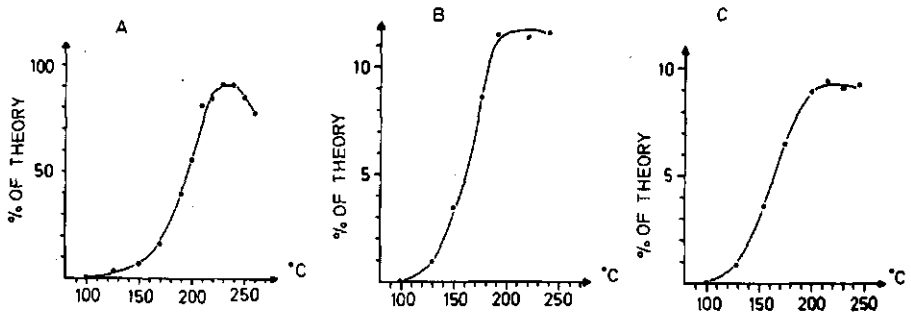

Fig. 7. Heat-induced decarboxylation of nitrosamino acids in silicone oil: relationship between nitrosamine yield and temperature. A. *N*-nitrososarcosine → *N*-nitrosodimethylamine. B. *N*-nitrosoproline → *N*- nitrosopyrrolidine. C. *N*-nitroso-3-hydroxyproline → *N*-nitroso-3-hydroxypyrrolidine.

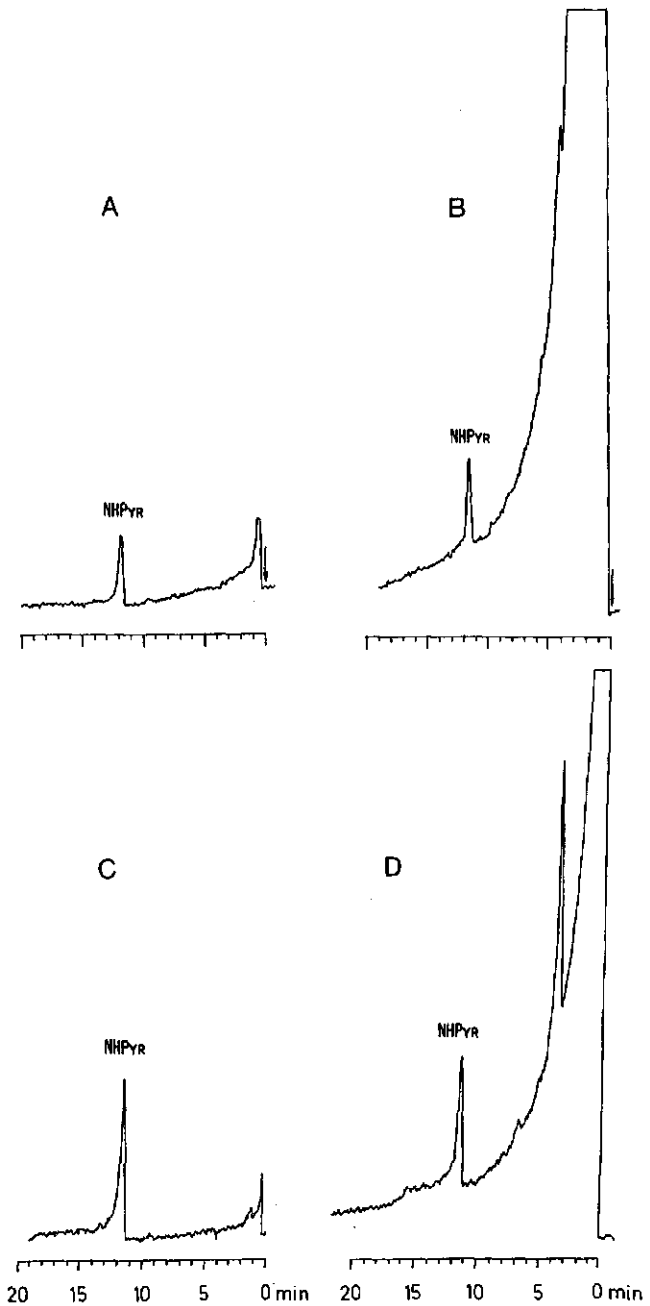

Fig. 8. *N*-Nitroso-3-hydroxypyrrolidine in fried ham. A. Reference, 1 ng. B. Sample (50 g), before column-chromatographic purification, 1 µl introduced. C. Reference, 4 ng. D. Sample (50 g), after column-chromatographic purification, 4 µl introduced.

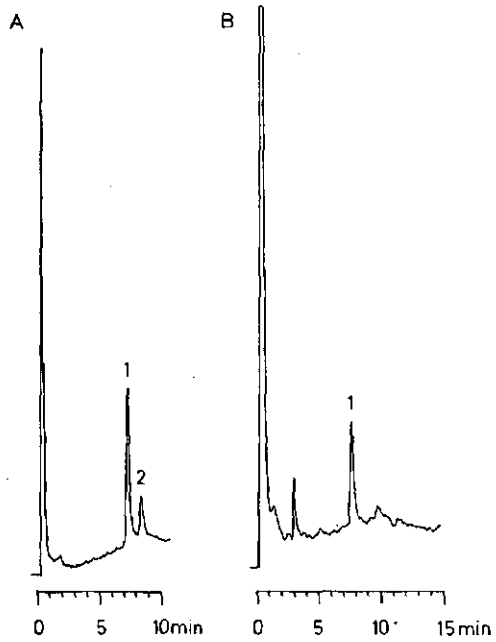

Fig. 9. Trifluoroacetyl-*N*-nitroso-3-hydroxypyrrolidine from fried ham. A. Reference, 1.26 μg of NHPyr (Peak 1) and 0.5 μg of Propylpropanolnitrosamine (Peak 2) in 1 ml *N*-methyl-bis-trifluoroacetamide (MBTFA)/acetonitrile. B. Sample concentrate (0.2 ml acetonitrile/MBTFA); 4 μl injected.

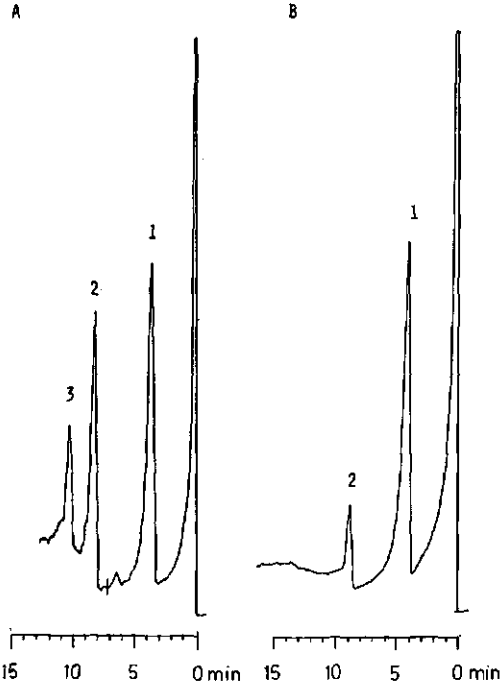

Fig. 10. Gas chromatogram of trimethylsilyl derivatives of *N*-nitrosamino acids. A. Reference mixture, 200 ng each of *N*-nitrososarcosine (Peak 1), *N*-nitrosoproline (Peak 2) and *N*-nitroso-3-hyroxyproline (Peak 3) in 200 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA); 4 μl injected. B. Concentrate of ham sample, fortified with *N*-nitrososarcosine at 65 μg/kg in 200 μl of MSTFA; 4 μl injected; Peak 2 (*N*-nitrosoproline) corresponds with about 20 μg/kg.

N-Nitroso-3-hydroxypyrrolidine contents in foods

Among 11 cured meat products (Table 5), only one of the uncooked samples showed traces of NHPyr. After frying (8 min at 180–190 °C), 7 of the samples were positive by TEA detection. Confirmation of these results by high-resolution GC-MS of the trifluoroacetyl derivative of NHPyr is under way. Figure 8 shows an example of the gas chromatography of a sample of ham, containing NHPyr at 9 µg/kg after cooking. The limit of detection with the TEA detector was below 0.25 ng NHPyr (signal/noise 4:1). See also Figure 9.

N-Nitrosoamino acid in foods

With ^{14}C-labelled reference substances, recoveries were about 70% for *N*-nitrososarcosine and about 80% for *N*-nitrosoproline; recovery of *N*-nitrosohydroxyproline was about 50% (detected by TEA).

As can be seen from the gas chromatogram of the trimethylsilyl derivatives of nitrosamino acids (Fig. 10), separations were satisfactory. The limit of detection with the TEA detector was well below 1 ng.

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A new type of N-nitrosation inhibitor

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Beside our work on nitrosamines in meat products (Groenen et al., This symposium), we are also studying the occurrence of nitrosamines in tobacco and tobacco smoke. Some of this work has been published in *Beiträge zur Tabakforschung* (Groenen & ten Noever de Brauw 1975). I now report in preliminary form that we have detected in tobacco smoke a compound which has the ability to trap NO_x vapours and to inhibit the formation of *N*-nitrosamines from secondary amines by competing for nitrite.

Though tobacco smoke and cured meat products have little in common, we consider it appropriate to describe some of the observations and conclusions from this work at this symposium, as it is conceivable that this new *N*-nitrosation inhibitor is active also in cured meat and other food products, and in vivo.

We will describe elsewhere how we became aware of this compound. We determined its identity and it was found to be pyrrole.

We then carried out a series of model experiments. The results were as follows:

1. Pyrrole in acidified aqueous solution (pH 1 or 3) is consumed extremely rapidly after addition of nitrite. A very fine black precipitate is formed.
2. New volatile or semivolatile nitroso compounds or other compounds are not formed in this reaction.
3. Experiments with an equimolar aqueous system of morpholine, pyrrole and nitrite at pH 3 showed that pyrrole can *completely inhibit* formation of *N*-nitrosomorpholine. A black carbon-like precipitate is again formed.
4. The extent of inhibition by pyrrole of the nitrosation of morpholine was compared with that of a series of acknowledged or potential nitrosation inhibitors under identical circumstances. The extent of inhibition was found to increase steadily in the order ammonium chloride $\leqslant$ phenol $<$ *iso*-amylmercaptan $<$ indole $<$ ascorbic acid $<$ guaiacol $<$ 4-methyl catechol $\leqslant$ pyrrole.

According to Challis & Bartlett (1975), 4-methylcatechol must be one of the best known scavengers of HNO_2. We now can add that pyrrole is at least as effective. Under the conditions of our experiment, 4-methylcatechol was also a highly effective nitrosation inhibitor. This is in contrast with the remarkable observations of Challis & Bartlett (1975), who report a 1 000-fold increase in the rate of nitrosamine formation, in spite of the rapid HNO_2 consumption of 4-methylcatechol.

There is suprisingly little literature on the nitrosation of pyrrole. *N*-Nitrosopyr-

role does not exist (Stevens 1957a); 3-nitrosopyrroles, however, do exist (Stevens 1957b). Indole compounds also are known to be *C*-nitrosated at the 3-position (Challis 1973). Pyrrole has the structure of a secondary amine, but it does not behave like one. The *N*-hydrogen atom can be replaced by potassium, so that the compound behaves rather like a weak acid (Karrer 1950), and has nothing in common with, for instance, pyrrolidine or piperazine. As early as 1917, it was known that a black polymeric precipitate is formed, called nitrosopyrrole black, on nitrosation of pyrrole (Angeli & Cusmano 1917).

Pyrrole and its derivatives occur in food products and in vivo (for pyrrole see, for instance, van Straten & de Vrijer 1973 and 1976) and it would certainly be worth while to study their inhibitory properties in more detail. It is conceivable that the type of reaction we have described plays a role in the partially unexplained loss of nitrite in cured meat products. Whether nitrosopyrrole black or similar products are really formed in small amounts in cured meat or tobacco smoke remains to be seen; of course other competitive compounds are at work too. If polymers like nitrosopyrrole black *are* formed, their toxicological properties should be elucidated.

Acknowledgment

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I gratefully acknowledge the assistance of Ms M. W. de Cock-Bethbeder and Ms R. J. G. Jonk.

Discussion on the session

Inhibition of nitrosation

In Dr Castegnaro's laboratory, the action of phenol on nitrosation of diethylamine at moderate phenol concentrations had been examined. A catalytic effect was found, increasing with the amount of phenol. Since phenol reacts with nitrite to give nitrosophenol, the effect of nitrosophenol was also tested. At concentrations of nitrosophenol a hundredth of those of phenol in the first experiments, an equivalent catalytic effect was observed. Although high concentrations of phenol were not used, it might well be that high levels compete with amine to give an inhibitory effect. Recents results obtained by Dr Davies confirm the findings of Castegnaro. He found that at pH 5.0, the only pH studied, *p*-cresol catalysed the reaction between nitrite and pyrrolidine to give nitrosopyrrolidine. The same effect was shown by some nitrosophenols at that pH.

General discussion on formation of nitrosamines

Contents of amines in bacon

Dr Roberts said that Patterson & Mottram (1974) give measurements of the contents of several amines (MA, DMA, TMA, *n*PA, *i*PA and DEA in decreasing order of 1 000 to 10 μg/kg), at different stages from slaughter of pigs through curing and storage as vacpacked bacon. Amounts changed before curing, conceivably by autolytic changes, arrested by salting. During storage, there was little change, despite rising contents of nitrite and increases in number contents of bacteria from perhaps $10^3 - 10^9$ g^{-1}. These bacteria were mostly lactic acid bacteria (not causing proteolysis) because of the vacuum packing. But even in uncured pork kept in air to the point of spoilage, with large production of NH_3, the levels of MA, DMA and TMA were similar in magnitude (Patterson & Edwards 1975).

Conclusions and recommendations of the chemical session on formation of nitrosamines, Wednesday 8 September

1. Ingestion of foods with high nitrate levels induce a rapid increase in salivary nitrite concentration, which remains for hours.

2. The intake of nitrite in food leads to a rapid increase in concentration of nitrite in the stomach. Low gastric nitrite levels persist when both the flow of saliva and food is stopped, suggesting the presence of another physiological source. Thiocyanate ion was conceived to catalyse the formation of *N*-nitrosamines.

3. Traces of *N*-nitrosopiperidine are found in the gastric contents of volounteers after ingestion of foods containing nitrite.

4. Many nitrosamines is found on nitrosation of spermidine. With three different types of detectors (ultraviolet, photohydrolytic cleavage of the N-NO bond followed by colorimetry, TEA) evidence was gained that about 30 nitroso compounds are present in the reaction mixture. The identity of most of them is unknown.

5. *N*-Nitrosoproline can be estimated in fried bacon with gas-liquid chromatography (as trimethylsilyl-*N*-nitrosoproline) and high-performance liquid chromatography. A good agreement in results (70 and 80 μg/kg) was obtained.

6. Dialkylcyanamides as well as monoalkylcyanamides were shown to be present in saliva. Under simulated stomach conditions, the monoalkyl compounds were nitrosated to *N*-nitrosoalkylcyanamids. Some of them are known to be highly mutagenic to bacteria.

7. Information about the formation of *N*-nitrosamines from amines and nitrite in model systems simulating stomach conditions should be obtained from tests under more specified circumstances.

8. In rats fed on *N*-nitrosodimethylamine, recoveries were low in their urine.

9. In a survey of *N*-nitrosamines in 52 cured meat products from the German local market, 30% of the samples were positive for trace amounts of NDMA, NPyr and

NPip by various methods of analysis. The contents of NPip and NPyr were always enhanced by cooking, but NDMA content in some samples decreased on cooking.

10. No correlation is found between residual nitrite level and contents of nitrosamines. Disagreements about this between various investigators might be explained by differences in frying operations.

11. Methods for the analysis of trace amounts of *N*-nitroso-3-hydroxypyrrolidine (NHPyr) and *N*-nitrosoamino acids have been given. Although not confirmed by MS, strong evidence was given for the presence of traces of NHPyr in various cured fried meat products.

Chemical session – Analysis of nitrosamines

Reporters: J. H. Dhont, P. J. Groenen

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Extraction, clean-up and estimation as methyl ether of 3-hydroxyl-l-nitrosopyrrolidine, a non-volatile nitrosamine in cooked bacon at mass fractions of μg/kg

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Abstract

A method was developed for the estimation of 3-hydroxy-l-nitrosopyrrolidine (HNPyr) in cooked bacon. The sample is extracted with acetonitrile and fats are removed by liquid-liquid extraction with *n*-heptane. Clean-up was by chromatography on an acidic alumina column. HNPyr is converted to its methyl ether which is estimated by combined gas-liquid chromatography and mass spectrometry. The limit of detection was about 2 μg/kg with recoveries of 51 to 102% when spiked with 10–100 μg/kg. Out of 13 cooked bacon samples, only 2 contained measurable (8 and 12 μg/kg) mass concentrations of HNPyr.

Introduction

Recent studies (Fiddler 1975; Sen 1974) have indicated that *N*-nitrosamines are an important class of environmental carcinogens. Traces of various nitrosamines have been demonstrated in certain foods such as cured meat products, cooked bacon, fish, cheese, spice-nitrite premixes, etc. (Fazio et al. 1971a, 1973; Sen 1972; Sen et al. 1973b; Eisenbrand et al. 1976b; Crosby et al. 1972). Most of the nitrosamines detected thus far are classified as 'volatile nitrosamines', mainly because of their easy volatility in steam. Many analytical methods utilize this property in the clean-up process during analysis of foods. There are many nitroso compounds, however, which are not volatile in nature, and therefore not amenable to clean-up by distillation. The non-volatility of these compounds also restricts them from direct analysis by gas-liquid chromatography (GLC) or combined GLC and mass spectrometry (GLC-MS).

Attempts have been made to analyse some of these non-volatile nitroso compounds by GLC or GLC-MS after converting them to volatile derivatives such as methyl esters, silyl ethers and trifluoroacetyl derivatives (Kushnir et al. 1975; Eisenbrand et al. 1976a; Dhont & Ingen 1976; Sen & Donaldson 1974). Although these methods work well with pure reference materials, there are many clean-up problems that are yet to be solved before these techniques can be used for trace analysis of foods.

Recently we (Sen et al. 1976b) showed that the conversion of a hydroxy-nitroso compound to a stable and volatile ether derivative may solve some of the problems. In this paper, we describe an extension of that work and report a procedure for the

analysis of hydroxynitrosopyrrolidine (HNPyr) in cooked bacon. Published reports (Bills et al. 1973; Gray & Dugan 1975a) suggested that traces of HNPyr may be present in cooked bacon. Moreover it has recently been shown to be a metabolite of nitrosopyrrolidine (NPyr), which is known to occur in cooked bacon and other cured meat products (Krüger & Bertram 1975).

Materials and methods

Reagents. All the reagents were of analytical grade and the solvents were distilled from an all-glass apparatus. HNPyr was prepared by nitrosation of 3-pyrrolidinol (Aldrich Chemicals) as described before (Krüger & Bertram 1975; Sen et al. 1976b). Acid aluminium oxide (anionotropic, 3% water) was purchased from M. Woelm (Eschwege, West Germany).

Bacon samples. Vacuum packaged sliced bacon was purchased from local stores, and samples of about 100 g were cooked in an electric frying pan until well done.

Extraction and clean-up. The cooked bacon sample, either the entire sample or the edible portion, was transferred in a blender and blended for 10 min with 250 ml acetonitrile. The mixture was allowed to settle for a few minutes and the supernatant was filtered through a funnel containing a heavy layer of glass wool. The filtrate was collected in a 1-litre volumetric flask. The residue in the blender was extracted once more with 250 ml acetonitrile. The whole mixture was poured onto the filter funnel and the filtrate was collected as above. The combined filtrate was made up to 1 litre with acetonitrile, mixed well, and half of the mixture was taken for the analysis. The sample solution was extracted with three 250 portions of *n*-heptane (to remove fats) and the heptane extracts were discarded. The acetonitrile layer was transferred into a 2-litre distillation flask and the solution evaporated to dryness under vacuum in a flash evaporator (water-bath temperature 45–50 °C). About 10 ml anhydrous ethanol was added to the residue and the evaporation step was repeated.

The residue in the flask was dissolved in about 25 ml ethylene chloride and the solution was passed through a column of acidic alumina (1 cm x 4 cm bed volume) which had a small (1–2 cm) layer of anhydrous sodium sulfate on top. The flask was rinsed twice with 15 ml portions of ethylene chloride and each wash was passed through the column. The effluents from the column were discarded. The flask was then rinsed successively with three 25 ml portions of ethyl acetate and the rinsings were passed successively through the alumina column. The combined ethyl acetate eluant contained the desired HNPyr fraction.

Derivative formation. The ethyl acetate eluant was evaporated under vacuum to about 2–4 ml, and the concentrated extract was quantitatively transferred into a 15 ml glass-stoppered test-tube. The solution in the test tube was evaporated to dryness under a gentle stream of nitrogen (water-bath, 40–50 °C). About 1 ml anhydrous ethyl acetate (dried over anhydrous $CaSO_4$) was added, and the mixture was evaporated again to dryness. Finally, 0.5 ml each of anhydrous ethyl acetate, methyl iodide, and about 20 mg sodium hydride (Sen et al. 1976b) were added to

the sample, the sample mixed thoroughly in a Vortex mixer, and the mixture was allowed to stand overnight at room temperature. The methyl ether derivative (NMPyr) was extracted and purified by vacuum distillation as described before (Sen et al. 1976c). The final preparation (in dichloromethane) was concentrated to 0.5–1.0 ml and 2.5 µl aliquots were analysed by GLC-ECD (Coulson electrolytic conductivity detector) or GLC-MS.

Additional clean-up. Usually the sample was then clean enough for direct analysis. Additional clean-up was, however, necessary for some samples.

If so, the concentrated extract from above was diluted with 1 ml *n*-pentane and the mixture passed through a second column of acidic alumina (1 cm x 4 cm, made in *n*-pentane). The column was washed with 50 ml of a mixture of equal volumes of dichloromethane and pentane, and the washing was discarded. Finally the column was eluted with 50 ml anhydrous ethyl acetate or 50 ml of a mixture containing 25% (by volume) anhydrous ethyl acetate and 75% dichloromethane. The eluant was concentrated to 0.5–1.0 ml first in a flash evaporator, then in a stream of nitrogen, as described above.

GLC-MS analysis. High-resolution (5 000) analysis by GLC-MS was as described before (Sen et al. 1976b). The instrument was operated in the specific-ion monitoring mode for either NO^+ or the molecular ion (M^+) peak of MEONPyr.

GLC-CECD analysis (pyrolytic mode). Aliquots of about 2–5 µl were analysed under conditions similar to those for analysis of volatile nitrosamines (Sen et al. 1972; 1974c).

Table 1. Presence or mass fraction (w) of 3-hydroxy-1-nitrosopyrrolidine (HNPyr) in cooked bacon. The limit of detection was 2 – 5 µg/kg; –, not detected.

Sample	Brand	Description	Method	w(HNPyr)/µg · kg^{-1}
1	A	lean	GLC–ECD, GLC–MS	– –
2	B	fat & lean	GLC–ECD, GLC–MS	– trace
3	C	fat & lean	GLC–ECD, GLC–MS	– trace
4	D_1	fat & lean	GLC–MS	trace
5	D_2	fat & lean	GLC–MS	–
6	E	lean	GLC–MS	–
7	F	fat & lean	GLC–ECD, GLC–MS	– trace
8	F	lean	GLC–MS	–
9	G_1	fat & lean	GLC–MS	12
10	G_2	fat & lean	GLC–ECD, GLC–MS	– –
11	H	fat & lean	GLC–ECD, GLC–MS	– –
12	I	fat & lean	GLC–MS	trace
13	J	fat & lean	GLC–MS	8

Preparation of reference NMPyr. Aliquots (1 μg or 10 μg) of HNPyr were converted as described above, the product extracted from the aqueous solution directly into dichloromethane (omitting the vacuum distillation), the extract dried and concentrated to 0.5–1.0 ml as described above. The concentration of HNPyr in the unknown samples was calculated by comparison of peak heights with that obtained with a known amount of the reference substance.

Results and discussion

About 13 samples of cured bacon were analysed (Table 1). The majority of the samples were either negative or contained traces of HNPyr. Only two samples contained significant mass fractions (8 and 12 μg/kg). These values are much smaller than of NPyr in cooked bacon as commonly detected in our laboratory.

The recoveries of various levels of HNPyr added to cooked bacon (Table 2) ranged 51–103%, depending on the samples and the spiking levels. A mass fraction of 10 μg/kg could easily be detected (Fig. 1 and 2). The minimum detectable mass fraction (twice the moise level) would be about 2–5 μg/kg.

The techniques described in this paper worked well with the samples analysed thus far. The extraction and the liquid-liquid partitioning techniques to remove fats were based on the procedure of Eisenbrand et al. (1969), which was suggested for estimation of nitrosamines but was never applied to foods. The alumina column clean-up technique was developed in our laboratory. The major loss in the entire procedure was found in vacuum distillation of NMPyr. Between the two GCL-MS techniques involving specific-ion monitoring for NO^+ and M^+, the former was more sensitive and specific than the latter. For unequivocal confirmation, the samples should be taken through all the clean-up steps described and then confirmed by

Table 2. Recoveries of 3-hydroxy-1-nitrosopyrrolidine (HNPyr) from cooked bacon. w = mass fraction (added). The non-nitrite bacon was prepared in the same way as normal commercial bacon but without sodium nitrite.

Sample	Type	Description	w(HNPyr added)/μg · kg^{-1}	Method	Recovery of HNPyr (%)
1	Non-nitrite	fat & lean	80	GLC–ECD,	80
				GLC–MS	80
2	Non-nitrite	fat & lean	100	GLC–ECD,	97
				GLC–MS	103
3	Non-nitrite	fat & lean	10	GLC–ECD	80
4	Non-nitrite	fat & lean	10	GLC–ECD	78
5	Non-nitrite	fat & lean	50	GLC–MS	57
6	Brand A	lean	10	GLC–ECD,	86
				GLC–MS	58
7	Brand B	fat & lean	50	GLC–ECD,	61
				GLC–MS	51
8	Brand C	fat & lean	10	GLC–MS	95
9	Brand D	fat & lean	20	GLC–MS	90
10	Brand E	lean	10	GLC–MS	102
11	Brand F	lean	10	GLC–MS	69

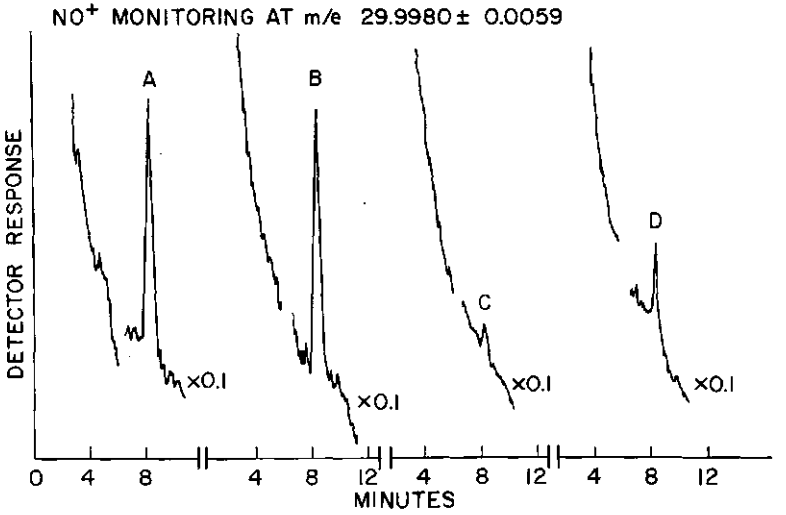

Fig. 1. Tracings of GLC-MS monitoring for NO^+. A, 5 ng hydroxynitrosopyrrolidine; B, a bacon (Table 2, No 5) spiked with a mass fraction of 50 μg/kg; C, a commercial bacon (Table 1, No 12); D, a bacon (Table 2 No 6) spiked with 10 μg/kg; *m*, ionic mass; *e* ionic charge. All samples were analysed after methylation. GLC column: 6 ft (about 1.90 m) long 1/8 in (about 3.18 mm) inner diameter stainless steel column packed with 10% Carbowax 20M on 60–80 mesh chromosorb W (HMDS). Column temperature 180 °C isothermal. The GLC column was connected through a Watson Bieman separator to a high-resolution mass spectrometer (Varian Mat 311A) and operated as described before (Sen et al. 1976b, c).

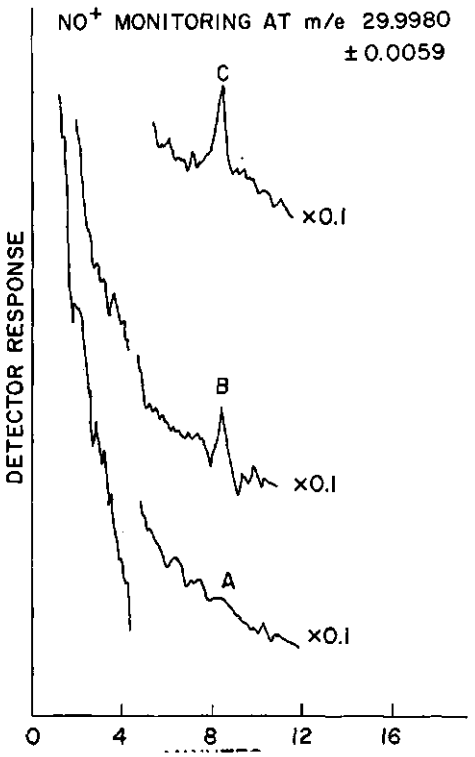

Fig. 2. Further examples of GLC-MS monitoring for NO^+. A, a commercial bacon (Table 1 No 6); B, a bacon (Table 2 No 10) spiked with a mass fraction of 10 μg/kg; C, 4 ng HNPyr; *m*, ionic mass; *e*, inonic charge. Conditions as in Figure 1.

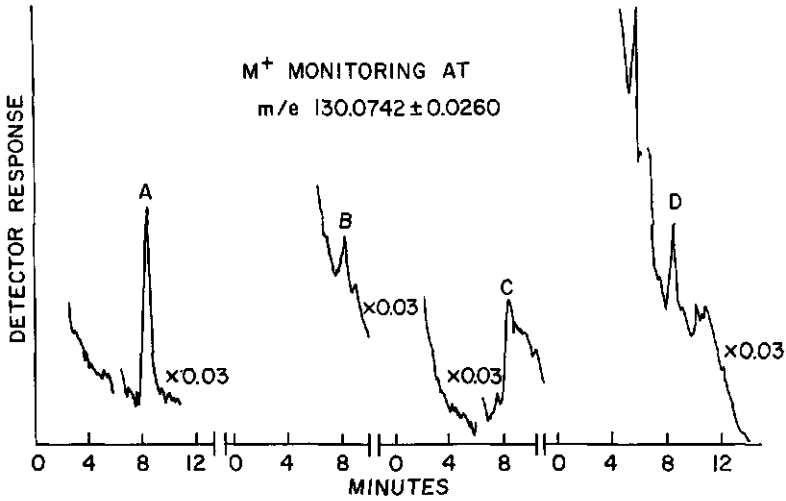

Fig. 3. Examples of GLC-MS monitoring for M^+ (3-methoxy-1-nitrosopyrrolidine, NMPyr). A, 5 ng HNPyr, B, a bacon (Table,2, No 6) spiked with a mass fraction of 10 μg/kg; C, a commercial bacon (Table 1, No 12); D, a bacon (Table 2, No 9) spiked at 20 μg/kg; *m*, ionic mass; *e*, ionic charge. Conditions as in Figure 1.

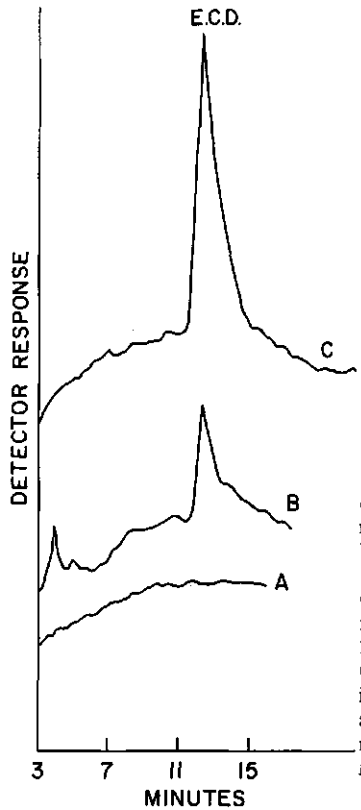

Fig. 4. Tracings of GLC-ECD (Coulson electrolytic conductivity detector) analysis for HNPyr (3-hydroxy-1-nitrosopyrrolidine) as the methyl ether. A, a commercial bacon (Table 1, No 11); B, 25 ng HNPyr; C, a bacon (Table 2, No 2), spiked at 100 μg/kg. GLC column 6 ft (about 1.9 m) long 1/8 in (about 3.18 mm) inner diameter stainless steel column packed with 10% Carbowax 20M on 80/120 mesh chromosorb W (high performance). Column temperature 140 °C for 3 min (effluent) vented in the air during this period), then programmed to 180 °C at a rate of 10 °C min^{-1}. ECD furnace (Coulson, pyrolytic mode); temperature 360-380 °C. Carrier gas (He); flow rate 30 ml/min.

both GLC-MS techniques. Although the GLC-ECD (pyrolytic mode) method worked satisfactorily with some samples, the sensitivity of the detector was inconsistent. Perhaps a Hall detector (Eisenbrand et al. 1976b) or Coulson ECD detector operating in the reduced mode would be more suitable. A few typical GLC tracings are shown in Figures 1–4.

Cooked bacon is a 'dirty' sample, consisting of many interfering compounds, so that analysis for nitrosamines has presented many problems (Wasserman 1974). Even the methods for volatile nitrosamines in bacon are lengthy and tedious, and at low levels give recoveries no better than here reported for HNPyr. In this light, our techniques are quite adequate for trace analysis of HNPyr in bacon. To our knowledge, this is the first reliable method reported for estimation of HNPyr in foods.

Although mass fractions of HNPyr in the samples were extremely low, a wider survey seems necessary ensure that cooked bacon does not contain significant levels. There is also a need to check other cured meat products and nitrate-treated fish and cheese for the presence of HNPyr. Since HNPyr is a metabolite of NPyr the technique will be useful for studying the metabolism of both NPyr and HNPyr.

Discussion on the session

Correlation hydroxynitrosopyrrolidine-nitrosopyrrolidine?

The authors had not measured nitrosopyrrolidine in their study. Dr Gough had tried to find a possible correlation between hydroxynitrosopyrrolidine and nitrosopyrrolidine, but had not found any.

Hydroxyproline in bacon

Dr Rubin remarked that hydroxynitrosoproline could be considered as a logical precursor for hydroxynitrosopyrrolidine. The nitrosamino acid itself could be formed from free hydroxyproline. Quoting from memory, he thought that free proline is present at a content of 30–80 mg/kg, and hydroxyproline at a content of about 3 mg/kg.

Mass spectrometric resolution

In Figures 1–3 of the submitted manuscript, the mass of the NO^+ ion is expressed to 4 decimal places. This suggested a higher resolution than was actually used. At a resolution of 5 000, it would seem more appropiate to give 2 (or at most 3) decimals. Dr Coffin agreed.

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Developments in nitrosamine analysis

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It is now twenty years since the paper by Magee & Barnes (1956) on the carcinogenic effect of nitrosodimethylamine first roused interest in this group of compounds as potential environmental carcinogens. For the analyst, the primary step was to seek methods for estimation at mass fractions in the mg/kg range. A variety of methods employed for this included polarography, colorimetry and thin-layer and gas chromatography (GC). Inconsistancies and lack of agreement in results led the International Agency for Research on Cancer to organize a meeting in London for biochemists and analysts to discuss the problems of analysis. That meeting recommended that analytical methods should be capable of measuring contents of 1–10 μg/kg in an environmental sample. At the second meeting in Heidelberg (1971), several methods for analysis of volatile nitrosamines were proposed and agreement was reached to conduct inter-laboratory studies on methodology. Since 1971, two further meetings have been convened and three collaborative studies conducted. These have demonstrated considerable progress in analysis of volatile nitrosamines.

In its contribution to solving analytical problems, the International Agency for Research on Cancer laboratory has established its own screening analytical method for volatile nitrosamines in food and alcoholic beverages. This paper briefly discusses current developments in the screening technique and the results of collaborative studies.

Analysis of volatile nitrosamines

Oxidation of nitrosamines to nitramines

The method of analysis employed has been published (Walker et al. 1975), but a modification has been introduced in the oxidation stage.

Our technique was originally based on the method described by Sen (1970), using 50% (by volume) hydrogen peroxide in water with trifluoroacetic acid. Nitrosamines are converted to nitramines in yields of about 50% after 18 h; Telling (1972) obtained a better conversion with peroxide 85% (about 85% in 3 h). This concentrated peroxide is difficult to obtain. However good yields may also be obtained with 50% peroxide by oxidizing in the presence of the extracting solvents

rather than first removing them. Vigorous shaking for $3\frac{1}{2}$ h then gives yields similar to those obtained with 85% peroxide. The results of this work are reported in Table 1, which compares the three techniques. The technique has the advantages that the solvents are distilled from the nitramines which are less volatile than the nitrosamines and that it eliminates the risks inherent in the more vigorous oxidant.

Confirmatory testing of nitramines

In our former technique, nitramines detected by electron capture were confirmed by a Coulson electrolytic conductivity detector, which has been superseded by mass spectrometry (MS) of the nitramines at a resolution of 8 000. Either the NO^+, the parent ions, or the nitrosamine ions by loss of an oxygen radical may be used. This method economizes considerably in effort as the fractions from column chromatography containing the respective nitramines are relatively clean and may be used directly for GC-MS detection after concentration.

Table 1. Comparison of efficiency of conversion (%) of *N*-nitrosamines to nitramines with 85 and 50% (by volume) hydrogen peroxide in water and different volumes (V) of solvent. Samples contained 0.5 μg of each nitrosamine. Method of Telling (1972). NDMA, nitrosodimethylamine; NDEA, nitrosodiethylamine; NDPA, nitrosodipropylamine; NDBA, nitrosodibutylamine; MPNA, methylpentylnitrosamine; NPip, nitrosopiperidine; NPyr, nitrosopyrrolidine. .

Peroxide 85%					50%		
V/ml	0	100	50	20	100	50	20
NDMA	82	81	79	83.5	58	62	58
NDEA	84	86	84	85.5	107	75	74
NDPA	87	106	90	88.5	87	84.5	74
NDBA	86	81	81	83	81	81	70
MPNA	91	93	91	89	81	80	72
NPip	52	57	53	47	73	74	68
NPyr	88	83	88	88	98	93	93

Table 2. Comparison of coefficients of variation (%) of standard deviations of means obtained by different laboratories. w, mass fraction; NDMA, nitrosodimethylamine; NDBA, nitrosodibutylamine; NDEA, nitrosodiethylamine; NPyr, nitrosopyrrolidine; MS, mass spectrometry; GC, gas chromatography.

$w/\mu g \cdot kg^{-1}$	20	5		
method	all	all	MS	GC
NDMA	39	66	34	63
NDEA	39	41	14	38
NDBA	53.1	37	26	33
NPyr	59.5	38	35	30

Cooperative studies

Three cooperative studies (i.e. studies of more than one method of analysis) for volatile nitrosamines have been organised. The first was for nitrosamines in reference solutions and two subsequent studies on analysis of four selected nitrosamines added to pork luncheon meat (Tables 2 and 3). Table 2 indicates that between and within laboratories, agreement is less precise at 5 μg/kg than at 20 μg/kg. Better agreement is obtained by mass spectrometry than gas chromatography at 5 μg/kg, particularly for nitrosodimethylamine (NDMA). Since NDMA is the most commonly reported nitrosamine in food, the closeness of averages by both techniques to the true value for samples spiked at 20 μg/kg but the higher values at 5 μg/kg for GC suggests that NDMA values by GC tend to be too high when measured at very low levels.(Table 3).

Even with specific-ion monitoring in MS, both the gas-chromatographic separation and the selection of ions for monitoring can be critical. Thus, for specific identification of NDMA, 70 000 resolution may be required to separate this ion from a silane ion introduced by the use of anti-foaming agents (Do et al. 1973; Gough & Webb 1973). Then a highly efficient gas-chromatographic column capable of separating the NDMA and silane is essential. This is an example well documentated, but how many examples exist of which we are unaware? A resolution of 80 000 may be required for specific identification of nitrosopiperidine (Table 4).

Table 3. Average contents (mass fractions, w) of nitrosamines in canned pork luncheon meat as calculated from amount added and from analysis, either averaged independently of method or separately for mass spectrometry (MS) or gas chromatography (GC). NDMA, nitrosodimethylamine; NDEA, nitrosodiethylamine; NDBA, nitrosodibutylamine; NPyr, nitrosopyrrolidine.

	Calculated from	NDMA	NDEA	NDBA	NPyr
Tests at about 20 mg/kg	amount added	18.9	15.6	19.8	19.8
	analysis, mean	15.21	13.83	14.52	13.5
Tests at about 5 mg/kg	amount added	4.15	4.22	4.83	5
	analysis, mean	6.75	4.54	3.63	3.90
	MS	4.39	3.29	2.75	2.81
	GC	7.93	5.23	4.17	4.51

Table 4. Ions interfering in the analysis of nitrosopiperidine. Combinations of C, H, N and O only. Interfering ions from Beynon 1969. M, molecular weight; z, charge number.

Ion	M/z	Resolution required
$C_4H_8N_3O^+$	114.103 2	9 000
$C_4H_{10}N_4^+$	114.127 0	10 190
$C_6H_{10}O_2^+$	114.104 5	10 100
$C_3H_8N_5^+$	114.114 4	81 511
Nitrosopiperidine	114.115 8	–

This was a problem encountered when mass spectrometry monitoring of the parent ion gave higher values than GC for both nitrosamine and nitramine. The problem was resolved by taking other ions.

An exciting innovation used in the last analytical survey was the thermal energy analyser. This highly selective detection system for *N*-nitroso compounds produced good results after only extraction and concentration. During the present collaborative study, a more precise judgment on this detector may be possible because of the increasing number of users. The nitrosopiperidine problem was eliminated by using this detector.

Conclusion

Though detection and measurement of *N*-nitroso compounds has progressed considerably, it remains a problem area in trace analysis. We hope that the interlaboratory collaboration will continue and will reduce the problems.

Discussion on the session

Relative importance of clean-up, GC resolution and MS resolution

The interference of $C_3H_8N_5$ ion with nitrosopiperidine even when high-resolution MS was used, had not been observed actually, but was put forward only as a theoretical possibility. Dr Gough said that, even if the ion exist and were to have the same retention behaviour as nitrosopiperidine, it could still be satisfactorily distinguished from the latter at a resolution of only 10 000. Mass spectrometry at this resolution was still the most reliable way of confirming nitrosamines, particularly when combined with packed and capillary GC columns operated in series.

Dr Wasserman said that the present state of clean-up procedures is such that the final concentrates are still too dirty for reliable low-resolution MS determination, and sometimes even at resolution 4000.

Dr Groenen said that the quantitative estimation of nitrosamines is a coherent chain of operations: clean-up, GC separation and detection/identification. These should be optimized together. Increasing GC resolution (use of capillary columns) contributes as much to the reliability of the identifications as increasing MS resolution (from 'medium' to 'high') or improving the clean-up procedures, and perhaps even more.

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Trace analysis of polar and apolar N-nitroso compounds by combined high-performance liquid chromatography and thermal energy analysis

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Abstract

Thermal energy analysis (TEA) has been combined with high-pressure liquid chromatography (HPLC). Performance characteristics, including sensitivity, linearity, reproducibility, selectivity and proportionality of response to amount of substance are discussed. The most useful feature of TEA-HPLC is unique selectivity to the nitrosyl functional group, allowing detection of mass fractions of less than 10^{-9} or mass concentrations less than $1\,\mu g\,litre^{-1}$, even in the presence of 'crude' environmental samples. TEA-HPLC allows, for the first time, routine analysis of non-volatile *N*-nitroso compounds in the environment.

Introduction

Although many *N*-nitroso compounds are known carcinogens that may be implicated in the etiology of human cancer (Lijinsky & Epstein 1970), assessment of their hazard to man has been hampered by the lack of adequate techniques for their assay in the environment (IARC 1972b, c). Combined methods of gas chromatography and mass spectrometry (GC-MS) are available for the 14 or so most volatile of the series (Issenberg & Essigman 1972), and procedures for thermal energy analysis (TEA) are available for all the volatile *N*-nitroso compounds (Fine & Rounbehler 1976b). However the majority of *N*-nitroso compounds likely to be present

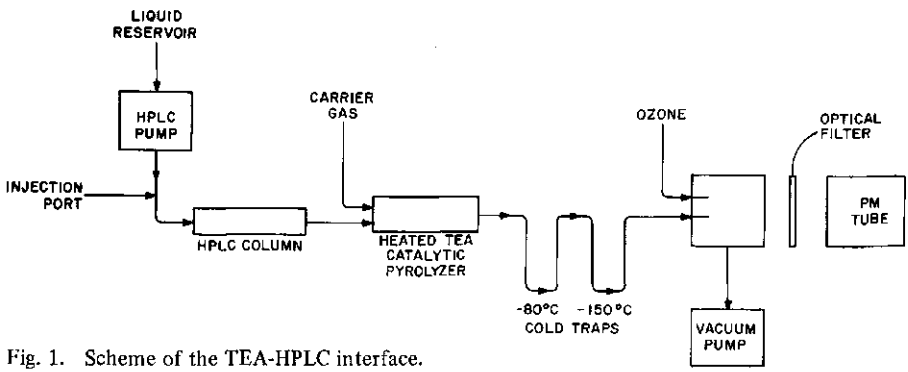

Fig. 1. Scheme of the TEA-HPLC interface.

in the environment would be non-volatile, and would therefore not be amenable to gas chromatography. Because of the lack of detectors specific for *N*-nitroso in liquid chromatography, the occurrence of non-volatile *N*-nitroso compounds in the environment has been largely ignored.

Fine & Rounbehler (1975) previously reported on the interfacing of a thermal energy analyser specific to *N*-nitroso compounds with a gas chromatograph (GC). For the first time, the combined TEA-GC system allowed routine analysis of all volatile *N*-nitroso compounds at mass fractions less than 1 μg/kg. This paper describes the interfacing of TEA with a high-pressure liquid chromatograph (HPLC), the combined TEA-HPLC system retaining all the sensitivity and selectivity inherent in both HPLC and TEA.

Apparatus

The principle of operation (Fine et al. 1975b), and the detailed design (Fine & Rounbehler 1975) of the TEA detector are described elsewhere.

The TEA-HPLC interface is shown schematically in Figure 1. Liquid effluent from the HPLC column enters the heated TEA catalytic pyrolyser, together with a small flow of argon or nitrogen carrier gas. The carrier solvent is instantaneously vaporized, carrying with it the more volatile compounds. *N*-Nitroso compounds, both volatile and non-volatile, are cleaved in the pyrolyser at the N-NO bond, splitting off the nitrosyl radical. Gas and vapours leaving the pyrolyser pass through two cold traps. The temperature of the first cold trap is maintained at a temperature sufficient to liquify the carrier solvent, but not solidify it. The second cold trap is maintained at −150 °C to remove all remaining solvent vapours and decomposition products. Only the nitrosyl radical and permanent gases survive the two

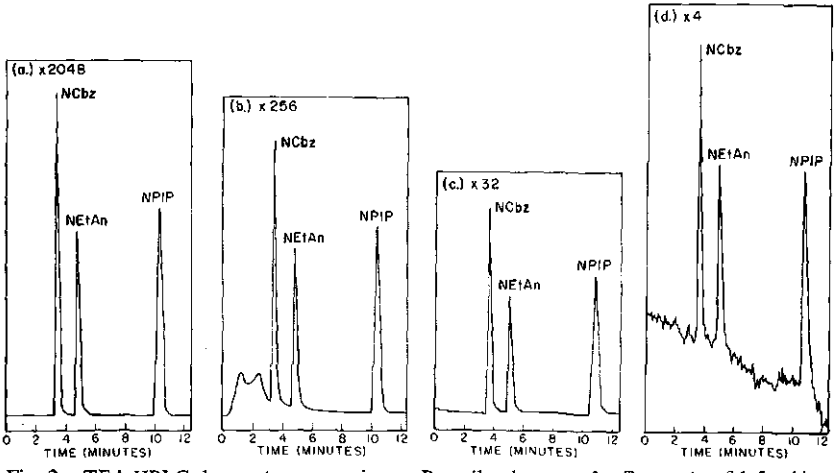

Fig. 2. TEA-HPLC chromatograms using a μPorasil column and a flow rate of 1.5 ml/min of 4% (by vol.) acetone in 96% 2,2,4-trimethylpentane. (a) Chromatogram of 10 μl of a mixture containing NCbz, NEtAn and NPIP each at mass concentrations of 0.1 g litre^{-1}; (b) 10 μl of mixture diluted 10-fold; (c) 10 μl of mixture diluted 100-fold; (d) 20 μl of mixture diluted 1000-fold.

cold traps and can enter the reaction chamber, where the nitrosyl radical is allowed to react with ozone, to give electronically excited nitrogen dioxide. The excited nitrogen dioxide rapidly relaxes to its ground state with the emission of radiation in the near infrared region of the spectrum. The radiation is monitored by an infrared-sensitive photomultiplier tube, the intensity of the emission being proportional to the number of nitrosyl radicals present.

The cold traps operate at low temperature and at low pressure so that both gas and liquid phases coexist in the traps. The traps are designed to remove large volumes of solvent up to 0.5 litre h^{-1}, while having an effective HPLC dead volume of less than 10 μl. This aim is achieved by reducing the pressure inside the traps from 760 mmHg (101 kPa) to less than 4 mmHg (0.53 kPa), thus reducing the volume of gas (calculated at 101 kPa and 25 °C) from 300 ml (the trap volume) to less than 1.7 ml. This volume (100 kPa; 25 °C) of solvent vapour corresponds to less than 10 μl of the liquid phase carrier in the HPLC (assuming a molecular weight of 100 and a mass density of 1). Thus although the traps have a capacity of about 300 ml of liquid, their effective dead volume for the chromatograph is less than 10 μl

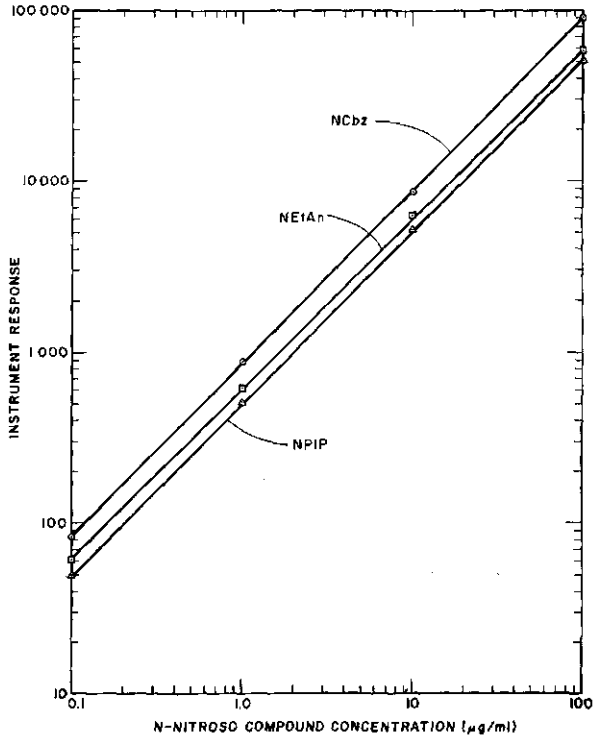

Fig. 3. TEA-HPLC calibration for NCbz, NEtAn and NPIP, with calibration data taken from Fig. 2.

Because the cold traps remove all solvent and solvent vapours, the performance of TEA is independent of the amount or nature of the solvent. This is an important feature when using solvent or flow programming.

The TEA-HPLC apparatus was constructed by combining two high-pressure pumps (Waters Associates, Model 6000) with a solvent programmer (Waters Associates, Model 2000) and a liquid-injection valve (Waters Associates, Model U6K). The thermal energy analyser (Thermo Electron, Model 502) was equipped with a TEA-HPLC interface (Thermo Electron, Model 502 GC-HPLC).

Solvents were obtained from Burdick and Jackson, and all were distilled in glass. Reference *N*-nitroso compounds were supplied by various collaborating laboratories. Reference solutions were made up gravimetrically.

Results and discussion

Linearity

The linearity of the TEA-HPLC was explored over four orders of magnitude, using a mixture of *N*-nitrosocarbazole (NCbz), N-nitroso-N-ethylaniline (NEtAn) and N-nitrosopiperidine (NPIP). The flow rate in the chromatograph was 1.5 ml/min of a mixture containing 4% (by vol.) acetone in 2,2,4-trimethyl-pentane on a μPorasil (Waters Associates) column. The TEA vacuum was maintained at 1.1 mmHg (0.13 kPa). A calibration plot for the three *N*-nitroso compounds, obtained from the peak heights in Figure 2, is shown as a log-log plot in Figure 3. The calibration is linear over the four orders of magnitude shown.

Sensitivity and flow programming

Because of the incorporation of the large-volume cold traps, TEA-HPLC can be used either for solvent or flow programming. In flow programming, the flow rate of liquid phase is systematically increased; because the same solvent system is used throughout, the column does not require equilibration at the end of the chromatogram. The technique therefore not only decreases the time for separation, but also allows a more rapid turn-around between chromatograms. Using a mixture of 6 *N*-nitroso compounds: NCbz, NEtAn, NPIP, *N*-nitrosopyrrolidine (NPYRR), *N*-nitrosodimethylamine (NDMA) and *N*-nitrosomorpholine (NMORPH), we demonstrated both the programming capability and the limits of detection of the technique. The compounds were separated on a μPorasil column, using a solvent system of 3% (by vol.) acetone and 97% 2,2,4-trimethylpentane. The flow-rate of the liquid phase was systematically increased 10-fold. The chromatograms of 100, 10 and 1 mg litre^{-1} do not exhibit detectable drift of the base-line. For the concentrations of 0.1 and 0.01 mg litre^{-1}, baseline drift is clearly visible, but is not sufficient to obscure the chromatogram (Fig. 4).

Figure 4d, the chromatogram of 40 μl of a mixture containing compounds at a concentration of 0.01 mg litre^{-1}, demonstrates the limitations of the TEA-HPLC interface. In this chromatogram only 0.4 ng (0.4×10^{-9} g) of each material was introduced. For the *N*-nitrosocarbazole, the signal-to-noise ratio was about 5. As

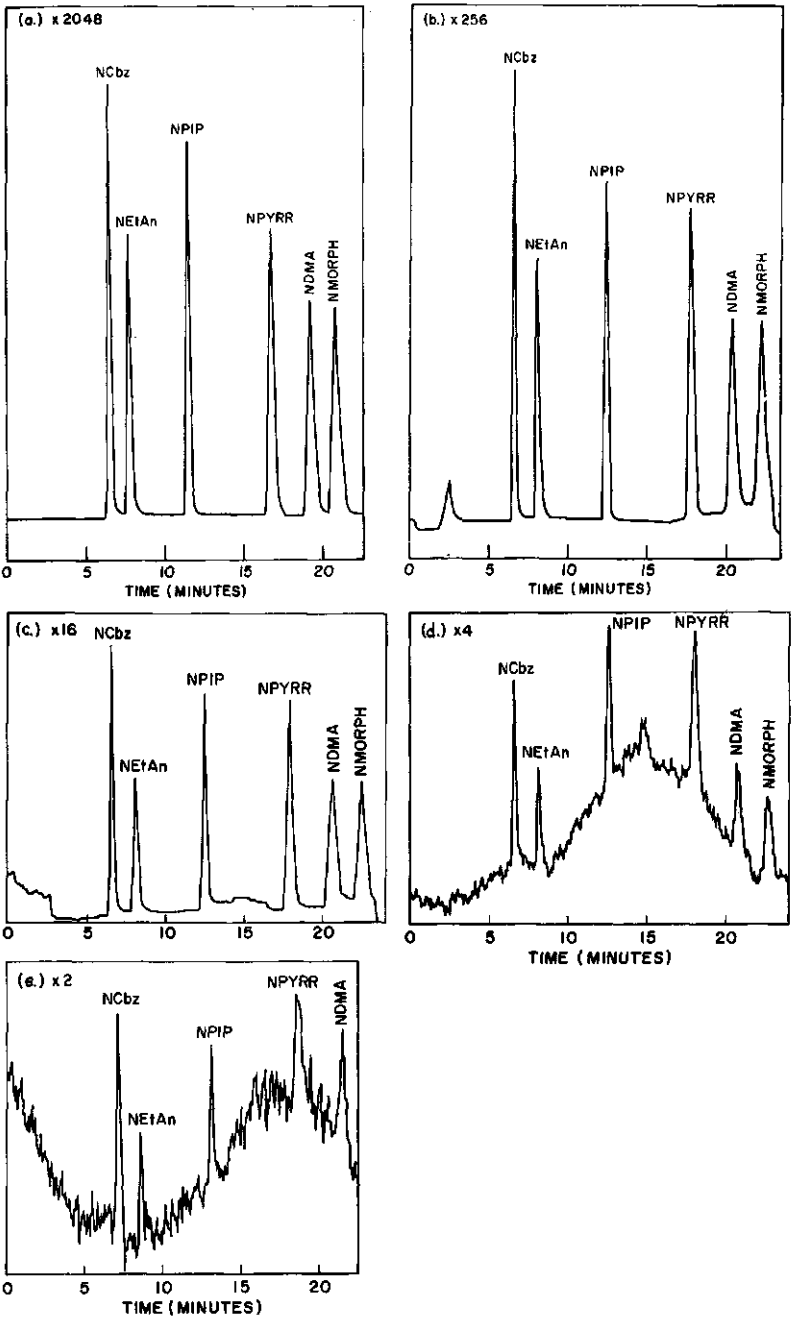

Fig. 4. TEA-HPLC chromatograms using a μPorasil column and programming of flow rate from 0.25 to 2.5 ml/min of 3% (by vol.) acetone in 97% 2,2,4-trimethylpentane. (a) Chromatogram of 10 μl of a mixture of NCbz, NEtAn, NPIP, NPYRR, NDMA and at mass concentrations of 100 mg litre^{-1}; (b) Chromatogram of 10 μl of mixture diluted 10-fold; (c) Chromatogram of 10 μl of mixture diluted 100-fold; (d) Chromatogram of 10 μl of mixture diluted 1000-fold; (e) Chromatogram of 40 μl of mixture diluted 10 000-fold.

may be seen for the other compounds in the chromatogram, about 0.4 ng of substance is the detection limit of the technique when using flow programming.

Polar N-nitroso compounds

As with other *N*-nitroso compounds, TEA response is of highly polar *N*-nitroso compounds such as *N*-nitrosamino acids and *N*-nitrosoureas. However conventional techniques for chromatographing polar compounds require buffered solvents; buffers would precipitate out and clog the TEA-HPLC interface. There are alternatives to buffered systems. We have used an anion-exchange column (Waters Associates), with a flow rate of 2.0 ml/min of a solvent system programmed from 10% (by vol.) acetic acid, 67.5% tetrahydrofuran and 2.25% 2,2,4-trimethylpentane program number 6 (linear) over 15 min. The chromatogram of 10 μl of a mixture containing about 300 mg litre^{-1} each of *N*-nitrosoproline (NProl), *N*-nitrosohydroxyproline (NHProl) and *N*-nitrosoiminodiacetate (NIDAcA) is shown in Figure 5. The baseline drift with this solvent system swamps the chromatogram at concentrations below about 20 mg litre^{-1}. Ideal chromatographic conditions for the polar *N*-nitroso compounds, which can fully utilize the TEA-HPLC sensitivity characteristics, have not yet been explored.

Comparison of TEA-HPLC with TEA-GC

The interfacing of a highly selective detector to both HPLC and GC allows confirmation of the identity of *N*-nitroso compounds amenable to GC by TEA alone. Confirmation is based upon four factors.

(a) Coincidence of retention time with known reference substances by gas-liquid

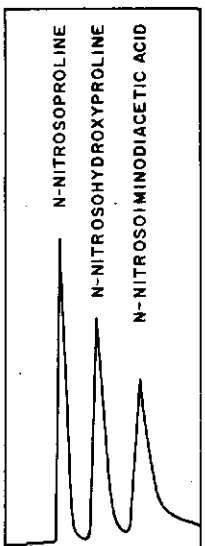

Fig. 5. TEA-HPLC chromatogram of 10 μl of a mixture of *N*-nitrosoproline, *N*-nitrosohydroxyproline and *N*-nitrosoiminodiacetic acid. Chromatographed on an anion-exchange (Ax) column, with flow rate 2.0 ml/min of a solvent system of acetic acid programmed from 10 to 90% (by volume), tetrahydrofuran from 67.5 to 6.75 and 2,2,4-trimethylpentane from 22.5 to 2.25%.

chromatography (GLC). In GLC, the elution order is dependent upon a combination of volatility and solubility characteristics.
(b) Coincidence of retention time with known reference substances by high-pressure liquid chromatography, where the elution order is based upon the relative polarity of the compound.
(c) The amount of TEA-responsive material found is identical by both GC and HPLC techniques.
(d) There is no risk of confusion between peaks because each chromatogram contains only one TEA-responsive peak. If more than one TEA-responsive peak is observed, the compounds are isolated on the TEA-HPLC and then reinjected onto TEA-GC.

Confirmation of identity by both TEA-GC and TEA-HPLC was used for the first time to confirm the presence of NDMA in urban air (Fine et al. 1976c) and water (Fine & Rounbehler 1976a); both findings were subsequently repeated by conventional mass spectrometry. The parallel TEA-GC and TEA-HPLC confirmatory technique requires only 1 ng of substance, which is considerably less than for confirmatory mass spectrometry. With materials such as foodstuffs and drinking water, confirmatory mass-spectrometry is always possible because larger amounts of starting material can be used. However for metabolic work with small laboratory animals and human beings, confirmation with only 1 ng allows work at true environmental levels (Fine et al. 1976e).

Selectivity

The selectivity of the TEA to the nitrosyl functional group has been established, both on model systems (Fine et al. 1975a) and on environmental samples (Fine & Rounbehler 1976). Compounds that give a response are *N*-nitroso compounds, organic and inorganic nitrites, some organic nitrates and other compounds with extremely thermally labile nitrosyl groups. Apart from compounds with the nitrosyl functional group, there are no known compounds that respond on TEA-HPLC. Because of the inherent selectivity of TEA, complex materials can be analysed at mass fractions less than 100 μg/kg without preparatory clean-up or concentration; the only requirement for the sample is compatibility with the column, pump and injector system. By TEA-HPLC, Fine et al. (1976a) have assayed complex foodstuffs such as cooked bacon and spiced meats for non-volatile *N*-nitroso compounds.

Conclusion

The TEA-HPLC system, like its TEA-GC counterpart is sensitive to mass concentrations less than 1 μg/litre and is uniquely selective to compounds with the nitrosyl group. Because it is truly selective, the instrument may be routinely operated at maximum sensitivity even with 'dirty' environmental samples such as agricultural crops, foodstuffs, and animal organs. TEA-HPLC turns analysis and detection of polar and non-polar *N*-nitroso compounds from being a formidable research undertaking to a task of routine simplicity. *N*-Nitroso compounds have become the first class of environmental carcinogens for which selective highly sensitive instruments now exist.

Acknowledgments

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Discussion on the session

Isolation of fractions from HPLC-TEA analysis

A T-split was inserted between liquid chromatograph and thermal energy analyser, to allow the collection of fractions for further analysis.

Memory effects of TEA

The author had not observed memory effects originating in the catalytic chamber of the TEA, not even after injection of large samples. Dr Gough added that injection of water and organic solvent after an injection of a solution of nitrosamine with 1 g/litre did not result in memory peaks.

The author presented many new results, not referred to the submitted manuscript. The discussion concentrated on these new results.

Nitrosamine, nitrite and nitrate contents of the meal, consumed before the blood analysis

The meal contained dimethylnitrosamine (2–3 μg/kg) and nitrosopyrrolidine (30–40 μg/kg). Nitrite and nitrate were not estimated.

Nitrosamine formation in rat stomach

At the request of the chairman of the session, Dr Telling recapitulated some findings from a rat feeding study (Telling et al. 1976). In the stomach of rats of the control group, fed only on the standard purified diet plus normal drinking water, 3–9 ng dimethylnitrosamine (mean 6 ng/stomach) were detected with GC and high-resolution MS. The purified diet and the drinking water by themselves did not contain volatile nitrosamines.

Nitrosamines in herbicide formulations

The author had detected very high nitrosamine levels in a product containing trichlorobenzoic acid – dimethylamino salt. The nitrosation was thought to be due to the antibacterial agent added to the formulation, which contained labile NO groups. Corrosion inhibitors on the inside of metal cans have also been shown to act as nitrosating agents. According to Gough, much higher levels of dimethylnitrosamine had been found in the United Kingdom in dead grass, presumably treated with a herbicide, than in the surrounding live untreated grass.

Conclusions and recommendations of the chemical session, analysis of nitrosamines, Wednesday 8 September

1. Analytical procedures have been developed for the determination of 3-hydroxy-1-nitrosopyrrolidine in cooked bacon. In a limited survey of samples, only low contents (less than 10 μg/kg) could be found in only a small proportion of the samples.

2. The nitramine method of nitrosamine analysis has been improved by the use of GC-MS.

3. The IARC collaborative study has shown that the accuracy and agreement of the GC-MS procedures are excellent, and that results are better than by other GC procedures. The TEA detector has produced results equivalent to GC-MS.

4. Liquid chromatography used in conjunction with the TEA detector can be used for the analysis of a wide variety of nitroso compounds, both volatile and non-volatile.

5. Low levels of DMN and DEN have been found in human blood following consumption of a meal. The method of detection is the TEA detector, the results have not been confirmed by GC-MS.

Chemical session – The occurrence of nitrosamines

Reporters: P. C. Moerman, W. J. Olsman

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Nitrosopyrrolidine in United States cured meat products

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Abstract

Nitrosopyrrolidine was found routinely in fried bacon and in some samples of severely fried (12 min at 170 °C) country-style ham in the United States. In fried US bacon purchased retail, its mass fraction declined steadily from 1972 to 1976 from an average of 67 to 17 μg/kg. There was no correlation of nitrosopyrrolidine after frying with content of residual nitrite in bacon at the time of frying nor with nitrite or nitrate content of country-style ham at the time of frying.

At the time of the First Symposium, scientific interest in nitrosamines in cured meats was increasing. Laboratories throughout the world had begun to examine the occurrence of the volatile nitrosamines in various cured meat products. One conclusion drawn by the First Symposium was the necessity for confirmatory mass spectrometry of nitrosamines detected by gas chromatography.

Subsequent analytical surveys in North America have demonstrated that most products with nitrite or nitrate as part of their manufacturing formulation occasionally contain one or more nitrosamines at μg/kg levels. Dimethylnitrosamine is the most common and occurs sporadically in a wide variety of cured meat products.

Nitrosopyrrolidine seems to have a special niche. Product surveys have demonstrated nitrosopyrrolidine routinely in fried (but not in raw or boiled) bacon. Though it is not found in raw commercial bacon, it is usually present after frying or broiling.

The only other product in which nitrosopyrrolidine has been detected in the United States is 'country-style ham', a dry-cured aged primal product indigenous to the southeastern United States and produced according to literally hundreds of time-honoured family recipes. Unlike bacon, 20 country-style hams surveyed included only 3 that contained nitrosopyrrolidine (Greenberg 1975). The 3 positive results occurred only after severe frying of slices 1/4 in (6 mm) thick for 12 min at 340 °F (171 °C). Raw, boiled and lightly fried samples contained no nitrosopyrrolidine.

Currently United States curing regulations call for a maximum mass fraction of added sodium or potassium nitrite of 211 mg/kg and of sodium or potassium nitrate in bacon of 700 mg/kg. Up to 624 mg/kg of KNO_2 or $NaNO_2$ and 2 184 mg/kg of KNO_3 or $NaNO_3$ can be used in formulating dry cured primal products. A maximum analytical mass fraction of $NaNO_2$ of 200 mg/kg is permitted in all

finished cured meat products.

As reported at the First Symposium, the simplest and easiest attainable way of preventing nitrosopyrrolidine production in bacon could be by adding increased proportions of ascorbate or isoascorbate (erythorbate). These materials are approved curing additives in the United States. Of the sodium salt, a mass fraction of 550 mg/kg is permitted by the United States Department of Agriculture – about twice the amount needed for the purpose (cured colour retention).

Early literature (Mirvish et al. 1972; Fiddler et al. 1973c) reports that ascorbate could block nitrosation in model systems and led to attempts in industry to solve the problem by decreasing concentrations of nitrite and increasing concentrations of ascorbate or erythorbate in curing pickle. Tests in pilot plants at American meat industry laboratories showed promising results (Greenberg 1974). Indeed, tests under commercial conditions during 1973 and 1974 showed that increasing erythorbate in the product from typical mass fractions (200–230 mg/kg) to maximum (500–550 mg/kg), reduced mass fraction of nitrosopyrrolidine in fried bacon (confidence level 95%). Reduction in mass fraction of added nitrite from the industrial average of 170 mg/kg to 120 mg/kg reduced nitrosopyrrolidine in fried bacon (confidence level 99.9%).

After this preliminary work, two commercial tests were set up in which bacon was produced with erythorbate at about 1000 mg/kg and nitrite at about 120 mg/kg. The product was produced under commercial conditions and distributed normally to retail markets, where it was collected by Department of Agriculture representatives and analysed for nitrosopyrrolidine. Control product

Table 1. Effect of extra erythorbate (Ero) and less nitrite in the cure formulation on nitrosopyrrolidine (NPY) content of fried bacon produced in industrial tests. The bacon was in slices $\frac{1}{8}$ in (3 mm) thick and was fried at 340 °F (171 °C) for 6 min. *w*, mass fraction. Nitrosopyrrolidine was confirmed by combined GLC–MS.

Treatment	$w(NaNO_2)/mg \cdot kg^{-1}$	$w(Ero)/mg \cdot kg^{-1}$	$w(NPY)/\mu g \cdot kg^{-1}$
Study 1			
Control	34	18	14, 19
Control	30	91	19, 17
Test	3	281	< 10, < 10
Test	5	324	< 10
Test	3	294	< 10
Test	12	269	15, 16, 12
Test	13	336	< 10
Test	6	271	< 10
Study 2			
Control	26	13	17
Control	31	16	17
Test	10	594	11
Test	7	675	12
Test	10	750	14
Test	11	663	11
Test	10	575	11
Test	10	516	11

Table 2. Decline in mass fraction (w) of nitrosopyrrolidine (NPY) in fried bacon from retail stores in Washington, D. C. (1971–76). Analysis by gas-liquid chromatography confirmed by mass spectrometry (US Food & Drug Administration laboratory, Washington). Samples were sliced $\frac{1}{8}$ in (3 mm) thick and fried at 340 °F (171 °C).

Brand	Earliest date of collection	w(NPY)/ $\mu g \cdot kg^{-1}$	Latest date of collection	w(NPY)/ $\mu g \cdot kg^{-1}$	Δw/ $\mu g \cdot kg^{-1}$	100 Δw/ w(earliest date)
1	1971–12	102, 106	1975–10	19, 20	–84	–81
2	1971–12	47, 106	1976–04	6, 7, 7	–70	–91
3	1972–06	100	1975–10	9, 11, 16	–88	–88
4	1972–04	108	1976–01	17, 20	–89	–82
5	1972–06	20	1975–11	5, 5	–15	–75
6	1973–03	57, 59	1975–11	17, 20	–39	–67
7	1974–04	45	1975–10	10, 10	–35	–78
8	1971–12	73	1975–11	20, 23, 48, 40	–40	–55
9	1972–06	18, 32	1976–05	20, 22	– 4	–16
Mean	1971–12 to 1974–04	67	1975–10 to 1976–05	17	–50	–70

(manufactured under each plant's normal operating procedures and formulations) was obtained from the same supermarkets and analysed. Though the test product in both experiments contained less nitrosopyrrolidine than the controls, the differences were insufficient to suggest that simple reduction of nitrite and increase of erythorbate would eliminate nitrosopyrrolidine from cooked bacon (Table 1).

In the meantime, some interesting information has emerged. You may recall a set of data which I reported at the First Symposium (Greenberg 1974, p. 185–186) which represented the first set of samples collected at the retail level by the Food & Drug Administration, fried under controlled conditions, 6 min at 340 °F (171 °C), and analysed for nitrosopyrrolidine. All eight brands showed no nitrosopyrrolidine in the raw state, but contained mass fractions of from 10 to 108 μg/kg after frying (Fazio et al. 1973). At that time, the only source of nitrosopyrrolidine analysis in the United States was the Food and Drug Administration laboratory in Washington. It remains today our central autority for nitrosamine analysis. However, other government laboratories have now become properly equipped (Eastern Regional Research Center of the USDA Agricultural Research Services at Philadelphia; and the USDA APHIS facility at Beltsville). Two commercial laboratories (the Midwest Research Institute, Kansas City, and the National Canners Association laboratory, Berkeley) have also recently set up analytical facilities for nitrosamine. The Food and Drug Administration's Washington laboratory has been analysing samples of retail bacon since December 1971. Table 2 lists the earliest and most recent values for nitrosopyrrolidine from laboratory analyses of nine brands of bacon picked up at Washington retail stores. The average mass fraction in the first samples of each brand is 67 μg/kg. The average of the most recent samples is 17 μg/kg. This reduction has taken place without any changes in United States government regulation of bacon curing or processing. Obviously, many commercial concerns have taken steps that have somehow improved their bacon manufacturing process.

Table 3 shows analyses of nitrosopyrrolidine made during the years 1973, 1974, and 1975 by the USDA ARS Eastern Regional Laboratory. These samples from Philadelphia supermarkets, after frying for 6 min at 171 °C averaged 13 μg/kg in 1973, 10 in 1974 and 5 μg/kg in 1975. Just as with the Washington retail bacon, a steady decrease in nitrosopyrrolidine content is evident.

Much speculation has been published on the mechanism of nitrosopyrrolidine formation. There is obviously something special about nitrosopyrrolidine since it occurs only in special cases. What is it about the physiology of bacon bellies which,

Table 3. Nitrosopyrrolidine (NPY) content (w) of United States fried bacon taken from retail markets in Philadelphia. Bacon was sliced $\frac{1}{8}$ in (3 mm) thick and fried for 6 min at 340 °F (171 °C). Data of A. E. Wasserman (USDA, ARS, Eastern Regional Research Center) from gas-liquid chromatography confirmed by mass spectrometry.

Year	n(samples)	$\bar{w}$(NPY)/μg · kg^{-1}
1973	8	13
1974	17	10
1975	13	5

after the introduction of nitrite and subjection to high temperatures in the frying pan, results in nitrosopyrrolidine? This question has been discussed at length in several recent articles (Kakritz et al. 1976; Nakamura et al. 1976) and in this Symposium.

I would like to consider one aspect: the relation of mass fraction of 'residual nitrite' at the time of frying and mass fraction of nitrosopyrrolidine in the product after frying. A recent publication by Havery et al. (1976) postulated that, 'Bacon with a low residual nitrite does not show an increase in nitrosamine formation after storage at room temperature, since there is an insufficient quantity of nitrite available to react with the additional amines formed. On the other hand, bacon with a high residual nitrite which can continue to react with the amines as they form at room temperature may show a greater formation of nitrosamines.' This seems a reasonable idea. However data available on commercially produced bacon picked up from retail stores, analysed for nitrite, and subsequently fried and checked for nitrosopyrrolidine, does not support it. Examples of such data are presented in the next two tables. Table 4 lists 12 market basket samples of bacon analysed by two government laboratories in 1974. Table 5 shows data on 14 samples of bacon taken from supermarkets in Ottawa, Canada (Sen et al. 1976c). Both sets of data show no correlation between residual nitrite and nitrosopyrrolidine.

There was a similar lack of correlation of both nitrite and nitrate in the twenty samples of country-style ham market baskets (Table 6).

Determining the fate of nitrite in substrates as complex as bacon and ham is extremely complicated. Residual nitrite, as such, is simply a measure of detectable nitrite at a particular time in the life of the product. The value continues to decline from manufacture to consumption. However, the antibacterial action, organoleptic

Table 4. Content (w) of residual sodium nitrite at time of frying and content of nitrosopyrrolidine after frying of bacon from United States retail market (1974). Data on NPY by gas-liquid chromatography confirmed by mass spectrometry at Food & Drug Administration (Washington) and the United States Department of Agriculture, Philadelphia. There was no significant relationship between nitrite and either FDA or USDA estimates of ON.Pyr at confidence level of 0.05. Correlation coefficients were 0.07, and 0.39, respectively. The bacon was sliced $\frac{1}{8}$ in (3 mm) thick and was fried for 6 min at 340 °F (171 °C).

Sample	$w(NaNO_2)/mg \cdot kg^{-1}$	$w(NPY)/\mu g \cdot kg^{-1}$	
		US–FDA	US–DA
1	16.0	14	7
2	14.8	38	16
3	47.2	28	21
4	24.8	23	31
5	17.6	47	17
6	42.8	16	14
7	5.6	8	7
8	23.0	28	13
9	24.8	28	17
10	42.0	29	29
11	24.8	15	12
12	27.0	26	28

Table 5. Content (w) of residual sodium nitrite at time of frying and content of nitrosopyrrolidine (NPY) after frying of bacon retailed at Ottawa, Canada (1976). Data from gas-liquid chromography confirmed, where asterisked, by mass spectrometry by Sen et al. (1976). There was no significant relationship between nitrite and NPY at 0.05 confidence level, correlation coefficient 0.01. The bacon was fried by various procedures to 'well-doen stage.'

Sample	$w(NaNO_2)/mg \cdot kg^{-1}$	$w(NPY)/\mu g \cdot kg^{-1}$
A	64	22**
A	69	15
A	30	14**
B	25	35
B	101	30**
B	25	25**
C	30	15**
C	86	44**
C	59	11**
D	90	20
D	10	21**
E	32	30**
F	35	40**
G	76	10

Table 6. Nitrosopyrrolidine (NPY) content (*w*) in fried country-style retailed in four United States areas (1974). There was no significant relationship between ON.Pyr and nitrite or nitrate at the 0.05 confidence level, correlation coefficients being −0.06 and 0.12, respectively. Data by gas-liquid chromatography confirmed by mass spectrometry. Ham was sliced $\frac{1}{4}$ in (6.3 mm) thick and was fried for 12 min at 340 °F (171 °C).

Market	$w(NaNO_2)/mg \cdot kg^{-1}$	$w(NaNO_3)/mg \cdot kg^{-1}$	$w(NPY)/\mu g \cdot kg^{-1}$
Atlanta	33	133	< 10
	51	89	< 10
	22	162	< 10
	48	112	< 10
	31	143	50
Raleigh	86	192	< 10
	72	167	< 10
	116	218	< 10
	52	153	< 10
	39	162	19
Baltimore	22	116	< 10
	38	75	< 10
	15	267	< 10
	32	79	< 10
	6	106	< 10
Nashville	334	86	< 10
	63	291	37
	24	487	< 10
	14	166	< 10
	13	190	< 10

qualities, and potential for nitrosopyrrolidine formation are functions of the initial mass fraction of nitrite added to the product at time of formulation.

By holding nitrite inputs close to those amounts actually required for the useful functions of nitrite, excluding nitrate wherever feasible, and determining the manufacturing conditions that minimize nitrosation and other nitrosopyrrolidine-generating reactions, we should be able to produce bacon and country-style ham with optimum appeal to the consumer and minimum risk to health.

Discussion on the session

Is there a relation between ingoing and residual nitrite?

The residual nitrite in the test samples in Table 1 was reduced roughly 3-fold with respect to the level in the control samples. Remembering Dr Kotter's suggestion that residual nitrite might be used as an index of input nitrite, Dr Ingram wondered whether this might be taken to suggest a simular reduction in input nitrite – say 200 to 70 mg/kg. Dr Greenberg clarified that the input nitrite was not so greatly different: controls 170 mg/kg versus test products 120 mg/kg. The lower residual nitrite in the test samples is due to the presence of erythorbate. It is obvious that the residual nitrite is not a general index of input nitrite; the two can be related only in a particular set of circumstances. Dr Ingram agreed that the lack of such a relationship, which has also been found by several other investigators, contrasts with the strong correlation observed when increasing amounts of nitrite are added to a particular product, a correlation implicit in the proposition that nitrosamine contents would be diminished if less nitrite were added. This again focuses attention on the difference between input and residual nitrite. Taking as a basis Dr Cassens' estimate that 50% of the nitrite which disappears goes into combined forms of interest and assuming a bacon with 200 mg/kg input nitrite and 50 mg/kg residual, Dr Ingram calculated that there would be an equivalent of 75 mg/kg in combined forms, the amount already outweighing the residual. If the residual had gone down to 10 mg/kg, the ratio combined: free might perhaps be nearly 10 : 1. It appeared to Dr Ingram that the important element in nitrosamine formation during cooking might be the *combined* nitrite.

Effect of ascorbic acid on nitrosopyrrolidine in fried bacon

Dr Preussmann recalled Dr Roberts' communication of the day before, that ascorbic acid might increase the nitrosamine content of the fat fraction of the meat product. He was anxious to know whether data were available on the nitrosopyrrolidine content in the cooked-out fat of bacon after frying in the last five years and, if so, whether these indeed showed that an increased ascorbate addition results in an increased nitrosamine content in the fat. Dr Greenberg replied that data on fried drained bacon and bacon fat indicate that both had reduced amounts of nitrosamines with lower nitrite or higher ascorbate treatments. The fat samples, however, were always higher in nitrosopyrrolidine content than the drained bacon.

Significance of differences in low levels of nitrosopyrrolidine

There is no real difference between, for instance, the levels of 11–14 μg/kg nitrosopyrrolidine in the test products and the level of 17 μg/kg in the control samples in bacon study II (Table 1).

Advice to manufacturers

The figures show that most companies are moving in the right direction, changing their processing in such a way that the nitrosopyrrolidine formation is considerably reduced. However some producers do not yet. Dr Tannenbaum asked what could be done about this. It was replied that manufacturers were advised on good manufacturing practices. This is done by way of newsletters and by way of practical instruction in a workshop.

Causes of decrease of nitrosamine content of bacon in recent years

It is a well-known effect in trace analysis, that the more experience one has, the lower the analytical results are. Dr Schuller wondered if a simular mechanism would be another contributory factor resulting in the decrease of nitrosamine content of bacon in the last five years. Dr Greenberg, however, preferred to regard the decrease of ingoing nitrite and the increased use of ascorbate as the determinant factors.

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N-nitrosopyrrolidine in bacon obtained from 10 commercial bacon production plants

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This experiment was conducted jointly by the US Department of Agriculture (USDA), the Food and Drug Administration (FDA) and the American Meat Institute. It was a test of the probable commercial result to be expected upon adoption of the proposed USDA regulations, which call for production of bacon with 125 mg ingoing sodium nitrite and 550 mg of sodium erythorbate or sodium ascorbate per kg product. It was also designed to determine if nitrosopyrrolidine content of fried bacon would be reduced if low levels of ingoing nitrite were utilized in conjunction with the maximum amounts of sodium erythorbate or sodium ascorbate (550 mg/kg) currently permitted by USDA regulations.

Procedures

The study parameters are presented in Table 1. At each of 10 commercial bacon production plants, 20 bellies were pumped with the amounts of nitrite and erythorbate or ascorbate shown.

Normal production amounts of other curing ingredients such as salt, phosphate and sugar were utilized at each plant. Bellies were carefully segregated and processed with the normal cook-smoke method of each plant. The bellies were numbered, and packages of sliced bacon were marked with belly number and position from shoulder to flank. The bacon was vacuum-packaged and held at normal refrigeration temperatures.

Analysis for nitrite, nitrate and erythorbate or ascorbate were conducted at each

Table 1. Experimental plan for treatment of 20 bacon bellies in each of 10 commercial bacon production plants, of which 2 used ascorbate (Asc) and the other 8 used erythorbate (Eryth).

Treatment	Added $NaNO_2$ (mg/kg)	Added NaEryth or NaAsc (mg/kg)
1	0	0
2	40	550
3	80	550
4	120	550

plant. Included were ingoing curing solution, bellies after pump and draining, initial finished bacon and bacon weekly thereafter for 3 weeks, and at time of frying. Complete records were kept of all processing procedures. These records included type and amount of phosphate, amount of salt, type and amount of sugar, smoke cycle, type and length of smoke, heat sources, type of smoke generator, weight of bellies, age and type of bellies (fresh or frozen), curing solution and belly temperatures, holding times and number of slices per pound (1 lb = 0.45 kg).

After refrigerated storage for 3 weeks, 20 1-lb subsamples were prepared for each treatment at each plant. They were selected as shown in Table 2.

After taking 2 slices from each package for nitrite, nitrate and erythorbate analysis, the bacon was fried at 340 °F (170 °C) for 3 min on each side. The fried slices were blotted on paper towelling to remove excess fat and then packaged and frozen for shipment. The grill or pan was cleaned thoroughly between fryings of individual 1-lb subsamples. The 20 fried subsamples were packaged individually.

The composites were prepared at each specified laboratory and analyzed for nitrosamines by the FDA procedure for combined gas chromatography (GC) and mass spectrometry (MS). This multidetection method is widely regarded as the best available technology, and is deemed to be accurate and reproducible at levels of 10 μg/kg or more. It was agreed, however, by the three government and two private laboratories that participated in the analysis, to attempt to confirm by MS any GC

Table 2. Preparation of composite 1–lb subsamples of the sliced bacon.

Composite	Position	Belly numbers
1	centre	2, 7, 9, 15, 18
2	centre	4, 8, 11, 13, 17
3	shoulder	1, 5, 12, 14, 20
4	flank	3, 6, 10, 16, 19

Table 3. Relation of content of added $NaNO_2$ in bacon to presence of *N*-nitrosopyrrolidine (NPy, content more than 5 μg/kg by GLC) after frying slices on each side for 3 min at 170 °C. Confirmation was by combined gas chromatography and mass spectrometry.

$NaNO_2$ added (mg/kg)	Number of samples						
	tested	NPy present (GLC) at ⩾ 5 μg/kg					
		total	confirmed by GC–MS			not confirmable	sample depleted or lost
			at ⩾ 2 labs	at 1 of several labs	at 1 lab only		
0	38	1	0	0	0	1	0
40	39	3	0	1	0	2	0
80	38	9	0	2	2	4	1
120	39	12	3	1	1	5	2
Total	154	25	3	4	3	12	3

value of 5 μg/kg or more. Different methods of confirmation by mass spectrometer were employed at each laboratory.

Results

The main results are presented in Table 3. Of the 160 bacon samples originally planned, 154 were completed satisfactorily. Six samples were either lost or prepared improperly.

Only 25 samples were found to contain presumptive nitrosopyrrolidine upon initial GC analysis at 5 μg/kg or more. Of these 25, just 10 samples were positively confirmed by MS as nitrosopyrrolidine: 1 with ingoing nitrite at 40 mg/kg, 4 with ingoing nitrite at 80 mg/kg and 5 with ingoing nitrite at 120 mg/kg. The highest MS-confirmed value was 9 mg/kg found in one sample prepared with ingoing nitrite at 80 mg/kg. The other 9 samples contained nitrosopyrrolidine levels ranging from 5–7 μg/kg.

Confirmation was unsuccessful for 12 of the 25 samples. Although the GC finding was 5 μg/kg or more, these samples did not contain MS-confirmable nitrosopyrrolidine.

Average contents of residual nitrite and erythorbate or ascorbate in the bacon at the time of frying are presented in Table 4.

Table 4. Average contents of residual $NaNO_2$ and sodium erythorbate (NaEryth) in bacon cured with different levels of added $NaNO_2$. Groups of samples as in Table 4.

$NaNO_2$ treatment (mg/kg)	Number of samples	Residual $NaNO_2$ (mg/kg)	Residual NaEryth (mg/kg)
0	38	3	0
40	39	9	286
80	8	15	264
120	39	20	232

Summary and conclusions

As might be expected, when attempting to extend the capabilities of GC-MS methodology to the low levels of nitrosopyrrolidine encountered, differences between laboratories were numerous. In spite of doubts arising from this, however, it seems reasonable to conclude the following from this experiment:

1. None of the samples tested were found to be positive for nitrosopyrrolidine at a level of 10 μg/kg or more.
2. Lowering the ingoing level of sodium nitrite from 120 mg/kg to 80 mg/kg does not appear to reduce the occurrence of very low levels (less than 10 μg/kg) of nitrosopyrrolidine.
3. Processing variables, such as salt level and cook-smoke cycle, did not influence on the results.

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Volatile nitrosamines in commercial Swiss meat products

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Abstract

In 112 samples of Swiss meat products including scalded and fermented sausage, cooked cured ham, and dried meat, combined gas chromatography and mass spectrometry failed to demonstrate demethylnitrosamine, methylethylnitrosamine, diethylnitrosamine, nitrosopyrrolidine, nitrosopiperidine and nitrosomorpholine. The detection limit was a mass fraction of about 1 μg/kg.

Introduction

Since 1971, the Laboratory of Food Chemistry of the Swiss Veterinary Office has been developing and operating a section to solve the problem of nitrosamines in meat products. The Swiss health authorities asked us to check the extent of contamination in the most important meat products. The first meat products we investigated were thus cured meat products already marked from standard recipes.

We have not found any volatile nitrosamine in any of the 112 samples from meat products on the Swiss market. The investigation included scalded sausage, uncooked (raw, fermented) sausage, cooked ham and cooked cured meat products. Tests were for dimethylnitrosamine, methylethylnitrosamine, diethylnitrosamine, nitrosopyrrolidine, nitrosopiperidine, and nitrosomorpholine by combined gas-chromatography and mass-spectrometry (for procedure see 'Method of analysis' below). The detection limit of each nitrosamine was about 1 μg/kg; the recovery of added nitrosamine was about 65–75%.

To permit conclusions from further studies on composition of ingredients and on the type and composition of the additives, I will describe standard recipes for meat products and their standard analysis.

In Switzerland, Germany and Austria, available cold meats can be classed into 4 groups:

Scalded sausage: Frankfurter, Wiener, Cervelas, Bologna, cold cuts.

2. Uncooked (raw, fermented) sausage: Salami (type Milano, air-dried), farmer's sausage (smoked).
3. Cooked ham and cooked cured meat products: ham and pork shoulder.
4. Uncooked cured meat products: cured dried beef, uncooked ham (air-dried and smoked).

Scalded meat products

Number of market samples analysed: 60 (20 Wiener, 20 Cervelas, 10 Bologna, 10 Bierwurst). Nitrosamine was not found in these samples.

Representative recipe: Cervelas.

Ingredients (% w/w)		Additives (g/kg)	
steer II	30	pepper	1
pigskin	18	nutmeg	1
bacon	24	coriander	1
ice	24	cloves	0.125
frozen blood plasma	4	garlic	0.25
		onion	2
		nitrite salt	18
		(0.6% $NaNO_2$) =	0.1 $NaNO_2$

Composition (average from about 500 samples):

Major components (% w/w)		Trace components ($w/10^{-6}$)	
water	56.0	$NaNO_2$	29.5
fat	27.4	$NaNO_3$	49.7
protein (N · 6.25)	14.5	hydroxyproline	0.7
collagen protein (Hypro x 7.1)	4.8	sodiumascorbate	40.0
'muscle protein' (3-4)	9.9		
salt	2.63		

The mixture is emulsified and put into a casing, smoked for 2 h at 70–80 °C, pasteurized 15–20 min at 75 °C, showered and immediately cooled to below 5 °C. Other important Swiss types of scalded meat products differ only in detail.

The 60 negative results for the 6 nitrosamines led us to investigate scalded meat products. The following 12 meat products have been produced:

1. Wiener with nitrite salt 1,8% and spices
2. Wiener with nitrite salt 1,8%, sodium ascorbate 0.4 g/kg and spices
3. Wiener with nitrite salt 1,8%, glucono-delta-lactone 3 g/kg and spices
4. Wiener with nitrite salt 1,8%, glucono-delta-lactone 3 g/kg and sodium ascorbate 0.4 g/kg, spices
5. Wiener with nitrite salt 1,8%, sodium pyrophosphate 3 g/kg, and spices
6. Bologna with nitrite salt 1,8%, sodium pyrophosphate 3 g/kg, spices
7. Bierwurst with nitrite salt 1,8%, sodium pyrophosphate 3 g/kg, spices, smoked, scalded
8. Cervelas with nitrite salt 1,8%, sodium pyrophosphate 3 g/kg, spices, smoked, scalded, home-grilled
9. Cervelas as No 8 but also with sodium ascorbate 0.4 g/kg
10. Cervelas as No 9 but grilled
11. canned Bologna with nitrite salt 1,8%, sodium pyrophosphate, 1 g/kg, spices, sodium ascorbate 0.4 and sodium caseinate 4 g/kg.

Samples 8–11 had high contents of rind which comprised a third of the meat. The content of hydroxyproline was 1% of the whole mixture. Even though there

were large contents of hydroxyproline, nitrosamine and nitrosopyrrolidine were not found in grilled sausages.

Cooked ham

Number of samples analysed: 20, 10 were fried as breakfast ham. None of the 6 nitrosamines was found.

Standard recipe of Swiss cooked ham:

Brine		Additives (g/kg)	
water	0.1 m^3	NaCl	17
nitrite salt	15 kg	sugar	3
glucose	3 kg	sodiumpyrophosphate	2
pyrophosphate	2 kg		

12% of this brine is injected and tumbled (24 h). Molding and cooking, core temperature = 70 °C; Q_2 = 4.0. Added nitrite corresponded to about 0.1 g $NaNO_2$ per kg ham.

Uncooked (fermented) sausages

Samples analysed: 20, 10 were air-dried (Salami Milano) and 10 smoked (farmer's sausage). There were no nitrosamines.

Recipes: Salami 'Milano' (air-dried uncooked sausage):

Ingredients (% w/w)		Additives (g/kg)	
pork meat	70	pepper broken	2
bacon	30	red wine with garlic	5
		nitrite salt	3
		KNO_3	5

Put the mixture into a casing, let it sweat for about 3 days at 22 °C, at 95% relative humidity, afterwards air-dry for 6 months (caliber 12).

Tourist sausage: smoked uncooked (fermented) sausage, cold-smoked for 24–48 h at 20–22 °C for about 14 days.

Ingredients (% w/w)		Additives (g/kg)	
steer	60	coriander	1
rind cooked	10	pepper	1
bacon	30	red wine	5
		rum	1
		glucono-delta-lactone	6
		sodiumascorbate	0.4

Standard Analysis (% w/w.)

water	26.0
fat	40.6
protein	27.3
collagen	4.47
muscle protein (3–4)	22.9
NaCl	4.83

Discussion for the results

Even though the six nitrosamines were clearly absent, the question arises why other workers have found nitrosamines in similar meat products. Perhaps nitrosamine can form with faulty or unhygienic processing: high concentration of amines, poor distribution of the nitrite in nitrite salt or in the emulsion, poor distribution of acidifying agents and therefore locally low pH, and high content of reagents.

Our last studies concentrated on the clarification of this question.

Method of analysis

Procedure

Weigh out 100 g of ground sample into a 500 ml conical flask with 50 ml methanolic KOH (15%) and reflux for 8 h. Measure 75 ml of digest material into a 2-litre conical flask with 150 ml H_2O and 100 ml KOH (5 mol/litre) and distill 50 ml over.

Add 8 ml HCl (6 mol/litre) and put into a 250 ml separatory funnel. Extract 3 times with 50 ml CH_2Cl_2, wash the combined extracts with 50 ml NaOH (1 mol/litre), collect into a 250 ml boiling flask and wash the alkaline layer with 20 ml CH_2Cl_2. Evaporate the bulked CH_2Cl_2 extracts to about 10 ml, dry with Na_2SO_4 and transfer into a 50 ml flask.

Weigh out 6 g Florisil activated with HCl 6 mol/litre and pack into a chromatographic column. Put the concentrated extract on column, rince with 0.5 ml CH_2Cl_2 and wash twice with 4 ml hexane. Rinse column with 100 ml hexane, elute the nitrosamine with 75 ml methanol and evaporate to 0.5 ml.

Analyse extract by gas chromatography and mass spectrometry.

Parameter

stat. Phase	UCON HB 5100 capillary column
length	21 m
diameter	0.31 mm
carrier gas	GC, flow rate of H_2 2.1 ml/min; excess pressure 0.47 atm
	MS, flow rate of He 1.4 ml/min; excess pressure 0.85 atm

Carlo Erba Fractovap. GI 450 selective for nitrogen and phosphorus detection. Finnigan 3100 D GC-MS System.

Finnigan 6100 Data System.
Quadripole Mass-Spectrometry.
High resolution was compensated by multiple ion detection up to 4 masses.

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Discussion on the session

Possible cause of negative results

Dr Eisenbrand asked whether the negatives could be due to the fact that methanol was used as a solvent in the chromatographic clean-up procedure. The concentration of the methanolic solutions to a small volume might cause high losses of volatile nitrosamines. However, Dr Hauser compared the methanol procedure with several other clean-up procedures and got simular results; recoveries were about 70%.

Difference with nitrosamine levels found in other countries

There is a remarkable difference between the negative nitrosamine findings in Swiss products and the levels found, for instance, in German and Dutch products. If this difference is real, it would be worthwhile to investigate the causes of the difference. To eliminate any doubts as to the analytical methodology, it would be advisable to participate in the IARC collaborative study on nitrosamine analysis.

One cause of the difference might be that in Switzerland it is mandatory to vitaminize all meat products by an addition of 400 mg/kg ascorbic acid. Dr Hauser said that sometimes nitrosamines were found in the fat fraction of these products. In the United States, work is in progress on a new series of market basket samples, and so far the FDA has not found any nitrosamines in the meat products other than bacon. These results seem to correspond with the Swiss findings.

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Development of a method of estimating N-nitrosamino acids and its use on some meat products

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Abstract

A clean-up procedure was developed for isolation of the nitrosamino acids, nitrosoproline and nitrososarcosine. Amounts obtained at the end of this procedure can be measured by gas chromatography, photolysis, or polarography.

Introduction

In contrast to analysis of volatile nitrosamines, the separation, identification and estimation of non-volatile nitrosamines was poorly developed. Only nitrosoproline had been studied in some detail.

Our institute has been developing a method for estimating the nitrosamino acids *N*-nitrosoproline, *N*-nitrososarcosine and *N*-nitrosohydroxyproline, which could be formed from nitrite ion and the corresponding amino acids. A preliminary version of our method has been described (Dhont & van Ingen 1976). Gas-chromatographic methods of estimating methyl esters and trimethylsilyl derivatives of *N*-nitrosamino acids have been reported recently (Ivey 1974; Kawabata et al. 1974a; Kushnir et al. 1975; Eisenbrand 1976a). A full description of the analytical method is in preparation.

Method

Clean-up procedure

The clean-up procedure is described step by step in Table 1. The methanol–water mixture used in Step 1 for the extraction of the sample was chosen because in this mixture fats have a very limited solubility and the bulk of proteins in the sample precipitates. After Step 2, the solution is often still turbid and is further cleaned up by adding zinc sulfate and NaOH to the extract. After Step 4, a clear, but usually coloured, solution is obtained. Extraction with ethyl ether in Step 7 removes the nitrosamino acids from the aqueous solution. For extraction, the solution must be strongly acidic: at pH ≥ 3, recovery of nitrosoproline was poor. At a very low pH, added amino acids (tyrosine, glycine, tryptophan, alanine) were not extracted into the ether layer.

Table 1. Description of the clean-up procedure

1. Mince 50–100 g of sample in an electric mixer with 2 g urea and 2 ml HCl (6 mol litre^{-1}) in 400 ml of methanol and water (3:1, by volume).
2. Separate solid material by centrifuging, pour off the supernatant and wash the solid material thrice more with the methanol–water mixture. Combine the extract and washings. Discard the solid material.
3. Add 5–10 ml of 12% zinc sulfate solution of mass concentration 120 g litre^{-1} to the solution and then 5–10 ml NaOH of substance concentration 0.5 mol litre^{-1}.
4. Remove precipitate by centrifuging and discard it.
5. Remove the methanol from the solution by distillation in a vacuum film evaporator.
6. Acidify the aqueous residue with 2 ml of concentrated HCl and make up to 200 ml with a saturated NaCl solution.
7. Extract this mixture continuously with ethyl ether for 10 h.
8. After extraction, add 25 ml of water to the ether extract and remove the ether by evaporation in a vacuum film evaporator.
9. Filter the aqueous residue through an Amberlite XAD-2 column.
10. Filter the eluate from the XAD-2 column through an acidified cation exchange resin. The eluate is clear and colourless, and can be used for quantitative analysis by the photolytic nitrite method, by polarography or by gas chromatography.

Pyrazines disturb estimation by photolysis since they form nitrite under the same conditions as the nitrosamino acids. As a check, we added 2,6-dimethylpyrazine, which gave a strongly positive nitrite reaction after photolysis. Small amounts of pyrazine were detected in the ether layer after 10 h continuous extraction. They were, however, entirely removed by filtering the aqueous residue obtained in Step 8 through an acidified cation-exchange resin.

Nitrosohydroxyproline was not quantitatively extracted, partly because of decomposition of the compound during extraction. Even reference solutions of nitrosohydroxyproline decomposed under conditions where nitrosoproline and nitrososarcosine remained unchanged.

In Step 9, the XAD-2 resin column efficiently removed the coloured matter present in many samples. This column probably removes some other compounds like bile acids and steroids too. The solution resulting from Step 10 is completely clear and colourless.

Remark In our earlier paper (Dhont & van Ingen 1976), ethylacetate was used as an extractant. This liquid, however, often gave very acid residues because of extracted HCl and water, which hydrolysed the nitrosamino acids. Ethyl acetate was therefore replaced by ether.

Measurement

For measurement by gas chromatography, the solution is evaporated to dryness in a vacuum film evaporator and the resulting residue dissolved in dry methanol containing 3–5% HCl (by mass). After esterification, water is added and the esters are extracted with dichloromethane. The dichloromethane extract is concentrated and analysed by gas chromatography through a 1 m x 4 mm (inner diam.) column packed with 3% (by mass) Carbowax 20M on 'Embacel' 60–100 mesh at 180 °C.

For measurement by the photolytic method, the eluate from Step 10 is made up to 200 ml with water, treated with 100 mg of K_2CO_3 and irradiated in a photoreactor.

If the sample contains only traces of nitrosamino acids, the solution becomes only slightly coloured by dyes formed in the Griess reaction. We therefore tried to concentrate the dye formed by adsorption on Polyamide, and to elute it into a small volume. This dye did not adsorb onto the Polyamide, in contrast with the behaviour of some azo dyes with the same general structure that are used for colouring food products. A dye with strong adsorption properties was obtained by reaction of the nitrite ion with naphthionic acid and *N*-1-naphthylethylenediamine. The dye was completely adsorbed on 300 mg Polyamide powder. If more than about 0.1 mg of nitrosamino acids are present in the sample, another 100–200 mg of the Polyamide powder is added until the dye is completely adsorped. The dye can be eluted from the Polyamide with methanol–ammonia.

Results

For an assortment of meat products with the clean-up method of Table 1, data from photolysis are shown in Table 2. The largest mass fractions of nitrosamino

Table 2. Mass fractions of total nitrosamino acids (expressed as nitrosoproline equivalent) in meat products. The meats are designated by the same code letters as in the paper by Groenen et al., page 227.

Product code	Total content of nitrosamino acids (mg/kg)	Product code	Total content of nitrosamino acids (mg/kg)
Smoked horse meat		Liver sausage	
J	1.1	H	1.9
K	1.2	J	1.8
L	0.8[+]	Cooked liver K	1.1
M	0.9	Paté de foie L	0.9
		Canned liver pie M	2.9
Luncheon meat			
H	0	Guelder-type ring sausage	
J	0	H	0
K	0.01	J	0.02
L	0.05	K	0.05
M	0.02	L	0.01
		M	0
Bacon			
J raw	0.6	Minced meat	
K raw	1.5	H fried*	0.1
L raw	0.5	J fried*	0.80
M raw	0.1	K fried*	0.65
N raw	0.4[+]	L fried*	0.09
N fried	0.03	M raw	0
		N fried*	0.95

*, Freshy prepared minced meat, fried in the laboratory.
[+], Nitrosoproline identified by MS.

acids were found in smoked horsemeat, raw bacon, liver products and fried minced meat; less was present in luncheon meats, fried bacon, Guelder-type ring sausages and raw minced meat.

The products listed in Table 2 were also analysed for volatile nitrosamines, nitrite and nitrate. Those results are reported by Groenen et al. in this symposium (next paper).

Acknowledgment

Much of this study was made under contract to the Netherlands Inspector-General of Public Health, which supervises foodstuffs and inspection of merchandise.

Discussion on the session

Recoveries

In the model systems, the recoveries of the nitrosamino acids range from 90 to 100%. In meats, recoveries were 80–110%.

Sydnones

In an attempt to hydrolyse the nitrosamino acids with strong mineral acid, recoveries of the parent amino acids were only 10–20% in the hydrolysate, and nitrosamino acids had disappeared completely. The only explanation would be the formation of sydnones, because the amino acids themselves were found to be resistant to the acid treatment. Dr Dhont drew attention to the possibility that sydnones could be made use of for GC detections. Other experts in the audience, however, expressed their doubts about the GC amenability of sydnones, because of their mesoionic character. They might also hydrolyse into hydrazines.

Specificity of detection technique

According to Dr Tannenbaum, photolytic decomposition followed by detection with the Griess reaction is not a sufficiently specific procedure and often produces results that are too high. Dr Dhont answered that all of the tested compounds that give a positive reaction were completely removed in the clean-up processing of the extracts. Only for nitromethane was a very faint positive reaction observed. Moreover, the results were confirmed with GC-MS.

Nitrosoproline levels

It was remarked that the nitrosoproline levels found are one order of magnitude higher than those reported by Dr Tannenbaum. Dr Wasserman's group too, found nitrosoproline levels in raw bacon of about 1 mg/kg, but these were only semi-quantitative values. But at that time he was mainly interested in demonstrating nitrosoproline for the first time (Kushnir et al. 1975). Recently he examined bacon with a new procedure. With products forming very little nitrosopyrrolidine on frying, he found little or no nitrosoproline. He is now looking for bacon with relatively high levels of nitrosopyrrolidine to determine whether the raw product will contain

nitrosoproline. Dr Tannenbaum said that Dr Dhont's data and his were not irreconcilable. Thus the lowest level reported by Dr Dhont is 100 μg/kg, whereas he found 80 μg/kg in a limited number of samples of a product of different type. However, this comment did not completely take away the feeling that there was a significant difference between the results of the two laboratories.

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Further studies on the occurrence of volatile N-nitrosamines in meat products by combined gas chromatography and mass spectrometry

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Abstract

Cured and uncured meat products of commercial origin were analysed for eight volatile nitrosamines by combined capillary gas chromatography and medium-resolution mass spectrometry on one or two columns.

Only dimethylnitrosamine was detected regularly; the contents found were 0.1–15.5 μg/kg. The highest contents of it were detected in luncheon meat and smoked meat. Nitrosopiperidine was next in frequency of occurrence; the contents found were 0.4–7.0 μg/kg. Diethylnitrosamine and methylethylnitrosamine were found or suspected occasionally, mostly at levels < 1 μg/kg, that is at or slightly above the detection limit. Methylpropylnitrosamine, methylbutylnitrosamine, dipropylnitrosamine and nitrosopyrrolidine were not detected in any of the products. The detection limit for nitrosopyrrolidine was higher than for the others, 10–20 μg/kg. One product was tested for γ-butenyl-(β-propenyl)-nitrosamine, but the compound was not detected.

The nitrosamine contents of the products were not correlated with their contents of residual nitrite or nitrate.

Introduction

At the 4th International Working Conference on the Analysis and Formation of *N*-Nitroso Compounds (Tallinn, USSR, October 1975), we reported on the occurrence of *N*-nitrosamines in cured meat products (Groenen et al. 1976), and described a method developed in our institute for estimating *N*-nitrosamino acids (Dhont & van Ingen 1976). Stephany et al. (1976) reported on estimation of volatile nitrosamines in cured meat products at the Netherlands National Institute of Public Health at Bilthoven. Ritskes (1975) described methods for volatile amines developed at the Institute of Fishery Products TNO in Ymuiden. These studies were parts of a survey of the occurrence of volatile nitrosamines and nitrosamino acids in cured meat products reaching the consumer in the Netherlands.

The present paper describes the estimation of volatile nitrosamines in another series of meat products, cured and fresh. Residual nitrite and nitrate were also estimated. Dhont (this symposium) describes the estimation of nitrosamino acids in the same products.

Methods and materials

Products

The products investigated were: 1. Cooked ham (gekochter Schinken; gekookte ham); 2. Smoked horse meat (Pferde-Rauchfleisch; paarderookvlees); 3. Luncheon meat (Frühstücksfleisch; boterhamworst); 4. Raw bacon; 5. Mildly fried bacon; 6. Liver sausage, cooked liver and liver pie (Leberwurst, gekochter Leber und Leberpasteten; leverworst, gekookte lever en leverpastei); 8. Guelder-type ring sausages (Gelderländer Rauchwurst; Gelderse rookworst); 9. Freshly minced meat, fried (Hackfleisch, gebraten; vers gehakt, gebraden): mixtures of beef or pork (or both) with fat and water (not cured), fried in the laboratory.

The samples were purchased in shops in Zeist and further afield. Thus, their manufacturing history and processing were unknown. To characterize the samples, salt, residual nitrite, nitrate (in only some products), protein, fat, moisture, and pH were determined at the Department Netherlands Centre for Meat Technology of our institute by Drs J. Meester and Mrs C. R. Star-Verkuil. Where possible, different brands of the products were purchased. For the unbranded products, we assume that random acquisition has resulted in samples from different processers.

Bacon, as before (Groenen et al. 1976), was fried mildly, at about 150 °C for $1\frac{1}{2}$ min on each side, in a flat open frying-pan, without added fat or oil. The samples of minced meat were fried for $2\frac{1}{2}$ min.

Samples were designated by letters F to N, to distinguish them from the preceding series of meat products, coded A–E (Groenen et al. 1976).

Isolation and concentration of nitrosamines

We have discontinued the use of the combined steam distillation and extraction procedure of Likens & Nickerson (1964) with the modifications described (Groenen et al. 1976). The reasons for this will be described under 'Results and Discussion'. Samples were now steam-distilled and extracted separately.

About 250 g of the sample were mixed with 50 g $MgSO_4$ (both for salting out the volatiles and to prevent foaming), some $K_2CO_3/KHCO_3$ (to obtain a pH of about 8), and 150–300 ml water, depending on the character of the product. We used no anti-foaming agents of the silicon type. The mixture was steam-distilled until 400–500 ml distillate had been collected; the distillates were then repeatedly extracted with dichloromethane by manual shaking at pH 1 after saturation with NaCl. The extracts were dried over Na_2SO_4 and were carefully concentrated to 500 µl as described (Groenen et al. 1975), without any further clean up.

Reference solutions of nitrosamines (concentration 10 µl/litre) in dichloromethane or hexane were used for measurements. *o*-Tolunitrile or benzonitrile was added to the extracts and nitrosamine reference solutions as an internal reference, at a concentration of 10 µl/litre.

Identification and measurement

The extracts were analysed on one or two stainless-steel capillary columns, 150 m long and 0.75 mm inter diameter, coated with UCON 50 HB 280X and SF 96, respectively. The columns for gas chromatography (GC) were coupled to a Varian MAT 731 high-resolution mass spectrometer (MS) through a slit separator. Experimental conditions were largely as described before (Groenen et al. 1976). Resolution ($m/\Delta m$) was about 4500. Sample volumes of about 1 μl were injected; with the wide bore of the capillary columns, there was no need for an injection splitter. Eight nitrosamines were measured in one GC-MS run by tuning the mass spectrometer from mass to mass between the retention times of the nitrosamines, as described by Bryce & Telling (1972) and Gough & Webb (1972).

Results and discussion

Composition of the products investigated

Table 1 summarizes the ranges of the contents of protein, fat and water and pH of the groups of products. Table 2 gives contents of NaCl, residual nitrite and nitrate in the products.

Nitrosamine contents of the products investigated

Table 2 also shows contents of the nitrosamines found in the meat products. In order of frequency, the nitrosamines detected were dimethylnitrosamine, nitrosopiperidine, diethylnitrosamine and, exceptionally, methylethylnitrosamine. Methylpropylnitrosamine, methylbutylnitrosamine, dipropylnitrosamine and nitrosopyrrolidine were not detected in any of the products. Contents of dimethylnitrosamine ranged from 0.1 to 15.5 μg/kg. The most was in luncheon meat, smoked horse

Table 1. Composition of the meat products investigated, expressed as range of mass fractions on a moist basis, and pH. Product F (cooked ham) was not analysed.

Category	Protein (%)	Fat (%)	Water (%)	pH
Smoked horse meat	21.2–27.4	0.2– 3.2	55.0–61.8	5.6–5.8
Luncheon meat	10.1–14.5	23.6–44.1	40.0–55.6	5.7–6.5
Bacon (raw)	16.9–21.7	16.2–34.0	46.3–57.0	5.3–5.8
Bacon (fried)[a]	28.2	28.5	33.0	
Bacon (same, unfried)[a]	18.3	22.2	53.0	
Liver sausages and pies	13.3–15.8	10.8–38.3	44.7–70.6	5.7–6.6
Cooked liver	21.9		65.4	5.2
Guelder-type ring sausages	11.8–13.9	31.0–39.1	44.9–50.1	5.3–5.7
Freshly minced meat (raw)[b]	14.8–20.2	9.9–29.4	53.4–67.6	
Freshly minced meat (fried)[b]	19.0–25.7	12.5–35.7	45.0–61.1	

a. Loss of mass during frying was about 35%.
b. Not cured.

Table 2. Contents of NaCl, residual nitrite and nitrate (expressed as $NaNO_2$ and KNO_3), and nitrosamines in meat products. Nitrosamines were determined by coupled capillary gas chromatography and medium-resolution mass spectrometry. Methylpropylnitrosamine, methylbutylnitrosamine, dipropylnitrosamine and nitrosopyrrolidine were never detected. Nitrosamine codes stand for: dimethyl, methylethyl and, diethylnitrosamine and for nitrosopiperidine, and are tabulated in that order, Nitrosamines were corrected for incomplete recovery. Values in parenthesis correspond to minute GC-MS peaks, at or only slightly above the detection limit; for the individual nitrosamines these detection limits were from a tenth to a few μg/kg, except for nitrosopyrrolidine, for which it was 10–20 μg/kg.
*, The identity of these peaks was confirmed with a second capillary GC column, also coupled to the mass spectrometer.

Category and code	Salt (%)	Residual nitrite (mg/kg)	Residual nitrate (mg/kg)	Nitrosamine			
				DMNA	MENA	DENA	NPip
Cooked ham F				2.3	–	(0.2)	–
Smoked horse meat							
F	5.8	13	1200	2.6	–	–	–
H	12.2	66	2480	7.4	–	–	–
J	10.8	54	1163	9.4*	1.1*	0.8*	–
K	11.4	138	2155	5.1*	–	0.5	–
L	13.2	220	387	5.1*	–	0.4	–
M	11.0	6	370	2.0	–	(0.1)	–
Luncheon meat							
H	1.9	8	52	(0.1)	–	–	–
J	2.5	7	25	9.5*	(0.2)	(0.4)	–
K	1.7	6	13	3.2*	–	(0.2)	0.7
L	1.8	3	< 5	15.5*	–	–	1.0
M	2.4	5	< 5	9.5*	–	(0.2)	–
Bacon							
F raw				1.8	–	(0.2)	–
G raw	3.7	9					
G fried				7.5	–	–	–
H raw	9.7	104	< 5	9.5*		(0.1)	7.0*
H fried				6.5*	–	–	6.5*
J raw	5.2	38	480	3.2*	–	–	5.6
J fried				1.2	–	–	1.2*
K raw	5.0	8	150	1.5	–	–	–
K fried				2.3	–	–	1.1
L raw	3.6	17	77	0.9	–	–	–
L fried				1.7 1	–	–	–

M raw	6.2	15	15	1.2	–	–	–
M fried				1.6	–	–	–
N raw	7.9	68		1.3	–	–	–
N fried	11.5	76		1.2	–	–	5.2
Liver products							
Liver sausage H	1.6	18		0.3	–	–	–
Liver sausage J	1.5	27		0.7	–	–	–
Cooked liver K	3.4	13		1.6	–	–	–
Paté de foie L	1.9	9		1.9	–	(0.4)	–
Canned liver pie M	1.8	14		0.9	–	–	(0.4)
Guelder-type ring sausages							
H	2.1	7		0.6*	–	–	3.5*
J	1.9	8		0.7*	–	–	3.4*
K	1.6	15		0.4	–	–	–
L	2.0	9		0.6	–	–	3.3
M	2.0	4		0.6*	–	–	1.9
Minced meat							
H raw	0.10	< 3					
H fried	0.08	< 3		0.4	–	–	–
J raw	0.03	< 3	5				
J fried	0.03	< 3		0.6	–	–	–
K raw	0.13	< 3	16				
K fried		< 3		0.6	–	–	–
L raw	0.12	< 3	16				
L fried	0.14	< 3		0.7	–	–	–
M raw	0.13	< 3	24				
M fried	0.16	< 3		1.9	–	–	–

meat, and, occasionally, raw and fried bacon. In the other products and in the other bacon samples, values were systematically low, about 0.1–1 μg/kg.

Nitrosopiperidine ranged from 0.4 to 7.0 μg/kg. The most was in some bacon samples. Four out of five Guelder-type ring sausages and two luncheon meat samples, too, contained some.

Very little diethylnitrosamine and methylethylnitrosamine, about 1 μg/kg or less, was found, or suspected, in some luncheon meat and smoked horse meat and, occasionally, in other products.

The nitrosopiperidine in Guelder-type ring sausages and luncheon meats may have been formed by reaction of nitrite with pepper. Much nitrosopiperidine has been found in mixtures of spices with nitrite in meat-curing agents (Sen et al. 1973b; Gough & Goodhead 1975), and the use of such premixes has therefore been stopped in some countries. But, as Sen et al. (1973b) pointed out, the reaction might occur in the finished meat product as well. We do not know whether spice extracts are added to brines used for curing bacon.

The present identification of the nitrosamines is certain and unambiguous, since the chromatographic capillary column had high resolving power and since detection was based on scanning of molecular mass at a resolution of about 4500. Trimethyl-[^{29}Si]silyl ion did not interfere in the identification of dimethylnitrosamine, because of the resolution of the GC column (Groenen et al. 1976) and because of the use of $MgSO_4$ instead of a silicon product as anti-foaming agent. Thus, it would not strictly be necessary to confirm the correctness of the identifications on a second GC column, whereas such a control is often adviseable with detectors of lower specificity. Nevertheless, it is certainly worth while, to substantiate any claims for the presence of compounds as undesirable as nitrosamines in foods, are to be confirmed with experimental evidence beyond the minimum required, in order to satisfy even the most critical observer. We therefore, checked some of the results on a different GC column (wall-coated with SF 96 instead of UCON 50 HB 280X as the stationary phase). The sensitivity of this particular arrangement was lower by a factor of 3–5 than that of the first GC-MS combination, so that marginally positive results were neither confirmed nor rejected. However, all definitely positive results from the first column that were checked on the second column were confirmed (Table 2).

As to the accuracy and the precision of results like those presented here, there is still a lack of information. The inter-laboratory studies, organized by the International Agency for Research on Cancer are certainly opportune. The results for nitrosamine given in Table 2 presumably suggest a higher precision than is warranted.

Like our earlier results (Groenen et al. 1976), our data for some of the same and some other types of meat products again show smoked meat and raw and fried bacon as products needing close scrutiny for the nitrosamines. Luncheon meat and Guelder-type smoked sausages now merit scrutiny too.

The present data do not show an obvious correlation between nitrosamine levels and residual nitrite and nitrate in the products. On the contrary, luncheon meat and smoked meat emerge from this study as products with the most dimethylnitrosamine, despite their divergent content of both nitrite and nitrate. In spite of much nitrite and nitrate in smoked meat and some of the bacon samples, dimethylnitro-

samine did not exceed 10 μg/kg in these products.

In our first study on nitrosamines in meat products (Groenen et al. 1976), we found diethylnitrosamine at 91 μg/kg in a smoked meat product, that contained residual nitrite at 218 μg/kg. That result was confirmed on two capillary columns coupled to the mass spectrometer, and by direct introduction of the extract into the mass spectrometer at a resolution of 15 000. It was also confirmed on a thermal energy analyser, specific for *N*-nitroso compounds by courtesy of Dr R. W. Stephany (National Institute of Public Health, Bilthoven, NL). Another sample, however, with nitrite at 100 μg/kg, contained no nitrosamine whatsoever. Evidence is ever increasing for a lack of correlation of nitrosamines with residual nitrite and nitrate. Probably the amounts of ascorbate or other inhibitors of nitrosamine formation play a decisive role.

Apart from the cured meat products, we studied formation of nitrosamines in uncured fresh minced meat after frying. The nitrite contents were estimated before and after frying; they were < 3 mg/kg. Nitrate was estimated in the raw products only and ranged from 5 to 24 mg/kg. Unexpectedly traces of dimethylnitrosamine were found in the fried product. This result is hard to explain. Perhaps minor amounts (of the order of 1 mg/kg) of nitrite or nitrate in fresh meat might lead to traces of the order of 1 μg/kg of nitrosamines. Data on nitrate in fresh meat are scarce. White (1975) studied the relative significance of dietary sources of nitrate and nitrite. He found only one casual estimate of nitrate in fresh meat, 0.9 mg/kg (Wright & Davison 1964). We therefore analysed some more fresh meat products (lean beef steak, pork chops and steaks, and pork fat) for nitrate and for salt and nitrite. 'Natural' salt levels were 0.06–0.12%, nitrite < 3 mg/kg and nitrate 2–6 mg/kg. Some mixtures of freshly minced meat and fat appeared to contain KNO_3 at $4-16 \times 10^{-6}$ mg/kg. These values are considered normal meat blanks (Fudge & Truman 1973; Follett & Radcliff 1963). Methods of estimating such traces are, however, open to improvement. According to Usher & Telling (1975), the method generally favoured (nitrate reduction with cadmium and measurement of nitrite formed as a diazo compound) is not ideal and is suspect for traces (10 mg/kg) of nitrate and nitrite in food samples.

The absence of nitrosopyrrolidine from the fried bacon samples is remarkable. The detection limit of our method has been improved, and is now about 10–20 μg/kg. Of all nitrosamines, this compound remains the most difficult to isolate from the food matrix. Anyhow, we conclude that mild frying of bacon does not form nitrosopyrrolidine in contents above 10–20 μg/kg.

As well as for volatile nitrosamines, the meat products of this study were analysed for nitrosamino acids (Dhont, these proceedings, p. 221).

Analytical aspects

We have discontinued the use of combined equipment for steam distillation and extraction (Likens & Nickerson 1964). Separate steam distillation at pH about 8 for 1–2 h, followed by a few manual extractions of the distillate at pH about 1 with dichloromethane after saturation with salt, gave equal or larger recoveries in a shorter time. Presumably, the amount of water needed to maintain a steady circulatory distillation and extraction in the equipment of Likens & Nickerson is too large.

Perhaps a fixed amount of water keeps circulating in the steam arm, thus hampering recovery of volatiles from the product. When we studied successive extracts from the equipment of Likens & Nickerson after 0–2, 2–4, and 4–6 h of operation, we found that dimethylnitrosamine, nitrosopiperidine and especially nitrosopyrrolidine, added to the product, were still being recovered during the third period. The higher aliphatic nitrosamines were recovered practically completely during the first 4 or even 2 h.

Scanlan (1975) has stated rightly that the term steam distillation is not appropriate for substances that are as soluble in water as some of the nitrosamines. Recoveries in such a distillation are negatively correlated with solubilities in water.

Even so, the equipment of Likens & Nickerson is very useful for isolation of trace components from complex mixtures. In our institute, it is widely used for studies on aroma and off-flavour.

During the present tests, with separate distillation and extraction, recoveries of nitrosamines at levels of 5–25 μg/kg were generally 50–100%, but only 0–55% for nitrosopyrrolidine. Goodhead & Gough (1975) report similar recoveries for nitrosamines from meat products. For nitrosopyrrolidine, recovery was 65%, but with a standard deviation of 32.9.

In GC-MS, it proved advantageous to use an internal reference in the extracts and in the nitrosamine reference solutions. The results thus become independent of the exact volume injected, so eliminating a source of error. As internal reference for meat extracts, we chose benzonitrile or *o*-tolunitrile. Our work on nitrosamines in cigarette smoke (Groenen & ten Noever de Brauw 1975; ten Noever de Brauw et al. 1976) showed that these nitriles occupy a convenient position in the nitrosamine chromatograms. Of course, another compound may be chosen, as long as it is not naturally present in the products. In foods, *o*-tolunitrile does not seem to occur naturally; benzonitrile has only been found in cacao, roasted peanuts and the like (van Straten & de Vrijer 1973, 1976). Recently dipropylnitrosamine has been used as an internal reference and for checking recovery (Goodhead & Gough 1975).

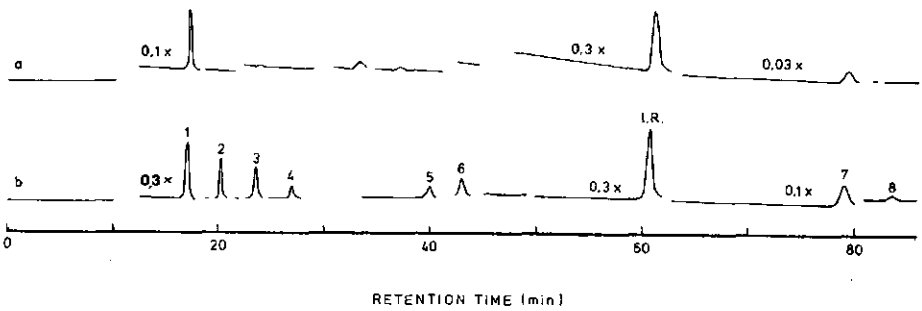

Fig. 1. Molecular ion mass chromatogram of extracts of (a) bacon H, raw; (b) the same product, with nitrosamines added (25 μg/kg). Peak 1, dimethyl; 2, methylethyl; 3, diethyl; 4, methylpropyl; 5, methylbutyl; 6, dipropyl nitrosamine; 7, nitrosopiperidine; 8, nitrosopyrrolidine. I.R. = *o*-tolunitrile as internal reference. Column stainless steel 150 m long; 0.75 mm inner diameter. Stationary phase: UCON 50 HB 280X. Column temperature: 110 °C. Mass spectrometer: VARIAN MAT 731.

The use of capillary columns instead of packed columns for GC-MS may be an additional contribution to identification. Foreman & Goodhead (1975) feared that the small capacity of a capillary column would tend to lower the limit of detection. We assume that they intended to say the converse. A small capacity of a column would raise the limit of detection, or, reciprocally, would lower sensitivity. Then, however, the statement in its generality, is not correct. In our institute, wide-bore stainless-steel capillary columns, of about 0.75 mm internal diameter, have been used for years, both in aroma research and for estimating nitrosamines. Without deterioration, these columns can take injected volumes of about 1 μl several times daily for at least a year. In contrast to narrow-bore capillaries, there is no need to split off a major portion of the injected sample, which would indeed proportionally decrease general sensitivity. Wide-bore capillary columns are used increasingly in aroma research and certainly merit wider use in work on nitrosamines.

Figure 1 shows mass chromatograms of about 1 μl of extracts of a bacon sample, blank and with nitrosamines added respectively. Interruptions in the base line correspond to adjustments of the mass spectrometer to the successive molecular masses of the nitrosamines.

A useful detail of our work has a bearing on safety for workers in the field. Instead of preparing reference solutions of nitrosamine by weighing the individual nitrosamines one by one on an analytical balance, with the corresponding risk for the analyst and his colleagues, we prepare reference solutions on a volumetric basis with microsyringes. This can be done easily in a safety hood. The reference solution we mostly used contained the nitrosamines at 10 μl/litre. Results were calculated in μg per kg product and were corrected for nitrosamines having a mass density different from 1.00 kg/litre (Table 3).

We want to emphasize that, though we have thoroughly searched some forty extracts of meat products for even traces of eight volatile nitrosamines, we may have overlooked other semivolatile nitroso compounds, perhaps in larger amounts. To indicate the range of retention times searched and unsearched. Figure 2 shows the gas chromatogram of the same extract of spiked bacon as in Figure 1. The chromatogram was obtained with a normal flame-ionization detector. The retention times of the eight nitrosamines are indicated. It is obvious that a large part of the volatiles and semivolatiles in the extract remain uninvestigated. Perhaps we have not been looking for the really important nitroso compounds. Apparent *N*-nitroso compounds have indeed been detected in food extracts with a thermal energy analyser,

Table 3. Mass density (volumic mass, ρ) of some nitrosamines, rounded to 2 decimal places.

Compound	ρ/kg · litre^{-1}	Source of data
Dimethylnitrosamine	1.01	US–CRC 1973
Diethylnitrosamine	0.94	US–CRC 1973
Dipropylnitrosamine	0.92	US–CRC 1973
Dibutylnitrosamine	0.90	IARC 1973, Vol 4, p. 197.
Nitrosopiperidine	1.06	US–CRC 1973
Nitrosopyrrolidine	1.10	Our estimate

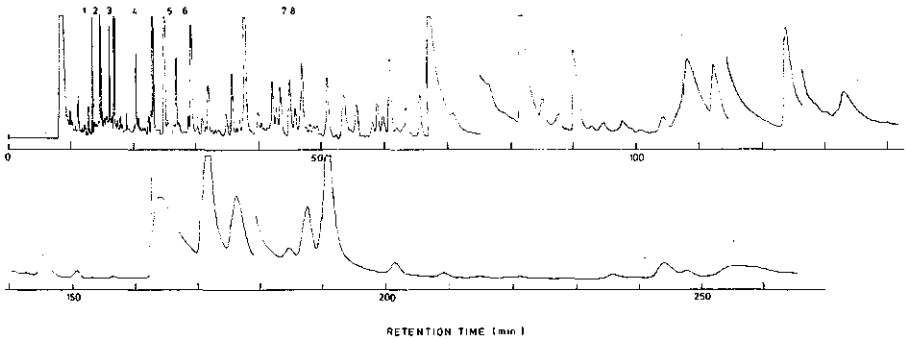

Fig. 2. Capillary gas chromatogram of an extract of bacon H (raw), with nitrosamines added at 25 μg/kg (same sample as in Fig. 1 b). Detection by flame ionization. Only a small region of the chromatogram (with numbered peaks) was searched for nitrosamines.

far behind the nitrosopiperidine peak (Stephany & Schuller 1976). This is disquieting, and is stimulus for further work.

As a first test, we have searched an extract of fried bacon for γ-butenyl-(β-propenyl)nitrosamine by GC-MS. The compound can be formed by nitrosation of spermidine and spermine (Hildrum et al. 1975). Though we did not know the exact retention time of the compound, we looked for some characteristic ions (*M*/*z* 123, 99 and 68) from its mass spectrum. The compound was not present in the extract in the range of retention times examined, that is the range of Figure 1. Much work remains to be done in this area of 'unusual' *N*-nitroso compounds.

Acknowledgments

Part of this study was under contract to the Netherlands Inspector-General of Public Health, charged with the supervision of foodstuffs and the inspection of merchandise. Ir M. C. ten Noever de Brauw provided advice and support with the mass spectrometry. We gratefully acknowledge the contributions of Drs J. Meester and Mrs C. R. Star-Verkuil (Department Netherlands Centre for Meat Technology).

Discussion on the session

Nitrate in fresh meat

Dr Groenen found some nitrate in raw meat samples. Several experts in the audience reported that levels of 10 to 20 mg/kg of nitrate, calculated as KNO_3, were not uncommon (by the method in which the nitrate was first reduced with cadmium, and the resulting nitrite then determined). Since nitrite absorbed in the intestinal tract appears in saliva, it might as well be present in other body fluids or the organs. Dr Tannenbaum and his coworkers found small amounts of nitrate (20–30 mg/kg) in almost all protein materials they examined.

If nitrates or other substances that could cause nitrosation be normally present in fresh meat, it would be useful to investigate the formation of nitrosamines in uncured meat products in more detail.

It was considered to be an unsatisfactory situation that, whereas nitrosamines can be determined at the μg/kg level, nitrate at the mg/kg level is still subject to uncertainties.

Preparation of standard solutions

The preparation of calibration standards using a microsyringe can be subject to errors of 3–4%. The volume delivered also depends on the surface tension of the liquid. In contrast, the overall random error in nitrosamine analysis is a few times higher.

On the recommendability of 'short' or 'long' retention times in GC-MS

Dr Freudenthal remarked that in Dr Groenen's procedure the whole range of nitrosamines is measured in one long GC run. Such a long run is needed to switch from one nitrosamine to the next in high resolution. The speaker preferred not to switch from one peak to the other. He first measures, for instance, only dimethylnitrosamine in a series of extracts. Then, the next nitrosamine is measured in the extracts, at a different temperature, and so on. In this way he can use short retention times, although more GC runs have to be done (for more nitrosamines). The advantage of the short retention times is that the signal-noise ratio improves.

Dr Groenen replied that switching from mass to mass in a GC-MS run was nothing new. It has been done before for nitrosamines by Bryce & Telling (1972) and by Gough & Webb (1972). He used long retention times in order to fully utilize the resolving power of the capillary column, to decrease the probability of interferences. Adjustment of the mass spectrometer from one nitrosamine to the other requires only a few minutes. This adjustment has to be stable also for only a few minutes, which it does easily and which can be checked immediately before and after the peak-by-peak matching with reference masses of perfluorokerosene fragments.

Dr Gough commented that mass spectrometer signal suppression by material co-eluted with a nitrosamine will affect measurement unless the peak-matching technique is used for detection. In this mode of operation, signal suppression can be observed and measured, and thus a correction can be made to the quantitative assessment of the level of nitrosamine present. Good-quality chromatography will minimize the occurrence of suppression.

Dr Groenen added that he had carried out a control experiment on the suppression of the MS signal by measuring nitrosamines in dichloromethane and added to a meat extract. There was no difference in MS response, thanks to the GC resolution and the efficiency of the ion source.

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Some critical remarks on the optimum resolution to use in trace analysis of nitrosamines with combined gas chromatography and mass spectrometry

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Abstract

The resolution of the mass spectrometer, the quality of its ion optics together with the properties of the gas chromatographic column are discussed as critical parameters in the analysis of *N*-nitrosamines with the combination GC-MS.

Introduction

At present, it has been accepted that the most reliable procedure for the unambiguous detection of traces of volatile *N*-nitroso compounds (NA) is a combination of gas chromatography and high-resolution mass spectrometry (GC-MS). There is still some disagreement between workers in this field about the best conditions for such analysis in practice. A short review of various GC-MS combinations reported in the literature is given elsewhere (Stephany et al. 1976a, b). Especially the question arises how high the resolution of the mass spectrometer (MS) should be to avoid all risks of obtaining false positive results. As will be discussed in this paper, the resolution of MS is only part of the requirements to be fulfilled to avoid false positive results in GC-MS.

Resolution of the mass spectrometer, quality of its ion optics and properties of the gas chromatographic column as most critical parameters in GC-MS

For quantitative analysis of a substance by GC-MS, the mass fragmentographic method is most frequently used. This means that the mass spectrometer serves as a selective and sensitive detector by tuning it to a characteristic relative mass of the substance under consideration. In addition, the substance is characterized by its GC retention time. A schematic representation of an analysis by GC-MS is given in Figure 1.

After injecting the sample, the components are separated in the gas-chromatographic column according to their retention time. The separated components are then successively eluted into the MS. The MS ionizes the eluted sample components and measures the various characteristic mass-to-charge ratios, M/z, of the resulting positive ions. In practice, most of the ions carry a relative charge of $^{+1}$ only and then M/z equals the mass of the ions, M. After being resolved into nearly parallel

ion beams, these discrete beams each consisting of ions with the same M/z finally appear at the narrow entrance slit of the ion detector of the MS (Fig. 2). Only ions with the relative mass M are allowed to pass the entrance slit of the ion detector, causing a signal proportional to the *total* number of ions entering the detector. Ions with the slightly different mass $M + \Delta M$ cannot enter the detector and are not detected. However, in practice, through imperfections the ion optics of MS, not all the ions with the same mass are positioned along the centre line of the corresponding ion beam but are partially scattered around this centre line. With increasing scatter, the ion beams significantly overlap.

Plotting the number of ions per volume unit as a function of the distance from the centre line of the beam of ions with the mass M, we get an ion density distribution plot. In such plots the distance along the axis also can be expressed as M/z. According to the ion optics of the MS various shapes of ion density plots are possible. Some frequently occurring shapes of such plots are shown in Figure 3.

With a fixed shape of the ion density distribution the overlap of two beams consisting of ions of slightly different masses M and $M + \Delta M$ is completely deter-

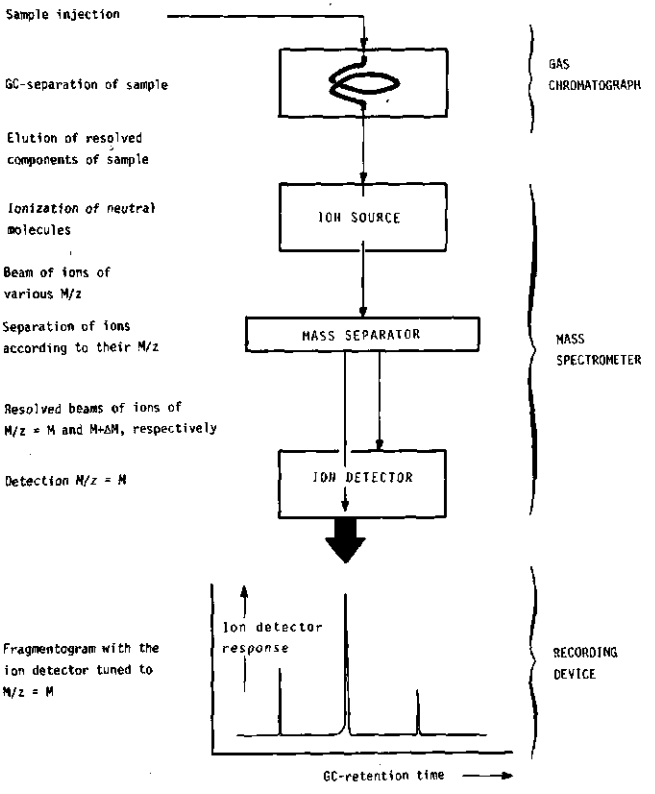

Fig. 1. Flow chart of analysis by combined gas chromatography and mass spectrometry. M, relative ionic mass; z, relative ionic change (charge number).

mined by the resolution of MS. For two equimolar ion beams, the resolution of MS is in general defined as the ratio $M/\Delta M$, if density plots of the two ions intersect mutually at 5% of the maximum ion density. If so, an ion density minimum of 10% exists, the '10% valley' between the two maxima. For two triangular ion density plots, the resolution and the overlap is demonstrated in Figure 4 for 'good' and 'bad' ion optics. As can be seen from Figure 4 with bad ion optics, the ion density plot is wide spread at the foot and the resulting overlap is much larger than with good ion optics. Still the resolution is the same! As will be clear now, MS resolution is not the most suitable parameter of overlap and thus of possible interference between ions of slightly different masses. The quality of the ion optics – a variable parameter characteristic for a particular instrument in combination with its operator – and *not* the resolution is at first instance responsible for possible interference obtained in a GC-MS analysis at medium to high resolution.

This statement is illustrated in the series of Figures 5–8, where mass fragmentography of ions with the mass M, for instance $^{14}N^{16}O^{\oplus}$, M/z 29 998 and ions with the mass $M + \Delta M$, for instance $^{12}C^{1}H_2\,^{16}O^{\oplus}$, M/z 30 011, are schematized as performed with 'good' and 'bad' ion optics. In each, the ion density plot is shown,

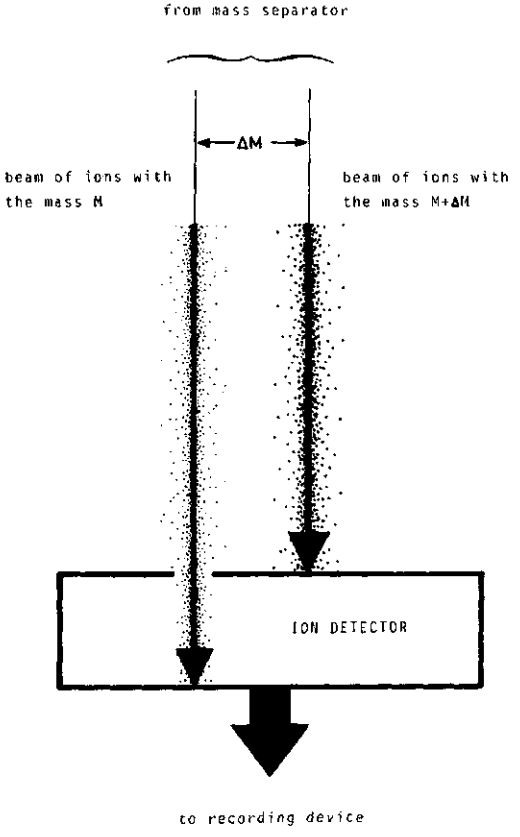

Fig. 2. Ion detector of mass spectrometer tuned to ions of mass M (assuming that z is 1). ΔM, any mass-spectral interval to another ion of mass $M + \Delta M$.

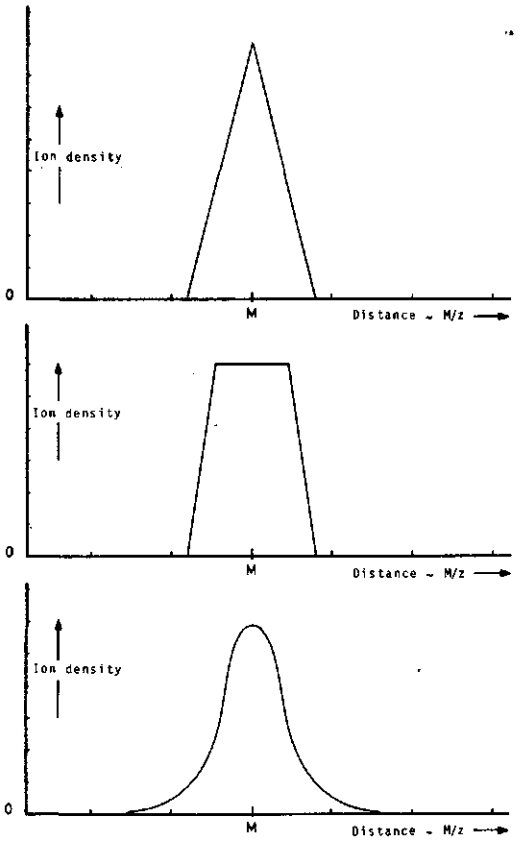

Fig. 3. Different ion density distributions possible for an ionic species of Mass M with z is 1.

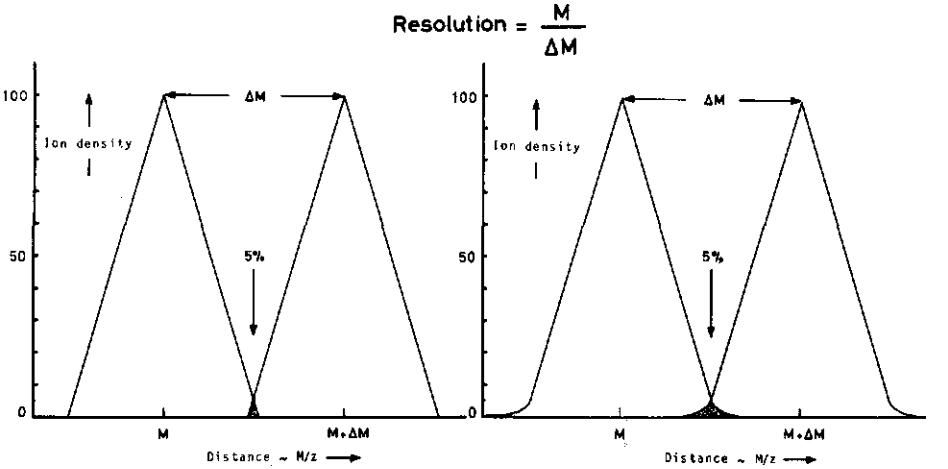

Fig. 4. Interference between equimolar amounts of two ionic species in a mass spectrometer with resolution $M/\Delta M$ and overlapping at 5% of maximum ion density. Left. 'Good' ion optics: peak area overlap ¼%. Right. 'Bad' ion optics: peak area overlap $> >$ ¼%.

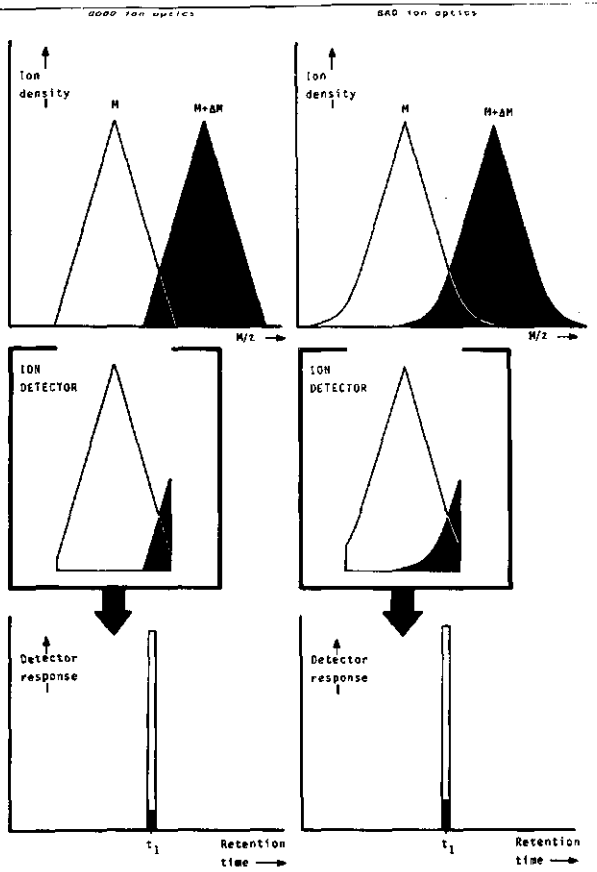

Fig. 5. Mass fragmentography at medium resolution of ions with mass M in the presence of equimolar amount of interfering ionic species of mass $M + \Delta M$. Left. 'Good' ion optics. Right. 'Bad' ion optics.

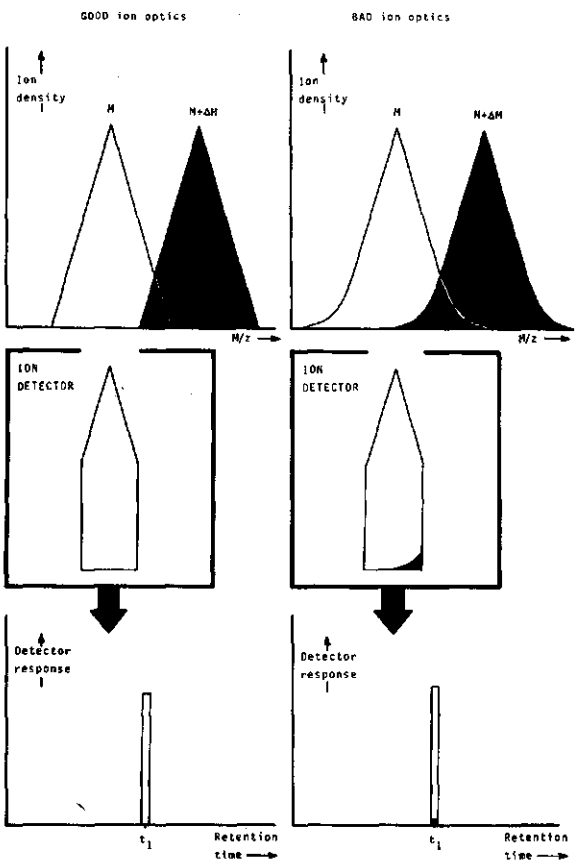

Fig. 6. Mass fragmentography at high resolution of ions with mass M in the presence of equimolar amount of interfering ionic species of mass $M + \Delta M$. Left. 'Good' ion optics. Right. 'Bad' ion optics.

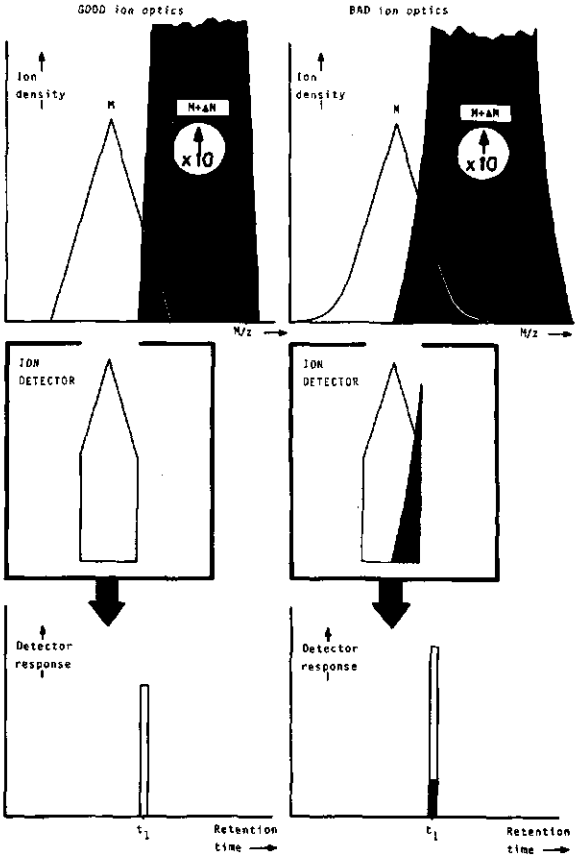

Fig. 7. Mass fragmentography at high resolution of ions with mass M in the presence of a large excess of interfering ionic species of mass $M + \Delta M$. Left. 'Good' ion optics. Right. 'Bad' ion optics.

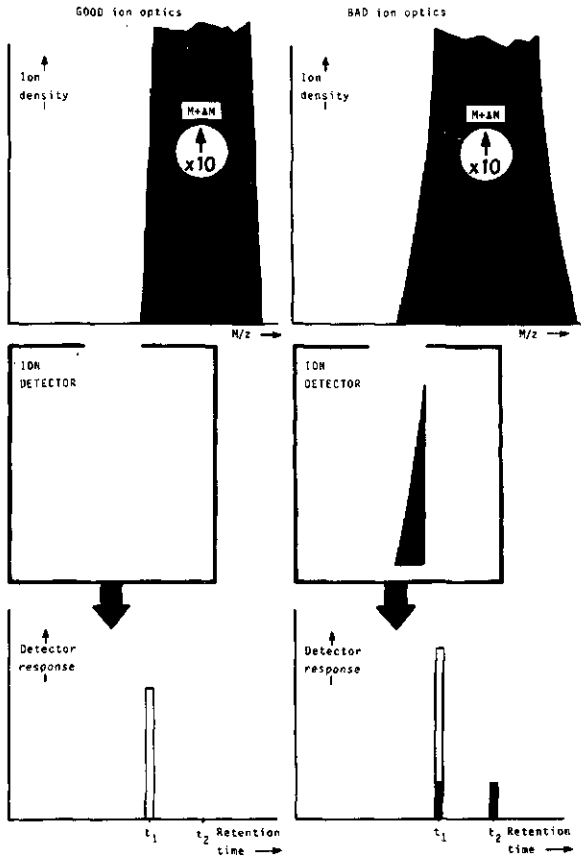

Fig. 8. Mass fragmentography at high resolution in the absence of an ionic species of mass M but with a large amount of an interfering ionic species of mass $M + \Delta M$. Left. 'Good' ion optics. Right. 'Bad' ion optics.

as well as that part of the ion density that passes through the entrance slit of the ion detector to yield a signal as shown in the resulting fragmentogram. We can now distinguish between four extreme situations. In Figure 5, a *N*-nitroso compound releasing $NO^{\oplus}$ and an interfering compound releasing $CH_2O^{\oplus}$ are eluted simultaneously from the GC column at retention time t_1. With equimolar amounts of both ions and with 'medium' MS resolution, for instance 2 000, in the fragmentogram, a contribution of the interfering $CH_2O^{\oplus}$ to the signal attributed to the $NO^{\oplus}$ is present with good ion optics as well as with bad ion optics. However with bad ion optics, this interfering contribution of the $CH_2O^{\oplus}$ is significantly larger than with good ion optics.

In Figure 6, the same situation is represented as in Figure 5. Only now the MS resolution is increased to 'high', for instance 10 000, by narrowing the entrance slit of the ion detector. Compared with the situation with 'medium' MS resolution, the selectivity also is largely increased and with good ion optics any interference by $CH_2O^{\oplus}$ is eliminated. However with this increase in selectivity, a concomitant loss of sensitivity has to be accepted. With bad ion optics, there still is, a small residual interference by $CH_2O^{\oplus}$, in spite of the increased resolution.

Closer to reality is the situation represented in Figure 7. Here the interfering ions are present in large excess with respect to the ions to be detected, for instance 10 times. Again no interference occurs with good ion optics, contrary to the situation with bad ion optics. In this situation, appreciable interference occurs in the

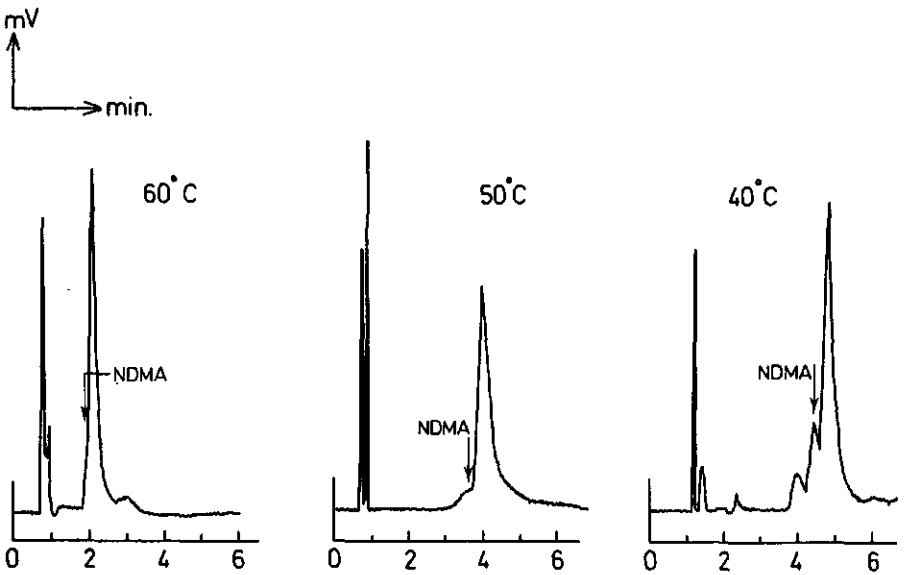

Fig. 9. Example of false positive result for *N*-nitrosodimethylamine (NDMA) by mass fragmentography after gas chromatography at 60 °C of an extract of luncheon meat (IARC, Can 52). Capillary column with polar coating UCON 50, 30 m long; resolution 3000; *M/z* (NDMA) 74, injected volume 0.25 µl equivalent to 220 mg sample. Reading implies 0.3 ng NDMA.

fragmentogram by the increased contribution of $CH_2O^{\oplus}$ to the total signal attributed to the $NO^{\oplus}$.

In Figure 8, the situation is represented at the retention time t_2. No *N*-nitroso compound but only a component of the sample releasing $CH_2O^{\oplus}$ is eluted now. Again there is no interference with good ion optics and at retention time t_2 there is *no signal in the fragmentogram*. However with bad ion optics in the fragmentogram, a signal appears at retention time t_2 originating exclusively from $CH_2O^{\oplus}$, but simulating the presence of $NO^{\oplus}$ in the ion detector. If the retention times t_1 and t_2 are very close together and especially if the stability of the retention times is poor, false positive results can easily be obtained, even at 'high' MS resolution. An improvement in GC conditions rather than a further increase in MS resolution will be the approach to avoid such false positive results.

A nice example of how one can get entangled with false positive results if one uses improper GC conditions is given in Figure 9, which shows the detection of *N*-nitrosodimethylamine (NDMA) in the fragmentograms of a blank extract of a luncheon meat sample at various temperatures of the polar capillary column. The fragmentograms were obtained at a MS resolution 3 000 with the MS tuned to the mass 74 048 of the parent ion of NDMA. At 60 °C, an interfering component simulated a strong false positive NDMA response.

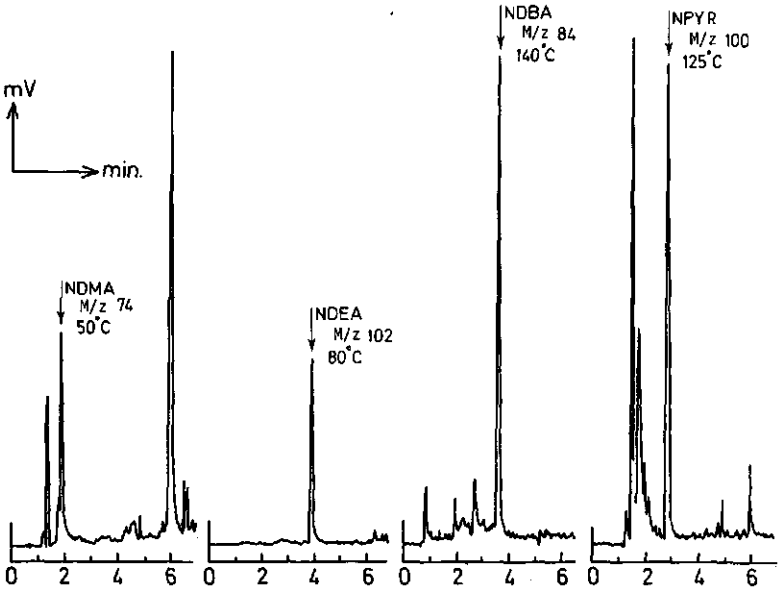

Fig. 10. Example of mass fragmentography of nitrosamines after gas chromatography under appropriate conditions of a spiked extract of luncheon meat (IARC, Can 52). Multiple apolar and polar capillary column coated with OV-101, 34 m long; MS resolution 3000; injected volume 0.25 µl equivalent to 220 mg sample; spiked with 2.5–3.0 ng of nitrosodimethylamine (NDMA, *M/z* 74), nitrosodiethylamine (NDEA, *M/z* 102), nitrosodibutylamine (NDBA, *M/z* 84) and nitrosopyrrolidine (NPYR, *M/z* 100).

Discussion and conclusions

Figures 5–8 demonstrated that, independently of the magnitude of MS resolution, one will always detect a small but finite *range* of masses. Too much emphasis is given to the advantages of increasing the MS resolution from a few thousands – so-called 'medium' resolution – to more than ten thousand – 'high' resolution – for unambiguous detection and identification of NA.

Every GC peak that releases even a few ions with masses close enough to the mass of detection will be recorded as a peak in the fragmentogram, at least in principle. In practice, however, only a very large amount of such interfering substances present in the extract will yield enough detectable ions to give peaks comparable in size with the peak originating from a specific ion of an *N*-nitroso compound. Unfortunately such a situation is common in extracts of biological material, even if additional clean-up procedures have been applied. Instead of wasting time searching for 'the ideal clean-up procedure' – which is strongly dependent on the kind of original material to be cleaned-up – one can better try to change the GC elution pattern of the components of an extract by using at least a polar and an apolar (capillary) GC column. We checked several methods of fragmentography for the analysis of NA in meat products (Stephany et al. 1976b) and in general the method with GC on multiple capillary columns combined with single parent-ion detection at medium resolution proved the most suitable.

Some typical examples of fragmentograms, obtained by the method just mentioned under proper GC conditions, of various NA in a spiked extract of a luncheon meat sample are shown in Figure 10.

The amounts of NA found with the different columns should agree of course. This agreement can be seen as an internal and additional check of the method. Quantitative disagreement between the results obtained with different columns suggests the presence of interfering substances. If so, positive results should be confirmed at a higher MS resolution (10 000–12 000) only for unambiguous confirmation, provided that enough sample is available. However with the GC-MS method we have used for 3 years to detect and measure NA down to 0.1 μg/kg, false positive results were never found.

Acknowledgments

The author is indebted to Dr P. L. Schuller for stimulating discussions and to Dr J. Freudenthal for valuable advice.

Discussion on the session

On the desirability of increasing GC resolution or MS resolution

Dr Freudenthal said that one of the most important parameters to be controlled is GC performance. The capillary column should have good separation qualities and stable retention times. If so, one can handle really dirty samples at a medium-resolution setting. Dr Groenen remarked that, when nitrosopyrrolidine is eluted from a capillary column in 3 min (as in Fig. 10), one should realize that 50–100 compounds are eluting in the same time-span. The resolving power of a capillary

column is not made use of in this way. Dr Freudenthal replied that with longer retention times, their stability and reproducibility becomes problematic.

Dr Groenen thought that resolution of GC and MS should both be optimized. Dr Gough communicated that he injects extracts into a packed column; narrow fractions from this column are introduced into a capillary column coupled to a mass spectrometer, thus allowing short retention times.

The reliability of peak matching methods in GC-MS

Dr Freudenthal was not quite sure whether combined GC and high-resolution MS using peak matching are reliable at low levels. Dr Groenen said that it took only a few minutes to switch from mass to mass. Long-time stability is not required. Dr Gough added that successive parent ion monitoring can be achieved provided there is a minimum of 2 min between the elution of each nitrosamine. Mass-spectral peak matching under high resolution should be used and this gives a detection limit (in the extract) of 0.2–2 μl/litre, depending on the nitrosamine and the state of the ion source (in other words 0.2–2 μg/kg in food).

Comparison of four techniques for nitrosamine determination

Dr Gough said that the enhanced possibility of positive results by mass spectrometry described by Dr Stephany has been demonstrated at the Laboratory of the Government Chemist (London). The various mass-spectral procedures published for nitrosamine determination and the TEA technique were compared using the same extracts on the same day, and some of these observations are shown in Table 1.

High-resolution mass spectrometry with peak-matching parent-ion monitoring and TEA always agree well. The other mass-spectral techniques sometimes gave erroneously high values for both nitrosamines. The conclusion, which is based on experience in analysing several thousand extracts by mass spectrometry, is that provided suitable care and precautions are taken, high-resolution peak-matching parent-ion monitoring mass spectrometry is comparable with TEA. A full description of these observations will be published shortly.

Table 1. Comparison of three mass spectrometric procedures and thermal energy analysis (TEA) for measurement of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPyr) in extracts. All extracts were analysed on the same day. –, not detected. Data from the Laboratory of the Government Chemist, London.

	Sample	Low-resolution MS; multi-ion monitoring	High-resolution MS; peak-matching parent-ion monitoring	High-resolution MS; precise-ion monitoring	TEA
NDMA	1	2	2	2	2
	2	4	3	4	3
	3	30	9	38	11
	4	100	8	80	9
NPyr	5	5	3	4	2
	6	9	19	18	22
	7	12	1	2	1
	8	10	–	3	–

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

How specific and sensitive is the thermal energy analyser?

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Abstract

Various aliphatic, alicyclic and aromatic C-nitroso compounds are reported to give a weak to strong molar response on the thermal energy analyser (TEA), depending on the structure of the compound considered. As a consequence, these compounds may interfere and give false positive results in the analysis of N-nitrosamines with TEA and GC-TEA.

Introduction

A new method for trace analysis of *N*-nitroso compounds, called thermal energy analysis (TEA), has been developed by Fine and his co-workers (Fine et al. 1975a and this symposium). This analyser, which has been commercially available since the beginning of 1976, has been claimed to exhibit high specificity and extreme sensitivity to *N*-nitroso compounds. This paper gives evidence that possible false positive results may be obtained with combined gas chromatography and thermal energy analysis (GC-TEA) as a result of interfering *C*-nitroso compounds.

Results

Besides the GC-MS method used at RIV to analyse *N*-nitrosamines (Stephany et al. 1976b), recently also the combination GC-TEA became available for the analysis of *N*-nitroso compounds. With this combination (GC Model 661 – TEA Model 502), we analysed extracts of a variety of meat products resulting from previous GC-MS studies (Stephany et al. 1976a) and fresh extracts from meat products.

In the resulting gas chromatograms, called TEA-grams, the majority of the extracts showed several large peaks besides the peaks originating from the NA present in the extracts and already confirmed by GC-MS. Except for a minor peak whose retention time matched that of *N*-nitrosomorpholine (NMORPH), none of these extra peaks could be assigned to one of over forty volatile reference *N*-nitrosamines available to us.

According to the applied isolation procedure (Stephany et al. 1976a, b) and as a consequence of the high specificity claimed for the TEA detector, we would have to believe that all these peaks originated from steam-volatile, heat-stable *N*-nitroso compounds amenable to gas chromatography but of unknown identity. In particu-

Table 1. GC-TEA conditions for Figures 1, 3, 4 and 5.

Column	4.3 m (14 feet) stainless steel, 3.2 mm (1/8 inch) outer diameter
Stationary phase	10% Carbowax, 20 M
Support	Chromosorb WHP, 80–100 mesh
Column temperature	125 °C, isothermal
Carrier gas	helium
Inlet pressure	5.4 atm (0.55 MPa)
Pyrolyser temperature	425 °C
Cold-trap temperature	−150 – −152 °C
Injected volume	5.0 µl

lar, the presence of several large peaks between the solvent front and the *N*-nitrosodimethylamine (NDMA) peak is hard to explain, because the number of *N*-nitroso compounds more volatile than NDMA on a polar Carbowax column would be very limited. Possible some very small cyclic *N*-nitrosamines or some small alkenyl, alkynyl or *t*-butyl substituted *N*-nitrosamines will meet the requirements to be eluted faster than NDMA within the applied GC conditions. The GC conditions for all figures in this paper are given in Table 1.

A TEA-gram of a luncheon meat extract showing several peaks between the solvent front and the NDMA peak is reproduced in Figure 1. The NDMA peak is situated at the foot of a large unknown peak. Under less favourable GC conditions, these two peaks will coincide, simulating a much too strong NDMA response. In the

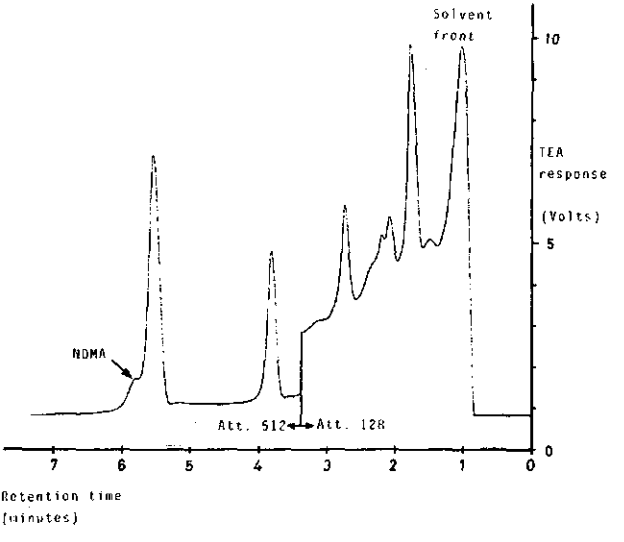

Fig. 1. TEA-gram of an extract of canned spiced luncheon meat (IARC, Can 96). The peak of nitrosodimethylamine (NDMA) lies at the foot of a large unknown peak, perhaps a *C*-nitroso compound. Injected volume 5 µl equivalent to about 6 g luncheon meat. The instrument was set at an attenuation (Att.) of 128 and then 512.

absence of NDMA, a false positive result would be obtained. The specificity of the TEA detector has been claimed to the nitrosyl functional group only (Fine et al. 1975a).

Besides *N*-nitroso compounds, organic nitrites ('*O*-nitroso' compounds), some organic nitrates, the nitrite and nitrate ion and all other compounds with thermally labile nitrosyl groups would give a response on the TEA.

Among such compounds with a labile nitroso moiety, *C*-nitroso compounds are probably the most likely to give a response on TEA. As far as we know, only a few aromatic *C*-nitroso compounds have as yet been tested for response on TEA. *p*-Nitrosodiphenylamine and 2-nitroso-1-naphthol are reported to show no significant molar response on TEA (Fine et al. 1975a). Of these two compounds, 2-nitroso-1-naphthol probably is not even a *C*-nitroso compound, but the isonitroso or oximido tautomer, 2-isonitroso-1-ketonaphthalene (Carunchio et al. 1976) (Fig. 2).

We studied various aliphatic, alicyclic and aromatic *C*-nitroso compounds for response on TEA. If injected directly into the catalytic pyrolyser of the TEA at 425 °C, all tested *C*-nitroso compounds so far showed a weak to very strong molar response, depending on the structure of the compound (see Note added).

With GC-TEA, only the aliphatic and alicyclic *C*-nitroso compounds showed a response. These results suggest the decomposition of the aromatic *C*-nitroso compounds on the column to products no longer able to release nitrogen monoxide under the applied GC conditions. Some of the *C*-nitroso compounds tested are listed in Figure 3. Some of the 2-nitroso-2-methylpropane derivatives that give a

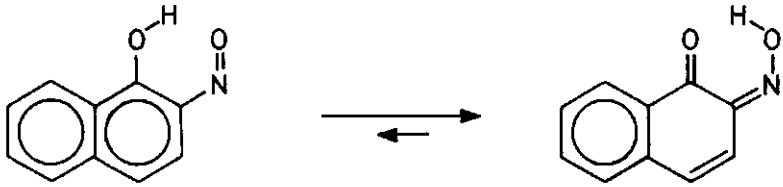

Fig. 2. 2-Nitroso-1-naphthol (2-nitroso-1-hydroxynaphthalene) and its oximido tautomer, 2-isonitroso-1-ketonaphthalene.

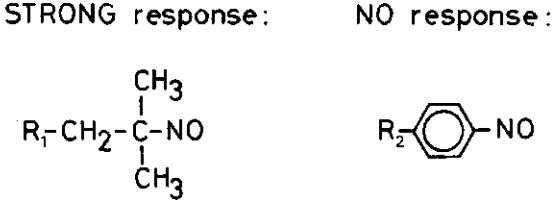

Fig. 3. Structure of *C*-nitroso compounds tested at the nanogram level for response on GC-TEA. All the *C*-nitroso compounds tested responded, differently according to structure, when injected directly into the catalytic pyrolyser of the TEA. R_1 was H-, Cl-, $CH_3CO{\cdot}O$-; R_2 was H-, Cl-, CH_3-, HO-, $(CH_3)_2N$-.

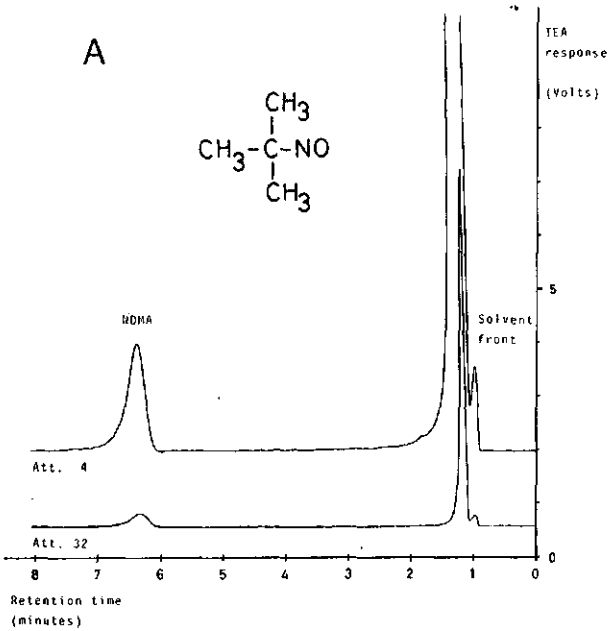

Fig. 4 A. 2-Nitroso-2-methylpropane (NMP, 65. ng): relative molar response of NMP to NDMA 7.5.

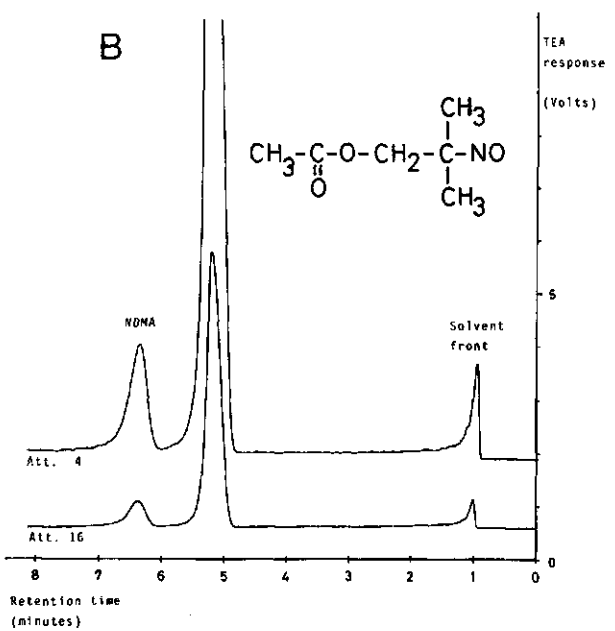

Fig. 4 B. 2-Nitroso-2-chloromethylpropane (NCMP, 9.0 ng): relative molar response of NCMP to NDMA 5.4.

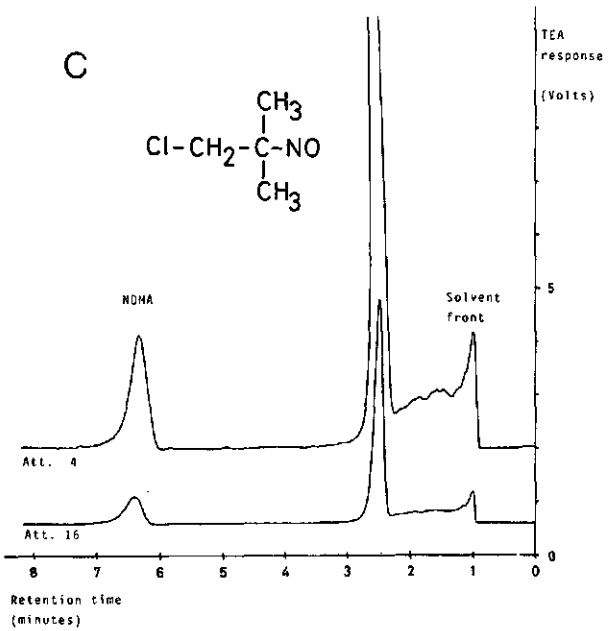

Fig. 4 C. 2-Nitroso-2-acetoxymethylpropane (NAMP, 11.0 ng): relative molar response of NAMP to NDMA 7.7.

Fig. 4. TEA-gram of equimolar solutions of *N*-nitrosodimethylamine (NDMA, 76 pmol equivalent to 5.6 ng) with different *C*-nitroso compounds.
The instrument was set at an attenuation (Att.) of 4 and either 32 (A) or 16 (B, C).

strong response and elute between the solvent front and NDMA are shown in Figure 4.

Although as yet, we could not prove the presence of any *C*-nitroso compound in the extracts of meat products analysed for *N*-nitroso compounds, their presence can not longer be excluded on grounds of specificity of the TEA detector.

As a consequence of our results, any unsignable peak can no longer be interpreted automatically as originating from an unknown *N*-nitroso compound. Besides the problem of the specificity of the TEA, we also found significant variations in the molar response of various common *N*-nitrosamines measured on our GC-TEA. According to the manufacturer of the TEA, the response should be stoichiometric and predictable and independent of the chemical structure of the *N*-nitroso compound under consideration. As shown in Figure 5, this is not true of our results with standard solutions of 7 common volatile *N*-nitrosamines: *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodi-*n*-propylamine (NDPA), *N*-nitrosodi-*n*-butylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosomorpholine (NMORPH). All reference solutions were made up gravimetrically and proved to be stable on storage at −19 °C in the dark. For NDMA, NDEA, NDBA and NPIP, purity was checked against high-purity reference nitrosamines obtained from Illinois Institute of Technology (IIT) Research Institute (Chicago).

Our starting nitrosamines proved to have at least the same purity as claimed for the NCI (National Cancer Institute. Bethesda) – IIT reference substances: 99% or

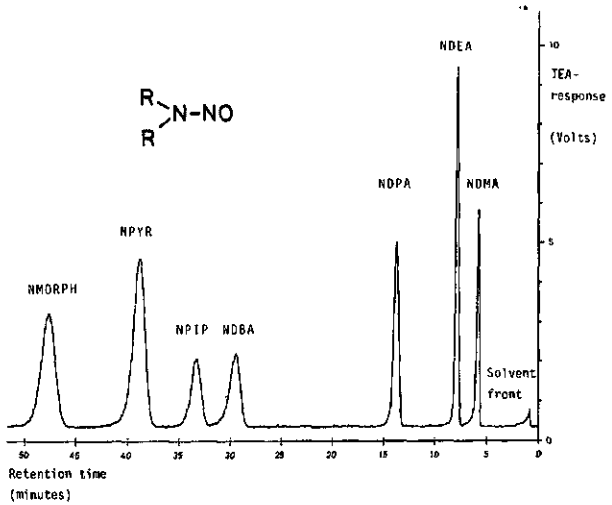

Fig. 5. TEA-gram of a reference solution of 7 *N*-nitrosamines (NA, each at a concentration of about 1 μg/litre) in dichloromethane. Molar response relative to nitrosodimethylamine (NDMA) was for nitrosodiethylamine (NDEA) 2.6, nitrosodipropylamine (NDPA) 2.8, nitrosodibutylamine (NDBA) 3.1, nitrosopiperidine (NPIP) 1.6, nitrosopyrrolidine (NPYR) 4.1, and nitrosomorpholine (NMORPH) 3.3.

better. According to Fine (pers. commun.), the molar responses of various *N*-nitroso compounds were measured by direct introduction of the compounds into the catalytic pyrolyser of the TEA only and should be stoichiometric. We checked this also recently for the same nitrosamines. Although we obtained less differences in the molar responses of the various nitrosamines than with GC-TEA, the differences are too large to be covered by experimental errors. As yet no satisfactory explanation is available for the discrepancies found between our results and those of Fine and his co-workers (but see Note added).

Conclusion

Besides GC-MS, GC-TEA or the combination of TEA with any other chromatographic device like, for instance, high-pressure liquid chromatography (HPLC) seem most suitable and reliable for fast trace analysis of *N*-nitroso compounds. However, the results obtained with this new analytical technique have also to be interpreted carefully and critically in qualitative and quantitative terms. As we have shown, possible presence of *C*-nitroso compounds in samples to be analysed for *N*-nitroso compounds increased risk of interference and might give rise to false positive results.

One has to be extremely careful to use the response of the TEA obtained in the direct mode as a measure of the total concentration of *N*-nitroso compounds present in the injected sample for two reasons: the non-stoichiometric response to the different *N*-nitroso compounds and the non-specificity of the TEA for NA. It is difficult or even impossible to express the amount of NA present as a meaningful

quantity like substance content of NO in mmol kg^{-1}, even if the sample has been cleaned up in such a way that all other compounds releasing NO than *N*-nitroso compounds are eliminated.

A lot of experimental work has to be done before the time required for analyses of volatile *N*-nitroso compounds at the μg/kg level in complex mixtures is reduced from days to only minutes by GC-TEA, as is claimed by the manufacturer of the instrument.

Acknowledgments

The authors are indebted to Dr D. H. Fine, Mr F. Campagna and Dr D. P. Rounbehler of Thermo Electron Corporation, Waltham, Mass., US, for valuable advice and assistance with the installation of the TEA, to Drs C. Schenk of the University of Amsterdam, Netherlands, for a gift of *C*-nitroso compounds, to Dr J. N. Keith of IIT Research Institute, Chicago, Ill., US, for a gift of high-purity *N*-nitrosamines and to Miss E. Egmond, Mr D. van den Bosch and Mr E. Th. Sahertian for technical assistance.

Note added after symposium

Evidence has been found that in our TEA the catalytic pyrolysers were not working properly. Exchange of the pyrolysers by the manufacturer resulted in the claimed stoichiometric response. According to Fine & Castegnaro (pers. commun.), the 2-nitroso-2-methylpropane derivatives we supplied to them also showed a strong response on their GC-TEA equipment. Recently we also tested 1-nitroso-2-naphthol and 2-nitroso-1-naphthol for response on our TEA. Both compounds showed no response if injected directly into the pyrolyser.

Discussion on the session

Comment of Dr Fine as the manufacturer of the TEA

As the authors themselves admit, the instrument, on which the data were collected was not functioning correctly. Because the equipment was malfunctioning, none of the data can be repeated in other laboratories. However the finding of a *C*-nitroso response on the TEA is too important to disregard. The authors should perhaps repeat their experiments, including mole response and TEA-chromatograms as soon as their instrument has been repaired. The new data should be included in the published paper (see Note added after symposium).

Conclusions and recommendations of the chemical session, occurrence of nitrosamines, Thursday 9 September

1. The presence of several volatile nitrosamines in the μg per kg range in meat products cured with nitrite and nitrate has been sufficiently confirmed by reproducible analytical investigations of high sensivity.

2. The findings of relatively high concentrations of some non-volatile nitrosamines should be confirmed by further investigations.

3. During frying of bacon or ham, nitrosopyrrolidine will form. Experiments on a practical scale have shown that the amounts of nitrosopyrrolidine may be significantly reduced by decreasing the nitrite levels in curing. An increase of ascorbate levels in curing seems to reduce the formation of nitrosopyrrolidine as well.

4. The question of how far the apparent presence of low levels of nitrate (and nitrite) occurring in fresh meats could contribute to the formation of nitrosamines merits further investigation.

5. In discussing the merits of various GC-MS methodologies for the analysis of trace amounts of *N*-nitrosamines the question was raised whether or not very high resolution must be pursued. The statement was made that the resolution of the mass spectrometer is not an adequate parameter to express specificity in mass chromatography. Some participants expressed the opinion that the reliability of the results obtained with GC-MS depends as much on optimum GC conditions – for instance highly resolving columns and very stable retention times – as on optimum MS conditions like adequate ion optics and the experience of the operator of the MS.

6. Like *N*-nitroso compounds, some *C*-nitroso compounds, depending on their molecular structure, also respond on the TEA and thus may interfere with the former.

Chemical session – Carcinogenicity of nitrosamines

Reporters: W. J. Olsman, P. J. Groenen

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Dose-response study with N-nitrosopyrrolidine and some comments on risk evaluation of environmental N-nitroso compounds

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Abstract

Results from a dose-response study are reported, in which rats were given *N*-nitrosopyrrolidine at daily oral dosages of 10, 3, 1 and 0.3 mg per kg bodyweight. The three highest dosages resulted in liver cancer in 46, 84, and 32% of the rats, respectively. With the lowest dosage (0.3 mg/kg), there was no statistically significant increase in tumor incidence over untreated controls. In the second part of the paper, some aspects are considered of evaluating risks from environmental *N*-nitroso compounds and the problem of synergistic effects is emphasized.

Introduction

In view of quickly accumulating and reliable data on the occurrence of small amounts of volatile nitrosamines in human food (Preussman 1975; IARC-SP–14 1976), air (Fine et al. 1976d) and water (Fine et al. 1976b), the problem of risk evaluation for populations exposed to such carcinogens is becoming more important. In the absence of data on carcinogenic effects of *N*-nitroso compounds in man, animal data must be used with all inherent difficulties. Though many qualitative experiments have demonstrated carcinogenic effects for the majority (80%) of about 130 *N*-nitroso compounds tested in different animal species, there are only few dose-response studies and low-dose tests. Such data, however, are a prerequisite for risk evaluations. In the rat only dimethyl and diethyl nitrosamine have been tested in several dose schedules including low ones (Terracini et al. 1967; Druckrey et al. 1963). Since nitrosopyrrolidine is important in view of its frequent occurrence in nitrite-treated food products, we decided to perform a dose-response study for carcinogenicity with this compound as a first step in a larger program in this field. The results of this study, which has just been finished, will be reported in the first part of this paper. In the second part, we will give some general comments on nitrosamine carcinogenesis as a risk factor for human health.

Dose-response in carcinogenicity of N-nitrosopyrrolidine

Material and methods

N-Nitrosopyrrolidine was synthesized from commercially available pyrrolidine by nitrosation with nitrite in dilute aqueous hydrochloric acid. It was purified by vacuum distillation (90 °C; 10 mmHg); its purity was checked by gas-liquid chromatography and ultraviolet spectrum. It was administered daily in the drinking water at concentrations ensuring the required dosage. Aqueous solutions were freshly prepared every second day. Untreated controls only received tap water.

Equal numbers of 100-day-old Sprague-Dawley rats of both sexes were used. Experimental and control rats were given Altromin® diet. All rats were carefully observed until they died or became moribund, death being induced with ether in the latter case. At autopsy, all major organs were examined then fixed in 4% formaldehyde solution and studied histologically by Prof. Dr K. Goerttler (Institute of Pathology, German Cancer Research Center).

Results and discussion

Four groups were given daily dosages of 10 (Group 1), 3 (Group 2), 1 (Group 3) and 0.3 (Group 4) mg per kg bodyweight. The treatment was tolerated well.

The results are summarized in Tables 1 and 2 and in Figure 1. Table 1 clearly demonstrates carcinogenic effects after the three higher dosages (Groups 1–3) with incidences of malignant tumors of 46%, 84% and 32%, respectively, as compared with 9.8% in untreated controls. For the lowest dosage (Group 4, 0.3 mg/kg), the χ^2 test (with one-sided alternative) did not indicate a significance at the 5% level and therefore a weak or marginal carcinogenic effect due to the treatment cannot be proven, although statisticians tell us that this test does not exclude effects due to the treatment. The median time of death of all tumor-bearing rats, also given in Table 1, shows the well-known phenomenon of an increase in latency period for tumor induction with decreasing dosage.

Table 2 shows the organ distributions of observed neoplasms and demonstrates in Groups 1–3 a clear prevalence of malignant liver tumors (mainly hepatocellular

Table 1. Effect of dosage of nitrosopyrrolidine (NPyr) *administered continuously by mouth to* Sprague-Dawley rats on final incidence of tumours. Δ, daily dosage in mg per kg bodyweight; *N*, number; δ, proportion; $\bar{t}$, median time of death of tumorous rats.

Δ/mg · kg^{-1}	*N*(rats)	Rats with malignant tumors		Rats with benign tumors		$\bar{t}$/day
		N	δ/%	*N*	δ/%	
0	61	6	9.8	5	8.2	(587)
0.3	60	12	20	8	13	664
1.0	62	20	32	9	15	685
3.0	38	32	84	3	7.9	533
10	24	11	46	6	25	444

carcinomas), with only few tumors in other organs. On the contrary, with the lowest dosage (Group 4) no malignant liver tumors were found; nevertheless 3 benign hepatocellular adenomas were observed, which usually did not occur in the untreated controls, indicating also with this dosage a certain tropism for the liver.

Survival of rats without tumors in the experimental groups and the control,

Table 2. Incidence of tumors in different organs in Sprague-Dawley rats given nitrosopyrrolidine by mouth. Δ, daily dosage in mg per kg bodyweight; for number of rats in the various treatment groups, see Table 1. Tumor incidence expressed as number of rats having a certain type of tumor.

Δ/mg · kg^{-1}	Malignant tumors				Benign tumors		
	liver	leukaemia	lympho-sarcoma	other	liver	mamma	other
0	0	2	2	2	0	4	1
0.3	0	5	2	5	3	4	1
1.0	13	1	1	6	2	3	2
3.0	30	0	1	1	1	1	1
10	9	0	0	4	5	0	1

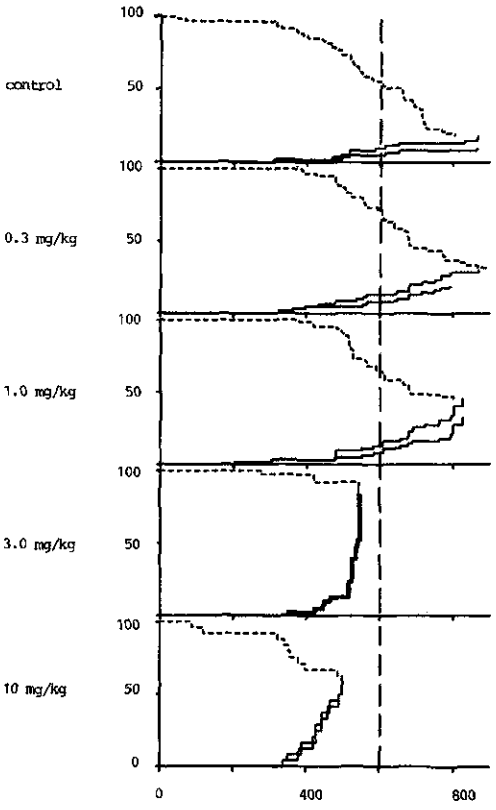

Fig. 1. Effect of nitrosopyrrolidine administered at different daily dosages per kg bodyweight on proportion of Sprague-Dawley rats (ordinate) dying with tumor (upper solid line), with malignant tumor (lower solid line) and dying without tumor (broken line plotted in reverse from 100%). The abscissa is time in days from the beginning of administration.

together with cumulative mortality of rats with benign or malignant tumors and also of those with only malignancies are shown in Figure 1. The survival times for controls and the two lowest dosages did not differ much; this demonstrates the absence of a significant shortening of life-span with carcinogen treatment. Nevertheless, at least with 1 mg/kg carcinogenicity is clearly indicated in the increasing incidence of tumors. But with the highest dosage of 10 mg/kg, some early deaths before the age of 400 days of rats without tumors (Fig. 1) suggest a cumulative toxicity of the rather high doses of *N*-nitrosopyrrolidine, which probably rendered the rats more susceptible to pneumonia, which caused death in these rats. These early deaths also explain the lower incidence of malignant tumors in this group (46%) than with lower dosages (84% and 32%) (Table 1).

The present results confirm earlier experiments on the carcinogenicity of *N*-nitrosopyrrolidine. First feeding experiments by our group (Druckrey et al. 1967) demonstrated hepatocarcinogenicity in BD rats (daily dosage 10–20 mg/kg). This has been confirmed with MRC rats by Greenblatt & Lijinsky (1972b), who reported a 100% incidence of liver carcinomas at a daily oral dosage of 16 mg/kg. In male rats, they also saw testis tumors (papillary mesotheliomas), a type of tumors not observed in our tests. This may perhaps be related to the lower dosages we used.

N-Nitrosopyrrolidine induced a rather low incidence of lung adenomas in Swiss mice after oral administration of about 0.25 mg per mouse (about 7–8 mg/kg) (Greenblatt & Lijinsky 1972). Tumors of the lungs and the trachea were induced by

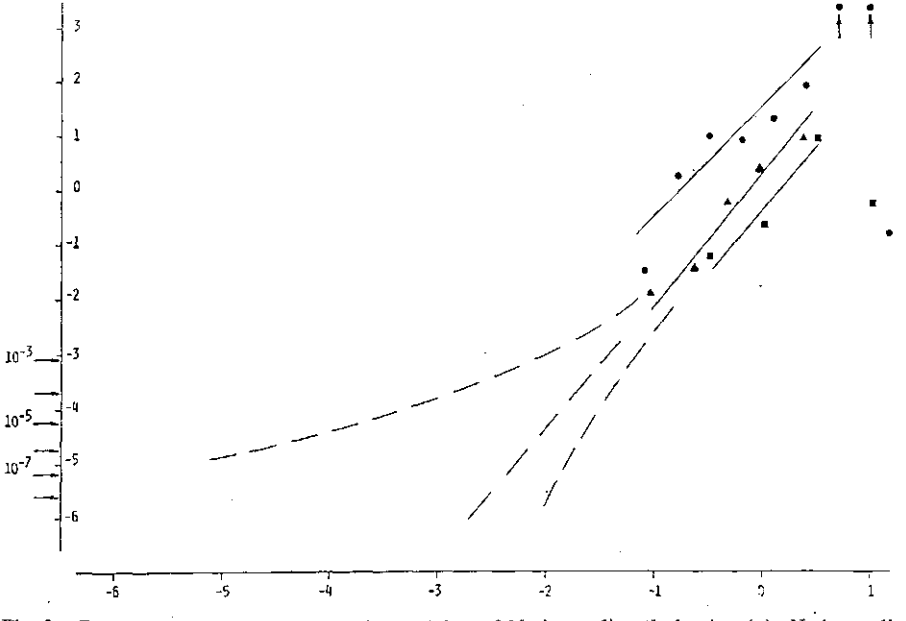

Fig. 2. Dose-response curves for carcinogenicity of N-nitrosodimethylamine (▲), *N*-nitrosodiethylamine (●) and *N*-nitrosopyrrolidine (■) with different possible extrapolations. Abscissa, logarithm of daily dosage in mg per kg bodyweight (given by mouth); ordinate, response rate (in normal deviates = probits − 5).

subcutaneous injection of *N*-nitrosopyrrolidine (Dontenwill 1968).

The results given here show that a 'no-effect daily dosage' is near 0.3 mg/kg bodyweight, corresponding to about 5 mg/kg in the rats diet. Whether such a level, after reduction with a certain 'safety factor' (100 to 1000 usually), can be considered 'safe' will be discussed in the second part.

The dose-response curve resulting from the experiment with nitrosopyrrolidine is given in Figure 2. The dotted lines show three choices in extrapolating the available data to lower dosages not covered by the tests. This will also be discussed later. Also shown in Figure 2 are the dose-response curves for carcinogenicity in the rat for dimethylnitrosamine and diethylnitrosamine, as taken from the papers by Terracini et al. (1967) and Druckrey et al. (1963). The 3 curves are almost parallel, as expected for carcinogens of the same chemical group, tested in the same animal species. The apparent 'no-effect levels' are 1–2 mg/kg for dimethyl and 1 mg/kg for diethylnitrosamine (Table 3).

Table 3. Estimated 'no-effect levels' (w) of carcinogenic nitrosamines in dose-response feeding studies with rats.

Nitrosamine	w/mg · kg^{-1}	Source
Dimethylnitrosamine	1 – 2	Terracini et al. 1967
Diethylnitrosamine	1	Druckrey et al. 1963
N-Nitrosopyrrolidine	5	Preussmann et al., unpubl.

Human health risk of environmental carcinogens: some thoughts on relevant questions

Let us start by stressing that scientific research always is and must be abstraction and that this is especially true for biomedical experiment. Therefore the attempt to transform such abstraction to conclusions about our complex ways of life will always be to a certain extent 'unscientific'. Nevertheless, this must be done, if science is not to remain in an ivory tower. It is especially necessary in biomedical research in general.

We have at least 2 reasons for doing so in nitrosamine research

– *N*-Nitroso compounds are potent chemical carcinogens

– Certain compounds of this group occur in the human environment or can be formed from precursors and therefore are a potential health threat for man.

Certain objections have been raised against this proposition.

First objection: Nitroso compounds have not been shown to be carcinogens in man

This is true. However, typical *N*-nitroso compounds like diethylnitrosamine have been shown to be potent carcinogens in about 20 different animal species including subhuman primates (Preussmann 1975) and no animal species is known today to be resistant. Why should the human species be so? Especially since Montesano & Magee (1970) have shown qualitative and quantitative similarities in nitrosamine

metabolism in human and in rat liver slices.

This is certainly only circumstantial evidence, though strong enough. Should we really wait to take action until direct and unequivocal evidence is available, until we can point to dead bodies to prove the case? Certainly not, especially since for other substances very quick action has been taken with doubtful 'carcinogens' like cyclamate or Red No 2.

Second objection: Environmental nitrosamines are liver carcinogens and liver cancer is very rare in Western countries and has not increased in the last two or three decades

Dimethylnitrosamine (DMNA) and *N*-nitrosopyrrolidine show a preference for liver as target organ in most animal species tested – at high dosages. However, there is good experimental evidence that this 'organ specificity' can change when low doses are administered. Two examples: High dosages of diethylnitrosamine (DENA) induce liver carcinomas in the rat, but at lower dosages increasing incidences of oesophageal tumors were observed (Druckrey et al. 1963). DMNA administered continuously induces liver cancer; single doses or short-term exposure almost exclusively induced kidney cancer (Magee & Barnes 1962; IARC 1972a). Furthermore there is considerable evidence of differences of organ specificity between animal species. Tumor sites for DMNA carcinogenesis in the mouse are lung and kidney as well as the liver. DENA in the mouse also produced tumors in the lungs, the oesophagus and the nasal cavity, in the hamster predominantly tumors of the trachea, larynx, nasal cavity and lung. With other nitrosamines, these examples could be confirmed and extended (Preussmann 1975).

Therefore, this objection to a role of nitrosamines in human cancer is not supported by the available experimental evidence.

Third objection: Environmental levels of nitrosamines are so low that they are unlikely to be of significance for carcinogenesis in man

Here the problem lies in what is meant by 'unlikely'. Does this mean a risk of 1:1 000 or 1:100 000? However at present it is impossible to answer the question 'How much is too much?' by experimental results. This is shown in Figure 2. Here the available dose-response results are shown as straight lines. Their extrapolation (dotted lines) into the low-dosage range, which cannot be covered by animal experiments, is arbitrary. There is no way of deciding which of the three extrapolations is correct. The curve pointing to the x axis would indicate a practical and theoretical threshold; the straight line would indicate a very low risk level; and the curve intersecting the y axis indicates a rather high risk of even low exposures to nitrosamines. We have emphasized already that at present there is no way to verify experimentally which of the three is correct. Even attempts to reach approximations by several mathematical and statistical methods (Mantel & Bryan 1961; Mantel & Schneiderman 1975; Mantel et al. 1975; Hoel et al. 1975) are difficult to interpret for biological relevance in the absence of any data to prove or disprove figures on 'acceptable' or safe levels of carcinogens.

This unsatisfactory state of affairs, however, is still further complicated by the

fact that man today is never exposed to a single environmental carcinogen but to large numbers of different chemical carcinogens (Preussmann 1975; 1976). Even for *N*-nitroso compounds, exposure is not only towards DMNA or nitrosopyrrolidine. Analysis of European meat products has demonstrated up to 5 volatile nitrosamines (IARC 1976; several papers in this book). *N*-Nitroso-3-hydroxy-proline as a non-volatile nitrosamine has been shown to occur in food products (Sen; Eisenbrand; both this book) while other non-volatiles have not been estimated because of difficulties in their chemical analysis. The formation of *N*-nitroso compounds from amines and nitrosating agents in the human gastro-intestinal tract is another possibility of exposure to be discussed at this symposium by Sander and by Lijinsky.

Cancer in man is usually due not to one factor but to many. Therefore additive or even multiplicatory interactions are likely. Synergistic effects have been demonstrated clearly in many experimental studies with different carcinogens (Nakahara 1975). In man, synergistic or additive effects of occupational carcinogens and tobacco smoke have been proven or made likely, as has been summarized recently by Hoffmann & Wynder (1976). For nitrosamine carcinogenesis, Schmähl et al. (1963) and Schmähl (1970) demonstrated synergistic effects in combination experiments, demonstrating either increased tumor incidence or decreased induction times. Another striking example of synergistic effects has been described by Hoch-Ligeti et al. (1968): while the hepatocarcinogenicity of DMNA was depressed by the simultaneous administration of 3-methylcholanthrene, the two carcinogens had a strong synergistic effect in lung tumor induction in the rat. At the dosages used, neither the nitrosamine nor the hydrocarbon alone was carcinogenic in the lung, but combined administration induced pulmonary tumor in 15% of the rats. Similar interactions by the same two agents have been demonstrated in mice (Cardesa et al. 1973). Many more similar results are known in the literature.

The consequences from this situation seem clear: even if there were 'no-effect levels' for individual carcinogens (meaning dosages that do not increase tumor incidence above that for controls within the normal life expectancy of the population at risk), this would *not* allow the definition of 'safe' levels of environmental carcinogens because of the possibility of syncarcinogenic effects. Therefore, everything reasonable within the limits of risk-benefit and cost-benefit evaluations must be done to reduce the amounts of carcinogens in the human environment.

Discussion on the session

Extrapolation of dose-response relations

The problem of extrapolating the observed regressions down to levels of practical significance far below 10^{-6} mg/kg/day occurs also in estimating risk from *Cl. botulinum*; in that case there is a generally accepted mathematical basis on which to proceed in many (but not in cured) foods. Dr Ingram asked if there were any corresponding mathematical criteria which could – for example – justify Dr Preussmann's suggestions that the three experimental relations represented in Figure 2 are in fact (i) different, (ii) linear and (iii) parallel. He said that, if this could be established, one could extrapolate along the central dotted line with more confidence. Dr Roberts wondered whether one could use the plot in Figure 2 as a means of establishing a 'relative toxicity index' of carcinogens. Dr Preussmann

thought that this would not be easy because in Figure 2 dose was related to effect. The induction time, however, is a second important factor to determine the effectiveness of a carcinogen. For a 'carcinogenicity index' both factors must be taken into consideration. Further difficulties are – according to Dr Preussmann – that results obtained in different animal species could not be compared in such an index.

No-effect levels for carcinogenic compounds

For any individual carcinogen, there is a no-effect level, which may be defined as the highest dose not inducing a significant increase in tumour incidence within the normal life expectancy of the population at risk. However, in Dr Preussmann's opinion there is no 'safe' dose for carcinogens in general, because the total body burden with carcinogens, including synergistic effects, seems to be decisive in environmental carcinogenesis. For the same reason, he considered a question from Dr Sleeth as academic: whether 5 mg nitrosopyrrolidine per kg food as a no-effect level in the daily food of rats could be translated (taking into account a safety factor of 1 000) as a 'safe' content in bacon of 5 μg per kg. Dr Tannenbaum disagreed with the opinion, for the proper reason that a decision *must* be taken about what is 'safe' (i.e. an acceptable risk).

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Possible formation of N-nitroso compounds from amines and nitrites

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Last year 365 000 people in the United States died of cancer, or 1 in 6 of all deaths. About 25% of the population, 600 000 people, developed cancer, so cancer cannot be considered a rare disease. The proportion is similar in Western Europe and in other industrial countries such as Japan. Perhaps 1–2% of these deaths can be attributed to known industrial compounds but the cause of the remainder remains a mystery. It has often been said that chemicals are responsible for as much as 90% of human cancer and, because of their properties, nitrosamines (or more properly *N*-nitroso compounds) seem likely to be major contributors to this chemical origin of cancer.

One of the properties that nitrosamines share with other types of carcinogen is that they act most effectively when administered in small frequent doses over a long period. This is precisely the way how most carcinogens are likely to act in man, since exposure to large doses must be rare and restricted to industrial events. In two other respects, nitrosamines differ from most other types of carcinogens. They induce tumors in all species of animals where they have been tested, and man is unlikely to be resistant to their action. They can induce tumors in certain target organs by whatever route of administration. That is, they show organ specificity and they have induced tumors in almost all organs and tissues of animals. In rodents more than three quarters of the 120 *N*-nitroso compounds tested have been carcinogenic. Tumors have been induced by them in liver, lung, kidney, pancreas, skin, oesophagus, nasal cavity, urinary bladder, tongue, nervous system, forestomach and, in some tests, glandular stomach. When considering man's exposure to *N*-nitroso compounds or, indeed, to any other carcinogen, it must be remembered that animals can be treated for only 2 years, whereas man's exposure can last 50 years or more. It is difficult to ascribe any particular human cancer to exposure to *N*-nitroso compounds, since we do not know which organ would be susceptible to exposure to a particular nitroso compound and they do show differences in the organ affected between one species and another.

N-Nitroso compounds are formed by reaction of secondary and tertiary amines with nitrite and therefore any situation in which these amines and nitrite occur together is a favourable one for formation of nitrosamines. However the optimum pH for formation of *N*-nitroso compounds is about 3 and the reaction rate drops off considerably on either side of that pH, particularly for tertiary amines, which

seem to react only very slowly with nitrite at pH below 3. The reaction of secondary amines with nitrite has been studied kinetically and it seems that the reaction of secondary amines with nitrite is second-order in nitrite, that is, proportional to the square of the nitrite concentration. For tertiary amines, the kinetics have not been well worked out but it appears that there too the reaction is about second-order in nitrite, between 1.5 and 2.5. This implies that if the nitrite concentration is high the formation of nitrosamines will occur to a much greater extent, much more than if the nitrite concentration is low. This is relevant when considering formation of nitrosamines in a sample of meat or in solution where the concentration of nitrite and of amines is likely to vary greatly from one place to another. Another relevant fact is that in a mixture of several amines with nitrite, each amine reacts independently of the others and produces more nitroso compound from a given amount of nitrite than in the presence of a single amine.

Some *N*-nitroso compounds, especially nitrosodimethylamine, have been found in samples of meat and fish cured with nitrite. The levels are very low, of the order of 10 μg/kg, although occasionally a little more, and not every sample even of the same type of material contains significant amounts of nitrosamine. However the carcinogenic effect of nitrosodimethylamine, even at 10 μg/kg cannot be considered insignificant. The potency of some *N*-nitroso compounds is such that a total dose of 0.2 mmol given in small fractions over an interval to rodents has given rise to a high incidence of malignant tumors. There is indirect evidence that nitrosoproline is formed in cured meats and fish since the carcinogen nitrosopyrrolodine is often found in fried bacon (although not in uncooked bacon) at levels up to 100 μg/kg. This compound is almost certainly formed by decarboxylation of nitrosoproline (Lijinsky et al. 1970).

Though the presence of *N*-nitroso compounds formed in food is a source of these carcinogens for man, a potentially much larger source of these carcinogens is secondary or tertiary amines reacting with nitrite in the body. The stomach with its acid contents is the place where this is most likely but it can also occur in the infected bladder in which one source of nitrite is nitrate reduced by the bacteria. Several experiments in chemical systems and in vitro have demonstrated the formation of *N*-nitroso compounds from both secondary and tertiary amines (Lijinsky et al. 1972). Some experiments have similarly shown that *N*-nitroso compounds are formed from secondary and tertiary amines and nitrite in the stomachs of animals (Sander et al. 1968; Lijinsky & Greenblatt 1972) although fewer of these have been performed because of technical difficulties. Furthermore, quantitative estimates of the nitroso compound formed are inaccurate because of absorption of the nitroso compound through the stomach wall (and of amines and nitrite also).

Long-term tests of combinations of secondary and tertiary amines with nitrite have been carried out in several laboratories. The first of these successfully carried out was by Sander & Bürkle (1969), who showed that feeding of *N*-methylbenzylamine and nitrite to rats gave rise to oesophageal tumors, the same as produced by feeding of nitrosomethylbenzylamine itself. Most of these tests have involved feeding of amines and nitrite at contents of about 1 g/kg in the diet or in drinking water to rats for periods of 6–18 months. Though many of these tests have led to no significant increase of the incidence of malignant tumors above the incidence found

in control rats, there was quite definite evidence in other tests of carcinogenicity of the combination.

One such experiment in my laboratory was to feed rats on heptamethyleneimine and nitrite, which gave rise to a high incidence of squamous carcinomas of the lung. This is the tumor produced by feeding nitrosoheptamethyleneimine, the product of the reaction of the amine with nitrite. Thus one source of carcinogen for cigarette smokers might be nitrosamines formed from nitrite in food or in saliva and amines in tobacco smoke, which contains large amounts of amines (Singer & Lijinsky 1976), many of which can interact with nitrite to form nitroso derivatives.

The question is often asked why we worry about nitrite in meat when there is nitrite in saliva. Dr Tannenbaum, in his extensive studies, has shown that nitrite is a common constituent of saliva and is derived from reduction by bacteria in the mouth of nitrate obtained usually from vegetables or from water. Salivary nitrite can reach quite high concentrations after a meal containing much nitrate. However it is normally not high in nitrite, and saliva is continuously secreted into the stomach, so that the concentration of salivary nitrite in the stomach at any one is rather small. This contrasts with the large amount of nitrite that can be introduced over a short time when cured meat is digested. This difference has a bearing, since the kinetics of the nitrosation reaction depend closely on the concentration of nitrite in the medium; the higher that is at any one particular moment, the more nitrosamine is formed. Hence the nitrite in meat is a more important source of nitrite for nitrosamine formation in vivo than the nitrite in saliva, although the latter cannot be ignored. Furthermore, it shows the unimportance of averaging intakes of nitrite.

The sources of nitrosatable amines are of great interest since these offer the possibility of formation of a great variety of nitroso compounds of both known and unknown carcinogenic activity. In my laboratory we have studied the reactions of many of these compounds, a large number of which are drugs and agricultural chemicals. Many of those for which reaction with nitrite to form nitrosamine is possible have been demonstrated in the laboratory (Lijinsky 1974; Elespuru & Lijinsky 1973; Eisenbrand et al. 1975b). For example, the insecticide carbaryl, a representative of a variety of methylcarbamate esters that are insecticides, reacts with nitrite to form nitrosocarbaryl which has been shown to be a powerful bacterial mutagen (Elespuru et al. 1974) and quite a potent carcinogen in rats (Lijinsky & Taylor 1976). Among the many tertiary amines that are commonly used drugs are oxytetracycline, aminopyrine, methapyrilene and chlorpromazine; dimethyldodecylamine is used as an anti-suckering agent on tobacco plants; all of these have reacted with nitrite in chemical systems to form the corresponding nitrosamines (Lijinsky et al. 1972).

Several of these compounds have been tested in vivo for formation of nitrosamine by feeding rats on them together with nitrite. Aminopyrine and nitrite, both at 250 mg/kg, has proved to be a potent combination which has given rise to liver tumors in all rats tested through the dimethylnitrosamine formed in vivo. That level of nitrite is not far above the level of 200 mg/kg currently allowed in many countries as the maximum for nitrite in meat products. Oxytetracycline given to rats together with nitrite for one year has given rise to liver tumors in four of a group of

30 rats (Taylor & Lijinsky 1975). This number of tumors is at the borderline of significance and we prefer to consider the experiment negative. However three other amine and nitrite combinations have given rise to what must be considered a significant incidence (Table 1). The three amines are chlordiazepoxide, dimethyldodecylamine and methapyrilene, which is a drug used as an antihistaminic in cold tablets and can be bought over the counter. These three compounds together with nitrite gave, respectively, 3 tumors of the nervous system (a significant incidence), 2 bladder tumors which are never found spontaneously in our rats, and 9 liver tumors produced by methapyrilene; this is a number of liver tumors greatly in excess of those which would be expected spontaneously in a group of 30 animals.

The amines tested by giving them to rats together with nitrite in drinking water were aminopyrine, arginine, chloropromazine, chlordiazepoxide, cyclizine, dimethyldodecylamine, dimethylphenylurea, hexamethylenetetramine, lucanthone, methapyrilene, methylguanidine, oxytetracycline, piperidine, tolazamide and trimethylamine oxide. Apart from those described above, none of the combinations gave rise to a significant incidence of tumors, even though a few tumors not normally seen in other than very low incidences in control rats were observed. It must be emphasized that even though the numbers of tumors produced in these experiments was not considered significant because of the small numbers of rats in each group, they cannot be considered definitive for safety of the combination. In particular, it cannot be assumed that no nitrosamine was formed in vivo in these experiments, but only that insufficient nitrosamine was formed during the lifetime of the rats to give rise to a significant number of tumors.

Therefore, it must be concluded that reduction of residual nitrite in meat (or of nitrite in saliva) would reduce exposure of people ingesting such food to nitrosamines and thereby reduce the total exposure to carcinogens. It must be remembered that nitrosamines do not act alone when formed in this way, but add to the burden of carcinogen exposure already experienced from other sources. Therefore the reduction of residual nitrite in meat which is being attempted and achieved by the meat-processing industry is encouraging and should be continued. Since the

Table 1. Incidence of non-endocrine tumors in rats fed on amines and nitrite each at 250 mg per kg feed. *N*, number (of rats).

Amine	*N*(rats)	Organ, type of tumor	*N*(rats affected)
Chlordiazepoxide	30	liver	3
		central nervous system	3
		heart, neurofibrosarcoma	1
		forestomach, papilloma	1
Dimethyldodecylamine	30	bladder	2
		forestomach, carcinomas	2
		papilloma	1
		liver	1
		brain, astrocytoma	1
Methapyrilene	30	liver, hepatocellular carcinomas	3
		haemangioendothelial sarcoma	1
		cholangiocarcinomas	5

cancers that we see today reflect exposures stretching back 30 or 40 years or more, we might expect present improvements by reduction in our exposure to nitrite and, thereby, in our exposure to nitrosamines, might lower the incidence of cancers in general and of some cancers in particular in the future. Through we do not know all of the sources of nitrosatable amines, those above are among the hundreds of secondary and tertiary amines that have been listed. Occasionally, surprising new sources of nitrosatable amines appear such as the formation of monoalkylcyanamides reported in saliva by Dr Tannenbaum. Those after nitrosation could give rise to nitrosoalkylcyanamides, which are potent glandular stomach carcinogens in rodents.

Discussion on the session

Carcinogenesis and the character of the exposure to ingested nitrite

Dr Ingram recalled that, in the discussion of Dr Walters' paper on the day before, Dr Lijinsky said he was especially unhappy about ingestion of large quantities of nitrite in a single meal, referring to the nitrosation reaction being a second-order function of nitrite concentration. Today, on the other hand, he began by emphasizing it is characteristic of nitrosamines that their carcinogenic action relates especially to long-term continuous exposure to low levels. Dr Ingram asked Dr Lijinsky to clarify the apparent difference between these viewpoints. He considered a statement in this matter to be important, because it could, for example, affect the estimate of the relative importance of nitrite intake from saliva as compared with food, or of nitrate intake from water as compared with cured meat. Dr Lijinsky replied that the 'high' doses he referred to yesterday, were high in nitrite only in relation to salivary nitrite levels, but not high in an absolute sense. The nitrosamines formed from such nitrite doses would be small, of the order of micrograms or milligrams, which are the levels acting cumulatively to induce tumors. Dr Lijinsky stated that both continuous low doses and incidentally high doses of ingested nitrite were important.

Occupational hazards

It was recognized that exposure to dimethylamine and trimethylamine could be a regular occupational hazard to fishmongers. It might be of interest to look at them as a distinct group. Dr Lijinsky reported, in connection to this, that trimethylamineoxide plus nitrite, to rats, gave no tumors.

Relative importance amine drugs as nitrosamine precursors

Dr Rubin and Dr Schuddeboom asked if it was justified to assume that the total load of nitrite and nitrate in the diet had not changed greatly in the last centuries, whereas – especially during the last three decades – a substantial increase in intake of nitrosamine precursors in the form of secondary and tertiary amine drugs had occurred, which might be of far greater relevance. Dr Lijinsky could confirm this, but said that nevertheless the total intake of nitrite and nitrate should not be neglected. On the other hand, statistics on type and incidence of cancer was not good enough 30 years ago to draw firm conclusions. Dr Preussmann questioned the suggestion that nitrite and nitrate intake did not change during the last century.

Nitrosamines in blood

Dr Fine remarked that, until recently, tumor induction was the sole indicator to depend on for nitrosamine formation from nitrite or nitrate and amines. However, at present the formation of *N*-nitrosamines in vivo can be detected in animals by determining their levels in the blood. Dr Fine detected the formation of dimethylnitrosamine in vivo from dimethylamine and nitrite or even 5 μg of nitrate in this way. His results will be published shortly.

Relative importance of individual carcinogens

Dr Tannenbaum granted Dr Lijinsky that the relative importance of individual carcinogens in human carcinogenesis is not known, but he considered it to be a fact that cigarette smoking is the major cause of lung cancer. Dr Lijinsky agreed, but remarked that the actual causative compounds in smoke were yet unknown.

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Carcinogenicity study on rats fed on canned heated nitrite-treated meat: preliminary communication

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Abstract

To detect possible formation of carcinogenic *N*-nitroso compounds from nitrite and secondary amines in meat, carcinogenicity was studied with 70 male and 140 female F_0 rats divided into 6 groups and 60, 100, 70, 60, 60 and 66 of their male and female offspring. One control group received casein and other groups chopped pork as the sole protein source 45% (mass/mass) on a fresh basis, either salted (NaCl) or not. For test groups, nitrite was also added to the meat before autoclaving and storing the diet, representing a mass fraction of 200, 1 000 and 4 000 mg/kg. Rate of weight gain, death rate and numbers of rats with tumors in general, or benign or malignant suggested no differences attributable to treatment with nitrite.

Introduction

In the early 1960s, dimethylnitrosamine was found in fishmeal treated with nitrite (Ender et al. 1964). Since dimethylnitrosamine and many other *N*-nitroso compounds have proved carcinogenic in animals (Magee & Barnes; 1956, Druckrey et al. 1967), formation of *N*-nitroso compounds in food might constitute a hazard to the human consumers.

Since also nitrite is widely used as a preservative and as a colour-preserving agent in meat products, we began a study in 1971 on rats were fed on canned heated nitrite-treated meat.

The purpose was to investigate possible formation of carcinogenic *N*-nitroso compounds in the meat or in the gastro-intestinal tract and their effect on the rats.

Materials

Meat products were prepared by adding to cut lean pork the calculated amounts of curing mix necessary to obtain a product with an initial content of sodium chloride of 25 000 mg/kg and of potassium nitrite 200, 1 000 and 4 000 mg/kg (expressed as sodium nitrite). The meat was chopped, vacuum-mixed and filled into cans, which were vacuum-sealed, and heated to 108 °C for 70 min. After being autoclaved, the cans were kept at room temperature for 1 month to simulate storage in shops, thereafter at 4 °C.

Animals SPF rats, Wistar/Af/han/Mol/(Han 67).

Diets The rats were given a balanced semisynthetic diet containing 45% (mass/mass) of the meat products as the only protein source (Table 1). The diet was prepared once a week and kept in refrigerator until use. Water was provided ad libitum as tapwater acidified with citric acid to pH < 3.5.

Experimental design

The carcinogenicity study was performed over 2 generations. The F_0 rats included 70 males and 140 females and were divided between six groups dosed from 10 weeks before mating as in Table 1. The F_1 rats were dosed throughout life and included 60, 100, 70, 60, 60 and 66 males and females in the respective groups. Groups 1, 2 and 3 served as controls. Weight gain and clinical observations were recorded for the F_1 generation as well as gross pathology and histopathology of all killed (or dead) animals during and at the end of the experiment.

Data on weight gain were analysed by Student's *t* test. Incidences of tumor and death rate were analysed statistically in accordance with guidelines recommended in the British Journal of Cancer by Peto (1974), using Group 3 as control.

Table 1. Protein sources, mass fraction of added nitrite in meat (calculated as $NaNO_2$) and NaCl in the diet given to rats.

Group	Protein source	Nitrite added to meat/mg · kg^{-1}	NaCl in diet/%
1	casein	0	0.38
2	meat	0	0.38
3	meat	0	1.15
4	meat	200	1.15
5	meat	1 000	1.15
6	meat	4 000	1.15

Table 2. Number of rats (*N*) with tumors, benign tumors and malignant tumors. For diets, see Table 1.

Group	Nitrit added/ mg · kg^{-1}	*N* (tumors)		*N* (benign)		*N* (malignant)	
		♂	♀	♂	♀	♂	♀
1	0	33	43	27	35	9	15
2	0	64	83	55	69	19	25
3	0	35	44	29	39	9	13
4	200	40	38	36	34	10	12
5	1000	37	41	30	37	10	11
6	4000	42	48	33	41	16	14

Results

The experiment lasted for 132 weeks, after which surviving rats (30–45%) were killed. Apart from a slight but significant ($P < 0.001$) decrease in weight gain for males in the highest-dose group during the first 54 weeks, no difference was found between the dosed groups and controls in weight gain and death rates.

The first tumors were observed at about 1 year of age and incidence increased with age. At the end of the experiment, about 60–70% of the rats had developed tumors.

No significant differences ($P < 0.05$) were found for males and females between the control group and the individual or pooled dosed group in the number of rats with tumors and with benign or malignant tumors (Table 2).

There was further no effect of dosage on total number of tumors in any organ or on number of any special tumor type. However a higher incidence of epidermal carcinomas in the preputial region in Group 6 than in Group 3 contributes to a slight, not statistically significant, increase in the number of males with malignant tumors in the highest dosed group.

No statistical differences ($P < 0.05$) between control group and dosed groups could be demonstrated in the number of rats with 1, 2, and more than 2 tumors.

Discussion and conclusion

Many experiments have been carried out on the formation of *N*-nitroso compounds in the stomach after simultaneous administration of nitrite and secondary amines (e.g. Sander 1971; Sander & Bürkle 1969; Shank & Newberne 1976). In other experiments, well defined precursors were used, whereas in our study nitrite was the only known precusor. Moreover, our study considered the summation of a possible formation of *N*-nitroso compounds in the meat and in the gastro-intestinal tract. Van Logten et al. (1972) in a similar study used pasteurized meat (80 °C for 70 min) and dosed only one generation, and our results support theirs.

Discussion on the session

Nitrite administration and nitrite level

Nitrite was added to the meat beforehand – instead of fed directly with the diet – in order to simulate the actual conditions in practice as much as possible. Dr Olsen admitted that a level of 4 000 mg/kg in meat is unrealistically high as compared to the normal levels in meat products. For the purpose of establishing the hazards of nitrite in foods the level is certainly not unrealistically high, however.

Nitrosamines detected

The product with an added level of 4 000 mg/kg nitrite was the only one to contain dimethylnitrosamine. The Laboratory of the Government Chemist (London) also analyzed the samples with the lower nitrite addition, but in none of them dimethylnitrosamine was formed. Dimethylnitrosamine was the only nitrosamine looked for.

Effect of citric acid

Dr. Vendeuvre asked attention for the finding that citric acid, added to meat products, would have an inhibitory effect on nitrosamine formation (A. Frouin et al., Proc. 21st Eur. Meet. Meat. Res. Workers, Bern 1975, p. 200).

Reason for a two-generation study

The reason was to determine any effects of in-utero exposure to nitrite or nitrosamine from nitrite.

Diet composition

The non-meat part of the diet was a standard basic diet.

Conclusions and recommendations of the chemical session, carcinogenicity of NAM, Thursday afternoon 9th September 1976

1. There are many *N*-nitroso compounds in the environment of man that are highly potent carcinogens acting at low levels.

2. A positive dose-response relationship exists for the induction of malignant tumors in rats by nitrosopyrrolidine, frequently found in nitrite-treated food products.

3. No evidence of tumor formation attributable to the ingestion of nitrite-treated meat was obtained from a 2-generation carcinogenicity study in rats fed on diets containing canned meat treated with various amounts of nitrite.

4. *In vivo* formation of carcinogenic *N*-nitroso compounds is probably a more important carcinogenic hazard to man than the presence of preformed *N*-nitroso compounds in food. Cured meat is one of the most important sources of nitrite for the *in vivo* formation of carcinogenic *N*-nitroso compounds.

5. Attempts should be made to decrease the level of nitrite in food products to the minimum, consistent with health and safety of man.

6. The existence of synergistic and additive effects in chemical carcinogenesis along with the now generally accepted principle of multifactorial cause of cancer in man emphasises the inadmissibility of setting safe levels for (complete, true) carcinogens such as many *N*-nitroso compounds.

7. Against the background of risk-benefit analyses it is unavoidable to accept a certain risk from carcinogens for the human population.

8. Appropriate epidemiological studies on the significance of carcinogenic *N*-nitroso compounds for cancer in man should be encouraged.

9. One might speculate that the presence of so many carcinogenic *N*-nitroso compounds in the environment of man is responsible for the background level of cancer occurring in the human population.

Epidemiological / Legislative session

Reporters: V. J. Feron, W. J. Olsman

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Intake of nitrate and nitrite of some Swedish consumers as measured by the duplicate portion technique[1]

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Abstract

The duplicate-portion technique was used to estimate the intake of nitrate and nitrite of 10 men and 10 women in an agricultural district in southern Sweden. The average daily intake of nitrate was for men 65.9 mg (35–111 mg) and for women 69.7 mg (49–109 mg). The average daily intake of nitrite was for men 4.6 mg (0.9–11.0 mg) and for women 6.6 mg (4.7–9.0 mg). The values, with one exception, were well under the maximum acceptable daily intake (5 mg per kg bodyweight for nitrate and 0.2 mg for nitrite) recommended by FAO/WHO. If the amount of nitrite produced in saliva is also taken into account, most of the values for nitrite intake exceed the recommended value.

Introduction

During the last few years, much interest has been paid to the content of nitrate and nitrite in food as these components may be precursors of carcinogenic nitrosamines. Despite this interest, relatively little has been written on the subject of nitrate and nitrite in various foodstuffs. Recently papers by Kolari & Aunan (1972) and by Fudge & Truman (1973) have dealt with meat products and one by Siciliano *et al.* (1975) was concerned with vegetables. Data on human intake of nitrate and nitrite still seems scarce. Phillips (1968) estimated that a typical Canadian meal (meat, potatoes, vegetables and desert) contained about 300 mg nitrate. Ashton (1970) calculated that the weekly nitrate intake from meat, vegetables and water was about 400 mg. In a recent survey, White (1975) estimated that the daily intake of a United States consumer is 106 mg nitrate and 8.6 mg nitrite. These data are average values obtained from analysis of food items and consumption per capita and do not represent the actual intake of individuals. Such data are, however, presented in this paper which reports the results obtained by a double-portion method.

1. The views and opinions expressed in this paper are those of the authors and do not necessarily reflect the views of FAO.

Material and methods

As part of a community care research programme, a study of food consumption was carried out in Dalby, a village in the south of Sweden situated in a markedly agricultural district. Ten men and ten women aged 25–60 years participated in the first part of this study. They underwent clinical examination and their dietary intakes of energy, proteins, fats and carbohydrate were estimated by chemical analysis of the cooked food collected by the duplicate portion technique (Borgström *et al.* 1975).

On seven different days corresponding to each day in a week everything consumed had to be duplicated and collected as exactly as possible. It was then put into a 2-litre plastic-lined carton which was stored in a refrigerator. The following day it was brought to the laboratory where it was homogenized in an Ultra-Turrax stainless steel homogenizer for 5 min. To facilitate emulsification of fat, 5 g sodium deoxycholate was added and the temperature raised to 40 °C. A sample of the homogenate was freeze-dried and the powder obtained was extracted twice with 200 and 100 ml chloroform. During extraction, the temperature was increased rapidly to 70 °C. The fat-free powder was stored at −20 °C. A pooled sample representing all the 7 days' collection for every participant was then analysed for nitrate and nitrite content.

To estimate nitrate and nitrite content[1], 0.5–1.0 g of the powder was dispersed in about 70 ml hot water in a 200 ml volumetric flask. A buffer, 5 ml pH 9.6–9.7, was added and the flask heated in a boiling water-bath for 15 min. The solution was then deproteinized with potassium ferrocyanide and zinc sulfate, chilled to room temperature, diluted to 200 ml with distilled water and filtered.

An aliquot was taken for estimation of nitrite by the method of Adriaanse & Robbers (1969). To estimate the nitrate content, an aliquot of the solutions was filtered through a column of cadmium, which reduced the nitrate to nitrite and was then estimated as described above.

Results

The daily intake of nitrate and nitrite, expressed in mg of their sodium salts is shown in Table 1. The values are averages for 7 days. Daily intake of nitrate ranged 35–110 mg for the ten men with a mean of 66 mg. For the ten women, the mean value was 70 mg with a range 51–110 mg.

The average daily intake of nitrite was for men 4.6 mg with individual values 1–11 mg. The individual values for the women's group lay between 4.7–8.4 mg with an average of 6.6 mg.

1. The analyses were carried out at the chemical laboratory of the Swedish Meat Research Centre, Kävlinge.

Table 1. Daily intakes of nitrate and nitrite by ten men and ten women. ±, standard deviation.

Subject	$NaNO_2$ (mg)	$NaNO_3$ (mg)
Men		
D 8	1.9	96.5
D 9	1.3	50.9
D 10	9.5	78.5
D 11	8.0	39.4
D 12	3.6	59.9
D 13	11.0	43.9
D 14	4.4	99.6
D 14	2.7	110.5
D 16	0.9	44.9
D 17	2.3	35.0
Mean	4.6 ± 3.6	65.9 ± 28.0
Women		
D 18	7.1	109.1
D 19	8.4	97.5
D 20	5.8	72.1
D 21	9.0	48.9
D 22	7.7	51.9
D 23	5.1	67.3
D 24	7.0	52.7
D 25	6.0	51.4
D 26	5.2	84.2
D 27	4.7	61.4
Mean	6.6 ± 1.5	69.7 ± 21.1

Discussion

It is well recognized that the main source of nitrate in the diet is vegetables and that most of the nitrite comes from cured meats. In some areas, water may also contribute considerably to the nitrate intake.

The aim of the present investigation was, however, not to show the influence of separate food items on the nitrate and nitrite content of the diet but to show how much of these compounds an ordinary Swedish diet contained. The difference between the two groups and within groups, especially in nitrite content of the diet, cannot therefore be explained quantitatively in terms of its ingredients. A semi-quantitative description certainly shows that the diet of D13 (11 mg nitrite) contained more cured meat than that of D16 (0.9 mg nitrite). In the former, cured meat was mentioned 21 times and in the latter only 6 times. By this method, however, we could not account for the differences in average nitrite intakes between men and women.

The present study shows that consumption of nitrate is below the highest intake accepted by FAO/WHO (1974). When the actual daily dietary intake of nitrate is calculated per kg bodyweight, values of 0.5–2.2 mg were found for men and 0.6–1.9 mg for women, which are values clearly below that of 5 mg given by

international experts as the highest acceptable daily intake.

When the intake of nitrite was calculated in the same way, daily intake for 7 of the men was less than 0.05 mg per kg bodyweight and for two of them around 0.1 mg. However, one of them (D13) had a consumption as high as 0.2 mg per kg bodyweight, which equals the maximum acceptable daily intake (FAO/WHO, 1974).

In the female group, daily intake of nitrite as calculated per kg bodyweight was around 0.10 mg for all members.

Dietary intake of nitrite seems well below the acceptable daily intake, but there is one important additional factor – the production of nitrite in saliva. Tannenbaum *et al.* (1974) have recently reported that human saliva contributes 6–12 mg nitrite per day to the diet. The amount does not vary widely for the individual nor does it seem strongly influenced by the composition of the diet.

If the salivary amount of nitrite used by White (1974) is added, mean 8.6 mg per day, the total intake of nitrite would increase considerably and the situation in relation to maximum acceptable daily intake would be quite different. Calculated in mg per kg bodyweight, all the values for the women's group would be above 0.2 mg. For the men, 4 of the values reach or exceed the limit.

It is outside the purpose of this paper to discuss implication of nitrite content of the diet for public health. But the concentration of nitrite in saliva is so low, 5–10 mg litre^{-1}, that the actual amount of nitrite in the stomach originating from saliva is low compared to that from cured meats. As to the risk of nitrosamine formation, it may therefore still be worthwhile to reduce or ban the use of nitrite in manufacturing such products.

Discussion on the session

Nitrate and nitrite intakes in Norway

The intake of nitrate and nitrite by the Norwegian population was studied in 1974, after the nitrite ban was introduced. The results indicated average daily intakes per person of 70 and 0.2 mg of potassium nitrate and sodium nitrite respectively (Høyem, T., Nitrate and nitrite contents in Norwegian food IV. Intern. Congr. of Food Sci. and Technol., Madrid, September, 23–27, 1974).

Average versus peak intakes of nitrite

Average values of nitrite intake are significant for evaluating the acute toxicity, but are not useful for assessing nitrosamine formation, which is dependent on the nitrite concentration when amines are present. Instead of determining average daily intakes it might be more useful to measure the salivary nitrite concentration in groups of people at various times, as well as variations in nitrite content of various food intakes (What bit of food is responsible for a high level of nitrite?). In general, peak intakes of nitrite are considered more hazardous to man in terms of possible exposure to carcinogenic *N*-nitroso compounds than average daily intakes.

Absence of nitrite in saliva of rodents

In extrapolating results from studies in experimental animals to human beings, it should be kept in mind that e.g. the saliva of rodents does not contain nitrite.

ADI's of nitrate and nitrite set by WHO

Acceptable daily intakes (ADI's) given by WHO for nitrate and nitrite are based on the results of acute and sub-acute toxicity studies, and do not take into account the possibility of nitrosamine formation. These ADI's should therefore, be considered in the light of new experimental information.

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

N-Nitroso compounds in products widely consumed in France: hams

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Abstract

Among 9 samples of cured ham purchased in France analysed by gas chromatography, only one sample contained a significant amount of dimethylnitrosamine, a mass fraction of 18.4 μg/kg. In other samples, the amounts found, < 5 μg/kg, were not confirmed by MS. Nitrates and nitrites were also estimated, ranging 50–363 mg/kg and 4–13 mg/kg, respectively; no correlation was between contents of nitrate, nitrite and nitrosamines.

Introduction

The carcinogenicity of various nitrosamines has been demonstrated in several species of laboratory animals (Magee & Barnes 1967). Although it has not been established for man, it is strongly suspected. The presence of trace amounts of these compounds in food for human consumption constitutes a health risk.

Numerous authors report that nitrosamines may be formed in cured products from nitrites or nitrates during processing. Fong & Chan (1973a) detected high levels (50–300 μg/kg), of dimethylnitrosamines in cured fish. Panalaks et al. (1973, 1974) identified dimethylnitrosamines in traces (2–12 μg/kg), in 57 of 197 samples of cured meats. Most of the offending samples were dried and fermented meats (like salami), while products for frying (like bacon or wieners) often contained nitrosopyrrolidine (Pensabene et al. 1974; Sen et al. 1974a).

Sen et al. (1976) recently reported the presence of nitrosamines after addition of spices during processing (possibly black pepper and paprika). While the use of nitrite-spice premixes results in formation of high levels of nitrosamines (> 300 μg/kg) (Sen et al. 1974b), the addition of these ingredients separately considerably lowers the average mass fraction of nitrosamines.

To our knowledge, no studies have been published on French cured meats. Our first step, therefore, was to examine mass fractions of nitrosamines in a widely consumed class of products, hams.

1. Study carried out under Environmental Contract No 7 492.

Materials and methods

Collection of samples

The samples were purchased from various commercial sources and directly from pork-butchers. They were taken rapidly to the laboratory and stored at −30 °C until further use.

Equipment and reagents

Gas chromatograph Girdel series 3 000 fitted with an electron-capture detector.
Mass spectrometer LKB series 90 215 coupled with gas chromatograph Pye 104.
Chromatographic column 1 x 30 cm with Teflon stopcock.
Concentrator Kuderna-Danish 500-ml flask with micro Snyder column, 1 chamber (local manufacture).
Basic and neutral alumina wash alumina with ether and pentane (1:1 by volume); dry in air, heat at 200–240 °C overnight in oven; cool in desiccator. Basic alumina activity II was prepared by adding 3 g distilled water per 100 g alumina and leaving overnight to equilibrate. Neutral alumina activity III was prepared in the same way by adding 6 g distilled water per 100 g.
Solvent analytical grade. Methylene chloride was glass-distilled.

Estimation of nitrosamines

The method used was developed by Telling (1972) and modified by Walker et al. (1975).

A 100g sample was steam-distilled in a neutral medium. The steam passed through an acid medium and was condensed. The distillate was made alkaline and extracted with methylene chloride. The organic phase was dried and concentrated in the concentrator and dissolved in hexane.

The extract was purified on a column of basic and neutral alumina (3 g each). The column was eluted with a gradient of ether in pentane. The first 25 ml constituted Fraction A and the succeeding 55 ml Fraction B. Each fraction was oxidized with a mixture of trifluoracetic acid and 90% hydrogen peroxide and left gently shaking over night. The solution was cooled in an ice bath, and a 20% potassium carbonate solution was added drop by drop to pH 10–11.

The nitramines were extracted with methylene chloride. The organic phase was dried and concentrated in the concentrator. The concentrate was dissolved in 5 ml pentane and fractionated on a chromatographic column similar to that described above. The eluate was then collected in 5 ml fractions.

Each part of Fractions A and B was analysed by gas chromatography. The results were compared with those obtained with a reference mixture in order to eliminate possible artefacts. The positive fractions were bulked and concentrated for quantitative analysis first by gas chromatography, then by mass spectrometry.

Estimation of nitrate and nitrite

Mass fractions of nitrate and nitrite were estimated by the spectrometric method of Griess modified by Volff et al. (1974) at 525 nm.

Results and discussion

The samples were examined for 5 volatile *N*-nitrosamines: nitrosodimethylamine, diethylamine, dibutylamine, piperidine and pyrrolidine. Fractions recovered for the 5 nitrosamines added to the ham samples ranged from 45 to 69%. Mass fractions of nitrate and nitrite ranged 50–363 mg/kg and 4–13 mg/kg, respectively. None of the samples exceeded the authorized limits: 1.5 and 0.15 g/kg, respectively.

There is no clear correlation between amounts of nitrate and nitrite, as was found also in a recent study on 1 468 samples (Durand, 1976). Presumably nitrate is converted only slowly to nitrite during processing and storage.

Rough values for nitrosamines, based solely on chromatography analysis with an electron-capture detector, suggest that dimethylnitrosamines were present in all the samples. Mass fractions by that method ranged from 1 μg/kg (limit of detection) to 18.4 μg/kg. Confirmation by mass spectrometry with specific ion detector was made on only 1 sample (No 4); for the other the amount of nitrosamines was to small.

Frouin et al. (p. 115) have shown that in the physico-chemical conditions within cured meat products (pH, redox potential) all added nitrite is reduced to nitrous oxide NO more readily than it could react to nitrosate amines. Nitrate could not be directly reduced to NO as the energy of activation is too high. It is first broken down into the intermediate NO_2^- by bacterial action. This theory, based on a mathematical model, seems to account for the absence of nitrosamines in products cured with nitrited salt (0.6% sodium nitrite in sodium chloride) added in concentrations below authorized limits and used without frying.

In fried products, in addition to nitrosopyrrolidine formed by decarboxylation of nitrosoproline (Pensabene et al. 1974), dimethyl and diethylnitrosamine can be present (Sen et al. 1974a; Groenen et al. 1976); however, the nitrosation reactions

Table 1. Mass fraction (w) of nitrate, nitrite and dimethylnitrosamine (DMNA) in various hams. DMNA was estimated by gas chromatography. J. M. Ziegler (Lab. Spectrometrie de Masse, Univ. confirmed the data by combined gas-liquid chromatography and mass spectrometry.

Nancy I Variety	Processing	$NaNO_2$ $w/mg \cdot kg^{-1}$	$NaNO_3$ $w/mg \cdot kg^{-1}$	DMNA $w/\mu g \cdot kg^{-1}$	Confirmation
Cooked ham	Pork-butcher	10	106	< 1	not tested
Cooked ham	Industrial	13	363	2.46	not tested
Smoked ham	Pork-butcher	12	123	< 1	not tested
Cooked smoked ham	Pork-butcher	10	50	18.4	positive
Bayonne ham	Pork-butcher	11	152	< 1	not tested
Westphalian ham	Pork-butcher	4	180	9	not tested
Westphalian ham	Pork-butcher	11	86	3.2	not tested
Parma ham	Pork-butcher	6	117	4.2	not tested
Parma ham	Pork-butcher	8	172	2	not tested

by which they are formed are not clearly understood. Neurath et al. (1976) suggested that NO_2 was formed from liberated NO or that free NO was fixed directly by the effect of heat.

In processing with nitrate or a mixture of nitrited salt plus nitrate, the nitrite freed during curing by the nitrate-reducing flora can also be used for a nitrosation reaction catalysed by the same bacteria (Fong & Chan 1973b). Similarly the lactobacillus flora, which is very active during fermentation of cured meats, can nitrosate the amines with the nitrite thus liberated (Hawksworth & Hill 1971).

The results in Table 1 are in agreement with the scarce data we have found in the literature on this type of product. Panalaks et al. (1973) report a study on 33 hams of all types, 4 of which contained dimethylnitrosamine at 2–3 μg/kg.

Confirmation by mass spectrometry of results obtained by gas-liquid chromatography, although costly, is indispensable since common methods of extraction and separation are not sufficiently selective and are subject to artefact. Without mass spectrometry, levels are likely to be overestimated.

In conclusion, mass fractions of nitrosamines in the samples were low or nonexistant. Nevertheless, even if nitrite is shown to be a harmless additive, technological processing is not neutral. While it is necessary to use treatment such as fermenting and maturing to preserve a product's original characteristics, it may be necessary to add substances inhibiting nitrosation such as organic acids (like ascorbic acid).

The dangers of transformation during home cooking being real, it is necessary to study problems of storage, processing and cooking by the housewife.

Discussion on the session

Effects of low-resolution mass spectrometry

The figure of 18.4 μg/kg dimethylnitrosamine given in Table 1 was confirmed on a low-resolution mass spectrometer. It has been shown several times that low-resolution techniques can give rise to incorrectly high figures. Therefore, the data in the table should not be regarded as confirmatory.

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

Experiences with nitrite and nitrate ban in Norway

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Abstract T. Høyem

Experiences in Norway with the ban on nitrite and nitrate are reviewed as communicated by the health authorities, the consumer organization, the food inspection service, and the food industry.

Since parts of the industry ignore the full implementation of the new regulations, and since nitrite or nitrous compounds are inevitably present in production areas, a practical limit is tolerated for nitrite in meat products where addition of nitrite is prohibited of 5 mg/kg by mass.

The ban is estimated to have reduced amounts of nitrite in meat products by 70%. No outbreaks of botulism have been reported since the ban was implemented.

Introduction

When Norway, as the first nation in the world, introduced a general ban on nitrite and nitrate in food from 1 January 1973, it was expected that problems would arise, of which the increased chance of botulism was worst feared. Other problems predicted were a decrease in shelf life and in quality (colour and taste).

These problems were thoroughly discussed in an *ad hoc* committee consisting of members from the authorities, research institutions, and the food industry. Discussions were based on a total ban on nitrite and nitrate, and the committee tried to foresee harmful effects and health risks of such a ban. Exemptions and some delays in implementing the ban were also negotiated in the committee.

Though the Norwegian food industry was informed about the ban well in advance, preparedness varied widely. So did willingness to accept the inevitability of the ban. This led to several problems after the ban was introduced, both for the food manufacturers and for inspectors.

The aim of this paper is to share our experiences in Norway with others who might be planning to restrict the use of nitrite and nitrate in other countries.

The information in this paper is based upon written and oral statements from health authorities, the consumer organization, the public food inspection service, and large parts of the Norwegian food industry.

Norwegian legislation on nitrite and nitrate

Nitrite had never been used in such large amounts in food in Norway as in many other countries. The same applied to some extent for nitrate. Nitrite had long been restricted for use only in combination with NaCl (0.5–0.6% by mass). According to Norwegian food regulations, nitrite and nitrate were regarded as food preservatives, and they still are, though scientific evidence and most practical experience exclude them as significant food preservatives in most meat products in the content used in Norway (Tjaberg & Kvåle, 1972), and though the levels now permitted allow only its action as a colouring agent. The regulations on nitrite and nitrate in meat products in Norway are summarized in Table 1.

Health authority

The health authorities have no information on health problems related to the nitrite ban. They reported a marked decrease in the use of nitrite since the ban, in spite of several exemptions granted.

Table 1. Permitted mass fractions of added nitrite and nitrate in illustrative meat products in Norway.

Year	Permitted	Products	lim mg/kg
Before 1973	KNO_3, $NaNO_3$	All products	500
	$NaNO_2$, only mixed (0.5–0.6% by mass) with NaCl and if no nitrate has been used	Cured or dried meat, sausages	Practical limits set by content of NaCl
1973	KNO_3, $NaNO_3$	Cured and dried meats, (e.g. bacon, dried ham, fermented sausages); semi-preserves	500
		Meat rolls, cooked ham saveloy	250
	$NaNO_2$, only mixed (0.5–0.6% by mass) with NaCl and if no nitrate has been used	Cured and dried meats, (e.g. bacon, dried ham, fermented sausages); semi-preserves	200
		Meat rolls, cooked ham, saveloy	120
1974	KNO_3, $NaNO_3$	Cured and dried meats, (e.g. bacon, dried ham, fermented sausages); semi-preserves	500
1974 on	$NaNO_2$, only mixed (0.5% by mass) with NaCl and if no nitrate has been used	Cured and dried meats, semi-preserves	200
		Meat rolls, cooked ham, saveloy	60
1975 on	KNO_3, $NaNO_3$	None	
1976		None as in 1974 and 1975	

The health authorities have the impression that the ban, though resulting in pale pink or gray sausages, was accepted by the consumers, but that some producers ignored the ban initially. After some time, however, products containing residual nitrite due to intentionally added nitrite were found less frequently.

The authorities felt that initial unwillingness among the producers to accept the ban was due to lack of information, but they had the impression that the ban is being generally accepted within the meat industry.

No information is available on the effect of the total ban of nitrate, implemented on 1 January 1975.

Within the fish industry, the ban is taking more time to implement. The fish industry was granted exemptions beyond the time limits anticipated.

The dairy industry still produces cheese where nitrate is added to the cheese milk. They are interested in avoiding this additive, but the necessary techniques are still under development.

Food inspection services

The official food inspection laboratories in most of the country faced numerous problems in trying to enforce the law. Even though many producers, omitting nitrite in their recipes, obtained grey to brownish products, others still produced red or pink products, among which analysis often suggested only traces of nitrite. The last group of producers ranged from those who simply refused to accept the nitrite ban, to those who could not prevent it because of the natural presence of nitrite or nitrous gases in the production area. Some producers also developed certain tricks to produce the desired colour in, or on, the surface of their products. Some of these are listed below:

- Products without nitrite and products with nitrite legally added are smoked together in the same chamber
- Salting of sausage skins in brine containing nitrite
- Nitrite brine placed in smoking chamber
- Nitrite salt added to sawdust for smoke generators
- Use of glucono-δ-lactone or ascorbic acid for maximum utilization of residual nitrite in water or food additives
- Storage of sausages for a few hours before cooking to utilize the natural contents of nitrate for microbial reduction to nitrite
- Increasing the contents of nitric oxide in the smoke by different methods.

After much confusion in the first year about the arbitrary limit between 'natural residual nitrite' and 'added residual nitrite', experience showed that the practical limit of residual nitrite ($NaNO_2$) could be set at 5 mg/kg. Nitrite, derived from natural sources, may occur in products in sufficient traces to produce a colour, at least on the surface of the products. Nitrite residues above 5 mg/kg are considered 'positive', indicating deliberate addition.

The food inspection points out a difference between smoking in old-fashioned and modern kilns, in that the old ones unavoidably give a reddish tinge at the surface of the products.

The food inspection claims to have the situation under control, and sees no serious problems.

Consumer organization

As some consumers expected a total ban on nitrite, they were confused that coloured products were still available in food stores. The term 'ban' created gross misunderstandings through lack of information to consumers.

The consumer's organization also claims that consumers wanted a choice between products with and without nitrite added.

Industry

Manufacturers had thorough technical discussion about the extent and implications of a nitrite ban before it became effective. Industry did not recommend a total ban, both because of the problem of inspection and of expected competition.

When the ban was implemented, most producers loyally adhered to the new regulations. Some producers did not, however, tow the line and developed various techniques to develop colour without really adding nitrite. This gradually lead to a situation where most producers, to keep up with their competitors, learned to utilize the natural environmental nitrite/nitrate to produce a faint red in their products, keeping residual nitrite below 5 mg/kg.

The ban initially lowered sales of sausage products by some 5%, but they soon returned to normal. Drastic reductions of nitrite in the products did not even affect sale, significantly as long as there was sufficient nitrite left to produce a reddish colour. According to one meat marketing organization, the consumer seemed almost indifferent towards gray-brownish products when there is no other choice; they selected the coloured products where both types were available.

Some producers unsuccessfully launched uncoloured products, clearly stating that no nitrite had been added. Such products had to be redrawn from the market.

Industry feels that the present situation, if not ideal, is satisfactory. Official food inspectors were sometimes blamed for ignoring violations, which may induce some producers to increased additions of nitrite.

Finally, there is no serious concern about the shelf life of 'nitrite-free' products.

Concluding remarks

Evidently, there was less concern for difficulties of the nitrite and nitrate ban among the health authorities than among producers and food inspectors. The parties concerned wish to let the matter rest and have reached a *modus vivendi* where coloured products are accepted as long as residual nitrite does not exceed 5 mg/kg.

A total ban on nitrate in all products was introduced from 1 January 1975. Again, exemptions were granted for some products, especially for fish preservatives. These exemptions will be terminated on 31 December 1976. It is yet not known whether the nitrate ban will be effective.

An estimate of annual intake of nitrite and nitrate per person in Norway (Høyem, 1974) indicated a 80% decrease of nitrite in meat through the ban. Estimates by industry after 3 years of experience show comparable results: 70%.

No botulism has occurred, even in products where nitrite has been completely excluded.

The Norwegian action has shown that a nitrite and nitrate ban can be effective. Its toxicological significance remains to be seen.

Discussion on the session

Extent of nitrite ban

The percentage of total cured meat production in Norway that has been exempted from the ban is estimated to be 15–20. The exempted products generally are the ones that are eaten cold (without home cooking). It cannot be said that the Norwegian climate favours a ban on nitrite; the temperature in summer can be as high as 25 to 30 °C. The products are kept strictly in the cold chain. At present the use of nitrited salt is still allowed in fish products, until 1 January 1977.

Consequences of the ban

No increase in price had occurred that may be associated with the ban. In the beginning, there was a decline of some 5% in the sellings of the products without nitrite, due to a decreased consumer acceptance, but later on this restored. The shelf-life accepted for cooked sausages without nitrite is 3 weeks. At the conclusion of this period, unsold products are removed from sale.

Enforcement

So far, officials have gone easy on violations of the ban, and try to advise rather than punish.

It was asked whether the nitric oxide myochrome content of a meat product would not be a better criterium for the control on the unpermitted use of nitrite than limiting the residual amount of nitrite to 5 mg/kg. In principle the NO-myochrome method was considered to be sensitive and easy to perform, but there are problems in attaining and storing the proper standards. However, a similar method is successfully used in France for detecting unpermitted addition of sulphite. The absorption spectrum of an acetone extract is said to be a better indicator for that purpose than the free sulphite content of the samples.

Subtle practices to obtain a cured meat colour

One of the means to obtain the desired red colour is increasing the NO-content of the smoke. This can be achieved by installing propane burners in the air intake of the smoking chamber.

General discussion on the Epidemiological/Legislative Session

Erythorbate an acceptable food additive?

There are indications in the literature (Hornig, D. and Weiser, H., 1976, Interaction of erythorbic acid with ascorbic acid catabolism. Int. J. Vit. Nutr. Res. 46: 40–47) that erythorbic (iso-ascorbic) acid may compete with ascorbic acid in human metabolism, which would make it undesirable as a food additive.

Urinary nitrate as an indication of nitrate intake

It is surprising that so little is known about the nitrate metabolism. There is some preliminary evidence that the nitrate content of the urine is a valuable indicator of the nitrate consumption in man.

Resolutions

The participants of the second International Symposium on Nitrite in Meat Products, held at Zeist, the Netherlands, from 7th till 10th September 1976, accepted the following resolutions.

1. As stated at the first Nitrite Symposium in Zeist in 1973, it seems highly advantageous to discuss different aspects of nitrite as an additive in meat products between scientists and public health authorities from various disciplines and countries.

Since 1973 many investigations have been made on the presence of nitrite, nitrate and nitrosamines in cured meat products. Toxicological work on nitrosamines has mostly been carried out on rodents, whereas the role of nitrite and nitrate in saliva has been studied in relation to the formation of nitrosamines in the human stomach.

2. More attention must be given to the reported presence of nitrate in meat and possibly in living mammalian tissues. At present a non-zero nitrate level in meat products seems inevitable. Satisfactory analytical procedures are still lacking.

3. As regards microbiological safety, especially from botulism; i) it is important to distinguish between input nitrite, residual nitrite which remains measurable as such, and the difference between the two representing nitrite which disappears partly by transformation into nitrosocompounds. This difference cannot yet be identified and estimated satisfactorily. There are few indications that residual free nitrite is related to longterm control of clostridia. Present data have suggested a possibly important role related to the nitrite which disappears. However, at present the most useful index of anti-microbial activity is the input nitrite concentration. None of these indices is wholly satisfactory in itself. ii) Special attention should be directed to nitrolic acids (reaction products of nitrite and the additive sorbic acid) as potential bacterial inhibitors, as well as to a possible action of hydroxymethylfurfural. iii) The influence of ascorbate on microbial growth in cured meat products requires further elucidation.

4. Various volatile nitrosamines are frequently detected at μg/kg levels using GC-MS

instruments of high sensitivity; research is now especially needed on the occurrence of non-volatile *N*-nitrosamines and of C-nitroso compounds.

5. In the formation of nitrosamines in cured meats, there is a lack of correlation between amounts of residual nitrite and nitrosamine; which contrasts with the correlation with input nitrite which is observed when increasing amounts of nitrite are added in a single experiment. This suggests that it may be the converted nitrite, rather than the residual, which is a determining factor in nitrosamine formation in meats. On this basis, the aim should be to reduce input rather than residual nitrite, and more research is needed to decide for each individual product, the lowest level consistent with human safety from the microbiological and toxicological viewpoints.

6. It would be helpful if information could be obtained about the relative importance for carcinogenesis in man of in vivo formation of carcinogenic *N*-nitroso-compounds compared with the presence of preformed *N*-nitroso-compounds in food.

7. In terms of 'risk benefit' it seems unavoidable that the human population must accept a certain risk at present from carcinogens, including many nitrosamines.

8. Appropriate epidemiological studies should be encouraged on the significance of carcinogenic nitrosamines for cancer in man.

9. In the immediate future, the obvious way to progress is to identify those products in which the use of nitrite and/or nitrate is not essential for safety, and to eliminate or diminish such use by appropriate processing of those products.

References

Adriaanse, A.; Robbers, J. E.; 1969. – Journal of the Science of Food & Agriculture 20:321.

AFNOR. See FR-AFNOR.

Ågren, G.; 1940. The interaction of amino nitrogen and carbohydrates. – Acta Physiologica Scandinavica 1:105–118.

Amerine, M. A.; Pangborn, R. M.; Roessler, E. B.; 1965. Principles of sensory evaluation of food. Academic Press.

Ando, N.; 1974. Some compounds influencing colour formation. – Krol & Tinbergen, p. 149–160. Discussion, p. 160.

Ando, N.; Nagata, Y.; Okoyama, T.; 1971. Effects of low molecular fraction of sarcoplasma from porcine skeletal muscle on the behaviour of nitrite and the formation of cooked cured meat color in rapid curing process. – EMMRW – 17, Paper C8.

Angeli, A.; Cusmano, G.; 1917. Nitrosopyrrole black. – Atti dell 'Accademia Nazionale dei Lincei. Rendiconti delle Sedute Solenni 26(1)273–278. Cited: Chem. Abstr. 1918 12:365–366.

Asan, T.; Solberg, M.; 1976. Inhibition of Clostridium perfringens by heated combinations of nitrite, sulfur, and ferrous or ferric ions. – Applied & Environmental Microbiology 31:49–52.

Ashton, M. R.; 1970. The occurrence of nitrates and nitrites in foods. British Food Manufacturing Industries Research Association, Leatherhead. – Literature Surveys No 7.

Ashworth, J.; Spencer, R.; 1972. The Perigo effect in pork. – Journal of Food Technology 7:111–124.

Ashworth, J.; Hargreaves, L. L.; Jarvis, B.; 1973. The production of an antimicrobial effect in pork heated with sodium nitrite under simulated commercial pasteurization conditions. – Journal of Food Technology 8:477–484.

Austin, B. L.; 1977. Unpublished results reported by Crowther et al.

Bacus, J. N.; Deibel, R. H.; 1972. Nitrite burn in fermented sausage. – Applied Microbiology 24:405–408.

Baird-Parker, A. C.; 1969a. Medical and veterinary significance of spore-forming bacteria. – Gould & Hurst, p. 517–548.

Baird-Parker, A. C.; 1969b. The use of Baird-Parker's medium for the isolation and enumeration of Staphylococcus aureus – GB-SAB-TS – 3, p. 1–8.

Baran, W. L.; Stevenson, K. E.; 1975. Survival of selected pathogens during processing of a fermented turkey sausage. – Journal of Food Science 40:618.

Barber, L. E.; Deibel, R. H.; 1972. Effect of pH and oxygen tension on staphylococcal growth and enterotoxin formation in fermented sausage. – Applied Microbiology 24:891–898.

Berg, B. C.; Forster, A. B.; Stacey, M.; 1956. Amino-sugars and related compounds. Part 1. The deamination of D-glucosamine hydrochloride. – Journal of the Chemical Society 4531–4535.

Beynon, J. H.; 1960. Mass spectrometry and its applications to organic chemistry. Elsevier, Amsterdam.

Bills, D. D.; Hildrum, K. I.; Scanlan, R. A.; Libbey, L. M.; 1973. Potential precursors of N-nitrosopyrrolidine in bacon and other fried foods. – Journal of Agricultural & Food Chemistry 21:876.
Block, S. S.; 1949-06-21. US Patent 2 474 139.
Board & Lovelock. See GB-SAB-TS – 8.
Bogovski & Walker 1974. See IARC-SP – 9.
Bogovski et al. 1972. See IARC–SP – 3.
Bogovski et al. 1976. See IARC–SP – 14.
Borgström, B.; Nordén, Å.; Åkesson, Å.; Jägerstad, M.; 1975. A study of food consumption by the duplicate portion technique in a sample of the Dalby population. – Scandinavian Journal of Social Medicine. Suppl. 10.
Bowen, V. G.; Deibel, R. H.; 1974. Effect of nitrite and ascorbate on botulinal toxin formation in Wieners and bacon. – US–AMIF, p. 63.
Boyland, E.; Walker, S.; 1974. Thiocyanate catalysis of nitrosamine formation and some dietary implications. – IARC-SP – 9, p. 132.
Bryce, T. A.; Telling, G. M.; 1972. Semiquantitative analysis of low levels of volatile nitrosamines by gas chromatography – mass spectrometry. – Journal of Agricultural & Food Chemistry 20:910–911.
Buchanan, R. L.; Solberg, M.; 1972. Interaction of sodium nitrite, oxygen and pH on growth of S. aureus. – Journal of Food Science 37:81–85.
Cantoni, C.; Renon, P.; 1973. Dimethylnitrosamine in meat products. – Atti della Societa Italiana delle Scienze Veterinarie 27:574–576.
Cantoni, C.; Cattaneo, P.; 1974. Reactions between nitrites and SH groups of food during digestion. – Industrie Alimentari 13(12)63–68.
Cantoni, C.; Renon, P.; Maccapani, M.; 1974. Nitriti liberi e legati nei derivati carnei. – Industrie Alimentari 13(9)129–131.
Cantoni, C.; Bianchi, M. A.; Beretta, G.; 1975. Stabilita di nitrosoderivati (nitrosotioli, nitrosofenoli e nitrosoemoglobina) a pH alcalino. – Industrie Alimentari 14(119)79–81.
Cardesa, A.; Pour, R.; Rustia, M.; Althoff, J.; Mohr, U.; 1973. The carcinogenic effect of methylcholanthrene and dimethylnitrosamine in Swiss mice. – Zeitschrift für Krebsforschung 79:98–107.
Carunchio, V.; Bedetti, R.; Tomassetti, M.; 1976. Formation constants for the cobalt(II) chloride – 1-nitroso-2-naphthol system in ethanol – benzene mixtures. – Talanta 23:479–480.
Cassens, R. G.; 1974. Nitrites and nitrosamines in processed meats: medical aspects. – EMMRW – 20, Rapporteurs papers, p. 38.
Cassens, R. G.; Sebranek, J. G.; Kubberød, G.; Woolford, G.; 1974. Where does the nitrite go? – Food Products Development 8(10)50–56.
Castellani, A. G.; Niven, C. F.; 1955. Factors affecting the bacteriostatic action of sodium nitrite. – Applied Microbiology 3:154–159.
Challis, B. C.; Bartlett, C. D.; 1975. Possible carcinogenic effects of coffee constituents. – Nature (GB) 254:532–533.
Challis, B. C.; Lawson, A. J.; 1973. The chemistry of nitroso compounds. Part 5. Encounter control for the nitrosation of indoles in dilute acid. – Journal of the Chemical Society. Perkin 2:918–925.
Chang, P.; Akhtar, S. M.; 1974. The Perigo-effect in luncheon meat. – Canadian Institute of Food Science & Technology Journal 7:117–119.
Chang, P. C.; Akhtar, S. M.; Burke, T.; Pivnick, H.; 1974. Effect of sodium nitrite on Clostridium botulinum in canned luncheon meat: evidence for a Perigo-type factor in the absence of nitrite. – Canadian Institute of Food Science & Technology Journal 7:209–212.
Chapman, G. H.; 1945. The significance of sodium chloride in studies of staphylococci. – Journal of Bacteriology 50:201–203.
Christiansen, L. N.; Tomkin, R. B.; Shaparis, A. B.; Kueper, T. V.; Johnston, R. W.; Kautter, D. A.; Kolari, O. J.; 1974. Effect of sodium nitrite on toxin production by Clostridium botulinum in bacon. – Applied Microbiology 27:733–737.
Collins-Thompson, D. L.; Chang, P. C.; Davidson, C. M.; Larmond, E.; Pivnick, H.; 1974. Effect of nitrite and storage temperature on the organoleptic quality and toxicogenesis by

Clostridium botulinum in vacuum-packed side bacon. – Journal of Food Science 39:607–609.
Cooper, C. C.; Cassens, R. G.; Briskey, E. J.; 1969. Capillary distribution and fiber characteristics in skeletal muscle of stress-susceptible animals. – Journal of Food Science 34:299.
Crathorne, B.; Edwards, M. W.; Jones, N. R.; Walters, C. L.; Woolford, G.; 1975. Use of isomers in the detection and estimation of volatile nitrosamines by combined high-resolution mass spectrometry-gas chromatography. – Journal of Chromatography 115:213.
Crosby, N. T.; Foreman, J. F.; Palframan, J. F.; Sawyer, R.; 1972. Estimation of steam volatile N-nitrosamines in foods at the 1 µg/kg level. – Nature (GB) 238:342.
Crowther, J. S.; Holbrook, R.; Baird-Parker, A. C.; 1977. Role of nitrite and ascorbate in the microbiological safety of vacuum-packed sliced bacon. – This symposium.
Dafaye, J.; 1964. Désamination nitreuse de la D-galactosamine: préparation des 2,5-anhydro-D-talose et 2,5-anhydro-D-talitol. – Societé Chimique de France. Bulletin 1964: 999–1002.
Dafaye, J.; 1970. 2,5-Anhydrides of sugars and related compounds. – Advances in Carbohydrate Chemistry & Biochemistry 25:181–228.
Daly, C.; la Chance, M.; Sandine, W. E.; Elliker, P. R.; 1973. Control of S. aureus in sausage by starter cultures and chemical acidulation. – Journal of Food Science 38:426–430.
DE-DFG, 1974. Forschungsbericht Rückstände in Fleisch und Fleischerzeugnissen. Bonn.
Densen, P. M.; Davidow, B.; Bass, H. E.; Jones, E. W.; 1967. A chemical test for smoking exposure. – Archives of Environmental Health 14:865.
Dhont, J. H.; 1977. Development of a method of estimating N-nitrosamino acids and its use on some meat products. – This symposium.
Dhont, J. H.; van Ingen, C.; 1976. Identification and quantitative determination of N-nitrosoproline and N-sarcosine and preliminary investigations on N-nitrosohydroxyproline in cured meat products. – IARC-SP – 13.
Dierick, N.; Vandekerckhove, P.; Demeyer, D.; 1974. Changes in nonprotein nitrogen compounds during dry sausage ripening. – Journal of Food Science 39:301.
Dontenwill, W.; 1968. Experimental studies on the organotropic effects of nitrosamines in the respiratory tract. – Food & Cosmetics Toxicology 6:571.
Dooley, C. J.; Wasserman, A. E.; Osman, S.; 1973. A contaminant in N-nitrosodimethylamine: confirmation by high resolution mass spectrometry. – Journal of Food Science 38:1096.
Drake, B. B.; Smythe, V. C.; King, C. G.; 1942. Complexes of dehydroascorbic acid with three sulfhydryl compounds. – Journal of Biological Chemistry 143:89–98.
Druckrey, H.; Schildbach, A.; Schmähl, D.; Preussmann, R.; Ivankovic, S.; 1963. Quantitative Analyse der carcinogenen Wirkung van Diëthylnitrosamin. – Arzneimittel-Forschung 13:841–846.
Druckrey, H.; Preussmann, R.; Ivankovic, S.; Schmähl, D.; 1967. Organotrope carnicogene Wirkung bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. – Zeitschrift für Krebsforschung 69:103–201.
Durand, P.; 1976. Les teneurs en nitrates et nitrites résiduaires des jambons cuits français. – Annales de la Nutrition et de l'Alimentation ... (in press).
Durand, P.; Rozier, J.; 1968–05. Evolution des nitrates et des nitrites dans la chair à saucisse en fonction de la température de conservation. – Recueil de Médecine Veterinaire de l'École d'Alfort 1968: 451–456.
Eisenbrand, G.; Preussmann, R.; 1970. Eine neue Methode für kolorimetrischen Bestimmung von Nitroaminen nach Spaltung der N-Nitrosogruppe mit Bromwasserstoff in Eisessig. – Arzneimittel-Forschung 20:1513.
Eisenbrand, G.; Marquardt, P.; Preussmann, R.; 1969. Trace analysis of N-nitroso compounds. 1. Liquid-liquid distribution in acetonitrile/heptane as clean-up method. – Zeitschrift für Analytische Chemie 247:54.
Eisenbrand, G.; Ungerer, O.; Preussmann, R.; 1974. Formation of N-nitroso compounds from agricultural chemicals and nitrite. – IARC-SP – 9, p. 71–74.
Eisenbrand, G.; Janzowski, C.; Preussmann, R.; 1975a. Gas chromatographic determination of N-nitrosoamino acids by trimethylsilylation and single-ion mass fragmentography. – Journal of Chromatography 115: 602–606.
Eisenbrand, G.; Ungerer, O.; Preussmann, R.; 1975b. The reaction of nitrite with pesticides.

Formation, chemical properties and carcinogenic activity of the N-nitroso derivative of N-methyl-1-naphthyl-carbamate (carbaryl). – Food & Cosmetics Toxicology 13:365–467.
Eisenbrand, G.; Janzowski, Ch.; Preussmann, R.; 1976a. Gas chromatographic determination of N-nitrosaminoacids by trimethylsilylation and single-ion monitoring in a GC/MS system. – IARC-SP – 13.
Eisenbrand, G.; v. Rappardt, E.; Zappe, R.; Preussmann, R.; 1976b. Trace analysis of volatile nitrosamines by a modified nitrogen-specific detector in pyrolytic mode and by ion-specific determination of heptafluorobutyramides in GC/MS system. – IARC-SP – 13.
Elespuru, R.; Lijinsky, W.; 1973. The formation of carcinogenic N-nitroso compounds from nitrite and some types of agricultural chemicals. – Food & Cosmetics Toxicology 11:807–817.
Elespuru, R.; Lijinsky, W.; Setlow, J. K.; 1974. Nitrosocarbaryl as a potent mutagen of environmental significance. – Nature (GB) 247:386–387.
EMMRW – 9 (9th European Meeting of Meat Research Workers. = 9e Réunion Européenne des Chercheurs en Viande); 1963. Budapest.
EMMRW – 16; 1970. Proceedings, Varna, Bulgaria, 1970.
EMMRW – 17; 1971. Proceedings, Bristol, 1971.
EMMRW – 18; 1972. Proceedings, University of Guelph, Ont., Canada, 1972. 2 vol.
EMMRW – 19; 1973. Proceedings, Paris, 1973. 2 vol.
EMMRW – 20; 1974. Dublin, Ireland, 1974.
EMMRW – 21; 1975. Proceedings, Berne, Switzerland, 1975.
EMMRW – 22: 1976. Proceedings, Malmö, Sweden, 1976.
Ender, F.; Ceh, L.; 1971. Conditions and chemical reaction mechanisms by which nitrosamines may be formed in biological products with reference to their possible occurrence in food products. – Zeitschrift für Lebensmittel-Untersuchung & -Forschung 145:133.
Ender, F.; Havre, G.; Helgebostad, A.; Koppang, A.; Madsen, R.; Ceh, L. 1964. Isolation and identification of a hepatotoxic factor in herring meal produced from sodium nitrite preserved herring. – Naturwissenschaften 51:637–638.
Epstein, S. S.; 1971. In vivo studies on interactions between secondary amines and nitrites or nitrates. – IARC-SP – 3, p. 109–115.
Erickson, F. J.; Fabian, F. W.; 1942. Preserving and germicidal action of various sugars and organic acids on yeasts and bacteria. – Food Research 7:68–79.
Fan, T. Y.; Tannenbaum, S. R.; 1971. Automatic colorimetric determination of N-nitroso compounds. – Journal of Agricultural & Food Chemistry 19:1267.
Fan, T. Y.; Tannenbaum, S. R.; 1973. Natural inhibitors of nitrosation reactions: the concept of available nitrite. – Journal of Food Science 38:1067–1069.
FAO-NMRS – 53 (FAO Nutrition Meeting Report Series No 53); 1974. Toxicological evaluation of certain food additives with a review of general principles and of specifications. Food and Agricultural Organization, Rome; World Health Organization, Geneva.
Fazio, T.; Damico, J. N.; Howard, J. W.; White, R. H.; Watts, J. O.; 1971a. Gas chromatographic determination and mass spectrometric confirmation of N-nitrosodimethylamine in smoke-processed marine fish. – Journal of Agricultural & Food Chemistry 19:250.
Fazio, T.; White, R. H.; Howard, J. W.; 1971b. Analysis of nitrite and/or nitrate-processed meats for N-nitrosodimethylamine. – Journal of the Association of Official Analytical Chemists 54(5)1157–1159.
Fazio, T.; Howard, J. W.; White, R.; 1971c. Multidetection method for analysis of nitrosamines in foods. – IARC p. 16.
Fazio, Th.; White, R. H.; Donsold, L. R.; Howard, J. W.; 1973. Nitrosopyrrolidine in cooked bacon. – Journal of the Association of Official Analytical Chemists. 56: 919–921.
Fiddler, W.; 1975. The occurrence and determination of N-nitroso compounds. – Toxicology & Applied Pharmacology 31:352–360.
Fiddler, W.; Piotrowski, E. G.; Pensabene, J. W.; Wasserman, A. E.; 1972. Some current observations on the occurrence and formation of N-nitrosamines. – EMMRW – 18, p. 416.
Fiddler, W.; Pensabene, J. W.; Kushnir, I.; Piotrowski, E. G.; 1973a. Effect of Frankfurter cure ingredients on N-nitrosodimethylamine formation in a model system. – Journal of Food Science 38:714–715.

Fiddler, W.; Pensabene, J. W.; Piotrowski, E. G.; Doerr, R. C.; Wasserman, A. E.; 1973b. Use of sodium ascorbate or erythorbate to inhibit formation of N-nitrosodimethylamine in frankfurters. – Journal of Food Science 38:1084 only.
Fiddler, W.; Piotrowski, E. G.; Pensabene, J. W.; Wasserman, A. E.; 1973c. Studies on nitrosamine formation in foods. – US-IFT 1973.
Fiddler, W.; Pensabene, J. W.; Fagan, J. C.; Thorne, E. J.; Piotrowski, E. G.; Wasserman, A. E.; 1974. The role of lean and adipose tissue on the formation of nitrosopyrrolidine in fried bacon. – Journal of Food Science 39:1070–1071.
Fiddler, W.; Pensabene, J. W.; Mergens, W. J.; Wasserman, A. E.; [1976?] Inhibition of nitrosopyrrolidine formation in bacon and in model systems using α-tocopherol. In preparation.
Fine, D. H.; Rounbehler, D. P.; 1975. Trace analysis of volatile N-nitroso compounds by combined gas chromatography and thermal energy analysis. – Journal of Chromatography 109:271–279.
Fine, D. H.; Rounbehler, D. P.; 1976a. N-Nitroso compounds in water. – Keith, p. 255–263.
Fine, D. H.; Rounbehler, D. P.; 1976b. Analysis of volatile N-nitroso compounds by combined gas chromatography and thermal energy analysis. – IARC-SP – 14.
Fine, D. H.; Rufeh, F.; Lieb, D.; Rounbehler, D. P.; 1975a. Description of the thermal energy analyzer (TEA) for trace analysis of volatile and non-volatile N-nitroso compounds. – Analytical Chemistry 47, 1188–1191.
Fine, D. H.; Lieb, D.; Rufeh, F.; 1975b. Principle of operation of the thermal energy analyzer for the trace analysis of volatile and non-volatile N-nitroso compounds. – Journal of Chromatography 107:351–357.
Fine, D. H.; Ross, R.; Rounbehler, D. P.; Silvergleid, A.; Song, L.; 1976a. Analysis of nonvolatile N-nitroso compounds in foodstuffs. – Journal of Agricultural & Food Chemistry ... (in press).
Fine, D. H.; Rounbehler, D. P.; Belcher, N. M.; 1976b. N-Nitroso compounds in air and water. – IARC-SP – 14.
Fine, D. H.; Rounbehler, D. P.; Belcher, N. M.; Epstein, S. S.; 1976c. N-Nitroso compounds in the environment. – US-IEEE-75-CH – 1004-1, Vol. 2, p. 307–312.
Fine, D. H.; Rounbehler, D. P.; Pellizzari, E. D.; Bunch, J. E.; Werkley, R. W.; McCrae J.; Bursey, J. T.; Sawicki, E.; Krost, K.; Demarrais, G. A.; 1976d. N-Nitrosodimethylamine in air. Bulletin of Environmental Contamination & Toxicology 15:739–746.
Fine, D. H.; Rounbehler, D. P.; Ross, R.; Song, L.; Silvergleid, A.; 1976e. Unpublished results.
Fine, D. H.; Rounbehler, D. P.; Silvergleid, A. S.; Ross, R.; 1977. Trace analysis of polar and non-polar N-nitroso compounds by combined high performance liquid chromatography and thermal energy analysis. – This symposium.
Follett, M. J.; Radcliff, P. W.; 1963. Determination of nitrite and nitrate in meat products. – Journal of the Science of Food & Agriculture 14:138–144.
Fong, Y. Y.; Chan, W. C.; 1973a. Dimethylnitrosamine in Chinese marine salt fish. – Food & Cosmetics Toxicology 11:841–845.
Fong, Y. Y.; Chan, W. C.; 1973b. Bacterial production of dimethylnitrosamine in salted fish. – Nature (GB) 243:241 only.
Food Chemistry News; 1975. Expert Panel [on Nitrite and Nitrosamines] gives O.K. to USDA curing proposal. – Food Chemistry News 17:39–47.
Foreman, J. K.; Goodhead, K.; 1975. The formation and analysis of N-nitrosamines. – Journal of the Science of Food & Agriculture 26:1771–1783.
Fox, J. B.; Ackerman, S. A.; 1968. Formation of nitric oxide myoglobin: mechanism of the reaction with various reductants. – Journal of Food Science 33:364–370.
Fox, J. B.; Nicholas, R. A.; 1974. Nitrite in meat: effect of various compounds on loss of nitrite. – Journal of Agricultural & Food Chemistry 22(2)302–306.
FR-AFNOR-NF – V – 04409; 1974–12. Détermination de la teneur en nitrate. Association Française de Normalisation, Paris – la Défense.
FR-AFNOR-NF – V – 04410; 1974–12. Détermination de la teneur en nitrate.
FR-SEC (Société des Experts Chimistes); 1976. Séance du 9 juin 1976.
Freudenreich, P.; 1976. Personal communication.
Frittoli, M.; Cantoni, C.; Merlino, M.; 1972. Quantitative variations in nitrate and nitrite contents during the salting of meat. – Scienza e Technologia degli Alimenti 2(4)227–231.

Frouin, A.; Thenot, M.; Bazile, J.; 1974. Pigment des viandes salées et nitrosamines: étude sur leur formation et leur composition. – Industries Alimentaires & Agricoles 91(II)1425.
Frouin, A.; Jondeau, D.; Thénot, M.; 1975. Etude sur l'état et la disponibilité du nitrite dans les produits de viande pour la formation de nitrosamines. – EMMRW – 21, 200.
Frouin, A.; Thénot, M.; Jondeau, D.; Barraud, C.; Grimault, M. L.; Durand, P.; Vendeuvre, J. L.; 1976a. Études sur l'état du nitrite dans les produits de viandes. Communication à la Journées sur les nitrates et nitrites, Dyon, 1976–05 – Annales de la Nutrition et de l'Alimentation ... (in press).
Frouin, A.; Thénot, M.; Jondeau, D.; Patt, K.; Barraud, C.; Grimault, M. L.; Durand, P.; Vendeuvre, J. L.; 1976b. Nitrates et nitrites: révision nécessaire de nos conceptions et méthodes d'analyses. – FR-SEC.
Fudge, R.; Truman, R. W.; 1973. The nitrate and nitrite contents of meat products – a survey by Public Health Analysts' laboratories in south Wales and the south west of England. Journal of the Association of Public Analysts 11:19–27.
Fujimaki, M.; Emi, M.; Okitani, A.; 1975. Fate of nitrite in meat-curing model systems composed of myoglobin, nitrite and ascorbate, Agricultural & Biological Chemistry (JP) 39:371–377.
Fürtig, W.; Pohloudek-Fabini, R.; 1965. Über das Problem der gebundenen Ascorbinsäure – Pharmazie 20:185–193.
GB-BFMIRA (British Food Manufacturing Industries Research Association, Leatherhead, Surrey).
GB-SAB-TS – 3 (Society of Applied Bacteriology. Technical Series No 3); 1969. Isolation methods for microbiologists. Edited by D. A. Shapton & G. W. Gould. Academic Press, London.
GB-SAB-TS – 8; 1975. Some methods for microbiological assay. Edited by R. G. Board & D. W. Lovelock. Academic Press, London.
GB-SAC; 1973. Official standardised and recommended methods of analysis. Edited by N. W. Hanson. Society of Analytical Chemists, London.
GB-US (University of Surrey, Guildford). See Woolford 1974.
Genigeorgis, C.; Martin, S.; Franti, C. E.; Rieman, H.; 1971. Initiation of staphylococcal growth in laboratory media. – Applied Microbiology 21:934–939.
Gilbert, J.; Knowles, M. E.; McWeeny, D. J.; 1975. Formation of C- and S-nitroso compounds and their further reactions. – Journal of the Science of Food & Agriculture 26:1785–1791.
Gilliland, S. E.; Speck, M. L.; 1972. Interactions of food starter cultures and food-borne pathogens: lactic streptococci versus staphylococci and salmonellae. – Journal of Milk & Food Technology 35:307–310.
Goodfellow, S. J.; Brown, W. L.; 1975. Incidence of S. aureus in commercial Wisconsin summer sausage. – Food Products Development 9:80–82.
Goodhead, K.; Gough, T. A.; 1975. The reliability of a procedure for the determination of nitrosamines in food. – Food & Cosmetics Toxicology 13:307–312.
Gough, T. A.; Goodhead, K.; 1975. Occurrence of volatile nitrosamines in spice pre-mixes. – Journal of the Science of Food & Agriculture 26:1473–1478.
Gough, T. A.; Webb, K. S.; 1972. The use of a molecular separator in the determination of trace constituents by combined gas chromatography and mass spectrometry. – Journal of Chromatography 64:201–210.
Gough, T. A.; Webb, K. S.; 1973. A method for the detection of traces of nitrosamines using combined gas chromatography and mass spectrometry. – Journal of Chromatography 79:57–63.
Gough, T. A.; Goodhead, K.; Walters, C. L.; 1976. Distribution of some volatile nitrosamines in cooked bacon. – Journal of the Science of Food & Agriculture 27:181–185.
Gould, G. W.; Hurst, A.; 1969. The bacterial spore. Academic Press, London.
Goutefongea, R.; 1973. Contribution à l'étude de la fixation du nitrite aux myofibrilles du muscle du porc. – EMMRW – 20, Abstracts & Communications, p. 88.
Goutefongea, R.; Cassens, R. G.; Woolford, G.; 1976. The effect of fat on loss of nitrite during curing. In preparation.
Graves, R. R.; Frazier, W. C.; 1963. Food microorganisms influencing the growth of S. aureus. Applied Microbiology 11:513–516.

Gray, J. I.; Dugan, L. R. Jr.; 1974. Formation of N-nitrosamines in low moisture systems. – Journal of Food Science 38:474.
Gray, J. I.; Dugan, L. R. Jr.; 1975a. Formation of N-nitrosopyrrolidine from proline and collagen. – Journal of Food Science 40:484.
Gray, J. L.; Dugan, L. R. Jr.; 1975b. Inhibition of nitrosamine formation in model food systems. – Journal of Food Science 40:981–984.
Greenberg, R. A.; 1974. Ascorbate and nitrosamine formation in cured meats. – Krol & Tinbergen, p. 179–188.
Greenberg, R. A.; 1975. Update on nitrite, nitrate, and nitrosamines. – US-MIRC, p. 71–76.
Greenblatt, M.; Lijinsky, W.; 1972a. Failure to induce tumors in Swiss mice after concurrent administration of amino acids and sodium nitrite. – Journal of the National Cancer Institute 48:1389–1392.
Greenblatt, M.; Lijinsky, W.; 1972b. Nitrosamine studies: neoplasms of liver and genital mesothelium in nitrosopyrrolidine treated MRC rats. – Journal of the National Cancer Institute 48:1687–1696.
Greenblatt, M.; Mirvish, S. S.; So, B. T.; 1971. Nitrosamine studies: induction of lung adenomas by concurrent administration of sodium nitrite and secondary amines in Swiss mice – Journal of the National Cancer Institute 46:1029–1034.
Grever, A. B. G.; 1973. Vorming van de Perigo factor in voedingsbodems. – Voedingsmiddelentechnologie 4:105.
Grever, A. B. G.; 1974. Minimum nitrite concentrations for inhibition of Clostridia in cooked meat products. Krol & Tinbergen, p. 103–109. Discussion, p. 109.
Grever, A. B. G.; 1975. Onderzoek naar de vorming van remstoffen voor Clostridium sporogenes in natriumnitriet bevattende voedingsbodems en in vleeswaren. Centraal Instituut voor Voedingsonderzoek, Zeist. NL-CIVO-R – 4692.
Groenen, P. J.; ten Noever de Brauw, M. C.; 1975. Determination of volatile N-nitrosamines in the vapour phase of the smoke from various tobacco products. – Beiträge zur Tabakforschung 8:113–123.
Groenen, P. J.; Jonk, R. J. G.; van Ingen, C.; ten Noever de Brauw, M. C.; 1976. Determination of eight volatile N-nitrosamines in thirty cured meat products with capillary GC/high-resolution MS: the presence of diethylnitrosamine and the absence of nitrosopyrrolidine. – IARC-SP – 14.
Groenen, P. J.; de Cock-Bethbeder, M. W.; Jonk, R. J. G.; van Ingen, C. Further GC/MS studies on the occurrence of volatile nitrosamines in meat products. – This symposium.
Grundmann, E. (Editor); 1975. Handbuch der algemeinen Pathologie. Band 6, Teil 6.2. Springer-Verlag, Heidelberg.
Haines, W. C.; Harmon, L. G.; 1973. Effect of selected lactic acid bacteria on growth of S. aureus and production of enterotoxin. – Applied Microbiology 25:436–441.
Halliday, J. H.; Wood, F. W.; 1966. The determination of salt in bacon by using a sodium-ion responsive glass electrode. – Analyst (GB) 91:802–805.
Hamm, R.; Dalrymple, R.; Honikel, K.; 1973. Post-mortem breakdown of glycogen and ATP in skeletal muscle. – EMMRW – 19, Vol. 1, p. 73–86.
Hansen, J. N.; Levin, R. A.; Effect of some inhibitors derived from nitrite on macromolecular synthesis in Bacillus cereus. – Applied Microbiology 30:862–869.
Hansen, T.; Iwaoka, W. T.; Archer, M. C.; 1974. A high-yield synthesis of ^{14}C-labelled nitrosoproline and nitrososarcosine. – Journal of Labelled Compounds 10:689–692.
Hanson, N. W.; 1973. See GB-SAC 1973.
Havery, D. C.; Kline, D. A.; Miletta, E. M.; Joe, F. L. Jr.; Fazio, T.; 1976. Survey of food products for volatile N-nitrosamines. – Journal of the Association of Official Analytical Chemists 59:540–546.
Hawksworth, Gabrielle M.; Hill, M. J.; 1971. The formation of nitrosamines by human intestinal bacteria. – Biochemical Journal 122:28–29.
Herring, H. K.; 1973. Effect of nitrite and other factors on the physico-chemical characteristics and nitrosamine formation in bacon. – US-AMIF 1973, p. 47–60.
Hildrum, K. I.; Scanlan, R. A.; Libbey, L. M.; 1975. Identification of γ-butenyl-(β-propenyl)-nitrosamine, the principal volatile nitrosamine formed in the nitrosation of spermidine or spermine. – Journal of Agricultural & Food Chemistry 23:34–37.

Hill, L. H.; Webb, N. B.; Moncol, N. Dolores; Adams, A. T.; 1973. Changes in residual nitrite in sausage and luncheon meat products during storage. – Journal of Milk & Food Technology 36:515–519.

Hoch-Ligeti, C.; Argus, F. M.; Arcos, J. C.; 1968. Combined carcinogenic effects of dimethylnitrosamine and 3-methylcholanthrene in the rat. – Journal of the National Cancer Institute 40:535–549.

Hoel, D. G.; Gaylor, D. W.; Kirschstein, R. L.; Saffioti, U.; Schneiderman, M. A.; 1975. Estimation of risks of irreversible, delayed toxicity. – Journal of Toxicology & Environmental Health (Washington, US) 1:133–156.

Hoffmann, D.; Wynder, E. L.; 1976. Smoking and occupational cancer. – Preventive Medicine 5:245–261.

Hofmann, K.; 1976. Eine einfache Methode zur Überprüfung des Nitritgehaltes von Nitritpökelsalz. – Fleischwirtschaft 56:486.

Holbrook, R.; Baird-Parker, A. C.; 1975. Serological methods for the assay of staphylococcal enterotoxins. – GB-SAB-TS – 8, p. 107–128.

Høyem, T.; 1974. Nitrate and nitrite content in Norwegian food. – ICFST

Huhtanen, C. N.; 1975. Some observations on a Perigo-type inhibition of Clostridium botulinum in a simplified medium. – Journal of Milk & Food Technology 38:762–763.

Huhtanen, C. N.; Wasserman, A. E.; 1975. Effect of added iron on the formation of clostridial inhibitors. – Applied Microbiology 30:768–770.

Hustad, G. O.; Cerveny, J. G.; Trenk, H.; Deibel, R. H.; Kautter, D. A.; Fazio, T.; Johnston, R. W.; Kolari, O. E.; 1973. Effect of sodium nitrite and sodium nitrate on botulinal toxin production and nitrosamine formation in Wieners. – Applied Microbiology 26:22.

Iandolo, J. J.; Clark, C. W.; Bluhm, L.; Ordal, Z. J.; 1965. Repression of S. aureus in associative culture. – Applied Microbiology 13:646–649.

IARC; 1972a. Monographs on the evaluation of carcinogenic risk of chemicals to man. Vol. 1. Proceedings of conference, Heidelberg, 1971. International Agency for Research on Cancer, Lyon.

IARC; 1972b. Second meeting on the analysis and formation of N-nitroso compounds. Edited by E. A. Walker & W. Davis.

IARC-SP – 3; 1972c. Third meeting on the analysis and formation of N-nitroso compounds. Edited by P. Bogovski, R. Preussmann & E. A. Walker. – IARC. Scientific Publications No 3.

IARC; 1973. Monographs on the evaluation of carcinogenic risk of chemicals to man. Vol. 4.

IARC-SP – 14; 1976. Environmental N-nitroso compounds: analysis and formation. Proceedings of the 4th International Working Meeting, Tallinn, Estonia, 30 September – 3 October 1975. Edited by E. A. Walker, P. Bogovski & L. Griciute. – IARC. Scientific Publication No 14. International Working Meeting, Tallinn, Estonia, 30 September – 3 October 1975. Edited by E. A. Walker, P. Bogovski & L. Griciute. – IARC. Scientific Publication No 14.

ICFST – 4 (4th International Congress of Food Science & Technology. = 4e Conggrrès International de Science et Technique des Aliments, Madrid); 1974-09-23 – 27.

Incze, K.; Farkas, J.; Mihályi, V.; Zukal, E.; 1974. Antibacterial effect of a cysteine-nitrosothiol and possible precursors thereof. – Applied Microbiology 27:202–205.

Ingram, M.; 1974. The microbiological effects of nitrite. – Krol & Tinbergen, p. 63–74. Discussion, p. 74–75.

ISAMS; 1976. 15th International symposium on the application of MS and NMR in the food industries, Bologna, Italy, 1975–11.

ISNMP (International Symposium on Nitrite in Meat Products, Zeist, 1973). See Krol & Tinbergen 1974.

ISO – 3091; 1975. Meat and meat products. Determination of nitrate content. International Organization for Standardization, Geneva. 5 pp.

ISO-DIS – 5551; 1976. Meat and meat products. Detection and enumeration of S. aureus.

Issenberg, P.; Essigman, J. M.; 1972. Gas chromatographic determination of volatile nitrosamines in foods. – Journal of Food Science 37:684–689.

Ivey, F.; 1974. The determination of N-nitrosoproline in cured meats. Thesis, Oregon State University, Corvallis. VI + 59 pp.

Iwaoka, W.; Tannenbaum, S. R.; 1976a. Liquid chromatography of N-nitrosoamine acids and

their syn and anti conformers. – Journal of Chromatography ... (in press).
Iwaoka, W.; Tannenbaum, S. R.; 1976b. Photohydrolytic detection of N-nitroso compounds in HPLC. – IARC-SP – 14.
Jarvis, B. (GB-BFMIRA). Pers. commun. to Roberts & Ingram.
Jedlicka, G. J.; Wilcox, J. C.; McCall, W. A.; Gagula, M. C.; 1975. Effect of various levels of nitrate and nitrite on S. aureus growth in peperoni. – Abstracts of the Annual Meeting of the American Society for Microbiology 1975, 75th Annual Meeting, Session P3, p. 200.
JP-NIG-AR – 24 (National Institute of Genetics, Misima. Annual report No 24); 1973.
Juszkiewicz, T.; Kowalski, B.; 1974. Passage of nitrosamines from rumen into milch in goats. – IARC-SP – 9, p. 173.
Kada, T.; 1973. *DNA-damaging products from reaction between sodium nitrite and sorbic acid.* – JP-NIG-AR – 24, p. 43–44.
Kakritz, L.; Spinelli, A. M.; Wasserman, A. E.; 1976. Effect of storage on the concentration of proline and other free amino acids in pork bellies. – Journal of Food Science 41:879–881.
Kamm, L.; McKeown, G. G.; Morison Smith, D.; 1965. New colorimetric method for the determination of the nitrate and nitrite content of baby foods. – Journal of the Association of Official Agricultural Chemists 48:892.
Kao, C. T.; Frazier, W. C.; 1966. Effect of lactic acid bacteria on growth of S. aureus. – Applied Microbiology 14:251–255.
Karrer, P.; 1950. Organic chemistry, 4th English edition. Elsevier, Amsterdam.
Kawabata, T.; 1974. *Inhibition of the formation of N-nitroso compounds by vitamin C.* – Vitamins (JP) 18:513.
Kawabata, T.; Mijakoshi, S.; 1976. Microbial formation of N-nitrosopyrrolidine from N-nitrosoproline. – IARC-SP – 14.
Kawabata, T.; Ishibashi, T.; Matsui, M.; 1974a. Quantitative determination of N-nitroso compounds by gas chromatography. – ICFST – 4.
Kawabata, T.; Shazuki, H.; Ishibashi, T.; 1974b. Effect of ascorbic acid on the formation of N-nitrosodimethylamine in vitro. – Bulletin of the Japanese Society of Scientific Fisheries (Tokyo University of Fisheries, Minato-ku, Tokyo) 40:1251.
Keith, L. H. (Editor); 1976. Identification and analysis of organic pollutants in water. Ann Arbor Science, Michigan.
Kenyon, J.; Munro, N.; 1948. The isolation and some properties of dehydro-l-ascorbic acid. – Journal of the Chemical Society 1948:158–161.
Kimoto, W. I.; Wasserman, A. E.; Talley, F. B.; 1976. Effect of sodium nitrite and sodium chloride on the flavor of processed pork bellies. – Lebensmittel-Wissenschaft und Technologie 9:99.
Kiss, G.; Neukom, H.; 1966. Über die Struktur des Ascorbigens. – Helvetica Chimica Acta 49:989–992.
Knowles, M. E.; McWenny, D. J.; Couchman, L.; Thorogood, M.; 1974. Interaction of nitrite with proteins of gastric pH. – Nature (GB) 247:288–289.
Kolari, O. E.; Aunan, W. J.; 1972. – EMMRW – 18, p. 422.
Kotter, L.; Möhler, K.; Prändl, O.; Preussmann, R.; Sander, J.; 1975. Ascorbinsäure antikarzinogenes Nahrungsadditiv für Fleischwaren. – Ärztliche Praxis 27:1815.
Kotter, L.; Fischer, A.; Schmidt, H.; Walters, C. L.; Hauser, E.; Heiz, H. J.; 1976. Zum Vorkommen von Nitrosaminen in Fleischerzeugnissen und Untersuchungen an schnittfesten Rohwürsten bei unterschiedlichen Zusätzen. – Fleischwirtschaft 56:997.
Krol, B.; Tinbergen, B. J.; 1974. Proceedings of the international symposium on nitrite in meat products held at the Central Institute for Nutrition and Food Research TNO, Zeist, the Netherlands, September 10-14, 1973. Pudoc, Wageningen. ISBN 90-220-0463-5. 268 pp.: 23 contrib. with discussions; conclusions and recommendations to 4 sessions; appendix summarizing national regulations.
Krüger, F. W.; Bertram, B.; 1975. Metabolism of nitrosamines in vivo. 4. Isolation of 3-hydroxy-1-nitrosopyrrolidine from rat urine after application of 1-nitrosopyrrolidine. – Zeitschrift für Krebsforschung 83:255–260.
Kubberød, G.; Cassens, R. G.; Greaser, M. L.; 1974. Reaction of nitrite with sulfhydryl groups of myosin. – Journal of Food Science 39:1228–1230.
Kushnir, L.; Feinberg, J. I.; Pensabene, J. W.; Piotrowski, E. G.; Fiddler, W.; Wasserman, A. E.;

1975.Isolation and identification of N-nitrosoproline in uncooked bacon. – Journal of Food Science 40:427–428.
Labots, H.; 1977. Effect of nitrite on the development of Staphylococcus aureus in fermented sausages. – This symposium.
Lechowich, R. V.; Evans, J. B.; Niven, C. F. Jr.; 1956. Effect of curing ingredients and procedures on the survival and growth of staphylococci in and on cured meats. – Applied Microbiology 4:360–363.
Lee, S. H.; Cassens, R. G.; 1976. Nitrite binding sites on myoglobin. – Journal of Food Science 41:969.
Lee, S. H.; Cassens, R. G.; Fennema, O. R.; 1976. Effect of muscle type on residual nitrite in cured meat. – Journal of Food Science 41:100–101.
Leistner, E.; Hechelmann, H.; Bem, Z.; 1974. Mikrobiologisch vertretbare Reduktion des Nitrit-Zusatzes zu Fleischerzeugnissen. – DE-DFG, p. 186.
Lijinsky, W.; 1974. Reaction of drugs with nitrous acid as a source of carcinogenic nitrosamines. – Cancer Research 34:255–258.
Lijinsky, W.; Epstein, S. S.; 1970. Nitrosamines as environmental carcinogens. – Nature (GB) 225:21–25.
Lijinsky, W.; Greenblatt, M.; 1972. Carcinogen dimethylnitrosamine produced in vivo from sodium nitrite and aminopyrine. Nature. New Biology 236:177–178.
Lijinsky, W.; Taylor, H. W.; 1976. Carcinogenesis in Sprague-Dawley rats of N-nitroso-N-alkylcarbamate esters. Cancer Letters 1:275–279.
Lijinsky, W.; Keefer, L.; Loo, J.; 1970. The preparation and properties of some nitrosamino acids. – Tetrahedron 26:5137–5153.
Lijinsky, W.; Keefer, L.; Conrad, E.; van de Bogart, R.; 1972. The nitrosation of tertiary amines and some biologic implications. Journal of the National Cancer Institute 49:1239–1249.
Likens, S. T.; Nickerson, G. B.; 1964. Detection of certain hop oil constituents in brewing products. – US-ASBC-P, p. 5–13.
Magee, P. N.; Barnes, J. M.; 1956. The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. – British Journal of Cancer 10:114 only.
Magee, P. N.; Barnes, J. M.; 1962. Induction of kidney tumours in the rat by dimethylnitrosamine. – Journal of Pathology & Bacteriology (GB) 84:19–31.
Magee, P. M.; Barnes, J. M.; 1967. Carcinogenic nitroso-compounds. Advances in Cancer Research (US) 10:163–246.
Manchot, W.; Linckh, E.; 1926. Über die Konsitution und die Absorptionsspektren der Schwefeleisen-Stickoxyd-Verbindungen (Roussin'schen Salze) und ihre Beziehungen zu den dissoziierenden Ferro-Stickoxyd-Salzen. – Berichte der Bunsengesellschaft für Physikalische Chemie 59:412–418.
Mantel, W.; Bryan, U. R.; 1961. Safety testing of carcinogenic agents. – Journal of the National Cancer Institute 27:455–470.
Mantel, N.; Schneiderman, M.; 1975. Estimating 'safe' levels, a harzardous undertaking. – Cancer Research 35:1379–1386.
Mantel, N.; Bohidar, N.; Brown, C.; Cimenera, J.; Tukey, J.; 1975. An improved 'Mantel-Bryan' procedure for 'safety' testing of carcinogens. – Cancer Research (US) 35:865–872.
Marshall, J. I. Jr; 1974. Carbonyl-amine reaction products as possible nitrosamines precursors. – Dissertation Abstracts International. Section B. Science & Engineering 35:1289.
Maruyama, S.; Muramatsu, K.; Shimizu, S.; Maki, S.; 1976. Reduction of nitrate with Bacillus coagulans in human saliva. – Journal of the Food Hygienic Society of Japan 17:19–26.
Maschka, A.; Mitna, A.; 1951. Kinetische Untersuchungen über den Zerfall der Äthylnitrolsäure in saurer Lösung. – Monatshefte für Chemie 82:84–94.
Meyer, V.; 1875. Über die Nitroverbindungen der Fettreihe. – Justus Liebigs Annalen der Chemie 175:88–140.
Meyer-Döring, H.; 1957-10-31. Verfahren zur Herstellung von wasserlöslichen therapeutischen Mitteln mit bakteriostatischer bis bakterieider Wirkung. DBP 1 008 741, Kl. 12 q.
Meyer-Döring, H.; Perkow, W.; 1958-01-02. Verfahren zur Herstellung von wasserlöslichen therapeutischen Mitteln mit baktericider bis bakteriostatischer Wirkung. DBP 1 012 607, Kl. 12 q.

Mirna, A.; 1970. Über die Umsetzung von Nitrit in Fleischwaren und dessen Verteilung in verschiedenen Fraktionen. – EMMRW – 16, Contrib. C16, p. 681–691.
Mirna, A.; 1974. Determination of free and bound nitrite. – Krol & Tinbergen, p. 21–27. Discussion, p. 27–28.
Mirna, A.; Coretti, K.; 1974. Über den Verbleib von Nitrit in Fleischwaren. Untersuchungen über chemische und bakteriostatische Eigenschaften verschiedener Reaktionsprodukte des Nitrits. – Fleischwirtschaft 54:507–510.
Mirna, A.; Hofmann, K.; 1969. Über den Verbleib von Nitrit in Fleischwaren. 1. Umsetzung von Nitrit mit Sulfhydryl-Verbindungen. – Fleischwirtschaft 10:1361–1366.
Mirvish, S. S.; 1970. Kinetics of dimethylamine nitrosation in relation to nitrosamine carcinogenesis. – Journal of the National Cancer Institute 44:633–639.
Mirvish, S. S.; Walleave, L.; Eagen, M.; Shubik, P.; 1972. Ascorbate-nitrite reaction: possible means of blocking the formation of carcinogenic N-nitroso compounds. – Science (US) 177:65–67.
Moeller, Th.; 1957. Inorganic synthesis. Vol. V., McGraw-Hill Book Company Inc., New York, p. 117–122.
Möhler, K.; Mayrhofer, O. L.; Hallermayer, E.; 1974. Possible mechanisms of nitrosamine formation in food and animal feeding stuffs. – IARC-SP – 9, p. 142.
Montesano, R.; Magee, P. N.; 1970. Metabolism of dimethylnitrosamine by human liver slices in vitro. – Nature (GB) 228:173.
Moran, D. M.; Tannenbaum, S. R.; Archer, M. C.; 1975. Inhibitor of Clostridium perfringens formed by heating sodium nitrite in a chemically defined medium. – Applied Microbiology 30:834–843.
Morton, I.; Rhodes, D. N. (Editors); 1974. The contribution of chemistry to food supplies. Butterworths, London.
Mossel, D. A. A.; Krugers Dagneaux, E. L.; 1959. Bacteriological requirements for and analysis of pre-cooked ('instant') cereals and similar foods. – Antonie van Leeuwenhoek: Journal of Microbiology & Serology 25:230–236.
Mottram, D. S.; Rhodes, D. N.; 1974. Nitrite and the flavor of cured meat. – Krol & Tinbergen, p. 161–170. Discussion, p. 170–171.
Mottram, D. S.; Edwards, R. A.; Patterson, R. L. S.; 1976. The distribution of N-nitrosopyrrolidine between lean, fat and vapour in frying bacon. – EMMRW – 22.
Mottram, D. S.; Patterson, R. L. S.; Rhodes, D. N.; Gough, T. A.; 1975. Influence of ascorbic acid and pH on the formation of N-nitrosodimethylamine in cured pork containing added dimethylamine. – Journal of the Science of Food & Agriculture 26:47–53.
Nagata, Y.; Mirna, A.; 1974. Einfluss der Verarbeitung auf die Bildung von Nitrosaminen in Fleischwaren. – Fleischwirtschaft 54(11), 1782–1786.
Nakahara, W.; 1975. Pharmacodynamic mechanisms in carcinogenesis. – Grundmann, Bd 6, Teil 6.2, p. 595–650.
Nakamura, M.; Baba, N.; Nakaoka, T.; Wada, Y.; Ishibashi, T.; Kawabata, T.; 1976. Pathways of formation of N-nitrosopyrrolidine in fried bacon. – Journal of Food Science 41:874–878.
Namiki, M.; Kada, T.; 1975. Formation of ethylnitrolic acid by the reaction of sorbic acid with sodium nitrite. – Agricultural & Biological Chemistry (JP) 39:1335–1336.
Neurath, G. B.; Dunger, M.; Pein, F. G.; 1976. The interaction of nitrogen oxides, oxygen and amines in gaseous mixtures. – IARC-SP – 14.
Newmark, H. L.; Osadca, M.; Araujo, M.; Gerenz, C. Z.; de Ritter, E.; 1974. Stability of ascorbate in bacon. – Food Technology 28(5) 30–31, 36.
Newth, F. H.; 1951. The formation of furan compounds from hexoses. – Advances in Carbohydrate Chemistry 6:83–106.
Nicholas, D. J. D.; Nason, A.; 1957. Determination of nitrate and nitrite. – Methods in Enzymology (Academic Press, New York, US) 3:981–984.
Niewiarowicz, A.; 1963. Occurrence and changes of some amines in stored fresh meat as well as in meat preserved by irradiation. – EMMRW – 9, Contrib. 34.
Niskanen, A.; Nurmi, E.; 1976. Effect of starter culture on staphylococcal enterotoxin and thermonuclease production in dry sausage. – Applied Microbiology 31:11–20.
NL-CIVO-R – 4030 (Centraal Instituut voor Voedingsonderzoek TNO, Zeist); 1973. See van Straten & de Vrijer.

NL-CIVO-R – 4692; 1975. See Grever.
NL-RIV-VG-VAR – 33/34 (Rijksinstituut voor Volksgezondheid, Bilthoven. – Volksgezondheid: Verslagen, Adviezen & Rapporten No 33/34); 1975.
Nordin, H. R.; 1969. The depletion of added sodium nitrite in ham. – Canadian Institute of Food Technology Journal 2:79–85.
Olsman, W. J.; 1974. About the mechanism of nitrite loss during storage of cooked meat products. – Krol & Tinbergen, p. 129–136. Discussion, p. 137.
Olsman, W. J.; 1975. (Stability of nitrite in meat products) N1 – Voedingsmiddelentechnologie 8:7–11. Cited: Food Science & Technol. Abstr. 1975 7, No 10S1382.
Olsman, W. J.; 1977a. Chemical behaviour of nitrite in meat products. 1. The stability of protein-bound nitrite during storage. – This symposium.
Olsman, W. J.; 1977b. Chemical behaviour of nitrite in meat products. 2. Effect of iron and ethylenediaminetetraacetate on the stability of protein-bound nitrite. – This symposium.
Olsman, W. J.; 1977c. The assay of protein-bound nitrite in meat products. – Zeitschrift für Lebensmittel-Untersuchung und -Forschung (to be published).
Olsman, W. J.; Krol, B.; 1972. Depletion of nitrite in heated products during storage. – EMMRW – 18, Vol. 2, p. 409.
Palumbo, S. A.; Smith, J. L.; Gentilcore, K. M.; Fiddler, W.; 1974. Investigation on the possible occurrence of nitrosamine in Lebanon Bologna. – Journal of Food Science 39:1257–1258.
Panalaks, T.; Iyengar, J. R.; Sen, N. P.; 1973. Nitrate, nitrite and dimethylnitrosamine in cured meat products. – Journal of the Association of Official Analytical Chemists 56:621–625.
Panalaks, T.; Iyengar, J. R.; Sen, N. P.; 1974. Further survey of cured meat products for volatile N-nitrosamines. – Journal of the Association of Official Analytical Chemists 57:806–812.
Pascal, P.; 1956. Nouveau traité de chimie générale. Azote-Phosphore.
Pate, T. D.; Shuler, R. O.; Mandigo, R. W.; 1971. The influence of glucono-delta-lactone on cured ham color and color stability. – Journal of Food Science 36:48.
Patterson, R. L. S.; Edwards, R. A.; 1975. Volatile amine production in uncured pork during storage. – Journal of the Science of Food & Agriculture 26:1371–1373.
Patterson, R. L. S.; Mottram, D. S.; 1974. The occurrence of volatile amines in uncured and cured pork meat and their possible role in nitrosamine formation in bacon. – Journal of the Science of Food & Agriculture 25:1419–1425.
Patterson, R. L. S.; Taylor, A. A.; Mottram, D. S.; Gough, T. A.; 1976. Localised occurrence of N-nitrosopyrrolidine in fried bacon. – Journal fo the Science of Food & Agriculture 27:257–260.
Pavel, O.; 1882. Über Nitrososulfide und Nitrosocyanide. – Berichte der Bunsengesellschaft für Physikalische Chemie 15:2600–2615.
Peat, S.; 1946. The chemistry of anhydro sugars. – Advances in Carbohydrate Chemistry 2:37–77.
Pensabene, J. W.; Fiddler, W.; Gates, R. A.; Fagan, J. C.; Wasserman, A. E.; 1974. Effect of frying and other cooking conditions on nitrosopyrrolidine formation in bacon. – Journal of Food Science 39:314–316.
Perigo, J. A.; Whitings, E.; Bashford, T. E.; 1967. Observation in the inhibition of vegetative cell of Clostridium sporogenes by nitrite which has been autoclaved in a laboratory medium, discussed in the context of sublethally processed cured meats. – Journal of Food Technology 2:377–397.
Peryam, D. R.; Pilgrim, F. J.; 1957. Hedonic scale method for measuring preferences. – Food Technology 11 (9, Supplement) 9–14.
Peterson, A. C.; Black, J. J.; Gunderson, M. F.; 1964. Staphylococci in competition. 3. Influence of pH and salt on staphylococcal growth in mixed populations. Applied Microbiology 12:70–76.
Peto, R.; 1974. Guidelines on the analysis of tumour rates and death rates in experimental animals. British Journal of Cancer 29:101–105.
Phillips, W. E. J.; 1968. Nitrate content of foods – public health implications. Canadian Institute of Food Technology Journal 1:98.
Preussmann, R.; 1975. Chemische Carcinogene in der menschlichen Umwelt. – Grundmann, Bd 6, Teil 6.2, p. 421–594.

Preusmann, R.; 1976. Chemical carcinogens in the human environment: problems and quantitative aspects. – Oncology (CH) ... (in press).
Ratner, S.; Clarke, H. T.; 1937. The action of formaldehyde upon cysteine. – Journal of the American Chemical Society 59:200–206.
Riha, W. E.; Solberg, M.; 1971. Chemically defined medium for the growth of Clostridium perfringens. – Applied Microbiology 22:738–739.
Riha, W. E.; Solberg, M.; 1973. Instability of sodium nitrite in a chemically defined microbiological medium. – Journal of Food Science 38(1) 1–3.
Riha, W. E.; Solberg, M.; 1975a. Clostridium perfringens inhibition by sodium nitrite as a function of pH, inoculum size and heat. – Journal of Food Science 40:439–442.
Riha, W. E.; Solberg, M.; 1975b. Clostridium perfringens growth in a nitrite containing defined medium sterilized by heat or filtration. – Journal of Food Science 40:443–445.
Ritskes, T. M.; 1975. The gas chromatographic determination of trimethylamine and dimethylamine in fish, fishery products and other foodstuffs. – Journal of Food Technology 10:221–228.
Roberts, T. A.; 1974. Inhibition of bacterial growth in model systems in relation to stability and safety of cured meats. – Krol & Tinbergen, p. 91–101. Discussion, p. 101.
Roberts, T. A.; 1975a. The microbiological role of nitrite and nitrate. – Journal of the Science of Food & Agriculture 26:1755–1760.
Roberts, T. A. (Meat Research Institute, Langford, GB); 1975b. Personal information to Crowther, et al.
Roberts, T. A.; Ingram, M.; 1973. Inhibition of growth of Clostridium botulinum at different pH values by sodium chloride and sodium nitrite. – Journal of Food Technology 8:467–475.
Roberts, T. A.; Smart, J. L.; 1976a. The occurrence and growth of Clostridium spp in vacuum-packed bacon with particular reference to Cl. perfringens (welchii) and Cl. botulinum. – Journal of Food Technology 11(3) 229–244.
Roberts, T. A.; Smart, J. L.; 1976b. The occurrence of clostridia, particularly Cl. botulinum, in bacon and pork. – Wolf et al. p. ...?
Roberts, T. A.; Jarvis, B. J.; Rhodes, Annette, C.; 1976. Inhibition of Clostridium botulinum by curing salts in pasteurized pork slurry. – Journal of Food Technology 11:25–40.
Rodd, E. H. (Editor); 1957. Chemistry of carbon compounds. Elsevier. Amsterdam.
Rosenberg, J. O.; 1879. Über die Nitrososchwefeleisen Verbindungen und ihr Verhalten zu den Nitroprussiden. – Berichte der Bunsengesellschaft für Physikalische Chemie 12:1715–1718.
Sander, J.; 1967-05. Les nitrites dans l'alimentation humaine: peuvent-ils être cancérigènes du fait de la formation de nitrosamines? – Archiv für Hygiene und Bakteriologie 151:22–28.
Sander, J.; 1967. Eine Methode zum Nachweis von Nitrosaminen. – Hoppe-Seyler's Zeitschrift für Physiologische Chemie 438:852–854.
Sander, J.; 1971. Untersuchungen über die Entstehung cancerogener Nitrosoverbindungen im Magen von Versuchstieren und ihre Bedeutung für den Menschen. – Arzneimittel-Forschung 21:1572, 1707, 2034.
Sander, J.; Bürkle, G.; 1969. Induktion maligner Tumoren bei Ratten durch gleichzeitige Verfütterung von Nitrit und sekundären Aminen. – Zeitschrift für Krebsforschung 73:54–66.
Sander, J.; Schweinsberg, F.; Menz, H. P.; 1968. Untersuchungen über die Entstehung cancerogener Nitrosamine im Magen. – Hoppe-Seyler's Zeitschrift für Physiologische Chemie 349:1691–1697.
Sander, J.; Schweinberg, F.; Ladenstein, M.; Benzing, H.; Wahl, S. H.; 1973. Messung der renalen Nitrosaminausscheidung am Hund zum Nachweis einer Nitrosaminbildung in vivo. – Hoppe-Seyler's Zeitschrift für Physiologische Chemie 354:384–390.
Saville, B.; 1958. A scheme for the colorimetric determination of microgram amounts of thiols. – Analyst (GB) 83:670–672.
Scanlan, R. A.; 1975. N-Nitrosamines in foods. – Critical Reviews in Food Technology (Chemical Rubber Co., Cleveland, Ohio, US) 5(4)357–402.
Schmähl, D.; 1970. Experimentelle Untersuchungen zur Syncarcinogenes. 6. Addition minimaler Dosen von 4 verschiedenen hepatotropen Carcinogenen bei der Leberkrebserzeugung bei Ratten. – Zeitschrift für Krebsforschung 74:457–466.

Schmähl, D.; Thomas, C.; König, K.; 1963. Versuche zur Krebserzeugung an Ratten bei gleichzeitiger Applikation von Diäthylnitrosamin und 4-Dimethylaminoazobenzol. – Zeitschrift für Krebsforschung 65:342–350.
Schmid, H.; Maschka, A.; Eipeltauer, E.; 1951. Photometrische Bestimmung der Äthylnitrolsäure. – Monatshefte für Chemie 82:79–83.
Schubert, M. P.; 1936. Compounds of thiol acids with aldehydes. – Journal of Biological Chemistry 114:341–350.
Schubert, M. P.; 1939. The combination of cysteine with sugars. – Journal of Biological Chemistry 130:601–603.
Scott, W. J.; 1957. Water relations of food spoilage microorganisms. – Advances in Food Research (US) 7:83–127.
Sebranek, J. G.; 1974. Studies on the ultimate fate and distribution of nitrite in a cured meat product. Dissertation, University of Wisconsin, Madison, US. Abstr. in Dissertation Abstracts International 1975 7:3381.
Sebranek, J. G.; Cassens, R. G.; Hoekstra, W. G.; Winder, W. C.; Podebradsky, E. V.; Kielsmeier, E. W.; 1973. ^{15}N tracer studies of nitrite added to a comminuted meat product. – Journal of Food Science 38:1220–1222.
Sebranek, J. G.; Cassens, R. G.; Hoekstra, W. G.; 1974. Fate of added nitrite – Krol & Tinbergen, p. 139–147. Discussion, p. 147–148.
Sen, N. P.; 1970. Gas-liquid chromatographic determination of dimethylnitrosamine as dimethylnitramine at picogram levels. – Journal of Chromatography 51(2)301–304.
Sen, N. P.; 1972. The evidence for the presence of dimethylnitrosamine in meat products. – Food & Cosmetics Toxicology 10:219.
Sen, N. P.; 1974. Nitrosamines. Liener 1974.
Sen, N. P.; Donaldson, B.; 1974. The effect of ascorbic acid and glutathione on the formation of nitrosopiperazines from piperazine adipate and nitrite. IARC-SP – 9, p. 103.
Sen, N. P.; McKinley, W. P.; 1974. Method of elimination of nitrosamines from meat curing agents. – ICFST – 4,27, 9c.
Sen, N. P.; Schwinghamer, L. A.; Donaldson, B. A.; Miles, W. F.; 1972. N-Nitrosodimethylamine in fish meal. – Journal of Agricultural & Food Chemistry 20:1280.
Sen, N. P.; Donaldson, Barbara,; Lyengar, J. R.; Panalaks, T.; 1973a. Nitrosopyrrolidine and dimethylnitrosamine in bacon. – Nature (GB) 241:473.
Sen, N. P.; Miles, W. F.; Donaldson, B.; Panalaks, T.; Iyengar, J. R.; 1973b. Formation of nitrosamines in meat curing mixture. – Nature (GB) 245:104–105.
Sen, N. P.; Iyengar, J. R.; Donaldson, B. A.; Panalaks, T.; 1974a. Effect of sodium nitrite concentration on the formation of nitrosopyrrolidine and dimethylnitrosamine in fried bacon. – Journal of Agricultural & Food Chemistry 22:540–541.
Sen, N. P.; Donaldson, B.; Charbonneau, C.; Miles, W. F.; 1974b. Effect of additive on the formation of nitrosamines in meat curing mixture containing spices and nitrites. – Journal of Agricultural & Food Chemistry 22:1125–1130.
Sen, N. P.; Iyengar, J. R.; Donaldson, B.; Panalaks, T.; Miles, W. F.; 1974. The analysis and occurrence of volatile nitrosamines in cured meat products. – IARC-SP – 9, p. 49.
Sen, N. P.; Donaldson, Barbara,; Seaman, St.; Iyengar, J. R.; Miles, W. F.; 1975-08. Inhibition of nitrosamine formation in fried bacon by propyl gallate and L-ascorbyl palmitate. – US-ACS-NM – 170.
Sen, N. P.; Iyengar, J. R.; Miles, W. F.; Panalaks, T.; 1976a. N-nitroso-amines in cured meat products. – IARC-SP - 14.
Sen, N. P.; Miles, W. F.; Seaman, S.; Lawrence, J. F.; 1976b. Trace analysis of 3-hydroxy-1-nitrosopyrrolidine, a nonvolatile N-nitrosamine, by combined gas chromatographic mass spectrometric method. – Journal of Chromatography ... (in press).
Sen, N. P.; Donaldson, B.; Seaman, S.; Iyengar, J. R.; Miles, W. F.; 1976c. Inhibition of nitrosamine formation in fried bacon by propyl gallate and L-ascorbyl palmitate. – Journal of Agricultural & Food Chemistry 24:397–401.
Sen, N. P.; Seaman, S.; Miles, F. W.; 1976d. Dimethylnitrosamine and nitrosopyrrolidine in fumes produced during the frying of bacon. – Food & Cosmetics Toxicology 14:167–170.
Shafizadeh, F.; 1958. Formation and cleavage of the oxygen ring in sugars. – Advances in Carbohydrate Chemistry 13:9–61.

Shank, R. C.; Newberne, P. M.; 1976. Dose-response study of the carcinogenicity of dietary sodium nitrite and morpholine in rats and hamsters. – Food & Cosmetics Toxicology 14:1–8.
Shank, J. L.; Silliker, J. H.; Harper, R. H.; 1962. The effect of nitric oxide on bacteria. – Applied Microbiology 10:185–189.
Shapton & Gould 1969. See GB-SAB – 3.
Shults, G. W.; Cohen, J. S.; Wierbicki, E.; 1976. Effect of sodium nitrate and sodium nitrite additions and irradiation processing variables on the colour and acceptability of corned beef briskets. 36th Annual Meeting of the Institute of Food Technology, Anaheim, CA, June 6-9, 1976, Abstr.322. – Journal of Food Science 1976 ... (in press).
Sicilliano, J.; Krulick, S.; Heisler, E. G.; Schwartz, J. H.; White, J. W.; 1975. Nitrate and nitrite content in some fresh and processed market vegetables. – Journal of Agricultural & Food Chemistry 23:461.
Singer, G. M.; Lijinsky, W.; 1976. Naturally occurring nitrosatable amines. 2. Secondary amines in tobacco and cigarette smoke condensate. – Journal of Agricultural & Food Chemistry 24:553–555.
Singh, B.; Dean, G. R.; Cantor, S. M.; 1948. The role of 5-(hydroxy-methyl)-furfural in the discoloration of sugar solutions. – Journal of the American Chemical Society 70:517–522.
Sisler, H.; Audrieth, L. F.; 1938. Potassium nitrilosulfonate. – Journal of the American Chemical Society 60:1947–1948.
Stephany, R. W.; Schuller, P. L.; 1975. De aanwezigheid van nitriet in menselijk speeksel en het N-nitrosamine probleem. – NL-RIV-VG-VAR – 33/34, p. 184–189.
Stephany, R. W.; Schuller, P. L. (Rijksinstituut voor de Volksgezondheid, Bilthoven, NL); 1976. Personal communication to Groenen et al.
Stephany, R. W.; Freudenthal, J.; Schuller, P. L.; 1976a. Quantitative and qualitative determination of some volatile N-nitrosamines in various meat products. – IARC-SP – 14.
Stephany, R. W.; Freudenthal, J.; Egmond, E.; Gramberg, L. G.; Schuller, P. L.; 1976b. Mass spectrometric quantification of traces of volatile N-nitrosamines in meat products. – Journal of Agricultural & Food Chemistry 24:536–539.
Stevens, T. S.; 1957. – Redd, p. 35, 47.
Stoja, W.; 1969. Vereinfachte Nitratbestimmung in Lebensmitteln, insbesondere Fleischwaren. – Deutsche Lebensmittel-Rundschau 65:144–147.
Swain, J. W.; 1973. Volatile flavor constituents of pork cured with and without nitrite. Dis sertation Abstracts International Section B. Science & Engineering 34(3)1143.
Tannenbaum, S. R.; Sinskey, A. J.; Weissman, M.; Bishop, W.; 1974. Nitrite in human saliva: its possible relationship to nitrosamine formation. – Journal of the National Cancer Institute 53:79–84.
Tarkow, L.; Fellers, C. R.; Levine, A. S.; 1942. Relative inhibition of microorganisms by glucose and sucrose sirups. – Journal of Bacterology 44:367–372.
Tarladgis, B. G.; 1962. Interpretation of the spectra of meat pigments. 2. Cured meats: the mechanism of color fading. – Journal of the Science of Food & Agriculture 13:485.
Tatum, J. H.; Shaw, P. E.; Berry, R. E.; 1969. Degradation products from ascorbic acid. – Journal of Agricultural & Food Chemistry 17:38–40.
Taylor, H. W.; Lijinsky, W.; 1975. Tumor induction in rats by feeding aminopyrine or oxytetracycline with nitrite. – International Journal of Cancer 16:211–215.
Taylor, A. A.; Shaw, B. G.; 1975. Wiltshire curing with and without nitrate. 1. Vacuum-packed sliced back bacon. – Journal of Food Technology 10:157–167.
Taylor, A. A.; Shaw, B. G.; Jolley, P. D.; 1976. Wiltshire curing with and without nitrate. 2. Vacuum-packed collar bacon and bacon from pigs with high ultimate pH. – Journal of Food Technology 11: ... (in press).
Telling, G. M.; 1972. A gas-liquid chromatographic procedure for the detection of volatile N-nitrosamines at the ten parts per billion level in foodstuffs after conversion to their corresponding nitramines. – Journal of Chromatography 73:79–87.
Telling, G. M.; Hoar, D.; Caswell, D.; Collings, A. J.; 1976. Studies on the effect of feeding nitrite and secondary amines to Wistar rats. – IARC-SP – 14.

ten Noever de Brauw, M. C.; van Ingen, C.; Groenen, P. J.; 1976. Identification and quantitative determination of trace amounts of nitrosamines in cigarette smoke and cured meat products. – ISAMS – 15, p. ... (in press).
Terracini, B.; Magee, P. N.; Barnes, J. M.; 1967. Hepatic pathology in rats on low dietary levels of dimethylnitrosamine. – British Journal of Cancer 21:559–565.
Tinbergen, B. J.; 1974. Low-molecular meat fractions active in nitrite reduction. – Krol & Tinbergen, p. 29–36.
Tjaberg, T. B.; Kvåle, O.; 1972. The effect of sodium nitrite on germination and growth of spores from Clostridium botulinum type B in a meat product. – EMMRW – 18.
Tozawa, H.; Sato, M.; 1974. Formation of dimethylnitrosamine (DMNA) in sea foods. 1. Effects of hemoglobin and ascorbate on DMNA-formation in Alaska pollock toe. – Bulletin of the Japanese Society of Scientific Fisheries 40:425.
Treadwell, F. P.; Hall, W. T.; 1935. Analytical chemistry. Vol. 2. 8th edition. Wiley, New York.
Troller, J. A.; 1972. Effect of water activity on Enterotoxin A production and growth of S. aureus. – Applied Microbiology 24:440–443.
Troller, J. A.; Stinson, J. V.; 1975. Influence of water activity on growth and enterotoxin formation by S. aureus in food. – Journal of Food Science 40:802–804.
US-ACS-NM – 170 (American Chemical Society. 170th National Meeting); 1975-08. Chicago, Abstracts of papers.
US-AMIF (American Meat Institute Foundation, Virginia, US); 1973. Proceedings of the meat industry research conference, Chicago, 1973-03-22 – 23. American Meat Institute Foundation, Virginia.
US-AMIF; 1974. Proceedings of the meat industry research conference, 1974.
US-AMIF; 1975. Proceedings of the meat industry research conference, Chicago, 1975.
US-AOAC; 1975. Official methods of analysis of the Association of Official Analytical Chemists. 12th edition. Washington, D.C.
US-ASBC (American Society of Brewing Chemists); 1964. Proceedings, 1964. Madison, Wisc.
US-CRC (Chemical Rubber Co.); 1973. Handbook of chemistry and physics. 54th edition, edited by R. C. Weast. Cleveland, Ohio.
US-DA (United States Department of Agriculture), Expert Panel on Nitrosamines; 1975-11-11. Report. – Federal Register 40(218)52614–52616.
US-IEEE (Institute of Electric & Electronic Engineers); 1976. International conference on environmental sensing and assessment. New York, Several volumes. – IEEE-75-CH – 1004-1.
US-IFT (Institute of Food Technology); 1973. 33rd annual meeting, Miami, 1973. Chicago.
US-IFT; 1975. 35th annual meeting, Chicago, IL, 8-12 June 1975.
US-MIRC (Meat Industry Research Conference). See US-AMIF.
US-OSU (Oregon State University, Corvallis). See: Ivey 1974; Wasthesen 1975.
US-UW (University of Wisconsin, Madison). See Sebranek 1974.
Usher, C. D.; Telling, G. M.; 1975. Analysis of nitrate and nitrite in foodstuffs: a critical review. Journal of the Science of Food & Agriculture 26:1793–1805.
van Logten, M. J.; den Tonkelaar, Engelina M.; Kroes, R.; Berkvens, Johanna M.; van Esch, G. J.; 1972. Long-term experiment with canned meat treated with sodium nitrite and glucono-delta-lactone in rats. – Food & Cosmetics Toxicology 10:475.
van Roon, P. S.; 1974. Inhibitors in cooked meat products. – Krol & Tinbergen, p. 117–122. Discussion, p. 122–124.
van Roon, P. S.; 1975. The influence of temperature on the formation of FE and NO containing complexes with bacteriostatic properties towards Clostridium sporogenes spores. – EMMRW – 21.
van Roon, P. S.; Olsman, W. J.; 1977. Inhibitory effect of some Perigo-type compounds in clostridium spores on pasteurized meat products. – This symposium.
van Straaten, S.; de Vrijer, F.; 1973, 1975-05. Lists of volatile compounds in food. 3rd edition; 7 cumulative supplement. Central Institute for Nutrition & Food Research TNO, Zeist. – NL-CIVO-R – 4030.
Volff, R.; Noyelle, G.; Gautrat, Cl.; 1974-11/12. Sur le dosage des nitrites et des nitrates dans les produits alimentaires. – Annales des Falsifications et de l'Expertise Chimique 67:599–608.

Walker & Davis. See IARC 1972.
Walker, E. A.; Castegnaro, M.; Pignatelli, Brigitte; 1975. Use of a clean-up method to improve specificity in the analysis of foodstuffs for volatile nitrosamines. – Analyst (GB) 100:817–821.
Walker et al. See IARC-SP – 14, 1976.
Walters, C. L. (GB-BFMIRA); 1974. Personal communication to van Roon & Olsman.
Walters, C. L.; Newton, B. E.; Parke, D. V.; Walker, R.; 1974. The precursors of N-nitroso compounds in foods. – IARC-SP – 9, p. 132–136.
Walters, C. L.; Dyke, Caroline S.; Saxby, M. J.; Walker, R.; 1976. Nitrosation of food amines under stomach conditions. – IARC-SP – 14, p. 181–194.
Warthesen, J. J.; 1975. Ph. D. Thesis. Oregon State University, Corvallis. Cited: Scanlan 1975.
Wasserman, A. E.; 1974. Trace nitrosamine analysis. – Morton & Rhodes.
Weast. See US-CRC 1973.
Weitz, E.; Achterberg, F.; 1933. Zur Kenntnis der stickoxyd-schwefeligen Säure. 1. – Chemische Berichte 66B:1718–1727.
Weitzel, G.; Geyer, H.-V.; Fretzdorff, A.-M.; 1957. Darstellung und Stabilität der Salze von Aminosäure-N-Glykosiden. – Chemische Berichte 90:1153–1161.
Weitzel, G.; Engelmann, J.; Fretzdorff, A.-M.; 1959. Darstellung und Bildungstendenz von Zucker-Cystein-Verbindungen und ihren Komplexsalzen. Stabilitäts-Konstanten der Zink und Kobalt(II)-Komplexe. – Hoppe-Seyler's Zeitschrift für Physiologische Chemie 315:236–255.
White, J. W. jr; 1975. Relative significance of dietary sources of nitrate and nitrite. – Journal of Agricultural & Food Chemistry 23:888–891.
White, R. H.; Havery, D.; Roseboro, E.; Fazio, T.; 1974. Isolation of volatile N-nitrosamines in edible vegetable oils and cooked bacon fat. – Journal of the Association of Official Analytical Chemists 57(6)1380.
Wierbicki, E.; Heiligman, F.; 1974a. Shelf stable cured ham with low nitrite-nitrate additions preserved by radappertization. – Krol & Tinbergen, p. 189–212. Discussion, p. 212.
Wierbicki, E.; Heiligman, F.; Wasserman, A. E.; 1974b. Cured meats with reduced nitrite preserved by radappertization. – EMMRW – 20, p. 100.
Wierbicki, E.; Heiligman, F.; Cohen, J. S.; Pensabene, J. W.; 1975a. Cured, smoked ham with greatly reduced nitrite and nitrate preserved by radappertization. – US-IFT 1975, Abstr. 194.
Wierbicki, E.; Howker, J. J.; Shults, G. W.; 1975b. Effect of salt, phosphate, and other curing ingredients on water-holding capacity of lean pork meat and the quality of radappertized ham. US Army Natick R & D Command, Natick MA 01760. – Tech. Rpt. TR-76-24-FEL, 1975-08.
Wierbicki, E.; Howker, J. J.; Shults, G. W.; 1976. Effect of salt, phosphates and other curing ingredients on shrinkage of lean pork meat and the quality of smoked processed ham. – Journal of Food Science 41:1116–1121.
Wirth, F.; 1973. Welche Konsequenzen hätte ein Verbot oder eine Reduzierung des Zusatzes von Nitrat und Nitritpökesalz zu Fleischerzeugnissen? Aus technologischer Sicht. – Fleischwirtschaft 53:363.
Wolf, J.; Barker, A. N.; Ellar, D. J.; Dring, G. J.; Gould, G. W.; 1976. Spore research. Academic Press, London.
Wolfrom, M. L.; Schuetz, R. D.; Cavalieri, L. F.; 1948. Chemical interaction of amino compounds and sugars. 3. The conversion of D-glucose to 5-(hydroxymethyl)-2-furaldehyde. – Journal of the American Chemical Society 70:514–517.
Wolfrom, M. L.; Schuetz, R. D.; Cavalieri, L. F.; 1949. Chemical interactions of amino compounds and sugars. 4. Significance of furan derivatives in color formation. – Journal of the American Chemical Society 71:3518–3523.
Woolford, G.; 1974. The fate of nitrite in meat curing and the determination of nitrosamines. Ph. D. Thesis. University of Surrey, Guildford, England.
Woolford, G.; Cassens, R. G.; 1976. The fate of sodium nitrite in bacon. In preparation.

Woolford, G.; Cassens, R. G.; Greaser, M. L.; Sebranek, J. G.; 1976a. The fate of nitrite: reaction with protein. – Journal of Food Science 41:585–588.
Woolford, G. C.; Walters, Cl.; Parke, D. V.; Gould, B. J.; 1976b. The fate of nitrite in meat products. In preparation.
Wright, M. J.; Davidson, K. L.; 1964. Nitrate accumulation in crops and nitrate poisoning in animals. – Advances in Agronomy (US) 16:197–247.

Appendix

Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist, 1976. Pudoc, Wageningen

International system of units (SI): an editorial note

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As from 1 January 1978, units of the International System will become the only legal way of expressing the results of measurements in several European countries including the Netherlands. The law includes several exceptions and escape clauses, for instance to permit the gradual phasing out of other commonly used units such as mmHg and kcal.

Application of the System in editorial policy is based on the following principles and authorities.

General principles are laid down in International Standard 1000 (ISO-1000, 1974: Rules for the use of units of the international system . . .) and in ISO-31.0 (1974: 'General principles concerning quantities, units and symbols'). The ultimate authority for the International System is the Bureau International des Poids et Mesures (1973: 'Le Système International d'Unités (SI)'.

Principles for the application of the System in analytical chemistry and biochemistry have been worked out within the International Union of Pure and Applied Chemistry (IUPAC):

- 1974. Quantities and units in clinical chemistry. – Pure & Applied Chemistry 37(4)517-546.
- 1975. Manual of symbols and terminology for physicochemical quantities and units. Butterworths, London. ISBN 0-408-70671-6.

Though the former was elaborated for medical science, it is also applicable to food science.

Analytical data is usually expressed in terms of 'amount' of a component and of the analysed material or 'system'. 'Amount' is here a general term for any of the quantities in Table 1.

For data on components, number and amount of substance are preferred, since they may indicate physiological and chemical relationships or pathways. For instance, number of bacteria is more meaningful than mass or volume of bacteria for most purposes, and amount of substance of nitrate is more useful than mass of nitrate or of sodium nitrate. Amounts of systems (materials) are usually in terms of mass or volume.

For volume, the SI base unit is the cubic metre but in analytical chemistry of solid and liquid systems, the litre is more usual and behaves like a supplementary base unit. It should not, however, be used in expressions including terms of length

or area, for instance molar absorbance, $A/l \cdot c$, has the base unit $m^2 \cdot mol^{-1}$, not litre$\cdot mol^{-1} \cdot m^{-1}$ (A being absorbance, l pathlength and c substance concentration).

On the basis of Table 1, analytical data may be expressed in terms of quantities in Table 2.

Large or small values may be expressed by attaching prefixes to a unit of the numerator, for instance mmol/kg, not mol/Mg. Division of units may be marked either by a solidus (mmol/kg) or by a negative power (mmol$\cdot kg^{-1}$ or mmol kg^{-1}).

For 'amount fractions' (Table 2, diagonal, commonly called proportions) and 'amount ratios' (for instance mass of a solute divided by mass of solvent), units are strictly redundant but expressions like ml/litre, g/kg or mmol/mol (all equal to 10^{-3}) are permitted for didactic reasons.

Expressions like %, ppm and ppb are not part of the system regulated by the Bureau International and are discouraged, for instance by IUPAC (1974). To avoid errors of factor 10, IUPAC (1974) discourages percentages altogether, preferring steps of 10^3, for instance mol/kg, mmol/kg, μmol/kg, nmol/kg. The expression ppb is ambiguous, meaning either 10^{-9} or 10^{-12}. A further reason for discouraging these three 'units' is slovenly usages like '1% solution' for 1 g per 100 ml or perhaps 1 lb per 100 gal.

The System has the advantage of the simple arithmetic relationship between a small number of base units; it is interdisciplinary and international. A minor disadvantage is the suppression of some units that were unique for one kind of quantity. It is therefore essential to designate the quantity (by name or symbol) as well as the unit in any item of data.

In fact, this was true of traditional ways of expression. For instance the expression 'Salt in meat was 4%' could mean:

- mass fraction (or 'content') of NaCl was 40 g/kg (or 0.04)
- (mass) concentration of NaCl in meat was 40 g/litre (or 40 kg$\cdot m^{-3}$)
- ratio of mass of NaCl to volume of water in meat was 40 g/litre.

In symbolic logic, one can say

$$\rho(\text{NaCl—meat}) = 40\ \text{g} \cdot \text{litre}^{-1} \quad (1a)$$

$$\text{quantity} = \text{number} \times \text{unit} \quad (1b)$$

Table 1. Ways of expressing amounts of a component or system.

Quantity		Base units	Examples
name	symbol		
number	N	1	number of *E. coli* or of specified molecules
volume	V	m^3; litre	volume of brine
mass	m	kg	mass of $NaNO_3$ or of meat
amount of substance	n	mol	amount of substance of $NaNO_3$ (or NO_3^-) added to a piece of meat

Table 2. Ways of expressing analytical data on liquid and solid systems. Each field includes the name (above) and symbol (below to the left of the solidus) of the quantity and the symbol (below to the right) for the base unit. Most terms are in line with IUPAC (1973, 1974), and ISO-31.8 (1973: 'Quantities and units of physical chemistry and molecular physics'). The term 'entitic' is provisional and is being considered within IUPAC; it is a widening of the concept implied by 'atomic' or 'molecular' in molecular or atomic mass. Terms in Column 4 ('molar') are used for quantities of the type $Q(1)/n(1)$ and $Q(B)/n(B)$ as well as for the analytical quantities $Q(B)/\Sigma n(A+B \ldots +N)$. Q is any amount; B any component; A a major component such as solvent; n amount of substance; concn concentration.

Amount of component	Amount of system			
	number	volume	mass	amount of substance
Number	Number fraction $\delta/1$	Number concn C/litre^{-1}	Number content .../kg^{-1}	Molar number L/mol^{-1}
Volume	Entitic volume .../litre	Volume fraction $\theta/1$	Volume content .../litre·kg^{-1}	Molar volume .../litre·mol^{-1}
Mass	Entitic mass M/kg	Mass concn ρ/kg·litre^{-1}	Mass fraction $w/1$	Molar mass M/kg·mol^{-1}
Amount of substance	Entitic substance .../mol	Substance concn c/mol·litre^{-1}	Substance content ν/mol·kg^{-1}	Substance fraction $x/1$
General term	Amount per 'entity' $Q(B)\cdot N(1)^{-1}$	Concn $Q(B)\cdot V(1)^{-1}$	Content $Q(B)\cdot m(1)^{-1}$	Molar 'amount' $Q(B)\cdot n(1)^{-1}$

It follows that

$$\rho(\text{NaCl—meat})/\text{g·litre}^{-1} = 40 \quad (2a)$$

$$\text{quantity/unit} = \text{number} \quad (2b)$$

The values in the body of a table or in a graph are numbers (right side of Eqn 2), so the headings of columns and the axes of graphs should be constructed as the left side of Equation 2 (IUPAC 1975; Codata 1974: 'Guide for the presentation in the primary literature of numerical data derived from experiments'). In text, data may be expressed in any natural grammatical order as long as the data elements quantity, component, system, number and unit are implicit.

The policy indicated here has not been applied rigidly in the present publication. It is indicated here for the guidance of readers and of future authors.