Elucidation of the Chemical Structure of Preformed Cooked Cured-Meat Pigment by Electron Paramagnetic Resonance Spectroscopy

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The preparation of cooked cured-meat pigment (CCMP) has previously been reported from our laboratories, but the exact chemical nature of this pigment has remained elusive. Electron paramagnetic resonance (EPR) spectroscopy was employed to help provide the answers sought. EPR spectra of CCMP in an acetone glass revealed that the molecule is paramagnetic and exists as a pentacoordinate mononitrosylheme complex. A well-resolved triplet in the g_3 region of the spectrum was due to hyperfine splitting of the ^{14}N nuclei of NO with an unpaired electron. In a pyridine glass, EPR spectra suggest that there was participation of a pyridine molecule with the nitrosylheme complex. Such spectra are typical of a hexacoordinate species and are similar to those of NO complexes of Fe(II) myoglobin and hemoglobin. Additionally, a loss in resolution of the hyperfine structure in the g_3 region was apparent. EPR spectra of cooked nitrite-cured and CCMP-treated meat, *in situ*, were similar and were identical to that of the preformed CCMP in acetone. Thus, the pigment formed in thermally processed nitrite-cured meat is identical to that of CCMP prepared outside the meat matrix and then applied to meat as part of a nitrite-free multicomponent curing system.

Keywords: *Electron paramagnetic resonance spectroscopy; cooked cured-meat pigment; nitrite; myoglobin; hemoglobin*

INTRODUCTION

The structure of the nitrosylheme pigment of cooked cured meat has long been a subject of dispute. In 1901, Haldane was the first to attribute the pink color of cured meat to a nitric oxide−hemoprotein complex. This hypothesis was based on the fact that the visible absorption bands of extracts of cured meat resembled those of the reaction product of nitric oxide and hemoglobin. On the basis of numerous spectroscopic investigations of nitrosylheme complexes, it is believed that during thermal processing of nitrite-cured meat, the globin portion of nitrosylmyoglobin denatures and subsequently detaches itself from the heme moiety. Yet, this is where the controversy exists. The resultant pigment either will be a pentacoordinate mononitrosylprotoheme or may acquire a second molecule of nitric oxide to form a hexacoordinate dinitrosylprotoheme (Figure 1).

On the basis of spectral studies using acetone extracts, prepared according to the method of Hornsey (1956), Tarladgis (1962) concluded that the pigment of thermally processed nitrite-cured meat was a low-spin ferrous−porphyrin coordination complex. The identity of the ligand in the vacant sixth coordination position of the cooked cured-meat pigment (CCMP) is uncertain, but Tarladgis (1962) suggested that both axial coordinate positions of the iron were occupied by NO groups. Further evidence for dinitrosyl ligation is available from studies by Lee and Cassens (1976) and Renerre and Rougie (1978) in which $Na^{15}NO_2$ was used to determine

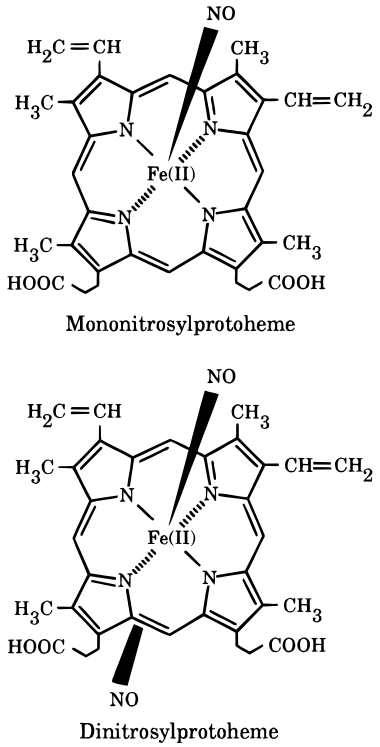

Figure 1. Proposed chemical structures of the CCMP, mononitrosylprotoheme or dinitrosylprotoheme.

the quantity of ^{15}NO bound to unheated as compared to heated solutions of myoglobin. These authors found, using a modified Kjeldahl analysis, that heated samples contained twice as much labeled ^{15}NO as their unheated counterparts. It was postulated that when the protein globin detached itself from nitrosylmyoglobin upon

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thermal processing, the protoheme complexed with a second NO molecule. Yet, the possibility that NO may bind with other constituents of the hemoprotein to account for the "consumption" of labeled ^{15}NO was not considered. In a series of papers by Bonnett and coworkers, these authors reported that the reaction of sodium nitrite with hemoproteins under mildly acidic conditions can occur at the ferrous ion to give the nitrosylheme pigment (Bonnett *et al.*, 1980b), in the porphyrin ligand (Bonnett *et al.*, 1978, 1980a) or in the protein (Bonnett *et al.*, 1979).

Andersen *et al.* (1990), using labeled ^{15}NO in aqueous model systems, reported that only one NO molecule coordinated to Fe(II) of purified equine myoglobin. However, heating the reaction mixture to temperatures in excess of 70 °C increased the ^{15}NO content of the product without resulting in any significant changes in the visible absorption spectrum (λ_{max}, ϵ_{max}: 546 nm, 12 500 L mol^{-1} cm^{-1}; 578 nm, 11 200 L mol^{-1} cm^{-1}). The authors stated that the increased labeling probably indicated nitrosation of the globin chain, since such a reaction is expected to have little influence on the spectral properties of the iron(II) chromophore, in contrast to the coordination of two NO molecules directly to iron(II). In fact, as a result of prolonged heating, Andersen *et al.* (1990) found that it was possible to increase the labeling of ^{15}NO to a level higher than the 2:1 stoichiometry required for dinitrosylation, thereby suggesting that further nitrosation reactions with the globin are of importance.

Wayland and Olson (1973, 1974) showed that toluene solutions of tetraphenylporphyrin (TPP^{2-}) iron(III) chloride, a high-spin Fe(III) complex ($S = 5/2$), reacted reversibly with NO to form a diamagnetic $Fe^{II}TPP(Cl^-)(NO^+)$ complex, but in the presence of methanol, $Fe^{III}TPPCl$ reacted with excess NO to produce $Fe^{II}TPP(NO)$. $Fe^{II}TPP(NO)$ was characterized as being a low-spin ($S = 1/2$) ferrous porphyrin complex with the odd electron in a molecular orbital with Fe (d_{z^2}) and NO (σ_N) character. The infrared (IR) spectrum of $Fe^{II}TPP(NO)$ had a strong ν_{NO} at 1700 cm^{-1}, and the EPR spectrum in a toluene glass showed three g values with ^{14}N hyperfine splitting of NO characteristic of a pentacoordinate heme complex. Additionally, $Fe^{II}TPP(NO)$ formed 1:1 adducts with nitrogenous donors such as pyridine and piperidine, but no adducts of phosphorus donors were observed. In a toluene glass, a rhombic g tensor was noted as well as ^{14}N hyperfine splitting in the g_3 region from both NO and the ring N donor. These spectra are typical of a hexacoordinate species and are similar to those of NO complexes of Fe(II) myoglobin and hemoglobin in which iron is axially coordinated by a NO moiety and a histidine residue. Wayland and Olson (1974) also reported that, in the presence of excess NO, $Fe^{II}TPP(NO)_2$ may form. Two IR N−O stretching frequencies were observed at 1870 and 1690 cm^{-1} [*i.e.* $Fe^{II}TPP(NO^-)(NO^+)$]. The band at 1870 cm^{-1} is in the range expected of a linear $Fe^{II}NO^+$ moiety, and the 1690 cm^{-1} band is consistent with a bent $Fe^{II}NO^-$ fragment. Moreover, the intensity of the 1870 cm^{-1} band decreased and eventually disappeared upon evacuation of excess NO from the system.

Bonnett *et al.* (1980b) attempted to characterize the pigment of cooked cured meat, nitrosylprotoheme, from the reaction of NO with protoheme dimethyl ester. A strong IR band at *ca.* 1660 cm^{-1} was diagnostic of the stretching mode of a bent Fe−NO moiety and a pentacoordinate complex. Scheidt and Frisse (1975) had reported a γ_{max} of 1670 cm^{-1} and an Fe−N−O angle of 149.2° for nitrosyltetraphenylheme. The EPR spectrum of nitrosylprotoheme 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.* $g_1 = 2.102$, $g_2 = 2.064$, $g_3 = 2.010$). Nitrosylprotoheme extracted with acetone from thermally processed nitrite-cured meat also had an EPR signal expected of a pentacoordinate nitrosylheme complex. When the pigment was dissolved in pyridine (Pyr), the EPR signal changed and the resulting spectrum was characteristic of a hexacoordinate system with a g_3 value of 1.98. Moreover, g_1, g_2, and the hyperfine structure at g_3 were no longer resolved, unlike the hexacoordinate $Fe^{II}TPP(NO)(Pyr)$ system reported by Wayland and Olson (1974). While these extraction experiments provided confirmation of the general chemical nature of the chromophore of cured meat, they did not reveal the coordination sphere *in situ*.

Bonnett *et al.* (1980b) examined various nitrite-cured meat samples directly, as opposed to their extracts, using EPR spectroscopy. They observed a signal with hyperfine splitting characteristic of a pentacoordinate nitrosylheme complex. These authors suggested that the color of cooked cured meat was due to nitrosylprotoheme which was physically trapped within a matrix of denatured globin, thereby offering some protection to the pigment against aerial oxidation. Killday *et al.* (1988) isolated and characterized an extract of the CCMP from thermally processed corned beef by IR and visible spectroscopies and thin-layer chromatography. They found that the pigment was a mononitrosyl ferrous protoporphyrin and further identified the pigment by fast atom bombardment mass spectrometry as having only one NO moiety. Burge and Smith (1992) prepared the CCMP from hemin and $Na^{15}NO_2$ according to the method of Shahidi *et al.* (1984) to model the pigment of nitrite-cured ham. They assumed that CCMP contained two NO groups, because the pigment was then referred to as dinitrosyl ferrohemochrome. Their ^{15}N NMR studies revealed that the preformed pigment contained only one NO group, and the IR spectrum (Nujol mull) exhibited a single NO symmetric stretch at 1801 cm^{-1}. Because uncertainty in visible absorption spectra existed, Burge and Smith (1992) never ruled out the possibility of initially having two NO groups in the molecule. Recently, Jankiewicz *et al.* (1994) provided further support for the view that the CCMP is nitrosylprotoheme from visible and IR absorption studies.

We have reported the preparation of CCMP directly from bovine red blood cells (Shahidi and Pegg, 1991a) or through a hemin intermediate (Shahidi *et al.*, 1985) in the presence of reductants and a nitrosating agent. This pigment, preformed outside the meat matrix, is used as part of a composite mixture for nitrite-free curing of meats, but its chemical nature has not been adequately elucidated. The purpose of this study was to investigate the chemical nature of the preformed pigment, itself, after its stabilization in carbohydrate-based wall materials by encapsulation and after its application to meat systems using EPR spectroscopy.

MATERIALS AND METHODS

Materials. All solvents used were of a high-purity grade and were nitrogen-flushed before use. Acetic acid and sodium hydroxide, used for preparation of the acetate buffer, sodium carbonate, acetone, benzene, and Whatman No. 3 filter paper

were obtained from Fisher Scientific Co. (Montréal, PQ). Hemin, sodium ascorbate, and pyridine were purchased from Sigma Chemical Co. (St. Louis, MO). Although any source of hemin is suitable, the bovine variety was used in all experiments because it is readily available as a byproduct in the production of plasma, an ingredient used in meat processing applications. Ultra-high-purity N_2 and NO were supplied by Canadian Liquid Air (St. John's, NF). Sodium tripolyphosphate (STPP) and sodium acid pyrophosphate (SAPP) were acquired from Albright and Wilson Americas (Toronto, ON). N-LOK, a modified starch used for encapsulating the preformed CCMP, was procured from National Starch and Chemical Corp. (Bridgewater, NJ), and ascorbyl palmitate (AP) was obtained from Hoffmann-La Roche Ltd. (Mississauga, ON). The AtmosBag and 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Quartz EPR sample tubes (types 700-PQ and 702-PQ) and an insertion Dewar flask were supplied by Wilmad Glass Company, Inc. (Buena, NJ).

Experimental Procedures. *Preparation of CCMP from Hemin and Nitric Oxide.* The CCMP was prepared from bovine hemin and NO. The method of Shahidi *et al.* (1985) was used with slight modifications. Briefly, 600 mg of hemin [chloroprotoporphyrin IX iron(III)] was dissolved in 100 mL of a 0.04 M Na_2CO_3 solution. The heme reagent was stored in the dark for 30 min before use and shaken periodically to ensure complete dissolution of the hemin. Sodium tripolyphosphate and sodium ascorbate were added to 50 mL centrifuge tubes at levels of 150 and 400 mg, respectively. To each tube was added 9 mL of a 0.2 M sodium acetate buffer, pH 6.5. The contents were mixed using a Vortex Genie 2 (Fisher) to ensure complete dissolution of the solids, and 1 mL aliquots of the heme reagent were added to each tube. Tubes were then transferred to an AtmosBag which was flushed twice with N_2 to remove oxygen from the headspace gases. Under a blanket of N_2, NO was bubbled into each tube for *ca.* 45 s. Tubes containing the CCMP were capped, removed from the AtmosBag, and stored in the dark until used. The CCMP was used within 3 days of its synthesis.

Lyophilization of CCMP and Preparation of the Powdered Cooked Cured-Meat Pigment (PCCMP). Pigment samples were centrifuged using an IEC Centra MP4 centrifuge (Fisher) at 2900*g* for 5 min; CCMP was recovered as a precipitate from the mixture. In a fumehood, tubes were opened, excess NO was released, and supernatant was discarded. Washing of CCMP, to ensure elimination of any traces of nitrite or nitrous acid from the system, was achieved by the addition of 3 mL of a 2% (w/v) sodium ascorbate solution to each tube. Samples were capped, vortexed for 30 s, and centrifuged at 2900*g* for 3 min, and supernatant was discarded. Pigment was either transferred to a freeze-drying flask, frozen using liquid N_2 (77 K), and then lyophilized by a Labconco 5 freeze-dryer (Labconco Corp., Kansas City, MO) for 12 h or used for production of PCCMP.

The PCCMP was prepared as described by Shahidi and Pegg (1991b) with slight modifications. Briefly, a mixture of CCMP and starch-based wall materials, which served as encapsulating agents, was formulated. The encapsulating agents consisted of N-LOK, STPP, SAPP, and AP (95:2:2:1 w/w/w/w) and were used at a 1.5% (w/w) payload (*i.e.* the ratio of the pigment to wall materials). When the system was prepared, wall materials were dissolved or dispersed in distilled water. The pigment was then introduced to the mixture together with sodium ascorbate at a CCMP/ascorbate ratio of 1:2 (w/w). The mixture was diluted with water to *ca.* 3.5% (w/w) and thoroughly stirred to ensure uniform dispersion of CCMP throughout. The vessel containing the mixture was covered with Parafilm and aluminum foil to minimize exposure of CCMP to oxygen and light. The mixture was then dried by means of a mini spray dryer (Model 190, Büchi Laboratory-Techniques Limited, Flawil, Switzerland). Nitrogen was used as the spray flow gas to minimize contact between CCMP and oxygen. Optimized spray-drying conditions were as follows: inlet and outlet temperatures, 150 and 98 °C, respectively; feed flow, 5.5 mL/min; N_2 pressure, 375 kPa(g). The PCCMP was transferred to amber-colored vials and stored under refrigeration conditions until used.

Preparation of Meat Systems and Application of CCMP. Beef round steak was obtained from a local supermarket, and all subcutaneous fat was trimmed. The meat was comminuted twice using a Braun meat mincer (Model KGZ 3, Braun Canada Ltd., Mississauga, ON) with an 8.0 mm and then with a 3.0 mm plate.

Ground meat was mixed with 20% by weight of distilled water and 550 ppm of sodium ascorbate. Sodium nitrite and preformed CCMP were added directly to meat samples at levels of 200 and 30 ppm, respectively. Beef slurries were thoroughly homogenized and then cooked at 85 ± 2 °C in a thermostated water bath for 40 min, with occasional stirring with a glass rod. After cooling to room temperature, meat samples were covered with Parafilm and stored in a refrigerator at 4 °C until used. Nitrosylheme pigments were extracted from treated cooked meat systems according to the method of Hornsey (1956) but at a much smaller scale.

EPR Experiments. Approximately 10 mL of acetone (or pyridine) was added to CCMP, after the supernatant wash was discarded, or to lyophilized pigment. A deep red solution formed and was centrifuged at 2900*g* for 3 min to ensure that no fines remained in the extract. Using a Pasteur pipet, a 1.5 mL aliquot of the supernatant was transferred to a reservoir connected to an EPR tube (i.d. 2.16 mm), as depicted in Figure 2. The pigment extract was frozen (77 K), and the glassware was attached to a vacuum line. The system was then placed under vacuum. Pigment samples were subjected to three freeze, pump, and thaw cycles in which N_2 gas was introduced into the line between pumping cycles. After the final pump, both stopcocks A and B were closed and the glassware was removed from the line (see Figure 2). Eventually the contents in the reservoir were thawed and then transferred to the evacuated EPR tube by opening stopcock B. The pigment extract in the tube was frozen in liquid N_2, forming an acetone glass. All spectra were recorded at 77 K by immersing the tubes in an insertion Dewar flask filled with liquid N_2. The flask was placed in the center of a resonant cavity of an EPR spectrometer. EPR spectra were recorded using a Bruker ESP-300 X-band spectrometer equipped with a rectangular ER 4102ST cavity at *ca.* 9.46 GHz. Field calibrations were made with DPPH dissolved in benzene (g = 2.0036). When spectra were obtained, the modulation amplitude and gain were varied depending upon the concentration of the paramagnetic compound in the tube. Generally, experiment conditions were as follows: modulation frequency, 3.13 kHz; field modulation intensity, 0.25−0.45 mT; gain, 4.0×10^4; time constant, 40.96 ms; microwave power, 2 mW.

In the case of PCCMP, a slurry was formed by the addition of a small volume of distilled water to the powder. Approximately 10 mL of acetone was added, and the mixture was vortexed and then filtered by gravity through Whatman No. 3 filter paper. Using a Pasteur pipet, a 1.5 mL aliquot of the extract was transferred to a reservoir connected to an EPR tube as described above.

In other experiments, moist nitrite-cured and CCMP-treated thermally processed meat samples were transferred to EPR tubes (i.d. 4.2 mm) directly, frozen (77 K), put under vacuum, and then examined as plugs (*ca.* 20 mm in height). Finally, acetone extracts of pigment recovered from nitrite-cured and CCMP-treated cooked beef systems were transferred to the reservoir and attached to the vacuum line as described above. Their EPR spectra were then recorded.

RESULTS AND DISCUSSION

The EPR spectral parameters of CCMP in an acetone glass are shown in Figure 3 and are compared to those of nitrosylprotoheme dimethyl ester investigated by Bonnett *et al.* (1980b) and to $Fe^{II}TPP(NO)$ reported by Wayland and Olson (1974). In all cases, the EPR parameters of these systems were similar and possessed characteristics recognized as those of a pentacoordinate nitrosylheme system. EPR spectra of the nitrosylpro-

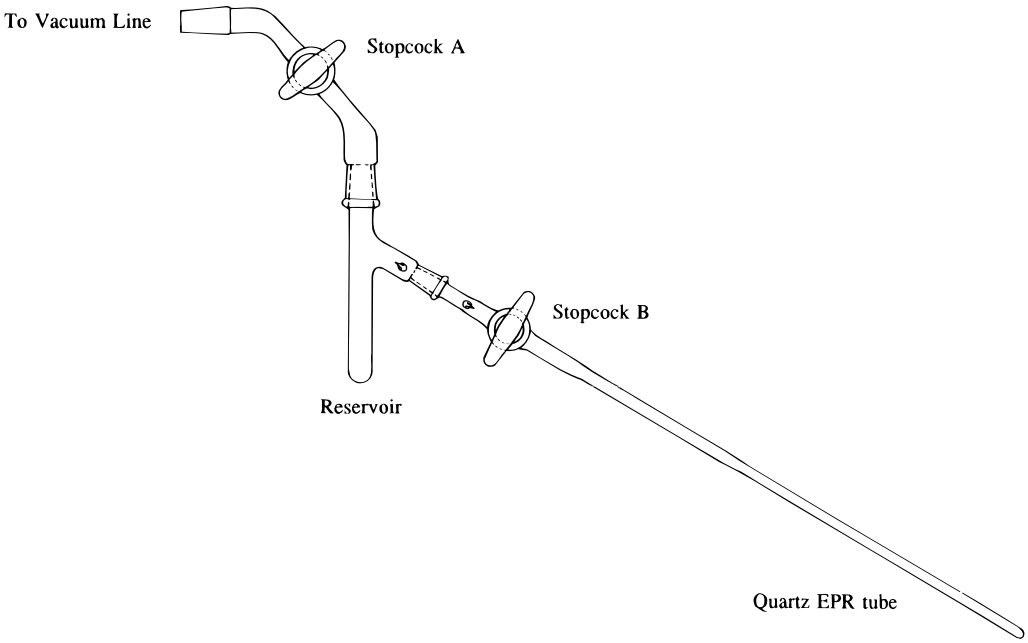

Figure 2. Design of apparatus used for preparing oxygen-free samples in EPR tubes, for either solvent glasses or solids.

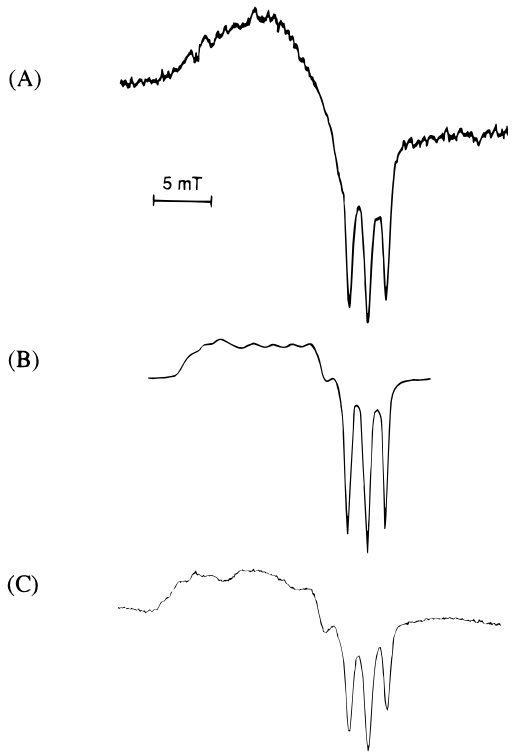

Figure 3. Characteristic EPR spectra of pentacoordinate nitrosylheme complexes: (A) nitrosylprotoheme dimethyl ester in acetone at 77 K; (B) $Fe^{II}TPP(NO)$ in toluene at 120 K; (C) preformed CCMP in acetone at 77 K.

toheme dimethyl ester, $Fe^{II}TPP(NO)$, and the preformed CCMP in a solvent glass (*i.e.* one not providing a nitrogenous ligand) showed g_1, g_2, and g_3 values characteristic of a rhombic g tensor due to the anisotropic nature of the system. Bonnett *et al.* (1980b) reported a solvent effect on g_3, noting that less polar aromatic solvents gave slightly lower values than aprotic polar solvents. EPR spectra also exhibited hyperfine splitting in the g_3 region from NO ligation. Hyperfine coupling by the single nitrogenous ligand ($I = 1$) produced a signal split into a triplet of equal line intensities (Figure 3C). According to Wayland and Olson (1974), this ^{14}N hyperfine coupling provides evidence for placing the odd electron in a molecular orbital with substantial iron d_{z^2} character. The odd electron of NO becomes highly delocalized onto the iron atom. Neto *et al.* (1988) investigated the appearance of pentacoordinate symmetry of myoglobin from various species after treatment with NO in both solution and powder form by EPR spectroscopy. They noted a significant temperature effect with regard to resolution of EPR spectra (289−112 K). This is routine, particularly when changes in the physical state of a paramagnetic sample (*i.e.* solution to solvent glass) are examined, but there was virtually no change in the EPR spectra at the lower temperatures examined (132 and 112 K), perhaps only a slight improvement in the signal-to-noise ratio. Although the EPR spectrum of $Fe^{II}TPP(NO)$ reported by Wayland and Olson (1974) was measured at 120 K while those of nitrosylprotoheme dimethyl ester and CCMP were measured at 77 K, the temperature effect at this range should not have significantly influenced the EPR signal with respect to the pentacoordinate symmetry of the molecule and the g values obtained.

Dissolution of CCMP in pyridine (*i.e.* a solvent providing a nitrogenous ligand) resulted in a dramatic change in its EPR spectrum. There was a difference in g_1 and g_2 values, and the hyperfine structure at g_3 was no longer resolved. In fact, the resulting signal from the solvent glass was similar to that observed for nitrosylprotoheme dimethyl ester in piperidine reported by Bonnett *et al.* (1980b). Loss of resolution of the hyperfine splitting in the EPR spectrum indicated that the N of pyridine occupied the sixth coordinate position on the central ferrous ion of the nitrosylheme complex. Nitrosylhemoglobin and nitrosylmyoglobin also showed this type of EPR spectrum (Maxwell and Caughey, 1976; Morse and Chan, 1980). A comparison between the EPR spectra of CCMP in an acetone glass, showing evidence of pentacoordination, and a pyridine glass, showing characteristics of hexacoordination, is provided in Figure 4.

The EPR spectrum of CCMP in an acetone glass was also compared to those spectra of lyophilized CCMP and PCCMP (Figure 5). Again, in all cases, spectra were

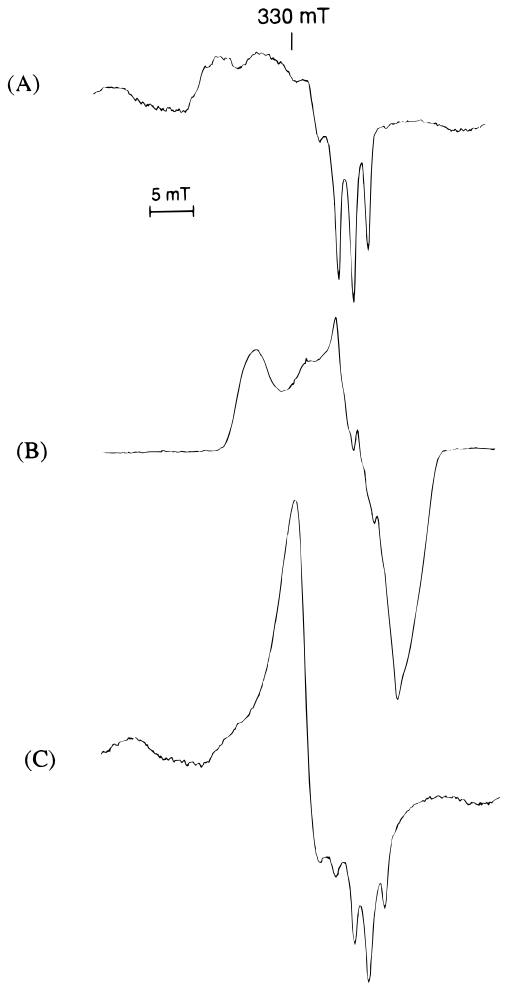

Figure 4. EPR spectra of solvent glasses at 77 K showing either penta- or hexacoordination: (A) preformed CCMP in acetone (*i.e.* after centrifugation to remove fines); (B) CCMP in pyridine; (C) CCMP in acetone (*i.e.* no centrifugation, fines present).

similar and EPR parameters of these systems possessed characteristics recognized as those of a pentacoordinate nitrosylheme system as described above. However, it should be noted that in preliminary investigations the EPR spectrum of CCMP in an acetone glass was somewhat different. The characteristic ^{14}N hyperfine splitting was evident in the g_3 region with an a_3 of 1.71 mT, but a small shoulder with two other splittings of low intensity and a single line with a prominent positive feature in the $g = ca.$ 2 region were also observed (Figure 4C). The existence of dinitrosylprotoheme complexes has been reported in the literature (Wayland and Olson, 1974; Olson *et al.*, 1982; Lançon and Kadish, 1983), but such complexes do not correspond with the observed EPR spectrum of CCMP. First, Wayland and Olson (1974) reported that FeTPP(NO) can reversibly coordinate with a second NO moiety when FeTPP(NO) is exposed to a positive pressure of NO. No EPR transitions were detected in the toluene glass medium (77 K) of such a system [*i.e.* $FeTPP(NO)_2$], which is consistent with the formation of an even-electron species. Solution magnetic susceptibility measurements, as a function of temperature, also concurred with this finding. In other words, $FeTPP(NO)_2$ is diamagnetic. Furthermore, if a second NO coordinated to nitrosylprotoheme and the free electrons in the π^* orbitals of each NO molecule occupied separate orbitals (*i.e.* no pairing in a d_{z^2} orbital), the hyperfine structure at g_3

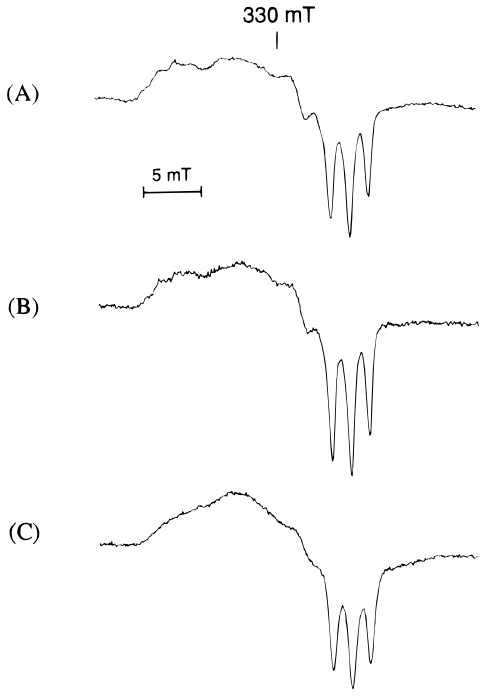

Figure 5. Characteristic EPR spectra of pentacoordinate nitrosylprotoheme in acetone at 77K: (A) CCMP; (B) lyophilized CCMP; (C) PCCMP.

should reveal a quintet (for $I = 1$ and $n = 2$) with line intensities in the ratio 1:2:3:2:1. Again, this was not observed. It is conceivable that the system was behaving as a triplet state, but when the extract was centrifuged to remove any fines and the EPR spectrum then rerun, these extra bands disappeared and only hyperfine splitting by a single ^{14}N nuclei was evident (Figure 4A). The hyperfine action in the EPR spectrum of the sample that had not been centrifuged was believed to be a combination of the EPR of the solid-state and dissolved sample. The intensity of the line at $g = 2.03$ also decreased. Interestingly enough, Bonnett *et al.* (1980b) also observed this signal in their EPR spectra of nitrite-cured meat samples. These authors noted that the species responsible for the signal was only evident in meat and was not extracted from the tissue by acetone. They could not explain the signal but suggested that it may be a radical arising from components of bound redox systems or from products of various oxidation pathways. Perhaps the meat matrix behaves as a diluter to spread out each signal arising from nitrosylprotoheme, but the line signal at $g = 2.03$ arises from an incomplete segregation of signals. Bonnett *et al.* (1980b) did suggest that thermal processing of nitrite-cured meat effectively breaks the iron−imidazole bond, thereby leaving the pentacoordinate nitrosylprotoheme physically trapped in a matrix of denatured globin. If one compares the EPR spectrum of uncooked bacon to that of its pasteurized counterpart, the presence of the hexacoordinate signal of nitrosylmyoglobin and the pentacoordinate signal of nitrosylprotoheme, resulting from some denaturation, is evident. However, after thermal processing, only the EPR signal of a pentacoordinate system is observed, and the intensity of the $g = 2.03$ signal decreases compared to that of its unpasteurized analogue. In other words, EPR signals arising from the nitrite-cured meat system might be occurring by a similar mechanism responsible for the signals occurring in the acetone extract of CCMP that had not been centrifuged.

Table 1. Parameters of EPR Spectra of Nitrite-Cured and CCMP-Treated Cooked Beef Samples and Their Acetone Extracts at 77 K

no.	system[a]	g_1	g_2	g_3	a_3 (mT)
1	nitrite-cured beef	2.112	2.070	2.009	1.69
2	extract of (1)	2.110	2.070	2.005	1.68
3	CCMP-treated beef	2.113	2.072	2.009	1.69
4	extract of (3)	2.107	2.061	2.006	1.70
5	extract of nitrosylprotoheme from ham[b]	2.102	2.064	2.008	1.66

[a] All beef samples contained 20% (w/w) distilled water and 550 ppm of sodium ascorbate. [b] EPR data reported by Bonnett *et al.* (1980b).

Finally, the EPR spectrum of CCMP was compared to those of nitrite-cured and CCMP-treated cooked beef systems *in situ* as well as their respective acetone extracts. Tarladgis (1962) reported that no EPR signal was obtainable from acetone extracts of cured meat. He proposed that the CCMP was dinitrosylprotoheme and therefore diamagnetic. Lack of an EPR spectrum, however, is no proof of dinitrosyl ligation. In this study, narrow EPR tubes and cryogenic temperatures (77 K) were employed to compensate for the high dielectric loss of acetone. Qualitatively, EPR parameters observed for these nitrosylhemes were similar in all cases, but no attempt was made to quantitate the extracts. The positions of the g_1 and g_2 signals (approximate) and the g_3 signal as well as the associated hyperfine splitting, a_3, are provided in Table 1. As Bonnett *et al.* (1980b) noted, the observed triplet in nitrite-cured meat systems, due to the hyperfine splitting of NO in the g_3 region, suggests that the iron−imidazole bond is effectively cleaved from nitrosylmyoglobin during thermal processing. If this was not the case, a second nitrogenous ligand (*i.e.* from imidazole) bound to the iron would provide EPR characteristics of a hexacoordinate system with a shift in the g_3 value. The spectra obtained, however, clearly indicate that the pigment of cooked cured meat is nitrosylprotoheme.

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