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Preface

It has long been known that small amounts of saltpetre are indispensable for the formation of the characteristic meat colour. About 1890, it was found that nitrite was in fact the active compound responsible for the colour, the mechanism of it being discussed by Haldane in 1901 in his classic publication.

Several decades passed before another characteristic of nitrite was discovered: a potent inhibitor of micro-organisms, among them pathogens, in many meat products. In particular the inhibition of toxin formation by *Clostridium botulinum* was established. More recently the role of nitrite in cured meat flavour was recognized.

For a few years, attention has been focused on the human health hazard arising from possible formation of carcinogenic *N*-nitroso compounds from nitrite and secondary amines in meat. Debate started in many countries on the pros and cons of the use of nitrite in meat products. Research was intensified in various Institutes all over the world.

We had the impression that it might be useful to bring experts together in an international meeting on nitrite in meat products. The reactions received from those currently engaged in the relevant problem indicated that such a conference was welcomed indeed by everybody.

Accordingly, the symposium took place at Zeist in September 1973 and was attended by experts from chemical, microbiological, toxicological and technological disciplines and some public health authorities.

All the participants felt that the papers and discussions had contributed to a better understanding of several problems. Their prompt publication together with resolutions of the meeting should assist others involved in the problem of nitrite in meat products.

We are grateful to the staff of Pudoc, Wageningen, for their competence in preparing the proceedings for publication so quickly.

B. Krol
B. J. Tinbergen

Word of welcome

H. de Boer

Research Group for Meat and Meat Products TNO, Zeist

I am glad to welcome you here on behalf of the Research Group for Meat and Meat Products TNO, under whose auspices this Symposium has been organized. For those not familiar with the structure of meat research in the Netherlands I may explain, that this Research Group includes the main governmental institutes and university departments for research on meat production, meat technology and meat hygiene.

Because of the significance of international co-operation of research workers, who work on the different aspects of this complex problem, the Dutch authorities decided to sponsor the symposium. We greatly acknowledge the financial support by the Commodity Board for Livestock and Meat, by the Ministry of Public Health and Environmental Hygiene, and by the Organization for Nutrition and Food Research TNO.

The organization of this Symposium is largely due to the work of the staff of the Central Institute for Nutrition and Food Research TNO, who are our hosts, and to the efforts of several staff members of the Netherlands Centre of Meat Technology.

This symposium brings together scientists working on the different aspects of the problem. I will not take too much time by extensive words of welcome but merely express how much we appreciate your response and the presentation of your latest research, thus providing material for a better understanding on the whole problem. We are grateful that Dr Aunan, Dr Rubin, Professor Kotter and Professor Mossel have accepted the invitations to act as chairmen in the different sessions. As you know, the problem of nitrite in meat products has various facets, each of which represents a specific field with its specific starting points. Even though it will be difficult after each session to synthesize each of the separate fields – analysis, microbiology, technology and toxicology – it will be more difficult to integrate these aspects into general conclusions.

I think we cannot expect this symposium to provide direct implications about the use of nitrite in meat curing. Our purpose may be to use this unique confrontation of knowledge and views from different facets for a better mutual understanding, allowing collaboration and leading to new approaches in some common framework.

I hope your work of the next days will be productive and provide prospects for the future.

Analytical session

Reporters: J. H. Houben, W. J. Olsman, P. S. van Roon

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Formation of curing pigments by chemical, biochemical or enzymatic reactions

K. Möhler

Abteilung Technologie der Nahrung – Bromatologie – Technische Universität München, 805 Weihenstephan bei Freising, W-Germany
Present address: Techn. Univ. München, Abt. Technologie der Nahrung, 805 Freising/Vöttung, W-Germany

Abstract

Chemical, biochemical and enzymatic reactions for the formation of curing pigment nitric oxide myoglobin (or nitric oxide hemoglobin) are discussed. All systems are derived from metmyoglobin which is the first product formed in the chain of reaction. It is reduced chemically by SH groups, biochemically by the co-enzymes NADH and FMN or FAD, or enzymatically by a NADH-dependent dehydrogenase system under co-function of ferrocytochrome c. Under similar or identical conditions nitrite is reduced to nitric oxide which is bonded to the myoglobin (hemoglobin).

Curing pigment – properties and nomenclature

Curing pigment is the red agent, which is formed from the muscle pigment myoglobin by addition of nitrite under various conditions. This pigment retains its colour after heating. The collective term of curing pigment includes the corresponding reaction products of the blood pigment hemoglobin, because blood is occasionally a component of sausages. Blood is also left behind in small, varying amounts in the muscle tissue after slaughter. A detailed illustration of the physical properties of hemoglobin (Hb) can be found in Weissbluth (1967). Calculations and theoretical conceptions as to the type of bonding of the oxygen onto the Hb-iron and the charge distributions in the oxymyoglobin (O_2-Mb) originate from Bayer and Schretzmann (1967). Such data are as yet not available for NO-myoglybin (NO-Mb) but a lot of basic data can be derived from the two publications mentioned. A publication of Smith and Williams (1970) is an important reference for the relation between the reactions of Hb and Mb and their spectra.

It is important for all further considerations, that all Mb-Bonds are of a complex nature. Protohaem IX is the active centre in the ferromyoglobin = deoxymyoglobin = Mb. The 5th coordination position is occupied by the tertiary imidazol-nitrogen of the so called proximal histidine. The divalent iron is penta-coordinated in Mb, i.e. the bonding of O_2 results without having to substitute a ligand. Similarly NO, with the characteristics of a radical, can be bonded. Metmyoglobin (MetMb) – iron is always hexa-coordinated, the 6th coordination position is occupied by a water molecule if no negative ions such as the nitrite ions are present. Through heating above the coagulation temperature of protein, the globin is denatured. There is no

proof, however, that through this the bonding conditions of the protoheme are changed. The Tarladgis hypothesis (1962): in cooked, cured meats two coordination positions of the protoheme are occupied by NO, could not as yet be confirmed. The solubility of curing pigment in acetone (and other organic solvents) is probably caused by the exchange of the imidazol-N-bond on the 5th coordination position with acetone.

The result of structural examinations definitely show that the name nitrosomyoglobin, and nitrosohemoglobin, is incorrect since curing pigment has no connection with nitroso bonding. The name nitrosyl myoglobin is also incorrect as nitrosyl bonds are considered as mixtures of anhydrides of nitrous acid and other acids (e.g. nitrosyl chloride). The only correct name can be nitrogen monoxide myoglobin (nitric oxide myoglobin = NO-Mb). The same is true for hemoglobin.

Fundamentals of NO-myoglobin formation

According to present opinion the first reaction of nitrite[1] with muscle pigment is an oxidation of Fe^{2+} in Mb to Fe^{3+} in MetMb. Simultaneously, nitrate is produced in an autocatalytic reaction. In model experiments with minced beef muscle, the amount of nitrate formed varies greatly (Möhler, 1967; Walters et al., 1968). However, from the statistical evaluation of more test material the following reaction equation can be formed (Möhler, 1967):

$$4\ MbO_2 + 4\ NO_2^- + 2\ H_2O \rightarrow 4\ MetMbOH + 4\ NO_3^- + O_2 \qquad (I)$$

Met-Mb and nitrate are formed in equimolecular amounts. Deviations can be caused by different factors, e.g. by varying the concentration of oxygen. This could be established with hemoglobin (Möhler & Baumann, 1971). In the next section it is implied that nitrate and MetMb are formed in equivalent amounts since the amount of MetMb has not yet been exactly determined. It is unknown whether or not MetMb appears always as an intermediate step in the formation of NO-Mb.

In meat products which are commonly manufactured with nitrite, usually a higher content of nitrate is found then would be expected with Equation I. This is due to the secondary oxidation (Möhler, 1967) in which the dismutation of nitrous acid could also play a role, as could the oxidation of NO, formed through nitrosothioles, according to Mirna & Hofmann (1969).

There are no specifications about whether other decomposition products of nitrite in commonly used concentrations have an influence on the formation or stability of curing pigments. In a balance of nitrite turnover during the formation of curing pigment, nitrite is constantly lost. In the study of these reactions NO and NO_2 were found, which were freed as nitrose gases. Another gaseous component is N_2O (Möhler & Ebert, 1971; Walters & Casselden, 1973). There is also a possibility that small amounts of ethyl nitrite are formed (Walters & Casselden, 1973).

1. When nitrate is used for curing in practice, the reduction to nitrite through micro-organisms in necessary.

The formation of curing pigments by chemical reactions

Application of gaseous NO

If oxygen is completely excluded, NO-Mb is formed in Mb-solutions by adding gaseous NO as occurred with NO-Hb in Hb-solution. After excess NO has been removed with N_2, the pigment solution is relatively stable to oxidation. According to Sancier and co-workers (1962) NO-MetHb is formed when NO is introduced to MetHb-solutions, whose spectrum coincides largely with that of NO-Hb but shows an overall higher extinction. Chien (1969) was able to show that the so-called NO-MetHb was actually NO-Hb; he always obtained the same end-product whether gaseous NO was added to Hb or MetHb. He therefore assumed that MetHb is reduced by NO whereby NO becomes NO_2^- and where Hb reacts with excess NO, NO-Hb is the result. From crystal suspensions of Mb or MetMb Dickinson & Chien (1971) obtained, by supplying sufficient amounts of gaseous NO, in both cases NO-Mb. Compared to that with Hb, this reaction was somewhat slower. If O_2 is excluded no reoxidation of NO can follow. Hence no special mechanism is required for the reduction of Fe^{3+} to Fe^{2+} if enough NO is available.

Application of nitrite

The formation of NO-Mb in vitro usually results from a reaction of Mb-solutions with nitrite, without exclusion of air oxygen and in the presence of a strong reducing agent such as sodium hydrosulphite ($Na_2S_2O_4$) e.g. according to Walsh & Rose (1956). The excess nitrite and hydrosulphite can be removed by dialysis. Comparable experiments with muscle mince are not available and would not be of use for practical purposes.

NO-Mb is formed in muscle mince by adding nitrite without any special reducing agents and by heating. In these two model tests, as in manufacture of frankfurters, luncheon meats etc., only chemical procedures will occur (Möhler, 1967). Denaturation caused by heating eliminates enzyme and co-enzyme activity. However conformational changes on proteins do occur in connection with the activation of SH groups, formation of S-S groups and H_2S.

The following mechanism-theory for the formation of NO-Mb begins with MetMb-nitrite. Through intermolecular reaction it is transformed to NO-Mb in one step, whereby the reactions of the SH groups act as electron donors. Since the SH groups of the myoglobins are probably not sufficient, other muscle proteins can act as reducing agents. However the effect seems to be connected with reactions such as $2\ SH \rightarrow S\text{-}S + 2\ H^+$ since, for instance, cystein or glutathion additives have no effect.[1]

One must consider however, that absolute proof of activity or inactivity of –SH groups is impossible under these conditions. Inhibiting the reaction by the usual SH

1. The positive findings of Reith & Szakály (1967) are due to a strong pH-reduction through use of cysteine hydrochloride. In my experiments these effects did not occur when the initial pH value was maintained. See text.

blockers has up-to-now been unsuccessful (exception: mercury acetate in high concentration according to Möhler (1967). A positive effect of SH groups can therefore only be noticed on a higher yield of NO-Mb which is normally about 70%.

Notable increases of the yield of NO-Mb can be achieved by addition of reducing agents such as ascorbic acid, erythorbic acid (synonym: isoascorbic acid) or thioglycollic acid and thioacetamide. This finding is not opposed to simultaneous intramolecular reduction of Fe^{3+} and NO_2^-. Steric conditions apparently play a role, since the formation of NO-Mb ceases when a large amount of water is added (10 parts H_2O to 1 part muscle meat). NO-Hb formation in blood is heavily diminished under similar conditions (Möhler & Baumann, 1971).

A transfer mechanism of pure chemical nature was discussed by Mirna & Hofman (1969). With SH groups nitrous acid forms nitrosothioles. These products, perhaps unstable, produce the curing pigment by splitting of NO and forming disulphide. The condition is, however, that at least temporarily there are sufficient hydrogen ions in the reaction so that nitrous acid can be formed from nitrite. If the pH value is near 3, red nitrosothiol can easily be formed from cystein and nitrite. As stated above, cystein has no effect on the formation of curing pigment in meat products at the usual pH value of 6. The question is now whether or not this transfer mechanism is possible under usual conditions of meat processing.

Fox (1966, 1968) assumed that a temporary bond exists between ascorbic acid and nitrite for the transfer of NO. Since additives such as ascorbic acid, glucono-δ-lacton etc. promote the formation of curing pigment but are not essential for the formation of NO-Mb, the reaction possibilities in reference to this subject will not be discussed.

Formation of curing pigments by biochemical reactions

Non-enzymatic reactions

Non-enzymatic formation of NO-Mb from MetMb was described by Koizumi & Brown (1971). NADH in presence of FMN or FAD was used as reducing agent. NADH by itself is not active. If oxygen is completely excluded, MetMb is reduced to Mb as shown in Fig. 1. Mb reduced nitrite to NO and is itself oxidized to MetMb. NO is immediately bonded to excess Mb, while MetMb returns to the circulatory system. Two points are especially important for the process of reaction. The system NADH/FMN or FAD cannot reduce nitrite to NO. The authors deduced from this that the co-enzyme mixture only affects the reduction of MetMb to Mb. Secondly, nitrite is reduced to NO by Mb under anaerobic conditions. This reaction was described for Hb by Brooks as early as 1937. The proof of a similar reaction with Mb relies upon spectrophotometric measurements of Koizumi & Brown (1971). It was admitted however, that under these test conditions NO-Mb and O_2-Mb are difficult to separate. A methylene blue-diaphorase system can be used instead of the co-enzyme mixture, whereby the enzymatically reduced methylene blue causes the reduction of MetMb to Mb. The reduction of nitrite to NO is impossible.

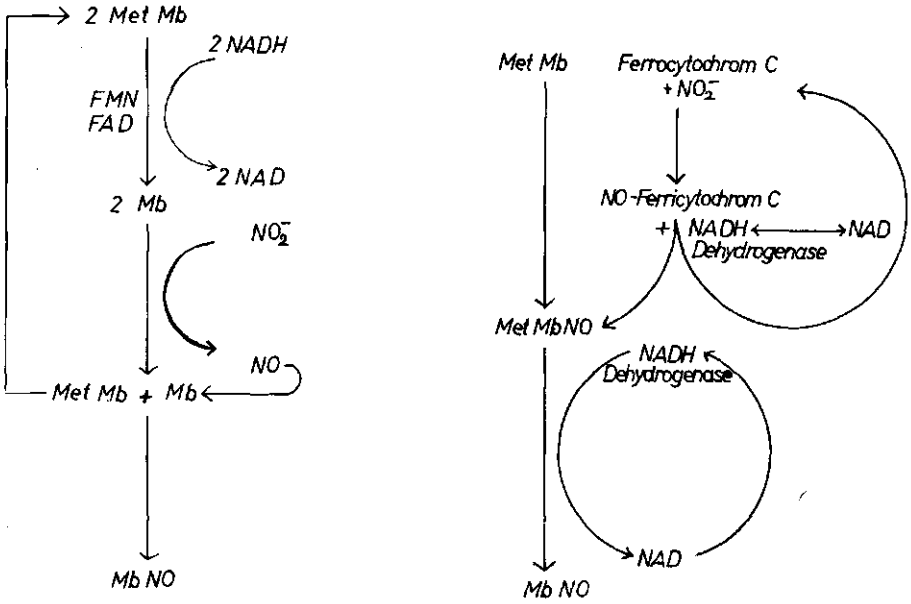

Fig. 1. Non-enzymatic and enzymatic formation of NO-Mb.
Left: formation of nitrosylmyoglobin, according to C. Koizumi and W. D. Brown.
Right: formation of nitrosylmyoglobin, according to C. L. Walters and coworkers.

Enzymatic formation of NO-Mb

The enzymatic biochemical interpretation of the formation of NO-Mb is closely connected with the name of Clifford Walters (1968). Principally his explanation states that nitrite takes the place of oxygen in a part of the respiratory chain. Then the oxidations caused hereby are repaired by the NADH-dehydrogenase system. Fig. 1 shows the somewhat abbreviated reaction-mechanism. The starting point here is also MetMb which should be produced when nitrite is added under anaerobic conditions. In addition, ferrocytochrome c has been oxidized by nitrite to ferricytochrome, and NO, as product of reduction, has been bound to cytochrome. Nitrosylferricytochrome c is now reduced by NADH with the aid of the dehydrogenase system. Ferrocytochrome c can return into circulation. Since nitrosylferrocytochrome is instable, NO separates and is transferred to MetMb. Thus, hypothetic NO-metmyoglobin developes. If the NADH-dehydrogenase system then reduces the MetMb, the desired curing pigment is produced. The initial reaction course of Mb is dependent on anaerobic conditions as they exist, according to Walters, e.g. in the interior of a muscle. If the hitherto discussed non-enzymatic system of Koizumi & Brown is compared with the enzymatic system of Walters and co-workers, two reduction steps can be observed in both systems. The first reduction step in each system is connected to NADH, whereby in one case the co-enzymes FMN or FAD are active, in the other the dehydrogenase system. Contrary to the reaction

according to Koizumi & Brown that according to Walters requires NADH also for the second reduction step. An analysis of both systems shows that two reduction equivalents are necessary for the formation of one NO-Mb.

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Discussion

Assessment of the reaction equation for pigment formation

The equation $4\ MbO_2 + 4NO_2^- + 2H_2O \rightarrow 4MetMbOH + 4NO_3^- + O_2$ is based on

experiments, in which nitrite was mixed with raw meat and which lasted for 1 – 2 h.

Between 100 and 1000 mg/kg nitrite, the amount of nitric oxide myoglobin formed is independent of the nitrite concentration, according to Möhler.

Action of reducing agents

Ascorbic acid improves the cured meat colour formation, but does not give a 100% yield of the pigment. Pigment formation also occurs at temperatures below 70°C, but at a slower rate. Reducing agents like thyoglycollic acid and thioacetamide enhance the colour formation when added to meat products; cysteine and glutathion, however, have no such effect. The reason for this discrepancy is unknown.

Nitrosation by nitric oxide myoglobin

According to Professor Roughton, University of Cambridge, the dissociation constant of nitric oxide hemoglobin is so small that it cannot be measured. Thus, it is unlikely that nitric oxide hemoglobin could nitrosate amines but this point is under study by Dr R. Bonnett of Queen Mary College, University of London.

Non-bacterial reduction of nitrate

Up to now no indication of a non-bacterial reduction of nitrate to nitrite in raw meat has been found. In vegetables however this can occur. The nitrite formed here is immediately converted to lower oxidation stages of nitrogen.

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Determination of free and bound nitrite

A. Mirna

Federal Institute for Meat Research, Department for Chemistry and Physics, Kulmbach-Blaich, W-Germany

Abstract

Procedures for the determination of free and bound nitrite are of interest for investigations into the different ways of nitrite reaction and its various reaction products. Compounds reacting directly with Griess reagent are referred to as free nitrite. Heavy metal ions such as Hg^{2+}, Cu^{2+} or Ag^{+} cause the cleavage of the nitrosyl ion(NO^{+}) from nitroso compounds, especially nitroso thiols. The NO^{+} formed is related to the amount of bound nitrite.

Extraction conditions (solvent, temperature, pH) have a distinct influence on the determination of the content of free and bound nitrite in meat products.

Introduction

The effect of curing additives and technological procedures on the curing process in meat products is mainly judged by the changes in the content of nitrite, nitrate and nitric oxide myoglobin (NO-Mb). In general the reliability of analytical methods is checked by recovery tests. Whether a compound found by analytical procedures was present in a product originally or was caused by artefacts during the procedures cannot always be proved adequately.

Apart from interests of mainly technological nature in this connection the question is raised, to what extent the presence of free and bound nitrite may bring about health risks.

Investigations on frankfurter and dry sausages were carried out to study analytical and compositional problems.

Methods

Free nitrite

The term 'free nitrite' is used for all compounds reacting directly with Griess reagent. For colorimetric measurement of the azo dye formed, a clear solution is required; therefore the influence of the solvent and that of the deproteination procedure on the nitrite content must be known. In most methods described in the literature, extraction with water of 100 °C is proposed. Acidic samples are adjusted to neutral or slightly alkaline pH values to prevent any further side-reactions of

nitrite as far as possible. For extraction 80% acetone was used (acetone-method); by means of this extract NO-Mb was determined as well (Mirna & Schütz, 1972).

Unfortunately, at present, non-destructive procedures for the determination of the nitrite content are unknown. The indirect way to oxidize nitrite to nitrate followed by electrometric measurement with ion-sensitive membrane electrode systems does not seem very promising at present (Gerhardt & Haller, 1973).

Bound nitrite

The term 'bound nitrite' refers to the content of nitrite detectable with Griess reagent after cleavage of nitroso compounds with Hg^{2+}, minus the free nitrite. This method was developed by Saville (1958) for the determination of small amounts of nitrosated sulphydryl groups. Acidic solutions of nitrosothiols are comparatively stable, in neutral and especially in alkaline media degradation occurs rather fast resulting in the formation of NO^+ (Mirna & Hofmann, 1969 and Saville, 1958). Experiments were carried out to investigate the stability of various nitroso compounds against cleavage with Hg^{2+} in aqueous acetone, following deproteinization with Carrez II solution (300 g $ZnSO_4$/1000 ml).

The amount of NO bound on structural proteins is determined after removal of components soluble in 80% acetone (Mirna, 1970). The method of Mirna, yields slightly lower values than the procedure as modified by Olsman & Krol (1972).

Results

Model experiments on bound nitrite

Various nitrosated compounds were checked for cleavage of the nitrosyl group by Hg^{2+} in aqueous acetone. According to these results the procedure is not specific for nitrosothiols exclusively. Among the nitrosation products of amino acids, the histidine derivative shows no reaction with Griess reagent, but that of arginine reacts strongly positive; *N*-nitrosoproline and *N*-nitrosohydroxyproline give only a faint reaction. The cleavage of the NO groups from *N*-dimethyl and *N*-diethyl-nitrosamine as well as from *N*-nitrosopiperidine, *N*-nitrosopyrrolidine and *N*-nitrososarcosine-ethylester is lower than that of *N*-nitrosomethylurea. The main product of the reaction between nitrite and creatinine (Archer et al., 1971) is creatinine-5-oxime (at 25°C) and l-methylhydantoin-5-oxime (at 0°C), respectively; both compounds do not split with Hg^{2+} and have no formation of NO^+.

The effect of Hg^{2+} on some *N*-nitroso compounds in an acetate buffer of pH 5.2 is a decreasing stability with time, in the order *N*-dimethylnitrosamine, *N*-nitrososarcosine-ethylester and *N*-nitrosomethylurea. After 10 days 16%, 22% and 32%, respectively of the NO was split off. Similar results were gained in a phosphate buffer solution of pH 5.5, although with somewhat lower conversion rates (Mirna, 1970).

Conditions of extraction

Cured meat products undergo very small change of colour after cooking in

Table 1. Influence of extraction solvents, temperature and addition of nitrite on the content of NO-Mb and nitrite both expressed in mg NO/kg in frankfurter and dry sausages. Nitrite added for recovery test: 22.0.

Extraction solvent	Temperature (°C)	Addition of nitrite	NO-Mb	Nitrite	Recovery (%)
Frankfurter					
Water	20	–	.	21.4	
	20	+	.	41.9	93
	65	–	.	21.1	
	65	+	.	41.8	94
80% Acetone	4	–	0.9	26.8	
	4	+	1.1	43.3	75
	20	–	1.3	27.9	
	20	+	1.4	46.6	85
	40	–	1.2	24.2	
	40	+	1.0	43.6	88
	50	–	2.2	23.5	
	50	+	2.6	41.8	83
	65	–	2.7	21.3	
	65	+	3.7	38.5	78
Dry sausage					
Water	20	–	.	14.8	
	20	+	.	37.0	101
	65	–	.	22.9	
	65	+	.	45.1	101
80% Acetone	4	–	2.0	15.3	
	4	+	2.3	36.9	98
	20	–	3.7	17.5	
	20	+	3.9	40.2	103
	40	–	2.8	21.3	
	40	+	3.0	41.5	92
	50	–	3.5	25.7	
	50	+	3.9	46.3	89
	65	–	6.0	34.4	
	65	+	5.8	53.3	86

water. The same treatment in slightly alkaline solution leads to a remarkable fading or even to the destruction of NO-Mb, NO bound in a NO-Mb complex becomes partly free and is therefore determined as free nitrite.

Extraction with water or 80% acetone, respectively, at various temperatures influences the nitrite values found in frankfurters as well as in dry sausages (see Table 1).

As a reference the nitrite values of frankfurters determined in water extracts

Table 2. Influence of pH on the content of NO-Mb and nitrite both expressed in mg NO/kg in frankfurter sausages and dry sausages.

Product	Extraction solvent	Temperature (°C)	Addition of saturated borax solution	NO-Mb	Nitrite
Frankfurter	water	100	+	.	10.6
	80% acetone	20	–	1280	15.8
	80% acetone	20	+	1230	16.3
Dry sausage	Water	100	+	.	2.1
	80% acetone	20	–	5030	1.7
	80% acetone	20	+	4330	2.9

were used; no influence of the temperature in the range of 20°C to 65°C on the nitrite content was observed. The recovery rates amounted to 93% and 94%, respectively. The nitrite content found with the acetone method was higher at lower temperatures than that found by extraction with water; at 65°C there was no difference between the two extraction procedures. The rates for the recovery values decreased in general with increasing temperature, the maximum being in the range of 20 to 40°C. On the other hand NO-Mb values showed an increase with rising temperature. Subsequent addition of nitrite was followed by only a small increase of the NO-Mb values.

Extraction of dry sausages with water at 20°C resulted in a lower nitrite content than with the treatment at 65°C. This was apparently caused by insufficient extraction of coarse particles in the sample. In recovery tests the added nitrite was found quantitatively to be independent of the temperature during the extraction. The nitrite values, determined by the acetone method, increased as the temperature was elevated whereas recovery rates decreased. In the range from 4°C to 20°C the recovery on the average was quantitative. A more pronounced increase of the NO-Mb values with higher temperature was observed with dry sausages than with frankfurters; a tendency toward higher NO-Mb values in the experiments with subsequent addition of nitrite could be observed.

The influence of alkaline media on the extraction results in a reduction of the NO-Mb content, but in an inconsistent increase of nitrite in both types of products (Table 2).

Curing pH

A sausage mixture (beef) was cured at various pH values; after protein precipitation with Carrez II a distinctly lower nitrite content was found than with the samples which had been previously treated with Hg^{2+}. The amount of nitrite depended not only on the pH, but also on storage time of the sausage mixture. To eliminate the influence of NO-Mb, which is always present in cured meat, an analogous experiment with myofibrils, not containing Mb, from bovine muscle was carried out. (Fig. 1). Results for sausage mixture and myofibrils were similar. For myofibrils the difference between free + bound and free nitrite on the first day was

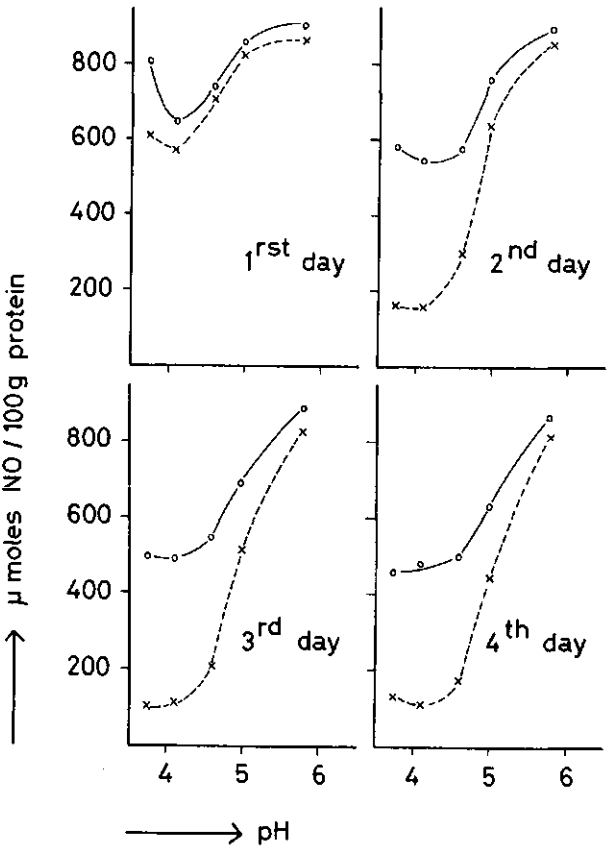

Figure 1: Influence of pH and time on the content of free and bound nitrite in myofibrils from bovine muscle. pH adjusted with acetic acid – sodium acetate – sodium hydroxide. Storage at 4°C.

———: Hg^{2+} Carrez II (free and bound nitrite)

- - - - - - - - -: Carrez II (free nitrite)

very small; for sausage mixture this difference was more pronounced.

At pH values above 5.8 a rather small amount of bound nitrite was found. Nitrosated compounds probably are only present in those cured meat products, e.g. dry sausages, which normally have a lower pH. The results of investigations on frankfurter sausages (pH > 6) showed nearly no differences in content of free and free plus bound nitrite. Any difference probably was the result of stability with time of some nitroso compound.

Discussion

Many investigations have been made on the influence of micro-organisms, food additives, temperature, pH and redox potential values and the length of storage on the decrease in nitrite content of meat products. In the interests of public health it is more important to know about different reaction products of nitrite than about the amount of nitrite added or remaining after processing.

Thorough work on the influence of pH on the content of NO-Mb and nitrite in sausage emulsions has been carried out by Ando et al. (1961, 1963). According to these investigations the amount of NO-Mb formed (colour formation value), the colour stability (colour retention value), as well as the content of sulphydryl groups decrease with increasing pH value, whereas the amount of nitrite increases. To avoid interferences in the nitrite determination an oxidation of reducing substances with potassium ferricyanide followed by the removal of excess reagent with lead acetate was proposed by Nagata & Ando (1967). Möhler (1970, 1971) has also shown that there is a statistical relationship between cured colour development, pH value and the amount of nitrite consumed. Among the reaction products of myoglobin the formation of a salt-like compound of metmyoglobin with nitrite is discussed. Because this kind of reaction product has a rather low stability in water or in deproteinizing agents, nitrite is quantitatively released (Ando et al., 1961). Investigations on complexes of iron nitrosyl compounds have shown that in stronger alkaline solution a cleavage of nitrous acid occurs (Swinehardt, 1967). The different trends in the NO-Mb and nitrite values of the tested groups of meat products lead to the conclusion, that depending on the composition of the sausage mixture and processing techniques, various reaction products are formed from nitrite.

In the literature so far few publications are known dealing with different binding forms of nitrite in meat products. Olsman & Krol (1972) observed that in heated fine ground meat products the amount of bound nitrite increased with lower pH values; during storage practically no further changes were observed. The addition of Fe^{2+} caused the amount of bound nitrite to increase during an 80-days storage period. This increase could be attributed only partly to the formation of nitrosothiols. Probably the iron adsorbed on the protein has the ability to bind NO^{+} as an iron nitrosyl complex. To what extent iron not bound in myoglobin can react with nitrite in a similar way needs further investigations.

Analogous experiments with dry sausage mixtures have shown that in the first five days of ageing about 15% of the added nitrite is protein bound. The addition of glucono-δ-lacton or sodium ascorbate caused a further decrease of the nitrite content (Mirna, 1970).

The previously discussed possibility of formation of iron nitrosyl complex is interesting for other reasons. Such compounds may react in alkaline solution with secondary amines to form *N*-nitrosamines, which might cause artefacts during purification for isolation of *N*-nitrosamines (Loach et al., 1973 and Maltz et al., 1971). On the other hand, in the presence of ketones or compounds with 'acidic' carbon-bound hydrogen, oximes of the corresponding organic compounds are formed (Swinehardt, 1967).

The determination of bound nitrite described in this method gives only a rough

characterization of the binding forms, because nitroso compounds of various structures are covered. The procedures of deproteinization applied in weak alkaline media lead to reactions which may systematically affect the values of bound nitrite as well as of free nitrite. In order to reduce the extent of such changes as far as possible, it is proposed to extract the sample with 80% acetone at 20°C in the dark.

In many cases the nitrate content is calculated from the nitrite values before and after reduction with cadmium; the errors as mentioned above are therefore also of influence on the reliability of nitrate determination.

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Discussion

Black Roussin salt as a curing agent

In the model system to study the formation of free and bound nitrite and the development of colour, black Roussin salt was used instead of nitrite. From the

content of free and bound nitrite, it could be concluded that the seven NO groups in the black Roussin salt are not bound in the same way. The amount of 5.8% free nitrite corresponds well to one NO group (theoretical value 5.2%). The bound nitrite amounts to 30.3% (theoretical value 30.9) based on the formula $NH_4[Fe_4S_3(NO)_6\ NO_2]. H_2O$.

Studies on colour formation showed, 15 to 30 minutes after grinding in mixtures with 82 mg/kg nitrite curing salt, the usual greyish colour, whereas with black Roussin salt at levels of 82 and 8 mg/kg, respectively a distinct red colour appeared. This pigment was only formed on the surface and remained stable for about 30 to 40 hours at 4°C.

It was not possible to extract the red pigment formed with black Roussin salt with 80% acetone. This fact indicates that the 'curing effect' of black Roussin salt is not caused by the reaction of nitric oxide with myoglobin.

Properties of black Roussin salt

The black Roussin salt is a complex coordination compound of Fe, NO and S^{2-}, the exact structure of which has not been completely elucidated. The toxicology of the salt is unknown too. Its use is certainly not allowed in countries with a positive list of food additives. It was remarked that there was a contradiction between the stabilities at acid pH of nitrosothiols and nitrite esters. There is no explanation for this at present.

Conditions of extraction for nitrite determination

The extraction liquid for the nitrite determination (80% acetone or water) is less important than the temperature of extraction. However the pH should be in the mildly acid region. One should be careful with the use of $HgCl_2$-containing agents for deproteinization, because bound nitrite can be released as free nitrite by mercuric ions. The results of the Griess-method for the determination of nitrite depends on the extraction procedure used. There is a strong need for accurate artefact-free methods for the determination of free and bound nitrite.

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Low-molecular meat fractions active in nitrite reduction

B. J. Tinbergen

Central Institute for Nutrition and Food Research TNO, Utrechtseweg 48, Zeist, the Netherlands
Dept.: Netherlands Centre for Meat Technology

Abstract

Buffered mixtures of sodium nitrite and low-molecular water-soluble fractions from minced beef muscle were anaerobically heated and stored at 18 °C. Subsequently, the free nitrite content was examined at regular intervals. The formation of nitrosomyoglobin was studied in vitro to screen the low-molecular fractions for their ability to reduce nitrite in the presence of ferric metmyoglobin.

The fractions strongly reduced the nitrite content in the heated mixtures during storage at 18 °C. The activity was pH- dependent.

The observed ability of the low-molecular fraction to reduce nitrite to NO to form nitrosomyoglobin under anaerobic conditions was found to be proportional to the concentration of the fraction.

It was found that an amino acid or a lower peptide – probably with an SH group – as well as a non-amino acid could be involved in the nitrite reduction.

Introduction

During heating and storage of a cured meat product the content of nitrite continuously decreases. The mechanism responsible for the nitrite depletion has only been partly elucidated. At the beginning of the heating process some nitrite is oxidized to nitrate (Möhler, 1970) and another part is used for the formation of the characteristic colour of the heated product. For the latter, nitrite has to be reduced to nitric oxide before it enters into the colour formation reaction with the ferrous muscle pigment myoglobin.

In an unheated minced muscle incubated with sodium nitrite, the remaining enzymatic reduction activity should be held responsible for a part of the nitrite depletion (Taylor & Walters, 1967). However during storage of the heated product this activity can be ruled out, in which case endogenous chemical reductants are considered active in nitrite reduction (Fox & Ackerman, 1968). The quantity of nitrite required for the formation of both nitrate and nitrosomyoglobin, however, is far less than that actually disappearing during heating and storage of the meat product.

Results obtained in a quantitative study of the effects of a number of factors on nitrite depletion in heated meat products during storage (Olsman & Krol, 1972; Olsman, 1973) and the recent findings of Ando, Nagata & Okayama (1971) that

substantial colour forming activity could be ascribed to the dialysable part of the sarcoplasm fraction brought us to the work described below.

Nitrite depletion during storage at 18 °C was studied in anaerobically heated model systems with the water-soluble low-molecular fractions from minced beef muscle. The formation of nitrosomyoglobin was studied in vitro to screen the fractions for their ability to reduce nitrite in the presence of ferric metmyoglobin.

Experimental

Water-soluble fraction

Two kg beef muscle, trimmed free of external fat and connective tissue, was ground twice and further homogenized in a high-speed laboratory mixer under addition of 8 000 ml demineralized water at 0 – 2 °C. The slurry was centrifuged at 15 000 X *g* and the supernatant volume reduced to 500 ml in a rotating vacuum evaporator at 10°C. The concentrate was dialysed against 10 000 ml demineralized water in cellulose tubing for 48h at 2 °C. The dialysate was concentrated by evaporation in vacuo at 10°C to a volume of 400 ml. The concentrated water-soluble low-molecular fraction was stored at –20 °C in 25 ml portions until further use. The fraction thus obtained is denoted below as D.

Fractionation by ion exchange

With a method for the quantitative separation of free amino acids and low-molecular peptides (e.g. glutathione) from organic non-amino acids, sugars etc., described by Salminen & Koivistoinen (1969), 30 ml of fraction D was adjusted to pH = 3.6 and passed through a column with Dowex 50 W X 4 (H^+ form, 50 – 100 mesh). The amino acids were sorbed on the column. Sufficient washing with demineralized water removed neutral compounds. The eluate and washings were combined and concentrated by evaporation in vacuo to the original volume of the dialysate, i.e. 30 ml. The obtained fraction is indicated below with D-A.

Amino acids were eluted from the resin with 10% ammonia and the effluent was evaporated to dryness. 5 ml 0.1 M NaOH was added and the evaporation repeated. The dry residue was dissolved in water, the pH adjusted to 5.5 and the volume brought to 30 ml. The amino acid fraction is denoted below as A. A recombined fraction was prepared by mixing equal volumes of D-A and A, followed by evaporation to half the volume in vacuo. The recombined fraction is denoted below as R.

All fractions D, D-A, A and R were tested for both nitrite depletion and colour forming ability in model systems described below.

Fractionation with ultrafilters

In order to determine whether the nitrite reducing activity of D was linked to a specific molecular weight range, the dialysate was passed through Amicon Diaflo® ultrafilters type UM2 and UM05, allowing the passage of solutes with molecular

weights below 1000 and 500 daltons[1], respectively. The fractions with mol. wt. below 1000 and 500 daltons and the solute retained by the UM2 filter and redissolved in distilled water (mol. wt. above 1000), were tested for their nitrite reducing ability.

Determination of free nitrite

The determination of free nitrite was carried out with Griess reagent according to the ISO method (1971). Extinction was measured at 527 nm.

Determination of SH content

The content of SH groups in the samples under study was determined by direct amperometric titration with 0.001 M $AgNO_3$ in a Radiometer autoburet assembly, as described earlier (Tinbergen, 1970).

Nitrite depletion in heated model systems

In 4 ml screw-capped glass vials 1 ml nitrite-phosphate buffer (0.3 M NaH_2PO_4, 9% NaCl and 0.03% $NaNO_2$) was mixed with 2 ml sample by passing through oxygen-free nitrogen via an injection needle. In all depletion experiments in heated model systems the buffer pH was varied between the values 5.5; 6.0; and 6.5. The vials were closed with a rubber stopper. The stopper had a cross-groove allowing the needle to remain in position during closing, thus avoiding air enclosure. The needle was then pulled out and the stopper held in position by tightly screwing the cap. The vials were heated for 20 min at 95 °C in a waterbath and cooled to room temperature. They were stored at 18 °C until further analysis. Control samples contained 1 ml nitrite-phosphate buffer + 2 ml H_2O.

Formation of cured meat colour in a model system

In a Thunberg tube, 2 ml of the sample under study (pH adjusted to 5.5), 0.1 ml antibiotics solution (40 000 iu Penicillin-G + 10 000 u Polymixin B sulphate per ml H_2O) and 0.2 ml horse-heart metmyoglobin (25 mg per ml H_2O) were gently mixed. The tube was closed with a hollow sidearm containing 0.5 ml $NaNO_2$ (560 mg/l) in H_2O. The tube was evacuated after which the contents were mixed and placed in an Optica CF4R spectrophotometer. The rate of nitrosomyoglobin (NO-Mb) formation was followed by the increase in absorption at 578 nm, corresponding to the maximum of the α-peak of NO-Mb.

Nitrite depletion in a comminuted beef product with added dialysate

A comminuted beef product was prepared with a composition of 1.5% fat, 17% protein and 73% water. In a similar product a part of the added water was replaced

1. 1 dalton = $1.660\,41 \times 10^{-27}$ kg.

by the dialysate D in such a way as to double the concentration of the low-molecular water-soluble meat fraction. Both products contained 0.02% $NaNO_2$ and were heated for 70 min at 100°C. Nitrite depletion as a function of the time of storage was studied.

Results and discussion

The nitrite depletion during heating and 24h storage at 18°C under oxygen-free conditions by the dialysate D and D fractions obtained by ion exchange and ultrafiltration is given in Table 1.

It can be seen that a water-soluble dialysable fraction D of beef muscle strongly reduces the $NaNO_2$ content in a heated anaerobic system, the activity being pH-dependent. When amino acids and lower peptides are removed from D the depletion is markedly diminished. However, as can be seen in Fig. 1, during further storage of the vials without amino acids (D-A), the nitrite content slowly decreases suggesting the possible reducing activity of a non-amino acid compound still present in the D-A fraction and not absorbed on the Dowex column.

From the data in Table 1 for the A fractions and the A lines in Fig. 1 it can be seen that A has a strong reducing activity although less than that of the recombined fractions R. Compounds active in nitrite depletion can be expected to have molecular weights not exceeding 500 daltons.

The ability of D to reduce nitrite to nitric oxide to form NO-Mb under anaerobic conditions is shown in Fig. 2. The NO-Mb formation is plotted as a function of time for the fraction D and dilutions with water 1:1, 1:4 and 1:8, respectively. The reaction rates for the corresponding dilutions can be compared by estimating the slopes of the linear parts of the curves. The resulting ratios are 1:0.56:0.24:0.1, respectively. This means that the reaction rate is proportional to the dialysate concentration. According to Fox & Ackerman (1968) this would make the involvement of a sulphydryl compound (e.g. cystein) unlikely, for they found the NO-Mb formation to be proportional to the square root of the concentration of cystein. However, when the SH-alkylating reagents vinylpyridine (VP) or N-ethylmaleimide (NEM) are added to a mixture of dialysate D,$NaNO_2$ and metMb, the formation of

Table 1. Percentage of free nitrite content remaining in buffered mixtures of nitrite and dialysate heated at 95°C for 20 min under oxygen-free conditions after 24 h storage at 18°C.

pH	Control	D	D-A	A	R	Mol.wt.>1000	Mol.wt.<1000	Mol.wt.<500
5.5	100	32	76	30	33	99	37	43
6.0	100	71	93	66	59			
6.5	100	89	98	79	81			

control : 1 ml nitrite-phosphate buffer + 2 ml H_2O
D : water-soluble low-molecular fraction of minced beef muscle
D-A : fraction D without amino acids and lower peptides
A : amino acid fraction of D
R : recombined fraction of D-A and A.

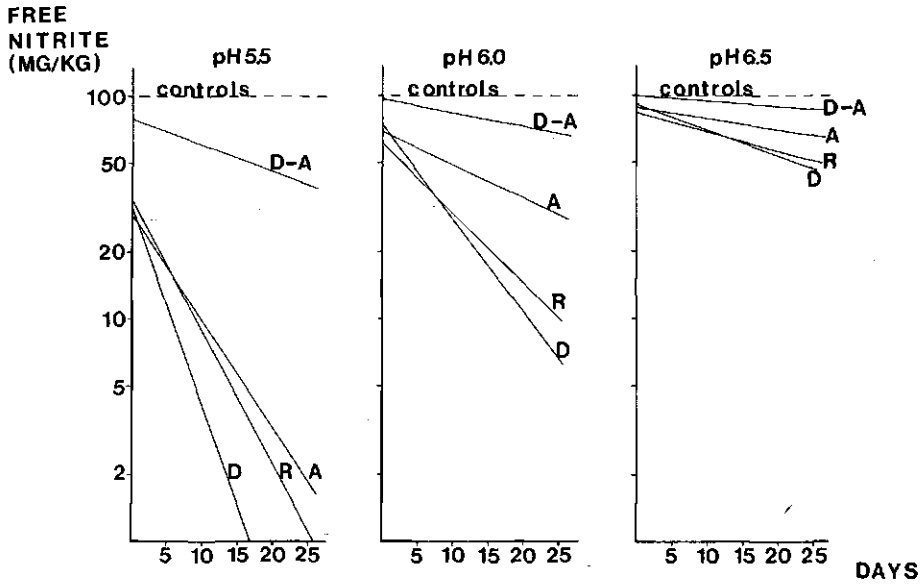

Fig. 1. Nitrite loss at different pH-values in solutions, stored at 18°C after cooking.

NO-Mb is partly inhibited, as can be seen in Fig. 3. It justifies the assumption that the SH/SS redox system is involved in the nitrite reduction. Cystein for example is known to be very reactive towards nitrite compared with other amino acids (Mirna & Hofmann, 1969; Olsman & Krol, 1972).

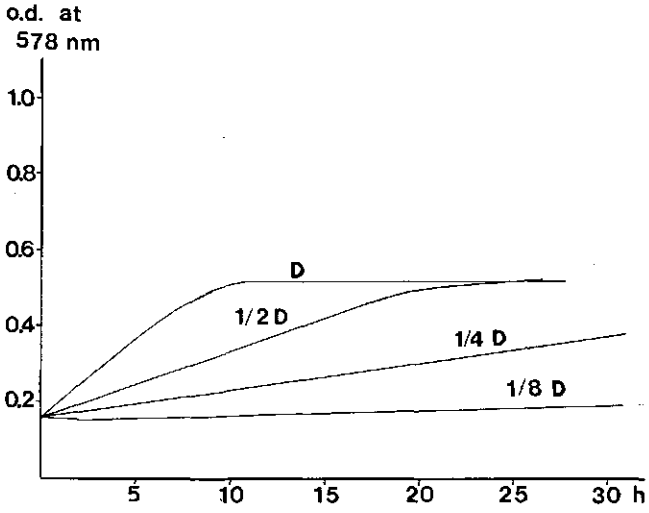

Fig. 2. Formation of NO-Mb under anaerobic conditions by different concentrations of dialysate D.

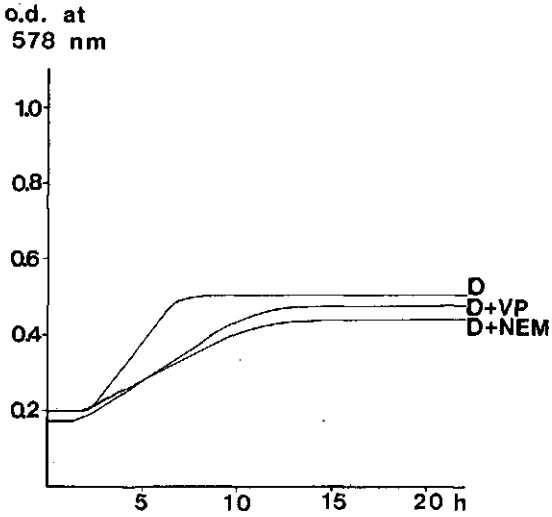

Fig. 3. The effect of SH-alkylation on the formation of NO-Mb under anaerobic conditions. D= dialysate; VP= vinylpyridine; NEM= N-ethylmaleimide.

The SH content of the dialysate, as determined by amperometric titration with $AgNO_3$ (Tinbergen, 1970), was found to be 2.3 – 2.5 mM SH. Reduction with 4 ml 5 mM $Na_2S_2O_5$ (pH adjusted to 7.5) raised the SH content to 4 –4.5 mM SH. This indicated the presence of 2 mM SS. If the dialysate is considered to have a

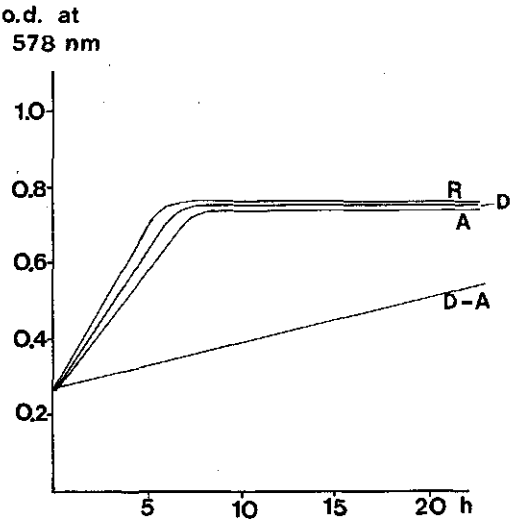

Fig. 4. Formation of NO-Mb under anaerobic conditions by D fractions obtained by ion-exchange.

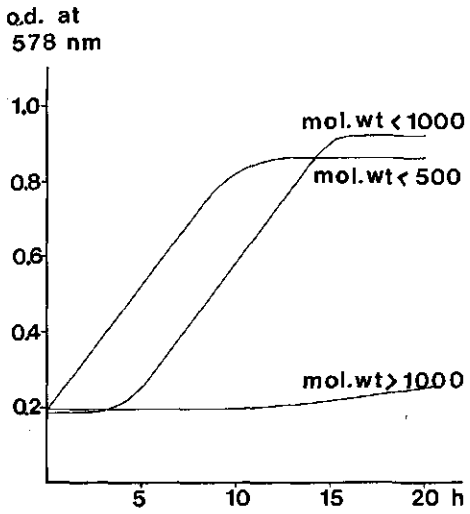

Fig. 5. Formation of NO-Mb under anaerobic conditions by D fractions obtained by ultrafiltration.

concentration 5 times as high as the water-soluble fraction of beef muscle, this content of 4 – 5 μmol SH + SS per ml dialysate is in fairly good agreement with the reported glutathione content in muscle tissue (Olsman & Krol, 1972). Only a part of the SH + SS content of D was detected in the fraction with a molecular weight below 500. No explanation for the observed reduction can as yet be given.

Removal of amino acids from D by ion-exchange resins strongly reduces the rate of NO-Mb formation as can be seen in Fig. 4. Recombination of the D-A and A fractions restores the original nitrite reducing activity. The residual activity of D-A demonstrates the presence of a non-amino acid compound involved in the NO-Mb formation in vitro.

As can be seen from Fig. 5 the colour forming ability of D is associated with a fraction with a molecular weight below 500. This is in good agreement with the findings on nitrite depletion in heated vials, reported above. The addition of dialysate to a comminuted meat product – thereby doubling the concentration of low-molecular water-soluble fraction – resulted in a significant increase of the reaction rate constant for the nitrite depletion. However, the increase was only 20%. Lowering the pH of the product from 6.24 to 5.75 did not change the effect. Olsman & Krol (1972) found that the addition of glutathione increased the reaction rate, although not to a significant extent.

The conclusion can be drawn that the nitrite reducing activity of the dialysate can be ascribed to both an amino-acid or lower peptide – probably with an SH group – and a non-amino acid compound.

Further investigations on the nature of the compounds involved should be carried out.

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Determination of volatile amines and amine oxides in food products

A. Ruiter

Institute for Fishery Products TNO, IJmuiden, the Netherlands

Abstract

A method for the analysis of trace amounts of volatile amines in foods is described. The amines are extracted and steam distilled; part of the concentrated distillate is fractionated by gas chromatography on Carbowax 400 + polyethylene imine. Amine oxides, after reduction with $TiCl_3$, can be determined in the same way. Under the conditions described, trimethylamine (TMA) and dimethylamine (DMA) could be easily separated. Traces of DMA were distilled quantitatively if ethylamine was added before distillation.

Some results of analysis in various foodstuffs are given.

Introduction

In many foodstuffs there are compounds that may be nitrosated if nitrite is present. In this study the possible presence of one group of substances that can be nitrosated: the volatile dialkyl and trialkylamines, in various foodstuffs was studied quantitatively. Amine oxides, which are easily reduced to the corresponding amines, were taken into consideration as well.

The rate of nitrosation for tertiary amines is much less than for secondary amines (Wolff, 1972). The nitrosation rate for amine oxides is not yet known, but, under certain conditions, these components tend to break down, e.g., to a secondary amine and formaldehyde (Sundsvold et al., 1969, 1971).

Until now, few concrete data have been available on the amount of these amines in food products, though it has been known for a long time that they are present in fish and fish products. Some years ago, Ito et al. analysed secondary amines in various foodstuffs. Their data were reviewed by Möhler (1972). The amines were converted into nitrosamines and analysed with the aid of a modified Griess reagent. Part of the nitrosamines were qualitatively identified by t.l.c.

Recently a method for determining both secondary and tertiary amines by gas chromatography was developed at our Institute. In fact, all volatile bases, with the exception of ammonia, can be analysed in this way. Amine oxides are determined by $TiCl_3$ reduction to the corresponding amines before gas chromatography.

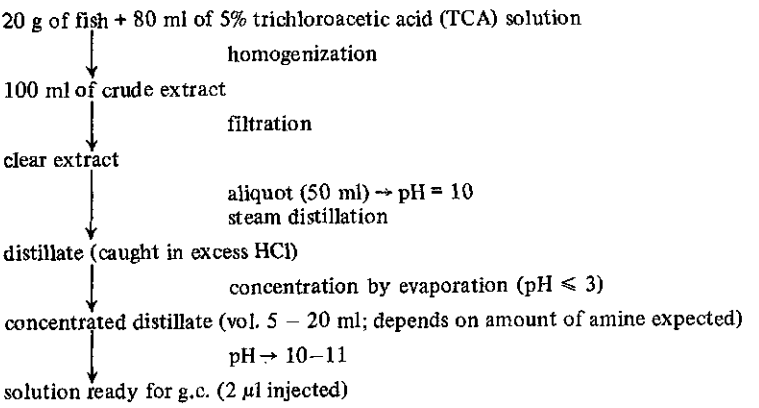

Fig. 1. Pre-treatment of fish samples for gaschromatographic determination of trimethylamine (TMA) and dimethylamine (DMA)

Description of the method

The method is a modification of the gas chromatographic determination of trimethylamine (TMA) and dimethylamine (DMA) in fish, and is described in detail elsewhere (Ritskes, 1973). The pretreatment of the sample is shown in Fig. 1. For the analysis a Hewlett-Packard Model 5700 gas chromatograph was used, the injector block temperature being 150°C and the detector temperature 200°C. Separation took place on a 1.83 m long glass column of 3.18 mm i.d. packed with 15% Carbowax 400 + 5% polythylene imine (Supelco, Inc.) on Chromosorb W-NAW (100-120 mesh), and provided with a ∿ 5 cm long pre-column of 3.18 mm i.d. (20% Dowfax 9N9 and 15% KOH on Chromosorb W-NAW 100–120 mesh). The column was maintained at 40°C and the pre-column at 150°C. The helium carrier gas flow rate was 20 ml/min. Retention times and peak areas were measured with a Hewlett-Packard 3370A electronic integrator.

A chromatogram is shown in Fig. 2. There is a satisfactory separation between the TMA and DMA peaks. Without the use of the pre-column the peaks are considerably broader and some overlap may occur.

If the procedure is not standardized thoroughly the injection of water on the column may cause some problems (e.g., splitting-up of TMA and particularly DMA peaks, and some loss of reproducibility). For the same reason it is necessary to keep the pre-column temperature at 150°C. The pH of the samples should be between 10 and 11.

It is worth mentioning that the use of an alkali flame ionisation detector (AFID) was found to be impracticable for several reasons (Ruiter & Ritskes, 1970):

– For optimum sensitivity (and for selectivity as well) the AFID needs a high detector temperature (400°C). For an acceptable ratio of signal : noise a relatively high (60 ml/min) flow rate is required. Both requirements are difficult to combine

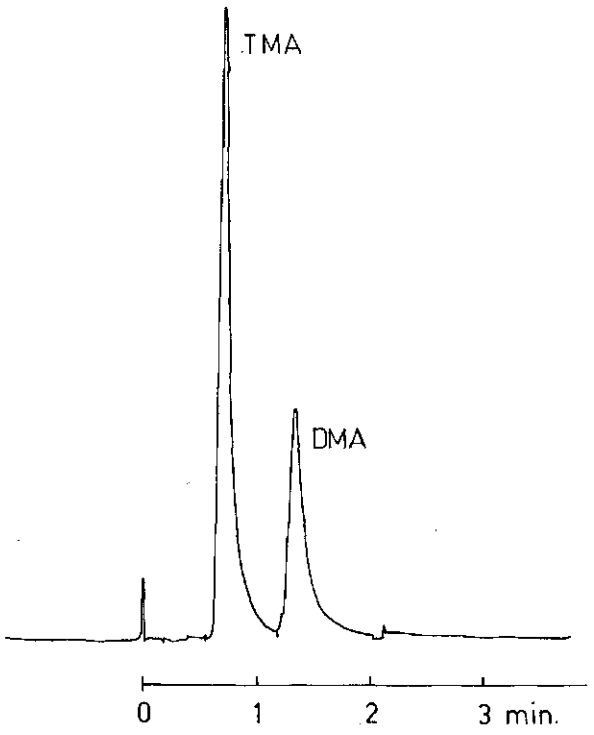

Fig. 2. Separation of TMA (45 ng) and DMA (30 ng). 15% Carbowax 400 + 5% polyethylene imine on Chromosorb W-NAW; FID; injection size 2 µl. Column temperature 40°C. He flow: 20 ml/min.

with the conditions necessary for optimum separation of the methylamines (column temperature 40–50°C; flow rate not exceeding 15–20 ml He/min).

– For amine separation, polyethylene imine was found to be an excellent column coating component, preventing tailing of the amines almost completely. However, it gave a large background signal with the AFID, thus making a quantitative determination of trace amounts impossible.

– We observed that injection of aqueous solutions strongly affect the base-line stability if an AFID is used.

TMA and DMA

With the method described, both TMA and DMA can be determined in concentrations down to about a concentration of 10 mg/kg in the product, which is sufficient for the analysis of these components in fish.

In this project, however, amine concentrations in the range of a few mg/kg had

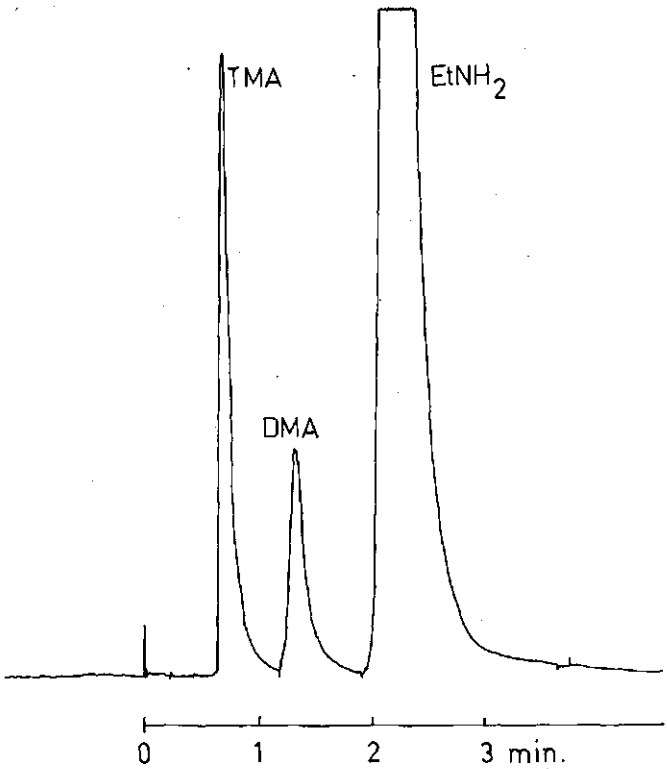

Fig. 3. Separation of TMA (45 ng), DMA (30 ng) and ethylamine. Conditions as in Fig. 2.

also to be taken into account and therefore the sensitivity of the method had to be increased. This increase could be easily achieved by a further concentration of the distillate (e.g., to 1 ml). Because of this concentration and the increased sensitivity of the detector system it proved possible to determine these amines even at a level under 1 mg/kg. However, steam distillation of microgram amounts of DMA in particular was found to be incomplete. Therefore a distillation aid had to be added to improve the yield of distilled DMA. It was found that the addition of another volatile nitrogen base before distillation satisfactorily solved the problem. Ammonia could be used for this purpose, but larger amounts of this component tend to influence the chromatogram. Ethylamine was found to be more suitable because of its good separation from the other amines, as is shown in Fig. 3.

Addition of 1.0 mg of ethylamine had the following effect on the yield of DMA:

Amount of DMA	Yield without $EtNH_2$	Yield with $EtNH_2$
10 µg	∿ 65%	∿ 115%
50 µg	∿ 75%	∿ 95%
100 µg	∿ 85%	∿ 95%

Obviously the method cannot distinguish between primary, secondary, and tertiary amine peaks. If unknown peaks are found beyond the TMA/DMA region, a further identification is necessary.

Amine oxides

The most common amine oxide, trimethylamine oxide (TMAO), was found to be easily reduced by titanous chloride. When small amounts of TMAO were left with 0.3% $TiCl_3$ (1 ml of 15% $TiCl_3$ in 50 ml of TCA extract) at 50°C for hours, more than 95% was reduced to TMA. Thus, amine oxides were reduced in a part of the TCA extract, before the pH was raised to 10 and the extract steam distilled. The results are compared with those obtained without the reduction step, and, from the differences observed, the amount of amine oxides can be calculated.

An alternative method, i.e., removal of volatile amines before reduction of the amine oxides, was not studied further.

Results and discussion

Some results of analysis are given in Table 1. It is shown that most of the products examined are extremely low in both TMA and DMA.

Other amines are not found in appreciable amounts except in orange juice which contains a volatile base with the same retention time as ethylamine. Calculated as $EtNH_2$ the concentration is about 5 mg/kg. I did not investigate this component further.

TMAO is found in all marine fish species. During loss of freshness it is partially reduced to TMA by bacterial action.

An enzymatic breakdown of TMAO to DMA and formaldehyde occurs in several gadoid species. This breakdown is not completely inhibited during frozen storage and, for this reason, frozen cod or coal fish may contain some DMA.

The DMA formation in some fish species was discussed, amongst other topics, in two recent reviews (Yamada, 1968; Ruiter, 1971).

Table 1. Results of analysis of TMA, DMA and TMAO (mg/kg) in various food products. The total amount of volatile bases (mainly NH_3) is also given, and expressed in mg nitrogen per 100 g.

	TMA	DMA	TMAO	Total volatile bases
Tomato juice	0.4	0.2	0.0	11
Orange juice	0.4	0.2	0.0	3
Old Gouda cheese	1.0	0.6	0.0	50
Banana	0.0	0.2	0.0	2
Minced meat (pork+beef 1:1)	2.5	0.4	0.0	5
Canned mackerel	550	145	265	50
Canned herring	345	100	520	36
'Maatjes' herring	50	20	1510	19
Fresh cod	8.4	16	3240	13.6
Cooked cod	195	83	2860	21
Fried whiting	840	67	570	39

The presence of DMA in some fishery products can be explained by partial TMAO breakdown during heat processing (Sundsvold et al., 1969, 1971).

Now the question arises whether because of the concentrations of DMA and TMA found in processed fish there is a risk that considerable amounts of dimethylnitrosamine will be produced in fishery products when nitrite is present.

To answer this question, it is important to look at the composition of the volatile bases as a whole. To these volatile bases TMA as well as DMA only contribute a small part. Most is ammonia, and this component has a strong affinity for nitrite. Ammonia and nitrite readily produce nitrogen and water under conditions that might permit nitrosation, thus possibly preventing, reducing, or retarding nitrosamine formation. Therefore the effect of ammonia in nitrosation should be studied both in model systems and in food before it is possible to give any opinion about the presence of DMA in heatprocessed fish products in relation to the nitrosamine problem.

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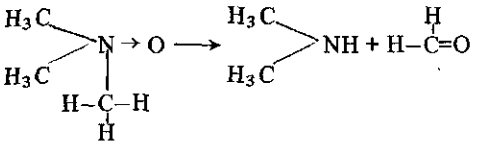
Discussion

Recovery of methylamines from fish

The recovery of TMA and DMA from meat products was not studied, but recovery from fish – even from fat fish – is complete, if the amines are allowed to protonize completely in the acid medium. In doing so, there is no risk that they are partially retained in the lipid phase.

Breakdown of TMAO

The breakdown of TMAO proceeds in the following way both chemically and enzymatically:



TMAO can also be reduced chemically to TMA under the same conditions.

TMA as an indicator for freshness

According to the author, TMA is not useful as a standard for the grading of fish freshness, although it has been proposed for this purpose several times. Too many factors influence the TMA value.

Amines of high molecular weight

Amines of higher molecular weight may be extracted more completely by perchloric acids, but their presence was not revealed in the trichloroacetic acid extract; they must be of minor importance.

Monoethylamine

Monomethylamine was detected in minute amounts in fish and fish products. Because of the addition of monoethylamine to the distillates of other foodstuffs including meat, it was impossible to detect monoethylamine. According to Mottram both DMA and TMA increase from 200 μg/kg in freshly slaughtered meat to about 600 μg/kg after curing, whereas monomethylamine reaches values of 2 mg/kg, which values decrease during curing.

Spores of nitrosamines

It was remarked that in fresh sablefish the presence of nitrosamines in amounts of some μg/kg has been confirmed twice by mass spectrometry.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen.

Determination of volatile nitrosamines: a review

G. Eisenbrand

Institut für Toxicologie und Chemotherapie, Deutsches Krebsforschungszentrum, Postfach 449, D6900, Heidelberg, W-Germany

Abstract

Volatile N-nitrosamines are of considerable interest to environmental toxicologists and the potential danger represented by their presence in foods today is fully recognized. Their presence at low concentrations has been demonstrated in a limited range of foods, especially of animal origin, such as meat products, fish and cheese (Fazio et al., 1971; Wasserman et al., 1972; Sen, 1972).

Despite the wide range of techniques available to the analytical chemist, the problem of separation and analysis of nitrosamines has proved to be far more difficult than originally anticipated. The greatest difficulties in the development of reliable analytical techniques have been encountered in the enrichment and isolation of nitrosamines from samples in sufficient purity to allow an undisturbed determination. This situation is reflected in the wide range of different methods that have been developed for purification and final analysis. It makes it desirable to review and assess the whole field.

Methods of isolation

Most published methods rely on a few basic techniques which are usually modified slightly.

Solvent-extraction techniques

Because of its superior properties, dichloromethane has become the standard solvent for nitrosamine work. It has been used either for direct extraction of food samples in a Soxhlet or blender (Marquardt & Hedler, 1966; Thewlis, 1967; Eisenbrand, 1971; Sen, 1971; Sen et al., 1972) or for liquid – liquid extraction of steam distillates (Crosby et al., 1971; Alliston et al., 1972; Sen et al., 1972; Telling, 1972).

Methods depending on distillation

Many investigators have taken advantage of the volatility of nitrosamines when attempting their isolation from biological materials. The first to use this technique were Heath & Jarvis (1955) when they separated dimethylnitrosamine (DMNA) from animal tissues by steam distillation of the alkaline homogenate.

Eisenbrand et al. (1970) examined the distillation rates of 16 different nitrosamines. No significant differences in the respective yields were found on distillation at reduced or atmospheric pressure from alkaline, acidic or neutral media. Casselden et al. (1969) applied fractional distillation to increase the concentration of aqueous solutions of nitrosamines after the addition of methanol and salt. The nitrosamine-rich fraction was collected between the alcohol and aqueous phase, concentration factors of up to 30 could be obtained for nitrosamines of low molecular weight. Fractional distillation has also been used by Crosby et al. (1971) when examining East African spirits for the presence of nitrosamines. Vacuum distillation has been applied with different results by many workers for nitrosamine enrichment (Lydersen & Nagy, 1967; Devik, 1967; Heyns & Koch, 1971; Williams et al., 1971; Scanlan & Libbey, 1971). One of the most advanced modifications is probably the vacuum distillation technique described by Telling et al. (1971) and Telling (1972). They distilled from an only weakly alkaline medium (4% potassium carbonate) at moderate temperatures (65°C). Recoveries of even highly volatile nitrosamines have been found to be very satisfactory.

An enrichment technique that is frequently used consists of a combination of an alkaline-methanolic digestion of the sample with subsequent dichloromethane extraction of the digest, followed by distillation of the dichloromethane extract (Howard et al., 1970; Fazio et al., 1972). This technique has also been shown to be very efficient for nitrosamine isolation from samples.

Methods of further purification

Chromatographic techniques such as preparative thin-layer chromatography and column chromatography on various sorbents have been frequently used. Marquardt & Hedler (1966) applied multiple thin-layer chromatography on silica-gel plates to isolate nitrosamines from extracts of flour. Other workers have stated that losses of volatile nitrosamines under these conditions are extremely high (Schuller, 1969; Sen et al., 1969; Eisenbrand et al., 1970). Under certain conditions, t.l.c. can be successfully applied as a purification step, resulting in a preseparation of nitrosamines in groups of different chromatographic mobility. Separations which are carried out at 4°C under light protection on a silica-gel layer of 0.6 mm thickness result in recoveries of better than 90%, even for highly volatile nitrosamines (Eisenbrand et al., 1970). Howard and co-workers (1970), when they examined smoked fish for the presence of dimethylnitrosamine, used an acid-treated celite column for purification of concentrates. This technique has been abandoned in this experiment in favour of a more versatile silica-gel column purification (Fazio et al. 1972). Wasserman et al. (1972) used acid-treated florisil instead of celite. Sen, in some of his investigations (1972), tried polyamide for the same purpose. Telling (1972) obtained good results by purifying concentrates on a column of neutral alumina. In the purified eluates the nitrosamines were oxidized to nitramines and these again subjected to column chromatography on neutral alumina. The resulting final solutions were very pure. Recoveries after every chromatographic step were from 77–98% for different nitrosamines.

Methods of detection and estimation

In view of the high significance for public health, identification and quantitative determination of nitrosamines in food must be reliable and unequivocal even at a concentration of say, a μg/kg. Various methods have been developed and applied but only a few fulfill the necessary requirements. They can be divided into two classes: 1. methods for direct determination of intact nitrosamines and 2. methods for derivative formation.

Direct determination of intact nitrosamines

Because of its simplicity, t.l.c. has often been used for nitrosamine detection. Preussmann and co-workers (1964) developed this method as a simple and convenient tool for rapid nitrosamine analysis. Detection was carried out by u.v.-irradiation and spraying with Griess reagent or diphenylamine-palladium (II) chloride. Kröller (1967) localized substances with ninhydrin. Sen et al. (1972) applied Griess and ninhydrin reagents for semiquantitative determination of volatile nitrosamines in alcoholic beverages.

Many methods have been used for quantitative nitrosamine determination, among them polarography, u.v. spectrometry, colorimetry and gas chromatography. U.v. spectrometry can only be used for pure nitrosamine solutions since u.v. spectra are very easily disturbed by interfering compounds, especially in the range of 230–240 nm. Polarography as a very sensitive method has been used by Heath and Jarvis (1955) for dimethylnitrosamine estimation in animal tissues. Some workers have tried to improve the specificity of the method by differential polarography of unirradiated and irradiated samples (Walters et al., 1970). Colorimetric methods relying on photolytic splitting of the N-NO bond were developed by Daiber & Preussman (1964) and by Sander (1967). Eisenbrand & Preussmann (1970) took advantage of the facile acid-catalysed denitrosation of N-nitrosocompounds for the development of a simple, rapid and sensitive colorimetric technique. It has proved to be superior to any other colorimetric method and may even be used to determine molecular weights of pure unknown nitrosamines.

The final determination of nitrosamines in purified sample extracts has been carried out mostly by gas-liquid chromatography. Stationary phases were of medium to strong polarity such as carbowax 1540, (Rhoades & Johnson, 1970), Reoplex 400 (Sen et al., 1969), polyethylene glycol 4000 (Serfotein & Hurter, 1966), Carbowax 1000 (Du Plessis et al., 1969), diethylene glycol adipate (Saxby, 1970), Carbowax-KOH (Howard et al., 1970), Carbowax 20-M-TPA (Fiddler et al., 1971), Ucon LB 550-KOH and Marlophen 87 LWH-KOH (Heyns & Röper, 1970).

Although g.l.c. is a very powerful technique for the separation of mixtures, it should be applied with great care for the identification of substances. For example, Devik (1967) claimed to have identified diethylnitrosamine in roasted potato starch spiked with glucose and amino acids. His findings caused great concern. Heyns & Koch (1971) repeated the experiment of Devik and demonstrated by capillary g.l.c. and mass spectrometry that pyrazines had been confused with nitrosamines.

The introduction of nitrogen specific detectors was a great step forward in nitrosamine trace analysis. Howard et al. (1970) used a modified thermionic

detector for DMNA analysis and Fazio et al. (1971) modified the procedure of Howard to a multidetection method for volatile nitrosamines, giving reproducible results with recoveries between 70–100% at a level of 10 μg/kg. Thermionic detectors have also been used by Fiddler et al. (1971), Crosby et al. (1972) and Wasserman et al. (1972).

Rhoades & Johnson (1970) used the Coulson electrolytic conductivity detector in the pyrolytic mode to analyse cigarette smoke condensates for the presence of nitrosamines by oxidation with peroxytrifluoroacetic acid. A very impressive the nitrosamines, but in the pyrolytic mode distinct nitrosamine peaks could be obtained. Sen (1972) also used the Coulson detector in the pyrolytic mode. He mentioned that although other nitrogen compounds also respond to the CECD, the technique is simple and useful for rapid screening. Crosby et al. (1972) used this detector in the reductive mode for detection of nitrosamines from fried bacon, fish and meat products. Despite the much better selectivity obtained with these specific detectors, all workers have emphasized the need for confirmation of positive results by mass spectrometry. Results have been confirmed in a number of cases, usually where the concentration of nitrosamine in the sample was at least in the range of 10 μg/kg (Fazio et al., 1972; Sen, 1972; Crosby et al., 1972; Wasserman et al., 1972). Concentrations far below this level were too low to be confirmed by Mass spectrometry.

Bryce & Telling (1972) reported on the use of high resolution mass-spectrometry for semiquantitative nitrosamine analysis. They monitored the g.c.-effluent for molecular ions or other characteristic ions of nitrosamines at a resolution of 10 000 and obtained much better sensitivities than which an earlier published method, where they monitored the NO^+ ion (Telling et al., 1971).

Formation of nitrosamine derivatives

Sen (1970) and Althorpe et al. (1970) developed procedures for the sensitive electron capture determination of N-nitro amines produced from the corresponding nitrosamines by oxidation with peroxytrifluoroacetic acid. A very impressive improvement of this method has recently been published by Telling (1972) who also utilized peroxytrifluoroacetic acid as the oxidizing agent and applied two column chromatographic steps for purification. Since the N-N bond of nitrosamines remains intact, the method ensures good selectivity. Another method to prepare suitable derivatives has been found by Eisenbrand and Preussman (1970) by the acid catalysed denitrosation of nitrosamines to secondary amines. These were reacted with heptafluorobutyryl chloride (HFB) and the resulting HFB-derivatives were detected by electron-capture gas chromatography (Eisenbrand, 1972). Since the amines could be selectively isolated by an ion-exchange step, interfering compounds were quantitatively removed. One advantage of the method is its suitability for gas chromatography – mass spectrometry measurements since HFB-derivatives can be easily detected in the mass spectrometer by monitoring the C_3F_7 fragment, which is abundant and characteristic for this class of compounds.

Alliston et al. (1972) split nitrosamines electrochemically under basic conditions and used essentially the same technique for HFB-derivative formation as Eisenbrand. Pailer and Klus (1971) when analysing tobacco smoke condensates for

nitrosamine content, split nitrosamines with cuprous chloride in hydrochloric acid and detected corresponding amines by electron-capture gas chromatography of their trifluoracetyl derivatives.

Reduction to hydrazines has also been used for nitrosamine analysis. Neurath et al. (1965) reduced with lithium aluminium hydride and prepared the corresponding 5-nitro-2-hydroxybenzal derivatives which could be separated by t.l.c., but the yields obtained from tobacco smoke condensates were extremely low. A modification has recently been proposed by Yang & Brown (1972) who condensed the hydrazines with 9-anthraldehyde to form highly fluorescent compounds, but this has only been tested with pure compounds. Hoffmann & Vais (1972) obtained better results in the reduction step by the use of diboran instead of $LiAlH_4$. They condensed with 3,5-dinitrobenzaldehyde and determined the resulting hydrazones by electron-capture gas chromatography.

To summarize the data reviewed here we can say that considerable progress in the analysis of volatile nitrosamines has been achieved during the last years. Some of the methods seem to be well suited as standard procedures; these are mainly those relying on nitrogen specific detection and on formation of suitable derivatives. In this context, the *N*-nitro amine formation seems very promising because it ensures a high degree of specificity. Mass spectrometry nevertheless remains the method of choice for final confirmation.

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Discussion

Methods of nitrosamine analysis

The usufulness of the Coulson electrolytic conductivity detector in the pyrolytic method was demonstrated in a limited number of examples. Very clean gas chromatograms can be obtained from crude extracts of steam distillates without any further purification. In addition some different methods of derivative formation were demonstrated: the Dansyl method and the HFB method.

The need for confirmation of positive results by mass spectrometry was stressed.

Decomposition of nitrosamines

Decomposition of nitrosamines can occur in visible light. This is attributed to the near u.v. radiation, which is part of the sunlight and artificial light sources. The stability of standard solutions of nitrosamines depends greatly on the solvent. Expecially water can promote decomposition. A satisfactory procedure is to use dichloromethane rather than hexane and acetone as a solvent and to prepare the solutions afresh every week.

Handling nitrosamine waste

There are several ways to destroy nitrosamine waste products and to decontaminate glassware, e.g. HBr in glacial acetic acid, aqueous alkaline solutions in contact with aluminium foil, CuCl together with HCl and sunlight.

Groenen stressed the necessity of adequate labeling, safe shipment and handling of N-nitrosamines. In the USA is has been recommended to handle N-nitrosamines in a way comparable to that for radioactive materials.

Nitrosylation of prolylglycine

Prolylglycine in vitro is nitrosylated in the proline ring and presumably also on the N of the peptide bond. This could be confirmed by low resolution mass spectrometry, but not at high resolution, according to Walters.

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The determination of total non-volatile nitrosamines in microgram amounts

C. L. Walters, D. G. Fueggle and T. G. Lunt

Biochemistry Section, BFMIRA, Leatherhead, Surrey, UK

Abstract

Nitrosamines and nitrosamides are denitrosated with thionyl chloride to form a product (presumably nitrosyl chloride) which can be volatilized in a stream of nitrogen and trapped in alkali as inorganic nitrite. At the 10 μg level, the yield of nitrite obtained usually approaches that obtained by denitrosation with hydrobromic acid in glacial acetic acid, which is virtually quantitative in most cases. Compounds tested in much higher amounts for their possible participation in this procedure have included a nitramine, nitramide, *C*-nitric and *C*-nitroso compounds, alkyl nitrites and nitrates, an oxime, azoxy compound, *S*-nitrosothiol, amine oxide and pyrazine; none gave significant release of nitrosyl chloride with thionyl chloride.

This procedure can be applied to the estimation in foods of total non-volatile nitrosamines. Water must be removed, preferably by freeze drying, and during the process volatile nitrosamines will be lost. Any non-volatile nitrosamines within the dried food matrix (for instance 500 g or more) suspended in dichloromethane will be denitrosated with thionyl chloride. Provided the suspension is not too viscous, the passage of nitrogen will volatilize nitrosyl chloride formed, though not always to a theoretical yield. In the absence of water, the percentage interference from inorganic nitrite is very small but detectable. The interference from nitrite increases with the addition of water up to a maximum of about 1.2% when about 1.0% water is present in the reaction medium.

Thus, this method provides a sensitive and selective procedure for the estimation of total non-volatile nitrosamines without the necessity for their extraction from the dried food or other matrix.

Introduction

Most secondary amines, tertiary amines and quaternary ammonium compounds recognized to be present in a biological matrix and to be potential precursors to nitrosamines after reaction with nitrite would give rise to non-volatile compounds. Unlike volatile nitrosamines, however, the non-volatile analogues possess no single physical or chemical property which permits their separation as a group, an essential requirement when estimating low levels of such compounds in the presence of a great excess of other aqueous and lipid soluble material.

An early attempt to separate non-volatile nitrosamines in aqueous extracts of foods, etc. took advantage of the fact that all such compounds examined were absorbed on to activated carbon (Walters et al., 1970). However, the efficiency of desorption of the adsorbed nitrosamines was by no means complete for all com-

pounds even in model systems and the presence of lipid could reduce markedly the ability of the carbon to adsorb.

The introduction by Eisenbrand & Preussman (1970) of the denitrosation procedure of nitrosamines and nitrosamides using hydrobromic acid in glacial acetic acid solution and the subsequent demonstration (Johnson & Walters, 1971) of its specificity to these compounds commended this reaction in the estimation of total non-volatile nitrosamines, since little difficulty should be experienced in removing interfering water from such compounds. Its application, however, would necessitate either preliminary extraction of the nitrosamines from the food or other matrix, a difficult procedure at low levels likely to co-extract interfering substances such as inorganic nitrite. Alternatively, the reaction of nitrosamines in situ in the dried food would require a volume of acetic acid sufficient to cover the matrix effectively and this would restrict markedly the sensitivity of detection of any *N*-nitroso compounds present.

Accordingly, attempts were made to convert non-volatile nitrosamines and nitrosamides into (a) volatile derivative(s) which could be separated readily from a food matrix and interfering compounds such as inorganic nitrite and which could preferably be concentrated in some way to enhance the sensitivity of detection. Initially, the reaction investigated was that with hydrobromic acid followed by distillation of the glacial acetic acid solvent in the hope of carrying over any nitrosyl bromide formed for trapping in alkali but this proved impracticable by virtue of the excessive volume of acetic acid to be distilled and trapped. As a result, attention was directed towards denitrosation to the more volatile nitrosyl chloride.

Materials and methods

Estimation of total non-volatile nitrosamines and nitrosamides

After the suspension and/or solution of the nitrosamine or nitrosamide or of a dried matrix such as a food in dry CH_2Cl_2 (e.g. 200 ml) in a threenecked round bottom flask (usually 500 ml – 1 litre) equipped with a dropping funnel, inlet tube for nitrogen and an exit tube, dry nitrogen is bubbled through the system to displace damp air. If the suspension in use is at all viscous, a stirrer is desirable to ensure as complete a reaction as possible. The addition of 5 ml of a 25% solution of thionyl chloride in dry dichloromethane is completed rapidly from the dropping funnel and the mixture is left to stand for 15 minutes at room temperature. At the end of the reaction period, a steady stream of nitrogen is bubbled through the mixture for 15 minutes, the gas leaving the flask through the third neck and passing through a trap containing 5.0 ml 100% w/v aqueous sodium hydroxide. In order to determine the amount of nitrite produced in the alkali trap, 1.0 ml sulphanilamide solution (5.0% in concentrated HCl diluted with three volumes of water) is added, followed by 1.0 ml concentrated HCl to lower the pH for diazotization. Colour development occurs following the addition of 1.0 ml *N*-(1-naphthyl) ethylenediamine dihydrochloride (0.1% in water) and is allowed to proceed for 15 minutes at room temperature. Then nitrite is determined by the optical density at 540 nm. Blank determinations without the presence of a nitrosamine should give no colour whatsoever.

Source of compounds used

N-nitrosoproline and -hydroxyproline and *N*-nitrososarcosine were prepared by a modification of the method of Stewart (1969). The preparation of *S*-nitrosothioglycolic acid was based upon that of Mirna & Hofmann (1969) for *S*-nitrosocysteine and -glutathione.

Results and discussion

Table 1 presents the percentage production of inorganic nitrite from a number of nitrosamines, nitrosamides and other related compounds after denitrosation with

Table 1. Yield of inorganic nitrite after denitrosation of compound with thionyl chloride in CH_2Cl_2.

Compounds	Amount	Yield(%)
Nitrosamine:		
N-nitrosoproline	10 µg	84
N-nitrosohydroxyproline	10 µg	90
N-nitrososarcosine	10 µg	54
N-nitroso-*N'*-phenylpiperazine	10 µg	81
N-nitrosodiphenylamine	10 µg	85
1,4-dinitrosopiperazine	10 µg	22
Nitrosamide:		
N-nitroso-*N'*-methyl urea	10 µg	40
Nitramine:		
N-nitrodimethylamine	100 µg	0.7
Nitramide:		
N-nitro urea	100 µg	0.0
C-nitro compounds:		
1-nitropropane	100 µg	1.2
C-nitroso compounds:		
1-nitroso-2-naphthol	100 µg	1.0
4-nitrosophenazone	100 µg	0.0
Oxime:		
cyclohexanone oxime	100 µg	0.0
Alkyl nitrites:		
n-bentyl nitrite	5.0 mg	0.5
isopropyl nitrite	5.0 mg	0.7
Alkyl nitrates:		
isopentyl nitrite	10 mg	0.0
ethyl nitrate	10 mg	0.0
Azoxy compound:		
cycasin	100 µg	0.0
Nitrosothiol:		
S-nitrosothioglycolic acid	1.0 mg	0.5
Inorganic nitrite:		
$NaNO_2$	10 mg	0.008
Amine oxide:		
3 hydroxy pyridine-1-oxide	100 µg	0.5
Pyrazine:		
methylpyrazine	100 µg	0.8

thionyl chloride. The percentage release of nitrite in the case of nitrosamines and nitrosamides is based upon that obtained by denitrosation in solution in glacial acetic acid with hydrobromic acid, which normally approaches the theoretical. That of compounds other than nitrosamines and nitrosamides is based upon the weight and nitrogen content.

At the 10 μg level, the recoveries of inorganic nitrite from nitrosamines and nitrosamides were not theoretical in all instances, but were greatly in excess of the yields obtained from the range of related compounds examined when tested at much higher levels. As can be seen from Table 1, virtually no response in terms of nitrite formation occurred with a nitramine, nitramide, *C*-nitro and *C*-nitroso compounds, oxime, alkyl nitrites and nitrates, azoxy compound, nitrosothiol, amine oxide or pyrazine. After drying thoroughly both dichloromethane and inorganic nitrite, the response from 1.0 mg $NaNO_2$ was equivalent to 0.009% falling slightly to 0.008% at 10 mg $NaNO_2$ and thence to 0.001% after the inclusion of 100 mg. The interference in the procedure provoked by an addition of 10 mg nitrite increased with the addition of water up to a value of 1.2% at a water concentration of 1.0% in the 500 ml volume of dichloromethane used. Above this water level, no further increase in the interference from 10 mg $NaNO_2$ was observed, but separation into two phases became apparent. No interference was observed in the Eisenbrand & Preussmann (1970) denitrosation procedure by 5% water but the reaction was completely prevented by 10%, probably by virtue of the easier protonation of water in preference to a nitrosamine.

No interfering chromogens have been found in the alkali trap after the displacement in a stream of nitrogen of nitrosyl chloride from the suspensions in dichloromethane of any of the dried food matrices so far examined. Unlike the denitrosation procedure with hydrobromic acid in glacial acetic acid solution, no interference has been detected in the spectrophotometric determination of liberated nitrite by compounds extracted from the food which sometimes cause asymetry of the peak at 540 nm through the formation of other colours absorbing at shorter wavelengths.

In studying the release of nitrosyl chloride from inorganic nitrite, use has been made of dried casein as a substrate. At a concentration of 0.3 g casein per ml dichloromethane, the suspension was found to be too viscous to allow the volatilization of nitrosyl chloride formed in a stream of nitrogen. However, a suspension of 0.1 g per ml CH_2Cl_2 was sufficiently mobile to permit ready volatilization of NOCl formed and, under these conditions, the estimation of 100 μg N-nitrosoproline was possible without interference from 500 mg sodium nitrite in 50 g casein.

One other method (Fan & Tannenbaum, 1971) has been proposed for the estimation of total non-volatile nitrosamines in a food extract and it has been applied to the autoanalyzer. This technique involves irradiation with ultraviolet light of an aqueous extract of the food etc., with release of inorganic nitrite. Many compounds other than nitrosamines release nitrite on photolysis but specificity has been claimed using monochromatic light of 360 nm wavelength in similar manner to Sander (1967), although some preliminary clean-up may be necessary to remove interference. Other compounds can also reduce or eliminate the formation of inorganic nitrite during photolysis, probably by acting as receptors of the nitrite

released. No interference was, however, apparent from salts, organic and inorganic acids, sugars, nucleoside bases and amino acids.

The desirable features claimed for our estimation of total non-volatile nitrosamines and nitrosamides are as follows:

– Sensitivity: provided the denitrosation system is sufficiently fluid to permit the volatilization in nitrogen of nitrosyl chloride formed, the amount of food or other sample in suspension in dichloromethane is almost immaterial. A concentration factor is thereby introduced equivalent to the ratio of the weight of the original sample to the volume of liquid in which nitrite released is determined.

– Specificity: in the absence of water, little or no interference arises from inorganic nitrite and many other types of compounds from which it is potentially possible to obtain inorganic nitrite. Such interference would give rise to false positives rather than prevention of the observation of nitrosamines present.

– No extraction of non-volatile nitrosamines is necessary with this procedure, thus avoiding a stage of variable efficiency.

– The volatility of nitrosyl chloride formed is such that is can be volatilized readily in a stream of nitrogen, a procedure so mild that the possibility is remote of carry over of food components which could interfere with the determination of nitrite.

Probably the only drawbacks of the technique are the necessity for the drying of the food, with the accompanying possibility of the destruction of nitrosamines and nitrosamides, and the fact that the nitrosamines and nitrosamides detected are not characterized individually. Nevertheless, it should provide a ready screening procedure from which samples with nitrosamine levels of concern can be pinpointed and studied further by appropriate procedures.

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Discussion

Labelling N-nitrosamines

In order to facilitate the observation of the release of non-volatile nitrosamines from meat products, studies have commenced using N-nitrosoproline and N-nitrososarcosine 'labelled' with ^{14}C. At an initial level of 20–50 µg/kg, good recoveries

have been obtained from a number of cured meat products; the products were extracted by agitation in a phosphate buffer with or without the addition of protease; radioactivity entered the aqueous solution.

According to the experience of Dhont (Civo) who had sued the proposed method for total non-volatile nitrosamines, recoveries were sometimes very low and the blank values very high. The author could not confirm this and emphasized that the method was still being studied. The possible interference of chloride and sulphite was discussed. Chloride appeared to have no influence, but the effect of SO_2 has not yet been studied.

Non-volatile nitrosamines

The determination of individual non-volatile nitrosamines is not yet possible, because no reliable isolation and separation are available.

Conclusions and recommendations of the analytical session, 11th September 1973

1. Sufficiently precise methods are lacking for the determination of nitrate and of free and bound nitrite. A number of collaborative studies has and is being carried out on nitrate and free nitrite analyses (e.g. in ISO, EEC, IUPAC, AOAC). It is recommended that the efforts of these various groups be co-ordinated and extended to include the determination of bound nitrite.

2. Further and more specific information is needed on the determination and level in foods of precursors of nitrosamines and their relation to nitrosamine formation.

3. It is now possible to determine volatile nitrosamines in meat products at levels of a few μg per kg (ppb). Positive results have to be compared using adequate methods, such as m.r.

4. More research is recommended on:
a. methods for the determination of non-volatile *N*-nitrosocompounds in foods,
b. the range of compounds of interest, and
c. the nature of their combination in the food matrix.

Microbiological session

Reporters: J. H. Houben, S. J. Mulder, W. J. Olsman, P. S. van Roon

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The microbiological effects of nitrite

M. Ingram

ARC Meat Research Institute, Langford, Bristol, BS18 7DY, UK

Abstract

The inhibitory effects of nitrite are discussed in relation to salt, pH, nitrate, number of bacteria, and storage temperature, in unheated and heated systems. The need for predictive generalisation is emphasized.

Introduction

Use and function of nitrite

Nitrite is used in meat curing, where it has at least the following four functions:
a. It is converted into nitrosomyoglobin which gives the characteristic pink colour to cured meat. It has been known for thirty years that a nitrite content as little as 5 mg/kg meat suffices to give a satisfactory colour for a limited time. It is believed that rather higher concentrations, perhaps up to 20 mg/kg, are necessary to provide commercially adequate colour stability, but detailed experimental confirmation of this is lacking.
b. Nitrite is essential for the characteristic flavour of cured meat; that which distinguishes ham and bacon for example from salt pork. The nature of the changes involved is still not known. To produce the characteristic flavour, some 50 mg of nitrite/kg are thought necessary; satisfactory quantitative evidence is lacking.
c. Curing meat provides an important degree of protection against botulism. It has been believed that more than 100 mg nitrite/kg is necessary to secure this protection, under commercial conditions. But the question proves to be very complex, because the necessary concentration of nitrite depends on several associated factors. Nitrite may provide similar protection against other food poisoning bacteria, e.g. *Clostridium welchii* or staphylococci, but the importance of this has never been assessed.
d. In raw fresh meat, there is protection against these kinds of food poisoning, since hazard does not usually arise until after the meat has gone putrid and the consumer rejects it. Cured meats, however, do not go putrid though they may carry equally large numbers of bacteria as putrid meat, because the curing salts inhibit putrefaction. It is not clear what are the relative contributions of nitrite and other curing

factors (especially salt) to the differences in spoilage; experience suggests that substantial concentrations of nitrite are involved, but again satisfactory experimental data are scarce. It seems important to understand these relations, because they make cured meat less 'perishable' than fresh.

Points (c) and (d) lead to the following general conclusions important to our present theme. In a situation where permissible concentrations of nitrite may be progressively reduced, the problems likely to arise first should be those involving the largest concentrations of nitrite, i.e. the microbiological problems. Though *Clostridium botulinum*, being the most dangerous, is the most important species, it is by no means the only one in question. We have to consider unheated systems, besides heated.

Chemical and biochemical properties

Nitrite has properties which seem important microbiologically:

– It reacts via nitrous avid with amino and other groups in proteins etc. Hence, undissociated form depending directly on the pH:
$\log_{10} \{ (HNO_2)/(-NO_2) \} = pK - pH$, where $pK = 3.4$. The concentration of HNO_2 thus increases roughly ten-fold for every unit fall of pH.

– It reacts via nitrous acid with amino and other groups in proteins etc. Hence, nitrite disappears when added to meat or to culture media, usually roughly exponentially, at rates increased by acidity or high temperature; in meat, Nordin (1969) observed the relation:
$\log_{10}$ (half life in hours) = $0.65 - 0.025 \times$ temp. ($^\circ$C) $+ 35 \times$ pH.
Because of this, it is necessary to add filter-sterilised nitrite in the cold, to obtain precisely defined concentrations in an experimental system. Because the nitrite gradually disappears, there might be opportunity for dormant organisms to grow out later (cf. Ashworth & Spencer, 1972); though it does not necessarily follow that the antimicrobial activity falls correspondingly, for nitrite might be converted into other substances with inhibitory properties.

– As a result of the balance between these two properties, there may be an optimum pH for the anti-microbial activity of nitrite, about 5.5.

– Nitrous acid decomposes to yield HNO_3 and NO, and the latter can be oxidised to HNO_3 by atmospheric oxygen in aqueous systems:

$$3HNO_2 \rightarrow HNO_3 + 2NO + H_2O$$
$$2NO + O_2 \rightarrow 2NO_2$$
$$2NO_2 + H_2O \rightarrow HNO_3 + HNO_2$$
$$\overline{3HNO_2 + O_2 \rightarrow 2HNO_3 + HNO_2}$$

Besides helping to explain how nitrate appears in systems to which only nitrite was added, this may be why nitrite is more inhibitory to microorganisms when oxygen is absent, or in presence of reducing agents.

– Ascorbic acid decomposes nitrite in acid solution. If present during acidification of nitrite-containing extracts for colourimetric estimation, therefore, the estimated quantities of nitrite are too small. This may explain reports that a given

microbiological effect is produced with less nitrite in the presence of ascorbate.

– Nitrite represents one step in a biochemical oxidation-reduction chain, potentially extending from nitrate to ammonia, and potentially reversible. Of the intermediate compounds, hydroxylamine and nitric oxide possess anti-microbial properties, the latter only weakly (Shank, Silliker & Harper, 1962).

$$HNO_3 \text{ —— } \begin{matrix} HNO_2 \\ NO_2 \\ NO \end{matrix} \text{ —— } \begin{matrix} H_2N_2O_2 \\ N_2O \end{matrix} \text{ —— } NH_2OH \text{ —— } \begin{matrix} NH_3 \\ NH_4NO_2 \\ N_2 \end{matrix}$$

Nitrous oxide and nitrogen are common end-products of bacterial denitrification.

– The biochemical mechanism whereby nitrite inhibits bacteria is not known in detail. It appears to be a general metabolic inhibitor (Dainty, 1971–2).

Unheated systems

Interest in the anti-bacterial effects of nitrite developed in the 1920's, from its preservative effect in raw cured meat. But attempts failed to demonstrate a corresponding inhibitory action of nitrite; concentrations of the order ten times greater being needed, even in the presence of 3.5% sodium chloride (Lewis & Moran, 1928; Evans & Tanner, 1934). From subsequent knowledge, this failure is most reasonably explained by assuming that the workers of those days used massive inocula and media of pH above 6 incubated near 37 °C, the importance of those factors not being understood at that time.

It had been observed by Grindley (1929) that nitrite is more inhibitory under acid conditions, and he suggested that this might be due to the presence of nitrous acid. But his observation passed virtually unnoticed, and was re-discovered by Tarr (1941a; 1941b; 1942). Tarr showed that the preservative action of nitrite in fish was greatly increased by acidification; and correspondingly, that the inhibitory effect against several species of bacteria depended on the pH of the medium and increased markedly at levels below pH 6.0. Tarr did not explain the phenomenon, and it was left to Jensen (1945) to repeat Grindley's suggestion that the inhibition might be due to undissociated molecular nitrous acid. The expected ten-fold diminution in the inhibitory concentration of nitrite for unit drop of pH was clearly demonstrated by Castellani & Niven (1955) with *Staphylococcus aureus;* and later confirmed by Eddy & Ingram (1956) using a *Bacillus* species, and by Perigo, Whiting & Bashford (1967) using vegetative *Clostridium sporogenes* PA 3679.

The implication of Tarr's work, and of subsequent investigations, has been that the inhibitory action of nitrite is rather general. Indeed, Tarr listed several genera as being inhibited by 0.02% nitrite at pH 6.0: namely *Achromobacter, Aerobacter, Escherichia, Flavobacterium, Micrococcus,* and *Pseudomonas.* Later investigations have revealed that some bacteria are more resistant: for example salmonellas (Castellani & Niven, 1955); lactobacilli (Castellani & Niven, 1955; Spencer, 1971); and *Clostridium perfringens* among clostridia (Perigo & Roberts, 1968). There is however a lack of detailed information of this kind.

The effects of acidity are confused at low pH levels by the reaction of nitrite

with constituents of the medium, so that its effect begins to diminish again at pH's approaching 5.5 (Henry, Joubert & Goret, 1954). This was confirmed by Shank, Silliker & Harper (1962) who observed that the anti-microbial effect increases with falling pH to a maximum near pH 5.0. Henry et al. (1954) also noted the importance of redox relations, and others have observed that nitrite is more inhibitory under anaerobic conditions (e.g. Castellani & Niven, 1955; Eddy & Ingram, 1956).

It has long been clear that pH influences the inhibitory effects of salt (sodium chloride) as well as of nitrite, smaller concentrations of salt being effective at lower pH (Ingram, 1948; Ingram, 1958). Because the sodium chloride can be partly substituted by other salts e.g. sodium nitrate, and because similar relations with pH apply to the inhibitory effects of sugars in pickling (e.g. Vas, 1957), it appears likely that the effect of sodium chloride relates mainly to water activity, and is only in minor degree specific. Such relations have recently been well discussed by Riemann, Lee & Genigeorgis (1972). A relation between the effects of sodium chloride and of sodium nitrite was implicit in some of the early work (e.g. Evans & Tanner, 1934; Riemann, 1963); but a clear demonstration was only given (so far a I know) some five years ago, when Pivnick, Barnett, Nordin & Rubin (1969) observed that their inhibitory effects were complementary. Though this demonstration was made with heated packs of canned pork luncheon meat containing *Clostridium botulinum* spores, recent research has amply confirmed similar relations in unheated systems. There are clear indications of a salt/nitrite interaction in a recent publication by Wood & Evans (1972) on their preservative effect in curing. Triple combined relations exist between the inhibitory effects of pH, nitrite, and sodium chloride, when inhibiting vegetative cells of several strains of *Clostridium botulinum*, or of *Clostridium perfringens* (Roberts & Ingram, 1973). Similar work by Bean & Roberts (about to be published) demonstrates similar inter-relations in inhibiting *Staphylococcus aureus;* and it appears that the phenomenon may be general.

The work of Roberts' group has further revealed a phenomenon like that observed with spores in heated systems, namely that the minimum inhibitory concentrations are smaller if they are challenged by a smaller number of vegetative cells. The differences have not been great until the number of vegetative cells fell below about 100 per gram; below this level, the effect of cell concentration has been marked. Little is known about the basis of this phenomenon, and more work is highly desirable.

A further important point, emerging in Bean & Roberts' experiments, is the very great influence of incubation temperature. Others have previously demonstrated that minimum inhibitory salt concentrations are less at lower growth temperatures (Segner, Schmidt & Boltz, 1966; Ohye & Christian, 1967; Alford & Palumbo, 1969; Matches & Liston, 1972); and that there is an analogous inter-relation between limiting pH and temperature (Baird-Parker & Freame, 1967). Similarly with the triple combined effects observed by Roberts & Ingram (1973) and Bean & Roberts (unpublished): the minimum inhibitory combinations of salt, nitrite and pH are similar at 25 and at 37 °C, but are much reduced at more normal temperatures of 20 °C and below. Even in unheated systems to which all the foregoing applies, quite high degrees of control are conceivable using practically realistic temperature conditions and combinations of salt, nitrite and pH. What we now need is some

established basis for interpreting quantitatively the combined effects of these several factors, to measure the effect of changing one of them upon the critical levels of the others. The anti-microbial effect of nitrite clearly can no longer be considered in isolation.

Heated systems

Because vegetative cells are destroyed by heating, interest in heated systems is specially related to inhibitory effects upon bacterial spores, especially those of *Clostridium botulinum* and of the more heat-resistant 'indicator' strains of *Clostridium sporogenes* like PA 3679.

This phase of the subject effectively began with the work of Stumbo, Gross & Vinton in 1945, and continued through a series of similar investigations with inoculated packs, conveniently reviewed by Spencer (1966), which together gave the following indications. The major inhibition is due to salt, with some supplementary effect of nitrite; nitrate and sugar were not important. The supplementary effect of heating, to the order F_0[1] = 0.1 to 1.0, is of critical importance. Systems which are inhibitory with 1–10 spores per gram fail when challenged with numbers 100–1 000 times greater. (See Table 1).

This phase culminated in an experiment briefly described by Riemann (1963),

Table 1. Processes shown experimentally to give stable and/or safe systems (after Spencer, 1966).

Reference	Heat process	Sodium chloride concentration (% on water phase)	Initial nitrite concentration (mg/kg total)	Spore level/g	Comments
28	$F_0 = 1$	about 4.5	150	5	spoilage inhibited for up to one year
15	$F_0 = 0.05$ to 1.0	about 4.5–5.0	not stated	natural	viable spores detected after heat processes but no spoilage in 9–18 months
25	150°F/70 min	about 3–3.5	200	5000[1]	no toxin after 30 days at 30°C
31	176°F/20 min	3.6	150 (83)	natural, 1	pH 5.8 ± 0.2. Failed when challenged with 50 spores/g
27	$F_0 = 0.08$	3.5	78 (10–20)	natural, 1	failed when containing only
	$F_0 = 0.10$	3.5	78 (10–20)	added, 2.6	38 mg nitrite/kg
12	$F_0 = 0.4$	4.02	not stated	3	

1. *Cl. botulinum*
The nitrite figures in brackets refer to post-processing levels
References:
28 – Stumbo, Gross & Vinton (1945)
15 – Vinton, Martin & Gross (1947)
25 – Steinke & Foster (1951)
31 – Bulman & Ayres (1952)
27 – Silliker, Greenberg & Schack (1958)
12 – Riemann (1963)

1. F_0 is a term normally used in process calculation work.
$F_0 = 0.5$ means a heat process in effect equal to a heating of 0.5 min at 121.1 °C (250 °F).

statistically planned on a factorial basis, the factors tested being salt, nitrate, nitrite, pH, F_0 value and spore level inoculated. After incubating six months at 30 °C, the number of spores developing was calculated from the numbers of swelled cans. Regarded singly, all the factors except nitrate had a statistically significant effect on the number of spores which developed. The following interactions also were statistically significant: $F_0 \times NaCl$; $F_0 \times NaNO_2$; $NaCl \times NaNO_3 \times NaNO_2$; $NaCl \times pH$; and $NaCl \times NaNO_3$. Several points are noteworthy. Though $NaNO_3$ was insignificant alone, it has a significant effect in interactions. pH alone had no effect, either in this, or in a parallel series of experiments reported at the same time. No interaction of pH with nitrite was revealed.

This kind of investigation, in which one observes spoilage or toxin formation in inoculated packs, has the advantage that it simulates realistic conditions. It has, on the other hand, the disadvantage that it provides little information about the nature of the inhibitory effects. In particular, it does not distinguish between four alternative possible explanations for the inhibitory effect of nitrite, first coherently enunciated by Johnston, Pivnick & Samson (1969):

The enhanced destruction of spores by heat

This possibility was suggested by Jensen & Hess in 1941. It is now largely eliminated, as a result of experiments in which spores have been heated in the absence or presence of curing salts and then transferred to separate growth media with or without salts. So far as sodium chloride and nitrate are concerned, the effect of heating in the presence of realistic concentrations is small, the important element being the presence of the salt in the medium on which the heated spores attempt to develop (Roberts & Ingram, 1966; Duncan & Foster, 1968). As regards nitrite, results are ambiguous; Duncan & Foster claimed that the presence of nitrite during heating increased the heat sensitivity of spores of PA 3679, especially at pH 6.0 (though this is not clear from the data presented); whereas Ingram & Roberts (1971), working with *Clostridium botulinum* strain 33 A at pH 6.0, failed to observe any corresponding phenomenon.

An increased rate of germination of spores during the heat process followed by death of the germinated spores from the heat process

This possibility was soon negatived by observations showing that some spores are in fact not killed as a result of the heating (e.g. Silliker, Greenberg & Schack, 1958; Riemann, 1963). Indeed, Silliker, Greenberg & Schack calculated that 20% of the inoculated spores survived in cured meat packs which nevertheless remained stable; and they then concluded that the failure of those spores to develop was probably the result of an inhibitory effect of the curing salts dependent on heat damage.

Prevention of germination of spores that survived the heat process

This third possibility, stated by Johnston and co-workers, was plainly demonstrated in the already cited work of Roberts & Ingram (1966) and of Duncan & Foster (1968). Large inhibitory effects were observed when spores, heated in

whatever system, were plated out on media containing salt or nitrite. With nitrite, this was especially so at acid pH values, and Roberts & Ingram remarked that the relations suggested the involvement of undissociated HNO_2, by analogy with similar phenomena with unheated bacteria.

Production of more inhibitory substances from nitrite

Up to this point, everybody had ignored the fourth possibility. Then Perigo, Whiting & Bashford (1967) observed that the inhibitory concentrations of nitrite in the experiments of Roberts & Ingram (1966) were of the order 100–500 mg/kg, some ten-fold greater than the concentrations effective in canning practice; and they noted that Roberts & Ingram added filtersterilized cold nitrite, instead of heating nitrite with the supporting medium as is done in canning. They therefore speculated that the nitrite might, when heated in the presence of the supporting medium, be converted into some substances which is a much more powerful inhibitor. To test this hypothesis, Perigo et al. developed an artificial culture medium, portions of which they could adjust to various pH values; to one set they added nitrite without heating, and to another they added nitrite and then heated, then challenged both with inocula of 8×10^6 vegetative cells of PA 3679. Much smaller concentrations of nitrite were indeed effective in the heated system. There were two other notable observations: first, that with medium at pH 6.0 heated for 20 minutes, it was necessary to heat in the temperature range 95–125 °C, but that in the range 100–110 °C the enhancement became ten-fold or more; and second, this effect is much less pH-dependent than that of nitrite. The conclusion was that a small proportion of the added nitrite turns into a different substance at least ten times more inhibitory than nitrite; and this presumed substance has since come to be called the Perigo Factor or Inhibitor (PF).

Not long after, Johnston, Pivnick & Samson (1969) addressed themselves to the same possibility, but in a different manner. They blended meat with the culture medium and heated with the various concentrations of nitrite up to 200 mg/kg, then challenged with *Clostridium botulinum*. The inhibition was found to be no greater than that expected from the residual nitrite in the substrate; in addition, the inhibitory substance in meat was dialysable, while PF in culture medium was not; these facts suggested that the inhibitory substance in the meat system was nitrite itself. Further, using a culture medium in which PF was formed by heating with nitrite for 20 min at 110°C, they found that additions of 20% or more of meat prevented the development of PF and that as little as 1% of meat interfered with its development. Finally, they observed that addition of meat to culture medium in which PF was already present had the effect of neutralising its activity. These observations, on the absence of PF in the meat system, and the ability of meat to neutralise pre-formed PF, have been confirmed independently by Dr T. A. Roberts. The conclusion seems to be that the Perigo Inhibitor is not produced during the heat treatment of meat containing nitrite, and cannot explain the role of nitrite in the stability of canned cured meats.

Since that time, there has been an apparent dichotomy between work in culture media and work in meat systems, and the two are best considered separately, because it is not clear what may be the relation of one with the other.

Table 2. Inhibitory concentrations (mg/kg) at pH 6 of sodium nitrite and heated sodium nitrite (Roberts, 1971).

	Spores/ml	Unheated nitrite			Heated nitrite		
		A	E	S	A	E	S
Unheated	10^6	240	160	200	20	15	10
	10^3	240	240	200	15	7.5	10
Heated	10^6	200	160	200	40	5	15
	10^3	200	240	200	10	5	15
Irradiated	10^6	240+	240+	240+	40	15	20
	10^3	240	200	200	40	7.5	20

A = *Cl. botulinum* Type A
E = „ „ Type E
S = *Cl. sporogenes*

Culture media Immediately after the demonstration of PF with vegetative cells it was demonstrated by Perigo & Roberts (1968) that a similar effect was observable with a variety of clostridia in the vegetative state. In subsequent work, which has been reported only briefly, Roberts (1971) went on to demonstrate a PF effect with spores of several types of *Clostridium* added to the Perigo medium heated with nitrite. As with vegetative cells, there was an about ten-fold enhancement of the effect of nitrite as a result of heating (Table 2). It is noteworthy that the number of spores (10^6 or 10^3 per ml), and heating or irradiation of the spores beforehand, made little difference to the inhibitory concentrations. In the meantime, there has been a good deal of work on the Perigo effect in artificial media, but it is difficult to discuss because little of it has been published, perhaps because of inconsistencies in the work of individuals and differences from the results of others. Almost the only point of general agreement is that a reducing agent is necessary in the medium: the thioglycollate used by Perigo et al. (1967) can be replaced by ascorbic acid or cystein. A protein hydrolysate is necessary, preferably of casein, but it is not decided which fractions are important. There have been suggestions that iron is necessary, to form Roussin salts, but my colleaques have had no consistent benefit from the addition of iron, and it seems that two inhibitors may be involved. There is however little profit in discussing these complexities, because of doubt whether they are applicable to the situation in the presence of meat.

Meat systems Since the work of Johnston, Pivnick & Samson (1969) demonstrated that the Perigo Inhibitor could be neutralised by meat, there have been two reports that an extra inhibitory effect does occur when nitrite is heated in the presence of meat. A positive response was observed by Pivnick's group, in work reported by Chang & Pivnick to the 1973 Meeting of the Canadian Institute of Food Technologists and to be published in their Journal. Chang & Pivnick canned commercially formulated luncheon meat with varying concentrations of nitrite and, after autoclaving, stored the cans until all residual nitrite had disappeared. Then, the cans were challenged with an inoculum of washed spores of *Clostridium botulium* previously heated to $F_0 = 0.4$ in a meat homogenate containing 4% NaCl and

150 mg/kg $NaNO_2$. In the now nitritefree meat, an inhibition was observed which was greater the greater the concentration of nitrite initially. This was interpreted as indicating the presence of an inhibitor derived from the original nitrite, as a result of reaction during autoclaving or storage. The inhibition was greater with smaller numbers of spores; but, with only 10 spores per can, and relatively high concentrations of nitrite initially (up to 200 mg/kg), one out of 16 cans showed growth. The other report was from Ashworth & Spencer (1972), who added nitrite directly to minced pork and heated, observing a greater inhibitory effect when nitrite was added before rather than after heating. The effect was observed in the range 150–300 mg nitrite/kg initially. But the enhancement factor was unusually small, less than 1.5; and practically independent of pH, unlike the situation in culture medium where Perigo et al. (1967) showed that the enhancement factor is much greater at higher pH values. Using more resistant strains of clostridia isolated from spoiled cured meats, Roberts (personal communication) has confirmed that there is an effect in pork, but only by using much higher initial nitrite concentrations up to 800 mg/kg.

The outcome of a substantial amount of work on the Perigo effect seems disappointing. In culture media, it is possible to get complete inhibition in heated nitrite with residual concentrations of the order 50–10 mg/kg which are frequently found in safe situations in practice. Yet it seems that the Perigo Inhibitor developed in culture media may not be the same as that in meat. In culture media, the enhancement factor is much greater, and sensitive to pH; its effect is little influenced by the number of spores or by heating or irradiating them beforehand, factors which are known to have a large influence in realistic meat systems; and finally, this inhibitor is neutralised by the presence of only quite small proportions of meat. For these reasons, it seems premature to speak of the Perigo Inhibitor or Perigo Factor in meat, and such terminology should be avoided. As to the inhibitor actually produced by heating nitrite in meat, to attain effective concentrations seems to require initial nitrite concentrations much greater than those observed to be effective in actual practice; and, indeed, Ashworth & Spencer (1972) themselves conclude 'from the work reported here no evidence has been obtained which would indicate that a Perigo effect is likely to be involved, under practical conditions, with the safety and stability of canned cured meats.'

Recent inoculated pack experiments

There has been administrative pressure in many countries recently to decide what concentrations of nitrite are necessary to provide protection against botulism. This has led in U.S.A. to a resumption of inoculated pack experiments, some of which have been described by Greenberg (1972) and by Christiansen, Johnston, Kautter, Howard & Aunan (1973). Ground pork ham was prepared with eight levels of nitrite (0–500 mg/kg as formulated), and inoculated with a mixture of spores from 5 Type A and 5 Type B strains of *Clostridium botulium*, at two levels namely 90 and 5 000 per gram. The canned materials were cooked in water to an internal temperature of 155°F (68.5°C); and the cans were then stored at 80°F (26.7°C) and observed over 6 months. The essential data are in Tables 3 and 4. Greenberg

Table 3. Effect of nitrite on botulinum toxin formation in canned ham (light inoculum). (Greenberg, 1972).

Nitrite added, mg/kg	Days until toxin first detected	Total number of toxic cans
0	7	45/80
50	7	32
100	28	16
150	68	2
200	>180	0
300	>180	0
400	>180	0
500	>180	0

Table 4. Effect of nitrite on botulinum toxin formation in canned ham (heavy inoculum). (Greenberg, 1972).

Nitrite added, mg/kg	Days until toxin first detected	Total number of toxic cans
0	7	63/80
50	12	18
100	13	33
150	42	13
200	70	5
300	168	1
400	21	2
500	>180	0

states that the observations correlated better with the initial concentrations of nitrite than with (calculated) residual concentrations. The problem is, how to interpret such observations?

First, it cannot be too strongly emphasized that they have no general validity. The limiting concentrations of nitrite refer only to the particular circumstances of the experiment: for example the salt concentration (2.5% or 3.7% brine); the particular numbers of spores (90 or 5 000 per gram), which were relatively high; and the particular heating regime, relatively low and not very precisely specified. Dextrose and ascorbate were present, but no polyphosphate; their likely effect is uncertain. The effect of pH (6.24) is uncertain. It does seem certain that, had the salt content, spore number of heating treatment been different, the limiting concentrations of nitrite would have been different. But we do not, unfortunately, know how the inter-relations observed in model systems, for example between salt, nitrite and pH, will remain valid in commercially heated packs; there are already indications (e.g. from Riemann, 1963) that the pH relations may not apply, which might perhaps be expected if a large part of the inhibition were due to substances other than nitrite. What we need at the present time, in my opinion, is not more inoculated pack experiments but a rationale for interpreting them.

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Discussion

Added or residual nitrite

The references to nitrite in the paper usually deal with the added nitrite. Some authors suggest a correlation of the inhibiton of clostridia with the amount of added nitrite other with the residual nitrite; there is no agreement on this point. Perhaps there is a relation with some reaction products of nitrite, i.e. with the nitrite which disappears.

The necessity for nitrite

The control of *Cl. botulinum* would be no problem without nitrite, if the storage temperature were always lower than 10°C or the pH lower than 4.5 or the NaCl content were higher than about 12%, but these are not realistic conditions in practice. The type of spoilage of raw and cured meats depends largely on the initial microflora, the curing conditions, the heating temperature and the storage conditions of a product.

Realistic numbers of spores in raw meat are about 10 per kg meat, and of these

about 1 on 10^4 is a *Cl. botulinum* spore, according to American investigations. At retail levels this may be somewhat higher.

In pasteurized comminuted products these numbers are much higher, counts of about 100 *Bacillus* and 10 *Clostridium* spores per gram are reported in Europe. Other authors have even found higher numbers occasionally.

Recent outbreaks of botulism in man due to unheated meat products without nitrite have been reported. No substitute for nitrite which is less hazardous has so far been found. If we knew the specific effect of nitrite on micro-organisms it would be easier to suggest a replacement.

Specific inhibitory action of nitrite

In the USA, experiments have demonstrated the ability of nitrite, but not nitrate, to retard or prevent botulinal toxin development in a series of cured meat products as manufactured currently.

Action of nitrosamines

Dr Walters examined the anti-clostridial effect of about a dozen nitrosamines; none of these had any effect at relevant concentrations. Others specifically looked for DMNA and DENA in culture media with which the Perigo effect had been demonstrated, but these nitrosamines were not found.

Action of aerobic sporeformers

Whether aerobic sporeformers can inhibit clostridia is not known. Bacilli can metabolize nitrite under certain conditions.

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The inhibition of Clostridium botulinum by nitrite and sodium chloride

A. C. Baird-Parker and M. A. H. Baillie

Unilever Research Laboratory, Colworth/Welwyn, Colworth House, Sharnbrook, Bedford, UK

Abstract

In a bacteriological growth medium (MRCM) most *Clostridium botulinum* type A and proteolytic type B and F strains will grow at pH 6.0 in the presence of at least 150–200 mg/kg sodium nitrite or 6% sodium chloride at 25°C. Under the same growth conditions (medium, incubation temperature and inoculum level), *Cl. botulinum* type E and non-proteolytic strains of types B and F are mainly inhibited by 4.5% sodium chloride or 100–150 mg/kg sodium nitrite. Less nitrite and salt are required to inhibit growth at lower storage temperatures. Sodium chloride and sodium nitrite are apparently synergistic in inhibiting the growth of *Cl. botulinum* in bacteriological media and 200 mg/kg nitrite in the presence of 3% sodium chloride inhibits the growth of almost all strains at 20 and 30°C as also does 100 mg/kg sodium nitrite in the presence of 4.5% sodium chloride at pH 6.0; these results were obtained in a bacteriological medium. However, in pork macerate broth (PMB) higher concentrations of nitrite and salt are required to inhibit the growth of the same number of cells as that used in the bacteriological growth medium. If smaller numbers of cells (10 or 10^3) are used to inoculate PMB containing nitrite most strains are inhibited by the presence of 200 mg/kg nitrite at pH 6.0 and a temperature of 20°C. L-Ascorbic acid (0.1 or 1.0%) in MRCM (pH 6.0) reduces the minimum inhibitory concentration (MIC) of sodium nitrite for all strains to less than 100 mg/kg. However, in PMB containing 0.1% L-ascorbic acid this enhancement of nitrite activity is not observed and the MIC of nitrite to cells of *Cl. botulinum* was the same in the presence as in the absence of ascorbic acid.

Preliminary experiments done in a laboratory cured bacon stored at 25°C show that the addition of 0.1% L-ascorbic acid to bacon containing 500 mg/kg sodium nitrate, 4.5% sodium chloride and 100 or 200 mg/kg nitrite (pH 5.6–5.8) does not affect the antibotulinum activity of nitrite.

Introduction

The microbial stability of a cured meat or fish product cannot be ascribed to any single attribute but depends on the interacting effects of a number of parameters. These include the type and quantity of the contaminating microflora, the a_W, pH, E_h and composition of the product, the degree of heat injury induced to micro-organisms surviving any thermal process used during manufacture, the packaging and storage conditions and the amounts and types of curing salts they contain (Riemann et al., 1972, Christiansen et al., 1973, Leistner et al., 1973, Pace & Krumbiegel, 1973).

There is a considerable amount of published data on the separate effects of most

of the above parameters on the growth of *Clostridium botulinum* in bacteriological media and some foods (Baird-Parker, 1969) and quite a lot of data on the effects of two or more combinations of these (Segner et al., 1966; Baird-Parker & Freame, 1967; Spencer, 1967; Ohye & Christian, 1967; Emodi & Lechowich, 1969; Pivnick et al., 1969 and Roberts & Ingram, 1973).

The present study was done to obtain further data on the individual and combined effects of curing salts, in particular sodium chloride and nitrite, on *Cl. botulinum* at concentrations which are relevant to production and sale of raw cured meat and fish products. Sodium chloride was included in these studies as it is believed to interact strongly with nitrite in preventing the growth of *Cl. botulinum* in cured foods.

The studies have been based on the use of a large collection of *Cl. botulinum* strains isolated from foods and natural environments in many parts of the world. The use of a large collection of strains was regarded as particularly important in these studies as it was considered that a major deficiency in almost all previous studies was that only a few strains had been studied. This is important, as it is well recognised that individual strains and toxigenic types of *Cl. botulinum* vary enormously in their tolerance to curing salts and other factors important to the safety and stability of cured meat and fish products.

Materials and methods

Sources of organism

Strains representing all toxigenic types of *Cl. botulinum* were used including the recently described type G (Gimenez & Ciccarelli, 1970). These were mainly obtained from workers and collections in North America, Europe and the United Kingdom but included a number of our own isolates. Most of the strains studied were toxigenic types A, B, E and F as only these have been incriminated unequivocally in human botulism.

Inoculum and inoculum levels

Vegetative cell inocula were used in all studies except for those involving bacon. In previous studies we have not observed any differences between the ability of spores and vegetative cells to initiate growth under growth limiting conditions (Baird-Parker & Freame, 1967; A. Baillie, unpublished). The vegetative cell inocula used were from overnight cultures (30°C) grown in modified Reinforced Clostridial Medium (see basal media) and subcultured from refrigerated stock cultures maintained in Robertson's Cooked Meat Medium. An inoculum level of approximately 10^6 cells/ml was used in all tests except those on the effect of inoculum level on growth. For the latter Thoma slide counts were done, and the cultures appropriately diluted in freshly steamed and cooled 0.1% peptone water to give inoculum levels of about 10^6, 10^3 and 10 cells/bottle of test medium.

Basal media

All media were dispensed in 20 or 25 ml amounts in 1 oz (30 g) screw-capped bottles and freshly steamed before making additions to the heat sterilized media. All pH adjustments were done with HCl or NaOH. The following two basal media were used.

a. Modified Reinforced Clostridial Medium (MRCM). The medium was that described by Baird-Parker & Freame (1967) except that ascorbic acid was omitted. It supports good growth of all toxigenic types of *Cl. botulinum* except for the fastidious type C strains which grow only moderately well in this medium.

b. Pork Macerate Broth (PMB). This medium was used to simulate a meat situation. Lean pork was diced and as much fat as possible removed. It was than minced, weighed into 1 kg aliquots and 1 litre of distilled water added to each aliquot. The macerates were then steamed for 30 minutes, with occasional stirring, and when cool the liquor was strained off through muslin bags; as much free liquor as possible was removed from the minced pork by squeezing the bags tightly. The meat was dispensed in 10 g amounts in wide necked 30 g screw-capped bottles. The liquor was clarified by adding about 50 g Celite 545 to each litre and filtering through Whatman No. 1 filter paper. It was then adjusted to pH 6.0 and 10 ml amounts were added to each bottle of minced pork. Appropriate additions and further pH adjustments were made after sterilizing the PMB at 121°C for 15 minutes. This medium supports excellent growth of all toxigenic types of *Cl. Botulinum*.

Additions

Sodium nitrite (Analar grade) was used for all experiments involving nitrite. This was made up as a stock solution in distilled water containing 50 times the highest final concentration of sodium nitrite required. It was sterilized by membrane filtration and diluted so that when 0.4 or 0.5 ml was added to the appropriate basal medium the required final concentration of sodium nitrite was obtained; all pH adjustments and other additions were made to the freshly steamed media prior to the addition of nitrite. Nitrite concentrations of 50, 100, 150 and 200 mg/kg were used. All nitrite additions were checked by chemical analysis in the final media. Nitrite was very stable in MRCM and analysed initial levels were within 5% of the calculated levels and after 28 days storage at the highest temperature used (30°C) the nitrite levels were still within 10% of the initial analysed levels. As would be expected nitrite was rather unstable in PMB. Analysed levels were all 10–20% below the calculated amounts added and during storage at 20°C for 28 days this dropped to 40–50% of the initial level.

Sodium chloride was added prior to sterilization of the basal media; the pH was adjusted prior to sterilization such that it was correct after sterilization. Concentrations of sodium chloride used were 1.5, 3.0, 4.5 and 6.0% w/v. All additions were checked analytically in the final media and were within 2.0% of the calculated amounts added.

L-Ascorbic Acid (Analar grade) was made up as stock solutions which were 50 times the required final concentrations in PMB or MRCM. The stock solutions

were adjusted to pH 6.0 and with NaOH and filter sterilized; 0.4 or 0.5 ml of each stock solution was added to the freshly steamed and cooled medium to give the final required concentration. The presence of L-ascorbic acid in MRCM and PMB resulted in a loss of the order of 75–85% of the initial nitrite concentration over the storage period of 28 days at 20°C.

Inoculation and storage

Five replicates of each combination of test medium and test organism were set up. In most experiments, 20°C was used as the storage temperature. To test the effect of temperature, experiments were also done at 15, 25 and 30 °C. Storage was done in jacketed incubators (accuracy ± 1°C) and inoculated media were stored for up to 28 days except for the 15°C storage experiments when the storage time was extended to 56 days.

Assessment of results

Throughout the storage periods bottles were examined at weekly intervals for visible signs of growth, i.e. turbidity, digestion of meat or gas production. At the end of the storage period contents of all bottles showing growth at the most severe test conditions were checked for purity by aerobic and anaerobic plating on MRCM agar; the contents of representative bottles were also tested for botulin by mouse toxicity tests (Baird-Parker, 1969). As it was often difficult to decide visually whether growth had occurred in PMB the contents of bottles were streaked out aerobically and anaerobically to assess growth. Growth is recorded in the tables, if one or more bottles of the 5 replicates tested showed growth.

Laboratory cured bacon

Bacon was slice cured in bags (3 rashers/bag; wt. c. 100 g) using a solution containing sodium chloride, sodium nitrate, and where appropriate sodium nitrite and L-ascorbic acid (adjusted to pH 6.0). The concentrations of curing salts and ascorbic acid used were calculated to give final concentration in the bacon of 4.5% sodium chloride on water phase, 500 mg/kg nitrate, 1000 mg/kg L-ascorbic acid, and 100 or 200 mg/kg nitrite. The bacon was then vacuum-packed and placed at 4°C for 2 days. Packs were opened and surface and deep inoculated (total inoculum 0.01 ml) with either a mixture of vegetative cells and spored of 7 proteolytic strains of *Cl. botulinum* (2, type A; 2, Type B; 2, Type F; 1, Type G) or 6 non-proteolytic strains (2, Type B; 2, Type E; 2, Type F). The inocula contained equal numbers of spores and vegetative cells. The inoculum levels were: proteolytic type A, B, F and G strains c. 260 cells/g, non-proteolytic type B, E and F strains c. 8 cells/g. The strains used were chosen to represent those strains which in PMB were most resistant to sodium chloride or sodium nitrite. The inoculated packs were revacuum-packed and then stored at 25°C. After 14 and 28 days storage 5 replicates of each combination of inoculum type and cure were examined for cells and toxins of *Cl. botulinum*.

Results and discussion

Inhibition by sodium nitrite

Results obtained in MRCM (pH 6.0) stored for 28 days at 20 and 25°C and 56 days at 15°C shown in Table 1. They show that although individual strains of *Cl. botulinum* vary markedly in their resistance to sodium nitrite they can be divided into two main groups on the basis of nitrite tolerance. The most resistant strains, generally growing in upto 150–200 mg/kg sodium nitrite in these tests at 25°C, occur amongst the heat resistant and physiologically closely related proteolytic and mesophilic types, i.e. *Cl. botulinum* type A and proteolytic types B and F. The most sensitive strains which are mainly inhibited by 100–150 mg/kg sodium nitrite are found amongst the heat sensitive, non-proteolytic and psychrotrophic types, i.e. *Cl. botulinum* type E and non-proteolytic types B and F. The mesophilic, non-proteolytic type C and D strains belonged to the more sensitive group. There was also a marked difference in the tolerance to nitrite of the proteolytic, mesophilic strains growing at 20°C and 25°C but virtually no difference in the nitrite tolerance of the non-proteolytic, psychrotrophic strains growing at these two temperatures. This difference may be a reflection of the fact that at 20 and 25°C the mesophilic strains are further from their optimum growth temperatures than the psychrotrophic strains and are therefore more subject to an influence of a small

Table 1. Effect of sodium nitrite on the growth of *Clostridium botulinum* in MRCM (pH 6.0) at 15, 20 and 25°C.

Toxigenic type	Storage temperature (°C)	No. of strains	Sodium nitrite (mg/kg)[3]			
			50	100	150	200
A	15[1]	6	4	4	3	2
B (proteolytic)	15	6	6	6	2	3
B (non-proteolytic)	15	6	4	2	0	0
E	15	3	3	3	0	0
F (proteolytic)	15	3	2	1	1	0
F (non-proteolytic)	15	3	0	0	0	0
A	20[2]	51	46	40	27	14
B (proteolytic)	20	12	11	8	2	1
B (non-proteolytic)	20	9	6	2	1	0
E	20	64	24	14	5	0
F proteolytic	20	3	2	2	1	0
F (non-proteolytic)	20	3	1	0	0	0
A	25[2]	50	50	50	46	30
B (proteolytic)	25	11	11	11	10	5
B (non-proteolytic)	25	9	7	5	3	2
E	25	64	51	19	5	2
F (proteolytic)	25	3	3	3	3	1
F (non-proteolytic)	25	3	3	0	1	0

1. Stored for 56 days.
2. Stored for 28 days.
3. Calculated amount of sodium nitrite added.

Table 2. Effect of sodium nitrite on the growth of *Clostridium botulinum* at 20°C in MCRM (pH 5.5, 6.0, 6.5 and 7.0).

Toxigenic type	pH	No. of strains	Sodium nitrite (mg/kg)[1]		
			50	100	200
A	5.5	6	0	0	0
	6.0	6	6	6	2
	6.5	6	6	6	6
	7.0	6	6	6	6
B (proteolytic)	5.5	6	0	0	0
	6.0	6	5	3	1
	6.5	6	6	5	5
	7.0	6	6	6	6
B (non-proteolytic)	5.5	6	0	0	0
	6.0	6	4	1	0
	6.5	6	5	5	2
	7.0	6	6	6	6
E	5.5	6	0	0	0
	6.0	6	1	1	0
	6.5	6	2	2	0
	7.0	6	5	5	5
F (proteolytic)	5.5	3	0	0	0
	6.0	3	2	2	1
	6.5	3	3	3	1
	7.0	3	3	3	3
F (non-proteolytic)	5.5	3	0	0	0
	6.0	3	1	0	0
	6.5	3	3	3	0
	7.0	3	3	3	3
G	5.5	1	0	0	0
	6.0	1	1	0	0
	6.5	1	1	0	0
	7.0	1	1	1	1

1. Calculated amount added.

temperature change. At 15°C, the proteolytic strains still remain most resistant to nitrite.

The results in Table 2 demonstrate the effect of pH on the inhibition of *Cl. botulinum* by nitrite. Thus at pH 5.5, none of the strains grew in the presence of 50 mg/kg nitrite whereas at pH 7.0 all strains grew in the presence of 200 mg/kg nitrite.

The results shown in Table 3 of the effect of nitrite on the growth of *Cl. botulinum* in PMB indicate, similar trends and differences to that noted for strains grown in MRCM although all strains grew to higher nitrite concentrations. This could either have been the result of the different growth substrate, redox potential, lower analysed nitrite initially and about a 50% drop in nitrite concentration during storage for 28 days or most likely to the production of the inhibitory substance

Table 3. Effect of sodium nitrite on the growth of *Clostridium botulinum* at 20°C in PMB (pH 6.0) without sodium chloride.

Toxigenic type	No. of strains	Sodium nitrite (mg/kg)[1]			
		50	100	150	200[2]
A	6	6	6	6	6
B (proteolytic)	6	6	6	5	6
B (non-proteolytic)	6	6	5	5	1
E	6	6	6	3	4
F (proteolytic)	3	3	1	2	0
F (non-proteolytic)	3	3	2	2	1
G	1	1	1	1	1
Total strains	31	31	27	24	19

1. Calculated amount added.
2. Analytical results of 200 mg/kg sample after storage at 20°C: 0 days 185 mg/kg $NaNO_2$, 7 days 164 mg/kg $NaNO_2$, 14 days 109 mg/kg $NaNO_2$, 28 days 92 mg/kg $NaNO_2$.

Table 4. Effect of inoculum size on the inhibition of growth of *Clostridium botulinum* by sodium nitrite in PMB (pH 6.0) at 20°C without sodium chloride.

Toxigenic type	No. of strains	Inoculum	Sodium nitrite (mg/kg)[1]		
			50[2]	100[2]	200[2]
A	2	10^1	2	0	0
	2	10^3	2	1	1
	2	10^6	2	2	2
B (proteolytic)	2	10^1	2	0	0
	2	10^3	2	2	0
	2	10^6	2	2	2
B (non-proteolytic)	2	10^1	0	0	0
	2	10^3	2	0	0
	2	10^6	2	2	1
E	2	10^1	0	0	0
	2	10^3	2	0	0
	2	10^6	2	2	2
F (proteolytic)	2	10^1	1	0	0
	2	10^3	1	0	0
	2	10^6	2	2	1
F (non-proteolytic)	2	10^1	0	0	0
	2	10^3	2	0	0
	2	10^6	2	2	1
G	1	10^1	1	1	0
	1	10^3	1	0	0
	1	10^6	1	1	1

1. Calculated amount added.
2. Analytical results (mg/kg):

	50 mg/kg	100 mg/kg	200 mg/kg
0 day	38	93	168
28 days	0	45	91

(reported by Van Roon, this symposium) in MRCM containing nitrite which enhanced the antibotulinum activity of nitrite in MRCM.

Table 4 demonstrates the effect of inoculum size on the ability of nitrite to inhibit the growth of *Cl. botulinum*. The effects are most marked. Inoculum size is an important consideration in determining the antibotulinum activity of nitrite, but results of high inoculum levels should not be ignored as such inocula contain the small number of resistant cells which occur in any population of cells and might also be expected to occur in nature.

Inhibition by sodium chloride

Results obtained in MRCM and PMB are shown in Tables 5 and 6 respectively. They show precisely the same differences as were noted for sodium nitrite. Thus *Cl. botulinum* type A and proteolytic type B and F strains generally grow in the presence of at least 4.5–6.0% sodium chloride at 20 or 25°C while most of the non-proteolytic and psychrotrophic types are inhibited by 4.5% sodium chloride in MRCM but some grow in PMB in the presence of 6% sodium chloride. At 20 and 25°C the effect of temperature on the level of sodium chloride inhibiting growth was only marked for the mesophilic proteolytic strains and was not apparent for the psychrotrophic strains; similar differences of the effect of temperature on the a_W tolerance of type A, B and E strains can be observed in the results reported by

Table 5. Effect of sodium chloride on the growth of *Clostridium botulinum* in MRCM (pH 6.0) at 15, 20 and 25°C.

Toxigenic type	Storage temperature (°C)	No. of strains	Sodium chloride (% w/v)			
			1.5	3.0	4.5	6.0
A	15[1]	6	1	1	0	0
B (proteolytic)	15	6	3	2	0	0
B (non-proteolytic)	15	6	4	1	0	0
E	15	6	6	2	0	0
F (proteolytic)	15	3	3	0	0	0
F (non-proteolytic)	15	3	3	0	0	0
A	20**	49	49	45	38	6
B (proteolytic)	20	10	10	10	7	0
B (non-proteolytic)	20	9	9	8	0	0
E	20	61	60	54	3	0
F (proteolytic)	20	4	4	2	1	0
F (non-proteolytic)	20	4	4	2	0	0
A	25[2]	50	50	50	50	38
B (proteolytic	25	11	11	11	10	7
B (non-proteolytic)	25	9	9	9	0	0
E	25	61	61	61	2	0
F (proteolytic)	25	4	4	4	2	0
F (non-proteolytic)	25	4	4	4	1	0

1. Stored for 56 days.
2. Stored for 28 days.

Table 6. Effect of sodium chloride on the growth of *Clostridium botulinum* at 20°C in PMB (pH 6.0).

Toxigenic type	No. of strains	Sodium chloride (% w/v)			
		1.5	3.0	4.5	6.0
A	6	6	6	5	5
B (proteolytic)	6	6	6	5	3
B (non-proteolytic)	6	6	6	2	0
E	6	6	6	4	3
F (proteolytic)	3	3	3	2	2
F (non-proteolytic)	3	3	3	2	0
G	1	1	1	0	0
Total strain	31	31	31	20	13

Table 7. The effect of sodium chloride on the growth of *Clostridium botulinum* at 20°C in MRCM (pH 5.0, 5.5, 6.0 and 7.0).

Toxigenic type	pH	Sodium chloride (% w/v)				
		0	1.5	3.0	4.5	6.0
A	5.0	–[1]	–	–	–	–
	5.5	+[2]	+	–	–	–
	6.0	+	+	+	+	–
	7.0	+	+	+	+	+
B (proteolytic)	5.0	–	–	–	–	–
	5.5	+	+	+	–	–
	6.0	+	+	+	+	–
	7.0	+	+	+	+	+
E	5.0	–	–	–	–	–
	5.5	+	+	–	–	–
	6.0	+	+	+	–	–
	7.0	+	+	+	–	–

1. No growth.
2. Growth.

Ohye & Christian (1967). At 15°C, 4.5% sodium chloride was inhibitory to all strains and 3% sodium chloride inhibitory to most.

Table 7 which is based on data presented in the paper by Baird-Parker & Freame (1967) shows that like nitrite, the inhibitory effect of sodium chloride against *Cl. botulinum* is highly dependent on pH.

Inhibition by sodium nitrite in the presence of sodium chloride

A synergistic effect of combinations of sodium chloride and sodium nitrite against *Cl. botulinum* is apparent from the results presented in Table 8. At both 20 and 30°C only one strain out of the 61 strains tested was able to grow in a

Table 8. Effect of sodium chloride in the presence of sodium nitrite on the growth of *Clostridium botulinum* at 20°C and 30°C in MRCM (pH 6.0).

Toxigenic type	Storage temperature (°C)	No. of strains	3% w/v sodium chloride + sodium nitrite (mg/kg)[1]				4.5% w/v sodium chloride + sodium nitrite (mg/kg)[1]			
			0	50	100	200	0	50	100	200
A	20	40	36	7	4	1	33	5	0	0
	30	10	10	7	4	1	10	4	1	0
B (proteolytic)	20	8	8	1	0	0	7	1	0	0
	30	4	4	3	0	0	4	1	0	0
B (non-proteolytic)	20	1	1	0	0	0	0	0	0	0
E	20	11	10	1	0	0	3	0	0	0
	30	6	6	0	1	0	1	0	0	0
F (non-proteolytic)	20	1	0	0	0	0	0	0	0	0
	30	1	1	0	0	0	1	0	0	0

1. Calculated amounts added.

combination of 3% sodium chloride and 200 mg/kg sodium nitrite in MRCM at pH 6.0. In the presence of 4.5% sodium chloride only one strain grew at 30°C in the presence of 100 mg/kg sodium nitrite. Very similar results are reported by Roberts & Ingram (1973) who in a study of single strains of *Cl. botulinum* types A, B, E and F found that at pH 6.0 a combination of 4% sodium chloride and 200 mg/kg sodium nitrite inhibited the growth of all strains at 35°C. However, these tests are on bacteriological media and preliminary data (unpublished) shows that higher concentrations of salt and nitrite will be required to inhibit the growth of high inocula (10^6/ml) of *Cl. botulinum* at high ambient temperatures in PMB.

Inhibition by sodium nitrite and sodium chloride in the presence of ascorbic acid

We have noted in previous unpublished studies that at pH 7.0 and an incubation temperature of 37°C 100–200 mg/kg sodium nitrite is inhibitory to all strains growing in MRCM containing 1% L-ascorbic acid; in MRCM and other media not containing ascorbic acid the inhibitory level is 1000–1500 mg/kg (unpublished and Perigo & Roberts, 1968). Results obtained at pH 6.0 in MRCM with and without 1% ascorbic acid are shown in Table 9. There was no effect of L-ascorbic acid on

Table 9. Effect of sodium nitrite and sodium chloride on the growth of *Clostridium botulinum* at pH 6.0 in MRCM and in MRCM + 1.0% L-ascorbic acid at 20°C.

Toxigenic type	No. of strains	Growth medium[1]	Sodium nitrite (mg/kg)					Sodium chloride (% w/v)			
			0	50	100	150	200	1.5	3.0	4.5	6.0
A	4	1	4	0	0	0	0	4	4	1	0
	4	2	4	4	4	1	1	4	4	2	0
B (Proteolytic)	4	1	4	0	0	0	0	4	4	3	0
	4	2	4	4	4	1	0	4	4	2	0
B (Non-proteolytic)	4	1	4	0	0	0	0	4	4	0	0
	4	2	4	2	1	0	0	4	4	0	0
E	4	1	4	0	0	0	0	4	4	1	0
	4	2	4	3	1	0	0	4	4	0	0
F (Proteolytic)	1	1	1	0	0	0	0	1	1	0	0
	1	2	1	1	1	1	1	1	1	0	0
F (Non-proteolytic)	2	1	2	0	0	0	0	2	2	0	0
	2	2	2	1	0	0	0	2	2	0	0

1. 1 = MRCM + 1.0% L-ascorbic acid, 2 = MRCM.

Table 10. Effect of sodium nitrite on the growth of *Clostridium botulinum* in the presence of ascorbic acid after 20 days at 20°C in MRCM (pH 6.0) without sodium chloride.

Toxigenic type	No. of strains	0 mg/kg ascorbic acid				1 000 mg/kg ascorbic acid			
		0	50	100	200	0	50	100	200
A	2	2	2	1	0	2	1	0	0
B (proteolytic)	2	2	2	1	0	2	1	0	0
B (non-proteolytic)	2	2	1	0	0	2	0	0	0
E	2	2	1	0	0	2	1	0	0
F (proteolytic)	1	1	1	0	0	1	0	0	0
F (non-proteolytic)	2	2	0	0	0	2	0	0	0
G	1	1	1	1	0	1	0	0	0
Total	12	12	8	3	0	12	3	0	0

sodium chloride tolerance but a very marked effect on tolerance to sodium nitrite, i.e. no strain out of the 38 strains tested grew in the presence of 50 mg/kg nitrite which was the lowest concentration tested. There can be little doubt that the principle reason for this pronounced inhibition of *Cl. botulinum* in MRCM containing ascorbic acid is due to the formation of a complex of the type described by Van Roon in this symposium. Similar results are obtained in MRCM containing 0.1% L-ascorbic acid (Table 10) but not in PMB containing the same level of L-ascorbic acid (Table 11). In the latter medium, the stimulatory effect of L-ascorbic acid on the antibotulinum activity of nitrite is not observed. This would be expected if the inhibitory factor formed in MRCM containing L-ascorbic acid and nitrite was a Van Roon type inhibitor as such inhibitors are inactivated by meat particles, a main component of PMB. It is important to note that whereas there is no stimulatory effect of ascorbic acid on the antibotulinum activity of nitrite in PMB there is no reduction in activity in the presence of ascorbic acid.

Table 11. Effect of ascorbic acid on the inhibition of *Clostridium botulinum* by sodium nitrite after 21 days at 20°C in PMB (pH 6.0 – 6.2).

Toxigenic type	No. of strains	No ascorbic acid, sodium nitrite (mg/kg)[1]					Ascorbic acid 1 000 mg/kg, sodium nitrite (mg/kg)[1]			
		0	300	400	500	600	0	300	400	500
A	3	3	3	3	3	3	3	3	3	3
B (Proteolytic)	2	2	2	2	2	2	2	2	2	2
B (Non-proteolytic)	2	2	2	2	1	0	2	2	2	0
E	2	2	2	1	1	1	2	2	2	1
F (proteolytic)	2	2	2	2	2	1	2	2	2	1
F (Non-proteolytic)	2	2	2	2	0	0	2	2	2	0
G	1	1	1	1	0	0	1	1	1	0
Total strains	14	14	14	13	9	7	14	14	14	7

1. Calculated amount added.

Inoculation trial with laboratory cured bacon

Results obtained of a storage trial at 25°C in which *Cl. botulinum* was inoculated into packs of bacon containing different levels of nitrite in the presence and absence of L-ascorbic acid are shown in Table 12. Bacon prepared with a level of c. 4.5% sodium chloride on water phase and an initial level of 500 mg/kg nitrate supported good growth and toxin formation by *Cl. botulinum*, i.e. 9 out of 10 packs were toxic after storage for 14 and also 28 days. This bacon was judged by the bacteriologists assessing the results as spoiled (putrid) by 14 days which was the first time the bacon was examined after inoculation. In contrast to this bacon containing the same levels of salt and nitrate but in addition 100 or 200 mg/kg nitrite did not readily support growth or toxin formation by *Cl. botulinum* either in the presence or absence of L-ascorbic acid. The bacon was also much more shelf-stable than the nitrite-free bacon and only slight souring of a few packs was observed after 28 days storage. Only one out of 40 packs examined after storage for 14 days and the same after 28 days, were toxic and contained cells of *Cl. botulinum*. The two toxic packs contained only 3.0% salt on water phase when tested and were therefore low in curing salts. *Cl. botulinum* type B cells were present at the 10^5-10^6/g level in the toxic packs but no cells of any toxigenic type were detected in the non-toxic packs; limit of detection was a most probable number count of 0.36 cells/g. Thus we conclude from this preliminary experiment that 0.1% ascorbic acid does not affect the antibotulinum activity of curing salts in bacon.

Table 12. Effect of L-ascorbic acid on *Clostridium botulinum* in vacuum packed bacon stored at 25°C.

Cure[1] additive	Number of days stored at 25°C	
	14	28
0 mg/kg ascorbic acid 0 mg/kg $NaNO_2$	9[2]	9
0 mg/kg ascorbic acid 100 mg/kg $NaNO_2$	0	1[3]
1 000 mg/kg ascorbic acid 100 mg/kg $NaNO_2$	0	0
0 mg/kg ascorbic acid 200 mg/kg $NaNO_2$	0	0
1 000 mg/kg ascorbic acid 200 mg/kg $NaNO_2$	1[3]	0

1. Bacon contained an average of 4.4% salt on water phase (range 3.0 – 5.8%) and an initial level of 500 mg/kg sodium nitrate (pH of bacon 5.6 – 5.8).
2. Number of toxic packs out of 10 tested (5 inoculated with proteolytic and 5 with non-proteolytic strains of *Cl. botulinum*).
3. Contained type B toxin (non-proteolytic inoculum).

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Discussion

Experimental details

The end pH in the experiments (initial pH 6.0) was 6.0–6.1. The end nitrite concentrations in MRCM + 1% ascorbic acid were 15–25% of the initial concentrations. At the 0.1% ascorbic acid level, the nitrite concentrations after 20 days at 20°C and initial levels of 50, 100 and 200 mg/kg nitrite were 4.4, 13 and 44, respectively and the corresponding ascorbic acid levels were 44, 37, and 65 mg/kg.

The experimental effect of ascorbic acid

The author does not think that one can explain the differences observed in MRCM with and without ascorbic acid solely in terms of differences in oxidation and reduction potential. It is likely that an inhibiting substance of the type II that van Roon described in his paper is formed.

The inoculum level

The author agrees that it is necessary to repeat experiments at lower inoculum levels. However, it is important not to disregard results obtained at high inoculum levels as populations of cells may contain only a few number of resistant organisms.

Dying out of spores

In the experiment reported on bacon no *Cl. botulinum* cells (limit of detection < 0.36 organisms/g) were detected in all non-toxic packs examined after storage of the inoculated bacon for 14 and 28 days. All toxic packs contained $10^5 - 10^6$/g cells of *Cl. botulinum*. The author believes that the *Cl. botulinum* cells (spores and vegetative cells) had died out in the non-toxic packs during the first few days of storage when the nitrite levels were high. We do not know what could have happened if further spores of *Cl. botulinum*, had been inoculated into the bacon after it had been stored and nitrite levels were low. Similar die out of spores has been observed in experiments with canned meat products containing curing salts.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen.

Inhibition of bacterial growth in model systems in relation to the stability and safety of cured meats

T. A. Roberts

ARC Meat Research Institute, Langford, Bristol, BS18 7DY, UK

Abstract

The interaction of inhibitory factors present in cured meats are known to be responsible for the bacteriological safety and stability of those products. Methods for the quantitative evaluation of these interactions are discussed with particular reference to pH, sodium chloride, nitrite and nitrate. Storage temperature is also considered.

Introduction

It is not the purpose of this paper to review the literature on the inhibition of growth of *Clostridium botulinum* in relation to cured meats: such reviews have already been provided by Ingram and Baird-Parker (this Symposium). I propose instead to describe the ways in which we have attempted to evaluate the extent of interactions between factors known to be important in inhibiting *Cl. botulinum* and *Staphylococcus aureus*. When an additional factor is added to a system its effect may be independent of those already acting, in which case it will be additive; or it may display synergistic or antagonistic interactions with one or more of the other factors. All such cases are included in the description 'interaction' in this paper.

Riemann (1963) demonstrated clearly that interactions of factors were significant in maintaining the bacterial stability of cured meat products, but made no attempt to determine their extent. Even a cursory consideration reveals the complexity of the subject. Within the commercially acceptable ranges of concentration of sodium chloride and sodium nitrite, higher values are known to inhibit bacterial growth more effectively than low, and nitrite is more effective in acid than in alkaline conditions (Roberts & Ingram, 1966). At given pH and concentrations of salt and nitrite, poorer growth results at sub-optimal storage temperatures, or if very low numbers of cells are used in the inoculum. Heat damaged spores are more sensitive to inhibition by curing salts than are unheated spores (Roberts & Ingram, 1966; Roberts, Gilbert & Ingram, 1966).

Already these five factors, if investigated at four levels each, lead to 1024 conditions without considering replication.

Medium

In addition the medium in which the tests are to be made is crucially important. Pork macerate closely resembles the practical situation and is excellent in respect of growth, but problems arise in handling large amounts and in obtaining uniform batches. Partial spoilage of raw pork macerate during, or even before, the test can be a real problem, and cooked macerate is in some respects easier to handle. If cooked macerate is used, the absence of the natural bacterial flora of pork might influence the effects of curing salts since no bacterial competition will be present. Different commercial products formulations should be considered, since surprisingly little is known of the effects on bacterial growth in the presence of curing salts of polyphosphates (which are difficult to study precisely because of variability in chemical composition), ascorbic acid, ascorbate, erythorbate or sugars, all of which may be used commercially and add to the interactions to be tested. Hence, for ease of experimentation, we initially used a bacteriological medium shown to give results very similar to pork macerate and are currently testing our conclusions in pork macerate itself.

Bacteria

A further problem arises in the choice of bacteria for the test. Clearly the toxigenic types of *Cl. botulinum* causing human botulism, types A, B, E and F should be tested, although type E may be omitted since it is associated with the marine environment and is already known to be much more sensitive than the other types to curing salts (Roberts & Ingram, 1973). However, within each toxigenic type the choice of bacterial strain could be important, since there is no guarantee that the strain most resistant under one combination of screening conditions will also be the most resistant under other conditions. Virtually no information is available on the variability of resistance of *Cl. botulinum* to salt and nitrite among strains and this all leads to considerable effort on screening. If spores are to be used, the manner in which they have been prepared and stored may be important, and, ideally it should be established that 'laboratory' spores are identical in relevant properties to those which occur in nature as contaminants of meat products. Again remarkably little relevant information is available.

It would also be prudent to make similar studies on other bacteria causing food poisoning e.g. *Cl. welchii, Staphylococcus* and *Salmonella,* although such studies could initially be a simple test of whether conditions inhibitory to *Cl. botulinum* are capable of inhibiting them. A range of strains of 'putrefactive anaerobes' could also be tested to determine whether they behave as 'indicators' of possible growth of *Cl. botulinum,* as they do in heat processed foods.

The problem is, therefore, enormous, and unlikely to be covered fully by any one laboratory, and it is time that a serious attempt was made to investigate inter-laboratory variation in experiments of this nature, with a view to sharing the work load. At present workers in different laboratories are using different media, methods and strains, and consequently it is difficult to correlate their results.

We have concentrated our effort on relatively simple systems which seemed

more likely to be reproducible and suited to the evaluation of the complex of interactions.

Evaluation of the inhibitory effects of mixtures of curing salts: method

Counts of viable bacteria in agar media

The simplest approach is to count the viable bacteria under test in agar media with a range of pH values and containing different concentrations and combinations of NaCl and $NaNO_2$ (or other relevant additives) and to compare them with counts in the same media containing no additives. We have used this method to show that a 12-D concept equivalent to that used in heat processing could be developed for pasteurized cured meats (Ingram & Roberts, 1971). These data also showed the absence of any effect of a mixture of NaCl and $NaNO_2$ on the heat resistance of spores of *Cl. botulinum* and demonstrated that the absence of growth was due to the inhibition of viable spores by the curing salts in the growth medium. This method is convenient when the incubation temperature is near optimal and growth consequently rapid, but at lower incubation temperatures, the slower growth necessitates prolonged incubation which is inconvenient when using anaerobe jars. The opening of anaerobe jars to examine the contents during prolonged incubation may cause changes in the redox conditions detrimental to spores surviving but remaining dormant, which could result in the test conditions being adjudged more inhibitory than in fact they are.

Growth – no growth tests

We have investigated in some detail the interactions of pH, NaCl and $NaNO_2$ in a medium based on a casein hydrolysate ('Trypticase', BBL) using *Cl. botulinum* types A, B, E and F. These tests were made in airtight screw-capped bottles, in which prolonged incubation without breaking anaerobiosis was possible.

Results and discussion

pH, NaCl, $NaNO_2$, storage temperature

In an initial experiment using spores of *Cl. botulinum* type B growth (at 35°C) was sporadic and not evidently related to the concentration of salt or nitrite. The reason for this was not discovered, and, although subsequent spore crops behaved more reproducibly and in a less unexpected manner, we eventually decided to work with inocula of vegetative cells arguing that, since toxin is elaborated during vegetative growth, inhibition of such growth would be an adequate safeguard against toxin production.

Results using vegetative cells of *Cl. botulinum* types A, B, E and F are in press (Roberts & Ingram, 1973). The four diagrams of that paper have been drawn together as one, representing the greatest growth of *Cl. botulinum* type A, B, E or F (Fig. 1). The same data may be expressed in tabular form (Table 1). In less extensive experiments, addition of 0.3% of a polyphosphate in common commer-

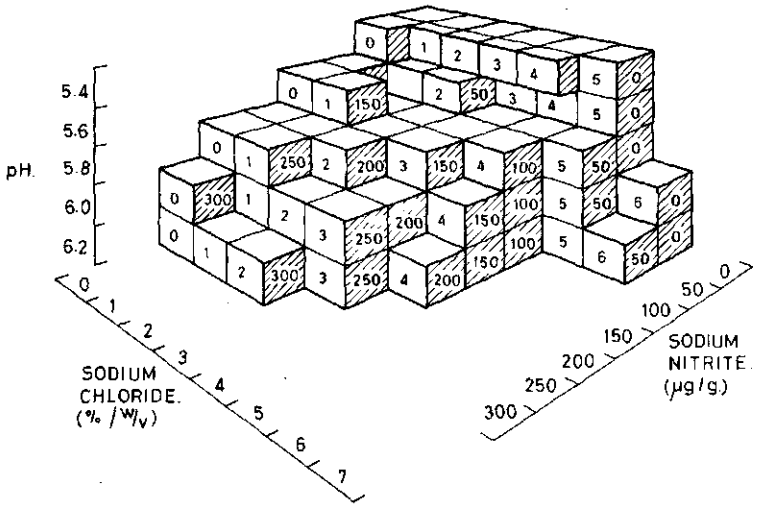

Fig. 1. The effect of pH, NaCl and $NaNO_2$ on growth of *Cl. botulinum* at 35°C. Composite diagram of types A, B, E and F. Blocks represent growth at the concentrations and pH indicated.

cial usage did not greatly affect the inhibitory levels of NaCl and $NaNO_2$. It is evident from Fig. 1 that the limit of the possibility of bacterial growth is expressed by a surface which bounds all effective concentrations of acid, NaCl and $NaNO_2$. Once this surface is defined, it is a simple matter to test the effect on it of other factors. After suitable mathematical transformation of the units of the axes, the surface describing the bulked data for types A, B, E and F approximates to $\frac{1}{8}$ of a sphere (Fig. 2) (although this might not be so when more data from current experiments at pH 7 are included). Although a diagram of this nature is of little

Table 1. Effect of sodium nitrite, sodium chloride and pH on vegetative inoculum of *Cl. botulinum* types A, B, E and F at 35°.

NaCl (% w/v)	a_W	Highest concentration of nitrite (mg/l) in which growth of any *Cl. botulinum* occurred at pH:				
		6.2	6.0	5.8	5.6	5.4
0	0.999	300+	300+	250	150	50
1	0.994	300	250	250	150	–
2	0.989	300	250	200	50	–
3	0.983	250	250	150	–	–
4	0.977	200	150	100	–	–
5	0.971	50	50	50	–	–
6	0.965	50	–	–	–	–

Note: The levels of nitrite are those initially added to the medium. – no growth with 50 mg/l sodium nitrite.

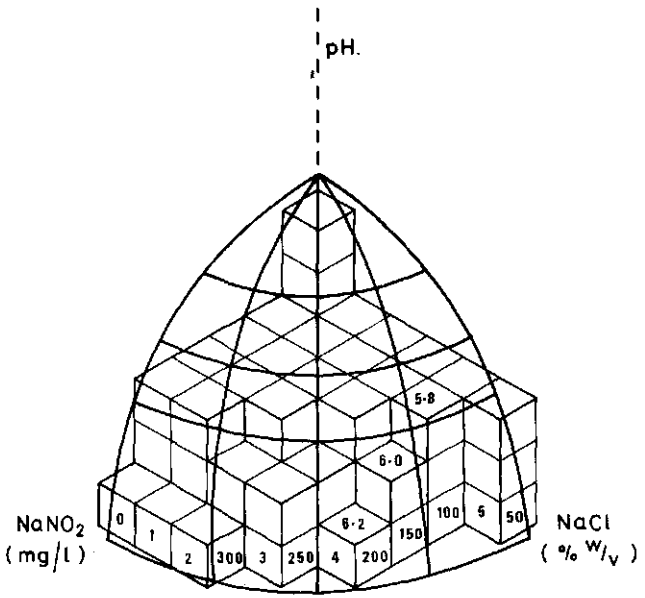

Fig. 2. Plot of growth zone of *Cl. botulinum* inside a $\frac{1}{8}$ sphere (using transformed co-ordinates).

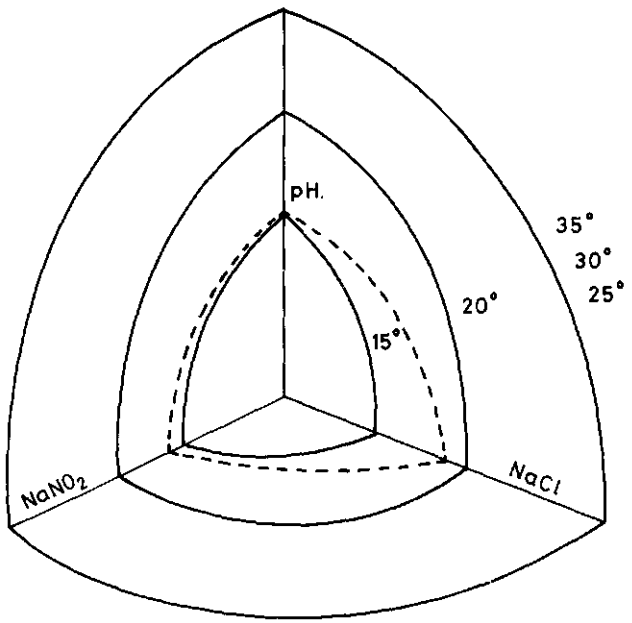

Fig. 3. Inhibition of *Cl. botulinum* by NaCl × $NaNO_2$ × pH. Effect of incubation temperature.

value to research or commerce, it may readily be converted into a nomogram which is simple to use.

We know that *Cl. botulinum* is inhibited more readily by curing salts when the incubation temperature is reduced, and under such conditions new definitions of the limiting surfaces will be needed. Preliminary tests indicate that temperatures from 25 to 35°C give essentially the same results; below 25°C temperature becomes increasingly important, and below 20°C inhibition of growth of *Cl. botulinum* may be achieved with commercially acceptable concentrations of NaCl and $NaNO_2$ provided that the pH is near 6.0. Such changes are represented diagrammatically in Fig. 3.

Similar detailed data on the effect of incubation temperature are available for the inhibition of *Staphylococcus aureus* by NaCl and $NaNO_2$ and resemble those described above (Bean, 1972; Bean & Roberts, in preparation) i.e. differences between 35 and 25°C are relatively small, but incubation at lower temperatures results in markedly greater inhibition.

All the above indicate that concepts of 'limiting water activity' are erroneous in not taking into account at least the pH of the growth medium.

$NaNO_3$

The effect of nitrate in the stabilizing of pasteurized cured meats has been little investigated, it is generally considered to be equivalent, weight for weight, to sodium chloride; however, Riemann (1963) suggested that it could be more than this.

Unpublished data kindly supplied by J. A. Perigo (Metal Box Co. Ltd., London) have been grouped and re-plotted in a manner resembling the data for *Cl. botu-*

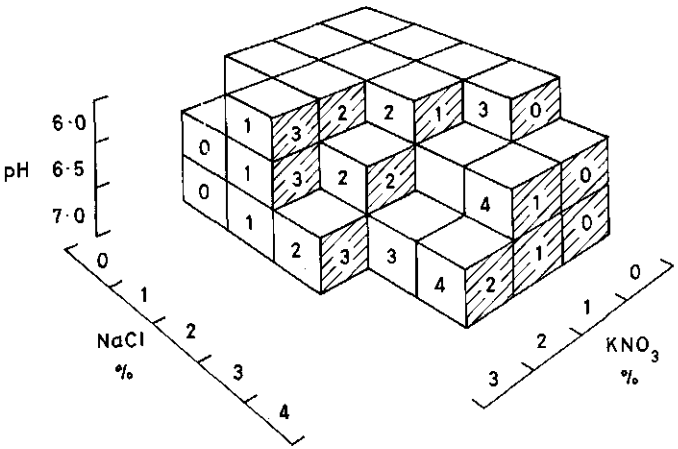

Fig. 4. The effect of pH, NaCl and KNO_3 on growth of *Cl. sporogenes* spores at 30°C ($NaNO_2$ absent).

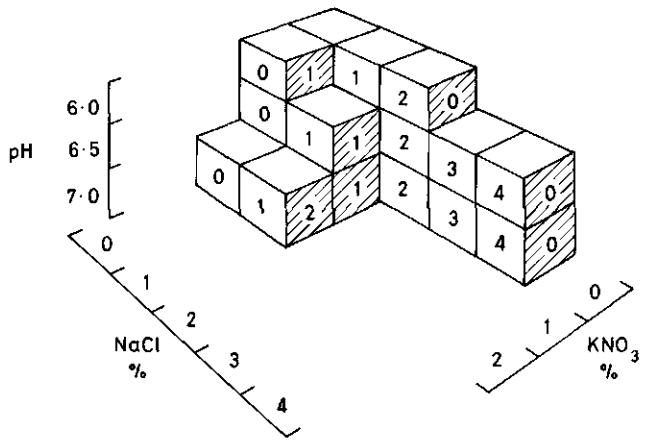

Fig. 5. The effect of pH, NaCl and KNO_3 on growth of *Cl. sporogenes* spores at 30°C ($NaNO_2$ 10 mg/l).

linum. The basal growth medium was that used in Perigo's studies of the heated nitrite inhibitor, and in these experiments, too, the nitrite was heated in the medium. Hence the concentrations limiting growth cannot be compared directly with those presented in Fig. 1 and Table 1 for *Cl. botulinum*. Three so far unconfirmed conclusions may be drawn:

1. the interaction of NaCl × KNO_3 reported by Riemann (1963) is demonstrated clearly both in the absence of nitrite (Fig. 4) and in the presence of 10 mg/l of heated nitrite (Fig. 5).

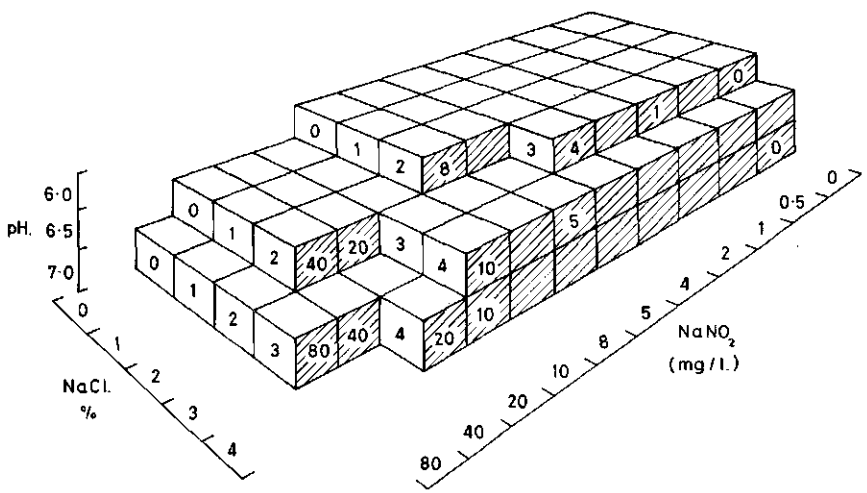

Fig. 6. The effect of pH, NaCl and $NaNO_2$ on growth of *Cl. sporogenes* at 30°C (KNO_3 absent).

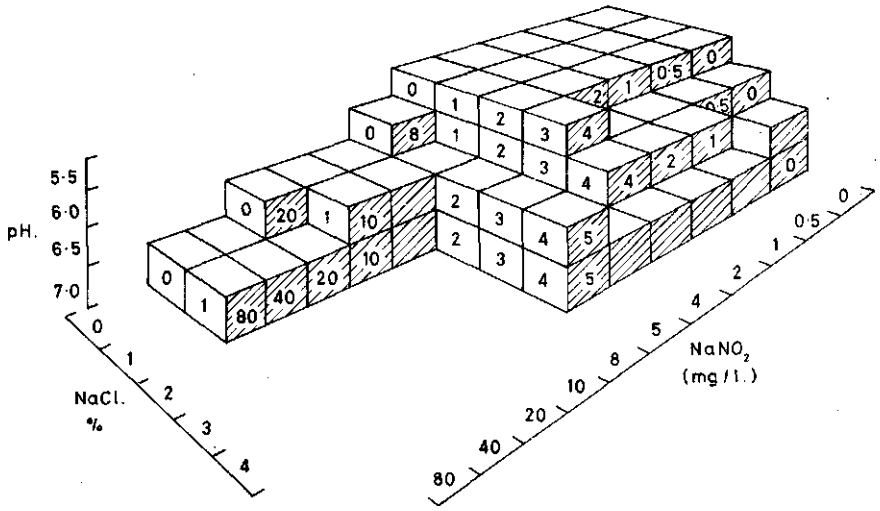

Fig. 7. The effect of pH, NaCl and $NaNO_2$ on growth of *Cl. sporogenes* at 30°C (KNO_3 1%).

2. if the data are arranged in 3-dimensions(pH × NaCl × $NaNO_2$)with and without 1% KNO_3, growth occurred, at constant pH, in higher concentrations of NaCl and $NaNO_2$ when nitrate was absent than when present, indicating an additional inhibitory effect of nitrate (Figs. 6 and 7).
3. further plotting of the data assuming 1% KNO_3 to have an effect equivalent to 1% NaCl (i.e. comparing 2% NaCl with 1% NaCl + 1% KNO_3), less growth occurred when nitrate was present (Fig. 8). The effect of 1% KNO_3 on water activity (a_W) is much smaller than 1% NaCl, hence a system containing 1% NaCl + 1% KNO_3 will

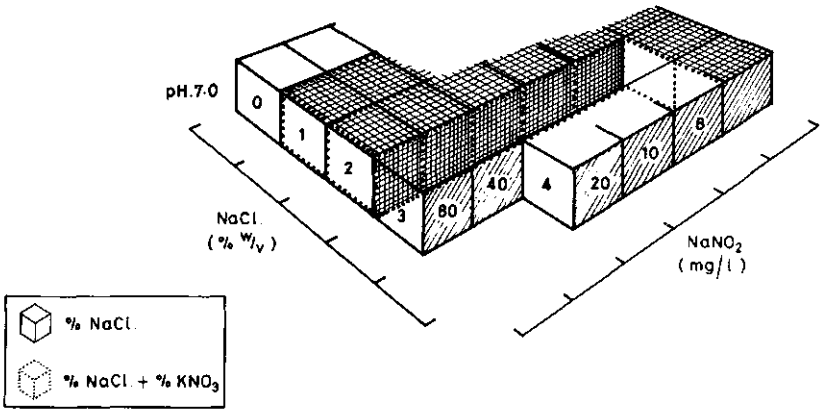

Fig. 8. The effect of KNO_3 on inhibition of *Cl. sporogenes* at 30°C by NaCl and $NaNO_2$.
Solid line = KNO_3 absent.
Broken line = sum of the concentration (%) NaCl + KNO_3.

have a higher a_W than a system containing 2% NaCl. Yet the former is the more inhibitory, indicating an effect of KNO_3 greater than can be accounted for by a_W alone. This occurred at pH 6.0, 6.5 and 7.0, although responses were not identical at these pH values. These data suggest that it might be unwise to insist upon the absence of nitrate from cured meats, at least until it has been established that products without nitrate are in no way less safe than those which contain it in low concentration.

Final remark

Viable counts by Most Probable Number (MPN) method

An alternative to viable counts in agar media is to make MPN counts in media containing different concentrations and combinations of curing salts. Although the method is inherently less accurate than plate counts, it has the advantage that incubation can be prolonged almost indefinitely, and examination for growth does not change the conditions of incubation. The results may be expressed conveniently to indicate the number of spores which are inhibited by a particular system. Results are accumulated as illustrated in Fig. 9, and when sufficient data become available, a surface may again be drawn covering the numbers of spores inhibited by different combinations of NaCl and $NaNO_2$. If experiments are made in media of different pH values, or at constant pH but incubated at different temperatures, a diagram similar to Fig. 10 will evolve, and it seems possible that they may be used to predict the safety margin of cured meat systems, in much the same way that the 12-D concept led to a safe canning process. A disadvantage of the method is that relatively large numbers of cells (or spores) are required to avoid unrealistic extrapolation to the level of 10^{12} spores. We are now concentrating on this type of

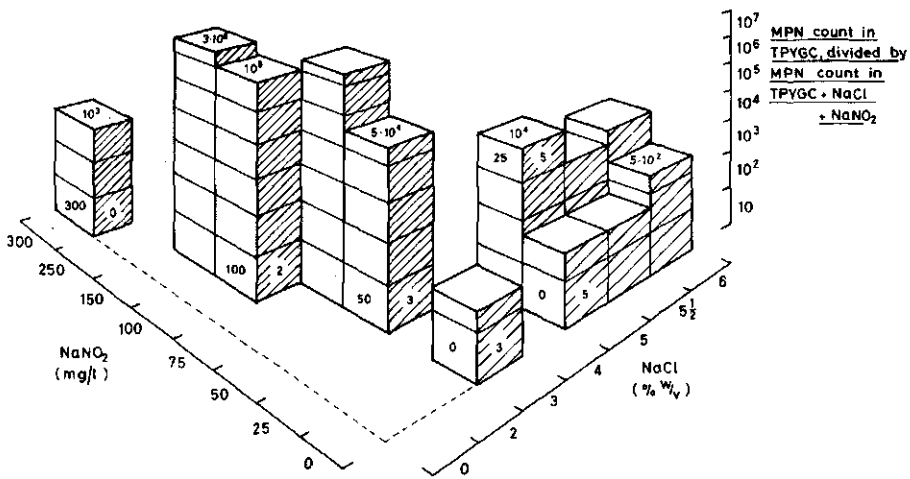

Fig. 9. Inhibition of *Cl. botulinum* in TPYGC (pH 6.2).

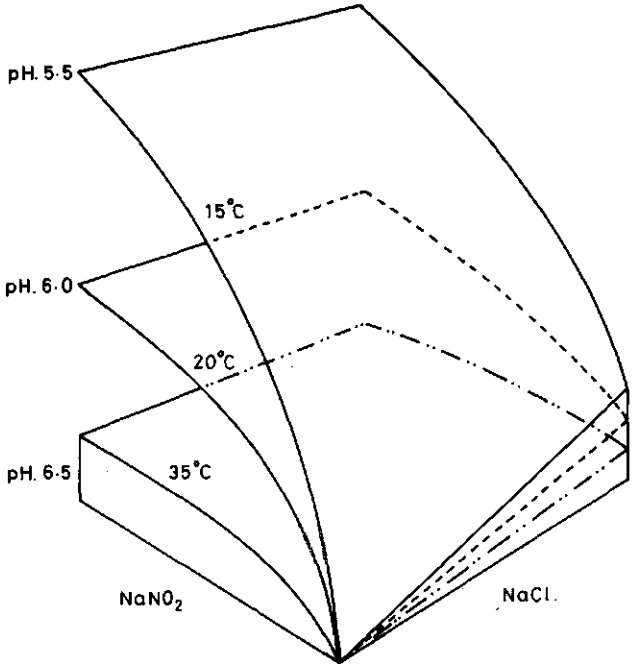

Fig. 10. Effect of pH or incubation temperature on inhibition of bacterial growth.

experiment, and particularly upon means of automating the rather tedious process of making very large numbers of MPN counts.

These investigations are beginning, for the first time, to lead to an understanding of the quantitative relationships between factors important in cured meats.

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Discussion

Petri dishes in plastic pouches

The use of Petri dishes sealed in plastic pouches containing alkaline pyrogallol as an absorbent was suggested as an alternative incubation method. Prolonged incubation and periodic examination is then possible without disturbing anaerobiosis.

Duration of incubation

The diagram obtained at near-optimal growth temperatures (35°C) using vegetative cell inocula (Fig. 1) did not alter greatly after 2 weeks' incubation, although incubation was continued for 3 months.

Nitrate concentrations

The nitrate concentrations in Figs. 4–8 are high, and tests should be made with commercially realistic concentrations to determine whether it has any antimicrobial effect prior to considering its prohibition.

Temperature dependence of pK of HNO_2

Experiments at different incubation temperatures must take into account that the pK of HNO_2 (presuming this to be the active agent) rises considerably with temperature.

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Minimum nitrite concentrations for inhibition of clostridia in cooked meat products

A. B. G. Grever

Central Institute for Nutrition and Food Research TNO, Utrechtseweg 48, Zeist, the Netherlands.
Dept.: Netherlands Centre for Meat Technology

Abstract

Emulsions of cooked sausage and of liver sausage prepared in a factory or a laboratory were tested for the amount of nitrite required to prevent growth of clostridia after pasteurization at 80°C and storage for 5 weeks at 24°C.

Endogenous and inoculated spores, and spores from contaminating spore-bearing materials were involved. It was concluded that 200 mg/kg nitrite must be added, provided that the maximum value of pH is 6.2.

Also the amount of nitrite needed for sterilized sausage emulsions was determined. Inoculated sausage emulsions heated at $F_0^1 = 0.5$ often showed inhibition without nitrite. This could be due to the inhibitory action of salt on spores damaged by heat.

Thus for meat products, heated in $F_0 = 0.5$, addition of 100 mg/kg sodium nitrite is sufficient to prevent growth of clostridia.

Simultaneously, growth possibilities for bacilli in pasteurized and sterilized sausage were studied. To prevent growth of bacilli more nitrite is required than to prevent growth of clostridia. The investigation also provided information on the degree of nitrite depletion during heating and storage.

Introduction

In order to restrict the use of nitrite in food as far as possible there is a need for information on the minimum nitrite concentration needed in meat products to prevent growth of clostridia. This was investigated for pasteurized factory-made sausages.

At least 200 mg/kg nitrite must be added when the pH is not above 6.2. This was verified with laboratory-made liver sausages contaminated in different ways. It was studied whether it was possible to decrease the required amount of nitrite by lowering the pH.

It could be expected that in appertized and sterilized meat products less nitrite is needed to prevent growth of clostridia. This was investigated for two meat products: liver paste and luncheon meat.

1. See p. 67.

Experimental

Factory-made emulsions

Six factories were requested to prepare emulsions of cooked sausage and of liver sausage in the usual way but without nitrite and salt. These were added at the Institute: the salt to a brine percentage of 3.5% and the nitrite to amounts of 0, 50, 100 and 200 mg/kg.

Small cans (size 76 × 35 mm) were filled with the emulsions, pasteurized (in the centre 10 min at 80°C) and stored at 24°C. After 1, 3 and 5 weeks the cans were tested for growth of spore forming bacteria. Residual nitrite content was estimated at the same time.

Laboratory-made liver sausage emulsions

Liver sausage emulsions were prepared in the usual way. The brine percentage was 3.5% and the amounts of added sodium nitrite were respectively 75, 125, 150 and 200 mg/kg. The pH was approximately 6.2. The emulsions were inoculated with various strains of clostridia and with spore bearing materials such as spices, soil, well-decayed cow-dung and dust. The emulsions were canned, pasteurized and stored just as for the factory-made products. After pasteurization the spore number was 30–300 per gram.

Non-inoculated emulsions were also included in the experiments. In spite of careful preparation under sanitary conditions the emulsions also contained some spores. The growth possibilities of these spores in the nitrite-containing emulsions were examined as well.

Laboratory-made cooked sausage with pH 5.8

A cooked sausage was prepared with an adjusted pH-value of 5.8. The brine percentage was again 3.5%. Inoculation, pasteurization and storage conditions were the same as for liver sausage emulsions.

Sterilized sausage emulsions

Luncheon meat and liver paste emulsions were prepared (brine percentage 3.5%) and 100 and 200 mg/kg nitrite added, respectively. They were inoculated with spore-bearing materials to 100 and 500 spores per gram. The emulsions were packed in small cans (76 × 35 mm) and heated at 95°C and 105°C with F_0 values of 0.05 and 0.5, respectively. The cans were incubated for 5 weeks at 30°C and examined for growth of spore-forming bacteria.

Nitrite depletion

To obtain information about the nitrite depletion in the various sausage products, nitrite concentrations were determined immediately after pasteurization and sterilization and during storage. The analysis was carried out simultaneously with

the bacteriological examination. To determine the non-bacteriological depletion of nitrite only the results for cans without observed bacterial growth were taken into account.

Results

Pasteurized sausage

Factory-made sausage emulsions As shown in Table 1, five out of twelve lots of sausage emulsions, failed to show growth of clostridia after pasteurization, even when no nitrite was added. Apparently the emulsions either did not contain clostridia, or, if present, the conditions were unfavourable for growth. One of the other lots was inhibited by 50 mg/kg nitrite, others only by 100 or 200 mg/kg nitrite. In two lots of liver sausage emulsions no inhibition was found even when 200 mg/kg nitrite had been added. The pH value of one of these emulsions was as high as 6.9 as is shown in Table 1. The table also demonstrates that the counts of *Clostridium* spores determined immediately after pasteurization were low.

Laboratory-made sausage emulsions In all experiments with liver sausage emulsions (pH = 6.2) inoculated spores were inhibited by 200 mg/kg nitrite. Growth occurred if the nitrite addition was less than 200 mg/kg.

For cooked sausage emulsions, 50 mg/kg nitrite did not inhibit inoculated spores in spite of the low pH-value of 5.8. The spores, originating from the spore-bearing materials, were not even inhibited by 200 mg/kg nitrite.

Nitrite depletion The degree of nitrite loss during pasteurization varied consider-

Table 1. Inhibition of clostridia in pasteurized, factory-made sausage emulsions.

Product	Factory code	Amount of nitrite needed for inhibition of clostridia (mg/kg)	pH after pasteurization	Count of *Clostridium* spores per gram after pasteurization
Cooked sausage emulsion	I	50	6.5	< 10
	II	100	6.3	2 × 10
	III	0	5.9	2 × 10
	IV	0	5.9	< 10
	V	100	6.4	2 × 10
	VI	200	6.5	< 10
Liver sausage emulsion	I	200	6.9	1 × 10
	II	> 200	6.9	2 × 10
	III	0	6.5	1 × 10
	IV	0	6.8	< 10
	V	0	5.8	1 × 10
	VI	> 200	6.5	1 × 10

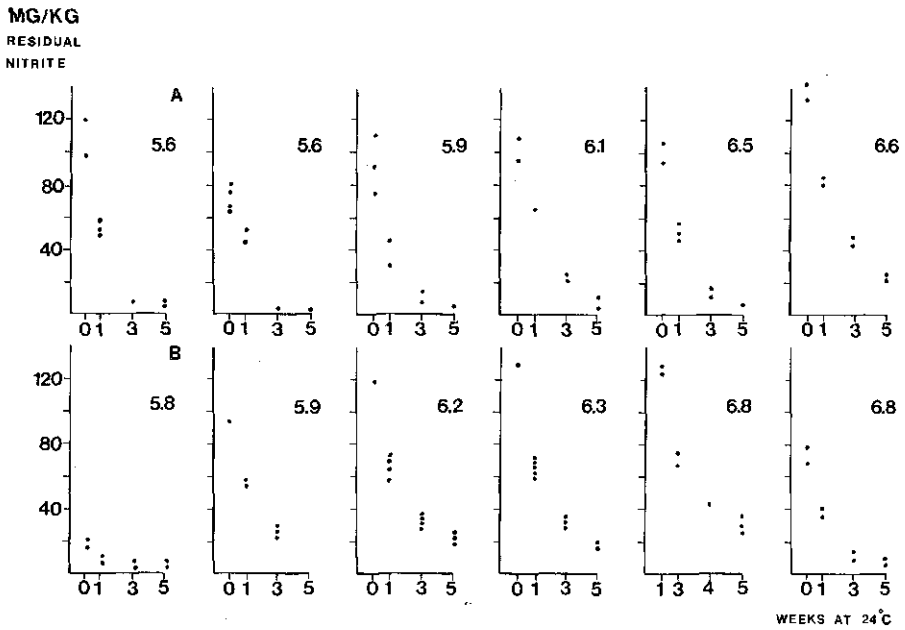

Fig. 1. Decrease of nitrite during storage at 24°C of pasteurized canned sausage emulsions made in the lab or from the factory, containing 200 mg/kg added nitrite. The figures refer to cans showing no growth of bacteria. A: cooked sausage; B: liver sausage.

ably. Nitrite losses were found to vary from 30 to 90%. The non-microbiological depletion of nitrite during storage is shown in Fig. 1.

Sterilized sausage emulsions

Inoculated *Clostridium* spores, after having survived a heat treatment in the meat product at 95°C ($F_0 = 0.05$), were not inhibited by 200 mg/kg nitrite during incubation at 30°C. However, the few spores in the non-contaminated meat products were inhibited by this amount of nitrite.

The contaminated meat products with 100 and 200 mg/kg of added nitrite which were processed at 105°C to an F_0 value of 0.5 did not show growth during incubation at 30°C. Surprisingly, growth often did not occur in the absence of nitrite either. Closer examination revealed this inhibition to be related to the action of salt on heat-damaged spores; the same meat product without salt did show growth.

This salt-sensivity has been established by Roberts & Ingram (1966), Roberts et al. (1966), Duncan & Foster (1968), Pivnick & Thacker (1970), Ingram & Roberts (1971), Van der Laan (1971) and Holwerda (1973).

Bacillus spores, surviving the heat processing of the meat product at 95°C (F_0-value 0.05), were not inhibited by 200 mg/kg nitrite. Inhibition by nitrite only occurred sometimes in the meat product which was heated at an F_0 value of 0.5.

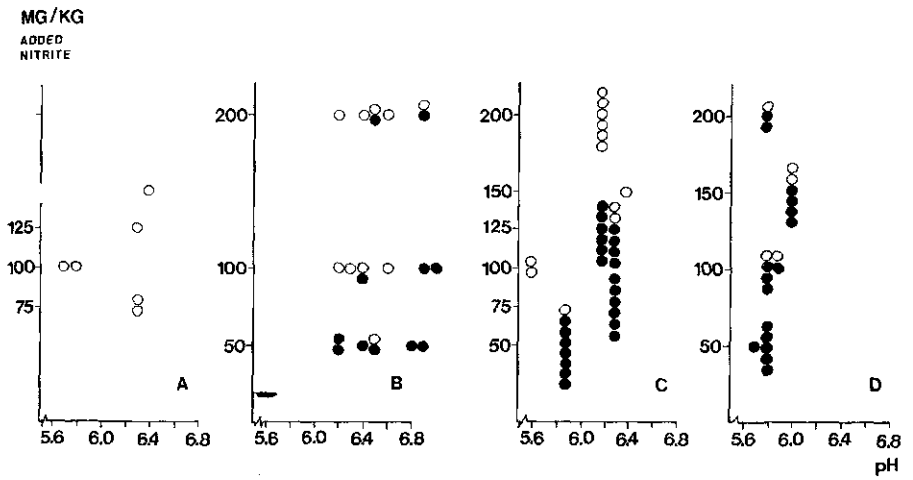

Fig. 2. Growth of clostridia after storage (5 weeks at 24°C) of pasteurized, canned sausage emulsions made in the lab or from the factory arranged according to added amounts of nitrite and pH values. A: endogenous spores from laboratory-made sausage emulsions; B: endogenous spores from factory-made sausage emulsions; C= inoculated spores; D: spores from contaminating spore-bearing materials. •= growth; ○= no growth.

Discussion

The results of all 350 pasteurization/storage experiments are graphically represented in Fig. 2.

It should be emphasized that all these experiments refer to those cases in which growth of clostridia occurred without added nitrite.

To guarantee complete prevention of growth of clostridia 200 mg/kg sodium nitrite should be added. Even then – with a heavy contamination – growth of clostridia might occur. For still better security, a maximum pH-value of 6.2 should be maintained.

This conclusion is also based on the work of others. Steinke & Foster (1951) established inhibition of 5 000 *Cl. botulinum* spores in liver sausage with 200 mg/kg nitrite when heated ag 70 °C. Bulman & Ayres (1952) reported the prevention of spoilage of pork trimmings, heated at 80 °C, containing 150 mg/kg of added nitrite and a natural level of spores below 1 per gram. However, no inhibition was osberved at an inoculation level of 50 *Clostridium* spores per gram. Mol (1970) inhibited growth of inoculated Clostridium spores in a meat mix, heated at 78 °C, by adding 144 mg/kg sodium nitrite as a pH-value of 6.2. Pivnick & Barnett (1965) inoculated Bologna sausage with *Clostridium botulinum* spores and obtained inhibition with approximately 100 mg/kg nitrite (10^4 spores per gram before cooking at 71 °C).

Bacilli are less inhibited by nitrite than clostridia as can be seen from Fig. 3. Only in the non-inoculated emulsions were bacilli always inhibited by 200 mg/kg nitrite.

As far as sterilized meat products are concerned, the present investigation indicates that less nitrite may be sufficient.

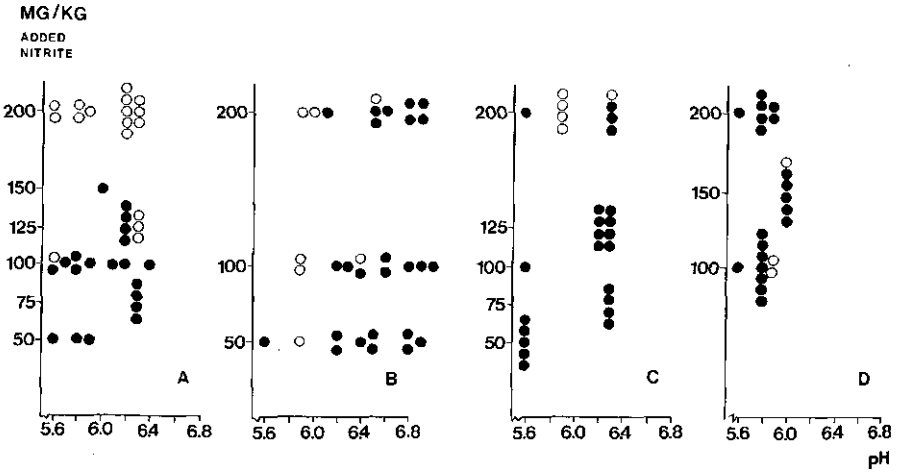

Fig. 3. Growth of bacilli after storage (5 weeks at 24°C) of pasteurized, canned sausage emulsions made in the lab or from the factory arranged according to added amounts of nitrite and pH values. A: endogenous spores from laboratory-made sausage emulsions; B: endogenous spores from factory-made sausage emulsions; C: inoculated spores; D: spores from contaminating spore-bearing materials. ●= growth; ○= no growth.

For a heat treatment resulting in a F_0 value of 0.5, clostridia were found to be inhibited by 100 mg/kg nitrite, provided that the brine percentage is 3.5 %.

The question arises whether this also applies to somewhat lower salt percentages and F_0 values.

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Discussion

Experimental details

The addition of salt and nitrite and the heat treatments were done in the pilot plant of the Institute, with the highest accuracy.

The data in Figure 2 refer to cans in which clostridia and in many cases also bacilli had developed.

Values of pH in practice

The pH range found in cooked, cured meat products is between 5.8 and 6.8. The growth of clostridia is better at the higher pH values. However the residual nitrite content after heating and during storage will remain higher.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen.

Perigo effect in pork

H. Pivnick and P-C. Chang

Food Research Laboratories, Food Directorate, Health Protection Branch, Health and Welfare Canada, Ottawa

Abstract

An inhibitor against *Clostridium botulinum* was formed when canned pork luncheon meat was processed to F_0=0.4. The meat was manufactured with 0–300 mg/kg of $NaNO_2$ and all of it was held at 35°C after processing until the highest concentration of nitrite declined to less than 2 mg/kg. Meat in cans was then inoculated with spores of *Cl. botulinum* that had survived a heat treatment of F_0=0.4 in a solution of raw meat juice, 4.5% salt and 150 mg/kg of $NaNO_2$. The inhibitory effect could be demonstrated by an increase in time required for inoculated cans to swell and by the number of spores required to initiate growth and cause swelling. However, the inhibitory effect was relatively small; meat made with 3.8% salt in the water phase and 200 mg/kg of nitrite inhibited 3.3 $\log_{10}$ of spores (3.3 units of inhibition). Salt contributed 1.86 units and the inhibitor formed from nitrite contributed 1.43 units.

Introduction

The value of nitrite as an inhibitor of *Cl. botulinum* in canned meat has been recognized for several decades, but is was thought that nitrite per se caused the inhibition. The discovery that nitrite could form an antimicrobial inhibitor (PF) when heated with some components of bacteriological media (Perigo et al., 1967; Perigo & Roberts, 1968) suggested that this inhibitor might be important in the safety of canned cured meat.

However, PF added to meat was inactivated: mixtures of PF and meat were not inhibitory to *Cl. botulinum* (Johnston et al., 1969). This discovery does not preclude the possibility that nitrite heated with meat might form a Perigo-type (inhibiting) factor (PTF) that is not identical with PF. Johnston et al. (1969) and Ashworth & Spencer (1972) showed that meat heated with nitrite could form an inhibitor that was not formed in meat heated without nitrite. The inhibitor was not very potent and, to demonstrate it, they had to add sterile nitrite to the heated meat or have appreciable amounts of free nitrite present after heating. Only recently, an inhibitor has been demonstrated in meat in the absence (<2 mg/kg) of free nitrite (Chang & Pivnick, 1973). The purpose of this report is to describe the experiments and to demonstrate a method for quantitating the inhibitory effect.

Materials and methods

Pork luncheon meat containing 0–300 mg/kg of sodium nitrite and 2.3% salt (3.8–3.9% in the water phase) was stuffed in cans of 3.00 × 3.08 inches (76.2 x 78.2 mm) and processed at 110°C for 85 min to F_0=0.4. All cans in a single experiment were then held at 35°C until the highest concentration of nitrite had decreased to < 2 mg/kg. Spores of *Cl. botulinum* 13983B were used to inoculate the meat. The spores were heated at 100°C to F_0=0.4 in an non-particulate extract of raw, sterile (γ-irradiated) pork supplemented after irradiation with 150 mg/kg $NaNO_2$ and 4.5% NaCl. Cans were pierced aseptically, and 0.1 ml of suitably diluted inoculum was introduced into the centre of the meat with a hypodermic syringe and needle. Thereafter, the cans were resealed, incubated at 30°C, and examined weekly for swelling. Meat in all cans that swelled contained botulinum toxin. No toxin was found in non-swollen cans.

Estimates of the numbers of spores that grew after introduction into the canned meat were made by the method of Fisher (1922) and Fisher & Yates (1963).

Results and discussion

Two main experiments were carried out. For each, canned meat and spore preparations were freshly prepared. In Experiment 1, the sodium nitrite (nitrite) added to the raw product varied from 0–300 mg/kg, and the heat damaged spores inoculated per can varied from $2.2 \times 10^1 - 2.2 \times 10^5$. Table 1 shows that with 22 spores per can there was little difference in the proportion of cans with toxic meat regardless of whether 0 or 200 mg/kg of nitrite was used; unfortunately we did not inoculate 22 spores into meat made with 300 mg/kg. In meat processed without nitrite and inoculated with 22 spores per can (440 spores in 20 cans), the estimated number of spores that grew was 11.86 and the ratio of spores added/spores that grew was 37.08 ($\log_{10}$ = 1.57); 97.3% of the spores failed to grow. This is about the amount of inhibition against spores heat damaged to F_0=0.4 that one would expect from 3.8% salt (Pivnick & Thacker, 1970); the brine % in the meat was 3.8%.

Table 1. Toxigenesis by Cl. botulinum in canned meat depleted of nitrite, Experiment 1[1]. Recorded are: number of cans toxic/number of cans inoculated.

$NaNO_2$ added (mg/kg)	Number of spores added per can				
	2.2×10	2.2×10^2	2.2×10^3	2.2×10^4	2.2×10^5
0	9/20	18/20	20/20	.	.
100	4/20	20/20	20/20	10/10	.
200	2/10	20/20	20/20	20/20	.
300	.	19/20	20/20	20/20	20/20

1. 11.8% protein; 59.7% water; 4.0% dextrose equivalent; 2.4% salt; 3.8% brine (salt in water phase). All cans held 15 weeks at 35°C until meat with 300 mg nitrite per kg contained less than 2 mg/kg.

Table 2. Weeks at 30°C for 50% of inoculated cans to swell (t_{50}). Experiment 1.

$NaNO_2$[1] (mg/kg)	Number of spores added per can				
	10^1	10^2	10^3	10^4	10^5
0	.	1.5	1.2	.	.
100	.	2.0	1.3	1.2	.
200	.	2.5	1.7	1.3	.
300	.	3.4	3.0	1.7	1.6

1. See footnote for Table 1.

Although we had used too many spores for most of the cans in Experiment 1, we did observe that the time for 50% of the cans to swell (t_{50}) increased with the concentration of nitrite added to the meat (Table 2). Thus, there was considerable indication that the nitrite, although no longer present at time of inoculation, had been converted into an inhibitor. As expected, the t_{50} decreased as the inoculum was increased. Christiansen et al. (1973) have observed similar results with canned meat inoculated before pasteurization, but their results, although suggesting the presence of a PTF, are far from conclusive: free nitrite was available after pasteurization.

Experiment 2 was similar to Experiment 1, except that we used smaller numbers of heat-damaged spores. Table 3 shows the composition of the meat and the nitrite level at time of inoculation (after 14 weeks at 35°C). Table 4 shows the number of cans that became toxic at each level of nitrite and spores. The numbers of heat-damaged spores added per can varied from 8 to 128 and the total numbers of heat-damaged spores added to the 80 cans at each nitrite level was 3968. The estimates (Fisher & Yates, 1963) of spores that grew at each level of nitrite is based on the total number of cans that became toxic divided by the total number of cans that were inoculated. These estimates were in good agreement with most probably

Table 3. Analysis of canned luncheon meat. Experiment 2[1].

Sodium nitrite (mg/kg)					Brine (%)
added	in raw product	after F_0=0.4	after storage at 35°C		
			6 weeks	14 weeks	
0	0	0	0	0	3.7
50	32	9	1.6	0.6	3.8
100	80	17	3.4	0.8	3.8
150	136	31	4.7	1.1	3.9
200	192	54	7.5	2.1	3.9

1. 24% fat; 12.1% protein; 58.2% water; 4.0 dextrose equivalent.

Table 4. Toxigenesis by *Cl. botulinum* in canned meat depleted of nitrite. Recorded are: number of cans toxic per 16 cans inoculated[1]. Experiment 2.

$NaNO_2$ added (mg/kg)	Number of spores added per can					Total toxic/ inoculated	Estimate[2] of total number of spores growing
	8	16	32	64	128		
0	1	6	5	9	12	33/80	54.25
50	1	2	6	7	9	25/80	35.34
100	0	1	2	5	10	18/80	22.63
150	0	3	4	7	7	21/80	27.59
200	1	0	0	1	0	2/80	2.01

1. 10 cans inoculated with sterile water did not swell.
Cans incubated 14 weeks at 35°C until $NaNO_2$ was < 2 mg/kg; then inoculated and incubated at 30°C until swollen, or maximum of 11 weeks.
2. 16(8+16+32+64+128) = 3968 spores added per level of nitrite. Estimate of number growing by method of Fisher and Yates (1963).

number calculations (Stumbo et al., 1950) for the various combinations of spores and nitrite (data not presented).

The time required for 50% of the cans that eventually swelled to be detected as incipient swells was calculated for those inoculated with 128 spores per can. The t_{50}'s were as follows: 0 mg/kg nitrite, 1.3 weeks; 150 mg/kg, 2.9 weeks. It was not possible to calculate the t_{50} for 200 mg/kg of nitrite.

Quantitative assessment of the Perigo effect

In studying inhibition by PF or Perigo-type factors (PTF), various workers have used fixed inocula while varying the concentration of nitrite added to the raw meat before processing plus supplemental nitrite added after processing. Additionally, they have used high or low numbers of vegetative cells, spores, and mixtures of the spores and vegetative cells. Their experimental approach, however, has not offered much aid in assessing one of the most pressing problems concerned with nitrite in canned, shelf-stable meat, i.e., how much does the PTF contribute to inhibition of the outgrowth of spores of *Cl. botulinum* that have survived thermal processing.

In an attempt to provide a simple quantitative approach to inhibitory properties of cured meats with respect to *Cl. botulinum*, we (Pivnick 1970a; Pivnick, 1970b; Pivnick & Petrasovits, 1973) have proposed an equation:

xPr=y Ds+z In

where Pr=Protection against *Cl. botulinum*, Ds=destruction of spores by heat (or other means), and In=Inhibition of those spores that survive the destructive process. Pr, Ds and In are expressed on the basis of 1 $\log_{10}$ i.e., protection against, or destruction or inhibition of, 90% of the spores.

In the experiments described here, Ds (destruction) is not considered; we are concerned only with inhibition. Inhibition can be expressed quantitatively by the ratio: spores added/spores that grew. For example, meat processed without nitrite permitted growth of 54.25 spores out of the total of 3968 spores added to the 80

Table 5. Number of spores inhibited by salt and by Perigo-type factor. Experiment 2[1].

$NaNO_2$ added (mg/kg)	Spores that grew	Ratio spores added[2] / spores that grew	Total units of inhibition in $\log_{10}$	Ratio spores growing in salt/ spores growing in salt and nitrite	Units of inhibition due to nitrite added
0	54.25	73.14	1.86		
50	35.34	112.28	2.05	1.54	0.19
100	22.63	175.34	2.24	2.40	0.38
150	27.59	143.82	2.15	1.97	0.29
200	2.01	1974.13	3.30	26.99	1.43

1. See footnotes for Table 4.
2. Spores added: 3968.

inoculated cans (Table 5); the ratio is 73.14 ($\log_{10}$=1.86 or 1.86 units of In). This could also be stated as follows: meat containing salt, but made without nitrite, inhibited outgrowth of 98,63% of heat damaged spores (compared with 97.3% in Experiment 1). The increased inhibition due to PTF is relatively small for meat made with 50–150 mg/kg nitrite, i.e., 0.19–0.38 units of In. With 200 mg/kg of nitrite, however there was some additional increase, 1.43 units of In above that obtained with meat made without nitrite. Assuming that cooked meat is the optimum medium for *Cl. botulinum* and that the only inhibitors were salt and PTF, then the total number of units of In for salt and PTF (in meat made with 200 mg/kg of nitrite) is 3.30.

We do not claim that the approach described above answers all of the questions concerned with inhibition of surviving spores, e.g., the combined action of nitrite, PTF, salt, etc., the long delay in swelling of cans inoculated before processing (Pivnick et al., 1969) which could be due to loss of PTF with time, etc. However, we do believe that our work: (1) demonstrates a PTF in the absence of nitrite and (2) presents a useful method of measuring the extent of inhibition in terms that are of use to scientists concerned with the safety of canned meat.

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Discussion

Inhibitory action of nitrite on spores

Earlier experiments showed that there was no inhibitor formed due to the presence of nitrite during heating of the spores in the raw meat extract, because spores heated in the same material devoid of nitrite behaved similarly.

In other experiments (Pivnick et al., 1969) spores were added to meat and then the meat was canned and heated; in some cans that never swelled or showed evidence of growth, viable spores were found 18 months later. Other workers have also observed these viable, but dormant spores in canned meat. However, in one experiment of the type described in the report spores could not be recovered from a few unswelled containers.

Nitrite the active compound?

Some American inoculated pack experiments with ham (Christiansen et al., 1973) showed that the antibotulinal effect during incubation at 27°C was fairly well correlated with the initial level of nitrite. This suggests the possibility of an inhibitor being formed during processing. However, because considerable free nitrite was also present after processing the inhibitory effect of nitrite per se could not be excluded.

The 'protection' equation

The equation $xPr = yDs + zIn$ can simply be written as $Pr = Ds + In$. This equation can also be applied to irradiation preserved products.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen.

Inhibitors in cooked meat products

P. S. van Roon

Institute of Food Hygiene, Department of Meat Technology, Faculty of Veterinary Sciences, University of Utrecht, Biltstraat 172, Utrecht, the Netherlands

Abstract

For several reasons it seems possible that iron-nitrosyl coordination complexes can be formed in canned, cured meat products during heating. Two complexes, the black Róussin salt, $[Fe_4S_3(NO_7)]^-$, and a nitrosyl-cysteyl-ferrate, were studied. Both inhibit the growth of clostridia spores. The Roussin salt is only produced when free Fe(II) ions and hydrogen sulphide are present. The second complex needs cysteine in place of hydrogen sulphide.

With a tentative assay method for the Roussin salt in sterilized meat products, this complex was not detectable in a luncheon meat. An assay method for the second complex is being studied.

The likelihood that the second complex is produced during heating of meat products is surveyed. It is also pointed out why the presence of such an inhibiting complex does not always imply that it inhibits growth.

Introduction

Perigo et al. (1967) and Perigo & Roberts (1968) described the inhibition of vegetative cells of several clostridia by very low amounts of nitrite, present in the growth medium during sterilization. The authors concluded that a potent growth inhibitor might have been formed with properties differing from nitrite.

Johnston et al. (1969); Johnston & Loynes (1971) and Roberts (1971) studied this Perigo effect in sterilized meat products and could not find such an effect. Addition of rather high amounts of ascorbic acid or cysteine before sterilization of the meat induced growth inhibition with small amounts of nitrite. The inhibiting effect which Perigo discovered in his medium, does not seem probable in meat under normal curing and sterilizing conditions. Johnston et al. (1969) were not able to find growth inhibition with Perigo's medium after addition of coagulated meat-protein. This protein effect was also found by Grever (1973).

These findings do not rule out the formation of an inhibiting agent in sterilized, cured meat, but tend to indicate that the activity of such a substance is perhaps inhibited by adsorption on to meat protein.

Grever (1973) studied the conditions for inhibiting growth of spores of *Clostridium sporogenes* after addition of a liquid containing low amounts of nitrite and cysteine in water, which was heated in closed cans for 20 min at 110°C. The inhibition by this fluid appeared to be irreproducible. The reason for this lack of

reproducibility may be found in trace metal ions like Fe, Sn and Pb originating from the can.

For several reasons the effect of Fe ions seems to be most important:
– Fe ions are present in the Perigo medium,
– Fe, being a transition metal, is able to form coordination complexes with nitric oxide and cysteine (Mc Donald et al., 1965; Vanin, 1967; Woolum et al., 1968),
– addition of small amounts of Fe^{2+} to a heated beef-product increases the rate of nitrite depletion (Olsman & Krol, 1972).

Another possible inhibitor might be the black Roussin salt, a complex of Fe, NO and S^-, which is formed from nitrite, sulphide and ferrous sulphate at 100 °C (Pawel, 1882). It appears to be a potent inhibitor for several microbes including *Bacillus* species (Dobry & Boyer, 1945; Candeli, 1949). In sterilized meat products hydrogen sulphide is produced (Hofmann & Hamm, 1966), which might react with nitrite or nitric oxide and Fe^{2+}, resulting in black Roussin salt.

The experiments described in this paper deal with:
– the synthesis of cysteyl-nitrosyl complexes,
– their growth inhibiting effect on clostridia,
– the occurrence and detection of one of these complexes in a meat product.

Experimental

All reagents were of analytical grade. Nitrogen was purified by washing with alkaline pyrogallol, nitric oxide by passing through a solution of potassium carbonate.

Black Roussin salt

The synthesis was according to the method of Pawel (1882).

Experiments with Na_2S, $FeSO_4$ and $NaNO_2$; cysteine, $FeSO_4$ and $NaNO_2$

The reactions were carried out in Thunberg vessels under vacuum in distilled water, presaturated with nitrogen. The added components were allowed to react for 30 min at 90 °C, after which the vacuum was replaced by nitrogen. The pH of the reaction mixture was 7.0.

Experiments with cysteine, $FeSO_4$ and NO

The reaction took place at room temperature for 15 min by introduction of nitric oxide (P_{NO} approx. 120 mm Hg) into the evacuated Thunberg vessel. The pH was 7.0.

Procedure for isolation and assay of black Roussin salt

The meat product (approx. 10g) was extracted twice with 60 ml of methanol in a Waring blender. The extract was separated from the mixture by filtration through a Buchner funnel. 10 g of anhydrous sodium sulphate was added to the filtrate and

it was placed for 1 h in a cold room (−20°C) to solidify the fat. The Na_2SO_4 and separated fat were removed by filtration. The dried defatted extract was concentrated in a rotating evaporator until a volume of V_1 was obtained. V_2 ml of this solution was applied to a column of aluminium oxide (activity state II–III) suspended in chloroform. The adsorbed Roussin salt was eluted with methanol and collected in a 50-ml fraction which was concentrated by evaporation in vacuo to a volume of V_3 ml. The Roussin salt concentration was estimated by spectrophotometry at 350 mm ($\epsilon_{10\ mm}$ in methanol 15.5×10^3. litre mol^{-1} cm^{-1}). This tentative method has a recovery of 87% with a pure solution of black Roussin salt. Losses mainly occurred during the chromatography.

For the meat product with a high fat content, a control without nitrite is required.

A_s and A_b being the absorption of the sample and the control respectively, the concentration of Roussin salt in the meat product is calculated as follows, x being the weight of the sample in grams:

$$C_{RS} = \frac{1000}{x} \cdot \frac{V_1 \cdot V_3}{V_2} \cdot \frac{A_s - A_b}{15.5} \ \mu mol/kg\ product$$

Preparation of the meat product (luncheon meat)

The emulsion was prepared in the usual way except that sometimes the salt containing nitrite was replaced by NaCl. The products were prepared in two main series with either 1.5 kg meat emulsion and 150 ml solution or 1.0 kg and 50 ml. All solutions contained ascorbic acid 10 mmol/litre. In the first series, one treatment also contained black Roussin salt 5 mmol/litre. In the second series, solutions contained also cysteine 10 mmol/litre or $FeSO_4$ 20 mmol/litre or both cysteine 20mmol/litre and $FeSO_4$ 20 mmol/litre. When a salt containing nitrite was used the initial nitrite content was approximately 1.6 mmol per kg (110 mg/kg). After mixing the emulsion was put into 57 x 75 cans and heated for 65 min at 112°C ($F_0 = 0.5$)

Isolation and assay of the cysteyl-nitrosyl-Fe coordination complex

The study of a method of analysis has recently been started.

Results

Inhibition of growth of Clostridium sporogenes by black Roussin salt

No growth of these spores in the medium used by Grever (1973) was found at concentrations of 50 μM (29 mg/l) and higher; no inhibition occurred with concentrations lower than 12.5 μM (15 mg/l).

Inhibition by the combined action of $NaNO_2$ (0.6 mmol/l), Na_2S (10 mmol/l) and $FeSO_4$ (1.0 mmol/l) after heating at 90°C

This combination gives growth inhibition after dilution (1:1) with the growth medium used by Grever (1973). Without heating no inhibition was detected. Therefore it is likely that the inhibition is due to formation of the black Roussin salt.

Inhibition by a solution containing $NaNO_2$ (0.6 mmol/l), cysteine (10 mmol/l) and $FeSO_4$ (1.0 mmol/l) after heating at 90°C

Only this combination of substances gives inhibition after a 1 : 1 dilution with growth medium. The solution becomes faintly yellow. Replacement of cysteine by cystine results in a colourless solution without inhibiting properties. Other experiments indicate that for complete growth inhibition the amounts of cysteine and Fe(II) can be reduced by a factor of 10. The yellow component which is very sensitive to oxidation seems to be a growth inhibitor.

Inhibition by a mixture of NO (P_{NO} approx. 120 mm Hg), cysteine (10 mmol/l) and $FeSO_4$ (1.0 mmol/l)

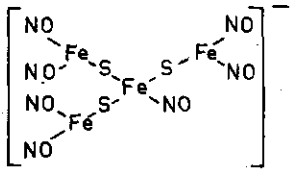
The reaction product with a bright yellow colour, appears to be an inhibiting agent. Neither nitric oxide alone, nor replacement of cysteine by cystine, giving a colourless solution, produced inhibition.

Detection of black Roussin salt in sterilized luncheon meats

Estimation of Roussin salt – 445 μmol added to 1 kg of the luncheon meat – by the assay method described above, resulted in a recovery of 215 μmol per kg product, i.e. 47%. In spite of the tentative character of this method it was used for analysis of normal luncheon meat and products with addition of $FeSO_4$ and/or cysteine as described above under preparation of the meat product. In all these cases no black Roussin salt could be detected.

Discussion

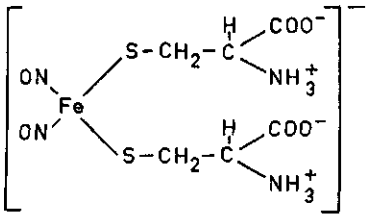
In the introduction it was suggested that one of the possible nitrosyl-Fe coordination complexes that might be formed during heating in cured meat is the black Roussin salt (I),



I

It appears to be an inhibitor for growth of Clostridium spores but it could not be detected in sterilized luncheon meat. Reasons for its absence could be:
– the rather low amount of H_2S developed during heating,
– the adsorption of the non-heme iron to myofibrillar protein (Hamm & Bünnig, 1972).
Addition of free Fe^{2+} and cysteine – being the main source for the pyridoxal catalysed production of H_2S (Gruenwedel & Patnaik, 1971) – or the combination of both additives gave no indication for production of complex I. Thus it does not seem very likely that the black Roussin salt contributes to growth inhibition.

Another possible growth inhibitor in cooked, cured meat products could be the yellow component formed by reaction of Fe^{2+}, cysteine and NO. According to McDonald et al. (1965) the probable chemical formula (II) is,



II

This inhibiting agent can be produced at room temperature, if nitric oxide, free Fe^{2+} and accessible SH-groups are present. Perhaps cooking temperatures are necessary to produce,
– higher amounts of NO,
– more available free Fe^{2+},
– more accessible free SH-groups, obtained by protein denaturation.

Vanin (1967) showed direct reaction of NO and Fe^{2+} with cysteyl groups of actomyosin. Because the inhibiting complex is tightly bound to the protein matrix, its inhibiting capacity may be unfavourably affected. More important for the formation of an inhibiting factor is the presence in meat of endogenous low-molecular weight substances, containing SH-groups like glutathione. Addition of Fe^{2+} and cysteine will act favourably on the production of complex II.

These considerations indicate why no Perigo-like effect could be detected in heated cured meat, assuming the Perigo factor is a complex like complex II.

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Discussion

Additional remarks

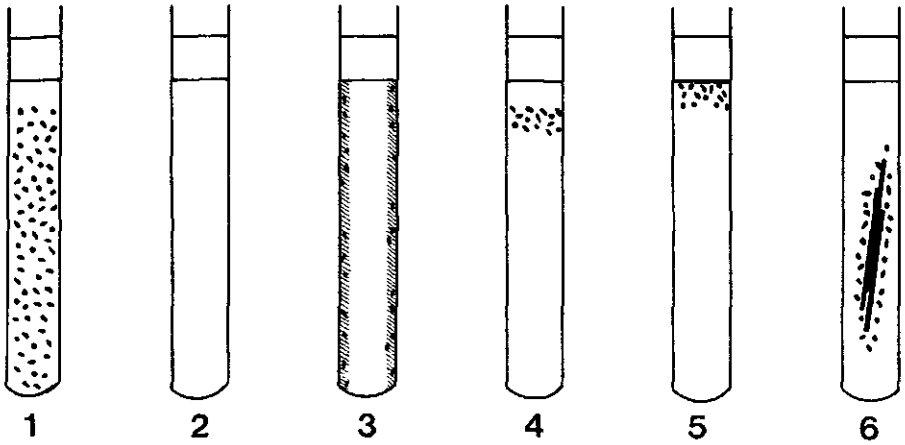
The author added that NO can react at room temperature with isolated myofibrillar protein or actomyosin preparations if enough Fe (II) has been added. This yielded a yellow protein, whilst the aqueous phase remained colourless. This finding may confirm that Fe (II) and NO are able to form type II complexes with cysteyl residues of the protein matrix (Vanin, 1967; Woolum et al., 1968). When free cysteine is also present during the reaction with NO, both the protein and aqueous fraction are yellow. By heating the protein fraction with the complex II bound to the cysteyl residues, after complete removal of free NO by evacuation, with added cysteine for 1 h at 90°C, the aqueous phase becomes yellow too. There appears to be competition between free cysteine and cysteyl residues of the protein matrix during the formation of complex II.

The bacteriological significance of these findings is now being tested.

Toxicological data about the black Roussin salt are in the publication of Dobry & Boyer (1945).

Grever added that the Perigo factor also may be formed in a very simple nutrient medium containing only tryptone and agar.

Heating 3% tryptone agar with 20 mg/kg nitrite for 20 min at 110°C, resulted in a medium that slightly inhibited a *Cl. sporogenes* strain. If 10% tryptone agar was heated with 20 mg/kg nitrite the medium completely inhibited this strain.



Growth phenomena in 1 1/2 % tryptone, 1% yeast extract agar in which the Perigo factor has been formed, inoculated with 1 ml of a spore suspension of *Cl. sporogenes* (10^7 spores per ml), in Miller-Prickett tubes.

1. Normal growth. At the top no growth due to the presence of oxygen.
2. Complete inhibition.
3. Inhibition, except in or near the liquid, caused by syneresis of the agar.
4. Inhibition, except in a zone at the top of the tube, due to inactivation of the inhibitor by the oxygen present.
5. Inhibition, except directly under the fat layer, due to some inactivation of the inhibitor by the fat. (There is no inactivation when the fat is replaced by parafin oil).
6. Inhibition except in the environment of a cooked-meat fibre.

The inhibiting effect could also be obtained by heating 3% tryptone – 0,05% cysteine agar with the nitrite.

When the formation of the inhibitor was studied by heating cysteine and nitrite alone it was found that the presence of iron was necessary.

After this was established it appeared that the inhibitor could be formed in 1.5% trypton, 1% yeast extract agar provided that iron was present (0.04% Mohr's salt).

The Perigo factor could also be formed in the mentioned media by heating at temperatures much lower than 110°C (60°C); 0.1% ascorbate must be added.

From observations of post-growth phenomena in parts of some inhibiting media various properties of the inhibitor could be derived: inactivation by oxygen, by fat and by a cooked meat-fibre.

Also it was observed that the Perigo factor is absent in the liquid formed by syneresis of the inhibiting agar medium.

Walters said that the results presented by van Roon and Grever correspond to those which are formed at BFMIRA, Leatherhead.

Acid hydrolysed casein has been used as an effective precursor to a Perigo type inhibitor and, after heating with nitrite, thioglycollate or ascorbate, one or more inhibitors have been extracted into lipid solvents. The black inhibitory extract

contains iron which added to casein hydrolysed with acid stimulates inhibitor production.

Inhibitory compounds are also obtained on heating nitrite iron salts and ascorbate with cysteine or reduced glutathione; these have been purified and are being studied.

The anti-microbial spectrum of action of the Perigo type inhibitor from acid hydrolysed casein is very similar to that of Roussin black salt. So far as toxicity is concerned, neither substance has been found to nitrosate amines such as morpholine or N-methylanilline at pH values relevant to meat products but some nitrosation was detected at pH 3.

It has also been demonstrated that some detergents inactivate the inhibitor.

Possible influence of oxygen on the Perigo factor

Roberts (1971) could show inhibition of clostridia by the Perigo factor for months. But after opening, removal of the spores and reinocculation of the medium no inhibitory effect lasted. This may be due to sensitivity to oxygen.

Influence of Fe

Addition of $FeSO_4$ in amounts of 100 and 200 mg/l to the Perigo medium produced no increase in inhibition. This may be due to other limiting factors like the content of thioglycollate and nitrite. Furthermore a very small concentration of the inhibitor will suffice. However, at the moment no information is available about the minimum amount of Fe (II) needed.

Stability of the inhibitor

The inhibiting compound according to formula II shows slow decomposition during storage under nitrogen gas at 6°C and in the dark. There is no information available at present about its properties when the complex is bound to the protein matrix.

Other inhibitors

It is not known which inhibiting substance is present in the cans used in the experiments of Pivnick. Johnston et al. (1969) could not find inactivation of the inhibitor with fat.

Conclusions and recommendations of the microbiological session, 12th September 1973

1. Outbreaks of botulism (5 per year) have been reported involving unheated cured pork. There have been none to our knowledge where levels of nitrite used in present commercial practices have been employed.

There is much experimental evidence that *Clostridium botulinum* can develop and form toxin in cured meat or model systems without nitrite. The presence of nitrite retards or prevents such toxin formation, depending on the amount used.

In controlled experiments with cured meat on the laboratory or commercial scale, initial nitrite concentrations of orders exceeding 100 mg per kg (ppm) have been necessary to retard or prevent development of *Cl. botulinum*. These experiments include trials with realistic numbers of inoculated cells or spores.

Experiments involving large inocula are more realistic than might appear from the small numbers actually found in meat, because it is usually necessary to include resistant cells which are only a small proportion of those which occur.

A few similar observations have been made with *Clostridium perfringens* and enterotoxinogenic staphylococci.

2. The necessary amount of nitrite is strongly dependent on related factors e.g. salt.

It is likely that the level of nitrite required will vary with many factors including the product, the geographical location, local practices and ambient temperature.

Experiments to simulate the situation in meat, besides adequately describing the changes in nitrite, should always been done under realistic and as far as possible, specified conditions in respect of water activity, pH, P_{O_2}, E_h, additives, number of cells involved, inactivating effect, of any processing treatment and storage temperature.

3. Cured meats include several different categories of foods, with distinctive microbiological and toxicological hazards. Each type of product should therefore be considered on its own merits.

4. We cannot for the foreseeable future wholly rely on current commercial refrigeration and demestic food handling practice to substitute for nitrite without the risk of botulism and possibly also of food poisoning from *Cl. perfringens* and staphylococci.

5. Any search for substitutes for nitrite should be guided by knowledge of its inhibitory action on non-sporing as well as on spore-forming organisms.

6. Experiments in ordinary microbiological culture media at present seem to have little direct significance in trying to assess the role of nitrite in meat products.

Chemical and technological session

Reporter: P. C. Moerman

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen.

About the mechanism of nitrite loss during storage of cooked meat products

W. J. Olsman

Central Institute for Nutrition and Food Research TNO, Utrechtseweg 48, Zeist, the Netherlands
Dept: Netherlands Centre for Meat Technology

Abstract

The effects of pH, temperature and addition of an alkylating reagent and ethylenediamine tetraacetate (EDTA) on the rate of loss of nitrite are discussed. The heat of activation of the depletion process is 13 to 14 kcal per mole of nitrite, which is equal to the value found by Fox & Ackermann (1968) for the nitrosomyoglobin formation from metmyoglobin in the presence of ascorbate. It is suggested that the small amount of endogenous ascorbic acid in meat plays a key role in the mechanism of the nitrite depletion by acting as an electron carrier in the reduction of nitrite by the sulphydryl group of the meat proteins. The pH dependence and the effect of alkylation can be explained on the basis of such a mechanism.

Introduction

The amount of nitrite used in manufacturing meat products generally exceeds that, needed for the formation of nitrosomyoglobin or nitrosomyochrome, by a factor 10 or more. This surplus of nitrite is considerd to have an important function by inhibiting the outgrowth of *Clostridium* spores. During cooking and, subsequently, during the storage at ambient temperatures most of this nitrite is lost, however. Oxidation to nitrate only occurs at the higher oxidation-reduction potentials before heating (Möhler, 1971). Nitrogen oxides, such as nitric oxide and nitrous oxide are formed as decomposition products, but cannot account for the complete loss of nitrite. The chemical mechanism of this nitrite depletion has hardly been studied, although it is generally accepted that it disappears by way of a reductive process in which the sulphydryl groups of the meat proteins play a role. This paper discusses some of the results of studies on the effects of pH, temperature, EDTA and SH group blocking reagents. These investigations are confined to the depletion during storage at ambient temperatures.

Experimental

The nitrite loss was studied in model comminuted beef products containing about 70% lean meat. As homogeneity of the system was considered to be an important prerequisite for a successful study of the reaction kinetics, jelly formation was prevented completely or reduced to a neglegible extent by the addition of

0.5% polyphosphates, 4% starch and 2% sodium caseinate. The added amount of sodium nitrite was 100 or 200 mg/kg. No ascorbate was added. The emulsions were filled into lacquered cans, 75 x 57 mm, and heated to such an extent that, on the one hand the keepability of the product was guaranteed for the duration of the experiment and, on the other, the residual concentration of nitrite immediately after cooking was high enough to allow its accurate measurement over a range of at least one log cycle.

The methods of analysis have been described elsewhere (Olsman & Krol, 1972).

Results and discussion

Depletion model

In a rough approximation the depletion rate may be considered as being proportional to the nitrite concentration:

$$\frac{d\,[NaNO_2]}{dt} = k\,[NaNO_2]$$

or:

$$\frac{d\,\log\,[NaNO_2]}{dt} = k \qquad \text{Eqn 1}$$

The relation between the logarithm of the nitrite content and the storage time t is, therefore, reasonably well represented by a straight line:

$$\log\,[NaNO_2] = a + kt$$

where k is the depletion rate constant and a is another constant. This relationship has also been found by Nordin (1969). If, however, the nitrite loss is followed over a period long enough for the concentration to drop by a factor 10 or more, significant deviations from this first order model may occur, especially with products of lower pH-values. The gross reaction kinetics is then between first and second order with respect to the nitrite concentration, and may be represented satisfactorily by the equation:

$$\log\,[NaNO_2] = a + bt + ct^2 \qquad \text{Eqn 2}$$

in which a,b and c are constants. Naturally, this parabolic model is theoretically incorrect. It is a working model only and applies to the range of nitrite concentrations, analysed during the period of storage.

Influence of pH

The dependence of the nitrite depletion on pH value was determined by

Table 1. Relation between $[k]_{40}$ and pH.

Experiment nr.	log $[k]_{40}^{1}$
1	8.50 – 1.37 pH
2	7.58 – 1.17 pH
3	6.81 – 1.06 pH
4	6.91 – 1.07 pH
5[2]	6.60 – 1.04 pH

1. $[k]_{40}$ in μM/min.
2. Pork products.

analysing a series of products of equal composition. They were prepared from one homogenized batch of meat, but different amounts of glucono-δ-lactone were added to obtain a range of pH values. The results of five experiments are summarized in Table 1. The $[k]_{40}$ is the time gradient of log $[NaNO_2]$ at a sodium nitrite concentration of 40 mg/kg and is calculated from the parabolic regression equation 2, fitting the results of analysis. Similar relations between the pH and log [k] values were found for [k] values at other nitrite concentrations. This pH-effect cannot be explained by differences in dissociation of HNO_2. Substituting the equilibrium equation

$$K_{HNO_2} = \frac{[H^+]\,[NO_2^-]}{[HNO_2]}$$

in Equation 1 gives:

$$\frac{d\,\{\log [HNO_2] + \log K_{HNO_2} + pH\}}{dt} = k$$

and, as both the pH and the equilibrium constant K_{HNO_2} are independent of the storage time t:

$$\frac{d \log [NaNO_2]}{dt} = \frac{d \log [HNO_2]}{dt} = k$$

One explanation for the pH dependence is that nitrite or HNO_2 reacts with a compound which itself is subject to an acid-base equilibrium. However, the protein sulphydryl group, being the most obvious reactant, is largely or completely in its acid form in the range of pH values (6.4 – 5.4) studied (Jocelyn, 1972). Reactions with amino groups hardly take place under these circumstances at ambient temperatures and, moreover, they proceed by way of N_2O_3, the anhydric form of HNO_2, and therefore should be of second order with respect to nitrite (Hughes et al.,

1958). Another possibility is, that, by way of a reversible reaction of nitrite with a meat constituent, some intermediate is formed whose decomposition rate is dependent on pH.

SH-alkylation and EDTA

In an earlier study (Olsman & Krol, 1972) it was shown that blocking of the SH groups results in a decrease of the nitrite loss in meat products. However, at the pH value concerned (6.27) the effect was unexpectedly small. A more extensive investigation was carried out covering the pH range of 6.4 to 5.4. The SH group was blocked by alkylation with 4-vinylpyridine (VP) (Friedman et al., 1970). This reagent was used in preference to *N*-ethylmaleimide because the latter was suspected to be nitrosated to a nitrosamine at lower pH values. The effect of adding EDTA was also studied, as well as the combined effect of VP + EDTA. In Table 2 the k or $[k]_{40}$ values are given for the various products. The effect of the added compounds on the depletion rate is shown by expressing k as a percentage of that of the control.

Typical depletion curves for the products with pH= 5.91 are shown in Fig. 1. The results in the table demonstrate the role of the SH groups in the mechanism of the nitrite loss. Alkylation retards the process considerably. The effect is greater when the pH is lower. Nevertheless, the inhibition was far less pronounced than had been anticipated. On the basis of the statement of Friedman et al.(1970), that alkylation with VP readily proceeds at room temperatures at pH values of 5 and upwards, it must be accepted that the alkylation of the SH groups (at an excess of reagent of 1.6) is virtually complete. The fact that the inhibition is nevertheless lower than 50%, except at pH= 5.4, would suggest that the SH group is not the only nitrite consuming reactant. The non-alkylating counterpart of VP, ethylpyridine, appeared to have no effect at all on the nitrite loss. EDTA does affect the nitrite depletion. It manifests its action by strongly bent depletion curves (see Fig. 1). The depletion kinetics deviated significantly from the first order model at all pH values. At three of them the loss rate during the first days or weeks of storage exceeded that of the corresponding control product. After some time this situation was reversed. The data given in Table 2 for the EDTA products are, therefore, strongly

Table 2. Effects of addition of VP, EDTA and VP+EDTA on the nitrite loss during storage, in meat products with different pH values.

pH	Control	VP		EDTA		VP + EDTA	
	$k_c \times 10^4$	$k \times 10^4$	$(k/k_c) \times 100$	$k \times 10^4$	$(k/k_c) \times 100$	$k \times 10^4$	$(k/k_c) \times 100$
6.38	108	82	76	103[1]	95	72	67
6.14	217[1]	153	71	182[1]	84	106[1]	49
5.91	363	202	56	410[1]	113	174[1]	48
5.63	496	276	56	667[1]	134	244[1]	49
5.41	1327[1]	446[1]	34	928[1]	70	444[1]	33

1. $[k]_{40}$ values calculated from the parabolic regression equation (2); in all other cases the depletion met the first order kinetic model satisfactorily.

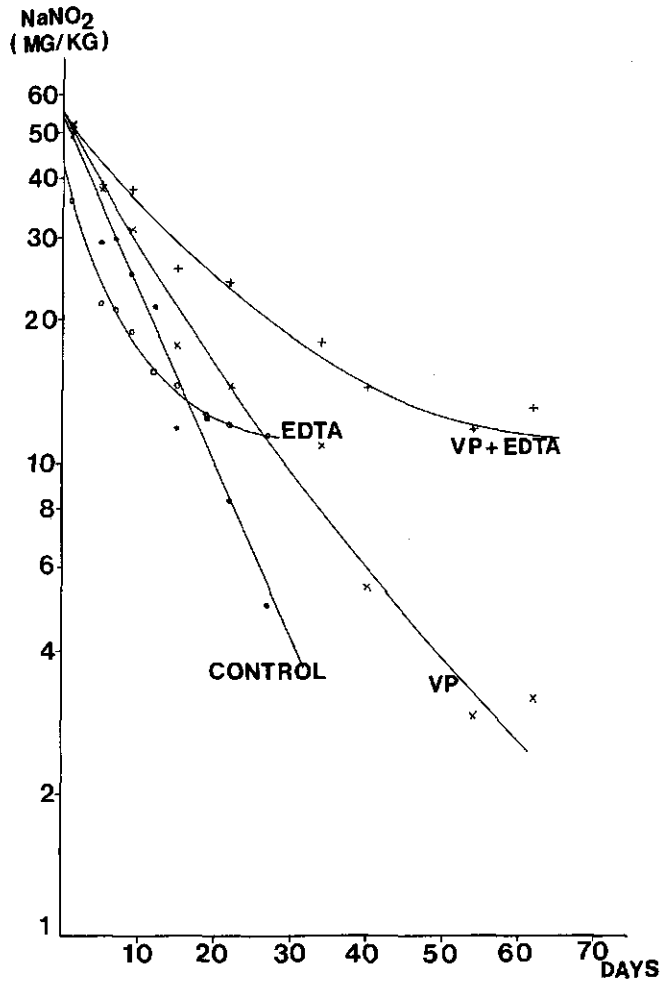


Fig. 1. Nitrite loss in products of pH 5.91 with VP and/or EDTA.

dependent on the particular nitrite concentration at which the [k] value was calculated and would be different for other values than $[k]_{40}$. This is also demonstrated in Fig. 1. These phenomena indicate that the nitrite loss is affected by metal traces which can be bound by EDTA (e.g. Fe, Cu and Sn, the latter originating from the tinplate of the can). As the nitrite breakdown is a reductive process, these materials obviously behave as electron carriers.

The products containing VP as well as EDTA behaved as expected from the separate effects of both compounds. The nitrite depletion in these products was even somewhat more retarded than in the ones with VP alone.

Heat of activation

In two experiments with different products the temperature dependence of the rate constant k of the nitrite depletion was determined. By means of the Arrhenius equation:

$$2.303 \log k = (\Delta H/RT) + C$$

(where R is the gas constant, T is the storage temperature in °K and C is a constant) the following values for the heat of activation ΔH were calculated:

Table 3. Heat of activation of the nitrite depletion.

Experiment nr.	Product pH	Δ H in kcal/mole	
		first order model	parabolic working model
1	6.30	13.9±0.3	14.0±0.4
2	6.31	13.3±0.3	13.5±0.3

The ΔH values calculated on the basis of the parabolic model are also given. Here the k of the Arrhenius equation was substituted by the slope d log $[NaNO_2]$/dt at a nitrite concentration of 40 mg/kg. The Arrhenius plots for the k values calculated on the basis of the first order model are shown in Fig. 2.

The heat of activation ΔH is equal to that found by Fox & Ackerman, (1968) for the rate limiting step in the formation of nitrosomyoglobin (NO-Mb) from metmyoglobin (MetMb) and nitrite in the presence of ascorbate as a reducing agent. By extrapolating their data, calculated for pH-values between 4.5 and 6.0, to the pH of the meat products of our study a value of ΔH= 13.6 kcal/mole was found. Fox & Ackerman postulated a chain of reactions, the slowest of which was:

$RNO \rightarrow R\cdot + NO$ (R· being the ascorbic acid radical)

This could imply that ascorbic acid which is naturally present in small amounts in muscle tissue acts as an electron carrier in the reduction of nitrite. Very different concentrations of endogenous ascorbic acid in beef are reported in the literature: Grau (1968) 10 mg/kg; Niinivaara & Antila (1972) 20 mg/kg; Souci et al. (1968) 8 mg/kg for lean beef, but 126 mg/kg for the Musculus semimembranosus. Recently Davidek et al. (1971) reported figures from 30 to 70 mg/kg dehydroascorbic acid in minced pork. Whether these discrepancies should be attributed to individual variabilities or to lack of reliability of the methods of determination is uncertain.

The ultimate electron donor, which reduces the radical and so keeps the nitrite reduction going, might be the SH group of the meat proteins, which is available in excess. Support for such an electron transfer chain operating in cooked meat products is given by the results of Klein & Davidek (1972). It is further known that dehydroascorbic acid can be easily reduced by cysteine and other SH-compounds

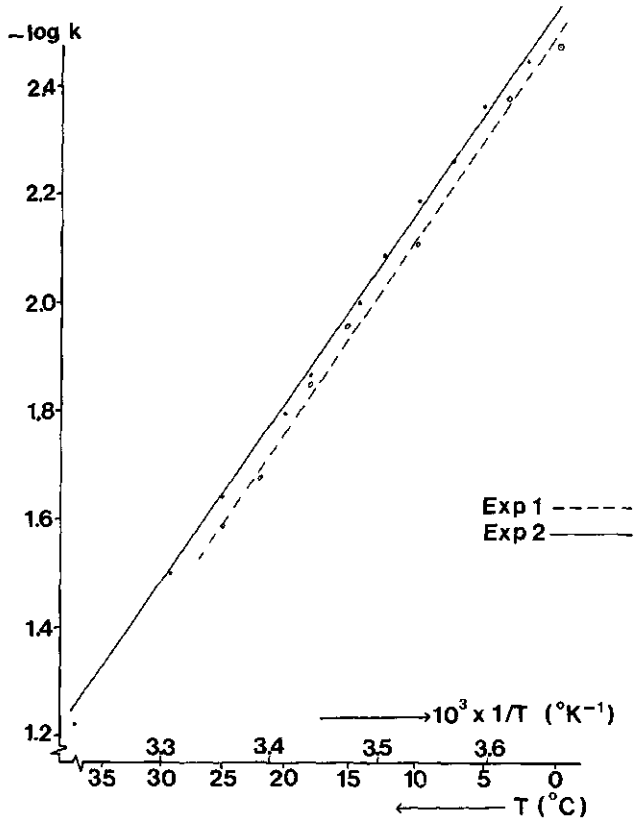


Fig. 2. Relation between reaction rate and temperature.

(Schmandke & Gohlke, 1966). As a consequence of the supposed mechanism there should be a proportionality between the rate of loss of nitrite in a meat product and its content of ascorbic acid. This is in agreement with our earlier finding that the depletion rate is proportional to the meat content divided by the water content of the product (Olsman & Krol, 1972). However, Fox & Thomson (1963) found the NO-Mb formation to be proportional to the square root of the ascorbic acid concentration. This discrepancy could be because ascorbic acid was the only reductant in their model systems, whereas in our meat products an excess of SH groups was available for reaction with the ascorbic acid radical. Tinbergen (1973) found a proportional relationship between the rate of NO-Mb formation and the degree of dilution of the dialysable fraction of a meat extract that provided the reducing system. In view of the ΔH of the nitrite depletion being 13 – 14 kcal/mole, it now seems improbable that nitrite is reduced directly by SH groups, because the ΔH of the reduction by cysteine is as high as 19.2 kcal/mole, and is dependent of the pH (Fox & Ackerman, 1968). This puts the effects of the alkylation into another light. The unexpectedly low inhibition of the nitrite loss

could now be explained by the disappearance of the excess of the electron donating SH-groups so that the rate limiting step is the reduction of the ascorbic acid radical rather than the cleavage of R-NO. The effect of the pH on the rate constant of the nitrite depletion could also be explained by the ascorbate-mediated mechanism, assuming that the cleavage of the NO-complex is pH-dependent, as is suggested by the findings of Fox & Ackerman (1968).

The first experiments for verifying this hypothesis, however, yielded rather negative results. Addition of 10, 20, 30 and 40 mg/kg ascorbic acid to a meat product which contained 12 mg/kg endogeneous ascorbic acid, did not accelerate the nitrite depletion significantly. This does not refute the hypothesis. An explanation for the finding could be that the rate-limiting reaction

$$RNO \rightarrow R\cdot + NO$$

does not proceed spontaneously, but only by mediation of a catalyst which could be a trace metal ion. It has been shown before that metal ions have an effect on the nitrite depletion.

Much work will be needed to elucidate the mechanism of the nitrite depletion.

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Discussion

Effect of pH on k

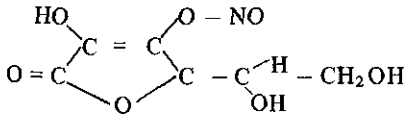
In cases where the nitrite depletion did not comply with 1st order kinetics, the $[k]_{40}$ was calculated. This is the change of log $[NaNO_2]$ with storage time at a sodium nitrite concentration of 40 mg/kg, which is a normal level in a cooked product. The effect of pH on the k value decreases as the nitrite concentration, for which the k is calculated, decreases. The data of Table 1 are only meant to demonstrate the presence of an effect of pH on the k value.

The alkylation effect

Many SH-groups can only be alkylated after they have been made available for reaction by heat denaturation. Hence, part of the alkylation takes place during cooking. At elevated temperatures the specificity of the alkylation decreases. Amino functions react competitively. Therefore, it may be possible that some of the thiol groups remain free, even with an excess of alkylating reagent with respect to thiol groups. The extent of inhibition found suggests that so few SH-groups are left, that not the cleavage of RNO but the reduction of the ascorbate radical is rate limiting, thus resulting in an inhibition of the nitrite depletion.

Ascorbic acid

The nitrosated ascorbate intermediate may be represented as:



Other reductones are supposed to behave like ascorbic acid. The widely different values for the ascorbic acid content of muscle tissue reported in the literature seem to indicate that the current assay methods are not sufficiently specific for meat and meat products. Presumably the need for a specific method was never felt because of the unimportance of meat as a vitamin C source. On the other hand, it is not ruled out that appreciable variation may exist between ascorbic acid levels of muscles of different animals.

Storage conditions

The storage conditions in this study can be considered as anaerobic, because the redox potential of canned meat products is known to drop drastically during cooking.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen

Fate of added nitrite

J. G. Sebranek, R. G. Cassens & W. G. Hoekstra

University of Wisconsin, Madison, WI 53706, USA

Abstract

The stable isotope of nitrogen was used to study the fate and distribution of nitrite in a cured meat product. The meat product was fractionated into water soluble, salt soluble (protein) and insoluble forms in order to conduct quantitative analysis for ^{15}N as a function of storage time and processing temperature. Residual nitrite, determined two days after processing, accounted for less than half of the label added in frozen samples and in samples processed at 71 °C, and the amount of label as nitrite decreased during storage. Samples processed at 107 °C were initially very low in residual nitrite compared to samples from other heat treatments. As residual nitrite decreased, the amount of label found in the non-nitrite water-soluble fraction and in the protein fraction (both soluble and insoluble) increased. About 5% of the label was lost as a gas during processing and 9–12% was present in the pigment fraction. Total recovery of label ranged from 72–86%.

Introduction

Sodium nitrite is used as a curing agent in meat. It causes the production of the typical cured meat color and distinctive flavor; it also specifically acts to inhibit outgrowth of *Botulinum* spores in the event of contamination and mishandling of certain products (Urbain, 1971; Greenberg, 1972). Nitrite is converted rapidly to forms undetectable as nitrite during processing and continues to be converted with time after processing until a fairly constant, low level is reached (Greenberg, 1972). This conversion depends on such factors as pH and processing and storage temperatures (Nordin, 1969). Some of the added nitrite forms nitrosomyoglobin and/or nitrosylhemochrome (Woolford et al., 1972; Bard & Townsend, 1971). Gaseous products such as NO and N_2O are formed (Woolford et al. 1972). The nitric oxide is believed to be involved in the nitrosomyoglobin and nitrosylhemochrome formation (Bard & Townsend, 1971). Some of the nitrite may also become bound to proteins (Mirna & Hoffman 1969, Olsman & Krol 1972).

A determination of the fate of the nitrite ion in cured meat is of importance in decisions concerning the addition of nitrite to meat products. We used the stable isotope of nitrogen (^{15}N) as a label for sodium nitrite in order to establish quantitatively the amount of nitrite nitrogen in various fractions of a typical cured meat as a function of processing temperature and storage time.

Materials and methods

A comminuted lucheon meat type product was prepared under conditions that simulated commercial production. The meat used was 80% lean picnic and included 3% water, 3% salt (sodium chloride), 0.25% dextrose and 0.25% sucrose. Sodium nitrite labeled with ^{15}N (96.1% enrichment from Prochem., Lincoln Park, New Jersey) was added at 156 mg/kg. Ascorbate was not used in the formulation because it accelerates breakdown of nitrite (Mirvish et al., 1972). Even though this may be desirable, ascorbate was purposely avoided in order to simplify our system.

The meat was ground and mixed with salt and sugar for 8 min. The nitrite and water were then added and mixing continued under vacuum for eight more minutes; product temperature after mixing was 2°C. The vacuum line to the mixer included two liquid nitrogen gas traps, connected in series to retain volatiles which might be produced during mixing.

The meat was stuffed into cans (11 ounce) which were closed under vacuum within 15 min of filling. Some of the cans were frozen at −55 °C immediately and the rest were held at 0 °C for 24 h. After 24 h half of the remaining cans were

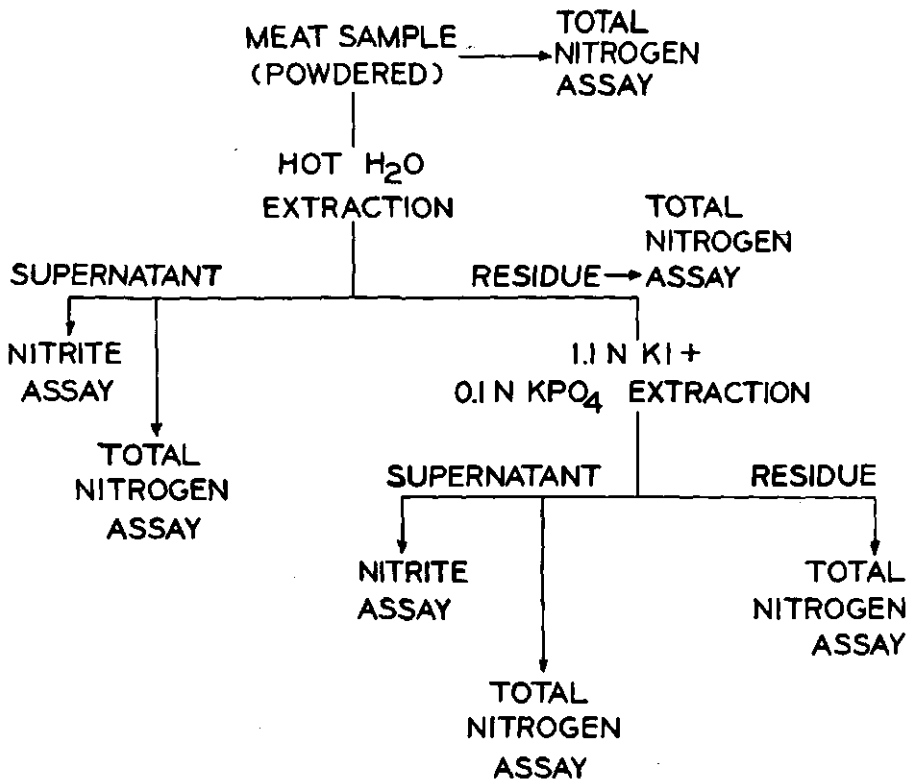


Fig. 1. Diagram for assay of meat samples.

cooked for 60 min in 71 °C water and half were retorted for 80 min at 107 °C. Following heat processing, the cans were chilled in 15 °C water. The frozen samples were stored in a freezer −18 °C. The 77 °C samples were stored at 5 °C and the 107 °C samples were stored at room temperature (22 °C).

The samples were fractionated as illustrated in Fig. 1; analysis for ^{15}N was conducted on each fraction by converting sample nitrogen to $(NH_4)_2SO_4$ which was in turn converted to N_2 on the mass spectrometer (Burris & Wilson, 1957; Bremner & Keeney, 1964; Davisson & Parsons, 1919). Sample analysis were conducted at 2, 23, 40, 49 and 65 days after processing.

The samples were extracted with water according to the AOAC procedure for colorimetric determination of nitrite. Nitrite assay was conducted on the water supernatant by the Griess reagent method (AOAC, 1970). Total nitrogen was determined on the water supernatant as a combination of the Devarda and Kjeldahl methods (Davisson & Parsons, 1919). The residue from the water extraction was analyzed by the total nitrogen method and then subjected to extraction with 1.1 *N*

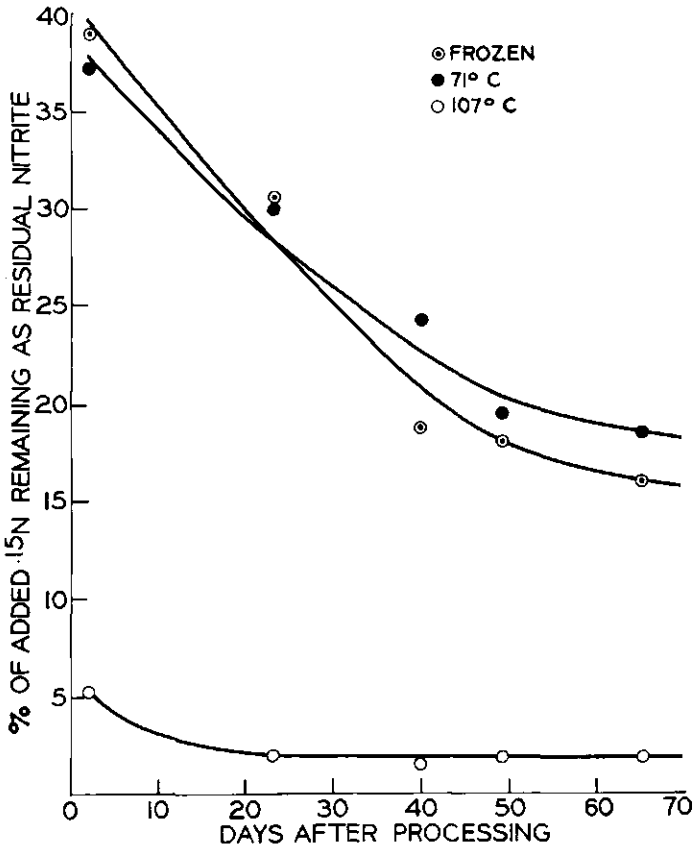


Fig. 2. Change with time of ^{15}N measured as residual nitrite.

KI and 0.1*N* KH_2PO_4 (Helander, 1957). The supernatant of the salt extraction was checked for residual nitrite and also subjected to the total nitrogen assay. The residue was also examined for total nitrogen.

The amount of label from nitrite that was associated with the pigment fraction was estimated by extraction with a solvent of 40 parts acetone and 3 parts water (Hornsey, 1956), followed by a simple Devarda reduction (Bremner and Keeney, 1964) which did not include the Kjeldahl digestion step.

Head space gases were analyzed by puncturing the can through an attached rubber septum with a Vacutainer test tube under vacuum. The collected gases were then injected directly into the mass spectrometer and analyzed for various nitrogen containing gases. Processing gas or volatiles generated during vacuum mixing were trapped as previously described and analyzed for various nitrogen containing gases.

Results and discussion

The first analysis was conducted two days after processing, and less than one-half of the added ^{15}N was identified as residual nitrite (Fig. 2). Residual nitrite

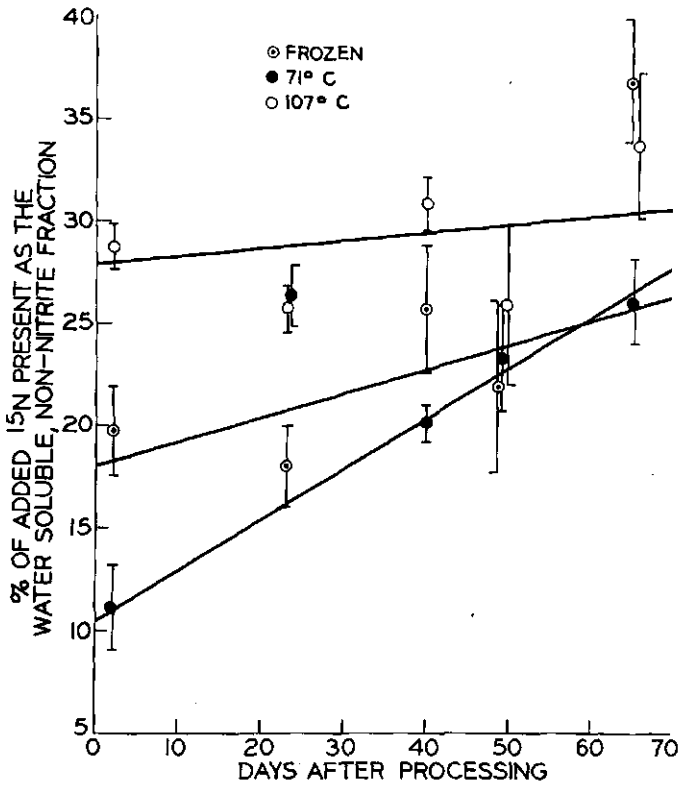


Fig. 3. Change with time of ^{15}N in non-nitrite water soluble compounds.

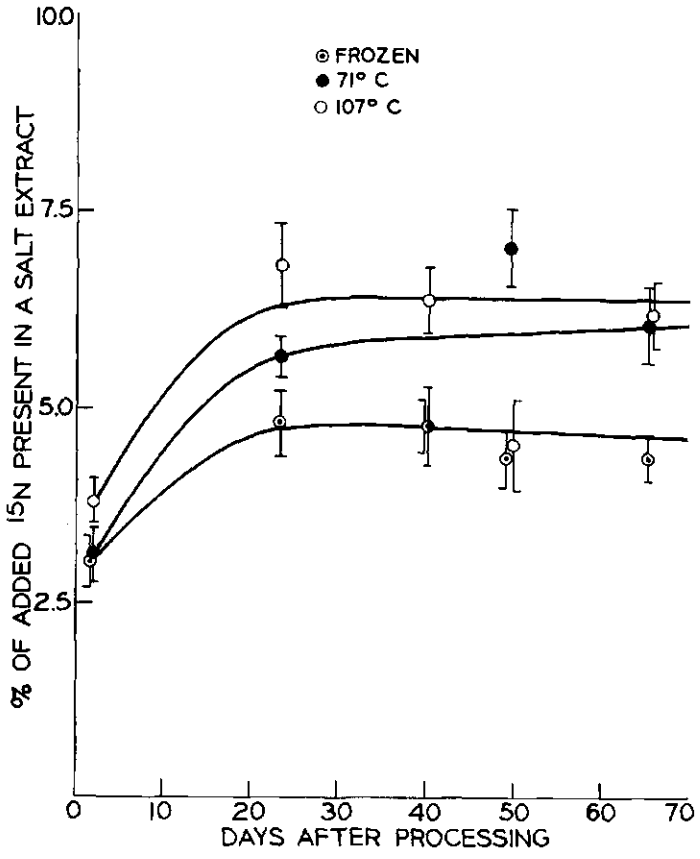


Fig. 4. Change with time of ^{15}N in compounds of a salt soluble extract.

continued to decrease with time as described by Greenberg (1972). The residual nitrite content and change during storage for both the immediately frozen (stored at −18 °C) and 71 °C processed samples (stored at 5 °C) paralleled each other closely and did not differ significantly. The residual nitrite content of the sample retorted at 107 °C and stored at 22 °C, however, was already very low two days after processing. Nordin (1969) found nitrite conversion closely related to temperature. This was confirmed by comparison of the 71 °C and 107 °C samples. The frozen sample, in our experiment, however, showed an unexpected rapid conversion of nitrite; the cause for this is not known but an increased concentration of solute due to freezing may have played a role.

The total nitrogen assay on the supernatant from the water extraction showed more ^{15}N than could be accounted for by the residual nitrite. This water soluble, non-nitrite fraction accounted for a substantial portion of the total recovered ^{15}N and showed a significant increase over time for the frozen and 71 °C sample (Fig. 3).

The salt extraction failed to solubilize much protein. This low protein solubility was expected since the samples were subjected to rather severe heat treatment, both in processing and in the hot water extraction. The protein that was extracted, however, showed a small but significant increase with time in the amount of ^{15}N (Fig. 4). The colorimetric test for nitrite in this supernatant was negative.

The residue from the salt extraction contained a small amount of ^{15}N initially which increased markedly and very significantly during the first 23 days of storage after which it plateaued (Fig. 5). The ^{15}N in this fraction of the frozen and 71 °C samples paralleled each other, while in the 107 °C sample it was significantly higher at 2 and 65 days of processing. The ^{15}N contained in this fraction, as well as that in the salt soluble extract, probably represents protein bound nitrogen. It has been suggested that nitrite binds to proteins through thionitroso bonding (Mirna & Hofmann, 1969; Olsman & Krol, 1972) as well as other means.

The amount of ^{15}N associated with the pigment fraction apeared at first to be surprisingly large. However, it was necessary to perform this extraction on a freshly

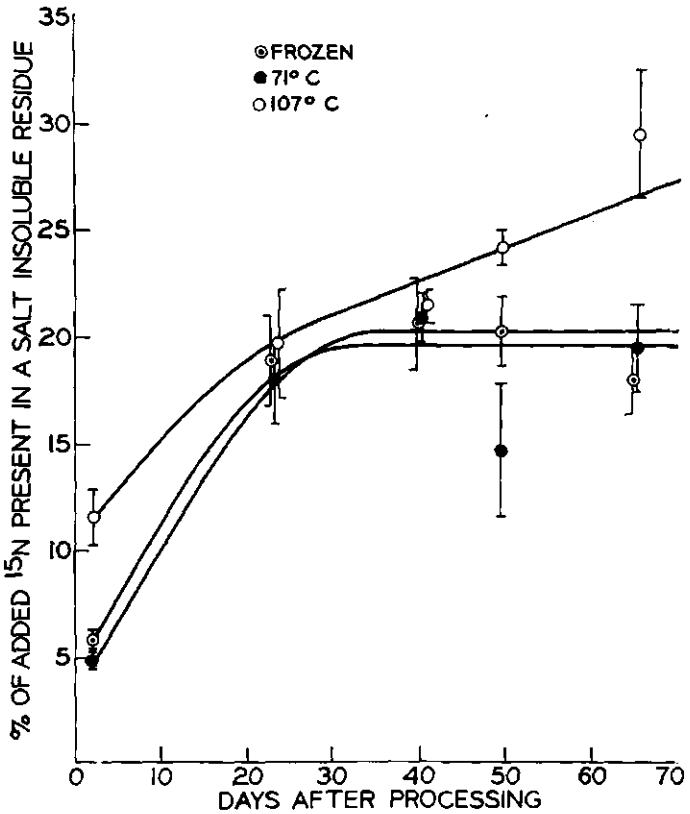


Fig. 5. Change with time of ^{15}N in compounds of a salt insoluble residue.

powdered sample and subsequent investigation showed that residual nitrite was carried along with the pigment in the small amount of water present. The amount of ^{15}N associated with the pigments was therefore corrected (for the 107 °C sample) by subtracting the ^{15}N of residual nitrite from the total ^{15}N in the uncorrected acetone-water extract and crediting the difference to pigment bound ^{15}N (Fig. 6). This correction may over-compensate, since nitrite may not be quantitatively recovered in acetone-water, but the error should be below 2% because less than 2% of the nitrite remained in the 107 °C sample beyond the first two days. This indirect method for pigment bound ^{15}N accounted for 8–9% of the ^{15}N in the 107 °C sample. The frozen sample should have little or no cured pigment formed, therefore all of the label in the uncorrected acetone water extract was presumed to be due to residual nitrite. Since residual nitrite levels were

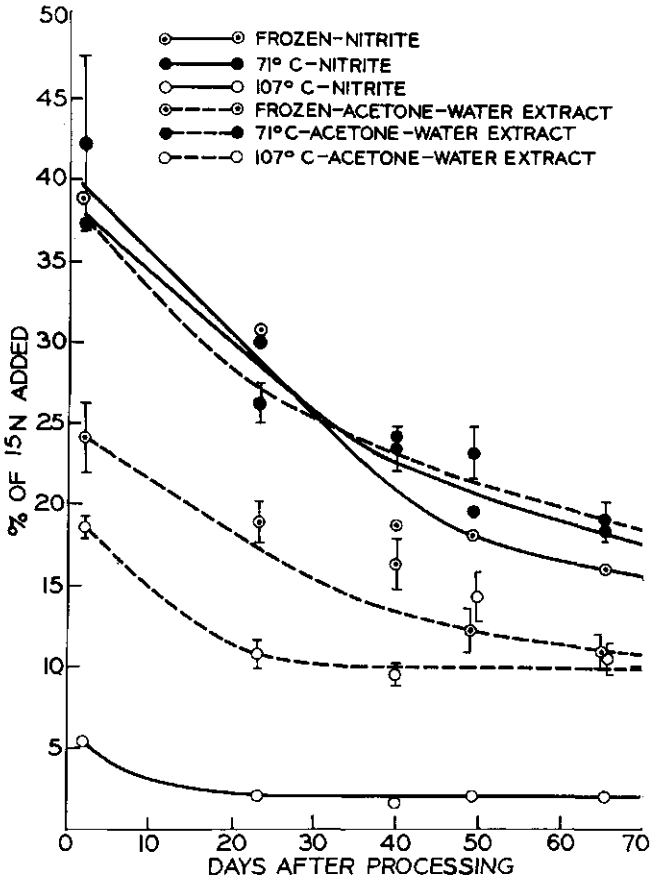


Fig. 6. Change with time of ^{15}N measured as residual nitrite and the change with time of ^{15}N in compounds in an uncorrected acetone water extract.

approximately the same for the frozen and 71 °C samples, the difference in ^{15}N content of the uncorrected acetone-water extract of two samples should be attributable to the pigment bound ^{15}N contained in the 71 °C sample. The difference was 9–12% which gave an estimation of the amount of pigment bound ^{15}N in the 71 °C sample.

Assuming that myoglobin comprises about 0.36% by wet weight of muscle (Lawrie, 1966) and the molecular weight of myglobin is 17,000, calculation reveals that on a mole of ^{15}N nitrogen per mole of myoglobin basis about 10% of the added label should be bound to myoglobin. Our results ranged between 9 and 12%. Meat will, however, contain varying amounts of myoglobin as well as some other heme pigments such as hemoglobin, cytochromes and vitamin B_{12}. Also, if cooked pigment has the capacity to bind two moles of ^{15}N nitrite as has been suggested (Tarladgis, 1962), then a maximum of 20% of the ^{15}N may be bound to pigments. Thus, our assumptions in correcting the pigment fraction seem reasonable.

Samples were also examined for ^{15}N in head space gases. Only a small amount of N_2 could be detected, which accounted for about 1% of the ^{15}N in the heat processed samples and none for the frozen sample.

Analysis of gas trapped during mixing showed a very small amount of N_2 and a somewhat larger amount of NO. Exact quantitation was not possible for the gas samples because of sampling limitations but estimates were made by comparison of peak heights with a reference of known concentration. The ^{15}N content in these two gases together was approximately 5% of the total ^{15}N added.

The sample processed at 107 °C also contained some exuded water and gelatin in the cans. This was examined by the total nitrogen assay and found to contain 2–3% of the total ^{15}N.

The total recovery of ^{15}N as determined by summing the individual fractions ranged from 72–86%.

Conclusions

It was confirmed that nitrite added to meat product is rapidly changed to other compounds during and after processing and that the rate of change slows until a rather constant low level of residual nitrite is reached. As the residual nitrite as such was changed we found an increase of ^{15}N occurred in two fractions: an apparent protein bound fraction and a non-nitrite, water-soluble fraction. The amount of ^{15}N in the pigment fraction was relatively constant during storage, did not vary greatly due to amount of heat processing and agreed with calculated predictions. Despite considerable effort, total recoveries could not be improved above the range 72–86%. However, because commercial conditions were being simulated, a low amount (156 mg/kg) of $NaNO_2$ was used. Use of larger amounts of nitrite might produce better recovery but result in artefacts and conclusions unrealistic for a commercial process. We have not attempted to quantitate nitrate which might be formed (Möhler, 1970) in the product since nitrate would be included in our total nitrogen assay.

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Discussion

Recovery of ^{15}N

It was remarked that the loss of ^{15}N was relatively high. Cassens answered that two years ago the recovery was only 30%. There is quite a lot of handling so that losses can be large. Walters confirmed that the losses in this kind of experiment are high. There was no indication that the type of heating or storage influenced the recovery: the data show too great a scatter. This can be explained since a small amount of ^{15}N had to be detected in a large amount of N-containing protein.

Loss of labelled ^{15}N at freezing

The suggestion that the unexpected rapid loss of nitrite ^{15}N during storage at −18 °C was due to concentration effects, was based on results presented by Tannenbaum and Fan (Proc. Meat Ind. Res. Conf. 1973, pp. 1–10) who obtained similar results at −6 to −46 °C.

Nature of the ^{15}N compounds

Are there any indications about the nature of the ^{15}N-compounds in the water-soluble and protein-bound fraction? The author answered that his laboratory is initiating work with Sephadex column chromatography in an attempt to separate ^{15}N containing compounds in the water soluble fraction. Very preliminary results have shown that the ^{15}N is associated with compounds of rather low molecular weight.

Walters said that in similar experiments, involving model curing experiments at low temperature, low-resolution mass spectrometric peaks were observed only in the presence of nitrite which could have been derived from either ethane or ethylene. Both gas chromatography and high-resolution mass spectrometry showed that the compound present was ethylene. No route of information can be put forward for its production in very small amounts, but ethyl nitrite has been recognized in extracts of bacon and can break down loss of nitrous acid.

Nitrate and nitrite in animal diets

Additional information was given by Cassens concerning the work of Wang and Hoekstra (Dept. Biochemistry, Univ. Wisconsin). They have conducted experiments on excretion and retention of ^{15}N labelled sodium nitrate and sodium nitrite following ingestion by rats. The rats (6 in each group) received a single meal that contained either 2% of the diet as sodium nitrate or 1.6% as sodium nitrate. The animals had been previously trained to consume such a meal. Urine and faeces were collected at 6-h intervals for 72 h and the animals were killed to terminate the experiment. Urine, faeces and whole body were analysed for ^{15}N and the results were expressed as a percentage of the original amount of N.

The bulk of the dose was excreted within 48 h.

In future experiments organs will be examined separately.

Distribution of ^{15}N, 72 hours after feeding.

Labelled component	^{15}N in urine (%)	^{15}N in faeces (%)	^{15}N in body (%)	recovery of ^{15}N (%)
$^{15}NO_2$	68.4 ± 7.9	12.4 ± 0.7	10.2 ± 1.4	91.0 ± 8.2
$^{15}NO_3$	58.0 ± 10.9	6.3 ± 1.6	10.7 ± 2.4	75.0 ± 10.5

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen

Some compounds influencing colour formation

Norihide Ando

Laboratory of Chemistry and Technology of Animal Products, Faculty of Agriculture, Kyushu University, Fukuoka, Japan
Present address: Takamiya 2–14–20, Minami-ku, 815 Fukuoka-shi, Japan

Abstract

The effects of some food additives and some metal ions on the behaviour of nitrite and those of some endogenous low-molecular compounds in sarcoplasm from porcine skeletal muscle on the behaviour of nitrite and the formation of cooked cured meat colour were studied.

Glutamate, succinate, nicotinic acid and nicotinamide enhanced the decomposition of nitrite in the presence of ascorbate; nicotinamide also favoured the formation of nitric oxide from nitrite in the presence of ascorbate.

Of the five metal ions: Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+} tested, only Fe^{2+} significantly decomposed nitrite in the absence of ascorbate. With ascorbate, however, Mg^{2+}, Ca^{2+} and Zn^{2+} enhanced the decomposition of nitrite to some extent, whereas the effects of Fe^{2+} and Fe^{3+} were considerable.

The subfraction most active in forming cooked cured meat colour was collected from the low-molecular fraction of sarcoplasm by gel filtration. The compounds in this subfraction were identified by thin-layer chromatography.

The results indicated that the low-molecular substances IMP, ATP, reduced glutathione, glutamate, Fe^{2+} and possibly ribose are probably at least partly responsible for the colour formation.

Introduction

In recent studies on the formation of cured meat colour the following compounds have been reported to favour colour formation: various nitroso-intermediates of such reductants as ascorbic acid, cysteine, reduced nicotinamide-adenine dinucleotide (NADH) and hydroquinone (Fox & Thomson, 1963; Fox & Ackerman, 1968), nitroso-intermediate of ferricytochrome c, mitochondria and NADH (Walters & Taylor, 1963; Taylor & Walters, 1967), nitrosothiol groups formed in meat proteins (Mirna & Hofmann, 1969), arginine and lysine (Miyake et al., 1969), NADH, reduced nicotinamide-adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD) and riboflavin (Koizumi & Brown, 1971), glucono-δ-lactone (Sair, 1963 and 1965; Meester, 1965), sodium ascorbate, sodium erythorbate, glucono-δ-lactone and sodium acid pyrophosphate (Sair, 1971).

In previous studies at our laboratory, it was observed that monosodium and monopotassium orthophosphates (Ando et al., 1961), disodium pyrophosphate and

sodium hexametaphosphate (Ando et al., 1963), and sorbic acid (Ando & Nagata, 1969) enhanced the formation of cooked cured meat colour. In the investigations of the rapid curing process, it has been shown that of the four fractions separated from porcine skeletal muscle, sarcoplasm, myofibrils, mitochondria and microsomes fractions, the sarcoplasm fraction was the most active in the decomposition of nitrite and colour formation (Ando & Nagata, 1970). When the low-molecular and high-molecular fractions were separated from sarcoplasm by dialysis, the low-molecular fraction was clearly more active in the decomposition of nitrite and in the production of colour (Ando et al., 1971). As in the rapid curing process of cooked sausage many food additives are generally used in Japan, the effects of various food additives on the behaviour of nitrite were also investigated with aqueous model sytems (Nagata & Ando, 1971 and 1972).

Recently, the possibility of the formation of carcinogenic nitrosamines from nitrite has become a serious problem in processing meat products. In Norway the use of nitrate and nitrite in processing meat products has been banned since January 1, 1973, with some exceptions. At the present stage, however, nitrite is still widely used in most countries.

In this paper, therefore, with special reference to the rapid curing process, the results of investigation about (1) the effects of some food additives and some metal ions on the behaviour of nitrite, (2) the effects of some endogenous compounds on the behaviour of nitrite and colour formation with aqueous model systems will be reported.

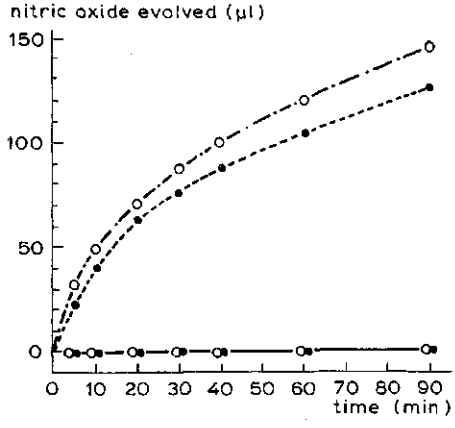

Fig. 1. Effect of nicotinamide on the formation of nitric oxide.

- - - ● - - - ● - $NaNO_2$ + Na-ascorbate
— ● — ● — $NaNO_2$ + Na-ascorbate + alkaline Na-sulphite (center well)
- - - ○ - - - ○ - - $NaNO_2$ + Na-ascorbate + nicotinamide
— ○ — ○ — $NaNO_2$ + Na-ascorbate + nicotinamide + alkaline Na-sulphite (centre well)

Warburg flask

Main compartment:	2 ml of 0.075% $NaNO_2$ solution in veronal buffer of pH 5.0 (0.05%)*
Side arm:	0.5 ml of 3% Na-ascorbate solution in veronal buffer of pH 5.0 (0.5%)*
	0.5 ml of 3% nicotinamide solution in veronal buffer of pH 5.0 (0.5%)*
Centre well:	0.2 ml of 10% Na-sulphite solution in 0.5N NaOH
Total volume:	3 ml
Atmosphere:	nitrogen
Incubating condition:	pH 5.0, 37°C, 90 min

( )*: the final concentration of additive used.

Materials and methods

Test solutions, prepared as described by Ando & Nagata (1969), contained sodium nitrite and one of the following: 0.3% monosodium glumate, 0.1% disodium succinate, 0.1% nicotinic acid, 0.1% nicotinamide, 0.02% $MgCl_2$, 0.02% $CaCl_2$, 0.02% $ZnCl_2$, 0.02% $FeCl_2$, 0.02% $FeCl_3$. Two samples from each solution were cooked at 75°C for 1 h, one immediately after preparation and one after standing for 72 h at 4°C. Before and after cooking, nitrite in the solutions was estimated by the method of Ando & Nagata (1969).

The effect of nicotinamide on the formation of nitric oxide was observed by a manometric technique under the experimental conditions specified in Fig. 1.

Experiments on some endogenous compouds

In the previous study (Ando et al., 1971), the low-molecular fraction of sarcoplasm obtained from porcine skeletal muscle was separated into 50 fractions of 5 ml each by Sephadex G–50 gel filtration; fractions 33 up to 43 (Region A in Fig. 2) were most active in decomposing nitrite, and had a high CFA (colour formation ability) and RA (reducing ability). They were also rich in ninhydrinpositive substance(s), carbohydrate(s) and unknown substance(s) with an absorption maximum at 248 nm.

In this work, therefore, Region A in Fig. 2 was collected first from the low-molecular fraction of sarcoplasm prepared from porcine skeletal muscle (Musculus adductores) just after slaughter, according to the procedures given in the previous

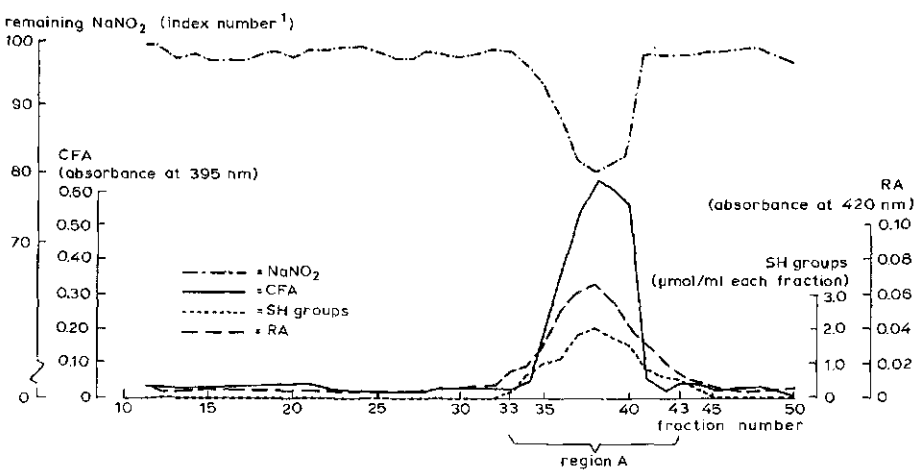

Fig. 2. Distribution patterns of colour formation ability (CFA), reducing ability (RA) and the amounts of SH groups and remaining $NaNO_2$ in the low-molecular fraction of sacroplasm from porcine skeletal muscle after cooking at 75°C, 1 h, under an aerobic condition, on Sephadex G-50.

1. Figures for index number were calculated on the basis of 25 mg/kg nitrite as 100.

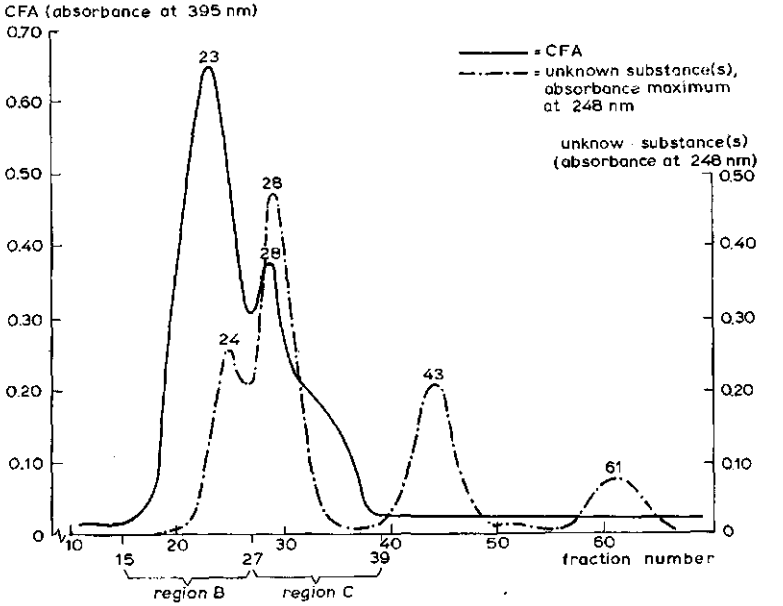

Fig. 3. Distribution patterns of colour formation ability (CFA) and unknown substances with an absorbance maximum at 248 nm in Region A in Fig. 2 on Sephadex G-15.

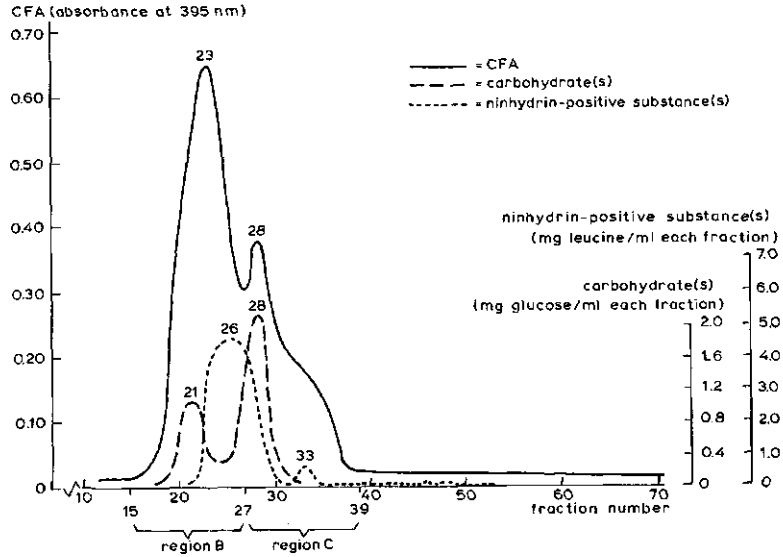

Fig. 4. Distribution patterns of colour formation ability (CFA), ninhydrin-positive substance(s) and carbohydrate(s) in Region A in Fig. 2 on Sephadex G-15.

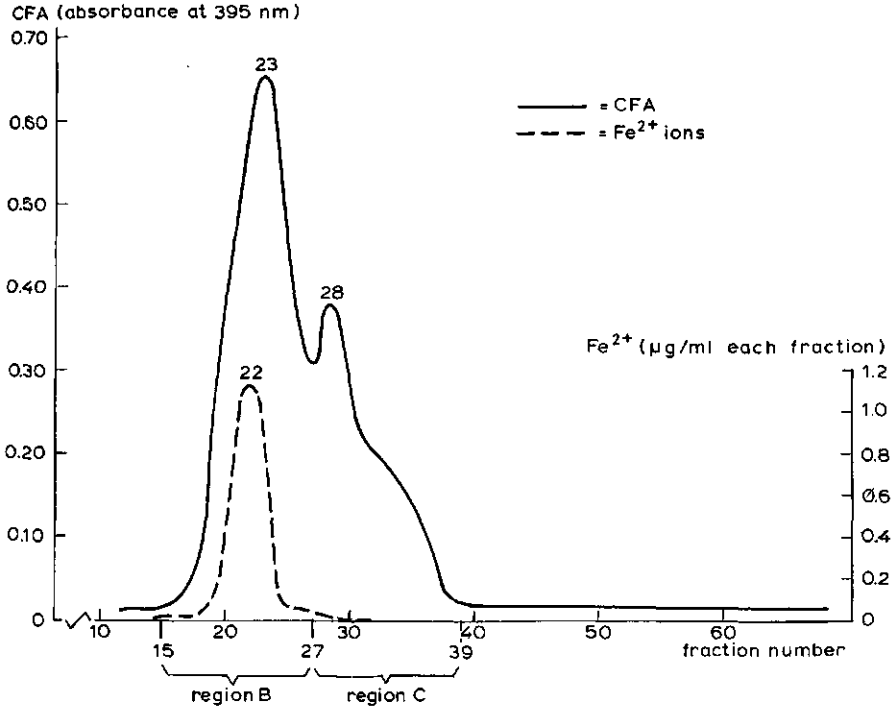

Fig. 5. Distribution patterns of colour formation ability (CFA) and Fe^{2+} in Region A in Fig. 2 on Sephadex G-15.

paper (Ando et al., 1971). Then the pooled fractions of Region A were again separated into 70 fractions of 5 ml each by Sephadex G–15 gel filtration. The distribution patterns of CFA, ninhydrin-positive substance(s), carbohydrate(s) and unknown substance(s) with an absorption maximum at 248 nm were determined by the method of Ando et al. (1971). The distribution of Fe^{2+} was determined with the o-phenanthroline-NH_2OH reagent (Jikken Nogei Kagaku, 1961).

As shown in Figs 3, 4 and 5, the fractions 15 up to 39 were found to be very active in colour formation. Two CFA peaks at the fractions 23 and 28 were observed. Consequently the fractions 15 up to 39 were divided into two regions, one comprising fractions 15 up to 27 (Region B in Figs 3, 4 and 5) and the other fractions 27 up to 39 (Region C in Figs 3, 4 and 5)

Since IMP has its absorption maximum at 248 nm, Region B and Region C were collected separately and then ninhydrin-positive substance(s), carbohydrate(s) and nucleotide(s) were identified by thin-layer chromatography (Randerath, 1963) on silica-gel plates (Merck), with the following developing solvent systems: phenol-water (75:25 w/w) for ninhydrin-positive substance(s), n-butanol-acetic acid-water (3:1:1 w/w) for carbohydrate(s), and isobutylalcohol-octylalcohol-ethylene glycol

monoethyl ether-NH_3-water (30:10:70:15:55 v/v)' for nucleotide(s). The ninhydrin-positive substance(s) were detected with ninhydrin-copper nitrate reagent, the carbodydrate(s) with anisaldehyde reagent, and the nucleotide(s) by looking at the spots on the silica-gel plate under ultraviolet light at 257 nm.

Results and discussion

Food additives

The results given in Tables 1 and 2 indicate that the food additives tested had more effect on the behaviour of nitrite in test solutions at pH 5.0 than at pH 6.0. Nitrite was not decomposed in any of the test solutions without ascorbate.

In the presence of ascorbate, however, as is shown in the test solutions at pH 5.0, the addition of glutamate, succinate, nicotinic acid and nicotinamide appreciably enhanced the decomposition of nitrite during cooking. In each test solution the amount of nitrite decomposed just after preparation was always larger than that after it had been kept for 72 h at 4 °C. This may have resulted from a decrease in the ascorbic acid due to autoxidation during the storage period.

The above fact is of importance when ascorbate is used in processing cooked sausage.

Glutamate and succinate are widely used as seasonings for processed meat

Table 1. Relative amount of $NaNO_2$ (%) after the influence of sodium glutamate and sodium succinate.

Test solution			Just after preparation at 0°C		After being kept for 72h at 4°C	
pH[1]	additive (%)		before cooking	after cooking[2]	before cooking	after cooking[2]
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Na glutamate	0.3				
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Na succinate	0.1				
5.0	$NaNO_2$	0.01	99.8	69.0	92.0	73.7
	Na ascorbate	0.1				
5.0	$NaNO_2$	0.01	99.8	61.7	90.9	68.0
	Na ascorbate	0.1				
	Na glutamate	0.3				
5.0	$NaNO_2$	0.01	99.8	61.4	89.6	71.0
	Na ascorbate	0.1				
	Na succinate	0.1				
6.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Na glutamate	0.3				
6.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Na succinate	0.1				
6.0	$NaNO_2$	0.01	100.0	94.1	98.7	95.0
	Na ascorbate	0.1				
6.0	$NaNO_2$	0.01	100.0	94.1	98.6	94.8
	Na ascorbate	0.1				
	Na glutamate	0.3				
6.0	$NaNO_2$	0.01	100.0	93.9	98.6	95.0
	Na ascorbate	0.1				
	Na succinate	0.1				

1. Adjusted with veronal buffer.
2. Cooked at 75°C for one hour.

Table 2. Relative amount of $NaNO_2$ (%) after the influence of nicotinic acid (NA) and nicotinamide (NAM).

Test solution			Just after preparation at 0°C		After being kept for 72 h at 4°C	
pH[1]	additive (%		before cooking	after cooking[2]	before cooking	after cooking[2]
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	NA	0.1				
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	NAM	0.1				
5.0	$NaNO_2$	0.01	99.8	68.7	91.5	75.5
	Na ascorbate	0.1				
5.0	$NaNO_2$	0.01	99.8	58.4	90.4	65.7
	Na ascorbate	0.1				
	NA	0.1				
5.0	$NaNO_2$	0.01	99.8	64.0	91.1	72.6
	Na ascorbate	0.1				
	NAM	0.1				
6.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Na	0.1				
6.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	NAM	0.1				
6.0	$NaNO_2$	0.01	100.0	93.8	98.7	95.5
	Na ascorbate	0.1				
6.0	$NaNO_2$	0.01	100.0	91.6	97.9	93.1
	Na ascorbate	0.1				
	NA	0.1				
6.0	$NaNO_2$	0.01	100.0	93.2	97.6	94.5
	Na ascorbate	0.1				
	NAM	0.1				

1. Adjusted with veronal buffer.
2. Cooked at 75°C for one hour.

products in Japan. Saleh & Watts (1968) reported that the addition of NAD or NADP and various substrates from the glycolytic and respiratory pathway promoted the enzymatic reduction. Of all the substrates they tested only the use of glutamate was economically possible in processing meat products. The presence of succinate in meat seems possible as an intermediate of the tricarbonytic cycle. In the present experiments with non-enzymatic model systems both glutamate and succinate enhanced the decomposition of nitrite in the presence of a reductant.

It has been observed that nicotinic acid (NA) and nicotinamide (NAM) form red hemochrone (Coleman et al., 1949 and 1951; Olcott & Lukton, 1961; Kendrick & Watts, 1969) and NAM, but not NA, forms pink hemochromes in canned tuna (Brown & Tappel, 1957).

NAM is now widely used in processing meat products in Japan together with nitrite and ascorbate or isoascorbate to enhance colour formation. However the role of NAM in colour formation is still unclear. It has been suggested that NAM may protect NAD in the tissues from destruction by nucleosidase (Severin et al., 1963; Bailey et al., 1964; Kendrick & Watts, 1969) so that it may play a role in colour formation.

As shown in Table 2, NAM enhanced the decomposition of nitrite in the presence of ascorbate. The result of the manometric experiment, described in Fig. 1, indicated that NAM also forms nitric oxide in the presence of ascorbate,

because no gas-evolution was observed in the presence of sodium sulphite, an absorbent for nitric oxide. Only nitric oxide may have been evolved from nitrite in this reaction system. These facts seem partly responsible for the better colour formation.

Metal ions

According to Weiss et al. (1953), Fe^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} enhanced the formation of cured meat colour in the presence of ascorbate. The favourable effect of Fe^{2+} was also observed by Siedler & Schweigert (1959) and Reith & Szakály (1967).

From Tables 3 and 4 it can be seen that Mg^{2+}, Ca^{2+}, Zn^{2+} and Fe^{3+} did not decompose nitrite in the absence of ascorbate, while Fe^{2+} alone significantly

Table 3. Relative amount of $NaNO_2$ (%) after the influence of Mg^{2+}, Ca^{2+} and Zn^{2+}. (All the metals were added as chlorides).

Test solution			Just after preparation at 0°C		After being kept for 72 h at 4°C	
pH[1]	additive (%)		before cooking	after cooking[2]	before cooking	after cooking[2]
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Mg^{2+}	0.02				
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Ca^{2+}	0.02				
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Zn^{2+}	0.02				
5.0	$NaNO_2$	0.01	99.8	68.9	90.8	72.2
	Na ascorbate	0.1				
5.0	$NaNO_2$	0.01	99.8	65.6	90.8	69.0
	Na ascorbate	0.1				
	Mg^{2+}	0.02				
5.0	$NaNO_2$	0.01	99.8	66.9	90.6	70.3
	Na ascorbate	0.1				
	Ca^{2+}	0.02				
5.0	$NaNO_2$	0.01	99.8	65.5	90.4	68.0
	Na ascorbate	0.1				
	Zn^{2+}	0.02				
6.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Mg^{2+}	0.02				
6.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Ca^{2+}	0.02				
6.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Zn^{2+}	0.02				
6.0	$NaNO_2$	0.01	100.0	97.9	98.7	97.0
	Na ascorbate	0.1				
6.0	$NaNO_2$	0.01	100.0	97.4	98.6	97.2
	Na ascorbate	0.1				
	Mg^{2+}	0.02				
6.0	$NaNO_2$	0.01	100.0	97.9	98.7	97.0
	Na ascrobate	0.1				
	Ca^{2+}	0.02				
6.0	$NaNO_2$	0.01	100.0	97.9	98.9	97.0
	Na ascorbate	0.1				
	Zn^{2+}	0.02				

1. Adjusted with veronal buffer.
2. Cooked at 75°C for one hour.

Table 4. Relative amounts of $NaNO_2$ (%) after the influence of Fe^{2+} and Fe^{3+}. (Both iron ions were added as chlorides).

Test solution			Just after preparation at 0°C		After being kept for 72 h at 4°C	
pH[1]	additive (%)		before cooking	after cooking[2]	before cooking	after cooking[2]
5.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
5.0	$NaNO_2$	0.01	99.8	67.0	100.0	71.7
	Fe^{2+}	0.02				
5.0	$NaNO_2$	0.01	99.8	100.0	100.2	100.0
	Fe^{3+}	0.02				
5.0	$NaNO_2$	0.01	99.8	68.9	90.8	72.2
	Na ascorbate	0.1				
5.0	$NaNO_2$	0.01	99.6	16.1	82.3	18.1
	Na ascorbate	0.1				
	Fe^{2+}	0.02				
5.0	$NaNO_2$	0,01	99.8	26.2	63.9	23.7
	Na ascorbate	0.1				
	Fe^{3+}	0.02				
6.0	$NaNO_2$	0.01	100.0	59.9	100.0	87.3
	Fe^{2+}	0.02				
6.0	$NaNO_2$	0.01	100.0	100.0	100.0	100.0
	Fe^{3+}	0.02				
6.0	$NaNO_2$	0.01	100.0	97.9	98.7	97.0
	Na ascorbate	0.1				
6.0	$NaNO_2$	0.01	100.0	47.2	98.9	40.0
	Na ascorbate	0.1				
	Fe^{2+}	0.02				
6.0	$NaNO_2$	0.01	100.0	73.5	95.0	73.3
	Na ascorbate	0.1				
	Fe^{3+}	0.02				

1. Adjusted with veronal buffer.
2. Cooked at 75° C for one hour.

decomposed nitrite in the absence of ascorbate at pH 6.0 as well as at pH 5.0. The enhancement of decomposition by the addition of Fe^{2+} seems to be in good agreement with the observations of Olsman & Krol (1972).

In the presence of ascorbate, however, Mg^{2+}, Ca^{2+} and Zn^{2+} showed similar tendencies to those of the food additives in Tables 1 and 2.

We observed in our previous work (Ando et al., 1973) that the addition of sodium chloride increased the content of free Mg^{2+}, Ca^{2+} and Zn^{2+} and as sodium chloride is usually added to meat in processing meat products, the increased content of free bivalent metal ions may conceivably enhance colour formation in the presence of the reductants in meat products. This may presumably be one explanation for the observations of Weiss et al. (1953) and Olsman & Krol (1972) that the addition of EDTA exerts an unfavourable effect on the formation of cured meat colour.

Fe^{2+} and Fe^{3+} clearly enhanced the decomposition of nitrite in the presence of ascorbate at pH 6.0 and at pH 5.0, as shown in Table 4. The Fe^{3+} is likely to be active only after it has been reduced to Fe^{2+} by the ascorbic acid. Therefore the combined effect of Fe^{3+} and ascorbate on the decomposition of nitrite, as shown in Table 4, is puzzling. In comparison with the solution containing Fe^{2+} and ascorbate, the loss of nitrite during the 72 hours is unexpectedly high. There is no obvious explanation for these findings.

According to Figs 3, 4 and 5, Region B contained nucleotide(s), ninhydrin-positive substance(s), and a fair amount of Fe^{2+}, while Region C was rich in nucleotide(s) and carbohydrate(s).

Thin-layer chromatography showed that Region B contained ATP, IMP, reduced glutathione (GSH), alanine, glutamate, histidine and Fe^{2+}, and Region C contained IMP, alanine, glutamate, glycine, histidine, tyrosine and ribose.

The results given in Table 5 indicate that GSH, in the system we used, enhances the decomposition of nitrite and colour formation. Neither ATP nor IMP enhanced the decomposition of nitrite and colour formation in the absence of GSH. In the presence of GSH, IMP seemed to enhance the decomposition of nitrite, whereas ATP seemed to enhance colour formation.

From the above observations, it may be suggested that in the fractions of Region B, colour formation may have been enhanced by the combined action of ATP, IMP, GSH, glutamate and Fe^{2+}, and in the fractions of Region C by the combined action of IMP, glutamate and possibly ribose, because it was observed in our previous studies that glucose and sucrose enhanced the decomposition of nitrite in the presence of ascorbate (Nagata & Ando, 1971).

Quite recently, Hamm & Bünnig (1972) reported that a fairly large amount of iron in porcine and bovine muscle was neither hemoglobin nor myoglobin bound. This nonheme-iron was as high as 68% of the total iron in porcine muscle and 29% in bovine muscle. As they had previously observed that no free iron ions existed in muscle tissue, they suggested that the above nonheme-iron may be bound to other components in muscle tissue. Olsman & Krol (1972) suggested that endogenous iron may exist in some co-ordination compounds.

As porcine skeletal muscle just after slaughter was used in the present experiments, there may have been a fairly large amount of nonheme-iron in the meat samples. Probably the Fe^{2+} found in Region B did not exist in the free state, but in some low-molecular co-ordination compounds in sarcoplasm.

Further detailed investigations are necessary to clarify the mechanism of the

Table 5. Effects of reduced glutathione (GSH), IMP and ATP on the behaviour of nitrite and the formation of cured meat colour.

Test solution[1]	Compounds added(%)	Relative amount of $NaNO_2$ decomposed after cooking[2] (%)	Absorbance at 395 nm
Control (C)	myoglobin 0.125 $NaNO_2$ 0.0025	3.2	0.065
(1)	(C) + GSH 0.1	14.4	0.365
(2)	(C) + IMP 0.2	4.4	0.065
(3)	(C) + ATP 0.2	3.2	0.098
(4)	(1) + IMP 0.2	19.6	0.281
(5)	(1) + ATP 0.2	14.0	0.416

1. The pH value of each test solution was adjusted to 5.5 with veronal buffer.
2. Cooked for one hour at 75°C.

promoting effects of the endogenous low-molecular compounds in sarcoplasm on the formation of cooked cured meat colour in the rapid curing process.

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Discussion

Succinate as a seasoning

In Japan succinate is used for seasoning; it enhances the decomposition of nitrite in the presence of ascorbate as well as glutamate.

Pre-rigor or post-rigor meat

Pre-rigor meat instead of post-rigor or aged meat was used because in aged meat the amount of low-molecular compounds is higher; so the situation is more complicated then.

Biochemical aspects

Some of the participants thought it likely that nitrite depletion could well be related to the biochemical process involved. However, Ando did not examine the effect of succinate and glutamate on the respiration of fresh meat. Only nitrite depletion and colour formation have been studied.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen.

Nitrite and the flavour of cured meat I

D. S. Mottram and D. N. Rhodes.

ARC Meat Research Institute, Langford, Bristol, BS18 7DY, UK

Abstract

The effect of varying the concentration of sodium nitrite used in curing pork upon the flavour of bacon has been investigated. A taste panel was used to identify the various flavour characteristics and to examine products cured under different conditions. As the nitrite concentration was increased from zero to 1000 mg/kg an almost linear increase in the intensity of bacon flavour was found but above 1500 mg/kg further increase in flavour was small. Salt was shown to make a major contribution to bacon flavour but sodium nitrite has no detectable taste at concentrations similar to those found in bacon. The differentiation between salt pork and bacon in blind comparisons by flavour or odour was remarkably uncertain.

Volatile odorous compounds, isolated from pork and bacon, were analysed by gas chromatography and gas chromatography – mass spectrometry; no compound directly responsible for bacon flavour was isolated.

Introduction

Curing has an age-old history but not until the end of the last century was it established that nitrite rather than nitrate is the agent responsible for cured colour formation and probably for cured flavour. The relationship of nitrite to flavour was first described by Brooks et al. (1940) who compared taste panel preferences for pork cured in brines of differing nitrite and nitrate composition and concluded that 'the characteristic cured flavour of bacon is due primarily to the action of nitrite on the flesh and a satisfactory bacon can be made with only sodium chloride and sodium nitrite.' No taste panel data were given, although it was observed that the differences in samples from different treatments were small. At the same time parallel work at the Research Association of the British Food Manufacturers (Macara, 1939) reached similar conclusions.

The first rigorous test of the essential part played by nitrite in cured flavour formation was made by Cho & Bratzler (1970) who compared porcine musculus longissimus dorsi cured experimentally in brines with and without nitrite. They established significant differences in blindfold triangular tests, and in a subsequent two sample test the nitrite-containing sample was judged to possess a more intense cured flavour. Nitrite-containing and nitrite-free samples could also be distinguished when the samples were smoked and when sodium chloride was omited from the cure. However, the results also revealed that on 116 occasions comprising 288 indivi-

dual tastings, no differentiation was made between salt pork and cured meat.

A similar conclusion regarding the role of nitrite in flavour formation in frankfurters was obtained by Wasserman & Talley (1972), although in a two-way scoring test the panel recorded similar scores for the flavour of smoked nitrite-free and nitrite-containing samples. Sausages prepared with and without nitrite were examined by Skjelkvale et al. (1973) who obtained significant differences between the samples in triangular tests, the differences being more easily distinguished in smoked than in unsmoked sausages.

In none of these studies was any relationship established between the amount of nitrite in the cure and the amount of cured flavour. Barnett et al. (1965) examined this among other factors which might affect the flavour of cured hams; his taste panels showed no preference for hams cured with nitrite levels over the range 100 to 1500 mg/kg, although 4500 mg/kg caused a bitter flavour which decreased the acceptability. No significant differences were observed between frankfurters cured with 150 or 75 mg/kg of nitrite (Wasserman & Talley, 1972) or sausages with 80 or 40 mg/kg (Skjelkvale, 1973).

Saltiness plays some part in the overall acceptability of bacon. The sensory appreciation of the saltiness of bacon is much less intense than that of aqueous solutions of equal strength, an effect due, probably, to fluid binding by the protein or to its slow release during mastication (Ingram, 1949).

A comprehensive study of the acceptability of Canadian and Danish bacon was carried out (in England) by the Canadian National Research Council in 1938–'39. They showed that the high salt content of Canadian bacon (7.5%) compared with Danish bacon (6.2%) was the major source of dissatisfaction (Winkler & Cook, 1941). These observations were followed by a laboratory taste panel study in which bacon containing salt in the range 4.5–9% was evaluated (Hopkins, 1947). A preference for 4.75% salt was observed with nitrate-free bacon, while with bacon containing 0.25% nitrate a 4% salt level was preferred. Nitrite at levels of 50–50 mg/kg did not apparently contribute to saltiness. Rhodes (1971, unpublished results) has also found a preference level of 3–4% salt in experimentally cured bacon, although 2% and 5% were only slightly marked down.

There are few reports concerning the actual constituents of cured meat responsible for flavour. Piotrowski et al. (1970) showed that precursors of basic meaty aroma are water extractable whereas components or precursors of the cured aroma are soluble in non-polar solvents. Gas chromatography of volatiles developed on heating these extracts showed that no single component had a meaty or cured flavour, but variations were observed in the pattern of volatiles among 6 types of ham studied. A number of volatile odorous compounds have been isolated from cured hams and sausages (Lillard & Ayres, 1969; Ockerman et al., 1964; Langner, 1969; Langner et al., 1970) most of which have been found in pork and other red meats. Cross & Ziegler (1965) also examined volatiles from cured hams and made a critical comparison with uncured pork. *n*-Hexanal and *n*-pentanal were found to be present in pork but almost absent from the cured product. It was suggested that the absence of these aldehydes was responsible for the flavour difference between ham and pork and that it was brought about by the modification of the course of the autoxidation of fat in the presence of nitrite.

The present work was undertaken to determine the effect of nitrite concentra-

tion on the flavour of bacon, and to investigate the nature of the volatiles contributing to flavour produced in pork and bacon.

Experimental

Taste panel studies on cured pork

Pilot-scale was carried out on pork middles with brines containing sodium chloride (20% w/v) and sodium nitrite at levels of 0, 250, 500, 1000 and 2000 mg/l. Pork middles (23–27 kg) were pumpted by repeated single needle injection to a 7 to 8% increase in weight, soaked in cover brines for 4 days at 4–5 °C and matured at this temperature for a further 6 days. Samples of twenty 3mm slices were vacuum packed and stored at −20°C until required. Salt and nitrite analyses were performed on random samples from each middle.

A standard cooking procedure was used in which the rashers were suspended from an aluminium frame in a covered pyrex casserole, and heated at 175 °C for 35 min. The samples were presented, one at a time to an experienced bacon tasting panel. Four samples were tasted at each sitting, the first on each occasion being salted pork (i.e. no nitrite in the cure) and the second a sample from the 2000 mg/kg nitrite cure. The components of the flavour of bacon and pork were decided on by the panel during preliminary tastings and discussions; they were pork flavour, bacon flavour, metallic flavour, saltiness, odour intensity and taste intensity when eating. While assessing taste intensity the panelists pinched the nose to eliminate the odour sensation. Samples were scored by marking the appropriate position on a 10-cm line calibrated at equally spaced intervals: nil, slight, moderate, strong, extreme. Numerical scores were subsequently obtained by measurement along the line (nil = 0, extreme = 10).

The colour difference between meat cured with and without nitrite made it essential to present the samples in such a way as to mask visual differences. Blindfolding of judges might have resulted in impaired ability to assess flavour. The use of green illumination in the panel booths eliminated the red colour of the meat, but bacon always appeared slightly darker when presented alongside pork samples. However, the samples were served separately, and the green illumination adequately prevented recognition of the identity of the meats.

Taste panel studies on nitrite solutions

Solutions containing different concentrations of sodium chloride and sodium nitrite were made up in freshly distilled water. The triangle taste test was used with the trained bacon tasting panel to determine if a difference could be detected between nitrite-free and nitrite-containing solutions. For each pair of solutions compared, tests were given with both the control and the nitrite solution as the odd sample. Samples were positioned randomly to prevent sample or positional bias.

The triangle test was also carried out using hot mashed potato as a carrier for nitrite and chloride. This potato was prepared from an 'instant' potato mix, reconstituted in the appropriate solutions.

Aroma panel studies on pork and bacon

Samples of pork and bacon (500 g of each) from the same animal were cooked in vacuum packs by immersing in water at 90 °C for 25 min. The cooked meat was minced and homogenised with 500 ml water and a few drops of an aqueous solution of a red food dye (Amaranth S) added to give similar colours to both homogenates. A two-way triangle test was used to determine if a difference could be detected in the aroma of the pork and bacon. Samples were served hot in pyrex beakers and were positioned randomly. Green illumination was employed in well ventilated taste panel booths.

The triangle odour test was also carried out with samples of grilled pork and bacon, presented minced in beakers covered with perforated aluminium foil to prevent selection being made on a visual basis.

G.l.c. analysis of volatile substances

One side of pork was cured by a normal commercial Wiltshire process and the other used as control; back fat and lean joints (approximately 500 g) were vacuum packed sealed in plastic bags and stored at −20 °C until required. The joints were cooked, by immersing the bags after thawing in water at 90 °C for 25 min and the whole content of the bag was minced. 500 g sample was homogenised with 1 l distilled water, 200 g sodium chloride added in a 3 l flask, and volatile compounds extracted for 12 h using a steam distillation-continuous extraction apparatus as described by Likens & Nickerson (1964). The solvent arm of the apparatus contained diethyl ether (50 ml) which had been re-distilled over a 350 X 15 mm column packed with glass helices. Any volatile material escaping from the apparatus was condensed in two cold traps connected in series to the condenser vent of the apparatus; the first trap was cooled with solid carbon dioxide/methanol, the second with liquid nitrogen. A slow stream of nitrogen was bubbled through the aqueous meat homogenate throughout the distillation.

The ethereal extract was dried over anhydrous sodium sulphate and concentrated to about 2 ml by distillation over a 150 × 10 mm column packed with glass helices, then by passing a slow stream of pure nitrogen over the surface of the chilled solution, to give a final volume of 250 µl. The concentrated extract was sealed in glass vials under an atmosphere of nitrogen and stored at −20 °C. Extracts were prepared from six bacon joints and combined for gas chromatographic analysis; similarly, the combined extract from six matching pork joints was prepared.

Gas chromatography of flavour extracts was carried out on a Pye 104 instrument equipped with a flame ionisation detector, using a 1.5 m × 6 mm glass column packed with 5% SE 30 on Chromosorb G (60–80 mesh) with an argon flow of 40 ml/min. The column temperature was maintained at 75 °C for 10 min, and then was programmed at 4 °C/min to 190 °C. The column effluent stream could be split before passage into the detector to permit evaluation of odours of components eluted from the column. The effluent splitter was also employed to allow the simultaneous use of the flame ionisation detector and a nitrogen-specific Coulson electrolytic detector.

Mass spectrometric analysis of the extracts was carried out with an LKB 9000

gas chromatograph – mass spectrometer, (electron energy, 70eV; ion source temperature, 250 °C; separator temperature, 200 °C). The gas chromatographic column (1.5 m x 6 mm, glass) was packed with 5% SE 30 on Chromosorb G (60–80 mesh) and operated with a helium flow of 20 ml/min. The column temperature was maintained at 75 °C for 5 min, and then was programmed at 4 °C/min to 190 °C.

Results and discussion

Taste panel data

No significant trends in the relationship between the taste panel assessments of metallic flavour, or of odour or taste intensity and the amount of nitrite used in curing were found (Table 1). The panel found a bacon flavour in salted pork (mean 2.54) and this increased with nitrite concentration almost linearly to a level

Table 1a. Taste panel evaluation of the flavour of cured pork prepared with brines containing different nitrite concentrations. Four samples served at each panel. Comparison of products from cures with 0, 500, 1000, 2000 mg/kg sodium nitrite (each figure is the mean of nine tasters and four replicates).

Characteristic	Treatment Means[1]				Analysis of variance: f-ratios		
	0	500	1000	2000	treatments	tasters	interaction
Bacon flavour	2.54	3.08	4.18	4.74	5.14**	2.53n.s.	2.73***
Pork flavour	3.03	2.35	1.33	1.00	5.23**	1.89n.s.	4.31***
Salt flavour	2.73	3.15	4.15	4.34	2.66n.s.	8.36***	1.86*
Metallic flavour	1.24	1.57	1.54	1.63	0.82n.s.	13.75***	0.58n.s.
Odour intensity	4.77	4.11	3.95	4.56	1.83n.s.	7.28***	1.85*
Taste intensity	5.24	4.78	4.98	5.37	1.46n.s.	9.80***	1.42n.s.

Table 1b. Comparison of products from cures with 0, 250, 1000, 2000 mg/kg sodium nitrite (each figure is the mean of nine tasters and two replicates).

Characteristic	Treatment means[1]				Analysis of variance: f-ratios		
	0	250	1000	2000	treatments	tasters	interaction
Bacon flavour	2.42	3.00	3.97	4.33	5.63**	2.69**	1.84n.s.
Pork flavour	3.35	2.36	1.46	1.24	9.41***	2.16n.s.	1.63n.s.
Salt flavour	2.57	3.39	3.80	4.00	2.27n.s.	4.45**	0.63n.s.
Metallic flavour	1.37	1.32	1.68	1.32	0.36n.s.	4.08**	0.77n.s.
Odour intensity	5.19	4.03	4.72	4.59	2.39n.s.	5.72***	1.39n.s.
Taste intensity	5.12	5.12	5.58	5.14	0.43n.s.	1.36n.s.	0.47n.s.

1. Means of scores of tasters derived from scale: 0 = nil; 2.5 = slight; 5 = moderate; 7.5 = strong; 10 = extreme.
*** significant at p = 0.001;
** significant at p = 0.01;
* significant at p = 0.05;
n.s. not significant.

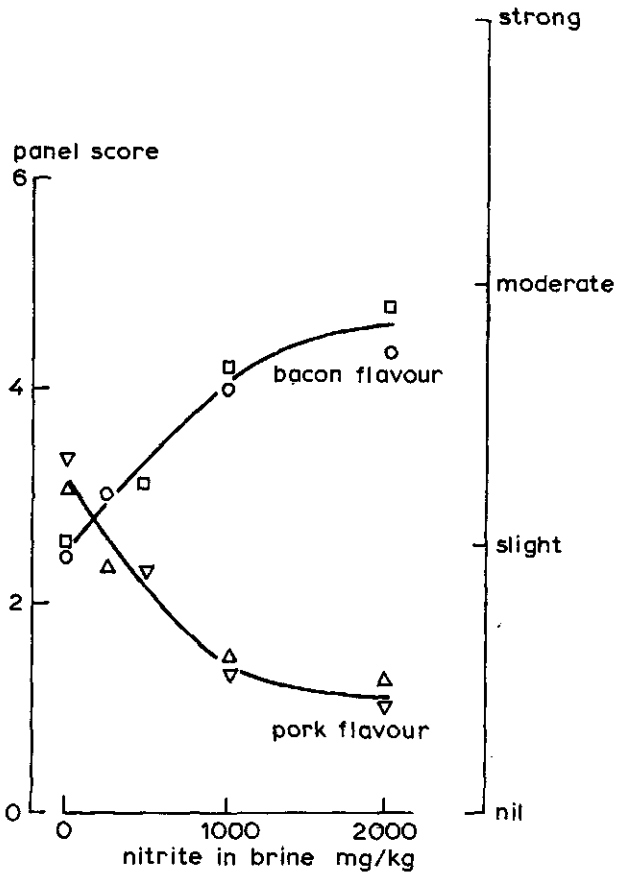

Fig. 1. The relationship between nitrite concentration in curing brines and panel assessment of bacon and pork flavours in the cooked bacon. (□ and ○ = bacon flavour, ▽ and △ = pork flavour from Tables 1a and 1b, respectively).

slightly above 1000 mg/kg; above this the increase in flavour fell off (Fig. 1). Thus a level of about 1500 mg/kg of nitrite was required to attain a near maximum effect on flavour: such a level contrasts with the much lower amount of nitrite needed to fully convert myoglobin to the nitroso-pigment characteristic of cured meat. This result does not imply that the 1500 mg/kg level is essential to produce satisfactory bacon; such a conclusion would require consumer marketing studies, but it establishes that a flavour-producing reaction between meat and nitrite is continuing well beyond the levels where colour formation is completed.

The panel mean scores for pork flavour displayed an inverse progression to those for bacon flavour, the relationship being extremely close (Fig. 2). The totals of the scores for pork flavour plus bacon flavour were, therefore, constant for all levels of nitrite with a mean of 5.5: this figure may be compared with the panel's assess-

Fig. 2. The relationship between taste panel scores for pork and bacon flavours in bacon cured with nitrite levels from 0 to 2000 mg/kg.

ments of taste intensity and odour intensity, neither of which varied with nitrite level (Table 1 a and b) and gave means of 5.2 and 4.5, respectively. Analysis of variance of the two sets of results (Table 1a and b) showed frequent interaction between testers and treatments indicating that a wide divergence of opinion existed amongst the panelists in their differentiation between bacon and pork flavour when making judgements without prior knowledge of the identity of the meat in the samples submitted.

The panel means for odour intensity showed the cured samples to be slightly lacking in odour compared with the salted pork control and this was tested by

Table 2. Comparison of aromas of pork and bacon by two-way triangle test.

Samples compared	Odd sample	Number of correct answers / Number of judges
Boiled bacon and boiled pork	pork	10/13**
	bacon	9/13**
Grilled bacon and grilled pork	pork	20/29***
	bacon	21/29***

**significant at p = 0.01;
***significant at p = 0.001.

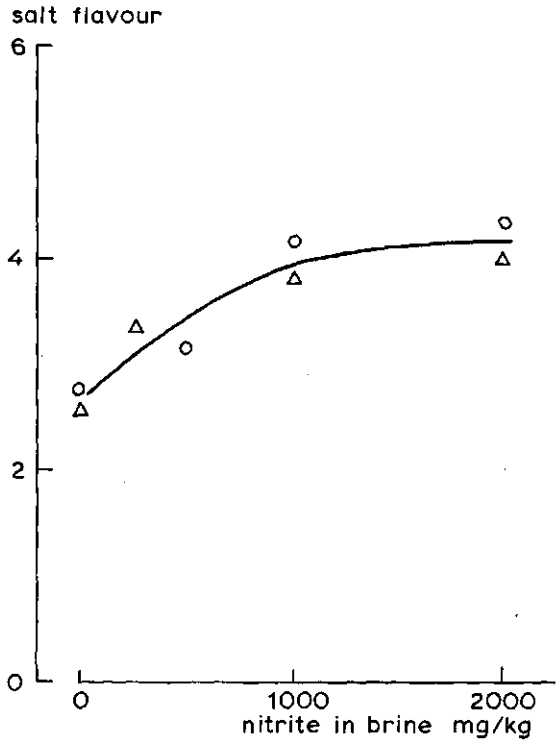

Fig. 3. Taste panel judgements of saltiness of bacon cured at different levels of nitrite. (○ and △ = results from Tables 1a and 1b, respectively).

triangle tests between pork and bacon, boiled and fried. An odour difference was then significantly established in the direct comparison; however, even under these critical circumstances, 30% of the judgements made were incorrect (Table 2).

Taste panel judgements of the saltiness of the samples were consistently higher in the cured meats than in the salted pork and showed a progressive increase with nitrite level (Fig. 3). The salt content of the samples varied between 3.9 and 6.1% but in a random way (Table 3) which could not account for the panel results. Nitrite itself has no detectable taste at 100 or 200 mg/kg in aqueous or 0.5% sodium chloride solution nor could it be detected when submitted in a neutral solid carbohydrate medium (mashed potato powder) (Table 4). Bacon is already known to exhibit a masking effect on saltiness (Ingram, 1949) and these results suggest that salt is bound into the cured meat structure more firmly than in untreated pork meat, though whether by a chemical or physical mechanism is not known. Unsalted pork was clearly recognised by the taste panel which gave it a mean mark of 0.32 for bacon flavour; whereas the presence of 4% salt led to the mark of 2.5 (Table 1).

These results underline the major part played by the presence of salt in the consumer's appreciation of bacon flavour; firstly, about one half of an expert panel's marking is accounted for by salt alone and secondly, about one third of

Table 3. Concentrations of curing salts in samples of cured pork served to taste panels.

$NaNO_2$ in brine (mg/kg)	Residual $NaNO_2$ (mg/kg)	Residual NaCl (%)
0	1	4.5
250	18	6.1
500	31	3.9
1000	72	5.0
2000	145	4.9

Table 4. Comparison of solutions with and without sodium nitrite by two-way triangle test.

Solution in:	$NaNO_2$ concentrations compared (mg/kg)	Odd sample	Number of correct answers number of judges
distilled water	0 and 100	0	7/14n.s.
		100	4/14n.s.
0.5% NaCl	0 and 100	0	6/14n.s.
		100	5/14n.s.
distilled water	0 and 200	0	7/14n.s.
		200	8/14n.s.
0.5% NaCl	0 and 200	0	5/14n.s.
		200	5/14n.s.
potato powder	0 and 200	0	5/12n.s.
		200	5/12n.s.
potato powder + 1% NaCl	0 and 200	0	6/12n.s.
		200	4/12n.s.

n.s. not significant

blind judgements on the identity of pork or bacon are in error. They show too that the moderate levels of nitrite in common usage for bacon production are enough to produce close to the maximum amounts of cured flavour attainable; reduction of these levels will, therefore, immediately result in loss of cured flavour.

Chemical analyses of volatiles

The aromas of pork and bacon are, by no means, distinguishable with certainty even by experienced judges (30% failures in our results; cf. 40% failures in taste testing by Cho & Bratzler (1970). Extracts of volatiles have been made and analysed and more than 40 peaks with retention times between 5 and 70 min have been found in gas chromatograms of both pork and bacon. Many of the peaks were complex and contained more than one substance and although observations of the odours of the more volatile peaks have shown some minor differences between cured and uncured

patterns, no clear differences have been indentified. Five peaks, in both samples, contained nitrogen, one of which was thialdine a compound formed from hydrogen sulphide, ammonia and acetaldehyde in boiled beef (Brinkman et al., 1972). No light has been thrown by the results so far obtained on the theory of Cross & Ziegler (1965) that autoxidation of linoleic acid in pork during cooking is inhibited by nitrite present in cured meats.

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Discussion

Tasting technique

It was stated that it was most unlikely that flavour components could have migrated from one sample to another during cooking or panelling.

Interpretation of results

Some participants thought it unjustified to draw further conclusions from the

high number of panelists unable to distinguish between pork and bacon, because the number of times the difference was noticed was highly significant. The only conclusion can be that the difference between bacon and pork was only subtle.

Practical concentration of nitrite

In commercial Wiltshire bacon production when no nitrate is present, the usual level of nitrite in the brine is 1 500–2 000 mg/kg. In the presence of 0.1–0.5% nitrate, which is used in the majority of cases, the nitrite level is 500–1 000 mg/kg.

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Nitrite and the flavour of cured meat II

A. E. Wasserman

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19 1 18, USA

Abstract

A review of the literature of the last 50 years shows very little interest in the effect of nitrite on flavor of cured meat products, particularly the relation of sodium nitrite to development of the flavor. Sensory evaluation studies indicate a need for the presence of nitrite; low concentrations may be sufficient to induce the flavor. Chemical analysis of head space vapors or extracts of cured hams has led to identification of up to approximately 50 compounds. Although many of them have been identified before in other meats or fowl, none has the characteristic cure aroma. Known reactions of nitrite and various meat components are discussed.

Introduction

Investigations into the role of nitrite in the cure process have been principally on the development of color and on the preservative, anti-clostridial effect. Authorization for the use of nitrite in cured meat as described in the U.S. Code of Federal Regulations (1971) is for the purpose of developing color. Recent reports, (Wolff & Wasserman, 1972), however, suggest a re-appraisal of the curing process because the nitrite may under some conditions react with amino compounds present to give small concentrations of nitrosamines. It is now being recognized what has been evident for many years: without the use of nitrite, the characteristic cured flavor is not developed in meat products. However, since 1940 very limited research has been carried out on this problem. The studies that have been reported can be classified either as processing and sensory evaluation products or chemical analysis of the reaction between nitrite and meat components. Insufficient information is available in either of these categories to permit a definitive discussion of the role of nitrite in the development of cure flavor, but this paper can serve to consolidate reported data and place them in a perspective that may encourage an increase in research activity.

Processing and sensory evaluation of meat products

The studies of Kerr et al. (1926), which established the basis for the use of nitrite in curing meat products, demonstrated that nitrite-cured meat was as acceptable as traditionally cured meat. Although flavor was not specifically men-

tioned in the report, it must be assumed that this was a consideration in the quality assessment of the products. Brooks et al. (1940) investigated the use of nitrite in the Wiltshire bacon curing process, particularly with respect to flavor formation. Although no sensory evaluation data were given and the responses appear vague, the authors concluded that the characteristic flavor of bacon and ham (as opposed to salt pork) is due to the reaction of nitrite with tissue constituents during curing or during cooking and that 10 mg NO_2^-/l cure was sufficient to give satisfactory flavor.

Barnett et al. (1965), in an extensive study of the development of ham flavor, found that hams pumped with pickle containing 0.1 g NO_2^-/l were as acceptable to a panel as those pumped with the normal pickle containing 1.5 g NO_2^-/l; the former value being equivalent to about 10 mg nitrite per kg. ham.

Unfortunately these authors did not compare hams without nitrite, but their data show that lower values can be used successfully. Tripling the normal concentration of nitrite in the pickle resulted in hams with bitter flavor.

Pork m. longissimus dorsi roasts, cured with 300 mg nitrite/kg but not smoked, could be distinguished in triangle taste tests from those cured without nitrite, and in paired comparison tests the panelists indicated the nitrite-treated products had more cure flavor (Cho & Bratzler, 1970). Smoking the cured roasts did not affect the outcome of the taste tests; nitrite-treated roasts were still differentiated in the triangle test and had more cure flavor.

More recently, Simon et al. (1972) found all meat frankfurters made with beef and pork were scored low in a hedonic taste panel evaluation when they contained no nitrite; 39 mg nitrite/kg, the lowest concentration tested, was sufficient to give an acceptable flavor. For some unexplained reason, however, all beef frankfurters were acceptable even in the absence of nitrite. It should be noted that in these studies the panelists were asked whether they liked or disliked the product – not how much cure flavor it contained.

In our laboratory, we (Wasserman & Talley, 1972) found the role of nitrite in frankfurter flavor to be complex, depending on the type of evaluation panel used. In triangle tests with both smoked and unsmoked frankfurters, there were significant differences between products prepared with or without 156 mg nitrite/kg (Table 1). However, in tests in which 'frankfurter' flavor was scored, the smoked, non-nitrite-treated sample was rated as highly as the cured sample. Analysis of variance showed an interaction between smoke and nitrite. Thus, while there is no question about the poor flavor of non-nitrite-, non-smoke-treated samples, cured franfurter flavor is also associated with smoke flavor.

Chemical studies

Ockerman et al. (1964) felt that since the aroma of dry cured ham was similar to its flavor, analysis of volatile components would identify the flavor factors. Vacuum distallates of ham cured with salt, sugar, and KNO_3 had a typical aroma and flavor; a number of carbonyls, acids, and bases were identified. While none were specifically associated with cured flavor, it was noted that the quantity of acids and carbonyls increased with the age of the ham.

Ockerman et al. (1964) felt that since the aroma of dry cured ham was similar to palmitoleic, oleic, and linoleic – decreased, first during curing and smoking, then

Table 1. Triangle test evaluation of the flavor of frankfurters prepared with cure in which sodium nitrite was either present or absent.

Experiment	Conditions	Number of correct answers number of judges
1	Cooked, no smoke; + NO_2 vs no NO_2	15/22***
2	Cooked, no smoke; + NO_2 vs no NO_2	28/36***
3	Cooked, no smoke; + NO_2 vs no NO_2	18/24***
4	Cooked, smoked; + NO_2 vs no NO_2	11/17**
5	Cooked, smoked; + NO_2 vs no NO_2	13/24*
6	Cooked, smoked; 50% NO_2 vs no NO_2	12/17**
7	Cooked, smoked; 50% NO_2 vs 100% NO_2	9/17n.s.

*p = .05;
**p = .01;
***p = .001;
n.s. = not significant

with aging. Flavor increased with age of ham. Flavor components in dry cured hams were also investigated by Lillard & Ayres (1969), who studied a number of hams of various ages from different parts of the United States. A total of 46 volatile compounds were identified. Although most of them had been reported previously in studies with chicken and other meats, a number of esters were unique to the aged hams. Large increases in amino acids were also noted, but variation due to age of the hams made it difficult to interpret these data. While chemical composition of uncured hams, unfortunately, was not compared to that of hams treated with nitrate, it was noted that the aroma and flavor of the country, or dry, cured hams was quite different from fresh pork. When the hams were cooked the flavor was intensified. The cured flavor was not water soluble but could be transferred by steam distillation at 60 °C under reduced pressure.

Volatile compounds of cured and uncured boiled hams were investigated by Cross & Ziegler (1965). Concentrating on the carbonyl compounds removed by stripping with nitrogen gas, they demonstrated the major difference between the two ham products was the absence of hexanal and valeraldehyde in the cured ham. Furthermore, after passage through a solution of 2,4-dinitrophenylhydrazine, the carbonyl-free volatiles of the uncured ham had a cured aroma, as did volatiles from cured and uncured chicken or beef. The cured aroma was removed by passage through solutions of mercuric chloride or mercuric cyanide. The authors conclude that cured ham flavor is the basic meat flavor derived from precursors other than triglycerides and that nitrite functions by inhibiting the oxidation of unsaturated lipids from which hexanal and valeraldehyde originate. Branched-chain aldehydes, derived from amino acids through the browning reaction, were found in equal concentrations in both cured and uncured hams, and thus, presumably, were not involved in the characteristic cured flavor.

Piotrowski et al. (1970) in our laboratory reported that a basic meaty aroma could be isolated in water extracts of cured ham similar to that from raw ham, beef, or veal. A fraction having the cured ham aroma was obtained by treatment of the cured ham residue of the water extraction with chloroform-methanol (2:1). This is

the lipid portion of the meat. It is difficult to decide whether the cured aroma is derived from the lipids or whether it is lipid soluble, but unpublished data show that cure aroma can be transferred from the lipid fraction to a bland vegetable oil by bubbling N_2 through the former. Smoke components undoubtedly play an important role in defining cure flavor; their interaction has been demonstrated in the section on Sensory Evaluation. However, we have found that nitrite treatment enhances smoke aroma of ham (Wasserman, 1973). Uncured hams, smoked simultaneously with cure-pumped hams, had only a faint smoke aroma. Although there has been a question whether smoke and cure flavor may not be the same, it is possible to distinguish between the two. On heating aqueous extracts of cured, smoked ham, aromas recognizable as smoky are volatilized at the beginning, leaving saltier, more cured notes to evolve as heating progresses. Gas chromatographic profiles of pyrolyzates of water- and lipid-soluble fractions from cured and uncured hams were complex and, although no identifications were made, it was concluded that differences resulting from nitrite treatment of the meat were more quantitative than qualitative (Piotrowski et al., 1970). None of the compounds eluting from the gas chromatograph had a meaty or a cured aroma.

More recently, Baily and co-workers (1973) have also analyzed uncured and nitrite-treated hams for aroma constituents by gas chromatography of extracts, They have identified about 50 components in the volatile fraction, many of them previously reported in meat. Although several previously unreported compounds were identified, Bailey indicated neither the new nor the known components had cured or hammy aromas. He also showed that, at this time at least, the major differences between cured and uncured hams are quantitative and appear to be associated with lower concentrations of cabonyl compounds derived from the unsaturated lipids in the cured hams.

Two studies from Russian workers also link carbonyl compounds with ham aroma. Iskandarian (1970) reported that pyruvic acid in cured pork was an intermediate for the formation of volatile carbonyl compounds that impart a pronounced ham aroma to meat. Solovyov et al. (1970) found that cured ham differed from cooked pork in having a greater content of volatile carbonyls, particularly methylglyoxal (pyruvaldehyde).

The effect of nitrite on the amino acid composition of hams has been investigated. The free amino acids have been implicated in the development of the basic meaty aroma and primary amines are known to react with nitrite via the Van Slyke reaction. Definitive data, however, have not been reported. Solovyov et al. (1970) reported higher total amino acids in cured ham, with losses of some free amino acids. Piotrowski et al. (1970) investigated the amino acid composition of raw, cooked, and cooked-smoked hams that were either uncured or treated with nitrite-containing cure and found variations in the different preparations but couldn't identify a trend due to treatment.

What is cured flavor? If the aroma of smoke, and possibly the effect of spices, are discounted, is there actually a cured flavor? Cross & Ziegler (1965) and Bailey (1973) suggest that nitrite acts to reduce oxidation of unsaturated lipids which from odorous carbonyls. Bailey (1973) has claimed that the odor of cured and uncured meats are the same immediately after processing and that 'cured' aroma develops after storage. This would mean the difference between aromas of nitrite-

treated and uncured products develops as oxidation compounds form in the uncured product. Thus 'cure' aroma actually would be a lack of certain odors that are associated with cooked pork.

On the other hand, it has been reported that the flavor of dry cured ham increases with aging (Lillard & Ayres, 1969); unfortunately these studies were not done with controls cured for the same period of time without nitrite. Oxidation under these conditions is extensive and the effect of nitrite could have been magnified.

Nitrite, however, reacts with other meat components as well. It combines with -SH compounds to form nitrosothiols. Cross & Ziegler (1965) found dimethyldisulfide in the mercuric salt trap that removed cure aroma from ham volatilies bubbled through it. They postulated this was derived from methanethiol. However, when Barnett et al. (1965) studied the effect of adding sulfur compounds to the meat prior to pickling, or curing meat from hogs fed sulfur compounds, they found, in general, there was no difference in preference between the treated and untreated ham. In some instances the sulfur compounds produced undesirable odors in the product even at low concentrations; methanethiol is an example.

Nitrite also reacts with primary amino acids, as described above; it is also known to react with secondary and tertiary amines. There are undoubtedly other meat components that react to some small extent with nitrite. It is not known whether these reactions affect the aroma.

The concluding remarks by Barnett et al. (1965) are still valid today. 'on a number of occasions we have compared hams prepared by present-day curing and processing practices with those generally employed 20 or more years ago, and almost invariably the 'modern' ham has been preferred. In spite of this, may old-timers recall the day when merely cutting a ham filled the room with tantalizing aroma, and the flavor on eating was equally as delightful'.

'However, perhaps hams never did taste like they used to.'

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Discussion

The study of nitrite and flavour formation

The experiments of Brooks were repeated by the British Food Manufacturing Industries Research Association with the same results. So already in 1940 it was clear that nitrite was necessary for the flavour. The gap between 1940 and 1965 can easily be explained by the lack of methods to study the nature of this flavour. Advanced techniques nowadays make a new approach possible.

Origin of cured meat flavour

Herring said that the flavour of cured meats varies according to product type. Cured flavour per se can only be measured on meat treated with nitrite and/or salt. He asked whether the flavour of all other products like cured primal cuts, wieners, dry sausages could be considered as a blend of cured flavour with that contributed by other factors such as bacteria, salt smoke and spices. A very typical example is the country style cured ham! Significant differences in flavour between frankfurters with and without nitrite were only obtained without smoke.

As long as the nature of the nitrite flavour is not known it is very difficult to look for other solutions. Further studies are necessary.

Cross and Siegler have inferred, and Bailey has indicated that cure flavour is due to the inhibition of oxidation of unsaturated fatty acids by nitrite. Cure flavour, therefore, would be a negative flavour.

NO_2 from smoke

Several speakers asked whether nitrogen oxides from the smoke plays a role. Wassermann stated that its role was proved by the identification of nitrosomyglobin in smoked frankfurters, prepared without nitrite. Furthermore it was stated that nitrite so formed may be a catalyst for a Maillard type reaction which is important both for smoke flavour and smoke colour.

Possible interaction of nitrite and fat in nitrosamine formation

Schram reported that the presence of lipid hydroperoxides was found to enhance the rate of nitrosamine formation in model systems. It is likely that the same would occur in bacon.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen

Ascorbate and nitrosamine formation in cured meats

R. A. Greenberg

Swift & Company, Research and Development Center, Oak Brook, Illinois 60521, USA

Abstract

Nitrite is an essential component of cured meat manufacture. It is responsible for cured flavor, color, prevention of 'warmed-over' flavor, and retards botulinal toxin development, particularly under conditions of product mishandling.

Nitrosamines can be formed from the reaction of nitrite and secondary or tertiary amines under acid conditions. Nitrosamines have been observed occasionally in virtually all types of cured meats. Only in bacon, after preparation for the table, has a nitrosamine been found to be generally present. It is probably that a precursor of nitrosopyrrolidine (nitroso-hydroxy-proline?) is formed during curing and/or subsequent storage. The heat of cooking apparently catalyzes conversion of the precursor to nitrosopyrrolidine.

Ascorbate has been a curing adjunct for many years. Recent observations in several laboratories have demonstrated the ability of ascorbic acid and sodium ascorbate to block nitrosation.

Work is under way to determine whether increasing ascorbate levels in bacon curing formulations can reduce nitrosopyrrolidine levels in cooked bacon. Preliminary results are quite promising.

Introduction

The observation that nitrosamines can be found in various processed meats has resulted in one of the most serious problems ever encountered by the meat industry. In the United States, about two-thirds of all pork is consumed in the cured form. Growing shortages of beef, poultry, and other traditional protein foods have made it mandatory that consumer acceptable cured meat products not be removed from the food supply without sound reason.

While nitrosamines have been observed to be occasionally present in other cured meats, the only meat product in which the positive result cannot be traced to excessive levels of nitrite, improper processing conditions, or questionable analytical technique, is bacon. Bacon is a popular food. (Its uncured counterpart, salt belly, is not). Commercial bacon samples fried according to typical U.S. consumer procedures have been found by analysts from the U.S. Food and Drug Administration (USFDA) to contain up to 108 μg/kg of nitrosopyrrolidine (Table 1).

Table 1. *N*-Nitroso pyrrolidine levels found in commercial bacon (μg/kg).

Sample	Raw	Pan-fried	Fat cooked out
1	0	30	45
2	0	95	96
3	0	106	207
4	0	106	142
5	0	10	81
6	0	20	68
7	0	32	61
8	0	108	100

(Fazio et al., 1973)

Ascorbate as a curing adjuvant

Ascorbic acid, isoascorbic acid, sodium ascorbate, and sodium isoascorbate have been used for many years to improve color characteristics of cured meats. US Department of Agriculture regulations permit about 470 mg/kg of the acid or approximately 550 mg/kg of salt (Table 2). The permitted level is about twice that actually necessary. Thus, most American processors use about 200–250 mg/kg in their products. Watts & Lehmann (1952) reported that color fixation, odor, and flavor were greatly improved when ascorbic acid was included in the formulation of cooked 'nitrited' ground pork. Mills et al. (1958) noted that frankfurters containing sodium isoascorbate or sodium ascorbate had a more desirable internal color than did control lots. Ascorbate-free control product was less uniform in color and tended to lose its color more rapidly when held in a lighted display case. Wirth (1973), in his recently published paper in 'Fleischwirtschaft' on the possible consequences of reducing or prohibiting nitrite in cured meats, showed that nitrite formulation levels could be reduced somewhat in several products, without organoleptic difficulty, when sufficient ascorbate was utilized.

Ascorbate as a nitrosation preventive

Because many modern drugs are secondary and tertiary amines, the possibility of

Table 2 US Regulations on addition of ascorbic acid, isoascorbic acid, sodium ascorbate, and sodium isoascorbate to cured meats.

	Per 100 gal pickle at 10% pump level	Per 100 lb meat or meat byproduct
Ascorbic acid or isoascorbic acid	75 oz	3/4 oz
Sodium ascorbate or sodium isoascorbate	87.5 oz	7/8 oz

in vivo production of nitrosamines has been under consideration for some time by the pharmaceutical industry. Kamm et al. (1973) have recently demonstrated a protective effect of ascorbic acid on hepatotoxicity for rats fed a combination of sodium nitrite and aminopyrine. Articles such as from Lijinsky et al. (1973) have postulated that amine drugs (or even cigarette smoke) could combine with free nitrite from food and form nitrosamines in the human stomach. Tannenbaum (1972) has shown that nitrite is present universally in human saliva. A man typically ingests as much nitrite each day from his own saliva (6–12 mg) as he would receive from the free nitrite present in three typical meals in which a cured meat was the main component. Thus, ascorbate appears to be an interesting candidate as a nitrosation preventive, from several points of view.

Dimethylnitrosamine has been observed to be present in occasional samples of commercial frankfurters at the retail level (Wasserman et al., 1972). However, attempts to purposefully produce DMNA in frankfurters under controlled laboratory conditions have required concentrations of sodium nitrite up to about ten times the legal limit (Fiddler et al., 1972).

Mirvish et al. (1972) and Fiddler et al. (1972a) have postulated that the ascorbate-nitrite reaction could be a possible means of preventing nitrosation in cured meats. Fiddler et al. (1973), studying the effect of various meat curing ingredients on DMNA formation in a model system, found that ascorbic acid, sodium ascorbate, and sodium isoascorbate markedly inhibited DMNA formation in systems containing high concentrations of sodium nitrite. The results of their experiments with frankfurters formulated with 1500 mg/kg sodium nitrite are shown in Table 3 (Fiddler et al., 1973a). Frankfurters formulated with no ascorbate or isoascorbate developed DMNA during moderate thermal processing. Product formulated with 550 and 5500 mg/kg of these salts did not contain DMNA. Even when subjected to severe overprocessing, the effect of increasing concentration of ascorbate and isoascorbate was to reduce the DMNA level in the finished product.

Table 3. Effect of ascorbate and isoascorbate on dimethylnitrosamine formation in frankfurters formulated with 1500 μg/kg sodium nitrite.

	Processed 2 h	Processed 4 h
Ascorbate (μg/kg)	dimethylnitrosamine (μg/kg)	
0	11	22
550	0	7
5500	0	4
Isoascorbate (μg/kg)		
0	10	11
550	0	6
5500	0	0

(Fiddler et al., 1973)

Table 4. Effect of ascorbate on nitrosopyrrolidine formation in fried bacon. (170 μg/kg sodium nitrite added).

Ascorbate (μg/kg)	Storage before curing at 4 °C (days)	
	1	13
	Nitrosopyrrolidine (μg/kg)	
0	7	18
250	6	11
1000	0	0

(Herring, 1973)

Ascorbate as an answer to the bacon problem

If nitrosation can be effectively blocked by ascorbate under commercial bacon manufacturing conditions, we would have a simple way out of the nitrosopyrrolidine dilemma. Obviously, the use of 200–250 mg/kg sodium ascorbate – the current practice – does not control the development of nitrosopyrrolidine precursors in bacon. The market basket survey from the US Department of Agriculture presented in Table 1 makes this abundantly clear. Increasing the use of ascorbate up to and beyond presently acceptable levels may be at least a partial solution. With this in mind, a series of tests were begun by the American meat industry, the US Department of Agriculture, and USFDA. The results of experiments studying the effect of increasing ascorbate formulation levels on nitrosopyrrolidine formation in bacon containing typical sodium nitrite cures are shown in Tables 4, 5, and 6. The data in Table 4 represent bacon produced in a commercial plant, stored before curing for 1 and 13 days at 4 °C. Although nitrosopyrrolidine was present after frying in bacon formulated with 0 and 250 mg/kg sodium ascorbate, no nitrosopyrrolidine was found in products formulated with 1000 mg/kg sodium ascorbate. The data in Table 5 show the results of a companion experiment in which the

Table 5. Effect of ascorbate on nitrosopyrrolidine formation in fried bacon. (170 μg/kg sodium nitrite added).

Ascorbate (μg/kg)	Stored after curing at 7 °C (weeks)		
	0	1	6
	nitrosopyrrolidine (μg/kg)		
0	0–13	5–11	5–10
500	0–5	0	0
2000	0	0	0

(Herring, 1973)

Table 6. Effect of ascorbate on nitrosopyrrolidine formation in fried bacon formulated with 170 μg/kg sodium nitrite.

Ascorbate (μg/kg)	Nitrosopyrrolidine (μg/kg)
0	20 – 92
250	14 – 26
500	0 – 7
1000	10
2000	0

(Wasserman, 1973)

product was stored up to 6 weeks at 7 °C after curing. Again, increasing ascorbate level in the cure formulation reduced nitrosopyrrolidine levels in the fried bacon. The data in Table 6 show the results of still another commercial plant experiment and demonstrate a decrease in nitrosopyrrolidine with increasing ascorbate.

More definitive large-scale commercial tests are currently under way. As of the writing of this paper, the results are not yet available.

Other aspects of increasing ascorbate concentration in bacon

We have observed no organoleptic difficulties with bacon formulated with up to 2000 mg/kg ascorbate.

Increasing ascorbate intake in the human diet should have a salutary rather than a harmful nutritional effect (Yew, 1973).

One of the considerations to be resolved should increasing levels of ascorbate prove a practical means of handling the bacon nitrosopyrrolidine problem is the effect on nitrite's ability to prevent botulinal toxin formation. Nitrite's antibotulinal efficacy has been demonstrated in canned comminuted ham (Christiansen et al., 1973) and in frankfurters (Hustad et al., 1973), as well as in bacon (Greenberg, 1973). Will high levels remove sufficient nitrite from the product so as to result in a botulism hazard?

Table 7. Effect of ascorbic acid on putrefactive spoilage of pasteurized lunch meat.

Sodium nitrite (μg/kg)	Ascorbic acid (μg/kg)	Putrid spoilage (%)
0	468	100
117	0	100
117	78	18
117	156	26
117	312	20
117	468	30

(Schack & Taylor, 1966)

Work is under way to answer the question. A suggestion that this may not be a problem can be deduced from some work on luncheon meat published in the Schack & Taylor patent (1966). Luncheon meat containing 117 mg/kg sodium nitrite and inoculated with *Clostridium sporogenes* P.A. 3679 spores did not show statistically different putrefactive spoilage at ascorbic acid formulation concentrations from 78 to 468 mg/kg. Complete and rapid spoilage occurred in products containing no sodium nitrite and 468 mg/kg ascorbic acid and in products containing 117 mg/kg sodium nitrite and no ascorbic acid. The data are shown in Table 7.

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Discussion

Trials on increase of ascorbate levels

Addition by the author

A large scale commercial plan test was conducted to further test the practicality of controlling nitrosamine in bacon by increasing ascorbate levels in the cure. Production size 20 000 pound lots each were manufactured, using four bacon pickles to test two levels of sodium ascorbate (330 mg/kg and 1 000 mg/kg) with and without 0.4% sodium tripolyphosphate. The pumped bellies were cured for one day at 4 °C (40 °F), processed about 22 hours to 55 °C (128°–130 °F) internal temperature. Chilled bellies were formed 3 to 4 days after smoking and vacuum packed one day after forming. Some products were fried in electric pans (average frying yield 33.5%), frozen and shipped for nitrosamine analysis to the laboratories of the US Department of Agriculture-Agricultural Research Service and the US Food and Drug Administration, Other products were frozen after manufacture and shipped to those laboratories for subsequent frying.

The samples fried at the packer and analysed by the USFDA laboratory were all negative (for unknown reasons). Similar samples tested at USDA-ARS showed a nitrosopyrrolidine reduction in both phosphated and non-phosphated bacon in the higher ascorbate level product.

Analysis of products fried at USFDA also showed reduced nitrosopyrrolidine levels in the higher ascorbate level product than that formulated with 330 mg/kg.

Analyses by USDA – ARS. US commercial plant bacon study.

	NPP (in μg/kg) assays of fried bacon (170 mg/kg NO_2)			
	with 0.4% TPP		without TPP	
	fried by packer	fried by ARS	fried by packer	fried by ARS
330 mg/kg ascorbate	4	11	15	3
1 000 mg/kg ascorbate	3	0	3	0

Analyses (three samples of each variable) by USFDA (all products fried at USFDA). US commercial plant bacon study.

	with 0.4% TPP	without TPP
330 mg/kg ascorbate	20 – 38 –20	20 – 15 – 18
1 000 mg/kg ascorbate	11 – 9 – 6	6 – 7 – 6

Residual nitrite level in cured meats at time of consumption (3 oz. portions, before home preparation).

	$NaNO_2$ in original formulation	$NaNO_2$ as consumed
Bacon	120 mg/kg	3.0 mg
Frankfurthers	150 mg/kg	1.4 mg
Ham	150 mg/kg	7.6 mg
Total		12.0 mg

(Tannenbaum – Proc. of 25th Annual Reciprocal Meat Conf. AMSA, 1972 – reports daily NO_2 excretion in saliva for all human subjects studied to be 6–12 mg per day).

Addition by Dr. Schram

Trials have been made with vacuum packed raw bacon with 500 mg/kg nitrate, stored at 25 °C. The followed results were obtained:

Added nitrite (mg/kg)	Added ascorbate mg/kg)	After 14 days			After 28 days		
		NO_2 mg/kg	DMN µg/kg	NNP µg/kg	NO_2 mg/kg	DMN µg/kg	NNP µg/kg
100	0	3.5	0.5	12	1.9	0.5	4.9
100	1000	2.0	0.4	6	2.6	0.3	4.9
200	0	7.5	1.4	24	2.1	0.3	4.5
200	1000	4.5	0.4	2	3.6	0.5	4.6
0	0	22	1.5	20	1.6	0.4	4.3

The effect of ascorbate was a rapid decrease of the amount of nitrite, so less nitrosamine was formed. Nevertheless it has a positive effect against *Cl. botulinum.* In inoculated packs without added nitrite 9 out of 10 appeared to be toxic, with only 100 mg/kg nitrite 1 out of 10 was toxic, the remaining packs showed no toxin formation.

The level of ascorbate was fairly constant between 500 and 600 mg/kg. In the

pouches without added nitrite the nitrite concentration showed a maximum after about two weeks.

Effect of ascorbate on DMNA formation

Work has been carried out at the Meat Research Institute in conjunction with the Laboratory of the Government Chemist to show the effect of ascorbate on the formation of dimethyl nitrosamine (DMNA). A reduction in nitrosamine formation in the presence of ascorbate has been found. Slices of pork eye-muscle, impregnated with dimethylamine (DMA) were cured in nitrite-containing brines, and the cured samples heated to 90 °C. In the absence of ascorbate at pH 5.8 a yield of about 0.1% of the theoretical amount of DMNA was found. At the same pH in the presence of ascorbate at 2.0, 1.0, 0.5 molar ratios to nitrite, this yield was reduced by about 80% i.e. a yield of 0.02%.

For pork middles similarily impregnated with DMA and cured, a similar effect of ascorbate was noticed in lean samples of the middle heated in a canning process, but in the case of fried samples the situation was more confused.

Minimum level of nitrite

Some results were given by Ingram concerning Wiltshire cure of backs with different amounts of nitrite, without nitrate. This was done in a factory under normal conditions. With 2000 and 1000 mg/kg nitrite in the brine there were no problems. With 500 mg/kg the colour was less good and also the flavour and keepability. With 250 mg/kg nitrite in the brine many problems occurred with respect to colour, flavour and keepability, there was even deterioration. These experiments were carried out with backs, which are more stable than shoulders, so it is expected that 100 mg/kg nitrite in the bacon (1000 mg/kg in the brine) will be the required level.

Role of ascorbate

Does ascorbate only act as a nitrite scavenger or does it interfere with the nitrosation reaction? No definite data are yet available, but probably ascorbate does not act only as a scavenger.

Alternatives for ascorbic acid

Alternatives are often themselves of questionable safety. It is known that several compounds like alloxane, dialluric acid and cyclohexanetrione, in very small concentrations can act like ascorbic acid. There is nothing known about their activity with respect to the nitrosation action, nor about their toxicity.

Effect of frying temperature on nitrosamine formation

It was stated that the temperature distribution during frying was extremely uneven. This has a considerable effect on the formation of nitrosamines. It was found that the concentration of nitrosamines in fat was much higher than in lean meat. Experiments have been carried out on this subject. Bacon was fried in a pan (starting with either a hot or cold pan), broiled on a grill, fried on a 'baconer' (direct heated with electric elements) or heated in a microwave oven. Little differences in the nitrosamine concentration were found, however lower levels were

obtained in the bacon cooked in the microwave oven. It was commented that controlling the cooking of bacon is difficult. It is possible to set up controlled grilling and frying, but even then the quantity of nitrosamines, found in samples of bacon, varies over very wide ranges. Schramm reported that in the Unilever laboratory it was found that N-nitrosopyrrolidine is formed only in the fatty tissue and hardly at all in the lean. Nitrosamines have never been found in cured meat products that have been boiled nor in raw bacon.

Sawyer supported the Unilever finding, that no evidence of volatile nitrosamines had been obtained in the examination of boiled bacon at the laboratory of the Government Chemist, London.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen.

Shelf stable cured ham with low nitrite-nitrate additions preserved by radappertization[1]

Eugen Wierbicki and Fred Heiligman

Irradiated Food Products Division, Food Laboratory, U.S. Army Natick Laboratories, Natick, Massachusetts, USA

Abstract

Radappertization (radiation sterilization) is effective in reducing the requirements for the curing agents, nitrites and nitrates, in cured ham. These agents benefit the organoleptic qualities of cured ham, and nitrite, among other of its functions, inhibits toxin production by *Clostridium botulinum*. The use of these agents, however, has been under reappraisal by the industry, the Food and Drug Administration, and the US Department of Agriculture. It has been found that under certain conditions nitrites may react with the free amines in food to form nitrosamines, which are carcinogenic.

The experiments reported in this paper were designed to learn whether or not the amounts of nitrites and nitrates, commonly used in combination with ascorbates/erythorbates in cured meats, could be reduced by use of radappertization. To this end a series of experiments was conducted to determine the minimum amounts of these additives needed to produce

1. The characteristic color of cured ham,
2. The cured ham flavor, and
3. The necessary control of *Cl. botulinum*.

Results showed that the levels of nitrites and nitrates commonly used by industry can be substantially reduced without impairment of any of the foregoing attributes.

An important factor in achieving this notable reduction is that radappertization destroys *Cl. botulinum*, thus eliminating the need for that amount of nitrite required for controlling *Cl. botulinum* in ham cured by non-radappertized processes.

Introduction

Background

Ham is the most important of cured meats from a commercial standpoint. In

1. Radappertization according to the definition given by Goresline et al. (Nature 204 (1964) 237–8) is: . . . . 'the application to foods of doses of ionizing radiation sufficient to reduce the number and/or activity of viable organisms to such an extent that very few, if any, are detectable in the treated food by any recognized method (viruses being excepted) while no spoilage or toxicity of microbial origin is detectable no matter how long or under what conditions the food is stored in the absence of recontamination'. . . The article continues: . . . 'the prefix rad discounts a heat treatment' . . . . .

The smokehouse processing as described in this article implies a heat treatment to inactivate enzymes and make the item ready-to-eat, but not shelf stable by the heat.

1972, over 584.7 million killograms (mil. kg) of ham was processed under Federal Inspection and 78.0 mil. kg of canned ham imported into the United States for a total of 662.7 mil. kg. Only 12.6 mil. kg (1.9%) of this total were canned shelf-stable hams packed in small cans (Anonymous, 1973b). Radappertization (radiation sterilization) processing of ham, to produce a shelf-stable product while preserving its organoleptic qualities, has been a part of the Food Radiation Program since its beginning (Wierbicki et al., 1965; Anonymous, 1968). It was one of the first radappertized meats of acceptable quality to be achieved even when processed with the relatively high radiation sterilizing dose of 4.5 – 5.6 Mrad (Anonymous, 1965; Wierbicki et al. 1965). Initially, the 12D dose was determined to be 2.9 Mrad when the product was in the non-frozen state (Anellis et al., 1967). With the advance of technology of radappertization of meats, it was shown that the quality of foods is improved by irradiating them in the frozen state (Wierbicki et al., 1965; Josephson et al., 1968). Continued investigation of irradiated foods at various product temperatures down to −196 °C has shown that as irradiation temperatures are lowered, higher irradiation doses are required to achieve the same biocidal effect under the 12D concept (Grecz et al., 1971). Moreover, the cost of processing increases as temperature is lowered below the limit of mechanical refrigeration. Therefore, the most favorable balance of quality, cost and effective irradiation dose appears to be −30±10 °C (Josephson et al., 1968; Josephson & Wierbicki, 1973). The minimum required irradation dose for sterilization (MRD) of ham (12D dose) at −30±10 °C is 3.7 Mrad. Table 1 lists the MRD for nine radappertized foods, including ham, under the 12D concept (Anellis et al., 1965, 1967, 1968, 1969 and 1972). Irradiation of ham in the frozen state is beneficial not only with regard to the organoleptic qualities of the product but also for significantly reducing the loss of vitamins by irradiation (Thomas & Josephson, 1970; Thomas & Wierbicki, 1971). Because

Table 1. Minimum required irradiation dose (MRD) for sterilization (Mrad)[1].

Food	Irradiation temperature (°C)	MRD
Bacon	5 to 25	2.9
Beef[2]	−30 ± 10	4.7
Beef[2]	−80 ± 10	5.7
Chicken[2]	−30 ± 10	4.5
Ham	5 to 25	2.9
Ham[2]	−30 ± 10	3.7
Pork	5 to 25	4.6
Pork[2]	−30 ± 10	5.1
Schrimp[2]	−30 ± 10	3.7
Codfosh cakes	−30 ± 10	3.2
Corned Beef	−30 ± 10	2.4
Pork sausage	−30 ± 10	2.7

1. 12D dose.
2. Preliminary data, Microbiol. Div., Fd Lab., NLABS.

Table 2. Chemical composition of non-irradiated smoked ham. Experiment 68/101: ground whole hams; average of duplicates

Lot No.[1]	Anal. Lab[2].	H_2O (%)	Protein (%)	Fat (%)	NaCl (%)	Ash (%)	Sugar (%)	Free nitrite mg/kg	Total P (%)	pH
I	Ind.	67.1	18.8	9.8	2.3	.	.	60	0.33	6.3
		66.8	20.1	9.8	2.0	.	.	80	0.35	6.5
	NLab.	67.1	19.2	7.9	2.2	4.6	0.78	60	0.35	6.6
		66.8	18.4	8.4	2.3	4.7	0.78	45	0.35	6.6
	Ave.	67.0	19.1	8.7	2.2	4.6	0.78	61	0.35	6.5
II	Ind	68.1	18.6	9.8	2.2	.	.	77	0.34	6.4
		65.4	21.0	9.9	2.2	.	.	33	0.35	6.0
	NLab.	64.9	18.9	11.6	1.9	4.0	0.24	3	0.34	6.4
		68.0	19.0	7.2	2.7	4.8	0.19	28	0.37	6.5
	Ave.	66.6	19.4	9.6	2.2	4.4	0.22	35	0.35	6.3
III	Ind.	69.6	17.4	7.8	2.6	.	.	10	0.37	6.2
		69.5	20.3	6.4	2.1	.	.	14	0.35	6.4
	NLab	65.4	19.2	11.4	1.8	3.9	0.22	3	0.33	6.4
		68.9	19.7	7.2	2.6	4.8	0.17	15	0.37	6.4
	Ave.	68.4	19.2	8.2	2.3	4.4	0.20	11	0.36	6.4

1. Lot I = Sugar (sucrose) cure; Lot II = No-sugar cure; Lot III = No-sugar + ascorbate.
2. Ind. = meat packing plant; NLab. = US Army Natick, Lab.

of the high organoleptic and nutritional quality of radappertized ham in the frozen state and because it does not require refrigeration during transportation and storage, the military expressed an interest as long ago as 1966 in using 7.1 mil. kg. radappertized ham per year, provided approvals from the U.S. Food and Drug Administration (FDA) and the US Department of Agriculture (USDA) could be obtained (Anonymous, 1966). Under the Food, Drug and Cosmetic Act of 1958, radappertized ham, like other irradiated foods, requires FDA's approval prior to umlimited human consumption. This requirement was the impetus for the intensive wholesomeness studies of radappertized meats.

In an initial study on the need of sugar in the cure, it was shown that sugar can be eliminated without affecting product quality of either irradiated or non-irradiated hams. In reviewing the data, it is interesting to note that our studies in 1968 showed that less residual nitrite is present in the ham cured without sugar than in the sugar-cured ham, and that further reduction of the residual nitrite can be obtained by adding sodium ascorbate (550 mg/kg) to the non-sugar cured ham (Table 2). The data reported in Table 2 were obtained on the ham with normally added nitrite (156 mg/kg) and nitrate (700 mg/kg).

In a succeeding study the objective was to investigate the addition of sodium chloride to ham during curing. Analysis of various commercial hams showed that the salt content ranges from 2 to over 6%. Deboned, rolled, smoked ham, with no 'added substance'[1] was procured from two meat packers, both using the same

1. 'Added substance' = (moisture + salt) – (protein × 3.79).

Table 3. Quality variability among and between smoked hams. Least significant difference test.

Experimental ham	Treatment	n	Ham No. I	II	III	IV	Radiation effect
68/98:	irradiated[1]	40	6.1[2]	5.9	6.1	6.4	6.1
Packer A,	non-Irrad	40	6.7	6.6	6.6	6.9	6.7[2]
2.5% salt	ham effect		6.4	6.2	6.3	6.6	
68/99:	irradiated[1]	40	5.5	5.2	5.5	.	5.4
Packer B,	non-Irrad.	40	6.3[3]	6.1[4]	5.3	.	5.9
5.5% salt	ham effect		5.9[4]	5.6	5.4		

1. 3.7–4.7 Mrad at −30 ± 10 °C.
2. Significantly preferred to irradiated ham.
3. Significantly preferred to samples rating <5.6.
4. Significantly preferred to samples rating <5.4.

additions of the curing ingredients except for salt. Both the irradiated and nonirradiated hams from the two procurements were evaluated by consumer-type panels and the data analyzed for acceptance. Table 3 shows the results. The responses confirm the fact that consumers prefer low-salt ham. As a result of this confirmation, we standardized the salt addition to radappertized ham at a level of 2.2 ± 0.3% in the finished product. The concentration of salt added to ham in relation to nitrite is an important factor in the control of the growth of *Cl. botulinum* (Duncan & Foster, 1968; Pivnick & Thacker, 1970) as well as for establishing the MRD required for radappertization (Rowley et al., 1968).

A series of acceptance tests were run on the non-sugar cured ham with the normal additions of nitrite (156 mg/kg), nitrate (500 to 700 mg/kg), and sodium chloride content of 2.2 ± 0.3%, radappertized in the close range of 3.7 – 4.7 Mrad

Table 4. Acceptance of 12D[1] radappertized ham. (Hams stored at room temp. for 1 to 12 months prior to serving; testing period: Oct. 1971-July 1973).

Experiment no.	Recipes	Number of men rating	Average acceptance rating
71/90	grilled	16	8.06
71/123	baked	16	7.46
71/123	baked	19	7.26
71/123	baked	22	7.40
72/80[2]	baked	17	7.71
72/80[2]	baked	45	7.06
72/80[2]	baked	17	7.85
72/101	baked	25	6.60
72/101	baked	24	7.08
73/17	baked	22	7.40

1. 3.7–4.3 Mrad at −30 ± 10 °C.
2. Apollo 17 ham.

at $-30 \pm 10^{\circ}$C. Very high acceptance ratings were given to this product. The 9-point hedonic scale devised by Peryam & Pilgrim (1957) was used in the evaluation. Table 4 presents representative results.

It is of more than passing interest that the ham from the Experiment No. 72/80 (Table 4) was used at the request of the National Aeronautics and Space Administration (NASA) by the astronauts of the Apollo 17 flight to the moon in December 1972. The ham slices, 12mm in thickness and weighing approximately 105 ± 5 g, were eaten at three meals in sandwiches made with radurized bread (50 000 rads) using radiation insect-disinfested rye flour (50 000 rads). The verdict of the astronauts was most encouraging. They reported: 'the juicy, chewy (irradiated ham and cheese on (irradiated) rye was one of the space culinary delights enjoyed by the Apollo 17 astronauts.' (Anonymous, 1973a).

The use of nitrate, nitrite and ascorbate/ erythorbate in cured meats

The use of nitrite, nitrate, ascorbate and erythorbate in cured meats is authorized by the US Meat Inspection Regulations (Anonymous, 1970b), the specific stipulation being: Nitrite, direct addition of 156 mg/kg, 'to fix cólor'; nitrate, as a 'source of nitrite'. When used in combination with nitrite, nitrate addition 'shall not result in more than 200 mg/kg nitrite in finished product'. Ascorbate and erythorbate, 'to accelerate color fixing or preserve color during storage.'

In 1969, the FDA, the meat industry and the USDA began to reappraise the use of nitrites and nitrates as the curing agents for meats, poultry and fish. The results of a number of investigations indicated that under certain conditions nitrites may react with free amines of foods or during digestion of cured meats in the stomach to form carcinogenic compounds known generally as nitrosamines (Lijinsky & Epstein, 1970; Wasserman et al., 1972; Wolff & Wasserman, 1972; Friedman, 1973). A government-industry working group was thereupon organized to study the safety of nitrites and nitrates in foods, the first meeting being held on January 21, 1970 (Friedman, 1973). Since that time, much information has been generated through coordinated government-industry research efforts.

In March 1972, Greenberg reported that sodium nitrite definitely inhibits toxin production by *Cl. botulinum* in perishable canned cured ham and that the level of nitrite at the time of product manufacture rather than the residual nitrite concentration is the key protective factor. In the same study it was shown that at a low inoculum of *Cl. botulinum* the allowable amount of sodium nitrite (200 mg/kg) is required to inhibit the toxin formation and that sodium nitrate did not demonstrate anti-botulinal activity (Greenberg, 1972; Christiansen et al., 1973). Similar results were reported in March, 1973, for wieners (Bard, 1973) and bacon (Herring, 1973).

Nitrosamines can be formed by the reaction of secondary or tertiary amines with free nitrite at a proper pH. The rate of the nitrosamine formation from secondary amines is directly related to the free nitrite concentration in the second power (Tannenbaum & Fan, 1973). This fact emphasizes the importance of reducing insofar as possible the free nitrite in cured meats. No nitrosamines have been found in canned cured ham or in wieners. Nitrosopyrrolidine was found in bacon after preparation for serving. Pan fried bacon showed the greatest amount and micro-

wave-cooked bacon the smallest amount of this nitrosamine. The use of ascorbate in the bacon cure showed some promise for the reduction of the formation of nitrosopyrrolidine during frying of bacon (Herring, 1973). Fiddler et al. (1973) have shown that the commonly used reductants (ascorbic acid, sodium ascorbate and sodium erythorbate) were effective in reducing the formation of *N*-nitrosodimethylamine in experimental (model) frankfurters; the inhibitory effect of the three reductants was practically the same. Studies have also shown that nitrite is important in the production of the characteristic flavor of cured meats (Bailey & Swain, 1973; Herring, 1973; Wasserman & Talley, 1972).

The amount of nitrite used in processing cured meat products exceeds the quantity needed for the formation of cured meats color nitrosomyoglobin, or its heat denatured form nitrosomyochrome. The surplus of nitrite is needed for the inhibition of the growth and toxin formulation by *Cl. botulinum*. The radappertization process destroys *Cl. botulinum* spores thus eliminating the need of nitrite in controlling botulism in radappertized cured meats. It is emphasized, however, that nitrite is needed for other purposes.

The series of experiments described in this paper was designed to determine the minimum amounts of nitrite and nitrate (in combination with ascorbate/erythorbate) needed for the characteristic color and flavor of the products while controlling *Cl. botulinum* by radappertization. The research was conducted on deboned, defatted, smoked ham.

In March 1972, samples of prototype radappertized hams were shown to meat industry personnel and to food distributors during a servey to learn whether the industry had a potential interest in food radappertization. Continuation of our efforts on research and development in this field (Josephson & Wierbicki, 1973) was encouraged.

Experimental

Raw material

Fresh, raw pork ham, shankless, weighing 6 to 8 kg was used. The fresh ham had not been frozen before processing. The salt was non-iodized, white refined sodium chloride, with or without anticaking agents. Food Grade sodium tripolyphosphate (TPP), sodium nitrite, sodium nitrate, sodium erythorbate and sodium ascorbate, conforming with the USDA requirements, were the curing agents.

Curing

Fresh, raw pork hams were skinned, deboned and all visible cartilage, ligaments, tendons, connective tissue, lymph glands and surface and internal fat were removed. The skinned, deboned, defatted hams were then sectioned into chunks 70 to 750 g and mixed in a food mixer with the curing brine. The brine was adjusted to a 15 percent level, i.e., 15 kg brine per 100 kg meat. The composition of the curing brines (pickles) for high and low nitrite/nitrate hams is given in Table 5. Deviations from this composition for individual experiments will be noted in the text. Meat and brine were mixed for 10 to 15 minutes to produce a tacky mixture. After

Table 5. Experimental curing pickles.

Composition	Curing pickles			Addition to ham[1]		
	1		2	1		2
Water	25	(kg)	25	0		0
NaCl	4	(kg)	4	2.4	(%)	2.4
TPP	750	(g)	750	0.45	(%)	0.45
Na-ascorbate	54	(g)	54	275	(mg/kg)	275
Na-erythorbate	54	(g)	54	275	(mg/kg)	275
$NaNO_2$	29.4	(g)	4.9	150	(mg/kg)	25
$NaNO_3$	117.6	(g)	9.8	600	(mg/kg)	50

1. Based on 15% pickle additions and 100% smokehouse yield-to-green ham.

mixing, the mixture was stuffed into drilled, prestuck, fibrous, easy-to-peel casings of a size to fit the primary container used for packaging the finished product. After stuffing, the casings were tied (or clipped) under pressure to remove entrapped air. The hams, stuffed into casings, were refrigerated for at least two (2) hours, but not more than twenty-four (24) hours, at 2 °C to 3 °C before smokehouse processing. No. 6–½ Union Carbide casings were used for making ham rolls to fit No. 404 x 700 (10.80 x 17.78 cm) or 404 x 202 (10.80 x 5.40 cm) metal cans, (ham experiments 70/86 and 72/111). No. 11 Union Carbide casings were used to make rectangular hams. The casings, loosely stuffed, were tightly packed into steel wire cages 9 x 13 x 75 cm in size. The rectangular hams were cut into 12 mm thick slices weighing 115 ± 5 g, for packaging into the flexible pouches.

Smokehouse processing

The cured raw product, stuffed into the casings, was processed in the smokehouse in accordance with the following schedule:

Time	Dry bulb temperature	Wet bulb temperature
1 hour without smoke	65 °C	49 °C
2 hours with smoke	65 °C	49 °C
5 hours with smoke	77 °C	57 °C
2 hours with smoke	82 °C	65 °C

Cooking continued without smoke, until the internal temperature of the ham was 68 °C, and then continued at a dry bulb temperature of 77 °C without steam until the weight of the finished product was in the range of 92 to 100 percent of the weight of the raw hams prior to curing. After cooking, the hams were chilled to 5 °C or less (internal temperature) within 12 hours, and then kept in a 1 to 5 °C refrigerator until cut and packaged.

Packaging (cutting)

Ham rolls were removed from their casings and cut into sections to fit the containers. The product was packaged either in beaded metals cans coated inside with enamel conforming to the provisions of the Federal Food Drug and Cosmetic Act, 21CFR121.2514, Resinous and Polymeric Coatings, specifically designated as the epoxy-phenolic type either V21 (American Can Co.) or 221 ALN (Continental Can Co.), or in flexible pouches with outside dimensions of 11.5 x 17.8 cm. Pouches were constructed from a multiple layer laminate material: the outside layer was 0.0025 cm polyiminocaproyl (Nylon 6), the middle layer 0.009 cm 1145 alloy aluminum foil, and the inside layer chemically bonded laminate of 0.001 cm polyethylene terephthalate and 0.006 cm medium density polyethylene, such as Scotchpak 9 or equivalent, with the polyethylene as the food contactant. Details regarding packaging of radappertized foods are available in a recent review paper by Killoran (1972). The filled cans or flexible pouches were sealed under a vacuum of not less than 25 inches gauge (not more than 125 mm Hg). Some samples (see 'Results and Discussion') were dipped (and immediately removed) in an aqueous solution containing 2.5 or 5% ascorbic and 2.5 or 5% citric acid prior to vacuum sealing in flexible pouches.

Irradiation and post-irradiation storage

Prior to irradiation, the ham samples were chilled to a temperature of $-40\ ^{\circ}C$ in the center of the hams. The irradiation was performed at the NLABS (U. S. Army Natick Laboratories) either in the 1 250 000 curie cobalt–60 isotope source (dose rate about 30 000 rad/min) or in the 10 Mev Linear accelerator (dose rate about 10^8 rads/sec). The product temperature during irradiation with cobalt–60 was $-30 \pm 10^{\circ}C$; during electron irradiation, the product temperature range was -40 to $-10\ ^{\circ}C$. The minimum dose received was 3.7 Mrad. The dose range, depending on the experiment, was from 3.7 – 4.2 to 3.7 – 4.7 Mrad. Before evaluation, the irradiated samples were stored at ambient temperature ($25 \pm 3\ ^{\circ}C$) and the non-irradiated controls in the $-29\ ^{\circ}C$ freezer.

Evaluation

Consumer-type panel Consumer-type tests were performed by the Food Acceptance Group, Behavioral Sciences Division, Pioneering Research Laboratory, NLABS. Selected samples were tested for preference using the 9-point hedonic scale (Peryam and Pilgrim, 1957) in which 9 = *like extremely* and 1 = *dislike extremely*. For ham and other meat products, a score of 5 (neither like nor dislike) indicates marginal acceptability, ratings of 6 to 7 indicate an acceptable product and ratings above 7, a highly acceptable product. The test data were statistically analyzed using analysis of variance and least significant differences (LSD) between the means.

Technological panel Some samples were sensory tested by an 8 to 12 member trained panel for preference, using the 9–point hedonic scale, and for color, odor and flavor, by means of a 9–points quality scale (See Table 6). Most ham samples were

Table 6. Technological examination for color, odor, flavor and texture.

Scale	Description
9	excellent
8	very good
7	good
6	below good above fair
5	fair
4	below fair above poor
3	poor
2	very poor
1	extremely poor

tested cold. Frozen samples were defrosted in a 3 to 5 °C refrigerator prior to serving. All irradiated samples were tested for the absence of *Cl. botulinum* toxin prior to sensory testing. If to be served hot, 12-mm thick ham slices were held in a 163 °C preheated electric oven until an internal product temperature of 63 to 65 °C was reached.

Color evaluation Most of the samples were visually evaluated for cured meat color only. Evaluations were usually made on both cold and hot samples. The 12–mm thick ham slices, vacuum sealed in flexible pouches, were held for 15 minutes in a 150 °C preheated oven. After heating, the samples were held at room temperature for 15 minutes before opening the pouches; they were then placed on white plates for evaluation. Evaluation was made at '0-Time' (within 5 to 20 minutes after removal of the ham slices from the packaging) and after 2 hours' exposure to light (room temperature 23 to 28 °C). The ham slices were wrapped in 0.0006 cm polyvinyl film, transparant to light, to prevent discoloration of the ham slices due to surface dehydration during the 2–hour exposure. The polyvinyl wrap was removed for color rating of the ham samples. In this series of evaluations for cured meat color, reference ham samples were shown to the panelists prior to the color rating by experts. The reference samples were of excellent color with assigned ratings of 8 or 9 (Table 6) by experts. The data obtained were statistically analyzed by the NLABS Data Analysis Office using Analysis of Variance and the Student's Range test.

Chemical analyses Ham samples were chemically analyzed for:
1. moisture, fat, protein, salt (NaCl), ash and total phosphate (AOAC Official Methods, 1970a);
2. residual sodium nitrite and sodium nitrate using ether extracted defatted samples (AOAC Official Method, 1970a);
3. thiobarbituric acid (TBA) values were determined by the method of Tarladgis et al. (1960);

Nitrosamines determination Samples from two ham experiments (hams 72/111 and 73/09) were analyzed for the following six nitrosamines:

dimethylnitrosamine (DMNA)
methylethylnitrosamine (MENA)
diethylnitrosamine (DENA)
nitrosomorpholine (NO-Mor)
nitrosopyrrolidine (NO-Pyr)
nitrosopiperidine (NO-Pip)

The analyses were performed at the USDA Eastern Regional Research Laboratory in Philadelphia, Pennsylvania, through the cooperation of Dr Fiddler and Wasserman.

Results and discussion

Ham 70/86 experiment

Various levels of nitrite, ranging from 10 to 200 mg/kg, were investigated, the objective being to ascertain the acceptance of the end product by the consumer-type panel. Four determinations were made; the first, 10 days after the ham processing, and the fourth, after nine months' storage. Results of these tests are given in Table 7. In this experiment, 350 mg/kg sodium nitrate and the maximum allowable amounts (550 mg/kg) of ascorbate (as 1 : 1 mixture of sodium ascorbate/sodium erythorbate) were used. As the data indicate, there are no significant differences in the preference ratings given the irradiated ham regardless of the nitrite added to the ham during curing. The non-irradiated samples received higher preference ratings, but significant differences for the same nitrite level (200 mg/kg were found only between the samples stored for one month before testing. Tests for residual nitrites (Table 8) revealed a rapid depletion in both irradiated and

Table 7. Ham 70/86: effect of the added nitrites on preference. (3.7–4.4 Mrad at −30 ± 10 °C). The numbers in brackets refer to the amount of $NaNO_2$ added to the hams (mg/kg).

Time at 21–25 °C	Number of panelists	Average preference scores[1]					LSD[3]
		B (10)	C (25)	D (50)	E (200)	F[2] (200)	
10 days	35	6.9	6.9	6.7	6.7	7.2	0.8
1 month	35	6.3	6.2	6.3	5.9	7.4	1.4
4 months	35	5.6	5.6	5.8	5.7	7.1	1.5
9 months	32	7.1	6.4	6.5	6.2	–	0.8

1. 9 = like extremely; 1 = dislike extremely
2. Non-irradiated control stored at −29 °C
3. Least significant difference.

Table 8. Ham 70/86: effect of the added nitrites on the residual nitrites. (3.7–4.4 Mrad at −30 ± 10 °C). The numbers in brackets refer to the amount of $NaNO_2$ added to the hams (mg/kg).

Time at 21°C	Residual nitrites in ham (mg/kg)					
	(0)	(10)	(25)	(50)	(200)	(200)[1]
10 days	4	5	4	8	12	34
1 month	3	3	2	2	3	17
4 months	–	2	2	2	5	–
9 months	–	3	4	4	6	–

1. Non-irradiated control stored at −29 °C.

non-irradiated samples. The ham sample without nitrite added showed 3–4 mg/kg residual nitrite (Table 8), the same amount as that found for the irradiated ham samples with added 10 to 200 mg/kg after one month of storage. This 3–4 mg/kg analytical nitrite is apparently the base (zero) line for the accuracy of the analytical methodology used. Even though the consumer-type panel found the ham samples with 10 mg/kg nitrite equally acceptable to 200 mg/kg, examination by expert technologists of the 10 mg/kg nitrite ham revealed some non-cured spots and a metallic flavor was detected.

Ham 72/108 experiment

In this experiment, various combinations of nitrite and nitrate were used to study their effects on the color of the ham. Ascorbate/erythorbate (550 mg/kg) level was kept constant. Table 9 gives the results. There were no significant differences in the color of the samples with the exceptions of: (a) non-irradiated

Table 9. Ham 72/108: effect of nitrite and nitrate on color[1]. (Technological panel: n = 10).

mg/kg added		Non-irradiated		Irradiated[2]	
$NaNO_2$	$NaNO_3$	cold	hot	cold	hot[3]
150	500	7.7 ± 0.7	7.1 ± 0.6	7.4 ± 0.5	7.4 ± 0.7
150	100	7.5 ± 1.0	7.3 ± 0.5	6.9 ± 0.9	6.6 ± 1.0
15	50	7.6 ± 0.8	7.3 ± 0.5	7.1 ± 0.8	6.1 ± 1.0
15	100	7.7 ± 0.7	7.0 ± 0.8	7.2 ± 0.6	6.6 ± 1.1
25	50	7.7 ± 0.7	6.7 ± 0.7	7.0 ± 0.9	6.1 ± 1.1
25	100	7.8 ± 0.6	6.9 ± 0.3	7.1 ± 1.0	5.2 ± 1.5
50	50	7.8 ± 0.6	7.5 ± 0.7	7.1 ± 0.7	6.8 ± 0.9
50	100	7.8 ± 0.8	7.2 ± 0.6	7.4 ± 0.7	6.8 ± 0.9

1. Determined 5 to 20 min after pouch opening.
2. 3.7–4.4 Mrad at −30 ± 10 °C.
3. Heated in sealed pouches for 15 min at 150 °C.

Table 10. Ham 72/108: Effect of nitrite and nitrate on color. (Irradiated: 3.7–4.4 Mrad at −30 ± 10 °C.

mg/kg added		n	Cold		Hot[1]	
$NaNO_2$	$NaNO_3$		0[2]	2[3]	0	2
25	100	3x11	7.2 ± 0.7	6.4 ± 1.2	7.2 ± 0.4	6.8 ± 0.9
25	50	3x11	7.1 ± 1.0	6.2 ± 1.2	7.2 ± 0.7	6.7 ± 0.7
15	50	2x11	6.7 ± 1.0	5.8 ± 1.1	7.2 ± 1.0	6.4 ± 0.7

1. Heated in sealed pouches for 15 min at 150 °C.
2. 0 = determined 5 to 20 min after pouch opening.
3. 2 = determined after 2 hours exposure to light at 23 to 28 °C.

cold vs. hot 25/50 (nitrite/nitrate) and 25/100 samples and, (b) the irradiated cold vs. hot 15/50, 25/50 and 25/100 samples. Therefore, these three irradiated samples were subjected to additional evaluations for color involving exposure to light (at '0'-time and after 2 hours). Table 10 gives the results. The data indicate that exposure to light has a significant effect on the color scores of these samples. The 15/50 nitrite/nitrate samples were scored significantly lower when evaluated cold. It was noted that the 15/50 samples had under-cured spots in some cases and a slight metallic flavor when analyzed closely by the expert panel. Based on this experiment, it was concluded that not less than 25 mg/kg should be used in curing radappertized ham.

Ham 73/29 experiment

In this experiment, attempts were made to eliminate the addition of nitrate to the low nitrite (25 mg/kg) ham (Cure 1) by varying the addition of ascorbate/erythorbate in the cure (Cures 2, 3, 4 and 5). The data (Table 11) were statistically analyzed and showed the following: (a) Cure 1 ham (with nitrate) received significantly higher color ratings than the other four cures (cures without nitrate); (b) no significant differences were found among the four no-nitrate samples which contained either no ascorbate/erythorbate or varying amounts from 250 to 1000 mg/kg; (c) the irradiation and the exposure to light under the extreme conditions used in this experiment (2 hours at 23 to 28 °C room temperature) significantly decreased the color of the ham samples, particularly in the cures without nitrate; (d) dipping the ham slices into an aqueous solution containing 5% ascorbic and 5% citric acid or evaluating the ham samples cold or hot had no significant effect on the ham color. It was particularly surprising not to find any effect on color when ham slices were dipped into the ascorbic/citric acid solution prior to vacuum packaging. USDA Meat Inspection Regulations, permitting the use of citric acid or sodium citrate for just this purpose, read: 'in cured products or in 10 percent solutions to spray surfaces of cured cuts prior to packaging to replace up to 50 percent of the ascorbic acid, erythorbic acid' or their sodium salts (Anonymous, 1970b). With this experiment, we have shown the need for nitrate, in association with nitrite, in the cures of radappertized ham in order to obtain the typical color of the product.

Table 11. Ham 73/29: Effect of ascorbate/erythorbate without nitrate on color. (Technological panel: n = 12).

	Cure no.				
	1 (25 NO_2 500 A/E 100 NO_3)[1]	2 (25 NO_2 0 A/E)	3 (25 NO_2 250 A/E)	4 (25 NO_2 500 A/E)	5 (25 NO_2 1000 A/E)
Irradiated[2]	6.5 ± 1.4	4.8 ± 1.5	5.1 ± 1.6	4.9 ± 1.5	5.2 ± 1.5
Non-irradiated	7.0 ± 1.1	6.7 ± 1.1	6.6 ± 1.2	6.9 ± 1.1	6.4 ± 1.1
Non-dipped	7.2 ± 1.0	5.6 ± 1.8	5.6 ± 1.6	5.8 ± 1.6	6.0 ± 1.5
Dipped[3]	6.4 ± 1.3	5.9 ± 1.4	6.1 ± 1.5	6.1 ± 1.6	5.6 ± 1.4
Evaluated cold	6.8 ± 1.4	5.8 ± 1.9	5.9 ± 1.8	5.9 ± 1.9	5.9 ± 1.6
Evaluated hot	6.8 ± 1.0	5.7 ± 1.4	5.8 ± 1.3	5.9 ± 1.3	5.8 ± 1.2
0-h exposure	7.2 ± 1.0	6.1 ± 1.5	6.2 ± 1.4	6.3 ± 1.5	6.0 ± 1.5
2-h exposure	6.3 ± 1.3	5.3 ± 1.7	5.4 ± 1.6	5.5 ± 1.6	5.7 ± 1.4
All treatments	6.8 ± 1.2	5.7 ± 1.6	5.8 ± 1.6	5.9 ± 1.6	5.8 ± 1.5

1. Additions (mg/kg) during curing of $NaNO_2$, ascorbate/erythorbate and $NaNO_3$.
2. 3.7–4.4 Mrad at −30 ± 10 °C.
3. See text.

Ham 73/39 experiment

In this experiment an attempt was made to show whether or not ascorbate/erythorbate can be eliminated from the nitrite/nitrate cures for radappertized ham. We used high (normal) nitrite (150 mg/kg)/nitrate (600 mg/kg) cure and low nitrite (25 mg/kg)/nitrate (50 mg/kg) cure as the reference (Table 12, Cures No. 1 and 2). The remaining three cures omitted either ascorbate/erythorbate or nitrate, or both.

Table 12. Ham 73/39: Effect of nitrite plus nitrate on color. (Technological Panel: n=12).

	Cure No.				
	1 (150 NO_2 600 NO_3 550 A/E)[1]	2 (25 NO_2 50 NO_3 550 A/E)	3 (25 NO_2 50 NO_3 0 A/E)	4 (25 NO_2 0 NO_3 550 A/E)	5 (25 NO_2 0 NO_3 0 A/E)
Irradiated[2]	7.5 ± 1.0	6.4 ± 1.3	5.6 ± 1.6	4.8 ± 1.8	4.3 ± 2.0
Non-irradiated	7.6 ± 1.1	6.9 ± 1.5	7.0 ± 1.3	7.5 ± 1.0	6.2 ± 1.8
Evaluated cold	7.9 ± 0.9	6.8 ± 1.5	6.3 ± 1.8	6.4 ± 2.1	5.9 ± 2.1
Evaluated hot	7.2 ± 1.1	6.6 ± 1.4	6.3 ± 1.4	5.9 ± 1.9	4.5 ± 1.9
0-h exposure	7.9 ± 1.0	7.2 ± 1.4	7.2 ± 1.2	6.8 ± 1.7	6.0 ± 2.0
2-h exposure	7.2 ± 1.0	6.2 ± 1.3	5.4 ± 1.5	5.5 ± 2.0	4.5 ± 2.0
All Treatments	7.6 ± 1.1	6.7 ± 1.4	6.3 ± 1.6	6.2 ± 2.0	5.2 ± 2.1

1. Additions (mg/kg) during curing of $NaNO_2$, ascorbate/erythorbate and $NaNO_3$.
2. 3.7–4.4 Mrad at −30 ± 10 °C.

The statistical evaluation of the data (Table 12) showed the following: (a) ham cured with Cure No. 1 had the best color; (b) the next best was Cure No. 2; (c) Cure No. 2 ham (containing both 50 mg/kg nitrate and 550 mg/kg ascorbate/erythorbate) had significantly better color than the No. 4 or 5 cure hams, neither containing nitrate; (d) Cure No. 2 ham, containing both nitrate and ascorbate/erythorbate suffered less discoloration by irradiation and the exposure to light than Cure No. 3 ham, which contained nitrate without ascorbate/erythorbate; (e) Cure No. 5 ham which omitted both nitrate and ascorbate had the poorest color. This experiment confirmed our previous overall observations that ascorbate/erythorbate is needed in association with nitrate and nitrite to achieve a radappertized ham of acceptable color.

Ham 73/36 experiment

In this experiment, five different cures of hams were evaluated by a 12-member panel not only for color but also for flavor, odor and preference. All hams received 25 mg/kg sodium nitrite during the curing process. The variables were: 100 and 50 mg/kg nitrate (Cures No. I and II); sodium citrate substituted for sodium erythorbate in the ascorbate/erythorbate mixture (Cures III and IV); and, sodium pyrophosphate and sodium acid orthophosphate substituted for TPP (Cure No. V) in equivalent amounts. Results of this investigation are given in Tables 13 and 14. In all instances, irradiated samples scored significantly lower than the nonirradiated controls. The effect of the individual cures differed, depending on the quality attribute of the ham. Color evaluations showed no significant difference between the Cures I and II (100 and 50 mg/kg nitrate). However, the color of the ham cured with Cure No. I significantly differed from the color of the hams cured with Cures

Table 13. Ham 73/36: Effect of various cures on flavor. (Constants: 25 mg/kg $NaNO_2$ added; technological panel: n=12). Served without previous exposure to light.

	Cure No.				
	I (P-1[1] 275 – Asc. 275 – Eryth. 100 – NO_3)[2]	II (P-1 275 – Asc. 275 – Eryth. 50 – NO_3)	III (P-1 275 – Asc. 275 – Cit. 50 – NO_3)	IV (P-1 275 – Asc. 275 – Cit. 0 – NO_3)	V (P-2[1] 275 – Asc. 275 – Eryth. 50 – NO_3)
Irradiated	6.4 ± 1.3	5.9 ± 1.5	5.8 ± 1.5	5.8 ± 1.5	5.9 ± 1.5
Non-irradiated	7.4 ± 0.8	7.3 ± 0.8	7.2 ± 0.8	7.2 ± 0.9	7.3 ± 1.1
Non-dipped	6.9 ± 1.2	6.4 ± 1.4	6.5 ± 1.4	6.4 ± 1.4	6.6 ± 1.5
Dipped	7.0 ± 1.1	6.8 ± 1.3	6.5 ± 1.4	6.6 ± 1.4	6.6 ± 1.5
Served cold	6.9 ± 1.4	6.3 ± 1.7	6.4 ± 1.7	6.5 ± 1.4	6.4 ± 1.5
Served hot	7.0 ± 1.0	6.9 ± 0.9	6.6 ± 1.0	6.5 ± 1.4	6.8 ± 1.5
All treatments	6.9 ± 1.2	6.6 ± 1.4	6.5 ± 1.4	6.5 ± 1.4	6.6 ± 1.5

1. P-1= 0.45% TPP added; P-2 = equivalent Na-Pyro P+NaH_2PO_4 added.
2. mg/kg added: Asc. = sodium ascorbate; Eryth. = sodium erythorbate; NO_3 = $NaNO_3$; Cit. = sodium citrate.

Table 14. Ham 73/36: Effect of various cures on preference. (Constants: 25 mg/kg $NaNO_2$ added; technological panel: n=12) Served without previous exposure to light.

	Cure No.				
	I (P-1[1] 275 – Asc. 275 – Eryth. 100 – NO_3)[2]	II (P-1 275 – Asc. 275 – Eryth. 50 – NO_3)	III (P-1 275 – Asc. 275 – Cit. 50 – NO_3)	IV (P-1 275 – Asc. 275 – Cit. 0 – NO_3)	V (P-2[1] 275 – Asc. 275 – Eryth. 50 – NO_3)
Irradiated	6.4 ± 1.2	5.9 ± 1.6	5.9 ± 1.6	5.7 ± 1.6	6.0 ± 1.4
Non-irradiated	7.2 ± 0.9	7.0 ± 1.0	7.1 ± 0.8	7.0 ± 1.2	7.1 ± 1.4
Non-dipped	6.8 ± 1.2	6.4 ± 1.5	6.6 ± 1.3	6.4 ± 1.6	6.5 ± 1.5
Dipped	6.9 ± 1.1	6.5 ± 1.3	6.4 ± 1.5	6.3 ± 1.5	6.6 ± 1.5
Served cold	6.7 ± 1.3	6.2 ± 1.7	6.3 ± 1.8	6.3 ± 1.6	6.4 ± 1.6
Served hot	6.9 ± 0.9	6.7 ± 1.0	6.7 ± 0.8	6.4 ± 1.6	6.7 ± 1.4
All Treatments	6.8 ± 1.1	6.5 ± 1.4	6.5 ± 1.4	6.3 ± 1.6	6.5 ± 1.5

1. P-1 = 0.45% TPP added; P-2 = equivalents Na-Pyro P + NaH_2PO_4 added.
2. mg/kg added: Asc. = sodium ascorbate; Eryth. = sodium erythorbate; NO_3 = $NaNO_3$; Cit. = sodium citrate.

No. III, IV and V. Previous observations were confirmed; namely: (a) color scores of the hams were significantly lower after 2-hour exposure to light; (b) there was no effect of dipping the ham slices into ascorbic/citric acid solution (this time 2.5% concentration of each acid was used) prior to packaging; (c) evaluating cold vs. hot did not affect the color scores; and (d) the ham cured without nitrate (Cure No. IV) suffered most in the loss of color by the irradiation and exposure to light.

There were no significant differences in flavor (Table 13), odor and preference (Table 14) of the hams due to differences in the curing pickles. However, in all quality attributes the hams cured with Cure No. I (25 mg/kg nitrite and 100 mg/kg nitrate) received slightly higher scores, and the effect of radiation on the scores was less pronounced than that of hams of the other four cures. The overall results have shown that: (a) the best low nitrite/nitrate cure for radappertized ham is Cure No. I; (b) there is no advantage in replacing erythorbate with citrate in the ascorbate/erythorbate mixture (Cure II vs III); and (c) there is no advantage in replacing TPP with sodium pyrophosphate and sodium acid phosphate (Cure II vs V).

Ham 73/30 experiment

The main objective of this experiment was to show the effect of nitrite and nitrate on the overall consumer preference for ham as well as the effect on flavor and odor characteristics of the product. There were five groups of ham samples, each processed with different curing pickles. All cures contained the same concentrations of salt, TPP, and ascorbate/erythorbate (Table 5). The cures varied in the nitrite/nitrate levels added to the ham during curing. They were as follows: 150/600, 25/100, 25/0, 0/100, and 0/0 mg/kg for cures, A, B, C, D, and E, respectively. There were four separate experiments for the five groups. In each

Table 15. Ham 73/30: Effect of nitrite and nitrate on preference. Consumer-type panel: n=35; samples (a)[1]

Cure	Variables (mg/kg)	Days of storage			
		1	7	14	21
A	150 NO_2 600 NO_3	6.8 ± 1.5	7.2 ± 1.3	7.0 ± 1.2	6.5 ± 1.5
B	25 NO_2 100 NO_3	6.1 ± 1.8	6.5 ± 1.8	6.2 ± 1.6	6.3 ± 1.8
C	25 NO_2 0 NO_3	6.1 ± 1.8	6.2 ± 1.8	6.0 ± 1.6	5.6 ± 1.9
D	0 NO_2 100 NO_3	4.5 ± 1.7	4.3 ± 2.1	4.2 ± 1.9	4.1 ± 2.5
E	0 NO_2 0 NO_3	4.2 ± 2.1	4.3 ± 2.0	4.1 ± 2.2	3.7 ± 2.1

1. Non-irradiated, no-vacuum, stored at 2 to 3°C.

experiment, hams were cut into 12 mm slices, each weighing 115 ± 5 g per slice, and packed in flexible pouches. The differences between the four experiments were as follows: (a) pouches unsealed (no vacuum), ham non-irradiated and stored at 2 to 3 °C for 1, 7, 14 and 21 days; (b): pouches unsealed, ham non-irradiated and frozen stored at −29 °C for 10, 30 and 90 days; (c): pouches vacuum sealed, ham irradiated (3.7 − 4.3 Mrad at −30 ± 10 °C and stored at 23 to 28 °C for 10 days and 3, 6 and 12 months; (d): pouches vacuum sealed and the ham frozen stored at −29 °C for 10 days and 3, 6 and 12 months (control samples for the sample used in

Table 16. Ham 73/30: Effect of nitrite and nitrate on preference. Consumer-type panel: n=35.

Cure	Variables (mg/kg)	Days of storage			
		10(b)[1]	30(b)[1]	10(c)[1]	10(d)[1]
A	150 NO_2 600 NO_3	7.3 ± 1.2	7.1 ± 1.0	6.4 ± 1.7	7.2 ± 1.2
B	25 NO_2 100 NO_3	7.2 ± 1.3	6.9 ± 1.1	6.3 ± 1.8	7.1 ± 1.6
C	25 NO_2 0 NO_3	6.8 ± 1.4	6.9 ± 1.4	6.0 ± 1.7	7.3 ± 1.6
D	0 NO_2 100 NO_3	5.5 ± 2.0	4.8 ± 1.8	5.8 ± 2.0	5.2 ± 2.0
E	0 NO_2 0 NO_3	5.2 ± 1.9	4.4 ± 1.7	5.5 ± 1.8	5.5 ± 1.5
LSD[2]:		0.7	0.7	0.7	0.7

1. (b): Non-irradiated, no-vacuum, stored at −29°C;
   (c): Irradiated, vacuum packed, stored at 23 to 28°C;
   (d): Non-irradiated, vacuum packed, stored at −29°C.
2. LSD: Least significant difference.

Table 17. Ham 73/39: Effect of nitrite and nitrate on odor and flavor. Technological panel: n=8; samples (a)[1].

Cure	Variables (mg/kg)	*Odor* days after processing				*Flavor* days after processing			
		1	7	14	21	1	7	14	21
A	150 NO_2 600 NO_3	7.4	7.3	6.6	7.5	7.6	7.3	6.5	7.4
B	25 NO_2 100 NO_3	7.0	6.6	6.3	6.8	7.1	6.4	6.0	6.0
C	25 NO_2 0 NO_3	6.6	6.5	6.8	5.4	6.6	6.1	7.0	4.9
D	0 NO_2 100 NO_3	5.3	5.3	4.5	4.9	4.6	3.6	3.6	4.0
E	0 NO_2 0 NO_3	5.4	4.3	5.1	4.8	5.1	3.5	3.8	3.9

1. Non-irradiated, no-vacuum, stored at 2 to 3°C.

(c) above). All evaluations were made on cold (3–5 °C) ham samples. As of this time, only samples (a) have been evaluated. The results are given in Tables 15–20. Consumer-type panel preference data are given in Tables 15 and 16. Similar data were obtained by the technological panel. Data clearly indicate the low quality of the hams cured without nitrite (Cures D and E). The differences were highly significant when compared with the ham with nitrite added (Cures A, B and C). In the samples (a) series (Table 15), the ham of Cure A was also significantly preferred to the hams of Cures B and C after 1, 7 and 14 days storage. After 21 days' storage,

Table 18. Ham 73/30: Effect of nitrite and nitrate on odor and flavor. Technological Panel: n=8; samples: (b), (c) and (d)[1].

Cure	Variables (mg/kg)	*Odor* days after processing				*Flavor* days after processing			
		10(b)	30(b)	10(c)	10(d)	10(b)	30(b)	10(c)	10(d)
A	150 NO_2 600 NO_3	7.3	7.3	6.6	7.0	7.4	7.4	6.5	6.6
B	25 NO_2 100 NO_3	7.5	7.1	6.4	6.8	7.5	7.1	6.4	6.6
C	25 NO_2 0 NO_3	6.6	7.4	5.9	7.0	6.9	7.1	5.3	6.8
D	0 NO_2 100 NO_3	5.1	5.9	6.0	6.1	4.6	4.9	5.9	4.6
E	0 NO_2 0 NO_3	5.1	5.3	5.6	5.6	4.9	6.1	5.0	4.6

1. (b): Non-irradiated, no-vacuum, stored at −29°C;
(c): Irradiated, vacuum packed, stored at 23 to 28°C;
(d): Non-irradiated, vacuum packed, stored at −29°C.

ham cured with 25 mg/kg nitrite without nitrate (Cure C) dropped significantly in preference. The first evaluations of samples (b), (c) and (d) are given in Table 16. Data confirm the need of nitrite to attain hams of high acceptance (Cures A, B and C vs D and E).

Tables 17 and 18 record the odor and flavor scores as determined by an 8-member technological panel using the scoring system given in Table 6. The data clearly indicate the need of nitrite for an acceptable odor and flavor (Cures A, B and C vs D and E). Lack of a typical odor and flavor as well as color were the main reasons given by the consumer-type panel for the low preference scores for hams of Cures D and E given in Tables 15 and 16. This response confirms the recently reported findings of Bailey and Swain (1973) that nitrite is needed for the flavor and taste of ham and other cured meats.

Odor and flavor scores for Cure C ham (25 mg/kg nitrite but no nitrate) were significantly lower when storage time increased from 14 to 21 days (Table 17). It appears that a small amount of nitrate (for example, 100 mg/kg, Cure B) might be needed for non-irradiated ham when the product is to be stored for an extended period of time. As shown previously, addition of 100 mg/kg nitrate to 25 mg/kg nitrite cure is needed to minimize the loss of the typical ham flavor and odor caused by irradiation (Table 18: samples (c) and (d), cures B and C).

Tables 19 and 20 show the effect of nitrite and nitrate on the TBA values (mg malonaldehyde per 1000 g). The significantly higher TBA values in the ham samples of Cures D and E stored in open pouches in refrigerator and the freezer (Table 19) clearly indicate the antioxidant effect of nitrite in ham; this is particularly observable in the samples stored for 7 days or longer. It is interesting that the ham cured with 25 mg/kg nitrite without nitrate (Cure C) showed a high TBA value after 21 days' storage (Table 19). This high value might be associated with the lower scores for odor and flavor (Table 17) and preference (Table 16) for the samples of the same group. Results suggest that a small amount of nitrate should remain in the cure for non-irradiated hams. Table 20 lists TBA values for irradiated and non-

Table 19. Ham 73/30: Effect of nitrite and nitrate on TBA. (a): non-irradiated, no-vacuum, stored at 2 to 3°C; (b): non-irradiated, no-vacuum, stored at −29°C.

Cure	Variables (mg/kg)	Days after processing				Days after processing	
		1	7	14	21	10	30
		samples (a)				*samples (b)*	
A	150-NO_2 600-NO_3	0.00	0.05	0.02	0.08	0.04	0.05
B	25-NO_2 100-NO_3	0.01	0.07	0.12	0.37	0.06	0.14
C	25-NO_2 0-NO_3	0.02	0.37	0.08	3.04	0.08	0.05
D	0-NO_2 100-NO_3	0.92	1.50	2.71	3.28	0.86	2.06
E	0-NO_2 0-NO_3	0.73	1.73	3.03	4.29	0.50	2.54

Table 20. Ham 73/30: Effect (duplicate samples) of nitrite and nitrate on TBA. (c): irradiated[1], vacuum packed, stored at 23 to 28°C for 10 days; (d): non-irradiated, vacuum packed, stored at −29°C for 10 days.

Cure	Variables (mg/kg)	(c)		(d)	
		1	2	1	2
A	150 NO_2 600 NO_3	0.26	0.26	0.06	0.05
B	25 NO_2 100 NO_3	0.23	0.23	0.12	0.07
C	25 NO_2 0 NO_3	0.21	0.21	0.06	0.09
D	0 NO_2 100 NO_3	0.23	0.25	0.29	0.31
E	0 NO_2 0 NO_3	0.38	0.40	0.33	0.30

1. 3.7–4.4 Mrad at −30 ± 10°C.

irradiated hams after 10 days of storage. Irradiation of hams with nitrite additions (Cures A, B and C) slightly increased the TBA values; however, the values are below the rancidity level. There was practically no effect of irradiation on the TBA values of the no nitrite hams (Cures D and E). These experiments are continuing and the final conclusion must await the end of the storage studies.

Ham 72/111 experiment

The main objective of this investigation was to determine the effect of adding nitrite and nitrate during curing on residual nitrite and nitrate in the finished product. The constant constituents of the cure for all hams were salt, TPP and ascorbate/erythorbate as given in Table 5. The variables were sodium nitrite and sodium nitrate. Five different combinations were used (Table 21). Proximate chemical analyses showed them to be in the range given for the hams in Table 2, except for pH, which was within the range of 6.0 to 6.2 for this experiment. The data in Table 21 indicate that no difference is apparent whether 25 or 150 mg/kg sodium nitrite are added to ham during curing since the residual nitrite approaches the analytical 'zero' line (0.9 to 1.9 mg/kg) after 10 days' storage. This finding confirms the results found for wieners (Bard, 1973). Non-irradiated frozen control ham cured with high nitrite/nitrate (Table 21, cure 01) had only 4.7 to 7.3 mg/kg free nitrite, thus indicating that the depletion of the added nitrite occurred mainly during the curing and smokehouse processing of the ham, probably by reacting with constituents of ham muscles other than myoglobin, as reported by Olsman and Krol (1972).

The amount of analytical sodium nitrate in the ham shows an interesting relationship to the addition of nitrite/nitrate to the hams during curing. Whereas the amount of the analytical (residual) nitrate in the high nitrate ham (Cure No. 01) is close to the amount added during curing in the low nitrite ham (Cure No. 02) and

Table 21. Ham 27/111: Added and residual nitrite and nitrate. (Averages of duplicate determinations).

Cure No.	mg/kg added		mg/kg residual			
	NO_2	NO_3	$NaNO_2$		$NaNO_3$	
			10	90	10	90[1]
	Irradiated[2]					
01[3]	150	600	2.4	2.2	477	515
02[3]	25	100	1.6	2.4	140	141
03	0	600	1.8	1.6	737	728
04	0	100	1.0	1.4	118	137
05	0	0	0.9	1.7	58	69
	Non-irradiated					
01[3]	150	600	4.1	7.3	572	666
02[3]	25	100	1.1	2.3	145	169
03	0	600	1.4	1.4	871	835
04	0	100	1.1	1.6	128	175
05	0	0	1.0	1.3	51	67

1. Days of storage: irradiated at 23 to 28°C, non-irradiated at −29°C.
2. 3.7–4.4 Mrad at −30 ± 10°C.
3. Samples used for nitrosamines determination.

no-nitrite hams (Cures No. 03 and 04), the amounts of the analytical nitrate is significantly greater than the amounts added during curing. Even the no-nitrate cured ham (Cure No. 05) contained 51 to 69 mg/kg analytical free nitrate. Bard (1973) reported similar results (3 to 47 mg/kg nitrate) in the wieners cured without nitrate. It might be an analytical error or it might be due to nitrate impurities in the water used for making the cures. This factor is being investigated.

Ham 73/09 experiments

The main objective of this experiment was to determine nitrosamines in high nitrite/nitrate (150/600 mg/kg) and low nitrite/nitrate (25/100 mg/kg) hams, irradiated by both the gamma rays from Cobalt–60 source and by electrons from the linear accelerator. The Cobalt–60 and electron irradiated samples were made from paired 12 mm ham slices, cut from the same ham rolls. The hams were of excellent quality as shown by the preference (acceptance) ratings in Table 22. It is of interest that in this experiment there were no significant differences between the preference ratings of high vs low nitrite/nitrate hams, but somewhat higher ratings were obtained for electron vs Cobalt–60 irradiated samples. There was no indication of the presence of the six nitrosamines in any of the samples of ham 72/111 and 73/09 analyzed.

Minimum radiation dose (MRD) for low nitrite/nitate ham

To complete the technology of low nitrite/nitrate ham the minimum radiation

Table 22. Ham 73/09: Acceptance of high and low nitrite irradiated Ham[1]. (Consumer-type panel: n=32).

Sample series No.	mg/kg added $NaNO_2$	mg/kg added $NaNO_3$	Irradiation Source	Acceptance ratings 10 days	Acceptance ratings 30 days
1	150[2]	600	Cobalt-60	6.8 ± 1.5	6.2 ± 1.7
2	25[2]	100	Cobalt-60	6.4 ± 1.5	6.0 ± 2.2
3	150	600	Electrons	6.5 ± 2.0	6.8 ± 1.4
4	25	100	Electrons	7.0 ± 1.2	6.8 ± 1.4
LSD (Least significant difference):				not significant	0.7

1. 3.7–4.4 Mrad at −30 ± 10°C; used also for nitrosamines determinations.
2. Paired sets of samples, Cobalt-60 vs electrons for both the low and high nitrite – nitrate hams.

sterilizing dose under the 12D concept (MRD) must be determined. The MRD for the high (normal) nitrite (156 mg/kg) /nitrate (700 mg/kg) ham at −30 ± 10 °C is 3.7 Mrad (Table 1). In the inoculated pack studies to determine the MRD for radappertized ham and other foods, enzyme-inactivated (pre-cooked) foods, diced into small pieces before inoculation and vacuum closing of the cans, were used (Anellis et al; 1965, 1967, 1969 and 1972). In the inoculated ham pack studies, *Cl. botulinum* spores are exposed only to residual levels of nitrite and nitrate, which are very low (Anellis et al., 1967; this report, Tables 8 and 21). The presence of salt (NaCl) in the inoculated ham pack studies has an effect on the surviving *Cl. botulinum* spores, whereas the residual levels of nitrite and nitrate have, or should have, little or no effect on the MRD for radappertized ham (Rowley et al., 1968). Since the salt content in the high and the low nitrite/nitrate hams is the same (2.2 ± 0.3%), little if any, deviation in the MRD from the high nitrite/nitrate ham (3.7 Mrad at −30 ± 10 °C) is expected. The inoculated pack study on the low nitrite/nitrate ham to determine the MRD has been in progress since December 1972.

Conclusions

1. Nitrite can be reduced from 156 mg/kg (the USDA maximum allowed and commonly used by the meat industry) to 25 mg/kg in radappertized ham.
2. A small amount of nitrate (100 mg/kg) and the USDA allowable amounts of ascorbate/erythorbate are needed for the characteristic flavor, odor and taste of cured meat color, formed by nitrite, in radappertized ham.
3. In addition to color, the use of 25 mg/kg nitrite, 50 to 100 mg/kg nitrate, and ascorbate/erythorbate are neede for the characteristic flavor, odor and taste of radappertized ham which determine the consumer acceptability of the product.
4. Small amounts of nitrate (50 to 100 mg/kg), in addition to nitrite and ascorbate/erythorbate, are needed apparently to prevent rancidity development in non-irradiated (and probably in irradiated) ham when stored in opened containers in a refrigerator. This factor should be investigated further.

5. Further reduction of nitrate from 100 mg/kg to 50 mg/kg in radappertized ham should be investigated in a paired comparison (50 vs 100 mg/kg $NaNO_3$) of irradiated and non-irradiated ham samples during a storage period of two years.
6. The mechanism of the prevention of discoloration of radappertized ham by nitrate and ascorbate/erythorbate is not known and should be investigated.
7. No nitrosamines were found in the radappertized, shelf-stable ham preserved by gamma rays from the cobalt–60 source or by the electrons from the linear accelerator. There was no indication, furthermore, of the presence of nitrosamines in the non-irradiated, frozen stored, control hams used in this investigation.

Acknowledgments

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Discussion

Influence of nitrite/nitrate on taste of ham

A taste panel of the Central Institute for Nutrition and Food Research TNO at Zeist gave the following marks for colour, odour and taste of irradiated ham with low and high nitrite levels (25 and 150 mg/kg): 7.8 and 7.9, 6.6 and 6.8, 6.1 and 6.1, respectively.

Nitrite seems to give a more firm product. Nitrate is necessary to prevent rancidity.

Influence of NH_4^-

It is known that nitrite as well as nitrate can be formed during irradiation of solutions of NH_4Cl with electrons.

However, Wierbicki did not determine the content of ammonia. Formation of nitrate and nitrite from ammonia in the irradiated ham samples is highly improbable since non-irradiated control samples contained comparable amounts of nitrite and nitrate.

Conclusions and recommendations of the chemical & technological session 13th September 1973

1. The general conclusion is that the problem of nitrite is so important that every effort must be made to fill gaps in our knowledge.

2. As to the loss of nitrite when added to meat only part of it could be accounted for by the proposed mechanisms. Much remains to be learned as to how nitrite, or related compounds, become bound to the meat fractions. In both of the above cases elucidation and quantification are needed.

3. It appears to be now possible to estimate the quantities of nitrite appreciably necessary to produce characteristic cured meat flavour. Disappointingly little progress has been made on the chemistry of cured meat flavour; the methodology is now at hand to make progress with this problem.

4. Bacon is currently the most critical product because, unlike other meat products, fried bacon has consistently been found to contain relatively large quantities of nitrosamine, particularly nitrosopyrrolidine.

5. Ascorbates at present are the best means to suppress or reduce the formation of nitrosamines in cured meat products. It is critically important to establish the optimal balance of nitrite and ascorbates in bacon.

6. Preliminary data indicate that the anti-botulinal effect of nitrite in cured meat products is not changed by the presence of at least 1000 mg per kg (ppm) of sodium ascorbate. Further work is required to establish this and to determine the general effect on the antimicrobial activity of nitrite.

7. Irradiation is a conceivable way of reducing the quantities of nitrite in processed meat products.

Toxicological session

Reporters: P. J. Groenen, V. J. Feron

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen.

Toxicity of nitrite and N-nitroso compounds

R. Preussmann

Institut für Toxicologie und Chemotherapie, Deutsches Krebsforschungszentrum, Postfach 449, D 6900, Heidelberg, W-Germany

Abstract

Toxic effects of chemical compounds may be roughly divided into acute, subchronic (or subacute) and chronic. There is general agreement among experts that the main toxicological problem of N-nitroso-compounds and nitrites is carcinogenesis, after chronic exposure to low quantities of N-nitroso-compounds, or the formation of such carcinogens from nitrite and nitrosatable amino compounds in the human gastrointestinal tract (see J. Sander p. 243–254). Therefore, in this short review, the *chronic* toxicity of the nitrite and N-nitroso compounds are stressed.

Nitrite, nitrates and nitrous gases

The main acute or subchronic toxic effect of ingested nitrites and nitrates (which are easily reduced to nitrites mainly by bacteria (Sander & Seif, 1969; Sander & Schweinsberg, 1972)) or are reduced in food samples to nitrite (Philips, 1971) is the formation of methemoglobin. Prominent symptoms are vomiting, cyanosis, shock and unconsciousness in man (Greenberg et al., 1945). Büsch (1952–1954), Pribilla (1965) and Bakashi et al. (1967) described nitrite poisoning in man due to illegal application of high concentrations of nitrite in meat products, mainly sausages. High nitrite reduction in infants under six months of age, due to low acid secretion in the stomach and the subsequent easy bacterial nitrate reduction, has led to serious intoxication and even death, especially after ingestion of nitrate-rich drinking water or spinach (Fassett, 1966; Phillips, 1971; Sander & Schweinsberg, 1972; all papers are summaries and contain further literature).

Chronic oral administration of nitrite has no carcinogenic or teratogenic effects: Druckrey et al. (1963) gave 100 mg/kg bodyweight daily to BD rats over the whole life-span of three generations. Except for a (probably not significant) shortening of the life-span and a reduction in hemoglobin concentration, no adverse toxic effects of the treatment were noted; reproduction of treated animals was not affected and no teratogenic effects were observed in the offspring. Sander (1971) added 1% $NaNO_2$ to the diet of rats for 117 days and observed the animals until natural death; he saw no tumors or other serious toxic effects. Lehman (1958) mentioned studies that showed sodium nitrate to be tolerated in the diet of rats for a lifetime at a level of 1% with no effects and only growth depression at 10%. Dogs tolerated

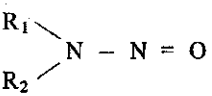
2% $NaNO_3$ for 3–4 months.

Chronic inhalation of NO_2 in rats did not lead to tumors (Ross & Henschler, 1963). The lung histology of rats inhaling air containing 2.9 mg NO_2/kg for 9 months was described by Arner & Rhoades (1973).

From such results a FAO/WHO expert committee (1962) considered a daily intake of 0.4 mg nitrite/kg bodyweight as acceptable; under special circumstances even doses as high as 0.8 mg/kg were considered tolerable. This evaluation was made at a time, when the possibility of a formation of carcinogenic N-nitroso-compounds in vivo had not been proven. In view of this, however, such a daily intake seems too high to be acceptable today and needs reconsideration.

N-nitroso compounds

According to their different chemical reactivities, organic N-nitroso compounds of the general formula



must be divided into two groups. The nitrosamines, in which R_1 and R_2 are alkyl, aralkyl and/or aryl residues, are chemically stable compounds, which are converted to reactive metabolites, very probably alkylating agents, by enzymatic processes in the mammalian organism (for review see Magee & Barnes, 1967). The nitrosamides usually have one alkyl residue R_1 and an acyl residue as R_2 and are chemically reactive compounds, which are split by hydrolysis more or less easily to form alkylating diazoalkanes.

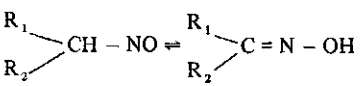
Chemical characteristics

The chemical and biochemical characteristics of the two groups also govern their toxicity. Nitrosamines are mainly systemically acting agents, while nitrosamides react locally as well as systemically. Acute toxicities range from a LD_{50} of 18 mg/kg bodyweight for the highly toxic methylbenzylnitrosamine to a LD_{50} of more than 7500 mg/kg for the practically non-toxic diethanolnitrosamine. *N*-methyl-*N*-nitroso-urethane had a LD_{50} of 4 mg/kg, while butylnitrosourea had an LD_{50} of 1200 mg/kg (Druckrey et al., 1967; Magee & Barnes, 1972). The main organ to be affected by nitrosamines is the liver (centrilobular necrosis) and the lungs (hemorragic oedema), while with nitrosamides damage of the bone marrow and lymphatic tissue is predominant (Druckrey et al., 1967).

Biological effect

The main biological effect of N-nitroso compounds without any doubt is their potent carcinogenic activity. More than 100 N-nitroso compounds have been tested until now and more than 80 of them were more or less potent carcinogens in experimental animals. Some of the important data are presented in Tables 1–5. The

Table 1. N-nitroso compounds. Structural requirements for carcinogenicity.

Formula	Carcinogenicity
	+++
	(+)
	(+)
	–
	?
	–
$NO_2(-)$	–
	–

studies have shown that this group of carcinogens has very versatile carcinogenic activities, inducing malignant tumors in almost all important organs, such as liver, lungs, kidney, urinary bladder, oesophagus, stomach, small intestine, brain and nervous system. The organ specifity of the action depends mainly on the chemical structure of the compound and to a minor degree on the animal species, the route of application and the dose. Many nitroso compounds are active in single dose experiments (Druckrey et al., 1967; Magee & Schweinsberg, 1972).

The carcinogenic activity of dimethylnitrosamine, for example, has been studied in mouse, hamster, rat, guinea pig, rabbit and rainbow trout. In all these different species, the compound is a potent carcinogen (IARC, 1972).

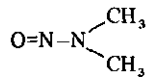
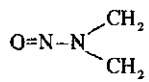
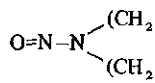
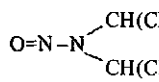
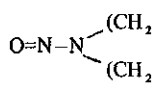
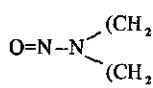
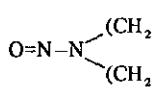
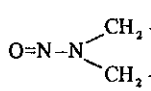
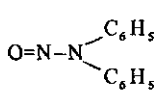
The next higher homologue, diethylnitrosamine, has been shown to be a carcinogen in 12 animal species, including subhuman primates. The 12 species were mouse, hamster, rat, guinea pig, rabbit, dog, pig, rainbow trout, the aquarium fish Branchydanio rerio, grass parrakeet and monkey. So far no animal species tested has been found to be resistant to the carcinogenicity of this compound (IARC, 1972; Schmähl & Osswald, 1967).

Methylnitrosourea, as an example of a nitrosamide, has been tested in mouse, hamster, rat, guinea pig, rabbit and dog. It is powerful carcinogen in all of these animals (IARC, 1972).

Many of the other N-nitroso carcinogens have not been studied as extensively, but for almost all of them reliable and reproducible animal data are available.

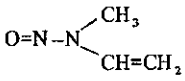
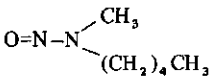
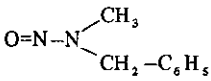
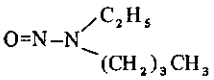
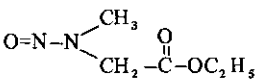
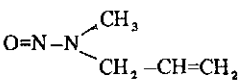
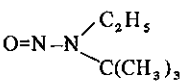
There are further data, which are relevant to the problem of extrapolation of animal data to man. Since nitrosamines most likely require enzymatic activation to form the proximate and/or ultimate carcinogen, comparative metabolism in animal

Table 2. Carcinogenicity of symmetrical nitrosamines.

Nitrosamine	Formula	Carcinogenic action	Application	Main target organ
Dimethyl-		+++	p.o.	liver
Diethyl-		+++	p.o.	liver
Di-n-propyl-		+++	p.o.	liver
Di-isopropyl-		+	p.o.	liver
Di-n-butyl-		+++	p.o.	liver (urinary bladder)
Butyl-butanol-n-		+++	sc. sc.	liver (urinary bladder) urinary bladder
Di-pentyl-		++	p.o. sc.	liver lung
Dibenzyl-		–	p.o.	– – –
Diphenyl-		–	p.o.	– – –

p.o.: by mouth
sc.: subcutaneous
iv.: intravenous

Table 3. Carcinogenicity of unsymmetrical nitrosamines.

Nitrosamine	Formula	Carcinogenic action	Application[1]	Main target organ
Methyl-vinyl-		+++	p.o.	oesophagus
Methyl-pentyl-		+++	p.o. sc.	oesophagus
Methyl-benzyl-		+++	p.o.	oesophagus
Ethyl-butyl-		+++	p.o. iv.	oesophagus
NO-sarkosinester		+++	p.o.	oesophagus
Methyl-allyl-		++	iv.	kidney
Ethyl-tert.butyl-		–	p.o.	– – –

1. Meaning of abbreviations: see Table 2.

and man can give further evidence to facilitate the discussed extrapolation of animal data to man. Montesano & Magee (1970) in a comparative study in vitro with dimethylnitrosamine showed that this compound is metabolized in a qualitatively similar manner in both human and rat liver. Quantitatively, it was shown that the rate of metabolism in human liver slices was comparable to that in rat liver and that similar levels of nucleic acid methylation occur in both species.

Last, but not least, there is one direct observation in man, although not related to carcinogenicity. Dimethylnitrosamine has induced acute toxic liver damage in workers in the chemical industry who were exposed to this compound (Barnes & Magee, 1954). Centrilobular necrosis is similarly produced in most animal species treated with high doses of nitrosamines in acute toxicity experiments.

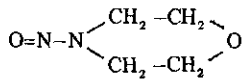
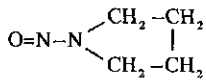
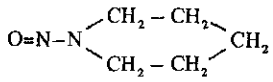
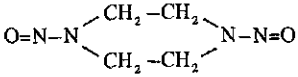
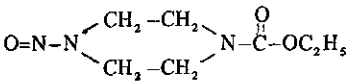
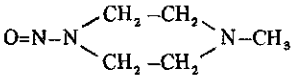
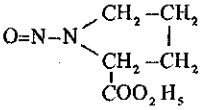
From the present evidence, it is clear that N-nitroso compounds act as carcinogens in many animal species, including subhuman primates. Until now no animal species has been found to be resistant to the carcinogenic effect. Metabolism as well as the acute toxic effects are similar or identical in man and experimental animals. Taking all facts into consideration, one can conclude that man will probably react in a manner similar to that of experimental animals, which means that N-nitroso

compounds are almost certainly carcinogenic in man. The opinion of Lijinsky & Epstein (1970) that nitrosamines and nitrosamides 'seem to be a major class of carcinogens that are likely to be causally related to human cancer' is now shared by many. The widespread concern about carcinogenic nitrosamines in the human environment is, therefore, justified.

A more difficult problem is, what are the levels of nitrosamines that should be considered dangerous to man. In general, there is agreement that for carcinogens only a 'zero tolerance' can be accepted in principle. However, it is well-known that this concept is not always feasible in general practice. Moreover, from the analytical point of view, 'zero' is always a matter of analytical methodology, which is not a constant. Therefore, a rough estimation of 'no-effect' doses from animal experiments is necessary.

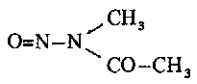
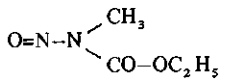
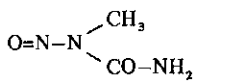
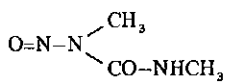
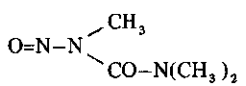
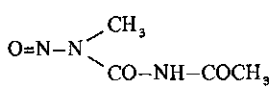
There is one dose-response study available for dimethylnitrosamine in the rat

Table 4. Carcinogenicity of cyclic nitrosamines.

Nitrosamine N-nitroso-	Formula	Carcinogenic action	Application[1]	Main target organ
-morpholine		+++	p.o. iv.	liver
-pyrrolidine		+	p.o.	liver
-piperidine		+++	p.o. sc. iv. sc. iv.	oesophagus nasal cavity
Di-nitroso-piperazine		+++	p.o. sc.	oesophagus
-N-carbethoxy-piperazine		++	sc.	liver
-N-methyl-piperazine		–	p.o.	– – –
-proline-ethylester		–	p.o.	– – –

1. Meaning of abbreviations: see Table 2.

Table 5. Carcinogenicity of nitrosamides.

Nitrosamide N-nitroso-	Formula	Carcinogenic action	Application[1]	Main target organ
-methylacetamide		++	p.o.	fore-stomach
-N-methylurethane		+++	p.o. iv.	fore-stomach lung
-methylurea		+++	p.o. iv.	fore-stomach brain
-dimethylurea		+++	p.o.	brain, nervous system, spinal cord
-trimethylurea		+++	p.o. iv.	periph. nerves spinal cord
-methyl-N'-acetyl-urea		+++	p.o.	glandular stomach

1. Meaning of abbreviations: see Table 2.

(Terracini et al., 1967). Dietary concentration ranged between 2 and 50 mg/kg. At 2 and 5 mg/kg, incidences of liver tumors among survivors at 60 weeks were 1/26 and 8/74, respectively. With higher concentrations more than 70% of the rats had liver tumors. Therefore, for continuous feeding studies, a concentration of 1 mg/kg in the diet could be considered a 'threshold dose'. Single doses of 20 mg/kg body wt. are carcinogenic in the rat (Magee & Barnes, 1959).

A dose-response study, involving oral administration of diethylnitrosamine in the rat, was done by Druckrey et al. (1963). Daily doses ranged between 14.2 and 0.075 mg/kg in nine dosage groups. Total doses administered until death were between 14 and 0.075 mg/kg. All daily doses higher than 0.15 mg/kg gave a tumor yield of 100%. Doses of 0.15 mg/kg gave a tumor incidence of 27/30 liver carcinomas. At 0.075 mg/kg 20 rats lived longer than 600 days; 11/20 had benign or malignant tumors of the liver and the oesophagus. All four of the animals living longer than 940 days at this dose level had tumors. Therefore, 0.075 mg/kg per day diethylnitrosamine, which corresponds approximately to 0.5 – 0.75 mg/kg in the diet, is clearly carcinogenic and above the 'threshold concentration'. The marginal effect dose could be estimated to be at 0.5 mg/kg.

An extremely low single dose of 1.25 mg/kg diethylnitrosamine leads to kidney tumors in the rat (Mohr & Hilfrich, 1972).

It is generally accepted international practice in establishing tolerated doses, to

observe a safety margin of 100 when extrapolating animal data to man. Therefore, a level of 5–10 µg/kg should be considered as a 'tolerable' dose of nitrosamines of low molecular weight. Hence this is also the concentration that must be detected and determined in a chemical analysis of such N-nitroso compounds in environmental media. This detection limit is generally accepted now.

It must be stressed, however, that such 'calculations' are rather unsatisfactory and can by no means lead to 'safe' levels of nitrosamines and nitrosamides. For one, it is well known that single-dose experiments with *N*-nitroso compounds have led to malignant tumors in animals. Unfortunately, no dose-response studies for such experiments are available. On the other hand, many experiments, especially those by Schmähl and his colleagues (1970) and Montesano & Saffiotti (1968), have shown that subthreshold doses of diethylnitrosamine still give rise to tumors when administered together with other carcinogens. This synergistic, additive effect of different groups of chemicals with the same organotropic carcinogenic effect (syncarcinogenesis) clearly is similar to the human situation, where a population almost certainly is never exposed to only one single carcinogen, but to minute quantities of many different carcinogenic compounds.

Extremely low doses of certain *N*-nitroso compounds have a transplacental carcinogenic effect, producing tumors in the offspring of mothers treated during pregnancy (Ivankovic & Druckrey, 1968). Many compounds of this group have also shown mutagenic and teratogenic effects.

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Discussion

Sources of nitrate and nitrite ingestion

From preliminary estimates it appears that in the United Kingdom half of the quantity of nitrate/nitrite ingested by humans comes from saliva, one quarter from the drinking water and one quarter from cured meat products and other foods.

Possible role of the diet

Tumours or other serious ill-effects have never been observed in animals after long-term oral administration of nitrite alone. Apparently no effective amounts of carcinogenic nitrosamines were formed in the stomach. This might be due to the absence of sufficient quantities of amines that can be nitrosated in the diets used.

More knowlegde about N-nitrosopyrrolidine

At present N-nitrosopyrrolidine seems to be a weaker carcinogen than many other volatile nitrosamines. It has, however, not yet been tested thoroughly, because the particular importance of this compound was not known until recently. Further experiments with N-nitrosopyrrolidine are planned in the Deutsches Krebsforschungs Zentrum.

Carcinogenicity of nitrosoproline

Whereas the ethyl ester of nitrosoproline had been found to be non-carcinogenic, the free acid was unstable and difficult to handle, and had therefore not been tested for carcinogenicity. It should be, however.

Realistic nitrite levels in experiments

The relevance of results obtained from studies, in which unrealistically high nitrite and amine concentration have been used, was questioned. The purpose of such model studies was, however, to find out whether the formation of nitrosamines from nitrite and amines took place at all in the stomach. This appeared to be the case. Further experiments must now be conducted under more realistic conditions.

Inhibiting effect of ascorbic acid on nitrosation

Recent experiments by Ivankovic, Zeller, Schmähl and Preussmann (Naturwissenschaften, in press) have shown that the carcinogenic effect observed in animals following the feeding of ethyl-urea and nitrite was prevented by the simultaneous feeding of ascorbic acid. Since the simultaneous feeding of ascorbic acid and ethylnitroso urea did not inhibit tumor formation, it is clear that ascorbic acid prevented the formation of nitroso compounds, but had no effect on preformed *N*-nitroso compounds.

Need for more research

Because most environmental carcinogens are present in low to very low concentrations, the possible synergistic effects of such carcinogens must be investigated.

Since, however, very little data are available on the carcinogenicity of non-volatile nitroso-compounds, which might be formed in food products, carcinogenicity studies with these compounds, also have high priority.

A tolerance level for nitrosamines?

Though the question of tolerance levels for nitrosamines is of current interest in many advisory committees, doubt was expressed whether in Germany a recommendation as to a tolerance level for nitrosamines will be given.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen.

Philosophy of 'no effect level' for chemical carcinogens

R. Kroes, G. J. Van Esch and J. W. Weiss

National Institute of Public Health, P.O. Box 1, Bilthoven, the Netherlands

Abstract

Several factors may have an effect on the setting of tolerance levels of carcinogens. Such factors are:
- modifying factors i.e. factors influencing the action of carcinogens,
- the existence of powerful and weak or possible carcinogens,
- the existence of a dose-response relationship of carcinogens,
- the question whether carcinogen-induced changes are always irreversible,
- epidemiological evidence especially in man for the carcinogenity of the compounds and
- the question of residues, especially with respect to the detection levels of carcinogens.

According to present knowledge the majority of human cancers are caused by chemicals. Therefore it is necessary to consider every step leading to the establishment of tolerance levels very carefully. However it seems illogical and inappropriate to ban every compound found to be a carcinogen in animal.

When evaluating tolerance levels we have to consider the possibility of the existence of many known and unknown factors which may augment or inhibit the carcinogenic action.

The fixing of tolerance levels for certain carcinogens, using a safety factor which is 10 times that used for toxic agents, is discussed for certain conditions.

Introduction

In the last decade numerous papers have been published about the threshold in carcinogenesis (Mantel, 1962; Weisburger & Weisburger, 1968; Roe, 1968; Bryan & Lower, 1970; Hatch, 1970; Goldberg, 1971; Hueper, 1971; Ledbetter, 1971; Shabad, 1971; Stokinger, 1971; Neiman, 1972; Shubert, 1972; Weil, 1972; Oser, 1973 and Shabad, 1973).

Although individual differences occur, most countries in the world have legislation prohibiting the use of carcinogenic compounds as food additives. These regulatory decisions were originally based on the Delaney Amendment, part of the Federal Food, Drug and Cosmetic Act (Public law 85–929).

The amendment reads as follows:

'No such regulation shall issue if a fair evaluation of the data before the Secretary– ..... fails to establish that the proposed use of the food additive, under the conditions of use to be specified in the regulation, will be safe: provided, that no additive shall be deemed to be safe if it is found to induce cancer when ingested by man or animal or if it is found, after tests which are appropriate for the evaluation

of the safety of food additives, to induce cancer in man or animal, exept that this proviso shall not apply with respect to the use of a substance as an ingredient of feed for animals which are raised for food production, if the Secretary finds (i) that, under the conditions of use and feeding specified in proposed labeling and reasonably certain to be followed in practice, such additive will not adversely affect the animals for which such feed is intended, and (ii) that no residue of the additive will be found . . . . in any edible portion of such animal after slaughter or in any food yielded by or derived from the living animal; . . . .'

It becomes immediately clear that this clause raises many problems especially if one realizes the diversity of opinions which exist in the world on the meaning of concepts such as 'induction of cancer', 'appropriate tests' and 'no residues'. Furthermore the clause does not indicate any dose, so that compounds fed at unrealistically high levels and found to be carcinogenic at that level, are banned from use as an additive in food. The different opinions about this clause and the concept of a 'safe' level for carcinogens can not be better illustrated than by quoting Hueper (1971): 'The concept of a 'safe' dose for carcinogens is without scientific basis, is deceptive and, if legally adopted, represents a potential highly dangerous public health policy' and Roe (1973): 'This regulation would make good sense (1) if it were possible to distinguish absolutely between carcinogens and non-carcinogens by means of animal tests (2) if it were possible to devise a diet that was free of naturally occurring carcinogens, and (3) if the effects of carcinogens were really irreversible' and 'The present preoccupation with new chemical additives to the exclusion of natural food constituents and natural contaminants is illogical if not absurd'.

When considering the threshold problem one must realize that there are many factors which may have an effect on the 'safe' level of a carcinogen.

1. Factors influencing the action of the carcinogen(s).
2. The existence of powerful or real carcinogens, and weak or possible carcinogens, as well as differences in action mechanism among the various carcinogens.
3. The dose-response relationship of carcinogens.
4. The existence of reversible effects of carcinogens.
5. Epidemiological studies in man, especially on the possible correlation of potential carcinogens already present in the environment and the cancer incidence in man.
6. The residue problem: what amount is detectable?

Factors influencing the action of carcinogen(s)

These factors, also known as modifying factors, may cause inhibition, augmentation or a qualitative change in a particular biological process (Berenblum, 1969).

Inhibition

Inhibition can manifest itself in a reduction of the tumor yield and/or a longer latency period. Suppression of carcinogenic action may be caused by nutritional factors, inhibition or stimulation of enzyme systems and hormonal and immunological status of the host.

With regards to nutritional factors, it is known that caloric restriction inhibits tumor development, as do dietary fat, protein and histamin deficiency. However excess of some constituent also seems to reduce tumor growth (Tannenbaum, 1958; White, 1961; Kalbe et al., 1968; Madhaven & Gopalan, 1968; Magee, 1969; Warwick 1971).

Inhibitory effects of the stimulation or suppression of enzyme systems are known for several chemical carcinogens. Inhibition may occur through increased metabolism to inactive derivatives (Miller & Miller, 1971). There are numerous examples of increased metabolism to inactive derivatives, sometimes via a stage in which the ultimate carcinogen is formed, but quickly metabolized (Wattenberg, 1966; Hadjiolov, 1971; Conney et al., 1971; McLean & Marshall, 1971; Yamamoto et al., 1971; Conney & Burns, 1972; Den Tonkelaar et al., 1972). Decreased metabolism to ultimate carcinogens was shown by Weisburger et al. (1972).

Hormonal status may affect the tumor induction by carcinogens. Although several reports are available demonstrating that estrogens are carcinogenic by themselves (Herbst et al., 1971), a few papers have been published on the inhibitory effect of anti-ovulatory drugs on tumor induction (Stern & Mickey, 1969; Kistner, 1969 and Thomas et al., 1972). Epidemiological studies in humans revealed a decrease of neoplasms in women treated with estrogen or anti-ovulatory drugs (Wilson, 1962 and Doll, 1972).

The importance of the immunological status of the host on inhibition of tumor development or tumor growth was shown by the work of, among others, Mathé (1973).

An interesting findings (Lacassagne et al., 1945) was that one carcinogen may inhibit the effect of another. Hill et al. (1951), Richardson et al. (1952) Miller et al. (1958) and Likhachev (1968) found similar effects.

Augmentation or co-carcinogenesis

Augmentation is an increase in tumor incidence or a shortening of the latent period or both. Augmentation can happen in many ways (Berenblum, 1969):
– by addition; i.e. cumulation of the effects of two or more carcinogens;
– by synergistic action, when the combined effect exceeds the sum of their separate effects;
– by incomplete carcinogenic action, either as initiator or as promotor;
– and by a change in specific properties such as sensitivity of organs, or systems (e.g. endocrines, transplacental exposure), the absorption rate of the carcinogenic compound, or by activation of certain viruses.

Augmentation may be induced by nutritional factors, changed metabolism of the carcinogen, immunological status of the host, certain compounds which are not carcinogenic by themselves, and many other factors. The age of the host at the time of exposure is very important. Transplacental exposure to some carcinogens yields very high tumor incidences (Napaikov, 1971 and Tomatis et al., 1971) while exposure to carcinogens at a young age also seems to enhance tumor incidence (Terracini & Testa, 1970, and Vesselinovitch et al., 1972). Augmentation is without doubt one of the most important problems that have to be considered when the possibility of threshold dosages or 'safe' levels is evaluated. Especially the promoting action of

certain compounds and the synergism among carcinogens are of importance. Bingham & Falk (1969) showed a 1000 fold enhancement of the potency of benzo(a)pyrene and benz(a)anthracene when the non-carcinogen-dodecane was used as diluent. The promoting effects of croton oil (Berenblum, 1969; Hecker, 1971, 1972) and tobacco smoke (Van Duuren, 1968 and Wynder & Hoffmann, 1969) are well documented. Some vegetables fats and oils are potent co-carcinogens (Sinnhuber et al., 1968). Interesting are the findings of Gaudin et al. (1972), who showed that co-carcinogens such as croton oil inhibit DNA repair, which might be the possible mechanism of their co-carcinogenic action.

Synergism has been observed in experimental animals as well as in humans. Schmähl et al. (1963) and Schmähl & Thomas (1965) indicated that carcinogens with the same target organ may act synergistically whereas carcinogens with a different target do not. When four different hepatocarcinogens were given to rats in daily doses which could be 'subthreshold' if given alone to rats, liver cancer occurred in 34% of the animals (Schmähl et al., 1970). Likhachev (1968), however, also noted a synergistic effect of carcinogens with a different 'target' organ. Synergistic action in skin carcinogenesis was extensively studied by Neiman (1967 and 1968), who found that painting of skin at one side with carcinogen sensitizes the whole skin for a second group of applications. Montesano (1970) reviewed the synergistic and additive effects in respiratory carcinogenesis. Of interest is the study of Deichmann et al. (1967) who gave four (weak) hepatocarcinogens to rats simultaneously. These compounds were Aramite, DDT, methoxychlor and thiourea or aldrin. Surprisingly no additive effect was noted. On the contrary an antagonistic type of effect was found because the survival time of these rats was longer and their liver tumor incidence lower.

In epidemiological studies in humans, synergistic or additive effects were found for uranium and smoking (Kuschner and Laskin, 1971), asbestos, tar and chromium (Bittersochl, 1971), asbestos and smoking (Gilson, 1973), and betelleaf chewing and smoking (Jussawalla, 1973) while the synergistic or additive effect in animals usually was no more than several fold, in human this increased tumor risk varied from twofold to thirty-fold.

Another form of augmentation of carcinogenic action can be induced by the immunological status (Roe & Rowson, 1968). Evidence is available that a suppressed immune system leads to increased tumor incidence in humans (Balner, 1970; Reis, 1971; Fudenberg et al., 1971 and Walder et al., 1971). In experimental animals treatment with immunosuppressive agents such as antilymphocyte serum or azathioprine was followed by an increase in tumor incidence and a shorter latency period of chemically induced tumors (Balner & Dersjant, 1969; Woods, 1969; Haran-Chera & Lurie, 1971; Sheehan et al., 1971 and Baroni et al., 1972). Thymectomy also proved to increase the tumor rate of chemical induced tumors (Sheehan & Shklar, 1971). Others, however, found no effect of immunosuppressants on tumor yield in chemical carcinogenesis (Frankel et al., 1970; Schmähl et al., 1971 and Wagner, 1971). There is evidence that certain carcinogens are immunosuppressive themselves, thus enhancing (or causing) their own action (Sternswärd, 1969; Ball, 1970; Barconi et al., 1970; Bluestein & Green, 1970). In a review Mäkelä (1972) states that immunosuppressive agents may provoke a higher tumor incidence but usually do not. There is however good evidence for a decrease in latency period and

the number of tumors per animal may be increased. In a recent review Gleichman & Gleichman (1973) state that the immunosurveillance theory (i.e. lack of cellular immune response against tumor-specific antigens) might not fit as a mechanism and that perhaps two other mechanisms: an alteration of tissue proliferation and a lowered resistance against viruses, may be involved.

Powerful or real carcinogens and weak or possible carcinogens

Although there is no doubt about the existence of powerful carcinogens, such as aflatoxin, nitrosamines, polynuclear aromatic compounds, aromatic amines and azo-dyes, there are numerous examples of compounds which have only a weak carcinogenic action. Weak carcinogenic action means that usually high doses of the compound are necessary over a long time to provoke a carcinogenic effect.

Cyclamate, carbon tetrachloride, metals like Pb and Cd, DDT, Aldrin, Dieldrin and several more of the pesticides and industrial chemicals investigated by Innes et al. (1969) can be considered as (weak) carcinogens. It may be asked whether these weak carcinogens are carcinogens at all, or just compounds which augment 'spontaneous' tumor incidence by indirect action. Calcium cyclamate or ethylene glycol (Golberg, cited by Roe, 1968) cause bladderstones which through irritation give rise to bladder papillomas and bladder carcinomas. DDT might be an immunosuppressant as are some of the dithiocarbamates tested by Innes et al. (unpublished results, Van Logten & Kroes, 1970). Recent findings of Hicks et al. (1973) indicate that saccharine most probably is a promoting agent. If we assume this is so, it might be suggested that saccharine fed at a rate of 7.5% in the diet which has induced some bladder tumors (cited by Oser, 1973) promotes irritation of the bladder epithelium due to concrements.

Special attention must be given to hormones and antihormonal agents as carcinogens. Do they act directly? Diethylstilbestrol (Herbst et al., 1971) seems to be a transplacental carcinogen in humans, although only at high levels. Some goitrogenic compounds have been named carcinogens because they induce nodular hyperplasia in thyroids subsequently giving rise to tumors. The same hyperplastic lesions and tumors are produced when rats are given a low iodine diet (unpublished results, Kroes, 1969). Is such a compound a carcinogen? Or an 'indirect' carcinogen?

There is a need for a classification on properties and 'direct' or 'indirect' action. This classification will not be easy at all, but that is no reason not to undertake it.

Such a classification has nothing to do with the classification of carcinogens in a distinction between primary or proximate carcinogens and secondary or procarcinogens, which refers to properties of action at the point of application or elsewhere (Weisburger & Weisburger, 1968).

The dose-response relationship of carcinogens

Especially the brilliant work of Druckrey (1967b) threw a light on the existence of a dose-response relationship of carcinogens. He noted that dependency of the induction time t on the dose d resulted in a linear function according to the formula dt^n= constant. The values of the exponent n were usually 2–4.

From these and other studies (Bock, 1968; Weisburger & Weisburger, 1968;

Shabad, 1971) it can be concluded that very low doses might not induce tumor in animals because the induction time exceeds the average lifespan of the animals. Weil (1972) quoted the work done on the dose-response relationship for radiation-induced cancer.

The dose-response curve however may vary in slope and intercept depending on several factors, such as type of animal, sort of neoplasm, distribution of the compound in space and time etc. That a dose-response relationship also exists in humans has been found in several epidemiological studies; prolonged exposure to a certain hazard increased the risk of tumor development (Case, 1966; Boyland, 1969; Gilson, 1973).

Another example is the existence or a correlation between the increased risk of lung cancer and the higher number of cigarettes smoked (Anon., 1970 and Wynder & Mabuchi, 1972).

In the evaluation of the carcinogenic action of compounds, when inducing an increased tumor incidence, it is important to establish any dose-response relation.

The existence of reversible effects of carcinogens

It is generally believed that carcinogens induce irreversible alterations (Weisburger & Weisburger, 1968; Bryan & Lower, 1970). However it is also believed that the concept of irreversibility might not always be valid. Recently Roe et al. (1972) showed evidence of reversibility in the initiation of carcinogenesis by dimethylbenz(a)anthracene.

The concept of irreversibility is also made less likely by the occurrence of spontaneous tumor regression and by the fact that DNA repair exists and immunosurveillance is present. Everson (1964) evaluated many cases of spontaneous regression in human cancer and concluded that in 130 cases spontaneous regression was probable.

Epidemiological studies in man, especially on the possible correlation of potent carcinogens already present in the environment and the cancer incidence in man

From the many epidemiological studies of human cancer it can be concluded that these studies are very important but also very difficult. So many factors may contribute to the observed results that is usually very difficult to establish evidence for a single factor influencing the tumor incidence. Nevertheless many relationships could be found from this type of study such as the relation between lung cancer and smoking, bladder cancer and the rubber industry, lung cancer and uranium, mesothelioma and asbestos, lung cancer and mustard gas, and liver cancer and aflatoxin (Epstein, 1972).

It is also clear that weak carcinogens will not be identified as easily as strong carcinogens by epidemiological studies. Special attention must be directed to epidemiological studies on people or groups of people who were involved in production of certain carcinogens or exposed in another way to carcinogens (i.e. on drugs). For example it would be of great importance if a good study on exposure to nitrosamines (time of exposure, direct or indirect contact) showed that nitrosamines are also carcinogenic in man.

The residue problem, what amount is detectable?

It is only in recent years that nitrosamines can be detected in smaller amounts than a mg/kg. Very delicate techniques to detect chemicals have been developed so that levels can now be measured in the range of 10^{-3} mg/kg, depending on the compound.

Nitrosamines can be detected now in amounts equal to 10^{-2} mg/kg and more. Aflatoxins at one time detected only when present at a level of a mg/kg can now be determined in amounts well below 10^{-3} mg/kg. The detection level of DDT has dropped from the range of a mg/kg to less than 10^{-3} mg/kg thanks to a delicate electron capture method (Oser, 1973).

It should be emphasized that according to present knowledge about 80–90% of human cancers are caused by chemicals (Clayson, 1967; Boyland, 1969; Bryan & Lower, 1970). Therefore it is necessary to consider every step leading to the establishment of safe levels very seriously and carefully. However we are faced with the fact that a number of known powerful carcinogens (such as benz(a)pyrene, nitrosamines) are already present in the environment and that their presence in common foods does not seem etiologically correlated with the incidence of human cancer (Oser, 1973). This might indicate that trace amounts can be tolerated. In fact some official statements are already available with regard to the setting of levels for carcinogens. In the WHO publication on International standards for drinking-water (1971), the concentration of polynuclear aromatic hydrocarbons (including benz(a)-pyrene) in drinking-water may not exceed 0.2 μg/1. Furthermore, in WHO report no. 406 (1968) the following is stated: 'On the one hand the establishment of safe levels is difficult in the absence of general principles for extrapolation. On the other hand the approach based on the assumption of no threshold is possibly too conservative'. In a postscript to the UICC[1] Technical Report on Carcinogenicity Testing (1969) some change of attitude can also be perceived towards the concept of 'safe dose levels' mainly because of the importance of weighing benefits of a certain product against its potential carcinogenic hazard and the fact that rigid legislation for exclusion of all carcinogenic products for use by man might prove selfdefeating. In a recent WHO report (Food additives series Vol. 4, 1972) on the evaluation of, among others, lead and diethylpyrocarbonate tolerable intakes and maximum concentrations are set, of both compounds, although lead and urethane (one of the products of diethylpyrocarbonate hydrolysis) have been proved to be carcinogenic under certain conditions.

In the evaluation of safe levels we have to consider the possibility of the existence of modifying factors which inhibit or augment the carcinogenic action. As mentioned earlier these factors can change the carcinogenic action several fold. In a few exceptions of which we can doubt the feasibility in practice, a 1000 fold increase was found.

Secondly, it is important to know if a compound is a carcinogen or not. How to distinguish them from cocarcinogens? Roe (1968) already pointed out that the definition of a carcinogen as used by legislation does not concern the mechanism

1. Union Internationale Contre le Cancer.

but solely the relationship between cause and effect. That cocarcinogens might be mistaken for carcinogens if this stand is taken is obvious. The Food and Drug Administration Advisory Committee says in a Panel on carcinogenesis (1971): 'What can be the significance of the incidence of 'spontaneous' tumors of susceptible strains when one is not certain about the presence of carcinogenic contaminants in the diet of which the animals have been maintained.' and 'are tumors ever induced that do not occur on control animals? '.

Hence, it is necessary to have better definitions of chemical carcinogens than those given by WHO (1972): 'a substance that is known conclusively to induce or enhance neoplasia'. We have to be aware that carcinogenicity of a compound is established only if a compound induces tumors in more than one species or, if only one species is observed, the compound induces a specific type of tumor, which is not common in the test species and a relation between dose and effect exists. Of course it must be realized that in determining carcinogenic activity the route of administration is important. It has been known for some time that subcutaneous testing of compounds may result in interpretational difficulties unless care is taken to define the goal of study (Roe, 1968; Goldberg, 1971). Next to the question whether a compound is a carcinogen we are faced with its activity. As mentioned earlier many weak carcinogens are known. For most of them epidemiological studies in humans provide little indication that they could be a hazard. For cyclamate and saccharin for example, no correlation has been found between their use and the occurrence of bladder cancer in man (Inhorn & Meisner, 1969 and Price et al., 1970).

Isonicotinic hydrazide has incuded lung tumors in mice (Toth & Shubik, 1966). It has, however, already been used for decades in the treatment for tuberculosis in man as a very valuable drug. The therapeutic doses are high, nevertheless no increased occurrence of neoplasms in treated patients has been observed as yet (Hammond et al., 1967; Boyland, 1969; Ferebee, 1970).

Of course we have to realize that latency periods are usually long and may vary. Wada et al. (1968) found an average latency period of 24.4 years for mustard gas induced lung cancer. Boyland (1968) and Bryan & Lower (1970) mention latency periods for naphthylamine- induced and benzidine-induced bladder cancer ranging from 15–40 years. Herbst et al. (1971) found latency periods for vaginal adenocarcinoma transplacentally induced by diethylstilbestrol from 14–22 years. Asbestos-induced mesothelioma has a latency period of 20–30 years (Selikoff, 1973).

It has been argued that a dose-response relationship exists for most, if not all carcinogens. Furthermore, there is evidence of a dose-response relationship of carcinogenic hazards in man. Thus, theoretically if dosages could be found low enough so that the latency period exceeds the lifespan of human beings, it should be safe to use them when transplacental carcinogenic action can be eliminated. Some compounds are tested in animals at unrealistic high levels. For comparison: saccharine was found to induce bladder tumors at a concentration of 7.5% in the diet. This concentration when extrapolated to man without any safety factor, would mean a total uptake of 1368 kg saccharine (eating 1000 grams a day and 50 years on the 'diet'). Lead acetate induces renal tumors in rats and mice (van Esch et al., 1962 and van Esch & Kroes, 1969) at levels of 1 and 0.1% in the diet. For humans this equals

without a safety factor, 182.5 kg or 18.25 kg lead acetate total intake over 50 years. It can never be said that man behaves in the same way as the tested spieces, moreover the sensitivity for the compound may be completely different. On the other hand almost all compounds which were found to be carcinogenic in man, were also found carcinogenic to animals. As however experiments with humans are immoral and impractical we are forced to extrapolate from animals to man.

Thus Weil's (1972) suggestion to introduce a safety factor for carcinogens does not seem illogical. He proposed a factor of 5000 using a 'minimum measured cancer producing level' (MIE) and not the 'no effect level'. This factor is 10 times higher than the usual safety factor on toxic compounds of 500 based on a MIE, or a 50-fold when based on a no effect level, which is 100 times a MIE. This additional factor is introduced especially on the assumption that carcinogenic action may be less reversible. Terracini et al. (1967) found that 2 mg/kg dimethylnitrosamine administered for lifetime to rats, still induced a liver tumor in one out of 26 rats. Druckrey (1967a) found that diethylnitrosamine (DENA) fed to rats at 0.075 mg kg^{-1} day^{-1} still induces tumors. Other experiments (Kroes et al., 1973) revealed no tumors after application during lifetime of DENA, 5 μg rat^{-1} day^{-1}, five times a week, in 58 surviving males and 40 surviving females. This represents about 0.175 mg/kg in the food which calculated without the safety factor would be an intake of 3193.6 mg of the compound in 50 years for man. Of course these extrapolations are very inaccurate because a tumor incidence as low as 1% may be missed even if only 108 rats are involved, and such a tumor incidence in human means 1000 cases per 100,000 individuals.

When using the information mentioned above for calculation of human risk for exposure to nitrosamines present in cured meat (taking DENA as representative for all nitrosamines) we can calculate that 18.9 mg of DENA should be the maximum total intake for humans. (Table 1).

In Table 1 some calculations are made for the total intake of 'nitrosamines' via cured meat when this meat contains 40, 20, 10 or 1 μg/kg of 'nitrosamines'. It is obvious that an average daily intake of 50 gram cured meat the total intake under certain conditions is already above the calculated maximum for humans. Moreover exposure to nitrosamines from other sources than cured meat is ignored. For this reason the levels of nitrosamines in cured meat should be kept as low as possible.

Table 1. Some calculations[1] for the total intake over 50 years (mg) of 'nitrosamines' via cures meat containing 40, 20, 10 or 1 μg/kg 'nitrosamine'.

Average daily intake cured meat (gr)	Nitrosamine level (μg/kg)			
	40	20	10	5
25	18.250	9.125	4.562	2.281
50	36.500	18.250	9.125	4.562

1. MIE DENA in rat is 0.075 mg kg^{-7} body weight day^{-1} (Druckrey, 1967). Calculated maximum tolerated intake 75/5000 μg/kg body weight day^{-1}, i.e. 18.9 mg/50 years.

Conclusion

It seems illogical and inappropriate to ban *every* compound, found to be a carcinogen in animals. Serious and careful judgement is required for each carcinogenic compound. It has to be established whether the compound is a real carcinogen, and what type of mechanism is involved by the induction of tumors. The dose levels have to be compared in relation with the levels of human exposure. It will be important to know whether the compound is stable or not. The compound has to be irreplaceable and essential for use. If alternative compounds are found which can replace the compound the use of the compound should be prohibited. Thus a 'safe level' should never be claimed for any possible carcinogen. In setting a safe level not only a safety factor and a MIE should be considered but available epidemiological information should equally be included. To enable calculations of tolerable exposure levels for potential carcinogenic stimuli epidemiological studies should be undertaken as well as correlation studies between cancer incidence and suspected stimuli. Such studies are especially indicated at the points of maximum possible human exposure such as production or use in concentrated form. More experience in 'low level' carcinogenesis is necessary. Also desirable are studies on interaction of a given test chemical with other materials likely to be used at the same time, or likely to be present in the environment.

It is a good thing to know that many laboratories are currently carrying out carcinogenicity studies with low levels of powerful carcinogens, thus providing more information about the possible hazard of these compounds.

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Discussion

The problem of setting human safety levels

It was stressed that the use of safety factors in calculating human risks for

exposure to toxic agents is a necessity, because experiments with humans are not permissible. The application of higher safety factors for carcinogens (1 000 or 5 000) than for non-carcinogenic agents (100 or 500) is based on an arbitrary assessment of a greater hazard with carcinogens, mainly arising from the possibility of the irreversibility of carcinogenic action.

If it is not possible to distinguish between carcinogen or co-carcinogen from the results of a carcinogenicity study, the substance tested has to be regarded as a carcinogen.

Proc. int. Symp. Nitrite Meat Prod., Zeist, 1973. Pudoc, Wageningen

Formation of N-nitroso compounds in laboratory animals. A short review

J. Sander

Hygiene-Institut der Universität, D–74 Tübingen, W-Germany

Abstract

For secondary, tertiary and quarternary amines, amidopyrine and alkylamides a survey is given of reactivity towards nitrite, in vitro and in vivo. Secondary amines of low basicity are particularly nitrosated in the stomach if nitrite is present. Feeding with slightly basic amines combined with nitrite induced specific carcinomata. The author estimated the nitrosation reaction equation of tertiary amines in vitro. Carcinogenicity of their nitroso compounds has not yet been reported. Quarternary ammonium compounds are nitrosated in vitro. They do not seem to hold a health risk. Reactivity of alkylamides in vitro varies. Various alkylureas combined with nitrite caused specific carcinomata. So far alkylurethanes and other alkylamides combined with nitrite have shown no carcinogenicity. Halide ions, thiocyanate and some aldehydes catalyse nitrosation and cause a shift in optimum pH for nitrosation. Thiocyanate acts as a catalyst in the rat's stomach. Ascorbic acid in vitro and in vivo effectively inhibits nitrosamine formation from several precursors. Quantitative data are so far inadequate.

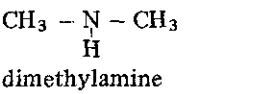
Introduction

In principle, carcinogenic nitroso compounds can be formed from secondary or tertiary amines, from enamines, from quarternary ammonium bases and from a variety of alkylamides. The amino-compounds react with nitrogen oxides which are usually derived from nitrite.

Secondary amines

$(R - \overset{H}{\underset{|}{N}} - R')$

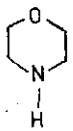
secondary amine



$CH_3 - \overset{H}{\underset{|}{N}} - CH_3$

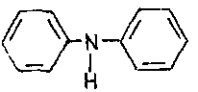
dimethylamine

high basicity, pK_A : 10.7



morpholine

moderate basicity, pK_A : 8.3



diphenylamine

low basicity, pK_A : 0.8

Reactivity in vitro

Secondary amines can be nitrosated in organic solvents or in water. In an organic solvent a high basicity will increase the reactivity of the amines towards the electrophilic nitrosating agent. In water the nitrosating mechanism is similar but the nitrosamine yield is strongly influenced by side reactions. It was shown that formation of a salt, or the addition of protons to the free electron pair of the amines, considerably inhibits the nitrosamine formation (Sander et al., 1968). In vivo a nitrosamine formation will mainly take place in an aqueous environment, but the organic phase must not be neglected.

While salt formation of amines is increased by lowering the pH of the solution, thus inhibiting the nitrosation, a low pH value allows formation of a greater proportion of free nitrous acid and its equilibrium products which are necessary for the nitrosamine formation. Maximum yields per time unit are therefore neither obtained at very high nor at very low pH values, but at a certain optimum pH. This was found for most secondary amines to be near pH 3.3. Some amino acids with a secondary amine group showed a maximum reaction near pH 2.25– 2.5 (Mirvish, 1972).

With respect to the optimum pH, good conditions for the formation of nitrosamines could be expected in the vertebrate stomach, although of course a synthesis in vitro cannot be directly compared with that in vivo where several other factors have to be taken into account.

Animal experiments

Animal experiments showed, although with very great differences in yield, that all secondary amines can be nitrosated in the stomach when nitrite is present. Secondary amines of a high basicity were, corresponding to the in vitro results, very poorly nitrosated, but the nitroso derivatives of amines of low basicity were easily formed.

Methods used to demonstrate the nitrosamine formation were the analysis of the stomach contents, the determination of nitrosamines excreted with the urine, the production of toxic effects typical for nitrosamines and, of course, the induction of tumors.

Despite the fact that the nitroso derivatives of highly basic amines could be detected in the stomach in very low yield, cancer was not induced by such amines and nitrite (Druckrey et al., 1963; Sander, 1971; N.P. Sen, 1973, personal communication). Feeding of less basic amines and nitrite, however, resulted in the induction of tumors typical for the corresponding nitrosamines. For example methylbenzylamine and nitrite caused tumors in the oesophagus of rats. Rats receiving morpholine and nitrite developed liver tumors (Sander & Bürkle, 1969). Methylaniline and nitrite caused a marked increase in the number of lung adenomas in mice (Greenblatt et al., 1971). Concurrent feeding of the amino acids proline and hydroxyproline and nitrite did not produce tumors in rats. This is probably because of a lack of in vivo decarboxylation of the obviously non carcinogenic nitrosoamino-acids (Greenblatt et al., 1973).

Tertiary amines

With tertiary amines experiments have not yet been carried out to the same extent as with secondary amines. They should be distinguished from enamines, because of different chemical behaviour.

tertiary amine enamine

Reactivity in vitro

Our experiments (Schweinsberg & Sander, 1972) with tertiary amines of low molecular weight demonstrated that the nitrosation in dilute aqueous solution at 100°C obeys the following equation:

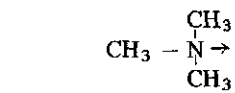
$$\text{rate} = k\,[\text{amine}] \times [\text{nitrite}]^3$$

The first step in the nitrosamine formation from tertiary amines is an oxidative desalkylation, the nitrosation of the resulting secondary amine being the second step. In our experiments tertiary amines formed nitrosamines much slower than secondary amines. This finding is partly opposite to results of Lijinsky et al. (1972), who described a quick nitrosamine formation out of a variety of tertiary amines. Lijinsky and his co-workers used much higher concentrations of the amines and nitrite and a much longer reaction time (16 h 30 min).

Feeding trials

Induction of tumors by concurrent feeding of tertiary amines and nitrite has not been reported so far. In our experiments, feeding of a diet containing 0.5% triethylamine and 0.5% $NaNO_2$ did not produce tumors within a year, nor did it cause a toxic reaction exceeding that of nitrite alone.

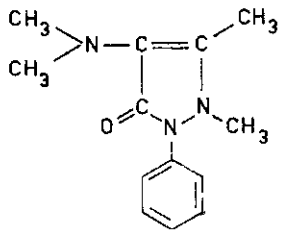
It should be kept in mind that the nitrosation of tertiary amines needs further investigation. The problem of nitrosamine formation from the corresponding amine oxides has also not received sufficient attention.



trimethylamine oxide

Enamines

Among the enamines, amidopyrin, a drug which has been widely used, easily reacts with nitrite in vitro and in vivo, leading to the formation of dimethylnitrosamine (Lijinsky & Greenblatt, 1972; Lijinsky et al., 1973).



amidopyrin

Quarternary ammonium compounds

Only a few experiments on the nitrosamine formation from quarternary ammonium compounds have been done. Fiddler et al. (1972) demonstrated the formation of dimethylnitrosamine from a variety of naturally occurring substances. Tetramethylammonium chloride reacted less readily than trimethylamine hydrochloride which in turn yielded less than 10% of the dimethylnitrosoamine produced from dimethylamine hydrochloride. Fairly high amounts of dimethylnitrosamines were produced from 2-dimethylaminoacetate, 2-dimethylaminoethanol, *N,N*-dimethylglycine and the methylester of the latter.

With respect to the feeding experiments done with diethylamine and triethylamine and nitrite, it seems unlikely that feeding of realistic amounts of the quarternary ammonium compounds tested and nitrite will induce cancer in test animals.

Alkylamides

Reactivity in vitro

There is a great number of alkylamides which also can react with nitrite to produce carcinogenic nitroso compounds. The reactivity of the amides towards nitrite varies as much as with the amines. Alkylureas were nitrosated very easily, while alkylurethanes showed a slow reaction. The same applies to methylguanidine and citrullin (Mirvish, 1971). Several other amides like methylacetamide did not show any measurable reaction in dilute aqueous solutions (Sander et al., 1971).

As the alkylureas do not readily form salts they are nitrosated best at higher acid concentrations. The main nitrosating agent probably is the nitrous acidium ion $(H_2NO_2)^+$. The reaction rate proved to be proportional to the concentration of the alkylurea, the nitrous acid and the protons.

$$\text{rate} = k\,[\text{alkylurea}] \times [HNO_2] \times [H_3O^+]$$

The k values of the N-alkylurethanes were about one-thirtieth of those for the corresponding alkylureas, the k values of the ethyl compounds being about one-fourth of the methyl derivatives.

Experiments in vivo

Tumors have been induced in animals by feeding various alkylureas and nitrite (Sander, 1970, 1971; Ivankovic & Preussmann, 1970; Mirvish et al., 1972). These tumors were similar to those which had been previously induced by N-nitroso ureas.

Alkylurethanes and other alkylamides have not been reported to induce tumors when concurrently administered with nitrite, although it seems very likely that at least with methyl and ethyl urethan a positive result might be obtained. A diet containing 5 000 mg/kg citrullin given concurrently with drinking water containing 5 000 mg/kg $NaNO_2$ for about half a year did not induce tumors after about two years of observation (unpublished data).

Catalysis and inhibition of the formation of N-Nitroso compounds in vivo

The nitrosation reaction may be catalysed as well as it may be inhibited by several agents. Catalysing agents are known to be halide ions and the pseudo halide thiocyanate (SCN^-). The latter arises from several goitrogenic substances which are contained in certain vegetables, and also are formed in vivo from cyanide which is inhaled with cigarette smoke. Some aldehydes may also be important as catalysts, e.g. formaldehyde (Roller, personal communication). The catalysts change the reaction rates of the nitrosation reaction. In the uncatalysed reaction the nitrosation of secondary amines obeys the following equation (Mirvish, 1970):

$$\text{rate} = k\,[R_2NH] \times [HNO_2]^2$$

But the catalysed reaction is expressed by another equation (Fan & Tannenbaum, 1973):

$$\text{rate} = k\,[HNO_2] \times [H_3O] \times [Hal^-] \times [R_2NH]$$

The catalysed reaction is linearly dependent upon the nitrite concentration while the uncatalysed reaction is dependent upon the square of the nitrite concentration. Catalysis also causes a shift of the pH-optimum. Boyland et al. (1971) described the pH-optimum of the catalysed nitrosation of methylaniline to be near pH 1; For morpholine Fan & Tannenbaum (1973) found pH 2.3, for diethylamine Schweinsberg (1973) found 2.5.

By kinetic studies it was recently demonstrated that in the rat stomach the nitrosamine formation normally occurs as a catalysed reaction due probably to the presence of thiocyanate. Thus additional catalyst does not significantly change the reaction (Schweinsberg, 1973).

The chemical analysis is in good accordance with feeding experiments in rats. Feeding a diet containing amines and nitrite and thiocyanate did not produce a higher toxicity than the corresponding mixture without the catalyst (Sander et al., 1972).

Several experiments have been concentrated on the problem of the inhibition of the nitrosamine formation by ascorbic acid which was first described by Mirvish et

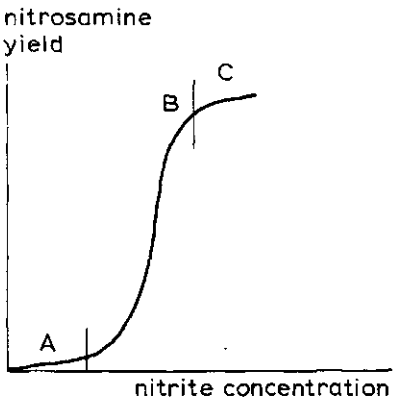


Fig. 1. Schematic description of the nitrosamine formation in the vertebrate stomach from a given amount of a secondary amine and different amounts of nitrite.

al., (1972). In vitro and in vivo, addition of ascorbic acid inhibited nitrosamine formation from dimethylamine, morpholine, piperazine, N-methylaniline and amidopyrin (Kamm et al., 1973; Greenblatt, 1973). With high doses of ascorbic acid the inhibition was complete. Feeding of piperazine or moropholine with nitrite induced lung adenomas, but was ineffective when administered concurrently with ascorbic acid (Mirvish et al., 1973).

The development of hydrocephali in the offspring of pregnant rats which can be induced by administration of ethylurea (100 mg/kg) and nitrite (50 mg/kg) to the mothers, was completely prevented by simultaneous application of a high dose of ascorbic acid (250 mg/kg) (Ivankovic et al., 1973).

Kinetic studies (unpublished data) demonstrate that the nitrosation of amines in the vertebrate stomach does not obey a simple equation, because inhibition by nitrite destroying agents and catalysis are active at the same time. In principle, the nitrosamine formation from a given amount of an amine can be described by a curve as shown in Fig. 1.

In Section A the nitrosamine formation is very low because a significant part of the nitrite is destroyed by ascorbic acid or related compounds. Destruction of nitrite becomes less important with greater amounts of nitrous acid. The steepness of the curve in Section B is highly influenced by the concentration of the catalyzing ions. The curve levels off (Section C) with amounts of nitrite much higher than equimolar to the amine.

To summarize the data reviewed here it can be said that experiments in vitro and in animals have contributed much during the last few years to the understanding of the nitrosamine formation in the stomach. We have good qualitative information on the nitrosation of amines and amides in vivo, but we need more quantitative measurements.

Some experiments in this direction are now underway in our laboratory.

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Formation of nitroso compounds in man: evaluation of the problem

J. Sander

Hygiene-Institut der Universität, D–74 Tübingen, W-Germany

Abstract

There are differences between the test animal and human stomach, implying a different suitability for formation of carcinogenic *N*-nitroso compounds. The possibility of nitrosamine formation in the human stomach is pointed out. Precursors, catalysts, but also inhibitors of the nitrosation reaction, like ascorbic acid, may easily be ingested in the daily diet.

To measure *N*-nitrosation in man, epidemiological investigations as well as with patients taking medical products, smokers and volunteers are feasible. In tests on his own body, the author did not find any dinitroso piperazine after ingestion of piperazine hexahydrate.

However, as long as the harmlessness of nitrite has not been proved, ingesting nitrite and amine should be held as risky. Ascorbic acid may play a protective role.

Introduction

There is little doubt that *N*-nitroso compounds are carcinogenic for man as they are for animals. It is also quite certain that, under suitable conditions nitrosamines or nitrosamides will be formed in the human stomach. But it is still unclear to what extent the ingestion of nitrosamine precursors may contribute to the induction of cancer in man.

There are several differences between the anatomy and biology of the stomach of man and experimental animals, therefore making it impossible to calculate the nitrosation of amines or amides in the human stomach just on the basis of animal experiments. Direct investigations in man will be necessary.

Differences in stomach anatomy and biology

Experiments on the nitrosation of amines or amides have to take into account that the vertebrate stomach is divided into functional sections. This division may or may not be strongly marked. The stomach of rodents for example is divided into a forestomach which serves mainly as a food store, and a glandular stomach where acid and gastric juice are produced. The human stomach does not show this distinct anatomic separation.

Experimental animals are normally healthy and usually young. In man, however, all ages and several diseases have to be considered. The gastric physiology in man is

known to change with age. For example, in babies acid secretion is very low. It is high in young adults but with old age the production of acid and other components of gastric juice is reduced. This has great influence on the nitrosamine formation for two reasons. One is the dependence of the nitrosation reaction on the pH of the medium. The second reason is the influence of the acid concentration on the metabolism of bacteria in the stomach. A neutral reaction allows bacteria to form nitrite from nitrate which is present in almost every meal. The amount of nitrite formed is dependent on the concentration of nitrate, the time available for the nitrate reduction and the number of bacteria present in the stomach contents. Food remains in the stomach only for a few hours. Therefore the multiplication of the bacteria in the stomach may be less important than the ingestion of nitrate reducing bacteria with food or saliva. Although complete anacidity yields maximum nitrite, the nitrosamine formation depending on bacterial nitrate reduction is favoured most by a moderate subacidity. Under these conditions at momentary optimum pH values nitrosation follows nitrite formation (Sander & Seif, 1969).

Precursors, *catalysts and inhibitors*

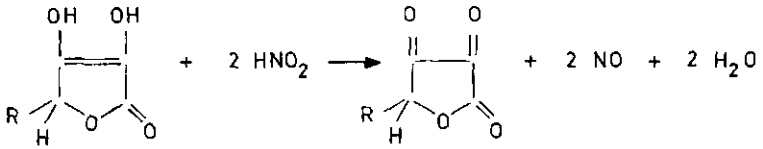
A great number of nitrosable amines and amides are known to occur in food. We do not yet have enough information, however, on the concentrations. Some nitrosable amino compounds are ingested in high doses as drugs (Sander, 1971; Lijinsky et al., 1972). Nitrite is found in the saliva and often also in gastric juice. It is formed, as mentioned, by bacteria in food or in the human organism from nitrate. Nitrate in turn is a component of drinking water and plant material. Besides this, nitrite and, to some extent nitrate, are used as food additives (Sander & Schweinsberg, 1972). It is quite likely that nitrite and nitrosable amino compounds do come together in the stomach in amounts sufficient to form a significant quantity of nitrosamines. The yield, however, will be strongly influenced by catalysts as well as by inhibitors.

As thiocyanate seems to be present in almost every human gastric juice (Schweinsberg, 1973), catalysis will always be an important factor in nitrosamine formation in the human stomach. Thiocyanate is not only formed in vivo in smokers from inhaled cyanide, but also independently from the smoking habit. Several precursors do occur in food, e.g., glucobrassicin which was found in cabbage (*Brassica* species). Savoy cabbage yields up to 300 mg/kg, cauliflower up to 100 mg/kg SCN^- (Lang, 1970).

Inhibition of nitrosamine formation is also very likely to be regularly an important factor in the nitrosation of amines or amides in the human stomach. The gastric juice contains very often substances which reduce 2,6-dichlorphenol-indophenol. This reduction may be due partly to the presence of ascorbic acid, partly to other lactones with similar structures, e.g. glucuronolactone and partly to other reducing agents (unpublised data). Ascorbic acid is also a constituent of many foodstuffs (Table 1). One mole of ascorbic acid destroys two moles of nitrite (Dahn et al., 1960).

Table 1. Amount of Vitamin C (mg/kg) in some selected food stuffs. (According to Souci-Fachmann-Kraut: Die Zusammensetzung der Lebensmittel, Stuttgart, 1962).

milk of cows	15
liver of cows	280
brain of pigs	180
fish	3–20
potatoes	120
kohlrabi *(Brassica oleracea,* var. gongyloides L.)	360
radish	180
asparagus	150
curly kale	540
Brussels sprouts	840
spinach	370
tomato	230
apple	110
grapefruit	110
bread	0
rice	0



ascorbic acid dehydro ascorbic acid

The reaction between nitrite and ascorbic acid is fast enough to inhibit the nitrosation of most secondary amines in vitro. In animals the inhibition is almost complete if equimolar concentrations of the amine, of ascorbic acid and of nitrite are applied (unpublished data). Very often the amount of ascorbic acid in the food exceeds the nitrite content. In these cases the nitrosamine formation will be very low. A very strong inhibition of the nitrosation of piperazine was also found in rats receiving ascorbate in a concentration lower than equimolar to nitrite (unpublished data). Because of the high reactivity of ascorbic acid towards nitrite, the concentration of Vitamin C and related compounds has to be measured in all experiments on nitrosamine formation in biological materials.

Ascorbic acid is very reactive not only towards nitrite but also towards a variety of oxidizing agents which may occur in food. Some foodstuffs contain a significant amount of Fe (III), e.g. spinach which readily is reduced by ascorbic acid to Fe (II). Very low concentrations of copper ions catalyse the autoxidation of ascorbic acid (Lehmann, 1971). The concentration of ascorbic acid in food is further strongly influenced by cooking, canning or storing.

Experiments in man

In addition to animal experiments, some experiments in man will be necessary, and they can be performed under certain conditions. Human gastric juice, urine or blood can easily be obtained for analyses. But who should be examined?

1. There are many patients who, for medical reasons, have to use certain drugs which may be nitrosated in vivo when ingested with normal food. Piperazine, for example, which is prescribed all over the world and which is taken orally in high doses as a vermicide offers an extremely good opportunity for examinations. Piperazine has the further advantage that it yields a relatively high amount of dinitrosopiperazine in urine, as has been shown in experiments with dogs (Sander et al., 1973). There are more drugs which can also be included in such an analysis (Lijinsky et al., 1972). Certainly the measurement of the corresponding nitrosamines in the gastric contents or in the urine will be the best analytical procedure in some cases, in others it will be more useful to look for metabolites. Besides that a toxicologic analysis of side effects of nitrosable drugs may help to solve the problem. There is extensive literature on the toxicity of such drugs. It is very likely that patients will co-operate in such a programme, as no disturbance of health will result from the examinations as such.

2. Volunteers are easily found among smokers who permit an analysis of their gastric juice. Experiments with smoking students which will soon be published, are underway in our laboratory to find out what is the meaning of nitrosamine formation from amines in tobacco smoke (Schweinsberg, unpublished).

3. There will be a few volunteers who will permit experiments with concurrent ingestion of amines and nitrite. I myself tried to demonstrate a nitrosation of piperazine in my saliva and in my stomach. In the first two experiments I took 10 mg of piperazine hexahydrate and the same amount of sodium nitrate. After ten minutes the saliva which was then strongly positive for nitrite, was alkalized and extracted twice with dichloromethane. Dinitrosopiperazine was not found, neither by thin-layer nor by gas chromatography.

Four more experiments were done to examine whether a nitrosation of piperazine in the stomach may be demonstrated. I swallowed 100 mg piperazine hexahydrate (Eraverm^{R}) twice with and twice without boiled ham (so called 'gekochter Schinken') which was always found to be exceptionally rich in nitrite. The highest amount was 60 g boiled ham. The nitrite content was 120 mg/kg. Ham and the drug were chewed and swallowed together. The gastric content was collected for 15 minutes, 25 minutes after swallowing the material. In the experiments without ham the gastric juice was collected by a stomach tube, in the other cases by vomiting. The pH value was in all cases below 3, although there were always some parts of the material, containing mainly saliva which reacted more or less neutrally. No dinitrosopiperazine was found.

4. Epidemiologic investigations may be among the most convincing examinations which can be done in man. They are, however, not easy to perform because they have to be based on a sufficient understanding of the interrelationship of the relevant chemical and biological factors influencing the nitrosamine formation.

Conclusions

The question as to what a possible nitrosamine formation in the human stomach may mean for the induction of cancer in man is far from being solved. Therefore we do not yet know how great the risk of ingesting nitrite and/or amines will be. Nitrite as a food additive has to be considered hazardous as long as its innocuous use has not been proved. Ascorbic acid may serve to inhibit nitrosamine formation not only in foodstuffs, but it is especially suitable to prevent nitrosation in vivo.

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Discussion

Nitrite formation in human saliva

Nitrite in the saliva of man is formed by bacterial reduction of nitrate taken in with food and drink. Whether nitrate gets into the mouth from the salivary glands is not known. Nitrite has not been found in saliva direct from the salivary glands. In saliva of dogs Sander could not detect nitrite.

Role of acidity of the stomach

Nitrosamine formation in the stomach, depending on bacterial nitrate reduction, is favoured most by a moderate sub-acidity. There might be a relation between this experimental finding and the epidemiological evidence that patients suffering from sub-acidity or anacidity more often develop tumours of the stomach and other organs than other people.

Inhibition of nitrosation in vitro

The nitrosation of secondary amines was not prevented by the simultaneous presence of primary amines. The same applied to amino acids and sugars. Ascorbate inhibited nitrosation very effectively. Comment of Dr Walters: cysteine and reduced glutathione did decrease nitrosamine formation, though with less efficiency than ascorbate. Dr. Sander had not tested these sulphydryl compounds.

Discussion of a contribution from E.O. Haenni[1]

Division of Chemistry & Physics, Office of Sciences, Bureau of Foods, Food & Drug Administration, 299 'c' St., S.W., Washington, D.C. 20204, USA

Drawbacks of high-resolution mass spectrometry

High-resolution mass spectrometry (resolution up to 14 000) was considered to be the most unambiguous method for the identification of nitrosamines. Comments of Dr Wasserman: high-resolution mass spectrometry failed to discriminate dimethylnitrosamine from the trimethylsilyl ion (difference in $^{m}/_{e}$ only 3 parts in 14 000). From existing literature it is known, that with low-resolution mass spectrometry dimethylnitrosamine may be erroneously assumed to be present, where in fact hydroxyacetone is present. In both cases, careful consideration of the complete low-resolution mass spectrum could prevent misidentification.

Nitrosamines and nitrite in bacon

In Dr Haenni's laboratory bacon samples are routinely examined for about 12 volatile nitrosamines. However, only dimethylnitrosamine and nitrosopyrrolidine have been found. This applies to fried bacon. None of these 12 nitrosamines have been detected in raw bacon.

It was not known, whether there was any difference in residual nitrite levels in the commercial bacon samples that contained widely differing amounts of nitrosopyrrolidine after frying. Nitrite had not been determined in these samples. In the literature there are no data, that indicate a correlation between levels of residual nitrite and of nitrosamines. Participants in the discussion strongly disagreed about this subject. There was a casual remark, that even 1 mg/kg of residual nitrite might perhaps be sufficient to produce nitrosamines in amounts of a few μg/kg.

It might well be, that nitrosoproline is present in raw bacon and that it is the precursor of the nitrosopyrrolidine in fried bacon, but both propositions remain speculative as yet.

1. This discussion was based on an article by Fazio, T., R. H. White & L. R. Dusold, 1973. Nitrosopyrrolidine in cooked bacon. JAOAC 56: 919–921.

The role of fat

Dr Haenni thought that nitrosamines tended to concentrate in the fat. Perhaps re-use of rendered fat was not advisable; perhaps it had better be discarded. In Dr Schram's view nitrosamines are actually formed in the fat, and probably in the fat only. When fat was rendered out of bacon fatty tissue at low temperature and then heated to 170°C, N-nitrosopyrrolidine was formed.

Conclusions and recommendations of the toxicological session 14th september 1973

1. Many N-nitroso compounds are powerful and versatile chemical carcinogens.

2. There is evidence that trace quantities of such carcinogens can be present in food among others in some cured meat products.

3. Carcinogenic N-nitroso compounds may be formed from nirosating agents such as nitrite and nitrosatable amino compounds in the gastrointestinal tract.
Model experiments with high concentrations of nitrite and certain amino compounds have shown to induce tumours characteristic for the corresponding N-nitroso compound. However additional chemical and biological experiments with more realistic amounts of precursors are lacking and have to be carried out.

4. There is a need for epidemiological studies concerning the human health hazard arising from N-nitroso compounds.

5. Where the continuous improving of the sensivity of analytical methods is leading to the revealing of so far undetectable quantities of carcinogens it is suggested to induce a safety factor for these carcinogens.
This safety factor should be much higher than that used for non-carcinogenic agents, having regard to unknown modifying factors such as synergism and promotion. Results from transplacental and single dose experiments and the knowledge that irreversible effects often occur, further strengthen this proposition.

6. From the present status of scientific knowledge it appears desirable to reduce the amount of added and residual nitrite where possible, but not in defiance of the relevant conclusions of other sessions, especially those of the microbiological session (protection against botulism hazard).

Resolutions

The participants of the International Symposium on Nitrite in Meat Products, held at Zeist, the Netherlands, from 10th till 14th September 1973, have accepted the following resolutions:

1. The discussion of problems concerning nitrite in meat products with a restricted number of scientists and public health authorities, from various countries and disciplines was considered fruitful.

2. All relevant information indicates that nitrite is currently an indispensable inhibitor of pathogenic microorganisms *(Clostridium botulinum)* in many meat products.
In addition nitrite plays a keyrole in colour formation and flavour development.
No adequate substitute is yet known.

3. The presence of nitrosamines in some meat products has been established. Sampling procedures and some analytical methods have to be improved to make results more representative and accurate.

4. Epidemiological studies related to ingestion of nitrosamines are desirable.

5. An inventory is needed of all meat products according to their nitrite content and relevant aspects of general composition, of manufacturing and merchandizing and of use by the consumer.
This could facilitate division of foods into categories appropriate for separate consideration as regards the necessity to use nitrite.

6. There are preliminary indications that the use of ascorbates decreases the risk of nitrosamine formation without impairing antimicrobial function.

Nitrate and nitrite allowances in meat products

Summary of some countries' regulations, made up September 1973

J. Meester

Central Institute for Nutrition and Food Research TNO,
Dept. Netherlands Centre for Meat Technology, Utrechtseweg 48, Zeist

Permitted	Products	Limits in mg/kg on addition = A in finished product = F
European Community (Directive Preservatives in Foods)		
KNO_3, $NaNO_3$, pure or mixed with NaCl $NaNO_2$, only mixed with NaCl	*no further specifications*	
Members European Community	*up to now no common regulation*	
Belgium		
KNO_3, $NaNO_3$	all	500, F, calc. as KNO_3
KNO_2, $NaNO_2$, only mixed (max. 0.6%) with NaCl	all	200, F, calc. as $NaNO_2$
Denmark		
KNO_3, $NaNO_3$	products not heated above 60°C	500, F, calc. as KNO_3
KNO_2, $NaNO_2$, only mixed (min. 0.4, max. 0.6%) with NaCl	all	100, F, calc. as $NaNO_2$
France		
KNO_3, $NaNO_3$, only mixed (max. 10%) with NaCl	all	no limit
$NaNO_2$, only mixed (0.6%) with NaCl	all	150, F, calc. as $NaNO_2$ (in freshly prepared brines mx. 210 g nitrite-salt per liter)

Permitted	Products	Limits in mg/kg on addition = A in finished product = F
West Germany		
KNO_3, $NaNO_3$	all, except raw unfermented comminuted products	600 KNO_3, A, or 500 $NaNO_3$, A, calc. on amount of meat and fat used
$NaNO_2$, only mixed (min. 0.5, max. 0.6%) with NaCl	all	no limit (combined use of nitrate and nitrate-salt only permitted in curing large pieces of meat, provided their ratio is max. 1 : 100)
Ireland		
KNO_3, $NaNO_3$	all	no limit
KNO_2, $NaNO_2$	all	200, F, calc. as $NaNO_2$, in cooked meat other than cured or pickled pork
Italy		
KNO_3, $NaNO_3$	all	250, F
$NaNO_2$, only mixed with NaCl	all	150, F
United Kingdom		
KNO_3, $NaNO_3$	bacon, ham pickled meat	500, F
KNO_2, $NaNO_2$	bacon, ham, pickled meat	200, F
Luxemburg		
KNO_3, $NaNO_3$	all	2000, F, calc. as KNO_3
$NaNO_2$, only mixed (max. 0.6%) with NaCl	all	no limit
the Netherlands		
KNO_3	all	2000, F
$NaNO_2$, only mixed (max. 0.6%) with NaCl (conditional exemptions for pure nitrite are granted)	all	500, F, calc. as $NaNO_2$
Some other European countries		
Austria		
KNO_3, $NaNO_3$	all	500 F
$NaNO_2$, only mixed (min. 0.5, max. 0.6%) with NaCl	all	200, F. calc. as $NaNO_2$ (combined use of nitrate and nitrite-salt only permitted in curing large pieces of meat, provided their ratio is max. 1 : 100)

Permitted	Products	Limits in mg/kg on addition = A in finished product = F
Finland		
KNO_3, $NaNO_3$	all	500, F
$NaNO_2$, only mixed (max. 0.6%) with NaCl (also $NaNO_2$ in max. 10% solution was allowed, but probably cancelled for 1973)	all	150, F
Norway		
KNO_3, $NaNO_3$	–some specified products (cured and dried meats e.g. bacon, dried ham; semi-preserves	500, A
	–some other specified products (e.g. meat-rolls, cooked ham, saveloy)	250, A
$NaNO_2$, only mixed (0.5–0.6%) with NaCl	–some specified products (cured and dried meats e.g. bacon, dried ham; semi-preserves)	200, A (provided no nitrate has been used)
	–some other specified products (e.g. meat-rolls, cooked ham, saveloy)	120, A (provided no nitrate has been used)
Sweden		
KNO_3, $NaNO_3$	all	500, F
$NaNO_2$, only mixed (max. 0.6%) with NaCl	all	200, F
Switzerland		
KNO_3, $NaNO_3$	all	60 g, A, per kg NaCl used in the product
$NaNO_2$, only mixed (max. 0.6%) with NaCl	all	200, F

Permitted	Products	Limits in mg/kg on addition = A in finished product = F
Some non-european countries		
Canada		
KNO_3, $NaNO_3$	*all*	*no limit*
KNO_2, $NaNO_2$	all	200, F, calc. as $NaNO_2$
Proposed revision (July 1973):		
KNO_3, $NaNO_3$	– dry and semi-dry sausage products	200, A, calc. as $NaNO_3$
	– slow-cure and specialty products (to be enumerated)	200, A, calc. as $NaNO_3$
KNO_2, $NaNO_2$	– cooked sausage products	200, A, calc. as $NaNO_2$
	– dry and semi-dry sausage products	200, A, calc. as $NaNO_2$
	– side bacon	150, A, calc. as $NaNO_2$
	– cured primal cuts, canned meats, slow-cure, and specialty products	200, A, calc. as $NaNO_2$
Japan		
KNO_3, $NaNO_3$	bacon, ham, sausage, corned beef	no limit
$NaNO_2$	bacon, ham, sausage, corned beef	70, F
United States		
KNO_3, $NaNO_3$	all	2188, A to meat (dry cure) 1719, A to chopped meat (in brines max. 8.4 g per liter, calc. at 10% pump level)
KNO_2, $NaNO_2$	all	625, A to meat (dry cure) 156, A to chopped meat (in brines max. 2.4 g per liter, calc. at 10% pump level) 200, F. calc. as $NaNO_2$