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0308-8146(96)00009-X

Food Chemistry, Vol. 56, No. 2, pp. 105–110, 1996

0308-8146/96 $15.00+.00

RAPID COMMUNICATION

A novel titration methodology for elucidation of the structure of preformed cooked cured-meat pigment by visible spectroscopy

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(Received 29 September 1995; revised version received and accepted 14 November 1995)

The chemical structure of preformed cooked cured-meat pigment (CCMP), which is believed to be the pigment of thermally-processed nitrite-cured meat, was investigated by a novel titration methodology. Electronic spectra of reduced haemin and its nitrosyl derivative were recorded in the visible region in an aqueous- or a dimethyl sulphoxide-medium. Changes in the electronic spectrum of reduced haemin upon gradual addition of aliquots of a saturated solution of nitric oxide (NO) revealed that only one molecule of NO ligates itself to the iron of the porphyrin molecule. A similar conclusion was reached from the infrared studies of CCMP, which revealed the presence of only one N–O stretching band. Thus, preformed CCMP is irrefutably a mononitrosyl, not a dinitrosyl, derivative of reduced haemin. This conclusion is further supported by an independent electron paramagnetic resonance study of CCMP, which showed that the pigment was a pentacoordinated paramagnetic complex. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The structure of the nitrosylprotohaem pigment of cooked cured meat has long been a subject of controversy. Based on many spectroscopic investigations of nitrosylhaem complexes, it is believed that, during thermal processing of nitrite-cured meat, the globin portion of nitrosylmyoglobin denatures and subsequently detaches itself from the haem moiety. The resultant pigment is either a five-coordinate mononitrosylhaem complex or a six-coordinate dinitrosylhaem compound, if it acquires a second molecule of nitric oxide (Fig. 1).

Tarladgis (1962) concluded, based on spectral studies using acetone extracts from Hornsey's method (Hornsey, 1956), that the pigment of thermally-processed nitrite-cured meat was a low-spin ferrous–porphyrin coordination complex exhibiting no electron paramagnetic resonance (EPR) signals. Thus, he suggested that the pigment was dinitrosylprotohaem. In later studies, Lee & Cassens (1976) and Renerre & Rougie (1979) used $Na^{15}NO_2$ and determined that the heated samples contained twice as much ^{15}NO as their unheated counterparts. These authors did not consider the possibility that NO may bind with other constituents of haemoprotein. In a series of papers by Bonnett and co-workers, these authors reported that the reaction of $NaNO_2$ with haemoproteins under mildly acidic conditions can occur at the ferrous ion to give the nitrosylhaem pigment (Bonnett *et al.*, 1980*b*), in the porphyrin ring (Bonnett *et al.*, 1978, 1980*a*) or in the protein (Bonnett & Nicolaidou, 1979).

Bonnett *et al.* (1978, 1980*b*) attempted to characterize the pigment of cooked cured meat, nitrosylprotohaem, as its dimethyl ester, obtained by the reaction of NO with protohaem dimethyl ester and with methoxyiron(III)-protoporphyrin dimethyl ester. A strong infrared (IR) band at ca. 1660 cm^{-1} was diagnostic of the stretching mode of a bent Fe–NO moiety and a pentacoordinate complex. The EPR spectrum of nitrosylprotohaem dimethyl ester in an acetone glass showed a triplet signal due to hyperfine splitting by a single axial nitrogenous ligand of NO indicative of a pentacoordinate system (i.e. $g_1 = 2.102$, $g_2 = 2.064$, $g_3 = 2.010$, $a_3 = 1.63$ mT). Nitrosylprotohaem extracted with acetone

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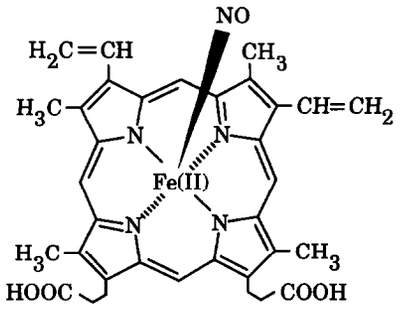
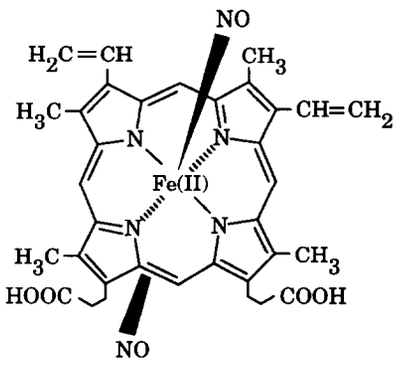


Mononitrosylprotohaem



Dinitrosylprotohaem

Fig. 1. Chemical structures of mononitrosylprotohaem and dinitrosylprotohaem.

from thermally processed nitrite-cured meat also had an EPR signal expected of a pentacoordinate nitrosylhaem complex.

Killday *et al.* (1988) isolated and characterized an extract of the CCMP from thermally processed corned beef by IR and visible (VIS) spectroscopies as well as thin-layer chromatography. They found that the pigment was a mononitrosyl ferrous protoporphyrin. The authors further identified the pigment by fast atom bombardment mass spectrometry as having only one NO moiety and suggested that a protein radical of globin reacts with a second molecule of nitrite, resulting in the stoichiometry found in the labelling experiments of Lee & Cassens (1976) and Renerre & Rougie (1979). Skibsted (1992) noted that on prolonged heating of haem model systems with labelled $^{15}NO_2$, it was possible to increase the labelling of ^{15}NO to a level higher than the 2:1 stoichiometry. Recently, Jankiewicz *et al.* (1994) provided further support to the view that the CCMP is mononitrosylprotohaem from VIS and IR absorption studies.

Our interest in the chemical nature of the cooked cured-meat pigment (CCMP) stems from our investigations of nitrite-free meat curing systems. We have reported the preparation of CCMP from haemin (Shahidi *et al.*, 1994) in the presence of reductants and NO. This pigment, preformed outside the meat matrix, is used as part of a composite mixture for nitrite-free curing of meat products, but its chemical nature has not been adequately elucidated. Based on EPR studies recently carried out, we reported that the preformed CCMP is a mononitrosylhaem complex (Pegg *et al.*, 1996), not dinitrosyl ferrohaemochrome as it has previously been described (Shahidi *et al.*, 1984, 1985). The purpose of this study was to investigate the chemical nature of CCMP via a novel titration methodology that uses a spectrophotometric procedure to detect the titration's endpoint, and to provide further support for the view that CCMP is mononitrosyl ferrohaemochrome.

MATERIALS AND METHODS

Materials

All solvents used were ACS grade or better and were flushed with nitrogen before use. Bovine haemin and dimethyl sulphoxide (DMSO) were acquired from the Sigma Chemical Company (St Louis, MO). Sodium dithionite was purchased from BDH Chemicals (Toronto, ON). Nitric oxide and nitrogen were supplied by Canadian Liquid Air (St John's, NF).

Model systems

Two model haemin systems were used. In the first set of experiments, an aqueous model system was employed and in the second set a DMSO model system was used.

Aqueous model system

A glass cuvette was filled with 3000 μl of a 0.04 M sodium carbonate solution. To the cuvette, a 75 μl aliquot of a 3.5 mg/ml haemin solution dissolved in the carbonate solution was transferred. A few crystals of sodium dithionite were added to reduce the Fe(III) haemin to its ferrous state. After capping the cuvette with a rubber septum, nitrogen was purged through the head space gases for ca. 30 s to remove oxygen. The absorption spectrum of the reduced haemin solution was recorded at 450–650 nm using a Hewlett Packard 8452A diode array spectrophotometer (Hewlett Packard Canada Ltd, Mississauga, ON). The sodium carbonate solution was used for background subtraction. At this point, a gentle stream of NO was bubbled into the system via a syringe needle for ca. 10 s forming the nitrosylhaem analog. Its absorption spectrum was recorded.

A saturated solution of NO was prepared in a test tube of deoxygenated distilled water. The tube was covered with Parafilm in an effort to prevent penetration of oxygen, and NO was purged into the system for 20 min via the syringe needle. A small magnetic stirring bar had been placed in the bottom of the tube to prevent supersaturation of NO in the medium. To a fresh preparation of the reduced haem system, 25 or 50 μl aliquots of the saturated solution of NO were added. The absorption spectrum was monitored at 450–650 nm. The titration proceeded until it was believed that all reduced haemin had been converted to its nitrosylated derivative (either mono- or dinitrosyl

compound). At this point, NO was bubbled into the cuvette and the absorbance spectrum read to ensure that the nitrosylation reaction had gone to completion.

Dimethyl sulphoxide (DMSO) model system

The nitrosylhaem derivative was prepared in the DMSO model system similar to that described by Jankiewicz *et al.* (1994), but with modifications. Briefly, haemin (ca. 6.2 mg) was dissolved in 80 ml of DMSO, whereupon the volume was made up to 100 ml with a solution of 100 mg of sodium dithionite dissolved in ca. 25 ml of distilled water. Upon addition of dithionite, the dissolved haemin turned from a black–brown colour to a bright red one denoting that reduction of the iron atom to the ferrous state had occurred. To a set of test tubes, aliquots of distilled water, ranging between 550 and 1000 μl, were added followed by a few crystals of dithionite. Nine millilitres of the reduced haem solution were added to each tube, which was then capped with a rubber septum. The headspace gases were purged from each tube with nitrogen for ca. 30 s. Aliquots of the saturated solution of NO, ranging between 0 and 450 μl, were transferred to the systems via piercing the septum of each tube with a Hamilton syringe. Tubes were vortexed and allowed to stand for 10 min before spectrophotometric measurements. Absorption spectra were monitored in the visible range between 450 and 650 nm, and derivative spectra were recorded using the aforementioned spectrophotometer. An 80% (v/v) DMSO–water mixture was used as the blank.

Infrared spectroscopy

Cooked cured-meat pigment was prepared as outlined by Shahidi *et al.* (1994). The pigment was dissolved in DMSO and then transferred to a sodium chloride infrared (IR) cell. The blank cell contained DMSO. Infrared spectral data were obtained using a Mattson Polaris Fourier transform IR spectrophotometer.

RESULTS AND DISCUSSION

Aqueous model system

The basic methodology underlying this experiment is a simple one, that is, forming a NO haem complex from the titration of a reduced haemin solution against a saturated solution of NO using the sensitivity of a spectrophotometer to detect the endpoint. Jankiewicz *et al.* (1994) prepared the nitrosyl derivative of haem and haem dimethyl ester in an 80% (v/v) DMSO model system using an aqueous solution of sodium nitrite as the nitrosating agent. These authors used 80% (v/v) DMSO as their solvent of choice because they noted that haemin and its nitrosyl derivative were insoluble in water. In preliminary studies, 3000 μl of distilled water were used instead of the carbonate solution. As aliquots of the saturated solution of NO were added to the reduced haemin system, the nitrosyl derivative formed and precipitated out of solution making spectrophotometric measurements impractical. However, both reduced haemin and its nitrosyl analog are soluble at the alkaline pH of a dilute carbonate solution (pH = 11.0). Because the preformed CCMP used for nitrite-free curing of meats is prepared in an aqueous medium with the use of NO rather than sodium nitrite, we elected to investigate the characteristics of the pigment in the carbonate buffer.

The visible absorption spectra of reduced haemin and its nitrosyl derivative were recorded (Fig. 2). The maximum change in absorbance between spectra of equal concentrations of reduced haemin and its nitrosyl

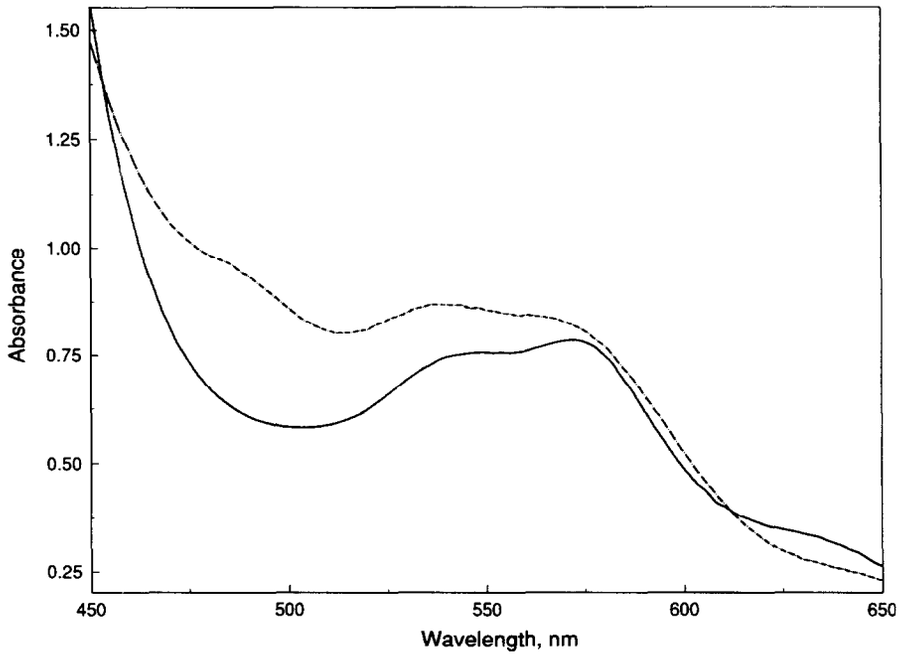


Fig. 2. Visible absorption spectra of reduced haemin, ——— ; and nitrosylprotohaem, – · – · – · – · –.

derivative occurred at 486 nm. Rather than bubbling NO directly into a fresh preparation of reduced haemin to obtain the nitrosylhaem complex, aliquots of the saturated solution of NO were added into the air-tight cuvette via a Hamilton syringe, and the change in absorbance at the 486 nm band was monitored.

The basic equation for the reaction of haemin ($ClFe^{III}P$) with NO in a protic solvent such as water or methanol, and in the presence of a reductant, is as follows (Bonnett *et al.*, 1980*b*): $ClFe^{III} + 2NO \xrightarrow{H_2O} ONFe^{II}P + HONO + HCl$, where P = porphyrin dianion.

Data provided by Young (1981) reported that the concentration of a saturated solution of NO in water at standard pressure and a temperature of 293 K is 2.02×10^{-3} M. Based on the micromoles of haemin present in the model system (ca. 0.40 μmol) and the stoichiometry of the above reaction, it was determined that ca. 400 μl of the saturated solution of NO would be needed to reach the endpoint of the titration if CCMP were a mononitrosylhaem complex; 800 μl would be required if the pigment were dinitrosylprotohaem. Based on data presented in Fig. 3, 350–400 μl of the saturated solution of NO were used to reach the endpoint of the titration, suggesting that only one nitrosyl group is ligated to the reduced haem. These results are in agreement with those of Killday *et al.* (1988) and Jankiewicz *et al.* (1994) who suggested that a 1:1 complex between NO and reduced haem is formed.

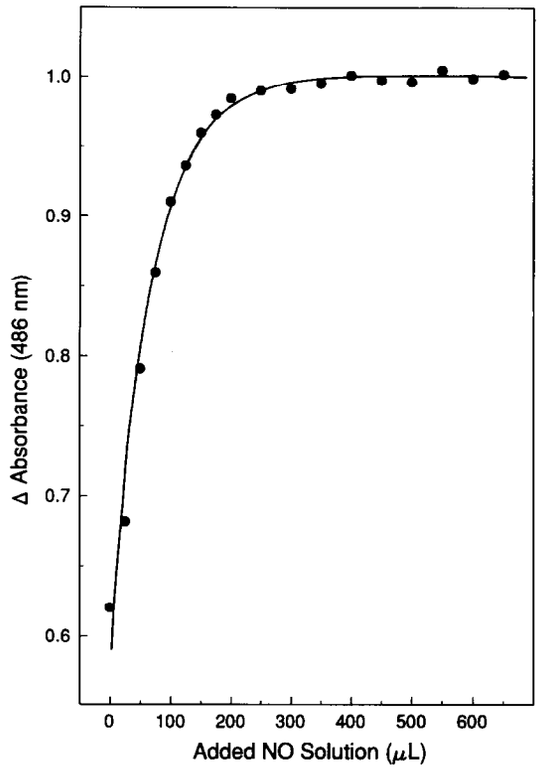


Fig. 3. Change in absorbance upon titration of reduced haemin with a saturated solution of NO. ΔAbsorbance at 486 nm, which was corrected for dilution, is between that of the nitrosylhaem complex formed and the reduced haemin. Addition of NO gas, at the termination of the titration, did not influence absorbance values.

Dimethyl sulphoxide model system

The absorption spectrum of reduced haemin in the DMSO system exhibited two absorption maxima at 524 and 554 nm and a minimum at 538 nm. This is consistent with absorption characteristics reported by Jankiewicz *et al.* (1994). As aliquots of the saturated solution of NO were added to the reduced haemin, a nitrosylhaem complex formed as evidenced by change in absorption spectra. A marked reduction in absorbance at the 524 and 554 nm bands was apparent for equal concentrations of reduced haem and its nitrosyl counterpart. Due to the formation of a new compound as increments of NO are added to the haem system, derivative spectroscopy was employed. Often derivative spectra reveal spectral details that are lost in the original spectrum. Furthermore, concentration measurements of an analyte in the presence of an interferent can sometimes be made more easily or more accurately (Skoog & Leary, 1992). Both first and second derivative spectra of the absorbance were recorded, but only the second derivative spectra are presented in Fig. 4. The minima in these spectra at 524 and 556 nm correspond with the local maxima of the absorbance spectra, and the maximum at 538 nm conforms to the local minimum of the absorbance spectra. As aliquots of the saturated solution of NO were added to the reduced haemin system and the nitrosylhaem derivative formed, a decrease in absorbance occurred at the 556 nm maximum. This translates to an increase in the second derivative of absorbance at 556 nm. Because the medium consists primarily of DMSO, the stoichiometry between the added nitrosating species and reduced haemin should be ca. 1:1. Based on the micromoles of haemin present in the model system (ca. 0.85 μmol), it was calculated that ca. 450 μl of the saturated solution of NO would be needed to form a mononitrosylhaem complex. By comparing the second derivative absorption spectrum of nitrosylprotohaem (i.e. reduced haemin system into which NO had been bubbled) and reduced haemin, after 475 μl of the nitrosating agent had been added, it was seen that over 96% of the reduced haemin was converted to its nitrosylhaem derivative. A plot of the logarithm of the ratio of the mononitrosylprotohaem concentration to that of reduced haemin (y) against the logarithm of the concentration of the NO solution (x) showed a linear relationship ($y = 1.17x + 2.0$, $r = 0.983$, Fig. 5). The slope of the plot gave a value of 1.2 which suggests that the haem:NO complex is 1:1 and that a mononitrosylhaem complex is produced.

Infrared spectra and other supporting evidence

The IR spectrum of CCMP exhibited a strong band at $\nu = 1659\ cm^{-1}$ which corresponds to the first nitrosyl group bound with the ferrous atom of the haem molecule as reported by Jankiewicz *et al.* (1994). Bonnett *et al.* (1980*b*) reported that this stretching frequency is consistent with the presence of a bent Fe–NO moiety and a pentacoordinate complex. Scheidt & Frisse (1975)

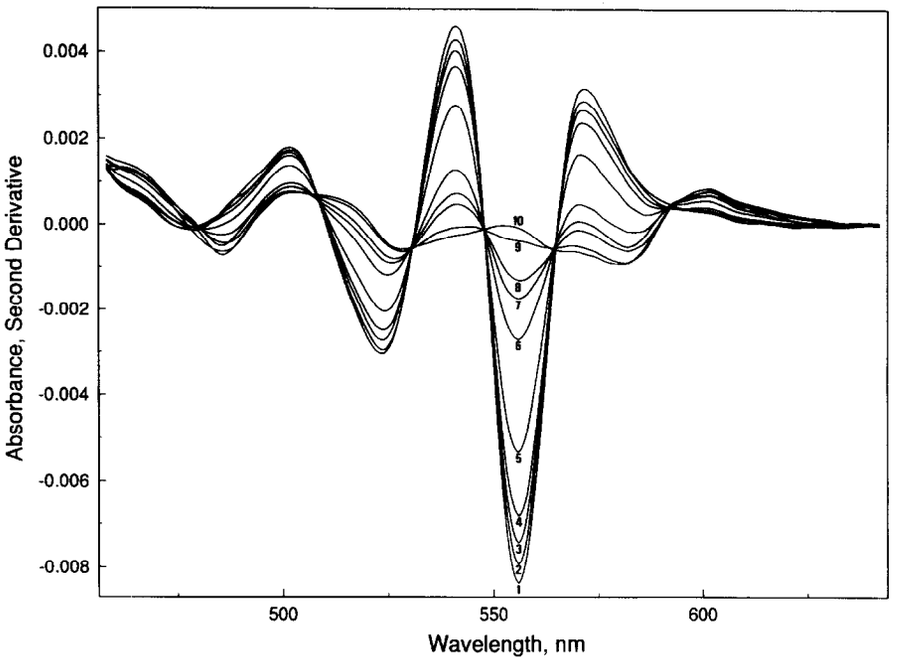


Fig. 4. Second derivative spectra of absorbance of reduced haemin in 80% (v/v) dimethyl sulphoxide and the change thereof as the nitrosylhaem complex forms upon addition of aliquots of a saturated solution of NO. (1) 0 μl; (2) 25 μl; (3) 50 μl; (4) 75 μl; (5) 125 μl; (6) 200 μl; (7) 275 μl; (8) 350 μl; (9) 475 μl; (10) NO gas.

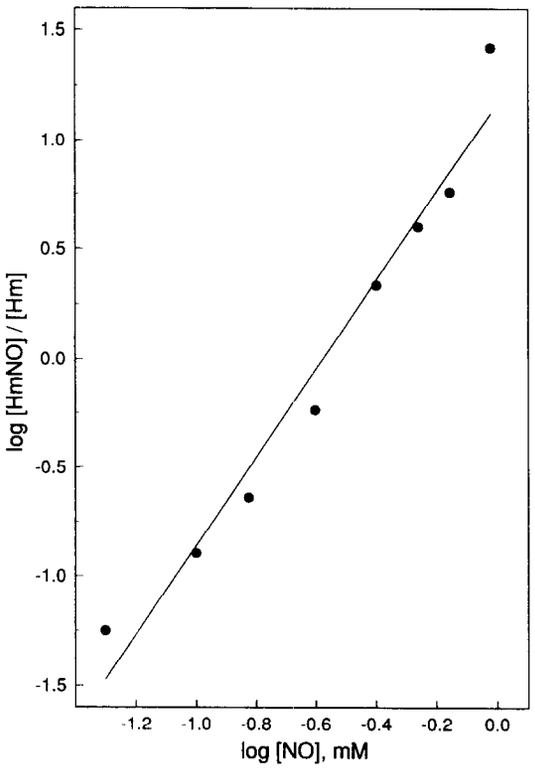


Fig. 5. Relationship between the log [HmNO]/[Hm] against the log [NO]. Hm = reduced haemin; HmNO = nitrosylhaem

had reported a ν_{max} of 1670 cm^{-1} and an Fe–N–O angle of 149.2° for nitrosyltetraphenylhaem. Furthermore, there was no stretching band at ν = ca. 1900 cm^{-1}, which according to Killday *et al.* (1988) would indicate the presence of a second nitrosyl group in CCMP.

Further evidence for a mononitrosylhaem complex as the pigment of nitrite-cured meat comes from EPR studies by several authors. The EPR spectral parameters of an acetone extract of a sample of CCMP were compared to those of nitrosylprotohaem dimethyl ester investigated by Bonnett *et al.* (1980*b*) and to Fe^{II}TPP(NO) reported by Wayland & Olson (1974). In all cases examined, the EPR parameters of these systems were similar and possessed characteristics recognized as those of a pentacoordinate nitrosylhaem complex; that is, the EPR spectrum of CCMP in an acetone glass showed ^{14}N hyperfine splitting in the g_3 region with a_3 of 1.71 mT (Pegg *et al.*, 1996). Together with the EPR studies cited, the titration experiments reported above, provide strong evidence for a mononitrosyliron(II) complex for the cooked cured-meat pigment.

REFERENCES

Bonnett, R., Charalambides, A. A. & Martin, R. A. (1978). Nitrosation and nitrosylation of haemoproteins and related compounds. Part 1. Porphyrins and metalloporphyrins. *J.C.S. Perkin*, I, 974–80.

Bonnett, R. & Nicolaidou, P. (1979). Nitrosation and nitrosylation of haemoproteins and related compounds. Part 2. The reaction of nitrous acid with the side chains of α-acyl-amino-acid esters. *J.C.S. Perkin*, **I**, 1969–74.

Bonnett, R., Hursthouse, M. B., Scourides, P. A. & Trotter, J. (1980*a*). Nitrosation and nitrosylation of haemoproteins and related compounds. Part 3. Attack at the vinyl groups of protoporphyrin dimethyl ester. X-ray analysis of 8^1(E)-8^2-nitroprotoporphyrin dimethyl ester. *J.C.S. Perkin*, **I**, 490–4.

Bonnett, R., Chandra, S., Charalambides, A. A., Sales, K. D. & Scourides, P. A. (1980*b*). Nitrosation and nitrosylation of haemoproteins and related compounds. Part 4.

Pentaco-ordinate nitrosylprotohaem as the pigment of cooked cured meat. Direct evidence from E.S.R. spectroscopy. *J.C.S. Perkin*, **I**, 1706–10.

Hornsey, H. C. (1956). The colour of cooked cured pork. I. Estimation of the nitric oxide-haem pigments. *J. Sci. Food Agric.*, **7**, 534–40.

Jankiewicz, L., Kwaśny, M., Wasylki, K. & Graczyk, A. (1994). Structure studies on the nitrosyl derivative of heme. *J. Food Sci.*, **59**, 57–9.

Killday, K. B., Tempesta, M. S., Bailey, M. E. & Metral, C. J. (1988). Structural characterization of nitrosylhemochromogen of cooked cured meat: Implications in the meat-curing reaction. *J. Agric. Food Chem.*, **36**, 909–14.

Lee, S. H. & Cassens, R. G. (1976). Nitrite binding sites on myoglobin. *J. Food Sci.*, **41**, 969–70.

Pegg, R. B., Shahidi, F., Gogan, N. J. & DeSilva, S. I. (1996). Elucidation of the chemical structure of preformed cooked cured-meat pigment by electron paramagnetic resonance spectroscopy. *J. Agric. Food Chem.*, **44**, 416–21.

Renerre, M. & Rougie, P. (1979). Influence du chauffage sur la fixation du nitrite à la myoglobine. *Ann. Technol. Agric.*, **28**, 423–31.

Scheidt, W. R. & Frisse, M. E. (1975). Nitrosylmetalloporphyrins. II. Synthesis and molecular stereochemistry of nitrosyl-$\alpha,\beta,\gamma,\delta$-tetraphenylporphinatoiron(II). *J. Am. Chem. Soc.*, **97**, 17–21.

Shahidi, F., Rubin, L. J., Diosady, L. L., Chew, V. & Wood, D. F. (1984). Preparation of dinitrosyl ferrohemochrome from hemin and sodium nitrite. *Can. Inst. Food Sci. Technol. J.*, **17**, 33–7.

Shahidi, F., Rubin, L. J., Diosady, L. L. & Wood, D. F. (1985). Preparation of the cooked cured-meat pigment, dinitrosyl ferrohemochrome, from hemin and nitric oxide. *J. Food Sci.*, **50**, 272–3.

Shahidi, F., Pegg, R. B. & Sen, N. P. (1994). Absence of volatile *N*-nitrosamines in cooked nitrite-free cured muscle foods. *Meat Sci.*, **37**, 327–36.

Skibsted, L. H. (1992). Cured meat products and their oxidative stability. In *The Chemistry of Muscle-Based Foods*, eds. D. A. Ledward, D. E. Johnston and M. K. Knight, Special Publication No. 106. The Royal Society of Chemistry, UK, pp. 266–86.

Skoog, D. A. & Leary, J. J. (1992). Chapter 8. Application of molecular ultraviolet/visible absorption spectroscopy. In *Principles of Instrumental Analysis*, 4th edn. Saunders College Publishing, Fort Worth, USA, pp. 150–73.

Tarladgis, B. G. (1962). Interpretation of the spectra of meat pigments. II. Cured meats. The mechanism of colour fading. *J. Sci. Food Agric.*, **13**, 485–91.

Wayland, B. B. & Olson, L. W. (1974). Spectroscopic studies and bonding model for nitric oxide complexes of iron porphyrins. *J. Am. Chem. Soc.*, **96**, 6037–41.

Young, C. L. (1981). Nitric oxide. In *Solubility Data Series. Volume 8. Oxides of Nitrogen*. International Union of Pure and Applied Chemistry. Pergamon Press, Oxford, UK, pp. 260–351.