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Nitrite in curing - microbiological implications

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When man first cured meat is not known but the salting and drying of meat was known in Mesopotamia around 3,500 B.C. (Jensen, 1954). Although solar salt, that is salt produced by the evaporation of sea water, invariably contains nitrate, the reddening effect on meat of nitrate was not recorded until late Roman times (Jensen, 1954). It was not until the beginning of the 20th century (Haldane, 1901, Hoagland, 1914) that it was realized that nitrite is responsible for the red colour of cured meat, the nitrite being produced by the microbiological reduction of nitrate.

Antimicrobial effect of nitrate

Whether nitrate has any antimicrobial effect in its own right has been a matter for conjecture and experiment for sixty years or more. Jensen (1954) commented "in the field of microbiology of meats, there were few subjects over which there was more controversy than on the effect of sodium nitrate on anaerobic bacteria in meat". The great majority of the evidence obtained during the past fifty years has been that it has no effect other than that resulting from its ability to depress the a_w (McBryde, 1911; Grindley, 1929; Tanner & Evans, 1933; Yesair & Cameron, 1942; Tanner, 1944; Mundt & Kitchen, 1951; Bulman & Ayres, 1952; Beerens, 1955; Gough & Alford, 1965). Moulton (1930) and Moulton & Lewis (1940) presented data on the effect of nitrate on certain clostridia in the presence and absence of other curing salts and concluded that nitrate alone, at a concentration of 0.9%, had some inhibitory effect. However, as Gross, Vinton & Martin (1946) have pointed out, the conclusions of Moulton (1930) and Moulton & Lewis (1940) are not in accord with the data they give. Similarly, among many experiments indicating that nitrate is, by and large, without effect, Jensen & Hess (1941) reported some from which they concluded that nitrate had an effect on putrefactive anaerobes. These conclusions have been challenged by Anderton (1963) as not strictly in accordance with the results given.

Under some circumstances nitrate may show some antimicrobial effect. Grindley (1929) found marked bacterial inhibition by nitrate at low pH values, and ascribed this to the formation, in the presence of reducing substances, of nitrous acid. In the presence of heat, too, it may have an inhibitory effect on clostridial spores (Jensen, Wood & Jansen, 1934; Riemann, 1963). At relatively high concentrations antimicrobial effects against clostridia have become apparent. In a concentration of 4.4%, it checked the proteolytic activity of Clostridium putrefaciens (Lewis & Moran, 1928) and in a concentration of 6.6% it inhibited the growth of a number of clostridia, including Cl. botulinum (Tanner & Evans, 1933). Beerens (1955) reported that some clostridia were inhibited at a nitrate concentration of 1.5%, an observation, carried out in culture media with pure cultures, which seems unique.

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Recent pure-culture experiments carried out on the influence of nitrate on micro-organisms associated with the spoilage of pork and of bacon, namely Pseudomonas/Enterobacter and Micrococcus/Lactobacillus respectively have confirmed the absence of any inhibitory effect of 0.1% sodium nitrate at pH values of 5.0 to 7.0 and temperatures of 5°C. to 30°C., and even in the presence of up to 400 p.p.m. sodium nitrite. Indeed, 0.1% nitrate stimulated the growth of micrococci in the semi-anaerobic conditions in the depths of the culture medium, presumably by acting as an electron donor in place of atmospheric oxygen (Riddle, Hibbert & Spencer, 1969).

The production of bacon without the use of nitrate has been under investigation in many laboratories for some time and several types of such bacon are now in commercial production. The production of "Wiltshire" bacon without the use of nitrate presents rather more complex problems but these are now being solved. Investigations into the spoilage of "Wiltshire" bacon produced on a limited scale and under closely controlled conditions without the use of nitrate have shown that neither when stored as backs nor when stored sliced in vacuum packs, at either 21°C. or 5°C. did such bacon differ in its spoilage characteristics from bacon produced by the use of traditional Wiltshire brines (Evans, Hibbert & Spencer, 1971).

It has been suggested that the incorporation of nitrate in cured meats may tend to inhibit clostridia by poising the food at a sufficiently "oxidized" level (high Eh) that the clostridia cannot grow, or alternatively that nitrate may be converted to hydroxylamine which will inactivate catalase in meat so that hydrogen peroxide is formed. This hydrogen peroxide will then inhibit clostridia (Jensen, 1954). There is however, little direct evidence to support these suggestions.

It would thus seem highly likely that nitrate, in the concentrations in which it is present in cured meats, is without direct effect on bacteria and thus does not contribute directly to the stability or safety of the product.

Antimicrobial effect of nitrite

Following the discovery at the beginning of this century that the typical red colour of cured meat was a result of the presence of nitrite, it was not long before it was proposed that nitrite could replace nitrate in the curing of meats (Lewis, Vose & Lowery, 1925; Kerr, Marsh, Schroeder & Boyer, 1926). An understanding of the antimicrobial effect of nitrite in cured meats was to come very much later, although it was foreshadowed by the observation of Grindley (1929) that nitrate was inhibitory at low pH values and the deduction that this was due to the presence of nitrous acid.

Lewis & Moran (1928) reported that Cl. putrefaciens was not inhibited by 0.05% sodium nitrite but was by 0.2%. Tanner & Evans (Tanner & Evans, 1934; Evans & Tanner, 1934) in general concurred, finding no inhibition of a number of species of clostridia, including Cl. botulinum, in various media, until concentrations of nitrite of around 0.5% were used, even in the presence of up to 3.5% sodium chloride. Yesair & Cameron (1942), however, claimed to have demonstrated some inhibitory effect

against Cl. botulinum in media containing mixtures of curing salts including 50 to 156 p.p.m. nitrite, over and above that explicable on the basis of the sodium chloride present. In the light of subsequent work it should be pointed out that the above investigations were conducted in media or meat either of a stated pH around 7, or where the pH was not reported, or determined, but might be supposed to be around 7, and not less than 6.

The first real indication of the inhibitory effect of nitrite was given by Tarr in the early 1940's (Tarr, 1941a; 1941b; 1942) when he showed that the inhibitory effect against several species of bacteria was dependent upon the pH of the medium and increased markedly at pH values below 6.0. Even so, Cl. botulinum was able to grow in 200 p.p.m. nitrite at pH 5.6. Tarr did not explain the mechanism of inhibition and the first suggestion that it might be due to the undissociated nitrous acid was by Jensen (1945). Castellani & Niven (1955) demonstrated with Staph. aureus that the pH of the test medium influenced the inhibitory concentration of nitrite in a manner conforming to the view that the active substance was undissociated nitrous acid and this was subsequently confirmed by Eddy & Ingram (1956) with a Bacillus species and more recently by Perigo, Whiting & Bashford (1967) with Clostridium sporogenes. The earlier observations of Henry, Joubert & Goret (1954) that the optimum pH for antimicrobial activity of nitrite is around 5.6-5.8 is in agreement with this view for at lower pH values nitrite tends to be fairly rapidly lost, possibly by reaction with amino groups (the van Slyke reaction). Shank, Silliker & Harper (1962) confirmed that the antimicrobial effect of nitrite increases with falling pH to a maximum effect at pH values of 4.5-5.5. They also showed that nitric oxide was relatively ineffective against micro-organisms.

The observations that nitrite is more effective when ascorbate is added to the medium (Henry, Joubert & Goret, 1954) or when glucose is autoclaved in the medium (Castellani & Niven, 1955), both of which procedures might be expected to lower the redox potential, and also under anaerobic conditions (Eddy & Ingram, 1956), indicate that another factor may be involved in the antimicrobial effect of nitrite.

Pure culture experiments on the influence of nitrite on micro-organisms associated with the spoilage of pork and of bacon showed there to be a marked antimicrobial effect of nitrite on Pseudomonas, Enterobacter and Micrococcus, when the medium was at pH 6 or below. The strain of Lactobacillus examined was, however, found to be little affected by nitrite, even at concentrations of up to 400 p.p.m. at pH 5.0 (Riddle, Hibbert & Spencer, 1969).

Nitrite and food poisoning clostridia: The demonstration by Tarr (1941b, 1942) that Cl. botulinum was not inhibited by 200 p.p.m. nitrite at pH 5.6 was confirmed by Steinke & Foster (1951) working with liver sausage at pH 6.1-6.5. Abrahamsson (1964) reported that the growth of Cl. botulinum type E at pH 7.2 and 30°C. was partially inhibited by 170 p.p.m. of nitrite and was completely inhibited by 340 p.p.m., while Schmidt & Segner (1964) reported that at a pH of 7.0 and at 8°C., 200 p.p.m. nitrite did not inhibit Cl. botulinum type E. Pivnick et al. (1967) reported that in vacuum-packed tongue at 20°C., 450 p.p.m. nitrite was insufficient to inhibit the growth of Cl. botulinum types A and B in the presence of around 4% salt. Gough & Alford (1965) reported that 500 p.p.m. nitrite were necessary to prevent

the growth of Cl. welchii. Perigo et al. (1967) give data relating pH value to the concentration of nitrite necessary to prevent the growth of Cl. sporogenes, for example the ED50 value (concentration of nitrite most likely to inhibit 50% of the inocula) at pH 7.0 was about 300 p.p.m., at pH 6.5 about 150 p.p.m., at pH 6.0 about 50 p.p.m.

Thus nitrite is a potent antimicrobial substance - to which lactobacilli, however, may be resistant - and is likely to play an important role in the stability of uncooked cured meats and in the stability and safety of cooked cured meats. So far as the stability and safety of cooked cured meats are concerned, however, it can not be fully responsible for it is present in too low concentrations to inhibit the growth of clostridia at the pH values usual in such meat. Some other factor or factors are involved, probably in conjunction with nitrite for it is common experience that when nitrite is omitted from such products, the mechanism of stability at least fails.

The influence of heat on the antimicrobial effect of nitrite:

Stumbo, Gross & Vinton (1945b) used an Fo = 1 process (i.e. a heat process equivalent in sterilizing effect to an exposure of 1 minute at 121.1°C.), an inoculum of spores of putrefactive anaerobe PA 3679 of 5 per g, and investigated the effects of sodium chloride, nitrate and nitrite. They concluded that a concentration of sodium chloride of 3.5g/100g meat (approximately 4.5g/100g water) markedly inhibited spoilage and that the addition of nitrate and nitrite had little extra effect.

Steinke & Foster (1951) worked with liver sausage containing various amounts of sodium chloride and nitrite. They inoculated the meat with various levels of Cl. botulinum spores, cooked it at 70°C. for 70 minutes, and incubated it at 30°C. They found that with an inoculum of 5,000 spores per g, 2.5g sodium chloride per 100g meat (approximately 5g/100g water), and 200 p.p.m. nitrite added to the uncooked meat, no toxin developed within thirty days.

Bulman & Ayres (1952) used raw pork trimmings to which were added various concentrations of curing salts and, in some cases, spores of putrefactive anaerobe PA 3679. The meat was heated in tubes which were held for 20 minutes at an internal temperature of 80°C. These workers found that a concentration of sodium chloride of 4.6g/100g meat (approximately 5.8g/100g water) was sufficient to prevent spoilage occurring within sixteen weeks at 37°C. with a inoculum of 50 spores/g; that 800 p.p.m. nitrite in the absence of sodium chloride and with an inoculum of 50 spores/g delayed spoilage, but that the nitrite content fell to 4 p.p.m. after sixteen weeks and that spoilage was then occurring; that 3.6% sodium chloride, with 150 p.p.m. nitrite (83 p.p.m. remaining after the heat process) prevented spoilage with a natural level of spores below 1/g, but failed at an inoculation level of 50/g.

Silliker, Greenberg & Shack (1958) used commercially-produced cured pork trimmings, naturally contaminated and with low added levels of spores of PA 3679, of known sodium chloride and initial nitrite concentrations, and processed to Fo values of 0.08-0.13. They showed that with a heat process of Fo = 0.13, and 3.5% sodium chloride (calculated on the water phase of the meat), 78 p.p.m. nitrite was effective against natural contami-

nation but 39 p.p.m. nitrite led to spoilage. When spore levels were increased to 2.6/g, an Fo value of 0.1 with a concentration of sodium chloride of 3.5% and 78 p.p.m. nitrite was again satisfactory. Post-processing nitrite levels were given as 10-20 p.p.m. They also found that 5% sodium chloride with no nitrite did not prevent spoilage at Fo values of around 0.1. These workers emphasized that the protective mechanisms broke down when challenged with large numbers of spores.

Lastly there is the unpublished work briefly described by Riemann (1963). In one series of experiments the spoilage of commercial luncheon meat containing less than 3 clostridial spores/g, 4g sodium chloride/100g meat, nitrite concentration not stated, was investigated after various processes. It was concluded that a process of Fo = 0.4 was necessary. Riemann also refers to a more detailed factorial experiment in which the factors tested were salt, nitrite, nitrate, pH and Fo values, using various inoculation levels of putrefactive anaerobic spores from spoiled canned luncheon meat. The number of spores germinating to give vegetative cells after various time intervals was determined. It was concluded that all the factors except nitrate had an effect on the number of spores which were able to initiate growth and that there were certain significant interactions, e.g. heat process with sodium chloride and heat process with nitrite. Details of the effective values of the various factors were not, however, given.

These investigations are summarized in the Table.

The antimicrobial effect of nitrite following a heat treatment of spores: Jensen & Hess (1941) suggested that those interactions between the heat process and curing salts were a result of an increase in the heat sensitivity of the spores due to the curing salts. It is now clearly established that this is not so. Yesair & Cameron (1942) were unable to detect any increase in heat sensitivity of spores of Cl. botulinum type A in the presence of curing salts, nor were Stumbo et al. (1945a, b), Silliker, Greenberg & Shack (1958), or Roberts, Gilbert & Ingram (1966) with sodium chloride alone, and with Cl. sporogenes. Additionally, the heat processes used in practice to achieve stability of those shelf-stable canned cured meats, Fo values of about 0.5 (Riemann, 1963; Spencer, 1966), do not succeed even in the presence of curing salts, in eliminating viable clostridial spores from the final, stable, product (Ingram, 1952; Brown, Vinton & Gross, 1960; Riemann, 1963; Steinkraus & Ayres, 1964). It thus appears likely that the form this interaction takes is one of the curing salts preventing the germination and/or outgrowth of the surviving spores which have in some way been made sensitive to the effect by the heat treatment (Brown et al., 1960; Riemann, 1963; Spencer, 1966). Recent investigations have indicated that this form of interaction is indeed an important one in the stability and safety of canned cooked cured meats.

Roberts & Ingram (1965) reported the results of an investigation into the effect of sodium chloride, potassium nitrate and sodium nitrite in the medium on the ability of heated spores of bacilli and clostridia (excluding Cl. botulinum and Cl. welchii) to develop and produce visible growth. This demonstrated unequivocally that these substances interfered with some stage in the germination and development of surviving heated spores, at

concentrations which would be ineffective with unheated spores. From the similarity between the effects of sodium chloride and potassium nitrate, it was considered that their action might in some way be osmotic, depending fundamentally on water activity. Nitrite, however, clearly operated on a different basis and its action was very dependent on pH value and may possibly have been due to undissociated nitrous acid.

The investigations of Roberts and his colleagues (Roberts & Ingram, 1965; Roberts et al., 1966) have been confirmed by Duncan & Foster (1968). Additionally, these workers demonstrated that heating spores in the presence of nitrite reduced their ability to give rise to growth subsequently in the absence of nitrite.

Curing Processes Shown Experimentally to be Stable and/or Safe

Reference	Heat Process	Sodium Chloride Concentration (% on Water Phase)	Initial Nitrite Concentration (p.p.m. on Total)	Spore Level/g	Comments
Stumbo et al. (1945)	Fo = 1	About 4.5	150	5	Spoilage inhibited for up to one year
Steinke & Foster (1951)	65.5°C /70 min	About 3-3.5	200	5,000*	No toxin after 30 days at 30°C.
Bulman & Ayres (1952)	80°C/ 20 min.	3.6	150/83	Natural 1	pH 5.8 ± 0.2. Failed when challenged with 50 spores/g
Silliker et al (1958)	Fo=0.08 Fo=0.10	3.5 3.5	78(10-20) 78(10-20)	Natural) 1, added 2.6)	Failed when containing only 38 p.p.m. nitrite
Riemann (1963)	Fo=0.4	4.02	Not stated	3	

* Cl. botulinum
The nitrite figures in brackets refer to post-processing levels.

Inhibitory effect of nitrite when heated:

A new chapter in the story of the antimicrobial effect of nitrite was commenced by the investigations of Perigo, Whiting & Bashford (1967). These workers used a model system of a complex bacteriological medium in place of meat and investigated the ability of nitrite to inhibit the growth of Cl. sporogenes at various pH values:

1. when nitrite was added as a sterile solution to the heat sterilised medium, and
2. when the nitrite was heated in the medium.

Perigo et al. found that 200 - 400 p.p.m. of nitrite was necessary to cause inhibition of Cl. sporogenes at pH values around neutrality in their experimental system when the nitrite

was added to the heat sterilised medium. However, when the nitrite was heated in the medium for 20 min. at 105 - 115°C. only 3 - 5 p.p.m. nitrite was necessary to inhibit growth and the inhibitory concentration was largely independent of the pH of the medium. Perigo et al. postulated that when nitrite is sufficiently heated in the medium it is involved in a reaction which results in the production of some unknown inhibitory substance (or substances) which differs from inorgance nitrite in three important respects:

1. its inhibitory activity is only slightly pH dependent,
2. it elicits a much less variable response from the test organism,
3. it is a far more important inhibitor than is inorganic nitrite.

They sug ested that this inhibitory substance may play an important role in the stability of certain sublethally processed cured meats. Perigo & Roberts (1968) subsequently examined the activity of nitrite heated in "Perigo" medium against a range of clostridia including various types of Cl. botulinum and obtained results similar to those obtained by Perigo et al. (1967) using Cl. sporogenes.

Johnston, Pivnick & Samson (1969) considered four possible roles for nitrite in the stability of cooked cured meats:

1. the enhanced destruction of spores by heat,
2. an increased rate of germination of spores during the heat process followed by death of the germinated spores from the heat process,
3. the prevention of germination of spores that survived the heat process,
4. the production of an inhibitory substance from the reaction of nitrite with some component of the meat during the heat process.

They investigated the latter role using a 50% w/v suspension of meat blended with a culture medium and heated with various concentrations of nitrite up to 200 p.p.m. This substrate was then challenged with Cl. botulinum. The inhibition found was no greater than that expected from the residual nitrite in the substrate. Additionally, they found that while the Perigo inhibitor in medium was not dialysable, the inhibitory substance in meat was dialysable, and suggested thatthis was nitrite itself. They concluded that a Perigo inhibitor was probably not produced during the heat treatment of meat which contained nitrite and hence was of little relevance to explaining the role of nitrite in the stability and safety of canned cured meats. Labbe & Duncan (1970) also expressed the view that nitrite does not combine with some component of meat during heating to produce an inhibitory substance, although they confirmed the Perigo effect in media, using Cl. welchii as the test organism.

The Perigo effect, both in media and in meat, has been investigated extensively at B.F.M.I.R.A. for several years. In experiments to determine whether a Perigo effect occurs in meat, minced lean leg pork was added in 25g. amounts to small screw-capped bottles so as to almost fill them. Some bottles were inoculated with a solution of sodium nitrite to give a concentration in the meat of 150-600 p.p.m. and heated at 115°C. for 20 minutes. Other bottles were heated and when cold were inoculated with a filter - sterilised solution of sodium nitrite to give a concentration in the meat of 75-300 p.p.m.

After overnight storage at 21°C. in the dark, bottles were inoculated with a vegetative culture of Cl. sporogenes, incubated and examined at intervals for growth. Invariably, lower residual concentrations of nitrite inhibited growth when the nitrite had been heated in the medium than when the nitrite was added after the medium had been heated. The diagram shows the results of a typical experiment.

The ED_{50} values, i.e. the concentration of residual nitrite necessary to inhibit growth in 50% of the challenged tubes, has been used to assess the effect of nitrite in different systems. The difference in the ED_{50} values between the nitrite concentrations added before and after heating was generally of the order of 30-60 ppm. The ED_{50} value of heated nitrite was of the order of 50-150 ppm residual nitrite, for which it was necessary to add 200-400 ppm nitrite to the meat before it was heated. The high initial concentration of nitrite necessary to produce an inhibitory effect suggests that the Perigo effect is not likely to be of importance in practice to the stability and safety of canned cured meats and provides an explanation for the failure of other workers, e.g. Johnston et al. (1969), to observe a Perigo effect in meat.

There still remains the intriguing question - what is the nature of the substance or substances responsible for the Perigo effect in media and in meat? Work at B.F.M.I.R.A. has shown that the effect might be caused by certain inorganic substances developed from nitrite the nature of which are currently under investiation.

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QUANTAL GROWTH RESPONSE OF CL. SPOROGENES

IN MINCED PORK · WITH ADDED SODIUM NITRITE