THE FATE OF NITRITE IN MEAT CURING

AND THE DETERMINATION OF NITROSAMINES

A thesis presented by

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SUMMARY

The metabolism of sodium nitrite by porcine skeletal muscle has been investigated in vitro under conditions relevant to the meat curing process leading to bacon and pasteurised ham, i.e. at pH 6.0 under reducing (anaerobic) conditions with nitrite at 3mM, (200 ppm). Other concentrations of nitrite were used to include the range relevant to curing and beyond. The rate of loss of nitrite from model cures has been studied.

In order to produce a 'balance sheet' of the nitrite added to model curing systems, the only stable isotope of nitrogen, $^{15}$N, was used as a tracer. Nitrite, labelled to 96.1% with $^{15}$N was found to be incorporated into both the non-water soluble fraction and the non-nitrite water soluble fraction of minced muscle cures. Analysis of the fractions revealed that enrichment was for the most part due to the formation of S-nitrosothiol derivatives and nitrosylmyoglobin. Analysis of the fractions also revealed the formation of nitrate, and non-volatile N-nitrosamines, the latter in concentrations up to 2 ppm. Total recovery of the $^{15}$N label was between 52 - 100%. Two methods of analysis of the $^{15}$N were employed, mass spectrometry and emission spectroscopy. Analysis of the headspace gases of the model cures by high resolution mass spectroscopy confirmed the formation of nitric oxide.

Volatile N-nitrosamines have been unequivocally identified by the development of two methods, both involving the use of high resolution mass spectroscopy and gas chromatography, in samples of foodstuffs preserved with nitrite prepared both commercially and in the laboratory.
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MATERIALS.

All chemicals, other than those listed below were obtained from Fisons Chemicals Ltd. Loughborough, Leics. and were Analar grade or equivalent.

N-1-Naphthylethylenediamine and sulphanilamide were obtained from Sigma (London) Chemical Co.

Plate count agar was obtained from Oxoid Ltd. London.

Chloromycetin (Chloramphenicol) from Parke-Davis and Co Ltd. Hounslow, London.

Gases were from British Oxygen Ltd. Croydon.

$^{15}$N labelled sodium nitrite was from Prochem Ltd. Croydon.

Copper wire, copper sheet and Molecular Seive was obtained from B.D.H. Chemicals Ltd. Poole.

Nitric Oxide from Matheson Gas Products, Rutherford N.J.

Heptacosfluorotributylamine was from Koch-Light Chemicals Ltd. Colnbrook, Bucks

Methylurea, allylurea, butyric acid hydrazide, N-2-aminoethylmorpholine and 1-piperazinecarboxaldehyde were obtained from R.N. Emmanuel Ltd. Wembley.

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PART 1

INTRODUCTION
INTRODUCTION

CHAPTER 1

1. Sodium nitrite and the curing of meats.

Cure: preserve (meat, fruit, tobacco) by salting, drying, etc. (from Latin curare 'to take care of') - Concise Oxford Dictionary.

The use of sodium nitrite as a preserving agent of food has as its origin the practice of adding rock salt to meat and fish that had to be stored. As an illustration of the antiquity of this process, tally lists of foods kept in temple precincts in early Sumarian times (3000 B.C.) show dried salted meat, dried and salted fish and various dried seeds. In the ancient Jewish kingdom, salt was in common use owing to the excellent supply from the Dead Sea. The use of salt as a preservative was long practised in ancient Greece. It was obtained from salt gardens, though the people also knew of rock salt. The ancient Romans learned the use of salt from the Greeks and they used it extensively in curing pork and fish. The desert salts, as produced by the ancients, often contained nitrate and borax as impurities, but the reddening effect of nitrate on meats is not mentioned in the literature of antiquity until late Roman times.

In 1891, it was discovered that nitrite present in cured products was produced through the reduction of added nitrate by organisms in the meat. (Polenski, 1891). This was soon followed by work from Lehmann in 1899, who established that the pink colour of cured meat was due to the action of nitrite and not nitrate. The mechanics of the colour reaction were finally clarified by Haldane; in 1901, as a combination of nitric oxide with meat pigments. Subsequently, the colouration of the meat was deliberately produced by adding sodium nitrite itself, because it improved
the speed and effectiveness of producing the requisite red or pink

It has been shown that nitrite exerts a definite preservative
action, and is critical to the safety and stability of cured meats,
(Pivnick et al., 1967; Tarr, 1961). The principal use of nitrite
is as a protecting agent against the growth of Clostridia (Spencer, 1966),
the development of which could lead to the production of toxins in cured,
vacuum-packed or certain canned meat products. The ingestion of these
toxins could be fatal to the consumer, (Creigh, Whiting and Bashford 1967;

Consequently, nitrite is used in many countries as a food additive.
In Britain, its use is permitted in cured and pickled meat products and
also in some cheeses, the maximum permitted concentration of sodium or
potassium nitrite allowed in meat products being 200 ppm and in cheese,
10 ppm (Preservatives in Food Regulations, 1962).

The limits were originally implemented because of the toxic actions
of nitrite. It is a vasodilator and hypotensive agent (Rubin, Zitowitz
and Hausler, 1963), and can reduce vitamin A stores in the liver and
disturb thyroid function (Emerick and Olson, 1962). Also of importance
is the ability of nitrite to oxidise haemoglobin to the ferric form,
methaemoglobin, which is incapable of transporting oxygen. Infants are
particularly susceptible to nitrite because foetal haemoglobin, present
in children under 3 years of age (Betke and Rau, 1952), is more readily
oxidised to methaemoglobin, than is adult haemoglobin, and they
may be deficient in the erythrocyte enzyme, glucose-6-phosphate dehydrogenase,
which facilitates reduction of methaemoglobin back to its ferrous form.
Methaemoglobinaemia (sometimes fatal) has been caused by the lack of this enzyme, after the ingestion of (a) water containing nitrate, (Wood, 1961), (the nitrate being reduced by microbial flora of the achlorhydric stomach), and (b) of food containing nitrate, (the nitrate being reduced to nitrite during preparation of the baby's feed or under unhygienic methods of storage) (Simon, 1966).

1.2 The discovery of nitrosamines in animal feeds.

The toxic actions of nitrite were, however, overshadowed by the occurrence, in 1961–2, of an outbreak of liver disease in ruminants and mink, now known to have been caused by the presence of a compound formed from sodium nitrite added to the feed of the ruminants, (Ender et al., 1964; Sakshaug et al., 1965). It was found that the feed, fishmeal, had been preserved by heating with high levels of sodium nitrite, and that the fishmeal contained N-nitrosodimethylamine – a hepatotoxic nitrosocompound, (Barnes and Magee, 1954; Magee and Barnes, 1956) (Eqn 1.1). Nitrosamines have been shown to produce tumours in many organs of the rat and have the capacity to induce cancer after a single administration.

\[
\begin{align*}
\text{CH}_3 & \quad \text{N} \quad \text{H} \\
\text{CH}_3 & \quad \text{N} \quad \text{NO} + \text{H}_2\text{O}
\end{align*}
\]

Eqn 1.1

Dimethylamine \quad N\text{-}nitrosodimethylamine

The metabolism of N-nitrosodimethylamine seems to be necessary for its toxic action as it appears that the active molecular species is a derivative of the parent nitrosamine (McLean and Magee, 1970). N-nitrosodimethylamine is an alkylating agent of nucleic acids in vitro and several carcinogens of this type are very powerful mutagens (Magee and Barnes, 1967). These observations have been extended to a considerable range of volatile and non-volatile nitrosamines by other workers.
KrucKrey et al., 1967; Weisburger et al., 1966), using various species of animals and routes of administration. N-nitrosodiethylamine was proved to be carcinogenic in all eleven animal species tested, including monkeys (Schmahl and Osswald, 1967).

It has been suggested that the formation of N-nitrosamines from the nitrosation of secondary and tertiary amines in vivo, from secondary amines and nitrite when mixed together in human gastric juice, may represent a greater danger than the ingestion of N-nitrosamines which may be formed in vitro. Hawksworth and Hill, (1971) have reported the formation of the nitrosamine derivatives of dimethylamine, pyrrolidine and piperidine, by human intestinal bacteria in the presence of nitrite and the above amines, at neutral pH values.

Tumours were not induced in rats when nitrite and dimethylamine were administered concurrently (Sander, 1968), although this may have been due to the high basicity of the amine since the substitution of dimethylamine by the less basic methylbenzylamine produced oesophageal cancers. Malignant tumours of the oesophagus were also induced by N-nitrosomethylbenzylamine, the corresponding nitrosamine, (Sander and Burkle 1969). Nitrosation of highly basic secondary amines is not favoured in the weakly acid conditions which prevail in the stomach. In fact, Mirvish (1970), has calculated from kinetic measurements that the formation of N-nitrosodimethylamine in the stomach after consumption of a meal containing dimethylamine (40ppm) and sodium nitrite (200ppm) would only be 3.3 μg/300g meal. Mirvish (1971) has also shown the relative nitrosation rate increased 180,000 times on proceeding from the piperidine (pKa 11.2) to piperazine (pKa 5.57) from the stronger to the weaker base, at the optimum pH for these nitrosation reactions. In the same study, Mirvish also showed that of the group of bases, morpholine, piperazine, N-methylaniline, ethylurea, methylurea and dimethylamine, only the strongest
base, dimethylamine, failed to induce lung adenomas in mice, to which each amine was fed along with nitrite in the food or drinking water.

Lijinsky and Epstein (1970) have postulated that cooking may liberate nitrosatable secondary amines, such as pyrrolidine and piperididine, and free amino acids such as proline and hydroxyproline. They further point out that both proline and hydroxyproline are nitrosated in dilute acid solution at 35° - 40° in the presence of an excess of sodium nitrite, in each case the yield exceeding 50% of the theoretical value. The possibility exists of N-nitrosoproline and N-nitrosohydroxyproline being decarboxylated, possibly bacterially, in the alkaline conditions prevailing in the duodenum and small intestine, yielding the highly carcinogenic N-nitrosopyrrolidine, (eqn 1.2).

Another possible source of nitrosatable amines is the large number of drugs and flavouring agents (Lijinsky and Epstein, 1970) that are secondary or tertiary amines. For example, the drugs piperazine (used as an anthelmintic) and desmethylimipramine (used as an antidepressant) are secondary amines. Greenblatt et al., (1972) have shown the in vivo conversion of phenmetrazine (3-methyl-2-phenyl morpholine), commonly used for appetite control, to its N-nitroso derivative at pH 3.5, from a mixture of
the amine and sodium nitrite injected through the stomach wall of rabbits.

Lijinsky and Greenblatt (1972) have also implied in vivo production of N-nitrosodimethylamine from nitrite and aminopyrine, (4-dimethylaminantipyrine) an analgesic. Mixtures of sodium nitrite and aminopyrine in solution were administered to rats and their livers examined after 68 hours. Severe centrilobular necrosis, typical of the effect of N-nitrosodimethylamine was seen in all cases.

It would seem that the possibilities of the production of nitrosamines from secondary and tertiary amines, introduced one way or another into the diet, are virtually endless. There is still doubt, however, as to the mechanism of formation of nitrosamines in cured products. Although the levels of secondary amines (notably dimethylamine) in fish are quite high, the levels of free amines in muscle cannot account for all the N-nitrosodimethylamine that can be produced. Archer et al. (1971), have published findings that creatine, and creatinine, both present in the muscular tissue of many vertebrates react with nitrite under acid conditions. Creatine was shown to react to produce first, sarcosine, and then N-nitrososarcosine, a weak carcinogen in the rat, according to the following possible reaction scheme (eqn 1.3)

\[
\text{NH}_2 \text{H}_2 \text{N}-\text{C}-\text{N}-\text{CH}_2-\text{CO}_2 \text{H} \xrightarrow{\text{HNO}_2} \text{H}_2 \text{N}-\text{C}-\text{N}-\text{CH}_2-\text{CO}_2 \text{H} \xrightarrow{\text{HNO}_2} \text{HO}-\text{C}-\text{N}-\text{CH}_2-\text{CO}_2 \text{H} \xrightarrow{-\text{CO}_2} \text{CH}_3 \text{N}-\text{CH}_2-\text{CO}_2 \text{H}
\]

\[
\text{Eqn. 1.3}
\]

Nitrososarcosine

Plumridge and Walters (1974) have observed the formation of N-nitrosodimethylamine as one of the nitrosation products of the nitrite-sarcosine reaction, so that a pathway for the synthesis of N-nitrosodimethylamine can be envisaged, with creatine, an abundant constituent of muscle, as a precursor.
It has been found by Telling et al. (1973) that cooking temperature had an effect on the amount of N-nitrosopyrrolidine found in fried bacon samples, most N-nitrosopyrrolidine being liberated at the highest temperature studied, 200°C. This observation can be linked to the reaction involving the decarboxylation of N-nitrosoproline to N-nitrosopyrrolidine, (eqn 1.2).

It should also be mentioned perhaps, that several naturally occurring nitrosamines have been discovered. N-nitroso-paramethylaminobenzaldehyde has been found in the edible mushroom *Clitocybe Suaveolens* (Hettmann, 1961) and N-nitrosodimethylamine has been reported in the fruit of an African solanaceous bush, the juice of which is consumed, (Du Plessis et al., 1969). The diabetogenic antibiotic, streptozotocin, isolated from *Streptomyces achromogenes* is a nitrosamide, and has given rise to kidney tumours in the rat, (Avason and Fendale, 1967). However, the identity of this compound has not been established unequivocally, for example, by the use of mass spectrometry.

The fact that nitrosamines may occur naturally does not lessen the potential hazard of adding nitrite to food products. Every effort must be made to judge the safety of cured foods from the point of view of both microbiological stability and carcinogenic potential. Working along these lines, the Toxicology Sub-Committee and the Food Additives and Contaminants Committee met at the British Food Manufacturing Industries Research Association in 1968, and a proposed programme of research was drawn up. The work reported in this thesis has evolved from some of the recommended lines of research.

Throughout the world, analyses of food products have been carried out, using various methods of detection of nitrosamines.
These methods are reviewed in the next section of this chapter.

1.3 The analysis of human foods for nitrosamines - methods

In spite of the wide range of techniques available to the analytical chemist, the development of sufficiently sensitive and specific analytical methods for the detection of nitrosamines, at the very low levels deemed to be necessary, has proved difficult. This is reflected in the large number of methods that have been used, with varying success, for nitrosamine enrichment, identification and quantitative determination.

1.3.1 Methods of isolation

a) Solvent extraction and partitioning methods

Dichloromethane is the standard solvent for nitrosamine work because of its superior properties. The partition coefficients using CH$_2$Cl$_2$ have been more in favour of the nitrosamine than with other immiscible solvents, particularly with the lower molecular weight nitrosamines. Dichloromethane has been used either for direct extraction of food samples in a Soxhlet or blender (Marquadt and Hedler, 1966; Thewlis, 1967; Eisenbrand, 1972; Sen et al., 1972), or for liquid-liquid extraction of distillates (Crosby et al., 1972; Alliston et al., 1972).

b) Distillation methods

Many investigators have taken advantage of the volatility of nitrosamines to separate them from biological materials. The first to use this technique were Heath and Jarvis (1955) when they separated N-nitrosodimethylamine from animal tissues by steam distillation of alkaline
homogenates Examination of the distillation rates of 16 different nitrosamines showed no significant differences in the respective yields on distillation at reduced or atmospheric pressure from acidic, alkaline or neutral media (Eisenbrand et al., 1970a). Because of this, steam distillation has become a basic step in most enrichment and purification procedures, although a steam distillation from an alkaline milieu would be a necessary preliminary before one from an acid environment, to prevent the formation of nitrosamines as an artifact from any nitrite present.

Casselden et al., (1969a) applied fractional distillation to concentrate nitrosamines from aqueous methanolic solutions. The method was found to be applicable to dialkyl, alkaryl and heterocyclic volatile nitrosamines with the notable exception of N-nitrosopyrrolidine. Crosby et al., (1972) used a similar technique to examine East African spirits for nitrosamines. Vacuum steam distillation has also been used for nitrosamine enrichment with varying degrees of success, (Lydersen and Nagy, 1967; Devik, 1967; Williams et al., 1971; Scanlan and Libbey, 1971).

An advanced modification of the vacuum distillation technique was described by Telling et al., (1971) and Telling, (1972). Since the distillation procedures used by these authors were mild (weakly alkaline medium, moderate temperatures), the resulting distillates were less contaminated with interfering compounds. Recoveries of even highly volatile nitrosamines have been found to be satisfactory under these conditions.

Another technique frequently used for nitrosamine extraction is an alkaline-methanolic digestion of the sample, followed by dichloromethane extraction of the digest. The resulting extract is further purified by steam distillation from alkaline medium (Howard et al., 1970; Fazio et al.,
This technique has been used by several workers and has also been found to be satisfactory, since it should ensure the rupture of any hydrogen or other bonds between nitrosamines and the components of the food matrix.

c) **Methods of further purification**

Thin layer chromatography and column chromatography on various sorbents have been utilised for further purification of extracts. Multiple thin layer chromatography on silica-gel has been applied in some cases (Marquardt and Hedler, 1966), but was shown by others to result in high losses of volatile nitrosamines (Schuller, 1969; Sen et al., 1969; Eisenbrand et al., 1970).

Thin layer chromatography, using strictly controlled conditions can also be used for quantitative work (Eisenbrand et al., 1970b). However, column chromatography is much more frequently used than thin layer chromatography, Howard and co-workers (1970) applied an acid treated celite column for purification of concentrates when they examined smoked fish for N-nitrosodimethylamine. A more versatile technique, consisting of a silica-gel purification, was introduced by Fazio et al. (1972), and facilitated the multidetection of a wide range of volatile nitrosamines. Acid treated florisil (Wasserman et al., 1972) polyamide and basic alumina (Sen, 1972; Sen and Dalpé, 1972) have also been used for purification purposes.

Telling (1972) has recently presented results, using a method of purifying concentrates first on a column of neutral alumina and then by oxidising the nitrosamines in the eluates to the corresponding nitramines. These were again purified on another column of neutral alumina to yield
1.3.2 Methods of detection and estimation

A wide range of methods has been developed and applied in the detection and estimation of nitrosamines, but only a few have the sensitivity and specificity now known to be required. The available methods can be divided into methods of determination of intact nitrosamines and methods of preparation and determination of nitrosamine derivatives.

a) Determination of intact nitrosamines

Preussmann et al., (1964) developed a thin-layer chromatography method as a simple and convenient tool for rapid nitrosamine analysis. The nitrosamines were detected as inorganic nitrite with the Griess reagent after irradiation with ultraviolet light, or with diphenylamine palladium (II) chloride followed on thin-layer chromatographs by reaction with ninhydrin. Sen et al., (1972) used Griess and ninhydrin reagents for semiquantitative determination of volatile nitrosamines in alcoholic beverages, after irradiation on a thin-layer chromatography plate. They also tested similar plates without irradiation to observe other ninhydrin reacting compounds in the extracts under examination.

Many methods that have been developed for quantitative nitrosamine determinations are only applicable if interfering compounds are absent. This is true of ultra violet spectroscopy, of polarography in its different modifications (Heath and Jarvis, 1955; Walters et al., 1970), as well as for gas chromatography with either a specific or non specific detector.
A colourimetric method described by Daiber and Preussman (1964) and modified by Sander (1967a), relying on the photolytic splitting of the N-NO bond at alkaline pH has been used mainly in simple model experiments. Many compounds other than nitrosamines can give rise to nitrite on irradiation and on the other hand, other components of biological origin can act as receptors to the liberated nitrite. Nevertheless the modification by Sander (1967) restricting the wavelength of irradiation to one higher wavelength (366 nm) has been claimed to enhance the specificity. An automated version of this method was published by Fan and Tannenbaum (1971).

Another colourimetric method takes advantage of the facile acid-catalysed denitrosation of nitrosocompounds by hydrogen bromide in glacial acetic acid (Eisenbrand and Preussman, 1970). This method is said to give consistent results, and may be used to determine the number of N-nitroso groups and/or molecular weights of pure nitrosamines. It requires, however, the virtual absence of water, alcohols, etc. This method has been shown by Johnson and Walters (1971) to be highly specific for nitrosamines and nitrosamides, provided adequate control is taken of compounds such as alkyl nitrites giving rise to inorganic nitrite in acetic acid in the absence of hydrogen bromide.

Gas-liquid chromatography, on various stationary phases of medium-to strong polarity, has been applied most frequently to the final separation and determination of nitrosamines. Positive results based only on retention times of chromatographic peaks must be regarded critically unless they are confirmed by other adequate techniques. Nitrosamines were claimed to be produced by the Maillard reaction between carbohydrates and amino-acids (Devik, 1967) which leads to browning in many foodstuffs.
Heyns and Koch (1971) repeated these experiments and were able to demonstrate by coupled gas-chromatography and mass spectrometry that pyrazines and acetylpyrroles had been confused with nitrosamines.

An important advance in the analysis of nitrosamines was made by the introduction of nitrogen specific detectors. Howard et al. (1970) were the first to use a potassium chloride thermionic detector for the detection of nitrosamines in food extracts. The same group of workers (Fazio et al. 1971) extended the original analytical procedure to a multidetection method for volatile nitrosamines, using for nitrosamine analysis the same type of detector. The method was reported to have worked well, giving reproducible recoveries on extracts of 70 - 100\% at a nitrosamine level of 10 - 20 parts per billion. It has since been successfully adopted by Fiddler et al. (1971), and Wasserman et al. (1972). A rubidium sulphate thermionic detector has also been used by Crosby et al. (1972), for the same purpose.

Another kind of nitrogen detector that has been applied to nitrosamine analysis is the electrolytic conductivity detector, described by Coulson (1965). The basis of the instrument is the catalytic reduction of nitrogen at 850°, in nitrogen containing organic compounds, to ammonia. The ammonia formed is then dissolved in a stream of water, and is passed through a small conductivity cell which measures the increased conductivity of the ammonia solution. Sen (1972), Sen et al. (1973), and Eisenbrand (1973) have used the Coulson detector for the analysis of various food extracts. Instead of using the reductive mode, however, they removed the nickel catalyst, and formed ammonia by pyrolysis at 400°. This, (Rhoades and Johnson, (1970), claim, gives much greater selectivity for nitrosamines and practically eliminates any response from any other compounds.
Crosby et al., (1972) used the Coulson detector in the reductive mode for the examination of nitrosamines from bacon, fish, cheese and meat products. Results at the low part per billion level of several nitrosamines were reported.

As mentioned earlier, results using only one method of analysis, and especially those relying solely on retention time can be regarded as highly suspect. If another complementary analytical technique is available to confirm the gas chromatographic evidence, the results obtained have far greater credulity. The most specific detector is the mass spectrometer, and when coupled to the gas-chromatograph provides the most sophisticated method of analysis to date.

One of the first published reports of the analysis of nitrosamines using coupled gas chromatography - mass spectrometry (GC - MS) was by (Telling et al., 1971). These workers monitored the ion at $m/z = 30$ (at a resolution of 12,000) due to NO⁺, that is produced by nitrosamines. They found, however, (Eyre and Telling, 1972) that they could achieve greater sensitivity by focussing on the molecular ion produced by each nitrosamine. Other early workers using this technique were Heyns and Roper (1970) and Fazio et al., (1971).

Gough and Webb, also focussing on the molecular ion of the nitrosamine evaluated two types of separators used to achieve the removal of gas chromatography carrier gas from samples entering the mass spectrometer. They found that the membrane separator, described by Hawes et al., (1969), gave a ten times greater sensitivity over the fritted glass separator (Biemann and Watson, 1956), used by Telling et al., (1971). They also constructed a peak cutting device, which enabled large volumes of sample to be introduced into the gas chromatograph linked to the mass spectrometer.
and were able to achieve a further ten fold increase in sensitivity. This made the limit of detection equivalent to 0.2 µg/kg of nitrosamine in the original foodstuff.

A further advance in the method of analysis described by Gough and Webb (1973) used pressure programming of the gas chromatograph column. By running the gas chromatograph oven isothermally at 145°, they were able to slowly increase the pressure of the carrier gas, or effectively the flow rate, through the analytical column, which lengthened the life of the column by lessening 'column bleed' and enabled a faster throughput of samples by keeping the gas chromatograph running at one temperature.

Gough and Sawyer (1973) have also reported the use of a palladium-silver tube mass spectrometer/gas chromatograph interface, based upon the ability of hydrogen to selectively effuse though the tube (Simmonds et al., 1970). Hydrogen was used as the carrier gas in the gas chromatograph, and Gough and Sawyer claimed that reduction of the compounds to be detected did not occur.

(b) Determination of derivatives from nitrosamines

Althorpe et al., (1970) and Sen (1970) have developed procedures for the sensitive electron capture determination of nitramines derived from the corresponding nitrosamines (eqn. 1.4) by oxidation with peroxytrifluoroacetic acid, itself prepared from hydrogen peroxide in dichloromethane and trifluoroacetic anhydride.
N-nitrosodimethylamine \longrightarrow \text{dimethylnitramine} \\
\begin{align*}
\text{CH}_3 & \quad N \quad N \quad 0 \quad + \quad \overset{\circ}{\text{H}} \\
\text{CH}_3 & \quad N \quad N \quad \text{O}_2 \\
\text{CH}_3 & \quad N \quad \text{NO}_2
\end{align*}
\text{Eqn 1.4}

About 16 pg of N-nitrosodimethylamine could be detected under the conditions used. This is about 1000 times more sensitive than the detection limit of nitrosamines by the hydrogen flame detector (flame ionisation detector). An improvement of this method has been published by Telling (1972), who also used peroxytrifluoroacetic acid as the oxidising agent and obtained gas chromatograms free from any interfering peaks by column chromatographic purification of concentrates on alumina columns before and after nitramine formation.

Another method of preparing derivatives was devised by Eisenbrand and Preussmann (1970) in the acid-catalysed de-nitrosation of nitrosamines. The resulting amines were converted into heptafluorobutyryl (HFB) derivatives and either detected by electron capture gas chromatography (Eisenbrand, 1972), or more specifically, by gas chromatography linked to mass spectrometry, monitoring the $C_7F_{17}^+$ fragment at $m = 169$, which is abundant and characteristic (due to its stability) for this class of compounds.

Alliston et al., (1972) cleaved nitrosamines electrochemically under basic conditions. The secondary amines this generated were converted into HFB-derivatives by a similar technique and determined by an electron-capture detector. Pailer and Klus (1971) cleaved nitrosamines with cuprous chloride in hydrochloric acid and detected corresponding amines by electron capture gas chromatography of their trifluoroacetyl derivatives.

The formation of strongly fluorescent derivatives from secondary amines, liberated from the corresponding nitrosamines by cleavage of the N–N bond with hydrogen bromide has been used in a limited number of analyses of spiked
wheat flour, (Eisenbrand, 1972). The extracts were reacted with 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride), a strong fluorescer. This provides a very sensitive screening method but, like other techniques involving formation of amine derivatives, it requires vigorous exclusion of contaminating amines.

Reduction of nitrosamines to hydrazines has also been used in some cases, Neurath et al. (1965), reduced with lithium aluminium hydride and prepared 5-nitro-2-hydroxybenzaldehyde derivatives which were separated by thin layer chromatography. The yields obtained when analysing tobacco smoke were extremely low, and a modification was proposed by Yang and Brown (1972) who condensed the hydrazines with 9-anthraldehyde to form fluorescent derivatives, but this has only been tested with pure compounds. Hoffman and Vais (1971) obtained better results in the reduction step by the use of diborane instead of lithium aluminium hydride. They condensed the hydrazines with 3,5-dinitrobenzaldehyde and determined the resulting hydrazones by electron capture gas chromatography.

Despite the much better selectivity obtained by the use of nitrogen specific detectors and the formation of suitable derivatives, the need for confirmation of positive results by mass spectrometry is vital.

1.4 The analysis of human foods for nitrosamines – results

The results obtained by previous workers can be divided into two classes according to whether or not mass spectrometry has been used for confirmation of results. This is necessary, since less emphasis is to be placed on results unconfirmed by mass spectrometry.
(a) Foods analysed without mass spectral confirmation of results

The presence of trace amounts of nitrosamines in white flour and cheese has been claimed by Marquardt and Hedler, (1966), using thin layer chromatography and the detection techniques of Preussmann et al., (1964). Kroller (1967) using both thin layer and gas liquid chromatography confirmed the results above. However Thewlis (1967), using the method of Marquardt and Hedler (1966) on wheat flour, failed to find any trace of nitrosamines, after ether extraction and thin layer chromatography.

McGlashan et al., (1968) reported finding N-nitrosodimethylamine (or a similar substance) in 8 samples of African alcoholic spirits, using polarography and thin layer chromatography. Levels of between 1-3 parts per million nitrosamine were said to be present, a level predicted to be carcinogenic to laboratory animals. Geographical studies have linked cancers of the oesophagus in Africa to the drinking of locally distilled spirits. Further analyses by Collins et al., (1972) using gas chromatography coupled to high resolution mass spectrometry did not find any evidence for the presence of N-nitrosodimethylamine. Sen and Dalpe (1972) also analysed various alcoholic beverages for volatile nitrosamines using thin layer chromatography and gas chromatography and were unable to find any nitrosamines. Analyses of apples and milk have also been carried out, (Newell and Sisken, 1972) using gas chromatography and a microcoulometric nitrogen determination. They found that apples and milk contained small traces of a compound with the retention time of N-nitrosodimethylamine; they pointed out, however, that this did not necessarily imply that milk and apples contained N-nitrosodimethylamine, but simply that some compound in these foods had the same retention time as N-nitrosodimethylamine. Although there are other examples of analyses of foods for nitrosamines, those presented show that mass spectrometry must be used to obtain consistent and, hopefully, unambiguous results.
spectrometers do, unfortunately, also have certain limits, and generally are less sensitive than some of the gas-chromatographic and spectrofluorometric methods that have been used. At a resolution of approximately 12,000, the limit of detection has been stated by Gough and Webb, (1972) to be of the order of 2 ng, whilst the nitramine method of analysis (Sen, 1970) claims to detect as little as 16 pg of nitrosamine.

The possibility of confusion has arisen over the identity of mass spectral peaks which appear to have the same mass as the molecular ion of N-nitrosodimethylamine. The peaks are, in fact, due to $^{29}$Si(CH$_3$)$_3$ and $^{28}$Si(CH$_3$)$_2$CH$_3$ (Dooley et al., 1973). A resolution of more than 37,000 is required to distinguish these peaks from that of N-nitrosodimethylamine, a specification beyond the range of most mass spectrometers. False identification of the nitrosamine could be avoided, however, by monitoring for two specific ion peaks by high resolution mass spectrometry.

(b) Foods analysed with mass spectral confirmation of results

Bacon

Results indicate that N-nitrosopyrrolidine has been found in many of the samples of bacon tested after frying. Crosby et al. (1972), analysed 24 samples of various types of bacon for four nitrosamines:

Traces of N-nitrosodimethylamine (less than 4 parts per billion) were found in 13 samples, less than 1 part per billion of N-nitrosodiethylamine in 1 sample, up to 9 parts per billion N-nitrosopyrrolidine in 12 samples and less than 1 part per billion N-nitrosopiperidine in 1 sample of bacon. In 1 sample of bacon there was between 16 - 40 parts per billion nitrosamine, and this was due to N-nitrosopyrrolidine. No corrections have been made to these figures for the large loss of N-nitrosopyrrolidine, known to occur in the initial steam distillation stage, and during extraction and concentration. No correlation
of levels of nitrosamines and nitrite concentrations was found.

Eight samples of bacon were analysed but only N-nitrosopyrrolidine, ranging from 10 - 108 parts per billion was found by Fazio et al., (1973). Although the raw bacon did not contain any nitrosamines, the rendered fat contained more N-nitrosopyrrolidine than the solid portion. Sen et al. (1973) detected either N-nitrosodimethylamine or N-nitrosopyrrolidine in 8 samples out of 16 tested, but only performed mass spectrometry as confirmatory tests for N-nitrosopyrrolidine on one sample and N-nitrosodimethylamine on another sample (though this showed 30 parts per billion N-nitrosodimethylamine in the uncooked bacon). Negative results ( < 25 - 65 parts per billion) were obtained by Telling et al. (1971), in an unspecified number of bacon samples tested for several nitrosamines. Telling et al. (1973) have also reported results on 29 samples of fried bacon containing 1 part per billion up to 28 parts per billion (mean 5.5 parts per billion) N-nitrosopyrrolidine and on 42 samples of fat recovered from cooked bacon, which showed traces of N-nitrosopyrrolidine between 1 part per billion and 100 parts per billion (mean 17 parts per billion). A sensitive method of analysis, (Alliston et al., 1972) in which the nitrosamine was reduced to an amine electrochemically, derivatised with heptafluorobutyl noyl chloride and detected by a gas chromatograph with electron capture detection, showed trace amounts of N-nitrosopyrrolidine in both samples of fried bacon tested, and 1.5 parts per billion in one sample raw bacon. Sen et al. (1974) have recently reported that fried bacon samples prepared with levels of nitrite from 50 to 200 parts per million, contained up to 5 parts per billion N-nitrosodimethylamine and up to 20 parts per billion N-nitrosopyrrolidine. The levels of the heterocyclic nitrosamine were found to correlate well with the initial levels of nitrite. In 12 commercial samples of bacon, only one was found to have more than 2 parts per billion N-nitrosodimethylamine, but all 12 were found to contain between 15 - 75 parts per billion N-nitrosopyrrolidine. Mass spectroscopy was used
as confirmation on several of the samples only, although Sen et al., claim they have found that the thin-layer chromatographic method they use gave results normally within 10 - 20% of those obtained by mass spectrometry.

2. Ham

Fazio et al., (1971) examined 5 samples of ham, and of these only one could be confirmed by mass spectrometry as containing as much as 5 parts per billion N-nitrosodimethylamine. Fiddler et al., (1972) studied 10 samples of ham and none were found to contain N-nitrosodimethylamine at levels above 25 parts per billion. Telling et al., (1971) using coupled high resolution mass spectrometry and gas chromatography did not detect any nitrosamines in 8 samples of ham, down to a limit of 1 - 2 parts per billion. Crosby et al., (1972) failed to find any N-nitrosodimethylamine or N-nitrosodiethylamine in ham.

3. Sausage and sausage products

Sen et al., (1972) examined 36 samples of commercially smoked sausage and salami and found 5 samples with N-nitrosodimethylamine ranging from 10 - 80 parts per billion. N-nitrosodimethylamine was found in 3 samples of frankfurters out of 40 studied (Wasserman et al., 1972). Crosby et al., (1972) found between 1 - 4 parts per billion N-nitrosodimethylamine and N-nitrosodiethylamine in 1 salami out of 6 tested for N-nitrosodimethylamine and N-nitrosodiethylamine. Telling (1973) analysed 4 samples of smoked sausage and found 1 with <1 part per billion N-nitrosodimethylamine. A range of sausage products (bologna, frankfurters, pepperoni, salami and thuringer) were analysed by Fazio et al., (1971), and it was found that none of the 18 samples contained sufficient N-nitrosodimethylamine to be confirmed by mass spectrometry (ie less than 2 parts per billion).
4. Luncheon meat

One sample of luncheon meat contained 1 - 4 parts per billion, N-nitrosodimethylamine, while 1 sample of chopped pork contained between 1 - 4 parts per billion of both N-nitrosodimethylamine and N-nitrosodiethyamine, (Crosby et al., 1972). Telling et al., (1973) analysed 12 samples of luncheon meat and found < 1 part per billion N-nitrosodimethylamine and no N-nitrosodiethylamine or N-nitrosopyrrolidine.

5. Fish

Crosby et al., (1972) examined 35 samples of fish (fresh, smoked, pickled, fried, baked) for N-nitrosodimethylamine and found 12 samples contained between 1 - 4 parts per billion nitrosamines and 3 samples contained between 5 - 9 parts per billion of nitrosamines. Telling et al., (1973) examined 88 samples of fresh fish and found low amounts of N-nitrosopyrrolidine in some of them. They also studied 16 samples of canned fish and found traces of N-nitrosodimethylamine and N-nitrosopyrrolidine in some of the samples.

6. Cheese

Crosby and coworkers (1972) analysed 12 varieties of cheese and found that 6 samples contained between 1 - 4 ppm N-nitrosodimethylamine.

The results show that not only are nitrosamines formed, or at least are present in various foods, but that their presence is sporadic (with the exception of N-nitrosopyrrolidine in fried bacon). Analyses of samples for residual nitrite content shows little, if any, correlation with amount of nitrosamine present.
Little was known of the fate of nitrite added to cured products, and because its disappearance might be linked with the formation of nitrosamines and possibly the formation of other hazardous compounds not yet discovered, it was thought necessary in this study to follow the added nitrite through the curing process. In the next section of this review an account is given of the possible reactions of nitrite in a meat system.

In any attempt to discover the source of the formation of nitrosamines from meat and nitrite, it is necessary to be able to account for the amounts of nitrite present in the system at any given time. In attempting to rationalise the decreasing concentration of nitrite in muscle with respect to time, it is essential to know primarily with which biological components the nitrite is likely to react. The review here, of the fate of the added nitrite, begins with reactions that can occur when nitrite is in the simplest of model systems applicable to meat, that of the aqueous solution.

Reactions of sodium nitrite in water

Sodium nitrite dissociates in water; the extent of dissociation of the salt and nitrous acid is governed by the relationship $pK_a = pH - \log \frac{(\text{salt})}{(\text{acid})}$.

Eqn 1.5 For nitrous acid, $pK_a = 3.4$, (Lange, 1946) and at the pH of post-mortem muscle tissue (pH 5.5 to pH 6.5) approximately 0.2 - 0.4% of the salt will exist as the undissociated acid. Inter-relationships of nitrous acid and its decomposition products involve the simultaneous oxidation and reduction of nitrite, called disproportionation or dismutation (Heslop and Robinson, 1960; Smith, 1921; Parkes and Mellar, 1939; Cotton and Wilkinson, 1966). Nitrate and nitric oxide are the suggested decomposition products, occurring in a 1:2
ratio. The general reaction is

\[ 3\text{HNO}_3 \rightarrow \text{HNO}_3 + 2\text{NO} + \text{H}_2\text{O} \quad \text{Eqn. 1.6} \]

Changing pH from 4 to 2.8 caused increasing nitrite loss from a solution originally containing 100 ppm nitrite, but above pH 4.0, losses were negligible (Ando and Nagata, 1963). In their work, analyses were limited to those for nitrite, as a result, it is not known what the decomposition products were.

In addition to pH, decomposition of nitrous acid is accelerated by a number of catalysts, including ferric, chromic and cupric salts.

While dismutation has been used to explain nitric acid production in solutions of sodium nitrite, alternative means for nitrous acid decomposition could be envisaged through the nitrosonium ion, \( \text{NO}^+ \) (Hein, 1963; Noller, 1957). This may be produced in the free form, \( \text{NO}^+ \) or arise from a carrier \( \text{NO}_X \), which can yield the \( \text{NO}^+ \) moiety (Hughes et al., 1958). One such carrier may be dinitrogen trioxide, \( \text{N}_2\text{O}_3 \), which can be formed by the self hydration of nitrous acid:

\[
\begin{align*}
\text{HNO}_2 & \rightleftharpoons \text{H}^+ + \text{NO}_2^- \\
\text{H}^+ + \text{HNO}_2 & \rightleftharpoons \text{H}_2\text{NO}_2^+ \\
\text{NO}_2^- + \text{H}_2\text{NO}_2^+ & \rightleftharpoons \text{N}_2\text{O}_3 + \text{H}_2\text{O} \\
\end{align*}
\quad \text{Eqn 1.7}
\]

Participation of \( \text{N}_2\text{O}_3 \) in the decomposition was examined by isotopic oxygen exchange between water and nitrous acid. It was found that the rate of exchange is proportional to the square of the nitrous acid concentration (Bunton et al., 1959, a,b). The mechanism suggested was:

\[
\begin{align*}
2\text{HNO}_2 & \rightleftharpoons \text{H}_2\text{NO}_2^+ + \text{NO}_2^- \\
\text{H}_2\text{NO}_2^+ + \text{NO}_2^- & \rightleftharpoons \text{N}_2\text{O}_3 + \text{H}_2\text{O} \\
\end{align*}
\quad \text{Eqn 1.8}
\]
Further evidence for the self hydration of nitrous acid as a mechanism for the production of a nitrosating agent was provided by the studies of reactions of nitrous acid and azide (Stedman, 1959; Bunton and Stedman, 1959b) nitrous acid and hydroxylamine (Hughes and Stedman, 1963), and organic nitrite hydrolysis (Allen, 1954).

Combination of nitrous acid and oxygen takes place in nitrite solution. The reaction results in the production of an acrid brown gas, nitrogen dioxide (Morecroft and Thomas, 1967; Grieg and Hall, 1966). The rate of formation depends on the concentrations of oxygen and nitric oxide but is between $10^3$ and $10^4$ litres$^{-2}$ mole$^{-2}$ sec$^{-1}$, (Sole, 1966). The reaction is third order and is therefore very slow at low levels of reactants.

**The reaction of nitrite with haem pigment**

It has already been stated that early researches observed similarities between nitric oxide treated haemoglobin and cured muscle tissue and accordingly they explored the relationship between the composition of curing agents and the colour of extracted tissue pigments (Haldane 1901; Hoagland, 1914). The evidence indicated that the bacterial flora, developed in the curing solution, caused reduction of sodium nitrate, a cure ingredient. The resulting nitrous acid subsequently may have been reduced to yield nitric oxide, which combined with tissue pigments to produce the colour associated with cured meats.

Spectrophotometric investigation of the quantitative relationship between nitrite and tissue pigments indicated that between 0.5 and 1 mole of nitrosylhaemoglobin was produced per mole of nitrite lost. (Meier, 1926; Barcroft and Muller, 1912; Austin and Drebkin, 1935; Van Slyke and Vollmond 1925). It was suggested that nitrate may be a by-product of the overall reaction (Meier, 1926).
Other workers, recognising the importance of residual nitrite analyses as well as pigment determination, reexamined the system. They reported that 1 mole of nitrite reacted with two moles of haemoglobin, but did not discredit Meier's suggestion that nitrous acid could be oxidised by atmospheric oxygen to form nitrate (Greenberg et al., 1943). From the early work, reactions involved in curing could be summarised as:

\[
\begin{align*}
\text{NaNO}_2 & \quad + \quad \text{acid conditions} \rightarrow \text{HONO} \\
\text{HONO} & \quad + \quad \text{reducing conditions} \rightarrow \text{NO} \\
\text{NO} & \quad + \quad \text{haemoglobin} \rightarrow \text{NO haemoglobin}
\end{align*}
\]

(Eqn 1.9)

(urbain, 1951).

Studies of the sedimentation rates of heart and skeletal muscle preparations revealed that a component of different properties than haemoglobin could be separated (Lemberg and Legge, 1949). It was determined that this compound, myohaemoglobin (now called myoglobin) had a molecular weight of about 17,000, one quarter that for haemoglobin. Since it is an intracellular pigment, it may comprise as much as 95 per cent of the total pigments in a well-bled carcase (Wilson, 1960). It was established that myoglobin reacted similarly to haemoglobin with nitrite, and it replaced haemoglobin in the restatement of the reactions involved in meat curing.

There are a number of postulated mechanisms concerning the production of the nitrosylmyoglobin complex, but nearly all start with the oxidation by nitrite of the Fe\(^{II}\) in myoglobin or oxymyoglobin to Fe\(^{III}\) in metmyoglobin. Simultaneously, nitrate is formed from oxymyoglobin in an autocatalytic reaction. In model experiments with minced muscle, the amount of nitrate formation is subject to large deviation (Mohler, 1970; Walters et al., 1968). However, statistical evaluation of a large number of experiments, led Mohler (1970) to propose the following reaction.
\[
4\text{MbO}_2 + 4\text{NO}_2^- + 2\text{H}_2\text{O} \longrightarrow 4\text{MetMbOH} + 4\text{NO}_3^- + \text{O}_2. \quad \text{Eqn 1.1}
\]

Metmyoglobin and nitrate are formed in equimolecular proportions.

Deviations can be caused, however, by varying various factors, and this was shown in the case of haemoglobin of Möhler and Bammann, (1971).

According to Möhler (1973) it is not known whether metmyoglobin appears everytime as an intermediate in the formation of nitrosylmyoglobin. In meat products which are commonly manufactured with nitrite Möhler states that in general a higher nitrate content is found than that which would be expected with Eqn 1.10. This, he claims, is due to secondary oxidation in which the dismutation of nitrous acid could also play a part, together with the product of the oxidation of nitric oxide, (itself formed from nitrosothiols, according to Mirna and Hofmann, 1969).

Several mechanisms for nitrosylmyoglobin formation involve the participation of various enzyme systems of the muscle tissue. The reduction of metmyoglobin to myoglobin in post-mortem tissue may occur via a portion of the respiratory enzyme system (Watts et al., 1966; Saleh, 1968). NO presumably is there to react with the myoglobin to form nitrosylmyoglobin. Koizumi and Brown (1971) describe the non-enzymatic formation of nitrosylmyoglobin from metmyoglobin. NADH in the presence of FAD or FMN was used as reducing agent. If oxygen was completely excluded, metmyoglobin was reduced to myoglobin as shown in diagram 1.1. Myoglobin then reduced nitrite to NO and is itself oxidised to metmyoglobin. NO is immediately bonded to excess myoglobin while Met-Mb returns to the circulatory system.

The proof for the reaction of Mb with nitrite relies upon spectrophotometric measurements by Koizumi and Brown, (1971).

A completely enzymatic pathway for the production of nitrosylmyoglobin has been proposed by Walters et al. (1967). In this case, nitrite takes the place of oxygen in part of the respiratory chain. Diagram 1.2 below shows the overall scheme of the reactions.

Diagram 1.2 Formation of nitrosylmyoglobin – Walters et al. (1967).
The starting point is metmyoglobin, formed from endogenous myoglobin, and nitrite. Ferrocytochrome c is oxidised by nitrite to nitrosylferri-cytochrome c. This compound can then be reduced by NADH with the aid, for instance, of NADH-dehydrogenase to yield ferrocytochrome c, which does not form a nitrosyl derivative. The NO then separates and is transferred to metmyoglobin. If the NADH-dehydrogenase acts again, this time reducing the nitrosylmyoglobin, the desired curing pigment is produced.

Other mechanisms for the production of nitrosylmyoglobin have been proposed by various workers. According to Van Assendelft (1965), metmyoglobin can react directly with nitric oxide to form nitrosylmetmyoglobin, with a subsequent reduction to nitrosylmyoglobin. The participation of nitric oxide directly in the formation of nitrosylmyoglobin has been demonstrated by Wodicka (1956), and Hashimoto and Tsutomu, (1958). The reaction occurs rapidly, an estimate for the rate constant was reported as $1.7 \times 10^{-7}$ moles$^{-1}$ sec$^{-1}$. This is about 1.5 times the rate for the reaction of oxygen and myoglobin, and 30 times the rate for the carbon monoxide myoglobin reaction (Gibson and Roughton, 1965). Formation of nitrosylmyoglobin is reversible and it is thought that one nitric oxide molecule is bonded to the central iron atom in the undenatured haem compound. A second molecule of nitric oxide may replace an imidazole moiety of the haem protein at the 6th co-ordination position after denaturation. These structures correspond to those present in fresh and cooked cured meats (Tarladgis, 1962).

With respect to pigment concentration, the conversion of myoglobin to nitrosylmyoglobin in aqueous solutions is zero-order. The relationship between nitrosylmyoglobin and nitrite concentration is linear up to a 5 to 1 ratio. Beyond this, the relationship changes, so that the rate for pigment formation is dependent upon the cube root of the nitrite concentration. A series of reactions was proposed to account for these observations. Nitrous acid undergoes dehydration to form dinitrogen tri-oxide which reacts with
undissociated ascorbic acid to form nitrosylascorbate, an assumed intermediate. Dissociation of this gave nitric oxide, which combined with myoglobin.

These reactions are drawn out below.

\[
\begin{align*}
2\text{HNO}_2 & \rightleftharpoons \text{N}_2\text{O}_3 + \text{H}_2\text{O} \\
\text{N}_2\text{O}_3 + \text{AH}_2 & \longrightarrow \text{AHNO} + \text{HNO}_2 \quad (A = \text{Ascorbic acid}) \\
2\text{AHNO} + \text{H}_2\text{O} & \longrightarrow 2\text{AH}_2 + \text{N}_2\text{O}_3 \\
\text{AHNO} & \longrightarrow \text{AH} + \text{NO} \\
\text{NO} + \text{MetMb} & \longrightarrow \text{NO MetMb} \\
\text{NO MetMb} + \text{AH} & \longrightarrow \text{NOMb}
\end{align*}
\]

(According to Fox and Thomson, 1963).

Further study of the reaction by Fox and Ackerman, (1968), has indicated that several reactions occur in a complex sequence, diagram 1.3.

Diagram 1.3 Formation of nitrosylmyoglobin - Fox and Ackerman (1968).
The data indicate that nitrosylmethmyoglobin is formed and subsequently reduced to nitrosylmyoglobin. While molecule R is not further identified, it is apparent that there are 3 ways for this molecule to participate in the formation of nitrosylmyoglobin.

Mirna and Hofmann (1969) suggested that -SH groups may play a part in the production of nitric oxide myoglobin, by the following reaction.

\[
2 (\text{protein - S-NO}) + 2\text{Mb} \rightarrow \text{protein - SS - protein} + 2\text{NO-Mb}.
\]

Nitroso thiols are formed according to:

\[
\text{RSH} + \text{HONO} \rightarrow \text{RS-NO} + \text{H}_2\text{O}
\]

Mirna and Hofmann have stated that the nitric oxide moiety attached to the thiol group may reside as 'activated nitrite' which facilitates the transference of the NO to myoglobin.

Nitric oxide, nitrate and nitrosylmyoglobin and nitrosylmethmyoglobin (and, of course, nitrosamines) however, are not the only products of the interaction of nitrite with muscle tissue. It has been known for many years that the difference between the initial and final levels of added nitrite in cured meat is far greater than can be accounted for by the production of the nitric oxide haem pigment.

Walters and Taylor (1963) used differential absorption manometry to show that nitric oxide was produced when pork muscle was incubated at 37°C with nitrite at a concentration of 2500 ppm. Möhler and Ebert (1971) showed the production of \(N_2O\) from beef and nitrite, in which the beef was mixed with nitrite and given a mild pasteurisation. Woolford et al. (1972), using \(^{15}\text{N}\) labelled sodium nitrite, and mass spectrometry, identified NO, \(N_2O\) and \(\text{C}_2\text{H}_4\).

Mirna and Hofmann (1969) demonstrated the formation of nitrosothiols in meat, a result which has been supported by Olsman (1973), who showed that
nitrite loss can be lessened, but not prevented, by blocking the thiol groups with vinyl pyridine.

Cysteine-nitrothiol has been shown, Incze et al. (1974), to have an antibacterial effect on strains of *Salmonella* and *Clostridia*; a greater inhibitory effect, it was reported, than nitrite has.

The loss of nitrite from model meat cures has been studied extensively for a long period. Lewis (1936) recorded a loss of nitrite, 22% - 34% on addition to ground beef, before thermal processing. Greenwood (1940), added 500 ppm nitrite to ground beef and recovered 250 ppm, a 50% loss rate, over several hours at 15°. These figures are essentially in agreement with those of Greenberg (1972), with losses of 25 - 50% of the added nitrite. Losses have also been noted during thermal processing. In an extensive study by Nordin (1969) a relationship was found between the final nitrite concentration, the initial nitrite concentration and the pH, temperature and time of storage. He was able to compute an equation \( \log_{10} \) (half life) = 0.65 - 0.025 (Temp °C) + 0.35 (pH). Nordin summarised his data by saying that the rate of nitrite depletion was exponentially related to both temperature and pH and doubles for every 1.2°C increase in temperature or 0.86 units decrease in pH. He also claimed that this rate was not affected by heat denaturation of the meat. The mechanism of the formation of nitrosylmyoglobin is still in dispute; the fate of added nitrite is unknown and it is undecided whether the hazard to human health, through the presence of nitrosamines, is to warrant banning the use of nitrite in food preservation. It is in these uncertain areas that this thesis was envisaged and it is hoped that the results make an advance in the knowledge of the curing process.
PART II

METHODOLOGY OF MASS SPECTROSCOPY AND SPECTROSCOPIC ANALYSIS
The importance of the identification of nitrosamines has been stressed in the previous chapter, and many methods of analysis for these compounds have been devised. Most chemical methods are, however, unspecific in so far as they only identify the nitrosamine group (-NNO) and cannot indicate the structure of the whole nitrosamine, a factor of great importance when the relative toxic and carcinogenic properties of the individual nitrosamines are compared.

It must also be borne in mind that the nitrosamines are likely to be present in extracts at a concentration in the parts per million range (even after concentration of the extract). The detection of nitrosamines or any other products from the interaction of nitrite with pork muscle in these trace concentrations with many other compounds present in a cured product is thus extremely difficult. The most comprehensive method of analysis used for the determination of nitrosamines is high-resolution - mass spectrometry linked to gas chromatography. This can provide both sensitive, quantitative analysis and structural information that allows the precise molecular formula of a nitrosamine to be determined.

Mass spectrometry has been used in this work to study not only the fate of nitrite in nitrosamine formation; but also the fate of nitrite, (enriched with $^{15}$N), to give other nitrogenous products. A faster method of analysis for isotopically enriched forms of nitrogen is emission spectroscopy; use of both mass spectrometry and emission spectroscopy are described.
CHAPTER 2

MASS SPECTROMETRIC METHODS FOR THE ANALYSIS OF NITROSAMINES
2.1. Introduction

The various experiments which were performed on low and high resolution mass spectrometers, together with the analytical methods for the analysis of N-nitrosamines which evolved from them, are described.
2.2. Experimental

Instrumentation for low resolution mass spectrometry - gas chromatography

Before the arrival at B.F.M.I.R.A. of a high resolution mass spectrometer, a low resolution mass spectrometer was used for analysis of nitrosamines in meat extracts. This instrument was an Hitachi-Perkin-Elmer model RMU-6E, which had a resolving power of approximately 2500. It was linked via an ohmically heated stainless steel capillary tube to a Perkin Elmer gas chromatograph model F11, equipped with a flame ionisation detector (F.I.D.). The effluent stream from the column was split in the ratio 15:1 so that the greater part of the effluent went into the mass spectrometer.

The mass spectrometer - gas chromatograph interface was of the fritted glass or Biemann-Watson type (Biemann and Watson, 1956). This is the most frequently used device for the separation of sample in the gas chromatograph effluent from the carrier-gas. It is not ideal, however, because the separator functions on the basis of Graham's Law, and the efficiency of separating compounds of low molecular weight from carrier gas is poor. It is, nevertheless, satisfactory for the vast majority of analyses that have to be performed by the mass spectrometer, and no attempt was made to study other kinds of interfaces for nitrosamine work, though use of a silicone membrane separator for nitrosamines has been reported (Gough and Webb, 1972).

Gas Chromatograph conditions

The nitrosamine analyses were performed on a stainless steel column, 2 metres x 3 mm i.d. packed with 15% 'Carbowax 20M' on 'Chromosorb W' (80 - 100 mesh). To separate the nitrosamines sufficiently, temperature programming of the gas chromatograph (G.C.) oven was employed, from 70° to 200° at 10°/min. The carrier gas was helium (for ease of removal by the separator), at a flow rate of 20 ml/min. The retention times of the nitrosamines studied were as follows.
<table>
<thead>
<tr>
<th>Nitrosamine</th>
<th>Retention Time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-methyl</td>
<td>7.5</td>
</tr>
<tr>
<td>Methyl ethyl</td>
<td>8.5</td>
</tr>
<tr>
<td>Di-ethyl</td>
<td>9.0</td>
</tr>
<tr>
<td>N-nitroso piperidine</td>
<td>15.0</td>
</tr>
<tr>
<td>N-nitroso pyrrolidine</td>
<td>15.5</td>
</tr>
<tr>
<td>Di-n-pentyl</td>
<td>19.5</td>
</tr>
</tbody>
</table>

**TABLE 2.1. RETENTION TIMES OF VARIOUS NITROSAMINES UNDER G.C. CONDITIONS IN TEXT**

**Analysis of meat extracts**

For the satisfactory recording of the spectra of G.C. effluents, at least partial resolution of the constituents is necessary. However, the separation of trace constituents from extraneous material present in substantially larger amounts in extracts is difficult to achieve. A typical gas-chromatogram of an extract spiked with volatile dialkyl and heterocyclic nitrosamines is shown in diagram 2.1., from which it is seen that the nitrosamines are completely masked. Diagram 2.2. shows a standard trace of dialkyl and heterocyclic nitrosamines. A mass spectrum run at the point at which di-methyl nitrosamine is expected, is clearly useless. The detection of traces of known materials can, however, be achieved even under such conditions of gross interference by monitoring a characteristic and abundant ion in the spectrum rather than by attempting to run a complete low resolution spectrum for characterisation. To facilitate rapid analysis of a series of nitrosamines, the m/e = 30 ion was chosen, which is caused by the NO$^+$.
Diagram 2.1. A typical gas chromatogram of a cured meat extract spiked with 6 nitrosamines. The arrow marks where N-nitrosodimethylamine was eluted.
Chart speed.....1 cm /min
Attenuation.....x 50
Injection.........1 μl, 6 Nitrosamine in hexane, each 100 ppm.

Diagram 2.2. Standard trace of 6 nitrosamines in hexane
and CH₄N⁺ ions from the fragmentation of nitrosamines. These ions can, of course, be formed from compounds other than nitrosamines; NO⁺ can arise from compounds containing −NO or −NO₂ groups and CH₄N⁺ is a prominent ion in the spectra of many amino compounds (Budzikiewicz, 1967). This meant that peaks were produced at m/e = 30 that were not due to nitrosamines, yet had to be examined, since the ion had the mass to charge ratio that was being monitored. Other combinations of C, H, N and O could also produce m/e = 30 peaks, and the resolution required to separate each combination from NO is calculated and given in the table below.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Exact mass</th>
<th>Approximate resolution to separate NO⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO⁺</td>
<td>29.9980</td>
<td>-</td>
</tr>
<tr>
<td>C¹⁸O⁺</td>
<td>29.9992</td>
<td>25,000</td>
</tr>
<tr>
<td>¹⁵N₂⁺</td>
<td>30.0002</td>
<td>13,600</td>
</tr>
<tr>
<td>¹³CHO⁺</td>
<td>30.0061</td>
<td>3,700</td>
</tr>
<tr>
<td>CH₂O⁺</td>
<td>30.0106</td>
<td>2,400</td>
</tr>
<tr>
<td>H₂N₂⁺</td>
<td>30.0208</td>
<td>1,300</td>
</tr>
<tr>
<td>CH₃¹⁵N⁺</td>
<td>30.0236</td>
<td>1,200</td>
</tr>
<tr>
<td>¹³CH₃N⁺</td>
<td>30.0309</td>
<td>900</td>
</tr>
<tr>
<td>CH₄N⁺</td>
<td>30.0344</td>
<td>800</td>
</tr>
<tr>
<td>¹³CCH₅⁺</td>
<td>30.0425</td>
<td>700</td>
</tr>
<tr>
<td>C₂H₆⁺</td>
<td>30.0469</td>
<td>600</td>
</tr>
</tbody>
</table>

TABLE 2.2. ELEMENTAL COMPOSITION OF IONS OF MASS m/e = 30, AND RESOLUTION REQUIRED TO SEPARATE THEM FROM NO⁺
Diagram 2.3. Gas chromatogram of 5 µl of a cured meat extract
Diagram 2.4. Trace obtained from mass spectrometer focussed on ion of $m/e = 30$ of meat extract in previous diagram, showing various compounds, other than nitrosamines, being detected.
The RMU-6 mass spectrometer had a maximum resolution of only 2,500, and extracts were first screened for compounds that produced m/e = 30 ions. This was done by introducing nitric oxide through the gas inlet of the mass spectrometer and then adjusting the main magnet of the spectrometer to focus exactly on the NO\textsuperscript{+} ions produced by the gas. When samples injected onto the G.C.-M.S. system produced peaks at m/e = 30 on the mass spectrometer this showed that either NO\textsuperscript{+} was being detected as a fragment ion or ions that could not be resolved from NO\textsuperscript{+}, i.e. \textsuperscript{13}CH\textsubscript{3}O\textsuperscript{+}, \textsuperscript{15}N\textsubscript{2}\textsuperscript{+}, C\textsuperscript{18}O\textsuperscript{+}, and possibly CH\textsubscript{2}O\textsuperscript{+}. If a sample did not give any peaks at m/e = 30, this was taken to mean the absence of any nitrosamines at the level at which they could be detected on this instrument. If, however, peaks at m/e = 30 were produced, a larger volume of sample (up to 25 \mu l) was injected onto the system. At the exact time at which a peak at m/e = 30 was previously produced, a complete mass spectrum was now run. The object of this was to try and identify the nitrosamine (if present) by its whole fragmentation pattern or several of the most prominent ions in the mass spectrum. Diagrams 2.3 and 2.4 show a gas chromatogram of a meat extract and the corresponding trace of ions produced at m/e = 30 by various compounds in the extract. It was found that for most nitrosamines, the smallest measurable peak on the m/e = 30 trace corresponded to approximately 50 ng of nitrosamine.

This method had several drawbacks, however, since interference was still likely from the other components of the meat extract. Although the resolution of the mass spectrometer was nominally 2,500 it was possible for abundant ions which were usually well resolved from NO\textsuperscript{+} to appear on the traces.

Diagram 2.5 shows NO\textsuperscript{+} and ion X of m/e = 30, normally well resolved. When the ion X becomes more abundant, interference occurs as in diagram 2.6. The result of the interference is that a small peak occurs at the position where NO\textsuperscript{+} would occur on the m/e = 30 trace,
Diagram 2.5. $NO^+$ and $X^+$ ions at similar concentrations
Diagram 2.6. $X^+$ ions far more abundant than $NO^+$ ions
and consequently more peaks than necessary have to be examined.
The only way of preventing this interference is by increasing the
resolution of the mass spectrometer.

**High resolution - mass spectrometry - gas chromatography**

The model RMU-6E was replaced by a double focussing Model RMU-7L
mass spectrometer. This instrument has a resolving power of approximately
20,000, and a much higher sensitivity than the RMU-6E, because of a
better signal amplification system. For nitrosamine work a resolution
of 10,000 was used, this being considered a satisfactory compromise
of the opposing requirements of sensitivity and ability to discriminate
against interfering ions.

**Analysis by a static method**

Instead of continuing work for nitrosamine analysis by focussing
on the m/e = 30 peak of NO⁺, it was known that greater sensitivity
and discrimination could be achieved by monitoring for a characteristic
ion which is more abundant than the NO⁺ ion. The molecular ion of each
nitrosamine was the obvious choice for all but one of the nitrosamines
studied. Di-n-butyl nitrosamine has a weak molecular ion, and a
relatively intense ion at m/e = 84, due to C₅H₁₀N⁺. Consequently,
this was the ion monitored for di-n-butyl nitrosamine.

For the analysis of meat extracts, the mass spectrometer was set
up focussed on the molecular ion of a particular nitrosamine. If a
standard sample of nitrosamines was injected onto the M.S.-G.C. system,
a peak at the retention time of the particular nitrosamine would
appear on a chart recorder linked to the output of the mass spectrometer.
The minimum amount detectable by this method was found to be
approximately 2 ng for dimethylnitrosamine, diethylnitrosamine,
dibutylnitrosamine, and dipropylnitrosamine; and 5 ng for N-nitroso
pyrrolidine.
While the mass spectrometer was used in this way, it was occasionally found that there was an appreciable steady voltage detected by the chart recorder, causing it to go off scale at high sensitivity settings of the amplification system. This current was due to background ions, in the source of the mass spectrometer, having an identical or near identical mass to the nitrosamines being analysed. This difficulty was overcome by putting a variable signal generator (made by the In-Line Process Control Department of B.F.M.I.R.A.) into the circuit between the mass spectrometer and the chart recorder. This provided an output signal which cancelled out the background signal and enabled the chart recorder connected to the mass spectrometer to be 'zeroed'.

The high gain of the amplification system meant that electronic noise was a problem and this was overcome by putting a 15000 μF capacitor across the output from the mass spectrometer. This cut out all small changes in voltage with a time constant of \( < \frac{1}{4} \) second. The peaks due to nitrosamine ions were sufficiently broad so as not to be affected by the capacitor.

A fault of this system, however, was that the capacitor took a long while to settle down, and it was virtually impossible to reset the focussing condition of the mass spectrometer quickly enough before the next nitrosamine emerged from the gas chromatograph into the mass spectrometer. This meant that a separate gas-chromatograph injection had to be made for each nitrosamine that had to be studied, and increased both analysis time and the amount of sample needed for analysis.

**Use of chemical isomers of nitrosamines**

Because of the health hazard of nitrosamines, I thought that whenever it was necessary to focus the mass spectrometer on a molecular ion of a nitrosamine, a chemical isomer that provided exactly the same ion could be used instead. This was found to be successful and the isomers
used are listed below in Table 2.3. for each of the six volatile nitrosamines studied.

<table>
<thead>
<tr>
<th>Nitrosamine</th>
<th>Isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-methyl nitrosamine</td>
<td>methyl urea</td>
</tr>
<tr>
<td>Di ethyl &quot;</td>
<td>butyric acid hydrazide</td>
</tr>
<tr>
<td>Di propyl &quot;</td>
<td>N-2-aminoethyl morpholine</td>
</tr>
<tr>
<td>Di butyl &quot;</td>
<td>tri-n-butylamine*</td>
</tr>
<tr>
<td>nitrosopyrrolidine</td>
<td>allyl urea</td>
</tr>
<tr>
<td>nitrosopiperidine</td>
<td>1-piperazine carboxaldehyde</td>
</tr>
</tbody>
</table>

*This did not give the molecular ion, but the $C_5H_{10}N^+$ ion at m/e = 84, the peak monitored for di-n-butyl nitrosamine.

**TABLE 2.3. CHEMICAL ISOMERS USED AS 'STAND-IN' COMPOUNDS FOR THE MOLECULAR IONS OF NITROSAMINES**

The compounds chosen each had to give an abundant ion at the required mass to charge ratio (m/e), in order to keep the ion source pressure to an acceptable level, and had to be volatile below 180°, this being the maximum temperature that the liquid inlet of the mass spectrometer could be heated to. The liquid inlet was chosen as the way of inserting these compounds into the mass spectrometer, since there is more control over the amount of isomer allowed into the mass spectrometer, the isomers could be easily removed, and there was no interference with the gas chromatograph. Because of inherent difficulties of this method i.e. the mass spectrometer had to be reset each time a different nitrosamine was studied, the mass spectrometer had to be kept thermally stable with no temperature changes in the room in which the analyses were done, and this method required several injections of extract for each extract studied it was decided to investigate another method of analysis.
The RMU-7L mass spectrometer is equipped with a peak matching system that allows the m/e ratio of an ion to be measured very accurately. The procedure for the measurement of ions that have been studied in this thesis is given below. This procedure is included here, since the description and equations are necessary to describe the scanning method of analysis of nitrosamines.

When a spectrum is recorded with the mass spectrometer either the magnetic field strength (H) or the ion acceleration voltage (V) must be scanned, as seen by the following formula:

\[ \frac{m}{e} = k \frac{H^2}{V} \]  

Equation 2.1.

where \( m \) = mass of ion, \( e \) = electronic charge of ion, \( k \) = constant i.e. to observe different m/e values, H or V must be altered. The peak matcher on the RMU-7L employs voltage scanning, which is adequate for precision measurement within a narrow mass range. Since double focussing instruments are designed so that the ion focussing conditions will not be disturbed even if the accelerating voltage V changes, the scanning must be done accurately with the deflection voltage Vs of the electrostatic field and also with V in proportion to Vs. Equation 2.1. can then be rewritten as equation 2.2, as in general cases \( e \) is unity. From

\[ m = k' \frac{V_s}{V_s} \]  

Equation 2.2.

where \( k' = k \cdot H^2 \)

equation 2.2., for changes in Vs, equation 2.3. is obtained.

\[ \frac{\Delta M}{M} = \frac{\Delta V_s}{V_s} \]  

Equation 2.3.

This means that the mass deviation \( \Delta M \), from the mass \( M \), (which corresponds to the voltage difference \( \Delta V_s \) of the voltage \( V_s \)) can be determined exactly if \( M \) and \( \Delta V_s/V_s \) are accurately known.
A mass spectrum such as in diagram 2.7a. is recorded on an oscilloscope screen when Vs is swept as in diagram 2.7b., with a saw-tooth wave, and the value of M can be easily calculated by equation 2.4., by measuring distances 'a' and 'x' if the values of Mo and M are known. In order to

\[ M = Mo + \frac{x}{a} (M_1 - Mo) \]

Equation 2.4.

measure 'a' and 'x' accurately, if Vs is suddenly changed by Vr as in diagram 2.7d. by superimposing a rectangular wave as in diagram 2.7e., the distance of Mo and M_1 on the screen can be artificially changed as shown in diagram 2.7c. If the amplitude Vr of the rectangular wave is precisely adjusted to match both peaks (Mo and M_1), the original peak interval can be expressed by the adjustment of Vr. The electronics of the peak matcher can then be arranged such that the ion with mass Mo and ion of mass M_1 are scanned alternately on the oscilloscope, i.e. the rectangular wave is added alternately. It is this which enables the peaks to be superimposed accurately or 'matched'.

M_1 and Mo are usually two ions from the spectrum of standard reference compounds such as perfluorokerosene or heptacosafluorotributylamine. M_1 and Mo are first matched to each other by means of two voltage adjusters, coarse and fine, and effectively calibrate the range of Vr. M, the ion to be measured, is then matched to Mo by means of another voltage adjusting control which enables the mass difference (Mo - M) to be read off in millimass units if Mo - M_1 is unity.

For each ion to be measured a suitable reference compound is chosen which has ions in its mass spectrum just above and below the mass of the unknown ion. If one reference compound is to be used to match ions from a series of compounds i.e. nitrosamines, then the reference compound must produce ions such that the masses of a pair of ions are above and below the masses of the ions to be measured. Usually
Diagram 2.7. Oscilloscope traces produced when matching ions and the form of electronic wave required to produce the trace.

(a) Normal scanning of Vs
(b) Sawtooth wave
(c) Effect of square wave on normal sweep
(d) Addition of rectangular wave to sawtooth wave
(e) Rectangular wave
and Mo are selected so that they can be easily matched together, i.e. have a similar peak height, or can be adjusted to a similar peak height by means of an amplification system provided for each reference ion when the ions are being scanned alternately.

High resolution mass spectrometry – use of scanning method for nitrosamines

It is not necessary to measure the masses of ions in this way when the mass spectrometer is being used as a detector. To detect nitrosamines, the following procedure was used. An ion, from an isomer, representing a particular nitrosamine and an ion from a reference compound were matched together and the positions of the voltage adjustment controls noted. The ions from the isomer were then pumped away, leaving the reference peak appearing on alternate sweeps of the oscilloscope. If a genuine nitrosamine was then injected onto the G.C., at the retention time of the nitrosamine (or just after, to allow time for the compound to get from the end of the G.C. column to the ion source of the mass spectrometer), the following pictures were seen on the oscilloscope screen (shown in diagram 2.8.) This is also what would be seen if the same nitrosamine was present in a meat extract, and the extract was injected onto the mass spectrometer. Diagram 2.8a. shows the reference ion being scanned and the fading trace of the scan for a nitrosamine ion. The next diagram (2.8b.) shows the trace of the reference ion now fading and the blank scan for the nitrosamine reappearing. Diagram 2.8c. is the same as diagram 2.8a. On the next scan for the nitrosamine, however, in diagram 2.8d, a small 'blip' has appeared matching exactly with the position of the fading peak of the reference ion. Diagrams 2.8e, f, g, and h then show the gradual appearance of the nitrosamine ion and its disappearance. The appearance of a peak, matching exactly with the peak of the reference ion at the retention time of the nitrosamine injected onto the G.C., is taken as proof of the presence of a nitrosamine. It is only possible to estimate the
Diagram 2.8. Appearance and disappearance of nitrosamine ion, while matched to reference ion, on consecutive sweeps of oscilloscope.
amount of nitrosamine by judging the height of the peak against the oscilloscope graticule. The diagram below shows what is seen when an ion of similar mass is present at the same time as the nitrosamine emerges.

Trace of 'matching standard ion' of nitrosamine

Fading trace of 'matching standard ion' with new trace of nitrosamine ion, in normal case

Fading trace of 'matching standard ion' with new trace of nitrosamine ion and ion with same integer $m/e$ value

DIAGRAM 2.9. View of trace on oscilloscope screen when ion of similar mass emerges with nitrosamine ion

The values of the adjusting voltages required to match each nitrosamine and its reference ion were measured and are recorded in Table 2.4. The values obtained were found to be reproducible over several months, although it was considered a useful precaution to check them each time a series of analyses were made.
<table>
<thead>
<tr>
<th>Nitrosamine</th>
<th>m/e observed</th>
<th>Reference compound</th>
<th>Reference m/e</th>
<th>Δ M</th>
<th>M Coarse</th>
<th>M Fine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-ethyl</td>
<td>102</td>
<td>HCTB²</td>
<td>100</td>
<td>0.94350</td>
<td>063</td>
<td>000</td>
</tr>
<tr>
<td>Di-propyl</td>
<td>130</td>
<td>HCTB</td>
<td>131</td>
<td>0.993705</td>
<td>330</td>
<td>000</td>
</tr>
<tr>
<td>Di-butyl</td>
<td>84</td>
<td>HCTB</td>
<td>82</td>
<td>0.96100</td>
<td>044</td>
<td>000</td>
</tr>
<tr>
<td>Nitrosopyrrolidine</td>
<td>100</td>
<td>cyclohexan-1one</td>
<td>102</td>
<td>0.99200</td>
<td>222</td>
<td>000</td>
</tr>
<tr>
<td>Nitrosopiperidine</td>
<td>114</td>
<td>HCTB</td>
<td>112</td>
<td>0.99020</td>
<td>090</td>
<td>000</td>
</tr>
</tbody>
</table>

1. It was found impossible to detect dimethyl nitrosamine in this way because with the high gain required to detect nanogram quantities of nitrosamines, an ion was present which obscured the position where DMN was expected. This meant DMN had to be detected by focussing on its molecular ion as described earlier.

2. HCTB: Heptacosa fluorotributylamine, a fluorinated hydrocarbon of high molecular weight.

3. It is not possible with the present peak matching system to superimpose or match ions that have a mass difference of less than one mass unit. This is due to a limit on the size of the voltage Vr, the 'saw tooth' wave. Heptacosafluorotributylamine has an ion in its mass spectrum at m/e = 99.99361 due to C₂F₄⁺, but this is too close to 100. The mass of the C₄H₆N₂O⁺ ion of nitrosopyrrolidine. The C₆H₁₀O⁺ ion of cyclohexanone at mass 98 was used to match the nitrosopyrrolidine ion instead.

When samples of meat extracts were examined, the peak matcher was set up by adjustment of the voltage controls and the main magnet so that the reference peak appeared in the centre of the oscilloscope screen on alternate sweeps.
Stainless Steel Bellows Valve

Fritted Glass Tube

Diagram 2.10 The Watson-Biemann Separator
Installation of a solvent diverting valve

A limiting factor in the detection of the nitrosamines was the amount of sample that could be injected onto the G.C. column (1 μl) since a large amount of solvent vapour is unacceptable in the ion source of the mass spectrometer. In an effort to bypass this problem, I decided to place a vacuum tight stainless steel bellows type valve in the oven around the Biemann-Watson separator, as shown in diagram 2.10. The valve chosen was a Nupro type 'H' all metal valve, with 1/8" Swagelock fittings. This was so that glass to metal seals could be fitted to the valve, allowing the whole assembly to be joined into the existing glass tubing.

With the valve, sample volumes of up to 10 μl were able to be injected onto the packed gas-chromatography columns used in the G.C. oven. This improved the sensitivity approximately ten times. While the solvent was being eluted, the valve was kept closed, and solvent that came into the separator was removed by the pump normally used to abstract the helium carrier gas. When the flame ionisation detector on the G.C. showed that the solvent had been eluted from the gas chromatograph, the valve was opened to allow the passage of the remaining constituents of the extract.

Gas chromatograph conditions used with high resolution - mass spectrometry

The gas chromatographic conditions used for the low resolution mass spectrometry were not necessary when the method of focussing on a molecular ion method of analysis was used. It was only necessary to know the retention time at a particular oven temperature, and the temperature was chosen so that the nitrosamine being monitored was eluted from the G.C. in about 3 - 5 minutes.
Diagram 2.11. Gas chromatograph produced of 6 nitrosamines in a standard solution run on a 2 metre packed column of D.E.G.A. on Chromosorb W. The temperature programme used was 10 min at 90° then 90°-115° at 5°/min.

1. N-nitrosodimethylamine
2. N-nitrosodiethylamine
3. N-nitrosodibutylamine
4. N-nitrosopyrrolidine
5. N-nitrosopiperidine
6. N-nitrosodipentylamine
For the peak matching method of analysis, it was necessary to have a good gas chromatographic separation of nitrosamines, to allow plenty of time to be able to reset the peak matching conditions for each nitrosamine being monitored. Another necessity was that the nitrosamine peaks were sharp, since the sharper the G.C. profile, the more concentrated the nitrosamine was and consequently the greater the deflection of the electron beam on the cathode ray oscilloscope of the peak matcher. This makes detection and estimation both easier and more accurate. A 2m x 3mm stainless steel packed column of 15% 'D.E.G.A.' on 'Chromosorb W' was found to give slightly sharper peaks than the 'Carbowax' column used previously. Temperature programming of the G.C. oven was used, the conditions being 10 minutes at 90° and then 90° to 115° at 5°/minute. The G.C. profile produced by this program is shown in diagram 2.11.
CHAPTER 3

ANALYTICAL METHODS FOR THE MEASUREMENT OF

THE STABLE ISOTOPES OF NITROGEN
3.1. Introduction

Although six isotopes of nitrogen are known, only those having mass numbers 14 and 15 are stable. The mass 13 isotope is the longest lived of the four radioactive nuclides, and it has been used as a tracer in chemical and biological research (Nicholas et al. 1961). The half life of this isotope (10.05 min.) is so short, however, that for most tracer investigations it is necessary to use nitrogen compounds enriched with the naturally occurring isotope $^{15}\text{N}$. In this study, most of the $^{15}\text{N}$ tracer work was carried out using $\text{Na}^{15}\text{NO}_2$ isotopically enriched to approximately 96.6 atoms % $^{15}\text{N}$. In one experiment the $^{15}\text{N}$ nitrite was diluted with $^{14}\text{N}$ nitrite. This was to avoid a 'memory' effect of $^{14}$-$^{15}\text{N}$ in a particularly sensitive mass spectrometer (AEI.MS20) that was used for some of the analyses.

Measurement of isotope enrichment can be made using mass spectrometry or optical spectroscopy. Both methods have been used in this investigation.

3.2. Methodology

The use of mass spectrometry for isotope enrichment analysis

To determine the isotopic composition of stable elements by mass spectrometry, it is necessary that the element under analysis be converted to a suitable gas. The gas should have a low molecular weight and be as simple as possible in structure from both the molecular and isotopic viewpoint. It should also be readily preparable from organic and inorganic compounds, and be easily pumped out of the mass spectrometer. In the case of nitrogen, the gas which best meets these requirements is molecular nitrogen, and the methods for nitrogen isotope-ratio analysis are accordingly based on the conversion of $^{15}\text{N}$ labelled nitrogen in the material under investigation to $^{14}\text{N} - ^{15}\text{N}$. In mass spectral analysis, the ions formed by electron bombardment of the nitrogen sample in the spectrometer are separated electromagnetically and the relative abundances are determined by measuring the currents they
produce when collected on an insulated electrode. The combinations of ions that are formed when nitrogen is bombarded by electrons are shown in Table 3.1. The isotopic composition of nitrogen is best determined by measurement of the ion currents corresponding to mass 28 \((^{14}N-^{14}N)\), mass 29 \((^{15}N - ^{14}N)\) and mass 30 \((^{15}N^{15}N)\).

### Table 3.1

IONS FORMED BY ELECTRON BOMBARDMENT OF MOLECULAR NITROGEN IN THE MASS SPECTROMETER

<table>
<thead>
<tr>
<th>Mass Number</th>
<th>Ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>((^{15}_N^{15}_N)^+)</td>
</tr>
<tr>
<td>29</td>
<td>((^{15}_N^{14}_N)^+)</td>
</tr>
<tr>
<td>28</td>
<td>((^{14}_N^{14}_N)^+)</td>
</tr>
<tr>
<td>15</td>
<td>((^{15}_N)^+ ) and ((^{15}_N^{15}_N)^{++})</td>
</tr>
<tr>
<td>14.5</td>
<td>((^{14}_N)^{++}) and ((^{14}_N^{14}_N)^{++})</td>
</tr>
<tr>
<td>14</td>
<td>((^{14}_N)^+ ) and ((^{14}_N^{14}_N)^{++})</td>
</tr>
</tbody>
</table>

Sample preparation for analysis by mass spectrometry

The conversion of \(^{15}N\)-labelled samples to gaseous nitrogen was effected using a Dumas high vacuum train, shown in diagram 3.1. This was based on a design from the Agricultural Research Council, Letcombe Laboratories at Wantage, Berks. Several methods of sample combustion have been tried by other workers. In most tracer investigations, the conversion of labelled-N to ammonium ion has been performed by the Kjeldahl method or one of its modifications, (Rittenberg, et al. 1939, 1948, Sprinson and Rittenberg, 1948, 1949). The Dumas method appeared more suitable because it converted combined nitrogen directly to \(N_2\), and contamination by amines was avoided.
Samples of meat, treated with $^{15}$N-enriched nitrite were analysed in the following way. The sample was first dried, either by evaporating water from solutions of samples in a rotary evaporator, or by freeze-drying. The dried sample (approximately 100 mg) was wrapped in a small square of copper foil and placed in one of the silica combustion tubes containing both copper oxide, and a coil of copper wire. The whole apparatus was then evacuated to a pressure of $10^{-3}$ mm Hg. This evacuation was made by a Speedivac 1" combined Rotary pump and Diffusion pump system (Edwards HiVac Co., Ltd.). The combustion tube was then heated to red heat ($900^\circ$) by means of a small hand-wound electric furnace. This procedure converted the sample to carbon dioxide, nitrogen and water. The function of the copper was to reduce oxides of nitrogen that were formed, and the copper oxide was to oxidise ammonia and amines. Gas production was observed by a fall in the level of the mercury in the manometer connected to the sample degradation section of the apparatus.

When the manometer ceased to show evolution of gas, the degradation of the sample to gaseous forms of nitrogen was taken as complete. The gases were then circulated through two furnaces, both at $800^\circ - 850^\circ$, one containing copper and the other copper oxide. This was to ensure the completion of the Dumas degradation to gaseous nitrogen, and to effect the total equilibration of the $^{15}$N atoms:

$$^{15}_N - ^{15}_N + ^{14}_N - ^{14}_N \rightarrow 2^{15}N^{14}N$$

The gases were pumped around the 'inner circle' of glass tubing by means of an all glass solenoid-operated circulating pump. One way valves were incorporated just above and below the pump, so that as it moved up and down in the glass tubing, similar to a piston, gas would be sucked into the pump through the lower valve, and then pushed out again through the upper valve. After 20 mins, the gas was allowed to expand into the liquid nitrogen-cooled trap. This was a precaution taken to ensure the
Diagram 3.1 Dumas degradation high vacuum train.

removal of any material that had still escaped degradation, and CO\textsubscript{2} and \textsuperscript{16}O\textsubscript{2} by freezing. Carbon monoxide and methylamine, both possible contaminants can cause serious errors in the isotope measurement of nitrogen, (Holt and Hughes, 1955; Steyermark, 1961). The mass to charge ratio of CO\textsuperscript{+} is 28, and methylamine can produce ions at m/e ratios of 28, 29 and 30.

The pure nitrogen gas was then adsorbed onto molecular sieve (calcium aluminium silicate), type 5A, which had been evacuated overnight whilst in the sample collection tubes. The molecular sieve was cooled with liquid nitrogen to improve its adsorption properties. The sample tubes containing the molecular sieve were then transferred to the mass spectrometer (RMU-6E) for analysis.
Mass spectral analysis

The mass spectral studies on samples prepared on the high vacuum train were all made using the Hitachi-Perkin-Elmer RMU-6E. For accurate measurement of peak heights, the usual amplification system of the mass spectrometer could not be used. This was because the electron multiplier was not completely linear in response. An alternative method, with the Faraday cup ion collector was used instead. This was many times less sensitive than the electron multiplier because the Faraday cup collector is effectively the first electron multiplier plate without any potential supplied to the other plates.

The change from electron multiplier to Faraday cup was made by removing an inspection cover on the ion detector system, and altering two of the connections inside. The amplification range of a variable amplification stage after the electron multiplier/Faraday cup assembly was found to be inaccurate after attempting calibrations with the known nitrogen isotope abundance of air samples. It was, therefore, necessary to have an accurate incremental attenuator built by the In-Line Process Control Section of B.F.M.I.R.A. that allowed the gain of the amplification system to be altered precisely. The alterations in amplification were necessary because of the vastly different peak heights from ions at m/e's 28 and 29.

The initial step in the $^{15}$N abundance measurement procedure was to magnetically scan over the peaks at m/e's 28, 29 and 30. This was repeated several times, so that an average reading of the heights of these background ions could be made. The gas in the sample tube was then connected to the mass spectrometer inlet via a 'U' tube, with ground glass joints. Enough gas was allowed out of the sample container to produce a pressure of 10 mm Hg on the inlet pressure gauge. This gas was then admitted into the mass spectrometer, and the new mass spectrum recorded four or five times as below in diagram 3.3. The m/e = 29
Diagram 3.3. Consecutive mass spectra of the m/e = 28 and m/e = 29 isotopes of nitrogen for abundance measurements.
peaks were then extrapolated so that the peak height of $^{14}\text{N}^{15}\text{N}$ was effectively measured at the same time as the m/e = 28 peak ($^{14}\text{N}^{14}\text{N}$). This was to overcome the loss in sample pressure while the gas was being pumped away, and which would have affected the result. The ratio of $m/e = 28 : m/e = 29$ was then calculated after subtraction of the background and an average value for the enrichment obtained.

This method of analysis would have continued for the measurement of all the enriched samples produced by this study had it not been for several serious drawbacks.

(a) The vacuum train suffered from leaks in various taps and joints, which lead to errors in the enrichment values obtained.

(b) It took an appreciable time to pump out the remains of the previous gas sample from the vacuum train. This was necessary to avoid 'memory' effects which would contribute to errors.

(c) The mass spectrometer was being frequently used for nitrosamine analysis. This meant that the background values for ions at $m/e = 28, 29, \text{ and } 30$ were relatively very high. Ideally, for isotope measurement, a mass spectrometer should have an internal pressure of $<10^{-9}$ mm Hg. The RMU-6E had a pressure of $1 \times 10^{-6}$ mm Hg because of the large amounts of extracts that were injected into it. The vacuum could be improved by 'baking out' the analyser tube, but this also slowed analyses.

Another method for analysis of $^{14}\text{N} : ^{15}\text{N}$ ratios was clearly needed, and about this time, an emission spectroscope had become commercially available, which could determine levels of enrichment of nitrogen gas samples. One of these instruments, a Statron NOI-15 was used through the courtesy of Mr R Mercer, Deputy Director ARC Wantage Laboratories, Berks.

The use of an emission spectroscope for $^{15}\text{N}$ abundance measurements

The analysis is based upon the relatively large isotope shifts in the molecular emission spectrum of gaseous nitrogen. The $^{14}\text{N} - ^{15}\text{N}$ band of the
transition $2 \rightarrow 0$ is separated by about 0.6 nm from that for $^{15}\text{N} - ^{14}\text{N}$.

Nitrogen gas in a sample tube is excited by a radiofrequency generator. This causes the nitrogen to emit light. The light is resolved by a sodium chloride prism on a turntable, which allows the sequential measurement of the bandhead intensities on a photomultiplier detector. The peak heights are graphically recorded, and the $^{15}\text{N} - ^{14}\text{N} : ^{14}\text{N} - ^{14}\text{N}$ ratio is then calibrated against a standard curve to obtain the abundance ratio in the sample.

**Sample preparation**

Samples from incorporation experiments were prepared for the analyser as follows. The samples were first converted to ammonia by the Kjeldahl method — (AOAC, 1970) which also converts NaNO$_2$; the resulting ammonium chloride solutions were taken to dryness in a rotary evaporator. A standard solution of NH$_4$Cl for each sample was then made up, so that a known amount could be added, via a syringe, to a small glass sample holder. It was necessary to add a predetermined amount of NH$_4$Cl, since there was an optimum amount that could be analysed; too little and no gaseous discharge could be created, too much and the discharge was quenched by the excess gas. Enough NH$_4$Cl solution was added to provide about 1 mm Hg pressure of gaseous nitrogen in the discharge tube. The sample holder, when filled with the sample to be analysed, was placed in an oven at 70° to evaporate the water. The sample holder was then placed in a discharge tube, containing between 0.5 and 1.0 g CuO. This is shown in diagram 3.4. The discharge tube was fixed to a glass T-piece with sealing wax. The T-piece was fitted with a quartz section, so that the CaO necessary for the combustion of the NH$_4$Cl could be heated strongly (to 900°) in order to activate it. This activation has been found necessary for the CaO to perform its function of absorbing H$_2$O, CO$_2$ and HCl produced in the degradation of the NH$_4$Cl (Perschke et al., 1971).
Diagram 3.4. Apparatus for the degradation of labelled NH₄Cl to N₂
The whole apparatus was evacuated, the discharge tube and the CuO were lightly heated to de-gas them, the CaO was then strongly heated. When no more gas was seen to escape (by reference to a vacuum gauge in the system), the discharge tube was sealed at the constriction and placed in an oven at 500° for 1 hour for the NH₄Cl, CuO and CaO combustion to take place. When cooled, the discharge tube was placed between two electrodes in the emission spectrometer and the $^{15}$N abundance measured. A typical spectrum is shown on the next page (diagram 3.5).

The peak heights a and b (i.e. the intensities of the bandheads) are read off and related to the same gain setting V.

Their ratio, $R = \frac{b (V_a)}{a (V_b)} = \frac{\sqrt{14}N_2}{\sqrt{14}N^{15} N}$ makes it possible to calculate the apparent abundance of the $^{15}$N isotope (in atoms %) from the formula

$$^{15}N \text{ (atom %)} = \frac{100}{2R + 1}$$

The value is then corrected by means of a standard curve. This correction is necessary because the $^{15}$N - $^{14}$N band lies on the shoulder of that of the $^{14}$N - $^{14}$N band (Leichmann et al. 1968).

A comparison of the mass spectrometry and emission spectroscopy methods

The emission spectroscope was found to have several advantages over the mass spectrometer.

(a) The time taken per sample was much less when analysed by the emission spectroscope than by the mass spectrometer.

(b) There was no 'memory' effect.

(c) The maintenance was much less since there were no fixed life filaments or elaborate vacuum systems. Instrument down-time was minimal.
Diagram 3.5. Emission spectrum of molecular nitrogen, partially labelled with $^{15}\text{N}$
(d) Although the lowest limits of detection were not needed for this study, it is possible to routinely measure the abundance of $^{15}$N in samples of approximately 5.0 μg (Faust, 1967). It is also possible to measure enrichments in samples of even lower mass, of the order of 0.1 g by using an inert gas, such as helium or xenon to sustain the discharge (Cook et al., 1967; Goleb and Middelboe, 1968).

The mass spectrometer, however, had the advantages of

(e) being able to measure lower levels of enrichment more accurately than the spectroscope, and

(f) giving an 'absolute' value for the enrichment; that is the emission spectroscope needed to be calibrated by reference to the abundances measured on identical samples by the mass spectrometer.
PART III

RESULTS
CHAPTER 4

THE DETECTION AND ESTIMATION OF NITROSAMINES
4.1 Introduction

The analyses for volatile-nitrosamines have been made on extracts supplied by the Biochemistry Laboratory of B.F.M.I.R.A. As the methods of analysis for volatile nitrosamines have been changed to suit demands for more sensitivity, the methods of sample preparation have also had to conform to this requirement. The samples which were prepared for analysis by the low-resolution mass spectrometer contained far more compounds than the samples which were supplied latterly for analysis on the high resolution instrument.

The need to identify 'non-volatile' nitrosamines from samples of cured meat meant that mass spectrometry might be needed for this, and a number of spectra were run of various nitrosamines and nitrosamides. This was to see if spectra of actual extracts from cured meats contained any similarities to the spectra of the pure compounds. The spectra of the nitrosamines and nitrosamides are shown, together with high resolution measurements made on some of the fragmentation peaks in order to confirm the tentative fragmentation pattern. It was not possible to identify all the peaks at low \( \frac{m}{e} \) values, such as below \( \frac{m}{e} = 50 \) and which would not be of any real importance in the identification of the nitrosamine, so it was decided to ignore the measurement of these ions.
4.2 The Preparation of Cured Meat and Fish Extracts for Low Resolution Analysis

The analyses were carried out as described in Chapter 2. The samples were first screened by monitoring the peak at $m/e = 30$ of NO$^+$. The mass spectrometer and gas chromatograph conditions were as follows:

A Perkin-Elmer F-11 gas chromatograph was used with a 2 metre x 3 mm i.d. stainless steel column, packed with 15% Carbowax 20M on 'Chromosorb W' (80 - 100 mesh). The effluent stream from the column was split, with effluent going to an F.I.D. detector and some being transferred via a heated stainless steel capillary to an Hitachi-Perkin Elmer RMU-6 mass spectrometer, (1:15 ratio).

Gas chromatograph conditions:

- Oven temperature: $70^\circ - 200^\circ$ at $10^\circ/\text{min}$
- Carrier gas: He, Flow rate = 16 ml/min
- G.C. Injector temperature: $200^\circ$
- Heated transfer line: $200^\circ$
- Separator oven: $200^\circ$
- Ion source temperature: $200^\circ$
- Glass heater temperature: $200^\circ$
- Accelerating voltage: 2400V
- Trap current: 60 $\mu$A
- Chamber voltage: 700V

Extracts were prepared by refluxing the cured meat product with methanol and then collecting a series of distillates from a spinning band fractionating column. (Casselden, Walters and Johnson, 1970)
4.3 Results

Low resolution analysis of extracts for volatile nitrosamines

The analysis of the samples supplied was a tedious process by low resolution mass spectroscopy, and the determination of the nitrosamines were limited to a qualification of their presence or absence only.

In the following tables, the symbols below represent:

- **DMN**: N-nitrosodimethylamine
- **DEN**: N-nitrosodiethylamine
- **DPN**: N-nitrosodipentylamine
- **NOPYR**: N-nitrosopyrroloidine
- **NOPIP**: N-nitrosopiperidine
- **ND**: None detected
- **P**: Present
<table>
<thead>
<tr>
<th>Description of cured product</th>
<th>Number of spinning band fractions analysed</th>
<th>DEN</th>
<th>DPN</th>
<th>NOIP</th>
<th>NOPYR</th>
<th>NOPy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial Corned Beef</td>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Spiked with 50 ppb DEN, DPN</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Commercial Corned Beef</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Spiked with 50 ppb DEN, DPN</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Commercial Corned Beef</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Spiked with 50 ppb DEN, DPN</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Description of cured product</td>
<td>Number of spinning band fractions analysed</td>
<td>DMN</td>
<td>DEN</td>
<td>DPN</td>
<td>NOPYR</td>
<td>NOPIP</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------------------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Commercial Luncheon Meat</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Commercial Luncheon Meat Spiked with 50 ppb DEN, DPN, DNOPIP</td>
<td>4</td>
<td>ND</td>
<td>P</td>
<td>P</td>
<td>ND</td>
<td>P</td>
</tr>
<tr>
<td>Luncheon Meat</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Luncheon Meat Spiked with DEN, DPN, NOPIP</td>
<td>4</td>
<td>ND</td>
<td>P</td>
<td>P</td>
<td>ND</td>
<td>P</td>
</tr>
<tr>
<td>Smoked Haddock</td>
<td>5</td>
<td>P?</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Description of cured product</td>
<td>Number of spinning band fractions analysed</td>
<td>DMN</td>
<td>DEN</td>
<td>DPN</td>
<td>NOPYR</td>
<td>NOPIP</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>-------------------------------------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Wiltshire unsmoked cured fore end bacon</td>
<td>7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Wiltshire unsmoked cured fore end bacon</td>
<td>7</td>
<td>ND</td>
<td>P</td>
<td>P</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Wiltshire unsmoked cured fore end bacon</td>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Wiltshire unsmoked cured fore end bacon</td>
<td>7</td>
<td>ND</td>
<td>P</td>
<td>P</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
The limit of detection of each nitrosamine, using this method of detection was found to be approximately 50 \( \mu g \). The low resolution mass spectrometer, however, was too insensitive and too indiscriminate. Consequently a high resolution instrument was required and when one was available, the alternative method of analysis, also described in Chapter 2 was carried out.

The following results were obtained using the 'static' method of analysis. This method was used because the volume of extract supplied was large enough to permit separate gas chromatograph injections for each nitrosamine. The static method also allows a permanent record to be kept of each analysis.

**Analysis of commercial spice/nitrite mixtures**

Volumes of 1\( \mu l \) of sample prepared from extracts of spice-nitrite mixtures were injected into the gas chromatograph-mass spectrometer system. If samples gave a positive response, the injection was repeated; if samples gave a negative response, 10\( \mu l \) of sample were injected. Checks were made before every injection to ensure that the mass spectrometer was focussed exactly on the nitrosamine ion under analysis. Analyses were made for N-nitrosodimethylamine, N-nitrosopyrrolidine and N-nitrosopiperidine, these being the nitrosamines thought most likely to be present. The results are given in concentrations of each nitrosamine originally present in the spice mix.
<table>
<thead>
<tr>
<th>Sample</th>
<th>N-nitrosodimethylamine</th>
<th>N-nitrosopyrrolidine</th>
<th>N-nitrosopiperidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spice mix 1</td>
<td>none detected</td>
<td>2 ppb</td>
<td>none detected</td>
</tr>
<tr>
<td>Spice mix 2</td>
<td>&lt;1 ppb</td>
<td>2 ppb</td>
<td>none detected</td>
</tr>
<tr>
<td>Spice mix 3</td>
<td>3 ppb</td>
<td>12-15 ppb</td>
<td>3 ppb</td>
</tr>
<tr>
<td>Spice mix 4</td>
<td>none detected</td>
<td>10 ppb</td>
<td>none detected</td>
</tr>
<tr>
<td>Spice mix 5</td>
<td>none detected</td>
<td>6 ppb</td>
<td>&lt;1 ppb</td>
</tr>
<tr>
<td></td>
<td>Nitrosamine</td>
<td>1 µl injection</td>
<td>10 µl injection</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------------</td>
<td>----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td><strong>Meat</strong> (1% NO₂, nitrosated) (cooked pork)</td>
<td>N-nitroso Pyrrolidine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DMN</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>N-nitroso Piperidine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Milk</strong> (1% NaNO₂) (pH 6.0)</td>
<td>N-nitroso Pyrrolidine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DMN</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>N-nitroso Piperidine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Egg</strong> (1% NaNO₂) (pH 6.0)</td>
<td>N-nitroso Pyrrolidine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DMN</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>N-nitroso Piperidine</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Analyses have been made using high resolution mass spectroscopy on several samples of cured meats from a commercial supplier, and on meat milk, cheese and eggs to which nitrite had been added. A 'breakfast' to which nitrite had also been added was analysed for N-nitrosopyrroloidine.
### Sample Nitrosamine Level

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nitrosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacon</td>
<td>5 ppb N-nitrosopyrrolidine</td>
</tr>
<tr>
<td>Bacon</td>
<td>5 ppb N-nitrosopyrrolidine</td>
</tr>
<tr>
<td>Nitrosated 'breakfast'</td>
<td>7 ppb N-nitrosopyrrolidine</td>
</tr>
<tr>
<td>Luncheon meat</td>
<td>30 ppb N-nitrosodimethylamine</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nitrosamine</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheese, 1% nitrite pH 3.0</td>
<td>DMN</td>
<td>80 ppb.</td>
</tr>
<tr>
<td></td>
<td>NPY</td>
<td>30 ppb.</td>
</tr>
<tr>
<td>Milk, 1% nitrite, pH 3.0</td>
<td>DMN</td>
<td>32 ppb.</td>
</tr>
<tr>
<td></td>
<td>NPY</td>
<td>15 ppb.</td>
</tr>
</tbody>
</table>
4.5 Detection of non-volatile nitrosamines

Spectra of a series of non-volatile nitrosamines were observed using the low-resolution mass spectrometer. This was to enable characterisation of nitrosamines from extracts of cured products, purified by thin layer chromatography. All the spectra were run under the following conditions:

- Accelerating voltage: 2400V
- Ion source temperature: 180°
- Chamber voltage: 70eV
- Trap current: 60 µA
N-nitrosoproline

The low resolution spectrum of this compound showed peaks at \( \frac{m}{e} \) values of 144, 99, 69, 44 and 30. (diagram 4.1). The probable pathway for their formation is:

\[
\begin{align*}
\text{NNO} & \rightarrow \text{NNO} \\
\frac{m}{e} = 144 & \rightarrow \frac{m}{e} = 30 \\
\text{NO}^+ & \\
\frac{m}{e} = 99 & \rightarrow \frac{m}{e} = 69
\end{align*}
\]

High resolution measurements were made on two of the major ions and gave the elemental composition of the ions.

<table>
<thead>
<tr>
<th>Mass</th>
<th>Measured</th>
<th>Calculated</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>144</td>
<td>144.05259</td>
<td>144.05347</td>
<td>( \text{C}_5\text{H}_8\text{N}_2\text{O}_3 )</td>
</tr>
<tr>
<td>99</td>
<td>99.05450</td>
<td>99.05574</td>
<td>( \text{C}_4\text{H}_7\text{N}_2\text{O} )</td>
</tr>
</tbody>
</table>
Diagram 4.1. Mass spectrum of N-nitrosoproline
N-nitrosohydroxyproline

The low resolution spectrum of this compound showed ions at \( \frac{m}{e} \) values 160, 115, 86, 56, 44, 41 and 30. (diagram 4.2). The probable mechanism of their formation is:

\[ \begin{align*}
(a) & \quad \text{HO-} \quad \text{CO}_2 \text{H} \\
& \quad \text{NO} \\
& \quad \frac{m}{e} = 160 \\
\downarrow \quad \text{NO}^+ (f) \\
(b) & \quad \text{HO-} \quad \text{NO} \\
& \quad \frac{m}{e} = 115 \\
& \quad \downarrow \\
& \quad \text{NO}^+ \\
& \quad \frac{m}{e} = 97
\end{align*} \]
Diagram 4.2. Mass spectrum of N-nitrosohydroxyproline
The mass spectrum of N-nitrososarcosine showed ions at $m/e$ 118, 88, 73, 44, 43, 42 and 30. (diagram 4.3). A possible mechanism for their formation is:

\[
\begin{align*}
\text{CH}_3^+ \text{N}-\text{CH}_2\text{CO}_2\text{H} & \rightarrow \left[\text{CH}_3\text{NCH}_2\text{CO}_2\text{H}\right]^+ \quad \frac{m}{e} = 88 \\
\left[\text{CH}_3\text{NCH}_2\text{H}\right]^+ & \rightarrow \left[\text{CH}_3\text{NCH}_2\text{H}\right]^+ \quad \frac{m}{e} = 73 \\
\left[\text{CH}_3\text{NCH}_2\text{H}\right]^+ & \rightarrow \left[\text{CH}_3\text{NCH}_2\text{H}\right]^+ \quad \frac{m}{e} = 43
\end{align*}
\]

High resolution measurement of the molecular ion gave

Measured \quad 118.0371 \quad \rightarrow \quad \text{C}_3\text{H}_6\text{N}_2\text{O}_3

Calculated \quad 118.0378
Diagram 4.3. Mass spectrum of N-nitrososarcosine

\[
\text{CH}_3 - \text{N} \cdot \text{CH}_2 - \text{COOH}
\]

\[
\text{NO}
\]
N-nitrososarcosinamide

The low resolution spectrum showed ions at 117, 87, 73, 59, 45, 44, 43 and 30. (diagram 4.4).
Diagram 4.4. Mass spectrum of N-nitrososarcosinamide
Nitrosated prolylglycine

The low resolution spectrum of this compound (diagram 4.6) shows the presence of more than one nitrosation product. Ions at $\frac{m}{e} = 185$ are due to nitrosation at one of the two secondary amino nitrogen atoms. The postulated mechanism below shows how the ions are probably formed.
Diagram 4.6. Mass spectrum of Nitrosated prolylglycine
Miscellaneous compounds

During the analysis for nitrosamines of volatile extracts from cured foods, as studied by low resolution mass spectroscopy, a number of mass spectra were obtained, and some of the components in these extracts were tentatively identified. These were:

methylethylpyridine  dimethylnaphthalene  phenylacetonitrile  o-methoxyphenol  benzaldehyde  methylcyanfibenzene  3-hydroxy 2-butanone  propylthiazole  methylthiazole

N-methyl toluidine  ethylpyridine  picoline  n-pentynitrite  ethynitrite

It should be emphasized that none of these compounds have been confirmed by high resolution mass spectroscopy.
CONCLUSIONS

The lack of results for the low resolution analyses of cured meat products illustrates the need for sensitive methods of nitrosamine detection.

Using high resolution mass spectrometry, volatile nitrosamines were found in samples of commercial spice/nitrite mixes. The production of these ready mixed cure ingredients has now been discouraged in the U.K.

Analysis of both bacon and luncheon meat has shown the presence of N-nitrosopyrrolidine and N-nitrosodimethylamine although the number of samples analysed was very small.

The possibility of nitrosamine formation in cheese, eggs and milk has been indicated.
CHAPTER 5

THE FATE OF NITRITE IN THE

MEAT CURING PROCESS
CHAPTER 5

THE FATE OF NITRITE IN THE MEAT CURING PROCESS

5.1 Introduction

Sodium nitrite in contact with muscle tissue is known to react to form nitrosylmyoglobin. From studies of the amount of residual sodium nitrite and the concentration of nitrosyl myoglobin in the cured meat product it is apparent that far more nitrite has been destroyed than can be accounted for by the production of nitrosylmyoglobin alone. The object of this study has been to identify the products of the muscle-nitrite interaction and to produce a 'balance sheet' between the nitrite added and the recoverable nitrogen (from the nitrite) in the cured product. To aid the location of the nitrogen, sodium nitrite labelled with $^{15}\text{N}$, the only stable isotope of $^{14}\text{N}$, has been used as a tracer as no radioactive isotope with an appreciable half life is available.

Commercial curing practices involve treatment of the muscle by different processes. In this study, for the ease of comparison of results only two processes have been simulated, one imitating bacon production and the other, pasteurised ham production.

5.2 Experimental

Source and preparation of muscle tissue

Since the processes followed, bacon and pasteurised ham production, both use pork, so the starting material all these series of experiments have been carried out using porcine skeletal muscle. This was, at first obtained from two sources, a normal commercial slaughterhouse and a local butcher. Ante-mortem histories of the pigs were not available from either source. Differences in the amount of nitrite utilisation by the muscle from the two sources were found to be so different that only muscle from
from the slaughterhouse was used in all the later experiments. In the initial experiments, the muscle used from the slaughterhouse was from the lumber part of the crura of the diaphragm. In the latter experiments a number of muscles from whole shoulder of pigs were used, since much larger quantities of tissue were necessary for all the analyses required.

The muscle from the slaughterhouse was removed from freshly killed pigs, placed in an ice-cooled container and immediately transported to the laboratory. As much fat as possible was removed from the sample. The muscle was then minced in a small hand tissue grinder (A. Gallenkamp & Co., London); larger quantities of muscle were put through a commercially available mincer with \( \frac{1}{2} \)" holes (Spong & Co).

**Incubation of muscle tissue**

All incubations were carried out in 0.2M phosphate buffer, (hereafter referred to as incubation buffer) prepared from \( \text{KH}_2\text{PO}_4 \) and \( \text{Na}_2\text{HPO}_4 \) in proportions to produce a \( \text{pH} \) of 6.0 (the overall average \( \text{pH} \) of post-rigor muscle tissue), containing sodium chloride (5mg/ml) as in commercial practice, and chloramphenicol (0.1 mg/ml) a broad spectrum antibiotic to inhibit bacterial action in the muscle.

Incubations were carried out anaerobically to simulate the conditions likely in a block of muscle tissue and which are thus relevant to the injection of meat with brines. Anaerobicity was usually achieved by repeated evacuation of the incubation vessels, and flushing with argon. This gas was used due to its inertness; nitrogen could not be used since several of the incubations were analysed for the production of gaseous nitrogen. The samples were then incubated under a reduced pressure of argon for various lengths of time.

Some experiments in which the headspace gases were to be analysed were
not incubated with any argon, the anaerobic condition being simply produced as well as possible by a vacuum pump. This was to prevent dilution of any headspace gases formed, and to increase the sensitivity of detection of the gaseous products.

The initial incubation mixtures were generally composed of 2.5 g muscle mince, 6.0 ml incubation buffer and sodium nitrite at various relevant concentrations. The incubations were carried out in Thunberg tubes.

The later experiments, using 50 or 100 g quantities of muscle mince to allow sufficient material for all the necessary determinations of both liquid and gaseous products, were carried out in 250 ml round bottomed flasks fitted with gas tight taps.

The most frequently used incubation conditions were at 4° for 40 hours with 200 parts per million NaNO₂, this being similar to a commercial curing of Wiltshire bacon. The pasteurised ham cure was carried out leaving the incubation mixture at 4° overnight and then heating up to 70° for 4 hours.

In order to achieve reproducibility of sample preparation and equal accessibility to the muscle by sodium nitrite, minced muscle was chosen as the best way of fulfilling these aims, whilst involving the least disruption of cellular pattern. Taylor (1963) has shown that mincing of muscle in a small hand mixer produced an homogenate with a greater respiration rate than other methods using slicing, macerating, or chopping.
The determinations of the products of the incubations were carried out as described below. All the determinations were performed as quickly as possible to prevent further reaction of the nitrite. When immediate analysis was impossible samples were stored at -30° for as long as necessary.

Nitrite determinations

At the end of the incubation the net weight of the total cured sample of muscle tissue was recorded. To an aliquot of the sample an equal volume of distilled water was added. The diluted sample was then centrifuged at 26,000g for 30 min to sediment the meat solids, leaving nitrite in the supernatant. It was felt that extraction of the nitrite could be improved by first macerating to tissue sample, and this was carried out using an Atomix homogeniser (M.S.E. Ltd, London). The homogenised sample was then centrifuged as before. The supernatant was decanted, and the meat pellet resuspended in distilled water. Centrifugation of the meat sample was repeated and the supernatants and washings were pooled. An aliquot of the combined supernatant was diluted by a known amount and the nitrite content assayed using an azo-coupling reaction with sulphanilamide and N-1-naphthylethylenediamine (Nicholas and Nason, 1957), after precipitation of any soluble muscle protein (capable of interfering in the determination of nitrite) with 1 ml of each of Carrez I and II reagents, (ZnSO₄ in acetic acid and potassium ferrocyanide respectively). Sulphanilamide (1 ml of a 5% (w/v) solution in approximately 2.5N, hydrochloric acid) was added to the nitrite solution, followed by aqueous N-1-naphthylethylenediamine solution (1 ml of 0.1% (w/v)). The pink colour was allowed to develop for 30 minutes and the extinction read at 540 nm on a Unicam SP800 spectrophotometer with scale expansion facilities, using as reference, a sample similarly prepared from a meat incubation without sodium nitrite.

The concentration of the nitrite present was calculated by reference to a standard curve.
Nitrate determination

To an aliquot of the solution obtained from the centrifugation of the homogenised muscle tissue, 3g of 'spongy' cadmium (prepared by displacing cadmium from aqueous cadmium sulphate solution with zinc) was added, together with 5 ml of NH$_3$ - NH$_4$Cl buffer solution (prepared by adding 40 ml conc HCl to 500 ml water, then adding 95 ml 880 (15N) aqueous ammonia and diluting to 1 litre). The solution and cadmium were shaken for several minutes, during which the cadmium reduced any nitrate present to nitrite (Follet and Ratcliff, 1963). The solution was then filtered and the filtrate analysed for nitrite as described previously. The concentration of any nitrate present was calculated from the additional amount of nitrite detected after subtraction of any nitrate in a blank, from the reagents and the supernatant of the incubation containing no nitrite.

Determination of nitrosylmyoglobin

This was carried out using the method of Hornsey (1956). Acetone was added to an aliquot of the incubation sample, dropwise, and with shaking to a final concentration of acetone to water of 4:1 (by volume), taking into account the water content of the meat sample (usually 70%). The only pigments extracted in quantity were nitric oxide-haem-acetone complexes, and as virtually all the pigment present in the muscle was myoglobin, this procedure can be assumed to be extracting only nitrosyl myoglobin and no other pigment of the meat sample.

After filtration, a drop of concentrated hydrochloric acid was added to the solution. The sample was then stoppered overnight during which time the nitrosyl myoglobin was converted to acid haematin. The extinction of this solution was read at 512 nm.
For the total pigment determination, concentrated hydrochloric acid was added to acetone while the latter was still in contact with an aliquot of the meat sample. All pigments were then converted to acid haematin and after leaving stoppered over night the suspension was filtered, and the extinction of the filtrate was read at 512 nm. The amount of nitrosyl-myoglobin formed, as a percentage of the total pigments present, could then be calculated by comparison of the extinction values obtained for the two solutions at 512 nm.

**Determination of nitrosothiol compounds**

The method used is based upon that of Saville (1958). A sample of the meat slurry (10g) was taken and acetone (45ml) added dropwise, with shaking to produce total extraction of the nitric-oxide haem pigment, and also to remove as much residual nitrite as possible. The acetone-meat mixture was left for 1 hour in the cold, and then filtered. The solid residue was extracted twice more with acetone, the acetone-meat mixture being left in the cold for 30 minutes each time. After this, the mixture was filtered and the excess liquid squeezed out of the solid tissue with a glass rod. The solid residue was then dried.

Five ml of borax solution (10% w/v) was slowly added to one-half of the dried residue. This was followed by 20 ml of water. Then 5 ml of mercuric chloride solution (5% w/v) was added dropwise. The mixture was then left for 15 minutes. Following this, 1 ml of Carrez I solution and 1 ml of Carrez II solution were added to precipitate the soluble muscle protein. The total volume of the liquid was made up to 100 ml and filtered. Then 1 ml each of the solutions used to determine the nitrite concentration were added, and the extinction read at 540 nm. The nitrosothiol content of the cured tissue was calculated from protein-
S-NO + HgCl₂ + H₂O → protein-S-HgCl + HCl + HNO₂, the extinction at 540 nm, effectively giving the nitrous acid concentration.

This procedure was repeated on the other half of the dried residue, omitting the addition of mercuric chloride. This provided a blank reading of any inorganic nitrite present in the nitrosothiol determination.

Preparation of cured sample tissue for further determinations

Determination of nitrogen content, non-volatile nitrosamine concentration and ¹⁵N incorporation required that the samples were free from moisture. This was achieved by macerating aliquots of the cured tissue in an Atomix (M.S.L. Ltd), and centrifuging the resulting tissue suspension, at 26,000g for 30 minutes. The supernatant was decanted, and the solid pellets resuspended and centrifuged as before. The supernatant solutions were pooled together, and freeze dried. The solid pellets were also freeze-dried.

Determination of Nitrogen content

Portions of both cured minced tissue and freeze-dried solid and supernatant fractions were analysed for nitrogen using the Kjeldahl method, (AOAC, 1970).

¹⁵N enrichment determinations

Portions of the freeze-dried solid and supernatant fractions of the model cures were first converted to ammonium chloride by the Kjeldahl method (AOAC, 1970). Dilute hydrochloric acid was used in the vessel receiving the ammonia gas instead of the customary sulphuric acid. The ammonium chloride solutions were then taken to dryness on a rotary evaporator.
The determination of $^{15}$N enrichment in ammonium chloride samples has already been described in chapter 3.

**Residual nitrite determinations**

Portions of the freeze-dried solid and supernatant fractions were taken and analysed for residual nitrite. A weighed sample of the freeze-dried material was extracted with distilled water and the residual nitrite concentration determined by the method already described. It is necessary to know the residual nitrite concentration to correct the value of $^{15}$N enrichment obtained from the samples for the contribution to the enrichment from this source.

**Non-volatile nitrosamine determinations**

The method employed was that of Lunt Fueggle & Walters (1973). Two grams of the freeze-dried solid fraction of the model cure was suspended in 200 ml of dichloromethane. Thicynyl chloride (5 ml of 25% w/v in dichloromethane) was added. Dry nitrogen was then bubbled slowly through the dichloromethane, and any nitrosyl chloride produced from reaction of nitrosamine with thicynyl chloride was swept into 5 ml of dilute (25%) sodium hydroxide. After approximately 15 minutes, 1 ml of sulphanilamide solution (in 2.5M HCl), 1 ml of concentrated hydrochloric acid, and 1 ml of N-1-Naphthylethlenediamine (0.1% w/v aqueous solution) were added. The solution was kept for 15 minutes in the dark for the colour to develop, and the extinction read at 540 nm. The nitrosamine concentration was calculated from the concentration of nitrite ion present formed by reaction of nitrosyl chloride with sodium hydroxide.

5.3 Results

**The loss of sodium nitrite**

The disappearance of nitrite from model cures has been studied with
respect to length of incubation times and temperatures and initial nitrite concentrations. The first result to become apparent was the difference in the amounts of nitrite utilised by different samples of muscle tissue.

Table 5.1 shows the amounts of residual sodium nitrite found in incubations of different samples of cured muscle tissue, at the same temperature, for the same length of incubation time, buffered at the same pH, and all starting with 200 ppm (2.7 mM) sodium nitrite, based upon the mass of meat used. Between 65 and 88% of the added nitrite was found to have disappeared during the 40 hour incubation period.

Within samples of minced tissue from the same shoulder of pork muscle, there was usually a linear relationship between the amount of added nitrite and that utilised by the muscle. Diagram 5.1 shows that the muscle in this particular experiment utilised the same percentage of nitrite at each level of added nitrite, a result also obtained by Walters et al. (1968) over a range of added nitrite from 200 to 2000 ppm of nitrite.

Both incubation time and temperature affect the level of sodium nitrite in model cures, the utilisation increasing with increase of time and temperature. Table 5.2 shows the percentage utilisation of nitrite for various times and temperatures. The individual effects of time and temperature were then studied further by setting up tissue incubations for a fixed incubation time. Two initial nitrite levels, 100 ppm and 200 ppm were used and sample flasks for each level of nitrite were held at 4°, 20° and 37°. The residual nitrite concentrations were then determined at the end of 20 hours. The results are displayed in diagram 5.2 and show the increasing rate of disappearance of nitrite at the higher incubation temperatures.
Table 5.1

SODIUM NITRITE CONCENTRATION BEFORE AND AFTER

INCUBATION WITH MUSCLE MINCE

<table>
<thead>
<tr>
<th>Initial NaNO₂ conc.</th>
<th>Final NaNO₂ conc. (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 ppm</td>
<td>44.0</td>
</tr>
<tr>
<td></td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td>72.0</td>
</tr>
</tbody>
</table>

All experiments with pH 6.0 phosphate buffer (0.2M), at 4° for 40 hours.
Diagram 5.1. The effect of initial NaNO₂ concentration upon the amount of NaNO₂ utilised by minced muscle tissue at pH 6.0 and 4°C for 40 hours
Table 5.2

THE RATIO BETWEEN THE AMOUNT OF NITRITE UTILIZED AND THE INITIAL NITRITE CONCENTRATION FOR A SERIES OF MODEL CURES AT VARIOUS TEMPERATURES AND TWO INCUBATION TIMES

<table>
<thead>
<tr>
<th>Temperature of incubation</th>
<th>Length of incubation (hr)</th>
<th>Percentage utilisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°</td>
<td>40</td>
<td>90</td>
</tr>
<tr>
<td>37°</td>
<td>20</td>
<td>64</td>
</tr>
<tr>
<td>37°</td>
<td>20</td>
<td>55</td>
</tr>
<tr>
<td>20°</td>
<td>20</td>
<td>49</td>
</tr>
<tr>
<td>4°</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>4°</td>
<td>20</td>
<td>38</td>
</tr>
</tbody>
</table>

All model cures in 0.2 M phosphate buffer at pH 6.0.
Model cures with muscle mince in 0.2 M phosphate buffer pH 6.0 for 20 hours.

*Diagram 5.2. The effect of temperature on the residual nitrite concentrations*
Incubations of minced muscle, from one shoulder of pork, in phosphate buffer at pH 6.0, and sodium nitrite have been set up to study the disappearance of nitrite at fixed temperatures over a time period of 30 hours. The utilisation of nitrite was found to be exponential with time. If the values of residual nitrite at various times are plotted on a logarithmic scale, the half life of the nitrite in the curing system at the incubation temperature can be calculated from the slope obtained. This is carried out in diagram 5.3. Half lives for nitrite of 38 hours and 14 hours were found for incubations at 4° and 37° respectively.

The effects of these various parameters on the disappearance of nitrite from model cures were investigated in order to find the extent to which nitrite was lost and hence the extent to which other compounds are formed from it. The most obvious reaction product of the muscle-nitrite interaction and the one noted centuries ago by the Romans, is the formation of the pink compound nitrosylmyoglobin. The next step in the elucidation of the nitrite reaction pathway, was therefore, to examine the myoglobin content of the muscle and to find out how much would combine with nitrite added at various levels.

**Formation of nitrosyl-haem pigment**

Incubations of muscle mince and sodium nitrite were carried out with phosphate buffer at pH 6.0, and 0.01% chloramphenicol and analysed for residual nitrite and haem pigment after 40 hours. The results of the experiment are set out in Table 5.3.
Initial nitrite concentration 200 ppm.
Incubation carried out in anaerobic Thunberg tubes at 4° and 37° using 10g minced muscle, 10ml phosphate buffer (0.2M pH=6.0) containing 5% NaCl (w/v) and 0.1% chloramphenicol.

Diagram 5.3. Graph showing the relationship between residual nitrite and time
<table>
<thead>
<tr>
<th>Initial NO$_2^-$ concentration (ppm)</th>
<th>Total pigment (mM)</th>
<th>Nitrosyl myoglobin (mM)</th>
<th>% of total conversion of pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.19</td>
<td>0.108</td>
<td>57.3</td>
</tr>
<tr>
<td>200</td>
<td>0.19</td>
<td>0.133</td>
<td>69.8</td>
</tr>
<tr>
<td>500</td>
<td>0.19</td>
<td>0.169</td>
<td>89.1</td>
</tr>
<tr>
<td>2000</td>
<td>0.19</td>
<td>0.1368</td>
<td>72.0</td>
</tr>
<tr>
<td>200</td>
<td>0.1</td>
<td>0.097</td>
<td>97.0</td>
</tr>
<tr>
<td>500</td>
<td>0.1</td>
<td>0.076</td>
<td>76.0</td>
</tr>
<tr>
<td>200</td>
<td>0.065</td>
<td>0.059</td>
<td>83.0</td>
</tr>
<tr>
<td>50</td>
<td>0.18</td>
<td>0.083</td>
<td>46.1</td>
</tr>
<tr>
<td>100</td>
<td>0.18</td>
<td>0.081</td>
<td>45.1</td>
</tr>
<tr>
<td>200</td>
<td>0.18</td>
<td>0.037</td>
<td>20.6*</td>
</tr>
<tr>
<td>500</td>
<td>0.18</td>
<td>0.077</td>
<td>43.5</td>
</tr>
<tr>
<td>750</td>
<td>0.18</td>
<td>0.130</td>
<td>71.9</td>
</tr>
<tr>
<td>1000</td>
<td>0.18</td>
<td>0.097</td>
<td>54.4</td>
</tr>
</tbody>
</table>

* Flask found to have leaked during incubation

**Myoglobin concentration found in samples of muscle minces and conversion of myoglobin to nitrosylmyoglobin with various concentrations of nitrite**

All incubations were carried out anaerobically in pH 6.0, 0.2M Phosphate buffer.
The results show that the myoglobin content of muscle varied considerably between samples of muscle. All the experiments were carried out with minced muscle from the shoulder of pigs, of unknown ante-mortem histories. The total pigment concentration varied from 0.065 mM to 0.19 mM, or 0.11% to 0.32% of the total muscle. The percentage conversion of the pigment to nitrosylmyoglobin also varied and this may have been due to the different ante-mortem histories of the pigs used.

The mean values for the conversion of myoglobin to nitrosyl myoglobin obtained from various experiments at pH 6.0, each of which used a different sample of pork muscle, are drawn in diagram 5.4. The percentage conversion to nitrosylmyoglobin was found to increase with increasing initial concentration to a maximum at 200 parts per million NO\textsuperscript{2-}, taken from the results of many experiments.

The formation of nitrosylmyoglobin is not, however, the only reaction that nitrite undergoes. The maximum concentration of myoglobin in pork is approximately 2.2 \times 10^{-4} mM. This means that for 100% conversion of myoglobin to its nitrosyl form in 100g of muscle tissue, 2.2 \times 10^{-3} moles of NaNO\textsubscript{2} are needed. This is equivalent to only 1.5 mg NaNO\textsubscript{2}, or 15 ppm—approximately 7% of nitrite added at 200 ppm, and 10% of the nitrite that 'disappears'.

Mirna and Hofmann (1969) discussed the possibility of NO-thiols after showing the reaction of nitrite with cysteine and glutathione at temperatures and pH relevant to meat curing. This led to the analysis of cured muscle in this study; for nitrosothiol compounds in which −SH groups on muscle proteins react with sodium nitrite to form compounds of the type −S=N−O.
Diagram 5.4. Graph of mean percentage conversion of myoglobin to nitrosylmyoglobin in cured muscle tissue at various levels of nitrite.
The analysis of cured muscle for nitrosothiol compounds

Analysis of the water-soluble and water-insoluble fractions from homogenised muscle showed that most of the S-nitrosothiol compounds were to be found in the water-insoluble fraction. The water-soluble fraction contained virtually no S-nitrosothiols.

Anaerobic incubation of minced muscle tissue with 0.2M phosphate buffer, pH 6.0, were set up, as before, with various levels of sodium nitrite and kept at 4° for 40 hours, and some at 4° for 18 hours and then 70° for 4 hours, to simulate bacon and pasteurised meat preparation conditions, respectively. The concentration of nitrosothiol compounds was then determined as described earlier.

The rate of reaction of NaNO₂ with sulphydryl groups was found to increase with increasing concentrations of nitrite, (diagram 5.5). The amounts of nitrosothiol compounds formed in this particular experiment were relatively small, however, and in other experiments the amounts of sodium nitrite (at an initial concentration of 1.36 moles/50g minced muscle) that has reacted with the thiol groups present in the muscle have varied from 0.0014 m moles to 0.395 m moles.

The results above apply to 'bacon' type, low temperature cures; nitrosothiol compounds are formed to a lesser extent under the pasteurisation conditions. Nitrosothiols are known to be heat labile (Mirna and Hofmann 1969) and breakdown of the -S-N=O compounds may be the reason why the nitrosothiol concentration was found to be lower. Comparative figures for nitrosothiol formation using identical samples of minced muscle at 4° and 70° are given in table 5.4.
Diagram 5.5. Graph of nitrosothiol formation in model cures at pH 6.0 in phosphate buffer at various levels of sodium nitrite.
<table>
<thead>
<tr>
<th>Initial level of sodium nitrite (ppm)</th>
<th>Temperature treatment</th>
<th>Pasteurised ham cure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacon cure</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40°, 40 hours</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.395</td>
<td>0.112</td>
</tr>
<tr>
<td>250</td>
<td>1.12</td>
<td>0.329</td>
</tr>
</tbody>
</table>

Table 5.4

mMOLeS NITROSO THIOL COMPOUNDS FOUND IN MODEL CURES WITH TWO LEVELS OF NITRITE AND TWO TEMPERATURE PROCESSES

Analysis of model cures using 50g muscle mince, 50 ml phosphate buffer pH 6.0, 0.2M containing 50mg/g NaCl and 0.1 mg/g chloramphenicol.
Analyses for non-volatile nitrosamines

The possibility of the formation of non-volatile nitrosamines in the model cures was investigated in order to ascribe part of the fate of the labelled sodium nitrite used in the next series of experiments. The results obtained, of analyses on the freeze-dried non-water soluble fraction of homogenised meat cures, using the method of Fueggle, Lunt and Walters (1973) are given in table 5.5. The figures show that the amounts of nitrite combined to form non-volatile nitrosamines are negligible in terms of the quantities of nitrite added.

<table>
<thead>
<tr>
<th>Temperature of cure</th>
<th>Nitrite added ppm</th>
<th>Concentration of Non-volatile nitrosamines (μM)</th>
<th>Equivalent as NaNO₂ (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40° for 40 hours</td>
<td>100</td>
<td>0.65</td>
<td>2.27</td>
</tr>
<tr>
<td>40° for 40 hours</td>
<td>1000</td>
<td>2.1</td>
<td>7.3</td>
</tr>
<tr>
<td>70° for 4 hours</td>
<td>100</td>
<td>none found</td>
<td>---</td>
</tr>
<tr>
<td>70° for 4 hours</td>
<td>1000</td>
<td>0.35</td>
<td>1.22</td>
</tr>
</tbody>
</table>

Table 5.5 RESULTS OF NON-VOLATILE NITROSAMINE ANALYSIS ON CURES OF 50 g PORK MUSCLE AT pH 6.0, ALSO CONTAINING 0.01 mg/g CHLORAMPHENICOL 50 mg/g NaCl
Labelled nitrite experiments

The first attempts to produce a 'balance sheet' of the added nitrite and nitrate, analysed by both chemical means and by measuring the $^{15}$N content of the cured muscle samples, were concerned only with the residual nitrite and nitrosylmyoglobin. Only limited success was attained by the attempts to convert the samples to nitrogen gas, and then to analyse the nitrogen using the Dumas high vacuum train and RMU-6 mass spectrometer (described in chapter 3), and no interpretable results were obtained. The first set of results quoted here were obtained using a Statron analyser (also described in chapter 3) and are shown in table 5.6. This shows the amount of incorporation of $^{15}$N into fractions derived from samples of cured minced muscle. Four different levels of sodium nitrite (labelled with $^{15}$N to 96.1 atoms %) were added to the phosphate buffered muscle cures, which were left anaerobic at 4°C for 40 hours. Chloramphenicol was added (0.1 mg/g buffer) to ensure the absence of any microbial intervention. The extent of incorporation of $^{15}$N into the fractions is given both in atoms $^{15}$N per 100 atoms ($^{14}$N + $^{15}$N) and as a 'sodium nitrite equivalent' so that the contribution of the labelled nitrogen can be easily seen in terms of added sodium nitrite.

Some of the samples failed to produce a molecular electronic spectrum, indicating the lack or overabundance of nitrogen gas in the sample tube. This could possibly have been caused by leakage while the sample tubes were being sealed. The most important points to emerge from this experiment were (a) the relatively large amounts of enrichment in the solid fractions, and (b) from the experiment involving the highest initial concentration of nitrite, a low level of enrichment was seen, based upon the level calculated to be present from the residual nitrite data, i.e. the $^{15}$N enrichment should have been of the order of 0.667 atoms% instead of 0.43 atoms %. This was taken to mean that the Kjeldahl method used on these samples was not completely converting sodium nitrite present in the
<table>
<thead>
<tr>
<th>Cure</th>
<th>NaNO₂ added</th>
<th>% ppm NaNO₂</th>
<th>Residual NaNO₂ (mg)</th>
<th>1⁵N enrichment as Na¹⁵NO₂ (mg)</th>
<th>Total NaNO₂ found by chem analysis (mg)</th>
<th>Total found by ¹⁵N measurements</th>
<th>Non volatile nitrosamines</th>
<th>% Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>.5</td>
<td>50</td>
<td>1.32</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>100</td>
<td>1.674</td>
<td>0.43</td>
<td>failed</td>
<td>1.834</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>200</td>
<td>4.66</td>
<td>failed</td>
<td>failed</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>50</td>
<td>500</td>
<td>10.36</td>
<td>0.44</td>
<td>0.43</td>
<td>3.13</td>
<td>1.164</td>
<td>10.36</td>
</tr>
</tbody>
</table>

**Table 5.6**

Model cures of 100g minced pork muscle with

100 ml incubation buffer containing

0.1 mg/g chloromycetin

50 mg/g NaCl

Temperature = 40 hours at 4°C
<table>
<thead>
<tr>
<th>Cure</th>
<th>NaNO₂ added</th>
<th>ppm NaNO₂</th>
<th>Residual NaNO₂ (mg)²</th>
<th>NO-Mb expressed as NaNO₂</th>
<th>¹⁵N enrichment</th>
<th>enricment as Na¹⁵NO₂ (mg)</th>
<th>Total NaNO₂ found by chem analysis</th>
<th>Total found by ¹⁵N measurements</th>
<th>% Recovery of ¹⁵N</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0.34</td>
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<td></td>
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<td>2.72</td>
</tr>
<tr>
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<td>0.18</td>
<td>0.42</td>
<td>0.44</td>
<td>0.49</td>
<td>3.88</td>
<td>1.44</td>
<td>0.70</td>
</tr>
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<td></td>
<td>5.32</td>
</tr>
<tr>
<td>D</td>
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<td>500</td>
<td>0.85</td>
<td>0.534</td>
<td>0.54</td>
<td>0.52</td>
<td>9.51</td>
<td>1.87</td>
<td>1.38</td>
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<td>11.38</td>
</tr>
<tr>
<td>E</td>
<td>100</td>
<td>1000</td>
<td>27.2</td>
<td>0.43</td>
<td>0.58</td>
<td>0.78</td>
<td>11.20</td>
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<td></td>
<td></td>
<td></td>
<td>16.71</td>
</tr>
</tbody>
</table>

Table 5.7 Model cures of 50g minced pork muscle with 50 ml incubation buffer containing

0.1 mg/g chloromycetin

50 mg/g NaCl
modified to ensure quantitative conversion of the nitrite, (AOAC, 1970) by the addition of methyl salicylate during the Kjeldahl digestion step.

Table 5.7 shows the results of a subsequent experiment in which sodium nitrite does not appear to have been completely converted to ammonia in two model cures, (D and E). In these cases, sodium nitrite may also have been lost as oxides of nitrogen when sulphuric acid was added to the freeze-dried supernatant fraction, in the first part of the Kjeldahl digestion. The amount of incorporation of $^{15}$N into the supernatant fractions of the other model cures is greater than can be accounted for by residual sodium nitrite and nitric oxide myoglobin alone. In all cures in this experiment relatively large amounts of incorporation occurred into the solid fraction. The levels of residual nitrite in the experiments in tables 5.6 and 5.7 vividly illustrate the ability of different samples of muscle to metabolise different amounts of sodium nitrite. In the first nitrite balance experiment the percentage utilisation ranges from 74% - 84% compared to the next experiment in which the nitrite utilisation figures are all well above 90%. The total nitrite that could not be accounted for by analysis for nitrite in the samples was high, ranging from 72% to 93%, and obviously the nitrite was reacting with one or more components of the meat not yet studied. It was known from the earlier work in this study on gas production that the amounts of nitrite appearing as gaseous oxides of nitrogen could not account for losses as large as these. The possibility of reaction of nitrite with thiol groups present in the meat was then studied in order to account for these large losses of nitrite. Analyses for the amounts of nitrosothiols formed on the addition of various concentrations of nitrite were carried out, as described earlier in this chapter; and then 'nitrite balance' experiments were performed, as before and including analyses for nitrosothiols.
The next 'nitrite balance' experiment (the results of which are shown in table 5.8), included analyses for nitrosothiol compounds present in the non-water soluble fraction of the meat. From the results obtained, it can be seen that these compounds are responsible for some of the loss of nitrite, though the $^{15}$N incorporation figures show that nitrosothiols are not the only additional contributors to the isotopic nitrogen enrichment. The amount of $^{15}$N enrichment in the non-water soluble fraction, above that due to nitrosothiols and nitrosylmyoglobin is proportional to the initial concentration of sodium nitrite, approximately equivalent to $13 \, \mu g \, Na^{15}NO_2$ per ppm $Na^{15}NO_2$ added. The results also show a greater proportion of 'unaccountable' $^{15}$N in the heat-treated samples. Possibly thermal degradation of the muscle protein produces or liberates reactive groups buried in the protein structure, such as phenols, which are known to react with NaNO₂ (Challis, 1973).

Enrichment in the supernatant fraction also occurs, increasing both with increasing nitrite concentration and with temperature, above that which can be related to the amount of residual nitrite.

Analyses for nitrate were carried out on these cured meat slurries. The results showed the formation of between 2-5ppm nitrate, depending upon the initial amount of sodium nitrite added to the model cure; more nitrate being found with increase in nitrite level in the cures.

The same trend of nitrogen enrichment was shown by 'nitrite balance' experiment (4), in table 5.9. The value for the amount of nitrosothiol formation in experiment B appeared to be too low, and gave rise to an anomalously high value for the amount of 'unaccountable' enrichment in the non-water soluble fraction. The amount of residual nitrite in B was higher than would be expected by extrapolation from the results of the two previous balance experiments; the value of the
<table>
<thead>
<tr>
<th>Cure</th>
<th>NaN(_2) added (mg)</th>
<th>ppm NaN(_2)</th>
<th>Residual NaN(_2) (mg)</th>
<th>NO-thiols expressed as NaN(_2)</th>
<th>NO-Mb expressed as NaN(_2)</th>
<th>15N enrichment</th>
<th>15N enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>144</td>
<td>2.5</td>
<td>2.73</td>
<td>0.60</td>
<td>0.35</td>
<td>0.355</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>342</td>
<td>9.3</td>
<td>7.71</td>
<td>0.46</td>
<td>0.38</td>
<td>0.457</td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>342</td>
<td>9.3</td>
<td>7.71</td>
<td>0.46</td>
<td>0.38</td>
<td>0.457</td>
</tr>
<tr>
<td>D</td>
<td>25</td>
<td>342</td>
<td>9.3</td>
<td>7.71</td>
<td>0.46</td>
<td>0.38</td>
<td>0.457</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
<td>185</td>
<td>1.58</td>
<td>0.77</td>
<td>0.60</td>
<td>0.395</td>
<td>0.467</td>
</tr>
<tr>
<td>F</td>
<td>25</td>
<td>403</td>
<td>2.6</td>
<td>2.26</td>
<td>0.60</td>
<td>0.51</td>
<td>0.635</td>
</tr>
</tbody>
</table>

**Table 5.8**

Model cures of 50g minced pork in incubation buffer containing

A, B, C at 4° for 40 hours

D, E, F at 70° for 4 hours
### Nitrite balance (4)

<table>
<thead>
<tr>
<th>Cure</th>
<th>NaNO₂ added (mg)</th>
<th>ppm NaNO₂</th>
<th>Residual NaNO₂ (mg)</th>
<th>NO-thiols expressed as NaNO₂</th>
<th>NO-Mb expressed as NaNO₂</th>
<th>¹⁵N enrichment (excess of 0.3647 atoms %)</th>
<th>enrichment is Na¹⁵NO₂ (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0072</td>
<td>0.009</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>100</td>
<td>3.57</td>
<td>0.29</td>
<td>0.17</td>
<td>0.0339</td>
<td>0.203</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>1000</td>
<td>26.13</td>
<td>9.47</td>
<td>0.30</td>
<td>0.976</td>
<td>2.16</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0144</td>
<td>0.009</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>100</td>
<td>1.23</td>
<td>0.613</td>
<td>0.51</td>
<td>0.0366</td>
<td>0.1049</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>1000</td>
<td>14.0</td>
<td>6.19</td>
<td>0.60</td>
<td>0.2473</td>
<td>1.8613</td>
</tr>
</tbody>
</table>

#### Table 5.9

Model cures of 50g minced pork muscle incubation buffer containing 0.5µg A, B, and C at 4°C for 40 hours D, E, and F at 70°C for 4 hours
residual nitrite level for C is approximately what would be expected, though this same cure had a much higher value for the concentration of nitrosothiols found. These comparisons are difficult to make, between different samples of pork muscle, but a table (5.10) of $^{15}\text{N}$ enrichment drawn from the results of these three 'nitrite balance' experiments showed some similarities in the amounts of enrichments for various nitrite levels added to the cures.

The amounts of enrichment appeared to be self-consistent for the $^{15}\text{N}$-incorporation into the solid and supernatant fractions before allowance was made for residual nitrite in the case of the supernatant fractions, and nitrosylmyoglobin and nitrosothiol compounds in the solid fraction. The levels of enrichment in the solid fraction added at nitrite concentrations of 100 p.p.m., 200 p.p.m. and 500 p.p.m. in all experiments were proportional to the added nitrite concentration. At nitrite concentrations above these, however, there appeared to be a maximum incorporation of $^{15}\text{N}$, corresponding to approximately 12 mg of sodium nitrite. Among the supernatant enrichments, there was no such proportionality to be found, and even after the amounts of residual nitrite had been subtracted from the supernatant enrichments, only when 25 mg of Na$^{15}$NO$_2$ was added was there any similarity between two samples of cured muscle. In the case of $^{15}\text{N}$ enrichment in the solid fractions, after taking into account the enrichment due to nitrosothiol compounds and nitrosylmyoglobin, the only obvious trend was for a higher incorporation of $^{15}\text{N}$ into cures with higher initial levels of sodium nitrite.

The next 'nitrite balance' experiment (number 5) was devised in order to try and pinpoint more accurately the $^{15}\text{N}$ enrichment caused by the presence of nitrosothiols, nitrosylmyoglobin and non-volatile nitrosamines. Pork muscle cures were set up as before, containing 100
<table>
<thead>
<tr>
<th>Nitrite Balance Experiment</th>
<th>NaNO₂ added (mg)</th>
<th>= p.p.m.</th>
<th>¹⁵N enrichment into SOLID and SUPERNATANT fractions</th>
<th>¹⁵N enrichment in supernatant - residual Na NO₂</th>
<th>¹⁵N enrichment in solid - enrichment due to nitrosothiols and nitrosyl myoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5</td>
<td>100</td>
<td>1.74</td>
<td>.98</td>
<td>0.97</td>
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<td>10</td>
<td>200</td>
<td>3.88</td>
<td>1.44</td>
<td>1.26</td>
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<tr>
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<td>100</td>
<td>2000</td>
<td>11.20</td>
<td>5.51</td>
<td>-</td>
</tr>
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<td>10</td>
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<td>3.95</td>
<td>2.71</td>
<td>0.21</td>
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<tr>
<td></td>
<td>25</td>
<td>342</td>
<td>12.70</td>
<td>10.19</td>
<td>0.89</td>
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<td>4</td>
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<td>100</td>
<td>1.61</td>
<td>3.91</td>
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<td>50</td>
<td>1000</td>
<td>12.40</td>
<td>27.44</td>
<td>1.31</td>
</tr>
</tbody>
</table>

1 Enrichment below level of residual nitrite, see text for explanation.
2 Nitrosothiol concentrations not analysed.
3 Judged to be inconsistent.

Table 5.10 ¹⁵N ENRICHMENTS INTO SOLID AND SUPERNATANT FRACTIONS OF CURED MUSCLE TISSUE FOR VARIOUS INITIAL LEVELS OF SODIUM NITRITE.
and 200 p.p.m. sodium nitrite and were kept anaerobic for 40 hours at 4°. The minced cured muscle was then fractionated as shown in the diagram 5.6. The results obtained from these analyses are set out in table 5.11. Two hundred gram samples of muscle tissue were used in order to provide sufficient material for all the analyses. Nitrite and nitrate analyses were carried out at each point in order to accurately describe the composition of the $^{15}$N enrichment in each residue. A proportion of the muscle was washed with mercuric chloride solution (5% w/v) in order to release $^{15}$NO from $-S^{15}$NO compounds and consequently obtain nitrosothiol-free cured muscle.

The results show that in this experiment, the amount of incorporation of $^{15}$N into B, which could be attributed to nitrosothiol compounds, was virtually the same as the amount which was measured chemically. The amount of enrichment due to nitrosothiols in C, however, is 0.62, expressed as mg NaNO$_2$, double that amount which was chemically determined. This high value would appear to be the correct one, since it has been found that the yield of nitrosothiol compounds increases with increasing levels of nitrite, and hence the amount of nitrosothiol compounds expected for a cure with 200 p.p.m. nitrite would be approximately 2 times that for a 100 p.p.m. nitrite cure, or 2 x 0.26 = 0.52 mg, expressed as sodium nitrite. In the case of cure B, approximately 5 mg NaNO$_2$ or 23% of the sodium nitrite has not been characterised, though some of this would appear as gaseous oxides of nitrogen. In cure C, 20% or approximately 8 mg of NaNO$_2$ is still uncharacterised.

The amounts of non-volatile nitrosamines analysed presented an anomaly in that the lower nitrite cure contained more non-volatile nitrosamines. This was possibly due to the fact that the method of
400g cured minced muscle slurry

(over page)

200g

washed with 700ml acetone to remove haem pigment

filtered

Supernatant

+HCl, leave for 24hr

Read optical density at 540mm to obtain nitrosylhaem pigment concentration.

residue (24g)

freeze dried

2g Nitrosothiol analysis (V) 4g Non-volatile nitrosamine analysis (Z)

6g Kjeldahl digestion to $^{15}$NH$_4$Cl

15N abundance measured

9g Kjeldahl analysis for total nitrogen

Diagram 5.6(a) FRACTIONATION OF MINCED CURED MUSCLE FROM BALANCE EXPERIMENT (5)
Diagram 5.6 (b)
Table 5.11 - Model cures of 200g minced pork muscle with 200ml incubation buffer containing 0.1mg/g chloromycetin, 50mg.

<table>
<thead>
<tr>
<th>Cure</th>
<th>NaNO₂ added (mg)</th>
<th>NaNO₂ = ppm NaNO₂</th>
<th>Residual NaNO₂</th>
<th>No - Thiols Expressed as NaNO₂ (mg)</th>
<th>NO - Mb Expressed as NaNO₂ (mg)</th>
<th>Total enrichment in SOLID residual NaNO₂ as (mg) NaNO₂</th>
<th>Total enrichment in SUPERNAT. residul NaNO₂ as (mg) NaNO₂</th>
<th>¹⁵N analysis of Nitrosamine using NaNO₂</th>
<th>Uncharacterised ¹⁵N as mg NaNO₂</th>
<th>Nitrosothiol formation by ¹⁵N enrichment as mg NaNO₂</th>
<th>Chemical analysis of non-volatile Nitrosamine as mg NaNO₂</th>
<th>NaNO₂ accounted for by chemical analysis (mg)</th>
<th>Na ac by mea ure Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>B</td>
<td>23.3</td>
<td>.116</td>
<td>16.46</td>
<td>0.26</td>
<td>1.2</td>
<td>3.31</td>
<td>5.083</td>
<td>3.097</td>
<td>2.989</td>
<td>0.21</td>
<td>0.108</td>
<td>18.03 (77%)</td>
<td>2.12</td>
</tr>
<tr>
<td>C</td>
<td>41.5</td>
<td>207</td>
<td>30.66</td>
<td>0.26</td>
<td>2.0</td>
<td>3.609</td>
<td>7.443</td>
<td>2.987</td>
<td>2.930</td>
<td>0.622</td>
<td>0.057</td>
<td>32.98 (80%)</td>
<td>4.18</td>
</tr>
</tbody>
</table>
analysis used (Lunt, Fueggle and Walters, 1973), occasionally produced unexplainably low results. Taking the figures, however, and the 'average molecular weight' of a non-volatile nitrosamine to be 100 (i.e. N-nitroso proline) the concentrations of non-volatile nitrosamines in B and C would be 0.78 p.p.m. and 0.41 p.p.m. respectively, in the non-water soluble fraction of the cured muscle mince. The $^{15}$N analysis of the nitrosamine fractions gave far higher enrichment than for non-volatile nitrosamines alone. The fractions analysed for nitrosamines also contained $^{15}$N from all compounds which were able to withstand the washing with mercuric chloride solution. If the biochemical analysis' values for non-volatile nitrosamine formation are subtracted from the values obtained by $^{15}$N analysis, the result gives the, as yet, uncharacterised enrichment of $^{15}$N. The values of the total enrichment expressed as NaNO$_2$, when added to the amount of residual nitrite gave recoveries of nitrite close to 100%.

5.4 Discussion

The results obtained for the 'disappearance' of nitrite from the model cures bear out the conclusions drawn by other workers using similar model systems. In cures in which the initial level of nitrite was the same, between 65 and 88% of the nitrite was found to have been utilised over a 40 hour incubation period. Walters et al. (1968) showed a loss of nitrite over a 21 hour incubation period with a 20% difference in nitrite utilisation between different samples. Kolari and Aunan (1972), Hill et al. (1973) and Sebranek et al. (1973) all report variable losses of nitrite from different samples of cured meat. This non-reproducibility of results between differing samples of cured muscle tissue, even when buffered to the same pH, means that comparison of results between samples is very difficult, and the description of the fate of the nitrite becomes qualitative rather than
quantitative. This does not mean that the study has failed however, since it is as important to know what compounds have been derived from nitrite as it is to know just how much of these particular compounds have been formed. The non-uniformity of the results may be due to the differing ante and post mortem histories of the porcine muscles employed. Although the samples were all buffered at pH6.0 when cured, the activities of the enzyme or enzymes responsible for metabolising part of the nitrite could be different for a different samples of muscle. Comparison of these results with those obtained by other workers shows trends in nitrite-muscle behaviour rather than similarity of the absolute values obtained for any property of the curing system. Hence, a linear relationship was found for the disappearance of nitrite with time from a sample of cured muscle, although the 'half life' of the nitrite was shorter than that found by Nordin (1969). In this study, values of 38 hours and 14 hours were found for cures at 4° and 37°, while Nordin obtained half lives of 440 and 66 hours for the same temperatures, respectively. The large difference could possibly be accounted for by the use of fresh meat in this study and also by more intimate mincing and mixing of the sodium nitrite.

Analysis of the model cures for haem pigment conversion to the nitrosyl form showed similar results to those produced by Walters et al. (1968), although those workers found an average maximum conversion of myoglobin to nitrosylmyoglobin at 120p.p.m. nitrite whereas in this study the maximum was found to be at 200p.p.m. nitrite.

Most of the nitrite that had reacted, however, seemed to be detectable as nitroso-thiol compounds. Total thiol groups are estimated to be approximately 21mm in muscle (Hamm and Hofmann, 1966).
Mirna and Hofman (1969), in one sample of pork muscle determined the thiol content to be about 18 mM. This would require about 1250 - 1700 p.p.m. NaNO₂ for complete conversion to nitrosothiol compounds, easily enough to explain the loss of the nitrite not accounted for by nitrosylmyoglobin formation. It was noted in the analyses of cured muscle mince for nitrosothiol compounds that, while the amounts of nitrosothiols formed were directly proportional to the amount of added nitrite at low levels of nitrite, the formation of these compounds levelled off when the initial nitrite concentration was 2000 p.p.m., suggesting saturation of the available thiol groups.

It was interesting to find that the results from model cures in which nitrite utilisation (i.e. the ratio between the amount of nitrite reacted to the amount of nitrite added) was low, a low percentage conversion of myoglobin to 'nitrosyl' myoglobin was observed, and that a comparatively low figure for nitrosothiol formation was also obtained. For cures in which the nitrite utilisation was high, high percentage conversions of the haem pigment to the 'nitrosyl' complex occurred, and a relatively high yield of nitrosothiols was observed. It has been suggested by Watts et al. (1955); Reith and Szakaly (1967); Fox and Ackerman (1968), and Mirna and Hofmann (1969) that sulphydryl groups and/or nitrosothiol compounds play a part in the conversion of myoglobin to nitrosylmyoglobin in muscle. The observation described above would seem to support these claims.

The amounts of nitrosothiol compounds found in the pasteurisation conditions tended to be lower than those formed in the 'bacon' type cures. The reason for this is not clear, although it has been found that nitrosothiols are heat labile. Hamm and Hofmann (1965) found that heating muscle protein to 70° had no significant effect upon the availability of thiol groups present in the protein, although further heating of the muscle protein did result in the loss of approximately 50% of the number of thiol groups. Heating the protein to 70° may account, therefore
for the lower quantities of nitrosothiol compounds formed in the pasteurisation process, because of their instability towards heat.

Not all investigators of the fate of nitrite have ascribed so much nitrite to forming nitrosothiols, however. Oldsman (1973) has shown nitrosothiol formation of the order of 0.01 m moles in 1000g muscle tissue. Using vinyl pyridine to alkylate the thiol groups, the determination was made by measuring the amounts of nitrite disappearing when muscle was reacted with nitrite in the presence and absence of the alkylating agent. This difference was then assumed to be due to nitrosothiol formation. The low result obtained by Olsman (1973) can be explained however, by reference to the work of Hamm and Hofmann (1965). These workers found that whereas amperometric determinations of thiol groups in muscle protein using silver nitrate gave an \(-\text{SH}\) concentration of 35 m molar; when they used \(N\) - ethylmaleimide to react with the thiol groups the thiol concentration was only 0.70 m molar in muscle protein. This suggests that relatively large molecules such as vinylpyridine and \(N\) - ethylmaleimide cannot enter far enough into the muscle protein to react or inactivate all the thiol groups, whereas small moieties such as silver ions and nitrite ions (or whatever is the nitrosating species) can gain access to the total number of thiol groups present.

The first \(^{15}\text{N}\) incorporation experiments showed that a large proportion of the added nitrite was not being accounted for as residual nitrite and nitrosylmyoglobin. Further chemical analysis showed that most of this 'unaccountable' nitrite had reacted to form nitrosothiol compounds, more than had been thought to be present by workers such as Olsman (1973), but consistent with the single result of Hamm and Hofman (1969). Consequently nitrosothiol analyses were performed in all the subsequent \(^{15}\text{N}\) incorporation experiments, the results of which showed that nitrosothiol formation
accounted for the largest proportion of the nitrite that did react.

The formation of non-volatile nitrosamines in the non-water soluble fraction of the muscle cures was studied, and it was shown that these did not account for any significant part of the nitrite disappearance. The amounts, however, were appreciable in terms of the levels of volatile nitrosamines that were being detected by other workers, especially since the method most often used here for the analysis of non-volatile nitrosamines, that of Lunt et al. (1973) has been found to give lower values, in general, than the Eisenbrand Preussmann method of HBr cleavage of nitrosamines. (Eisenbrand and Preussmann, 1970). Few results have been published of analyses of cured products for non-volatile nitrosamines, but of those that have, Newton (1974) has shown that nitrosation of pork muscle under conditions prevailing in the stomach gave similar quantities of non-volatile nitrosamines. Druckrey et al. (1967) have shown that nitrosoproline ethylester and N-nitrosarcosine are carcinogenic when injected into rats, so the formation of non-volatile nitrosamines in these cured meat samples could have great significance.

It is possible to show where most of the nitrite has been used. The diagram following gives the summary of this chapter, together with possible routes for the still unidentified nitrogen compounds that gave rise to the enrichment in cured pork samples.
Diagram 5.7 The fate of nitrite in pork muscle

- Residual nitrite, usually <50% added
- Nitrosylmyoglobin
- Nitrosylmetmyoglobin
- Nitrosamines
- Nitrosamides
- Gaseous products NO, N₂O, possibly N₂
- C-nitrosocompounds + phenols
- eg. Nitrosophenols
- Na NO₂
- + oxygen
- + been pigments
- volatile
- eg. N-nitrosodimethylamine
- non-volatile
- possibly nitroso amino acids, nitroso peptides and proteins
- + amine and amino acids
- + thiol compounds
- salts similar to Roussin's iron thionitrosyl
- possible routes of unidentified nitrite
- Nitrosothiol compounds
- eg. S-nitroso cysteine
CHAPTER 6

THE ANALYSIS OF THE HEADSPACE GASES OF MODEL CURES
CHAPTER 6
THE ANALYSIS OF THE HEADSPACE GASES OF MODEL CURES

6.1 Introduction

Evidence had previously been provided that nitric oxide was one of the products of the nitrite-skeletal muscle interaction, and this study describes the confirmation of nitric oxide as a product of model cures, using medium and high resolution mass spectrometry and gas chromatography. Also described are analyses made on minced skeletal pork muscle-nitrite systems for gases, (other than nitric oxide) that may be formed.

The kinetics of the production of nitric oxide in a model cure were also studied.

6.2 Experimental

Low resolution mass spectrometric analysis of headspace gases with and without isotopically labelled nitrite

The model cures used in the analysis of the headspace gases were usually set up in a similar fashion to those model cures of the previous chapter. Minced pork muscle tissue, in the incubation buffer (0.2M phosphate buffer with NaCl (5% w/v) and chloramphenicol (0.1% w/v) at pH 6.0, was incubated anaerobically at 37° for 40 hours in round-bottomed flasks, with either 200 parts per million (ppm) Na¹⁴NO₂ or 200 parts per million Na¹⁵NO₂. The flasks were fitted with gas tight glass taps, to enable both the withdrawal of atmospheric gases when making the contents anaerobic, and for the easy analysis of the headspace gases formed. Mass spectra were then run on the headspace gases in the vessels.

Analysis of headspace gases for ethylene

Subsequent experiments analysing the headspace gases revealed a further
component. Low resolution mass spectra showed that small peaks at $m/e$ values of 24, 25, 26 and 27 were present, corresponding in relative intensities to the mass spectrum of ethylene. With low resolution mass spectrometry, the molecular ion of ethylene would be obscured by the $^{14}N - ^{14}N$ ion, and consequently would not be seen. The identity of the ethylene was confirmed using the following procedure.

Incubations of 100g minced skeletal pork muscle tissue and 100 ml incubation buffer (pH 6.0) were used in the experiments to show the presence of ethylene. Sodium nitrite was added to a concentration of 1000 ppm. A control experiment with no sodium nitrite was also carried out. The flasks were made anaerobic by evacuating the air using a vacuum pump. Each of the two-necked, sealed, round-bottomed flasks, was equipped with an air-tight septum in the neck of the flask. This was to enable samples to be withdrawn and injected onto a gas chromatograph without the admittance of the atmosphere. At the end of the incubation period, water was run into the flasks to compress the gaseous fraction to atmospheric pressure.

Using a hypodermic syringe, 10 μl of the gaseous headspace was injected onto a Perkin-Elmer F11 gas chromatograph. The gaseous sample was detected by flame ionisation. A two metre, $\frac{3}{8}$" diameter stainless steel column containing carbon molecular sieves was used to resolve the gas mixture. Nitrogen was used as the carrier gas, and the column temperature was 150°.

With a gas mixture of CO$_2$, CH$_4$, C$_2$H$_4$, CO, C$_2$H$_6$, H$_2$ and O$_2$ in nitrogen, standard retention times of 0.6 minutes for CH$_4$, 2.7 minutes for C$_2$H$_4$ and 4.2 minutes for C$_2$H$_6$ were obtained. No flame ionisation response was obtained for CO$_2$, CO and H$_2$. The results of the gas analysis on the standard mixture and the flask headspace with and without nitrite, are shown in diagram 6.2.
Model cures to show the effect of various nitrite levels on the production of ethylene were prepared, in the manner previously described. The gaseous headspace was examined by gas chromatography as in the previous experiment. Cured minced muscle from these experiments was plated out on blood agar and counted after 48 hours incubation, with the assistance of the Microbiology Dept. of B.F.M.I.R.A. This was necessary in order to discount bacterial intervention in the production of ethylene.

Measurement of the rate of production of headspace gases

Attempts were made to analyse the total headspace gases by gas chromatography, using thermal conductivity detection, similar to the method of Wilhite and Hollis (1968). Two 2 metre ½" diameter stainless steel columns, one packed with Porapak Q and the other with Porapak R, as stationary phases, were joined together and used at ambient temperature on a Perkin-Elmer gas chromatograph. Mixtures of gases were injected onto the column, using a gas sampling device, (Perkin-Elmer Ltd). The gases N₂O, C₂H₄, CO₂ and N₂ were well resolved. Nitric oxide, however, did not appear to be detected. This was assumed to be due to the high reactivity of NO, and the subsequent reaction of NO with metallic parts of the sampling system and analytical columns. Large volumes of NO were injected through the system in the hope of saturating the reactive sites, and although small peaks of NO were eventually detected, even then, however, the sensitivity of detection of NO was inadequate. Mass spectrometry was therefore used to analyse the headspace gases.

Anaerobic incubations of minced pork muscle tissue in incubation buffer at pH 6.0 were carried out, with and without the inclusion of sodium nitrite at 500 ppm. The muscle tissue, (in sealed round-bottomed flasks fitted with gas tight glass taps) was stored at 4°C for the duration of the experiment, this temperature being most commonly used during bacon manufacture.
Aliquots of the sample headspaces were introduced into the mass spectrometer at each time interval and intensities recorded of ions at m/ε's of 28, 30, and 44. The results were then calculated taking into account the aliquot removed during each analysis.

**Kinetic study of nitric oxide production**

In order to measure the rate of nitric oxide production more accurately, a different method from the one mentioned above was employed. A separating funnel was modified by fitting a vacuum tight glass tap to the main body of the vessel, replacing the lower quality tap already fitted. In place of a stopper, a glass tube with a Quickfit joint at one end to fit the top of the separating funnel led to a vacuum tight tap at the other end. The completed apparatus is shown in diagram 6.1. Both gas and muscle mince slurry samples could be withdrawn from the container. The gas was sampled by connecting the end of the upper-tap to the gas inlet system of the mass spectrometer (RMU-7L). The residual nitrite concentration was analysed by connecting the lower tap to a vacuum pump via a glass pipette. When the pump was switched on and the lower tap opened, minced muscle slurry was drawn out, along the pipette, where it was possible to determine the volume of sample being taken. The pipette and the tubing from the lower tap were washed and dried after each sample was taken. Sodium nitrite, was placed on an aluminium foil 'shelf', taped to the inside of the separating funnel. The whole apparatus was then evacuated using a rotary pump, the tap closed and the vessel tipped up and shaken well to mix the nitrite and muscle mince slurry added to the vessel. The funnel was also shaken frequently during the experiment to try to ensure that bubbles of gas reached the upper part of the container. Initially, fresh muscle tissue (400g) was minced finely and incubation buffer at pH 6.0 added in the ratio of 2 parts buffer to 1 part muscle mince. This was to produce a slurry of fine enough consistency to be sucked through the glass tap without any blockages occurring. Sodium nitrite, to give
Diagram 6.1. Apparatus for the measurement of nitric oxide production.
analysed for nitric oxide after 10 hours at ambient temperature. Hardly any nitric oxide was seen, however, and it was thought that this was due to the gas dissolving in the large quantity of liquid available. The solubility of nitric oxide in water at 20° and 1 atmosphere pressure is 4.7 ml/100 ml water. The water is easily able to dissolve the nitric oxide produced in this experiment. Another problem that contributed to the lack of nitric oxide was caused by muscle tissue sticking in the travel of the glass tap. Air was able to leak round the sides of the tap, and the headspace gas would have been diluted. The only way to overcome this was to take the utmost care when turning the tap and to try and take the more liquid part of the slurry.

The experiment was then repeated at 0°, since this temperature could be attained by immersing the separating funnel in a bucket of ice, and was nearer to 4°, the usual temperature of bacon brining, and hence more relevant. The ratio of muscle mince and buffer was changed to 1:1. Sodium nitrite was added to produce a concentration of 1000 ppm relative to the muscle tissue. Samples from the separating funnel were then analysed for nitric oxide and residual nitrite. The response of the mass spectrometer was calibrated with pure nitric oxide, which enabled the partial pressure of the nitric oxide in the separating funnel to be calculated. Knowing the volume of the headspace in the vessel then enabled the mass of nitric oxide to be calculated. Samples of the headspace and minced muscle slurry were extracted at intervals over 25 hours.

**Muscle cures containing amytal**

In order to show the involvement of muscle enzymes in the production of the headspace gases, incubations of muscle tissue and sodium nitrite were carried out in which the respiratory chain inhibitor amytal (Ernster, Dallner and Azzone, 1963) was added. Four flasks containing 25g minced pork muscle tissue and 25 ml incubation buffer, together with sodium nitrite at
a concentration of 500 ppm were incubated anaerobically at 4° for 60 hours.

Two of the flasks also contained amytal at a concentration of 20 mM.

The headspace gases were then analysed by mass spectrometry. The results are shown in diagram 6.6. The peak heights were converted to show the partial pressure of each gas as a percentage of the total pressure excluding the pressure due to water vapour.
6.3 Results

Low resolution mass spectrometric analysis of headspace gases

Table 6.1 shows the relative peak heights over the background peaks of ions from \( \frac{m}{e} = 12 \) to \( \frac{m}{e} = 88 \), from incubations containing either \( \text{Na}^{14}\text{NO}_2 \), initially at 200 ppm, after 40 hours at 37°. Although the flasks had been evacuated when making them anaerobic, nitrogen, argon and carbon dioxide were all present to an appreciable extent in the headspace gases. The notable ions in the spectrum are marked in table 6.1 by asterisks. The data below describes the possible source of some of the ions.

\( \frac{m}{e} 15 \): Possibly the ion \( (15\text{N}^{15}\text{N})^{++} \), this would indicate the formation of 15N-15N in the model cure. Also possible in the unlabelled nitrite cure is the formation of \( ^{14}\text{NO}^{++} \).

\( \frac{m}{e} 15.5 \): This is probably due to the ion \( ^{15}\text{NO}^{++} \).

\( \frac{m}{e} 29 \): The production of \( ^{14}\text{N}^{15}\text{N} \) by Van Slyke degradation could give rise to this ion, although it is possible that its presence is due to leakage of nitrogen into the cure.

\( \frac{m}{e} 30 \): In the case of the unlabelled cure the peak is undoubtedly due to \( ^{14}\text{NO}^{+} \).

\( \frac{m}{e} 31 \): This confirms the production of nitric oxide by the ion \( ^{15}\text{NO}^{+} \).

\( \frac{m}{e} 45 \): The peaks in the mass spectrum of both headspaces are possibly due to \( ^{14}\text{N}^{15}\text{NO}^{++} \).

\( \frac{m}{e} 46 \): \( ^{15}\text{N}^{15}\text{NO}^{+} \) gives rise to peaks at \( m/e = 46 \). The corresponding peak at \( m/e = 44 \) for the unlabelled cure is probably obscured by a large carbon dioxide ion. \( m/e 37, 41, 47, 48, 49, 51, 84, 86, 88 \): These were thought to be due to the inclusion of chloramphenicol in the cures, and experiments omitting the antibiotic showed this to be the case.
Relative heights of peaks over background peak for incubation containing labelled and unlabelled nitrite.

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<tr>
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<td>18</td>
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Table 6.1

ANALYSIS OF HEADSPACE GASES IN MUSCLE MINCE CURE AT 37°C

WITH 200 ppm NITRITE

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**ANALYSIS OF HEADSPACE GASES IN MUSCLE MINCE CURES AT 37°C**

**WITH 200 ppm NITRITE**
Analysis of headspace gases for ethylene

A in diagram 6.2 shows an injection of 10 μl of the headspace of the control incubation, run without sodium nitrite. B shows an injection of 10 μl of the headspace of the cure containing sodium nitrite, and the appearance of a peak with a retention time of 2.78 minutes. C shows a 10 μl injection of the gas mixture mentioned before, in which methane, ethylene and ethane are present. Ethylene has a retention time of 2.75 minutes. When 5 μl of the headspace from B was added to 5 μl of the gas mixture, the height of the ethylene peak rose in proportion to the CH₄ and C₂H₆ peak, supporting the contention that the incubation including sodium nitrite had produced ethylene.

The effect of nitrite level on ethylene production was then studied, and diagram 6.3 shows the proportion of ethylene in the headspace formed with respect to increasing levels of nitrite. The results of bacterial count on blood agar plates at the various nitrite levels are also displayed.

Measurement of the rate of production of headspace gases

The peak heights of ions from gases in the headspace of model cures with and without nitrite at 500 ppm were plotted for various time intervals over 74 hours. Diagram 6.4 shows the heights of peaks from ions at $\frac{m}{e} = 28$, produced by ethylene and nitrogen, $\frac{m}{e} = 30$ from nitric oxide and at $\frac{m}{e} = 44$ from carbon dioxide and nitrous oxide. Although the absolute values obtained for the ions vary, the rising trend in the quantities of ions and thus gases, can be easily seen in the incubations which included nitrite. A more thorough investigation of the rate of production of nitric oxide was made, however, using the RMU-7L mass spectrometer, in which the instrument scanned over the mass of nitric oxide at a resolution of 10,000, thus discriminating nitric oxide from any other ions at $\frac{m}{e} = 30$. The result of this experiment is described below.
Diagram 6.2(a).

Injection of 10 μl control incubation headspace

Retention time 2.78 min

Injection of 10 μl sample of incubation headspace
Retention time 2.75 min

Retention time 0.58 min

Retention time 0.6 min

Injection of 10μl standard gas mixture

Injection of 5μl standard gas mixture + 5μl sample incubation headspace

Diagram 6.2.(b).
Diagram 6.3. Graph of bacterial count and ethylene production for various nitrite levels.
Diagram 6.4. The production of $CO_2$, $N_2O$, NO, $C_2H_4$ and $N_2$ in incubations of minced muscle tissue with (A & B) and without (C) sodium nitrite.
During the sampling of the headspace gases of the model cure in the separating funnel (diag 6.1) the total pressure in the system was measured each time. On the basis of the size of the peak produced by the nitric oxide ions from the sample and from the size of the peak produced from pure nitric oxide at a known pressure, it was possible to calculate the partial pressure of nitric oxide in the headspace. From a knowledge of the volume of the headspace, absolute amounts of nitric oxide in the headspace could be determined. In the graph below (diagram 6.5) the rate of nitric oxide production has been corrected for the withdrawal of sample headspace gases during each analysis. The points also include a correction for the solubility of nitric oxide in the slurry. It can be seen that nitric oxide was produced approximately linearly with time, over a period of 26 hours at a rate of 13 ng/minute or 0.6 nmoles/min at 0°C. Expressed per gram of tissue, the result is 0.0015 nmoles/g tissue/ min.

There were, however, a large number of difficulties involved in the measurement, and limits the accuracy of the result. These drawbacks are listed below:

(1) The sampling of the gas by the mass spectrometer removed nitric oxide from the system and this meant that nitric oxide may have come out of solution to take its place and gave a false reading for the amount of nitric oxide produced when the next analysis was made.

(2) The solubility of nitric oxide in a meat slurry system is not known. The solubility of nitric oxide in pure water was used as an approximation to that within a meat slurry.

(3) Gas produced at the bottom of the separating funnel may not have found its way to the headspace before the composition of the gas was analysed, although
Diagram 6.5: The rate of production of nitric acid at 0°C in a model cure.
the flask for nitric oxide production was shaken vigourously during the experiment. It could still have been possible for bubbles of gas to have been trapped in the meat slurry.

Notwithstanding these criticisms, the experiment does give some indication of the rate of nitric oxide evolution in a model curing system.

Muscle cures containing amytal

Proof that nitric oxide and ethylene were at least partly enzymically produced was obtained with incubations in which the respiratory chain inhibitor amytal (Ernster et al. 1963) was added. Analysis of the headspace gases by mass spectroscopy gave the results drawn below in diagram 6.6. The peak height at $m_e = 27$ was measured to enable discrimination between $N_2$ and $C_2H_4$, both of which produce ions at $m_e = 28$; the ethylene concentration was assumed to be directly proportional to the height of the peak at $m_e = 27$. The known ratio between the $m_e = 27$ and $m_e = 28$ ions of ethylene allowed the contribution to the $m_e = 28$ peak due to ethylene to be subtracted, to give a value for nitrogen alone.

The graph shows the proportion of each gas in the headspace mixture and since the headspace reached the same pressure in each incubation flask, the peak height of each component can be compared with the peak height of the same component in the other flasks. The presence of amytal was seen to inhibit both nitric oxide and ethylene production. It cannot be commented whether the production of these two gases is both chemical and enzymic, since amytal only inhibits completely in pure cytochrome systems.
Diagram 6.6. Incubation of nitrite with muscle mince at 3° with and without the addition of amytal
Discussion

The experiments performed in this chapter, on the presence of nitric oxide as a product of the nitrite-skeletal pork muscle interaction confirmed the results found, using infra-red, differential absorption manometry and low resolution mass spectrometry, of Walters and Taylor (1963). The combination of the low resolution mass spectrometric evidence involving the use of $^{15}$N, together with high resolution measurement of the nitric oxide ion from the model cures, unambiguously identified nitric oxide as the major gaseous component of the headspace gases produced at 4° and 37° at various nitrite levels. The presence of nitrous oxide as a product in model bacon cures, though to a far smaller extent than NO, was confirmed by mass spectrometry. Möhler and Ebert (1971), have also shown the presence of nitrous oxide in mixtures of minced beef and nitrite after heating both to 37° and 75°, using nitrite levels of up to 400 ppm.

The pathway for the production of nitric oxide involves several theories, discussed in the introductory chapter. Results in this thesis, arising from the markedly decreased output of nitric oxide in the presence of the respiratory chain inhibitor, amytal, which has been found by Ernster et al. (1963) to block flavoprotein-cytochrome c interaction in mitochondrial preparations, suggest that nitric oxide evolution was at least partly associated with the respiratory oxidation-reduction systems in the muscle tissue.

In the anaerobic system present in the model cures, evidence has been provided (Walters and Taylor, 1964) that it may be possible for nitrite to act as an electron acceptor in the electron transport chain, and be reduced to nitric oxide. The oxidation reduction potential for the change

$$\text{HNO}_2 + \text{H}^+ + \text{e} \rightarrow \text{NO} + \text{H}_2\text{O}$$

is + 0.99 volts (Charlot et al., 1958), close to that for the reduction of oxygen to hydroxyl ions, so that the system is not precluded electronically
from providing a ready acceptor for the electrons of the mammalian respiratory chain normally directed aerobically towards oxygen.

The rate of production of nitric oxide in model cures has been found to be approximately linear with respect to nitrite concentrations up to 1000 ppm at 0°C over a period of 26 hours. The value obtained (though derived from only one experiment), 0.0015 nmoles/g tissue, compares well with the result of Walters and Taylor (1964) of 0.005 nmoles/g tissue. These latter workers used a higher temperature (37°C) and a higher initial nitrite concentration (2300 ppm) in the muscle and buffer slurry.

The production of nitrous oxide was found to increase with nitrite concentration, although an experiment of the complexity of the one mentioned above was not performed, and the result must have less significance. The combination of two nitrogen atoms as in NO could proceed through the labile dimerisation of any nitroxyl (NOH) formed, followed by elimination of water from the resulting hyponitrous acid. NO may also be formed from the action of nitrous acid with quaternary ammonium ammonium compounds and tertiary amines. (Fiddler et al. 1972) and with NADH (Evans and McAuliffe, 1956) or thiol groups (Mohler and Ebert, 1971)

The most surprising result of the headspace analyses and the most difficult to account for was the presence of ethylene. First observed by peaks in the headspace mass spectrum at m/e values of 26 and 27, the identity of the gas was proven both by gas chromatography and high resolution mass spectrometry. Ethylene production increased with nitrite concentration up to 50 mM, and as the bacterial count with nitrite present was very low, the formation of C_2H_4 was apparently not associated with bacterial action. The production of ethylene was also inhibited by the addition of amytal, showing evidence of the influence of the respiratory oxidation-reduction systems.
in the formation of \( \text{C}_2\text{H}_4 \). It was first thought that ethylene production might proceed along similar lines to \( \text{C}_2\text{H}_4 \) production in fruit (Mapson, 1970), i.e. from methionine or linolenic acid; both mechanisms involve the presence of oxygen and the concurrent production of ethane, neither of which are present in the incubation vessels. Ethylene may possibly be produced by the loss of HNO\(_2\) from alkyl nitrites (several of which are reported in this study to be present in volatile extracts of cured meats), though no published evidence is available in support of this pathway.

In conclusion, several points may be noted; the reaction between minced pork muscle and nitrite produces an assortment of gases: nitric oxide, nitrous oxide and ethylene, the origins of which are not totally understood. However, as far as the object of describing the fate of nitrite is concerned, the gaseous products account for only a minute proportion of the available nitrite, and a much larger quantity of nitrite is still untraced.
PART IV

CONCLUSIONS
The present investigation was concerned primarily with the products of the interaction of sodium nitrite and porcine skeletal muscle under conditions as relevant as possible to the curing process. In so far as one of the more important products was likely to be the highly carcinogenic group of compounds, N-nitrosamines, two methods of analysis of these compounds by mass spectrometry have been developed.

The unequivocal identification and measurement of N-nitrosamines at very low levels in extracts of cured foodstuffs is a difficult and elaborate procedure. Initially, use was made of low-resolution mass spectrometry linked to gas-chromatography. This was found to have several important drawbacks as far as sensitivity and specificity were concerned. The smallest quantities of nitrosamines that could be identified by the low-resolution mass spectrometer (RMU-6E) were of the order of 50 ng, much higher than the amounts that could readily be extracted and concentrated from cured products when added at parts-per-billion—a level considered to be of potential epidemiological significance. The confidence that could be placed in results from low resolution mass spectrometry, (based upon the NO⁺ fragment common to all the nitrosamines studied), was small, since many compounds can also give ions of mass to charge ratio = 30 and ions of the same mass to charge ratio as the molecular ions of the N-nitrosamines. Spectra of N-nitrosamines in extracts of cured products could easily be disturbed by the other volatile components of the food extracts.

However, while this was the only method of analysis available, analyses were made of extracts from commercial samples of corned beef, luncheon meat, bacon and smoked fish. No volatile N-nitrosamines were found, with the exception of samples to which several of the N-nitrosamines had been added at a level of 50 parts per billion.
During the examination of the low resolution mass spectra for evidence of the presence of N-nitrosamines in the cured meat, several other compounds were tentatively identified. Among the compounds present, containing nitrogen, were methyl-ethyl-pyridine, n-pentyl-nitrite, methyl-thiazole and propyl-thiazole. Many more compounds were present that could not be identified. The mass spectra obtained were ill-defined and the identification of the compounds involved was outside the scope of the present study.

The method of analysis currently accepted as providing the least ambiguous identification at low concentrations is the use of high resolution mass spectrometry coupled to gas chromatography, and two ways of detecting N-nitrosamines using this combination have been developed. One, the 'scanning' or 'peak matching' method involved the use of the accurate mass measuring equipment of the mass spectrometer. The other, 'the static' method comprised the focusing of the magnetic sector of the mass-spectrometer upon the exact mass of the ion to be detected, (usually the molecular ion of the N-nitrosamine). This was achieved using chemical isomers of the nitrosamine studied. The isomers produced ions of the exact mass required, without the hazards associated with the use of nitrosamines. The 'static' method was used preferentially, since a permanent record of the analysis was automatically kept. The two methods gave similar results as far as sensitivity was concerned, the minimum quantities of N-nitrosamine injected through the gas chromatograph that could be detected were generally of the order of 1 - 2 ng.

Using the 'Static' method of analysis, N-nitrosopyrididine was found in several samples of spices that had been mixed and stored with NaCl and NaNO₂. N-nitrosodimethylamine and N-nitrosopiperidine were also shown to be present in several of the samples. Analyses for volatile N-nitrosamines were also performed on extracts from nitrosated pork, milk, and eggs. No volatile N-nitrosamines (above 2 parts per billion) were
Further analyses of extracts of luncheon meat have also been made and N-nitrosopyrroloidine has been shown to be present, together with N-nitrosodimethylamine. Volatiles from milk and cheese subjected to nitrosation contained N-nitrosodimethylamine, N-nitrosopiperidine up to 80 parts per billion and N-nitrosopyrroloidine up to 30 parts per billion.

To aid identification of non-volatile N-nitrosamines that may be formed in cured products, mass spectra, together with high resolution measurements of the major ions, were obtained for a number of authentic nitrosamines considered to be likely products. The compounds studied included N-nitrosoproline, N-nitrososarcosine and N-nitrososarcosinamide. The product of the attempted nitrosation of prolyl glycine was also studied; the mass spectrum obtained did not, however, point unequivocally to the formation of N-nitrosoprolly glycine.

N-nitrosamines are only one of the products of the addition of sodium nitrite to foodstuffs, and another facet of this investigation has been to produce a 'balance sheet' of the fate of nitrite added under conditions similar to the curing process: at pH 6.0 (the post-mortem pH of muscle), in an anaerobic (reducing) environment which could exist in the interior of a block of meat, using relevant concentrations of nitrite. To detect the products in which added nitrite had been involved, the $^{15}$N labelled salt was used, and the nitrogen isotope enrichment in model cures observed using both mass spectroscopy and emission spectroscopy.

Analyses were made for nitrite, nitrate, nitrosylmyoglobin, nitroso-thiol compounds, non-volatile N-nitrosamines and the gaseous headspace product in model curing systems.

Nitrite was found to be used by model cures simulating bacon production (as measured by both residual nitrite, and $^{15}$N enrichment studies), leaving
between 12% and 35% residual nitrite, depending upon the original level added. Nitrite was used by the model cures at higher temperatures (simulating the products of pasteurised ham condition 4 hours at 70°), to a greater extent. This was a genuine reflection of nitrite 'metabolism' by the muscle, the nitrite utilisation occurred in the presence of the antibiotic chloromycetin, which was added to prevent bacterial metabolism of the nitrite. The rate of nitrite disappearance was exponential with time, at both 4° and 37° over 30 hours.

Nitrate was found in both the bacon (4°) and pasteurised ham model cures (7°), though only to a very small extent (2-5 parts per million). It probably derived from the oxidation of nitrous acid by traces of oxygen, and did not contribute significantly to the loss of nitrite.

Some of the nitrite utilised was found ultimately as nitric oxide in combination with muscle myoglobin, and the formation of nitrosylmyoglobin was studied at several levels of nitrite; the maximum concentration of nitrosylmyoglobin appeared to be formed at initial nitrite concentrations of 200 parts per million, (2.7 mM). Up to this concentration of nitrite nitrosylmyoglobin formation was linearly proportional. At 200 parts per million nitrite, and above a plateau of the nitrosated haem pigment concentration appeared to have been reached.

The major pathway of the nitrite disappearance was found to be the formation of nitrosothiol compounds and accounted for up to 25% of the total nitrite added. The relationship between nitrite concentration and the formation of nitrosothiol groups at pH 6.0 was shown to be approximately linear at the lower temperature used (4°). Less nitrosothiol formation was found in the pasteurised ham model cures. Nitrosothiol compounds have been reported to be implicated in the formation of nitrosylmyoglobin, and during this investigation it was
found that, in general, those model cures in which a high yield of nitrosothiols were to be found the highest concentrations of nitrosylmyoglobin were also to be seen.

The occurrence of non-volatile N-nitrosamines was investigated, primarily to ascertain the relevance of these compounds when determining the fate of nitrite. Although the amounts of nitrite which reacted to form N-nitrosamines was very small, the quantities of N-nitrosamines which were produced were significant in terms of carcinogenic activity. It should be borne in mind that two precursors of N-nitrosopyrrolidine and N-nitroso-dimethylamine are thought to be non-volatile nitrosamines (N-nitrosoproline and N-nitroso-creatinine, respectively) and that the compounds, proline and creatine, are abundant in the biological system studied here.

Analyses of the gaseous headspace of the model cures confirmed the presence of nitric oxide and nitrous oxide by low and high resolution mass spectrometry with and without the use of $^{15}\text{N}$ labelled nitrite. Another component, ethylene, was shown by both high resolution mass spectrometry and gas chromatography. The production of both nitric oxide and ethylene was partially inhibited by the use of the respiratory chain inhibitor, amytal, showing that the production of both gases was, to some extent at least, linked to the action of the electron transport chain of the porcine muscle. The kinetics of the production of nitric oxide in a model curing system were studied using mass spectrometry; and the rate of nitric oxide production was found to be 0.6 moles/min at 0°C with an initial nitrite level of 1000 parts per million. The quantities of other nitrogen containing gases found in the headspace of the model cures was small enough for their contributions to the use of nitrite to be ignored.

This investigation has helped to elucidate the pathways of the fate of nitrite added to porcine muscle under conditions closely resembling those of modern curing practices. The main products of the nitrite–muscle interaction,
together with some of the products formed in minute quantities have been identified and measured, so that an account of the fate of practically all of the nitrite can be given. Although occurring at very low levels, N-nitrosamines, both volatile and non-volatile, have been detected as unambiguously as possible.

Despite the fact that several nitrosothiols have already been shown to have an antibacterial effect, the significance of nitrosothiols in cured products, as to whether they constitute a health hazard, has yet to be evaluated. Until this is undertaken, it seems we must continue to utilise the bacteriostatic action of nitrite (especially in the context of botulinum) in certain foodstuffs while the demand for cured products continues.
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