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The fate of nitrite in food processing: isolation of dinuclear and tetranuclear iron–sulphur nitrosyl complexes from cysteine and methionine sources

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In the presence of iron(II) salts nitrite reacts with cysteine, and sources of cysteine including hydrolysed caseins, under a range of experimental conditions relevant to food processing, to yield the anti-microbial iron–sulphur nitrosyl salt $Na[Fe_4S_3(NO)_7]$ in isolable quantities: under certain conditions, the conversion of nitrite to $Na[Fe_4S_3(NO)_7]$ is quantitative. In similar reactions, methionine and some of its derivatives yield the neutral dinuclear complex $[Fe_2(SCH_3)_2(NO)_4]$, a known tumour promoter, where the SCH_3 groups have been incorporated intact from methionine.

INTRODUCTION

The mode of action by which nitrite, NO_2^-, inhibits the development of *Clostridium* species and other food spoilage organisms in preserved foodstuffs has been the subject of investigation for a number of years (Walters & West, 1984; Woods *et al.*, 1989). One key feature is the loss of nitrite observed (Perigo *et al.*, 1967; Mirna & Hofmann, 1969; Fox & Nicholas, 1974; Roberts *et al.*, 1981) alongside the formation of a bacterial inhibitor more potent than nitrite itself (Perigo *et al.*, 1967; Ashworth & Spencer, 1972).

A possible clue to the anti-clostridial action of nitrite is the observation (Reddy *et al.*, 1983) that treatment of vegetative cells of *Clostridium botulinum* with nitrite in the presence of ascorbate caused replacement of the electron paramagnetic resonance (EPR) signal at g = 1·94, characteristic of the reduced form of a {4Fe—4S} iron–sulphur centre by a signal at g = 2·035, characteristic of iron–nitrosyl complexes of general type $[Fe(NO)_2X_2]$, most likely in this case of type $[Fe(NO)_2(SR)_2]^-$. Subsequent work (Butler *et al.*, 1985*b*) using synthetic models for both the {4Fe—4S} and {2Fe—2S} centres of redox proteins showed that treatment with aqueous nitrite readily gave the complex $[Fe(NO)_2(SH)_2]^-$, identifiable by EPR spectroscopy, as an intermediate in the formation of the tetra-iron complex $[Fe_4S_3(NO)_7]^-$, isolable in yields of around 40%.

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The formation of the dinuclear iron–nitrosyl complex $[Fe_2(SMe)_2(NO)_4]$, isolable from plant materials exposed to aqueous sodium nitrite solutions (Wang *et al.*, 1980; Baty *et al.*, 1987) and demonstrated (Cheng *et al.*, 1981; Li & Cheng, 1984; Liu & Li, 1989) to be a promoter of the tumorigenic activity of environmental carcinogens such as polycyclic aromatic hydrocarbons and *N*-nitrosamines has similarly been interpreted (Butler *et al.*, 1988) in terms of a reaction between nitrite and a preformed iron–sulphur cluster.

Earlier chemical work (Ashworth *et al.*, 1974; Moran *et al.*, 1975) had suggested, however, that added iron salts, as well as some source of sulphur, were necessary for the formation of clostridial inhibitors, but the identity of the inhibitor was not established. Material with properties similar to those of $[Fe_4S_3(NO)_7]^-$ salts was extracted (Ashworth *et al.*, 1974) from a limited range of substrates, including casein hydrolysates, cysteine, and sodium thioglycolate, after treatment with nitrite with or without added iron salts. However, the analytical and spectroscopic evidence adduced (Ashworth *et al.*, 1974) in support of the identification of this material was indicative only, and by no means definitive: for example the analytical data supported an atomic ratio Fe:S:N of 4:3:6 rather than the 4:3:7 required for the sodium salt of $[Fe_4S_3(NO)_7]^-$ or the 4:3:8 ratio required for the ammonium salt.

We have now reinvestigated the reactions of sodium nitrite with a range of cysteine and methionine sources in the presence of iron(II) salts, under a range of heating regimes relevant to food-processing in order to characterise further the fate of the nitrite. By use of a combination of analytical techniques including, in addition to elemental analysis, Fourier-Transform infra-red (FTIR)

spectroscopy, high resolution mass spectrometry with appropriate use of isotopic labelling (in particular H/D and $^{14}N/^{15}N$ substitutions), and ^{15}N nuclear magnetic resonance (NMR) spectroscopy on samples highly enriched (99%) with ^{15}N, we have achieved definitive identification after isolation of the tetranuclear complex $[Fe_4S_3(NO)_7]$ formed by sulphur-capture from a range of cysteine sources, in yields which sometimes represent 100% conversion of the available nitrite: similarly we have isolated and identified the neutral dinuclear complex $[Fe_2(SMe)_2(NO)_4]$ formed from methionine sources by incorporation of intact SMe groups from the side chains.

MATERIALS AND METHODS

Materials

Amino acids and their esters and *N*-acetyl derivatives, thioglycolic acid, sodium thioglycolate, and glutathione were all purchased from Aldrich. Sodium nitrite, enriched to 99% in ^{15}N was purchased from MSD Isotopes, and methionine-methyl-d_3, enriched to 99% in deuterium, was purchased from Aldrich. Haemin, bovine haemoglobin, and acid-hydrolysed and enzyme-hydrolysed caseins were obtained from Sigma.

Literature methods were used for the preparation of *S*-nitroso-*N*-acetylpenicillamine (Field *et al.*, 1978) and for the production of authentic samples of $Na[Fe_4S_3(NO)_7]$ (Brauer, 1960) and $[Fe_2(SMe)_2(NO)_4]$ (Glidewell *et al.*, 1989). Perigo medium was prepared, and used, according to the literature (Perigo *et al.*, 1967).

Standard aliquots of pork slurry were prepared by homogenizing 250 g of manually defatted pork gigot with 750 cm^3 of water in which were dissolved systematically varied quantities of iron(II) sulphate heptahydrate from 0 g to 25 g, sodium nitrite from 0·10 g to 5·0 g and sodium ascorbate equivalent to 4 times the weight of sodium nitrite.

Instruments

Infra-red spectra were recorded using a Perkin–Elmer model 1710 Fourier-Transform spectrophotometer: spectra were recorded in tetrahydrofuran or methanol solutions using 0·5 mm pathlength cells. NMR spectra were recorded at 298 K using a Brüker AM-300 instrument: ^1H spectra were recorded at 300·133 MHz relative to internal tetramethylsilane; ^{13}C spectra were recorded at 75·469 MHz relative to internal tetramethylsilane; ^{15}N spectra were recorded at 30·424 MHz relative to external nitromethane (50% v/v solution in acetone). Mass spectra were recorded using an AEI MS50 instrument, at an ionising potential of 70 eV. Atomic absorption analyses for iron were done using a Pye–Unicam model PU9000 spectrophotometer. The autoclave employed had a capacity of 14·3 dm^3.

Heat treatments

(a) Reactions of sodium nitrite and iron sulphate with characterized sulphur sources

(i) Autoclave reactions. Iron(II) sulphate heptahydrate (0·50 g, 1·80 mmol), sodium nitrite (0·10 g, 1·45 mmol) and the sulphur source (16·5 mmol) were dissolved with sodium ascorbate (3·3 g, 16·5 mmol) in water (1 dm^3), and the mixture was autoclaved at 118°C for 20 min: duplicate runs were conducted omitting the sodium ascorbate. Selected runs were repeated using $Na[^{15}NO_2]$, and with different molar ratios of reactants.

(ii) Microwave heating. Mixtures as in (i) above were heated in 500 cm^3 aliquots in Erlenmeyer flasks loosely closed with cottonwool and thermostatted at 90°C for 1 h, using 650 W microwave power.

(iii) Anaerobic reactions. Iron(II) sulphate heptahydrate (1·70 g, 6·1 mmol), sodium nitrite (1·0 g, 14·5 mmol) and the sulphur source (6·7 mmol) were dissolved with sodium ascorbate (2·7 g, 13·6 mmol) in deoxygenated water (100 cm^3): the mixture was bubbled continuously with N_2 gas and either stirred at room temperature, or heated under reflux, for 2 h.

(b) Reactions of sodium nitrite with Perigo medium

Sodium nitrite (0·10 g, 1·45 mmol) and iron(II) sulphate heptahydrate (0·50 g, 1·80 mmol) were added to Perigo medium (1 dm^3) and the mixture was autoclaved at 118°C for 20 min: duplicate runs were carried out omitting the iron sulphate.

(c) Reactions of sodium nitrite with casein hydrolysates

Sodium nitrite (0·10 g, 1·45 mmol) was dissolved in water (1 dm^3) and either acid-hydrolysed casein (150 g) or enzyme-hydrolysed casein (100 g) was added. The mixtures were autoclaved at 118°C for 20 min. Similar runs were carried out for each mixture using the following additives:

(1) iron(II) sulphate heptahydrate (0·50 g, 1·80 mmol);
(2) sodium ascorbate (2·0 g, 10·1 mmol);
(3) iron(II) sulphate heptahydrate (0·50 g, 1·80 mmol) and sodium ascorbate (2·0 g, 10·1 mmol).

Selected runs were repeated using $Na[^{15}NO_2]$.

(d) Reactions using haemin or haemoglobin as potential iron sources

Reaction mixtures as in (a) above, using either cysteine or methionine as sulphur source but substituting haemin or bovine haemoglobin for iron sulphate were

(1) autoclaved at 118°C for 40 min, or
(2) heated under reflux, in a nitrogen atmosphere, for 2 h.

(e) Reactions of sodium nitrite and iron(II) sulphate with pork slurries

(i) Autoclaved reactions. Standard aliquots of pork slurry were autoclaved at 118°C for 20 min. For the mixture containing 1·0 g iron(II) sulphate heptahydrate and 0·20 g sodium nitrite, autoclave times of 20 min, 40 min and 70 min were employed.

(ii) Microwave heating. A standard aliquot of pork slurry was mixed with sodium nitrite (0·20 g, 2·90 mmol), iron(II) sulphate heptahydrate (1·0 g, 3·60 mmol) and sodium ascorbate (0·80 g, 4·0 mmol). The mixture was thermostatted as above (a(ii)) for 30 min at 90°C (650 W microwave power).

(iii) Anaerobic reaction. A standard aliquot of pork slurry was mixed with sodium nitrite (5·0 g, 72·5 mmol), iron(II) sulphate heptahydrate (8·5 g, 30·6 mmol) and sodium ascorbate (13·5 g, 68 mmol) and the mixture was heated under reflux, in a nitrogen atmosphere, for 2 h.

In all systems studied, there are many concentrations which can be independently varied, including those of nitrite, any added iron salts, the sulphur source, and ascorbate, as well as the nature of the heat treatment. Consequently, a complete and systematic study would hardly be practical. The experiments described here are intended to provide a representative survey of a wide range of experimental conditions relevant to food processing procedures, which in turn demonstrate the wide range of conditions under which nitrite can be easily converted into iron sulphur nitrosyl complexes.

Chromatography

The reaction mixtures from each of the procedures (a)–(d) inclusive, above, were cooled, filtered through Hyflo-supercel where necessary, and extracted exhaustively with diethyl ether: the combined ether extracts were washed with water and dried over magnesium sulphate. Where the initial ether extracts were colourless, the whole mixture was evaporated to dryness, and the residue was extracted exhaustively with methanol. The mixtures in procedure (e), above, separated during heat treatment into solid bolus and supernatant liquor. The supernate was extracted with diethyl ether, but the ether remained colourless: the bolus was broken up and stirred with acetone (four successive portions, each of 200 cm^3). The acetone extract was filtered through silica and evaporated to small volume and the residue was extracted exhaustively with diethyl ether: this extract was washed and dried as described above.

The ether extracts obtained above contained $Na[Fe_4S_3(NO)_7]$ and/or $[Fe_2(SR)_2(NO)_4]$ where R represents a non-polar side-chain. The solvent was removed and the residue was dissolved in the minimum volume of chloroform: this solution was applied to an 8 cm × 1·5 cm silica chromatography column. Elution with chloroform yielded spectroscopically pure $[Fe_2(SR)_2(NO)_4]$, while elution with dry acetone yielded spectroscopically pure $Na[Fe_4S_3(NO)_7]$. The methanol extracts obtained above contain $Na[Fe_4S_3(NO)_7]$ and/or $[Fe_2(SR)_2(NO)_4]$ where R represents a polar side-chain containing NH_2 and/or COOH functions: all attempts to purify the extracts by chromatography caused the conversion of polar derivatives $[Fe_2(SR)_2(NO)_4]$ to $[Fe_4S_3(NO)_7]$ salts.

Characterization of isolated $Na[Fe_4S_3(NO)_7]$ and $[Fe_2(SMe)_2(NO)_4]$

Isolated samples of $Na[Fe_4S_3(NO)_7]$ were identified by:

(a) Elemental analysis. Found Fe, 40·6; N, 17·5; S, 16·9%: $Fe_4N_7NaO_7S_3$ requires Fe, 40·4; N, 17·7; S, 17·4%. Derivatives formed by metathesis with (i) aqueous $(Ph_4As)Cl$—$(Ph_4As)[Fe_4S_3(NO)_7]$, found C, 31·2; H, 2·1; N, 10·6%: $C_{24}H_{20}AsFe_4N_7O_7S_3$ requires C, 31·6; H, 2·2; N, 10·7%; and (ii) aqueous $[(Ph_3P)_2N]Cl$—$[(Ph_3P)_2N][Fe_4S_3(NO)_7]$, found C, 40·3; H, 2·7; N, 10·3%: $C_{36}H_{30}Fe_4N_8O_7P_2S_3$ requires C, 40·5; H, 2·8; N, 10·5%.

(b) Infra-red spectral comparison with authentic samples. In tetrahydrofuran solution, $Na[Fe_4S_3(^{14}NO)_7]$ has $\nu(^{14}NO)$: 1795 (w), 1742 (vs), 1707 (m) cm^{-1}; $Na[Fe_4S_3(^{15}NO)_7]$ has $\nu(^{15}NO)$: 1760 (w), 1708 (vs), 1673 (m) cm^{-1}.

(c) ^{15}N NMR comparison with authentic samples. In acetone solution $Na[Fe_4S_3(^{15}NO)_7]$ has $\delta(^{15}N)$: 11·8 (s, 1N), 39·5 (d, J 3·8 Hz, 3N), 80·6 (d, 3·8 Hz, 3N). (Butler *et al.*, 1985*a*).

Isolated samples of $[Fe_2(SMe)_2(NO)_4]$ were identified by:

(d) Infra-red spectral comparison with authentic samples. In tetrahydrofuran solution, $[Fe_2(SMe)_2(^{14}NO)_4]$ has $\nu(^{14}NO)$: 1776 (s), 1751 (s) cm^{-1}; $[Fe_2(SMe)_2(^{15}NO)_4]$ has $\nu(^{15}NO)$: 1739 (s), 1714 (s) cm^{-1}.

(e) NMR spectral comparisons (^1H, ^{13}C, and ^{15}N) with authentic samples. In $CDCl_3$ solution $[Fe_2(SMe)_2(NO)_4]$ has $\delta(^1H)$, 2·83 (s); $\delta(^{13}C)$, 27·5 (q): in toluene-d_8 solution, $\delta(^1H)$ 2·17 (s), 2·23 (s): in CD_2Cl_2 solution $[Fe_2(SMe)_2(^{15}NO)_4]$ has $\delta(^{15}N)$ 23·1 (d, J 2·8 Hz), 30·5 (s), 36·2 (d, J 2·8 Hz).

(f) Mass spectral comparisons with authentic samples. $[Fe_2(SCH_3)_2(^{14}NO)_4]$ has m/z: 326, 296, 266, 236, 206, 191, 176; $[Fe_2(SCD_3)_2(^{14}NO)_4]$ has m/z: 332, 302, 272, 242, 212, 194, 176; $[Fe_2(SCH_3)_2(^{15}NO)_4]$ has m/z: 330, 299, 268, 237, 206, 191, 176.

RESULTS AND DISCUSSION

Cysteine and its derivatives

When sodium nitrite and iron(II) sulphate heptahydrate (1·45 mmol and 1·80 mmol respectively) were autoclaved at 118°C in dilute aqueous solution with DL-cysteine (16·5 mmol), the sodium salt of Roussin's

black anion, $Na[Fe_4S_3(NO)_7]$ was isolated in 18% yield. In the presence of an excess of sodium ascorbate, the yield rose to 77%. An autoclaved mixture of iron(II) sulphate, sodium nitrite and large excess of cysteine ($3{\cdot}6 \times 10^{-3}$, $0{\cdot}72 \times 10^{-3}$, and 66×10^{-3} mol dm^{-3} respectively) has been demonstrated to be extremely inhibitory towards growing cultures of both *Clostridium perfringens* (Moran *et al.*, 1975) and *Clostridium sporogenes* (Payne *et al.*, 1990): we have now found that from such a mixture autoclaved at 118°C, the salt $Na[Fe_4S_3(NO)_7]$ can be isolated in 39% yield, based upon sodium nitrite as the limiting component. When sodium ascorbate was present in the same molar concentration as the cysteine, the conversion of nitrite to $Na[Fe_4S_3(NO)_7]$ was quantitative. Since this inhibitory mixture showed the same level of inhibition, based upon the concentration of NO fragments as pre-formed but unautoclaved $Na[Fe_4S_3(NO)_7]$ (Payne *et al.*, 1990), it can be suggested that this tetranuclear salt, or one of its dissociation products (Butler *et al.*, 1988) is the effective inhibitor.

In a similar manner, several cysteine derivatives including the methyl and ethyl esters, and *N*-acetyl cysteine, as well as homocysteine, cystine and the tripeptide glutathione also gave $Na[Fe_4S_3(NO)_7]$ under autoclave conditions, although the last two required the presence of sodium ascorbate. The isolated, purified yields ranged from 6·5% (from homocysteine) to 52% (cysteine methyl ester).

The interaction of cysteine with iron(II) sulphate and sodium nitrite was also investigated under a range of other conditions of relevance to food processing, using always a molar excess of cysteine over sodium nitrite. Under microwave heating at 90°C quantitative Fourier-transform infra-red (FTIR) spectroscopy indicated *c.* 1% conversion of nitrite to $Na[Fe_4S_3(NO)_7]$ after 1 h in the absence of sodium ascorbate, compared with *c.* 3% conversion when sodium ascorbate was present. Boiling of similar mixtures in anaerobic conditions, with sodium ascorbate present gave *c.* 3% conversion of nitrite to $Na[Fe_4S_3(NO)_7]$, while even at room temperature, in air, with no ascorbate, the formation of $Na[Fe_4S_3(NO)_7]$ was detectable, although the conversion of nitrite was less than 0·1%.

The capture of sulphur from cysteine by iron(II)/nitrite mixtures, to form the tetranuclear anion $[Fe_4S_3(NO)_7]^-$ is therefore extremely facile. It appears to be promoted by a reducing environment (anaerobic atmosphere; sodium ascorbate) but most importantly by high temperatures: under such conditions, low levels of nitrite can be converted quantitatively to $[Fe_4S_3(NO)_7]^-$ salts. The capture of sulphur from cystine requires sodium ascorbate, probably to reduce cystine to cysteine, although the mechanism of this capture is not proven.

In acid-hydrolysed casein, there is typically *c.* 0·035% by weight of sulphur incorporated as cystine: in enzyme-hydrolysed casein, the cystine content typically provides 0·045–0·08% by weight sulphur. The casein hydrolysates used in this work were analysed by atomic absorption spectrophotometry and each was found to contain *c.* 20 ppm iron, presumably iron(II) sequestered by the coordinating sites of the side-chains. In our runs using acid-hydrolysed casein, the maximum available iron was 0·054 mol, while in runs using enzyme-hydrolysed casein, it was 0·036 mol: clearly, in both experiments iron was the limiting component. In the presence of sodium ascorbate, required to reduce the protein-bound cystine to cysteine, autoclaving of acid-hydrolysed casein with sodium nitrite produced $Na[Fe_4S_3(NO)_7]$, identified by FTIR, and by ^{15}N NMR spectroscopy in runs using $Na[^{15}NO_2]$: no $Na[Fe_4S_3(NO)_7]$ was formed in the absence of sodium ascorbate. That the reduction of cystine was the limiting factor, rather than the state of the casein-bound iron, was demonstrated by repeating the experiments with the addition of 500 ppm of iron(II) sulphate heptahydrate: again $Na[Fe_4S_3(NO)_7]$ was produced only when sodium ascorbate was present. The results using enzyme-hydrolysed casein were essentially identical, with the identification of $Na[Fe_4S_3(NO)_7]$ confirmed by use of $Na[^{15}NO_2]$ and ^{15}N NMR spectroscopy, as with the acid-hydrolysed casein.

The results with casein hydrolysates show that even with very low levels of available sulphur (0·035%) and adventitious iron (20 ppm) a simple brief heat treatment with sodium nitrite under reducing conditions readily forms $Na[Fe_4S_3(NO)_7]$.

Closely related to cysteine, $HSCH_2CH(NH_2)COOH$, is the dimethyl analogue penicillamine $HSC(CH_3)_2CH(NH_2)COOH$. Both penicillamine and its *N*-acetyl derivative can act as sulphur sources for the formation of $Na[Fe_4S_3(NO)_7]$ in reactions with sodium nitrite and iron(II) sulphate. However the stable and long-lived *S*-nitroso derivative, *S*-nitroso-*N*-acetylpenicillamine (SNAP) does not act as a sulphur source under these conditions: instead it acts as a source of NO groups in reaction with iron(II) salts and an additional sulphur source such as cysteine. In the presence of sodium ascorbate, yields of up to 55% $Na[Fe_4S_3(NO)_7]$ were obtained, based upon NO groups as the limiting component.

Methionine and its derivatives

When an aqueous mixture of sodium nitrite, iron(II) sulphate heptahydrate and methionine was autoclaved in the presence of sodium ascorbate, the neutral dinuclear iron complex $[Fe_2(SCH_3)_2(NO)_4]$ was isolated in *c.* 4% yield: no $Na[Fe_4S_3(NO)_7]$ was detected. The source of the methyl groups was established by use of methionine specifically and fully deuterated in the *S*-methyl group: in this case the product was $[Fe_2(SCD_3)_2(NO)_4]$, demonstrating the incorporation into the product of the SMe fragment, from methionine, as an intact entity. Just as methionine $CH_3S(CH_2)_2CH(NH_2)COOH$ gave $[Fe_2(SCH_3)_2(NO)_4]$, so the analogous ethionine $C_2H_5S(CH_2)_2CH(NH_2)COOH$ gave $[Fe_2(SC_2H_5)_2(NO)_4]$ again confirming the transfer of intact SR fragments into $[Fe_2(SR)_2(NO)_4]$. Methionine ethyl ester similarly

gave $[Fe_2(SCH_3)_2(NO)_4]$ while *S*-methyl cysteine, $CH_3SCH_2CH(NH_2)COOH$ gave a mixture of dinuclear $[Fe_2(SR)_2(NO)_4]$ and tetranuclear $Na[Fe_4S_3(NO)_7]$. In all of these runs, sodium ascorbate was necessary for the formation of $[Fe_2(SR)_2(NO)_4]$ whether R = CH_3 or C_2H_5.

When the reaction between sodium nitrite, iron(II) sulphate and methionine was conducted at 100°C, under anaerobic conditions at ambient pressure, rather than at 118°C in an autoclave with air present, the isolated yield of $[Fe_2(SCH_3)_2(NO)_4]$ rose reproducibly from *c.* 4% to *c.* 10%. Regardless of the temperature, this indicates that the establishment of a reducing environment is more critical for the formation of $[Fe_2(SCH_3)_2(NO)_4]$ from methionine and its analogues than for the formation of $Na[Fe_4S_3(NO)_7]$ from cysteine and its derivatives. Provided such conditions are established, the capture of SCH_3 groups proceeds readily: aqueous methionine/iron(II) mixtures also yield $[Fe_2(SCH_3)_2(NO)_4]$ in the presence of sodium nitrite at 90°C under microwave power, and at room temperature in the presence of nitric oxide, NO, a reduction product from the reaction between nitrite and ascorbate (Dahn *et al.*, 1960). As well as cysteine sulphur, hydrolysed caseins contain sulphur also as methionine: acid-hydrolysed casein typically contains *c.* 0·40% by weight of sulphur as methionine, while enzyme-hydrolysed casein contains 0·3–0·4% of methionine sulphur. In autoclave reactions with sodium nitrite and iron(II) sulphate, both casein hydrolysates yielded $[Fe_2(SMe)_2(NO)_4]$, from methionine, as well as $Na[Fe_4S_3(NO)_7]$, from cysteine. Unhydrolysed caseins provided neither the dinuclear nor the tetranuclear iron complexes.

Perigo medium

The principal sulphur source in Perigo medium (Perigo *et al.*, 1967) is sodium thioglycolate, $HSCH_2COONa$: hence the reactions of sodium thioglycolate, thioglycolic acid, and methyl thioglycolate, $HSCH_2COOCH_3$, with sodium nitrite and iron(II) sulphate were studied prior to investigation of the entire Perigo medium.

The behaviour of methyl thioglycolate is straightforward: under autoclave conditions at 118°C, the known binuclear complex $[Fe_2(SCH_2COOCH_3)_2(NO)_4]$ (Glidewell *et al.*, 1990) was formed: in the absence of sodium ascorbate, the yield was 52%, and in its presence the yield rose to 72%. A control experiment using authentic preformed $[Fe_2(SCH_2COOCH_3)_2(NO)_4]$ showed that this material was fully stable under the conditions of the autoclave procedure. The behaviour of sodium thioglycolate is similarly straightforward; from reaction under autoclave conditions, $Na[Fe_4S_3(NO)_7]$ was isolated in 21% yield. In unbuffered aqueous solution, thioglycolic acid produces initially the binuclear complex $[Fe_2(SCH_2COOH)_2(NO)_4]$, but attempts to separate and purify this material by chromatography led to its decomposition giving $Na[Fe_4S_3(NO)_7]$ as the sole isolable iron–nitrosyl complex. By contrast when the autoclave reaction was conducted in buffer solution, pH 4, the tetranuclear complex $Na[Fe_4S_3(NO)_7]$ was formed in 75% isolated yield.

Since Perigo medium contains both sodium thioglycolate and enzyme-hydrolysed casein, the observed formation of $Na[Fe_4S_3(NO)_7]$ was expected. In the absence of added iron, the level of $Na[Fe_4S_3(NO)_7]$ formed was very low, and it could not be satisfactorily separated from the rest of the mixture: nevertheless, its formation was confirmed by ^{15}N labelling and ^{15}N NMR spectroscopy. As expected, in the presence of added iron, the yield was increased.

Haems and pork slurries

Under autoclave reaction conditions, neither haemin nor bovine haemoglobin gave either $Na[Fe_4S_3(NO)_7]$ or $[Fe_2(SCH_3)_2(NO)_4]$ when reacted with sodium nitrite, in the presence of sodium ascorbate and, in the case of haemin only, added cysteine or methionine as sulphur source, and in some runs with haemoglobin, added iron(II) sulphate. Hence haemoglobin does not act as a sulphur source in the presence of added iron(II) for the formation of iron–nitrosyl complexes, while neither haemin nor haemoglobin acts as an iron source. These results indicate that only non-haem iron is likely to give rise to the complexes $[Fe_2(SCH_3)_2(NO)_4]$ or $Na[Fe_4S_3(NO)_7]$ under conditions likely to be relevant to food processing.

A series of experiments using slurries of de-fatted pork leg muscle showed that added iron was essential to the formation of $Na[Fe_4S_3(NO)_7]$. Under autoclave conditions the tetranuclear complex was readily detectable at added iron levels down to 0·004% by weight iron(II), similar in fact to the levels of iron typically encountered in beef products (Holland *et al.*, 1991). However, below these levels, $Na[Fe_4S_3(NO)_7]$ could not be identified with certainty. At all levels of sodium nitrite addition, iron was the limiting factor: control reactions using iron(II) chloride in place of iron(II) sulphate gave identical yields and confirmed the pork slurry as the source of the sulphur. The yields of $Na[Fe_4S_3(NO)_7]$ appeared, from the limited time-course studies made, to be independent of whether the autoclave period was 20 min, 40 min or 70 min. A limited investigation under both microwave heating conditions and boiling at ambient pressure under a nitrogen atmosphere confirmed the formation of $Na[Fe_4S_3(NO)_7]$ in the presence of added iron(II) but no added sulphur source. In none of the runs did we obtain any evidence for $[Fe_2(SCH_3)_2(NO)_4]$ formation.

CONCLUSIONS

We have identified two iron complexes, readily formed under a range of experimental conditions relevant to food-processing procedures, whose formation can account for nitrite loss during processing: in some circumstances, complete conversion of added nitrite to

$Na[Fe_4S_3(NO)_7]$ was observed. We note that with beef slurries (Fox & Nicholas, 1974), some nitrite loss could be attributed to reduction to free nitric oxide, NO; however, the proportion of nitrite so converted was generally fairly small, particularly when cysteine was present. This is probably because, in such experiments, the cysteine sulphur is captured to form $[Fe_4S_3(NO)_7]$, as demonstrated here. Although the role of cysteine has been postulated to involve formation of *S*-nitrosocysteine (Mirna & Hofmann, 1969), the evidence for this is sparse and ambiguous (Fox & Nicholas, 1974). We have found no evidence for the formation of any *S*-nitroso compounds for cysteine or any of its derivatives: control experiments on the direct nitrosation of *N*-acetyl cysteine demonstrate that these *S*-nitroso derivatives are labile and short-lived, and unlikely to survive in many of the experimental systems where they have been postulated (van Roon & Olsman, 1976; van Roon, 1980).

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