Nitrosation and Nitrosylation of Haemoproteins and Related Compounds. Part 4.[1] Pentaco-ordinate Nitrosylprotohaem as the Pigment of Cooked Cured Meat. Direct Evidence from E.S.R. Spectroscopy

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The pigment of cured meat, nitrosylprotohaem, was obtained by synthesis and by extraction from nitrosylhaemoglobin and from cured meats. The e.s.r. spectrum of nitrosylprotohaem was observed in two types of solvent; those which do, and those which do not, provide an effective nitrogenous sixth ligand: spectra characteristic of hexa- and penta-co-ordination are observed, respectively. Similar e.s.r. spectra were detected in the direct examination of a variety of cured meats, and are interpreted in terms of pigment structure.

THE reaction of sodium nitrite with haemoproteins under mildly acidic conditions can occur at the metal (to give the nitrosylhaem), in the porphyrin ligand,[1,2] and in the protein.[3] In Part 1 [2] of this series we described the characterisation of some nitrosylhaems, and here extend this theme with particular reference to the application of e.s.r. spectroscopy to investigate the nature of such pigments in cured meats.

The formation of the rather stable pink colour of cured meats is associated with the use of sodium nitrite in the curing liquor. A characteristic taste also results, but, most importantly, nitrite confers resistance to the growth of toxin-producing bacteria (*Clostridium botulinum*). The possibility of the concomitant formation of *N*-nitrosamines, some of which are known to be carcinogenic, has led to an upsurge of interest in this area in recent years.[4]

In 1901, when the primary structure of protein was just becoming apparent, and the structure of haem was still unknown, Haldane [5] attributed the pink colour of cured meat to a nitric oxide–haemoprotein complex. Haldane's observation was essentially that the visible absorption bands of extracts of cured meat resembled those of the reaction product from nitric oxide and haemoglobin. In recent years the structures and spectroscopic properties of nitrosylhaems [6-8] and of nitrosylhaemoproteins [9,10] have been investigated, although relatively little attention has been accorded to the pigments of cured meat as such.

RESULTS AND DISCUSSION

The substance which is generally regarded as the pigment of cured meat, nitrosylprotohaem (1), has recently been characterised as its dimethyl ester (2), which has been obtained by the reaction of nitric oxide with protohaem dimethyl ester [7] and with methoxoiron(III)–protoporphyrin dimethyl ester.[2] The corresponding diacid (1) may be prepared in an analogous way as in equation (E1) and has spectroscopic properties

$$ClFe^{III}P + 2\,NO \xrightarrow{MeOH} ONFe^{II}P + MeONO + HCl \quad (E1)$$

P = porphyrin dianion

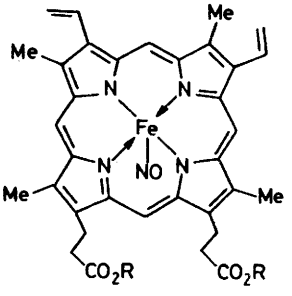
which are very similar to those of the dimethyl ester, as shown in Table 1. The strong i.r. band at *ca.* 1 660 cm^{-1} is consistent with the presence of a bent Fe–NO moiety [8] and a pentaco-ordinate complex.[7] The visible spectra are also similar to one another (and resemble that of a thin film of a cured meat, see Experimental section). However, observation of visible spectra is complicated by the fairly ready aerial oxidation of the nitrosylhaems, a factor which becomes especially important at the dilution needed to observe the Soret band. We have, therefore, turned to e.s.r. spectroscopy, where more concentrated solutions can be routinely examined in closed, oxygen-free systems at low temperatures.

TABLE 1

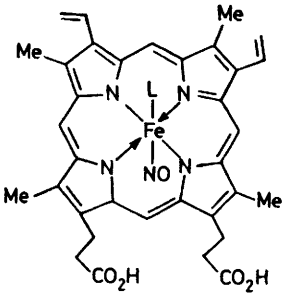
Spectroscopic properties of nitrosylprotohaem (1) and its dimethyl ester (2)

	(1)	(2) (ref. 2)
ν_{Fe-NO}(CsI)/cm^{-1}	1659	1660
$\lambda_{max.}$ ($CHCl_3$)/nm	478, 543, 567	(398),* 487, 546, 570
E.s.r. (acetone, 77 K) g_3	2.009	2.010
a_3/mT	1.62	1.63

* Maxwell and Caughey [7] quote $\lambda_{max.}$ ($ClCH_2CH_2Cl$) 418, 542, and 566 nm; we believe that the difference in Soret position is due to aerial oxidation during dilution of our sample (see text).



(1) R = H
(2) R = Me



(3) L = nitrogenous ligand

The e.s.r. spectrum of the nitrosylhaem (1) in an acetone glass resembled that of the diester (2), possessing a characteristic appearance (Figure 1) with parameters which are now recognised [7,10] as those of a pentaco-

ordinate nitrosylhaem system.* The triplet nature of the high-field feature, due to hyperfine splitting by the single axial nitrogenous ligand (*i.e.* NO), is particularly striking.

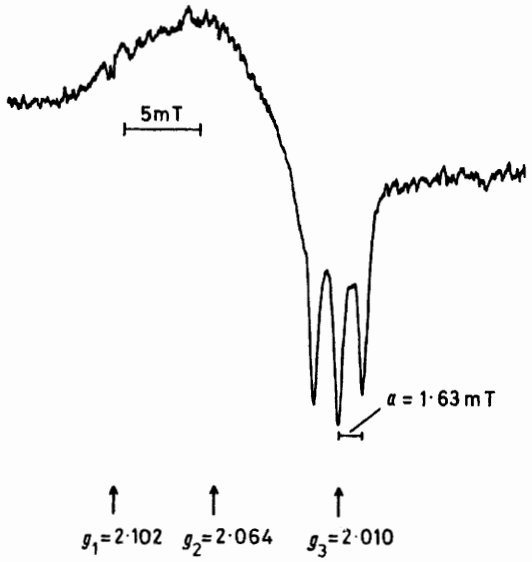


FIGURE 1 Typical e.s.r. spectrum of a pentaco-ordinate nitrosylhaem: synthetic nitrosylprotohaem dimethyl ester in acetone at 77 K

For a sample kept in the e.s.r. tube (*i.e.* a sealed, oxygen-free system) at room temperature in the dark the signal remained virtually unchanged over a two-year period, reflecting the considerable thermodynamic stability [11] of the nitrosylhaems. Similar spectra were observed

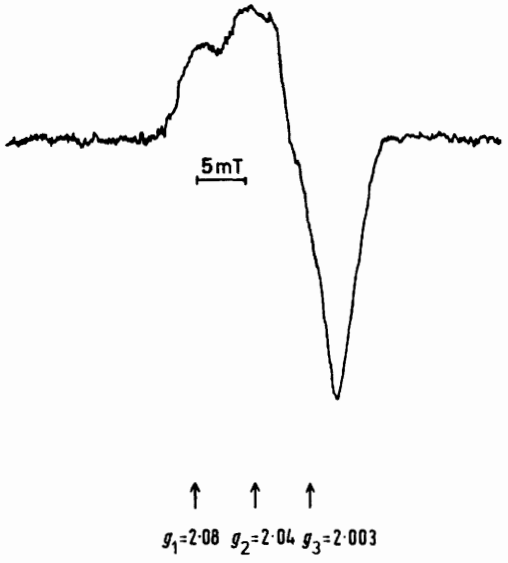


FIGURE 2 Typical e.s.r. spectrum of hexaco-ordinate nitrosylhaem, the sixth ligand being nitrogenous. Nitrosylprotohaem in piperidine at 93 K

for a number of solvents which did not provide a nitrogeneous ligand (Experimental section): a small solvent effect on g_3 was detected, less polar (aromatic) solvents giving lower values than aprotic polar solvents. However, when the solvent provided a second nitrogenous ligand there was a characteristic change in g_1 and g_2 values (compared with those observed in polar solvents such as acetone, see Table 2) and the hyperfine structure at g_3 was no longer resolved. The resulting spectrum (*e.g.* nitrosylprotohaem in piperidine, Figure 2) with the minimum of the broad high-field feature located at an effective g value of 1.98, is characteristic of the hexaco-ordinate system (**3**) [nitrosylmyoglobin (**3**, L = imidazole of globin) also shows this type of e.s.r. spectrum [12]]. With benzene–diethylamine both sets of high-field signal were present, and persisted even when diethylamine alone was employed as solvent. This difference in behaviour compared to piperidine is attributed to the more stringent steric requirements of the diethylamine ligand.

Samples of nitrosylprotohaem with the same e.s.r. characteristics as those shown in Figure 1 can be obtained from solutions of nitrosylhaemoglobin by treatment with acetone. The protohaem system cannot be extracted from haemoglobin under the same conditions, and we attribute the relatively easy removal of nitrosylprotohaem from nitrosylhaemoglobin to the structural *trans*-effect of the nitrosyl ligand, which results in the weakening and lengthening of the bond between iron and the co-ordinated nitrogen atom of the imidazole ring. Such lengthening of the bond *trans* to the nitrosyl function has been observed in analogous tetraphenylhaem complexes [8] and in nitrosylpenta-amminecobalt(III) dichloride.[13] Similarly, nitrosylprotohaem can be extracted from cured meat samples with acetone,[14] and the extract shows the e.s.r. spectrum (as in Figure 1) expected for a pentaco-ordinate nitrosylhaem.† A variety of cured meats (see Experimental section) has been extracted in this way with the same general result. The extraction is preferably made in the presence of a reducing agent (as suggested by Hornsey [14]) to avoid oxidation of the small quantities of nitrosylhaem involved. In parallel with the results with the pure haemoproteins, protohaem is not effectively removed from fresh meat samples under these conditions.

TABLE 2

E.s.r. parameters for nitrosylhaems

	g_1	g_2	g_3	a_3/mT
Nitrosylprotohaem dimethyl ester (synthetic, in acetone)	2.102	2.064	2.009	1.63
Nitrosylprotohaem (extracted from ham, in acetone)	2.102	2.064	2.008	1.66
Nitrosylprotohaem (in piperidine)	2.08	2.04	2.003	

While these extraction experiments provide confirmation of the general chemical nature of the chromophore of cured meat, they do not reveal the details of the co-ordination sphere *in situ*. We find that it is possible to obtain information on this by the direct examination of cured meat samples by e.s.r. spectroscopy. This appears to be the first report of a characteristic e.s.r.

* Throughout this paper penta- and hexa-co-ordination refer to nitrogenous ligands.

† We are aware of one earlier attempt to observe the e.s.r. spectra of acetone extracts of cured meats, but no signal was detected, (B. G. Tarladgis, *J. Sci. Food Agric.*, 1962, **13**, 485).

signal in cured meat preparations. The cured meat samples were examined at low temperature, and similar spectra were obtained with desiccated and with moist samples. Various nitrite-treated meat preparations have been examined, and four spectra from cooked or pasteurised cured meats are illustrated in Figure 3: certain parameters derived from the spectra of these and other samples are given in Table 3.

Although the spectra differ in detail, they show two features in common in the $g = ca.$ 2 region. Firstly there is a single line (referred to here as r) of variable relative intensity at g_r *ca.* 2.03. The origin of this signal is not known. The species responsible for it is not extracted from the tissue by acetone: the spectrum of the pale residue after extraction retains this signal as the predominant feature. This radical does not appear to be the same as another that we have detected (g = 2.004) in vacuum-dried fresh meat. These signals may arise from components of bound redox systems, or they

TABLE 3

Parameters of sharp high-field feature (g_3) and second component (g_r) in e.s.r. spectra of cured meat samples

	Sample weight/g	g_3	a_3/mT	g_r	Figure
Corned beef (cooked, tinned)	0.089	2.011	1.65	2.033	3(*a*)
Ham (pasteurised, sliced, plastic sachet)	0.089	2.012	1.63	2.032	3(*b*)
Bacon (pasteurised, sliced, plastic sachet)	0.094	2.009	1.63	2.032	3(*c*)
Lap cheong [a]	0.087	2.009	1.67	2.032	3(*d*)
Luncheon meat (beef, tinned)	*ca.* 0.09	2.010	1.62	2.033	
Paté (pork and liver, tinned)	*ca.* 0.09	2.010	1.65	2.033	
Bacon (uncooked)	0.091	2.012 [1.990] [b]	1.65	2.032	4(*a*)
Previous sample, after 30 min at 50°C		2.010	1.65	2.033	4(*b*)

[a] A sausage of oriental origin, but purchased in London. [b] Effective g value (g_e) at the highest field minimum in the spectrum. The corresponding value for (piperidine)nitrosylprotohaem is 1.982 (Figure 2).

may be due to products of different auto-oxidation pathways. Clearly they deserve further study.

Secondly, those cured meats which have been subjected to heat treatment show an e.s.r. signal characteristic of the pentaco-ordinate nitrosylhaem. The position of the g_3 signal (2.009—2.012) and the associated hyperfine splitting a_3 (1.62—1.67 mT) are given in Table 3: the g_1 and g_2 values cannot be experimentally determined with accuracy, but the observed spectra are consistent with the g_1 and g_2 values observed for nitrosylprotohaem in acetone (Table 2). Thus in the cooked and pasteurised cured meat samples examined, the e.s.r. spectra suggest that the iron–imidazole bond is effectively broken. We therefore ascribe the colour of cooked cured meat to the pentaco-ordinate nitrosylprotohaem, which we suppose to be physically trapped in a matrix of denatured globin, a situation shown diagrammatically in the Scheme. In view of the aerial

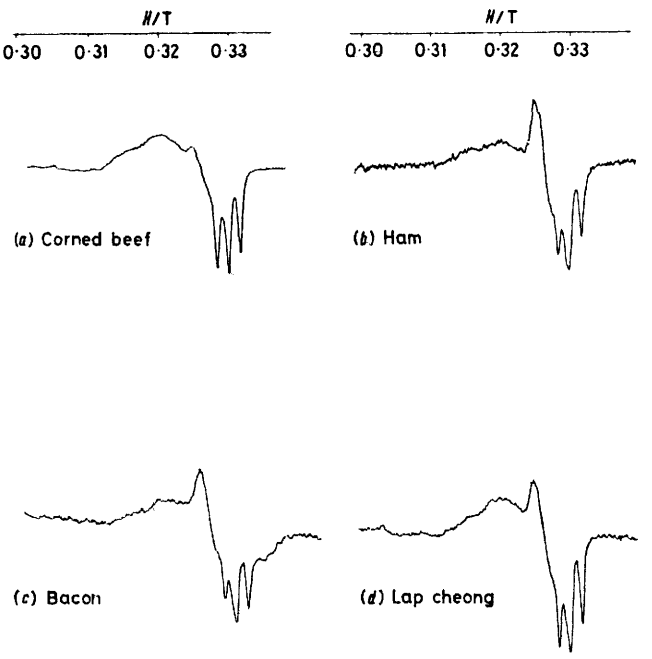
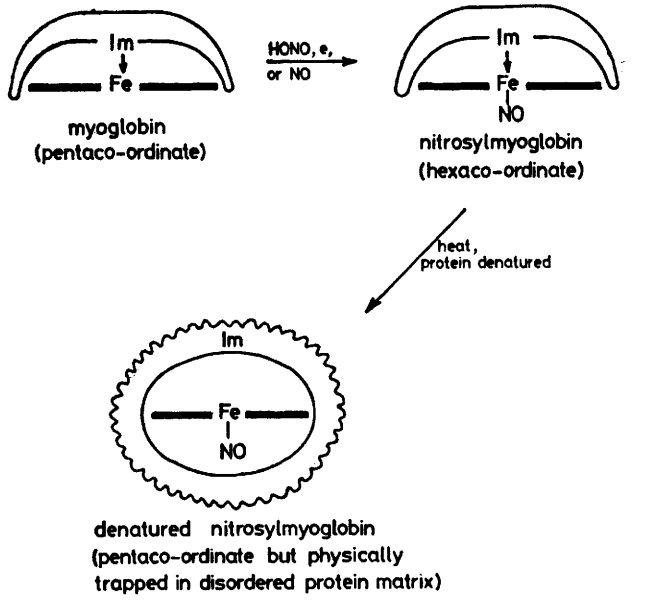


FIGURE 3 E.s.r. spectra of samples of cured meats at 93 K. The spectra were measured under the same conditions, except that the signal response for samples (*b*), (*c*), and (*d*) was twice that for sample (*a*). The unidentified radical gives a line with a prominent positive feature at about 0.325 T (g_r = 2.03, see text)

oxidation referred to earlier, we also suppose that this situation affords some protection of the nitrosylhaem towards aerial oxidation, possibly reinforced by the presence of natural or added reductants.

Support for this view comes from the e.s.r. spectrum of bacon which has not been subjected to the heat treatment



SCHEME Diagrammatic representation of chemical modifications of myoglobin during the curing process. If the cure proceeds without heating, denaturation will be incomplete. Reactions of nitrous acid with α-amino-acid side chains [3] may also contribute to denaturation.

generally used in the pasteurisation of cured meats presented in plastic sachets.[15] The e.s.r. spectrum of uncooked bacon indicated the presence of *both* penta- and hexa-co-ordinate nitrosylhaems. However, after heating the sample gently in the e.s.r. tube (50 °C, 30 min) the broad high-field feature located at an effective g value of 1.990 had disappeared while the features characteristic of the pentaco-ordinate species had become sharper and had increased in intensity, as shown in Figure 4. Our interpretation of these observations is as

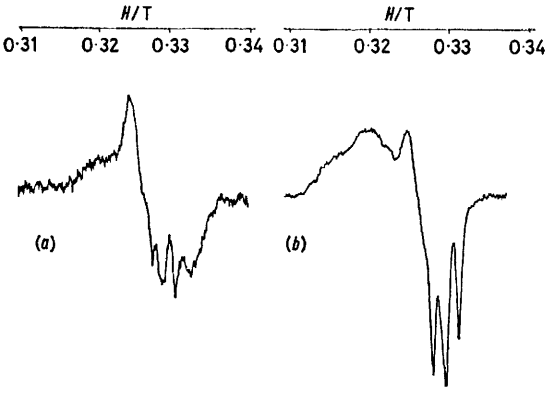


FIGURE 4 E.s.r. spectra of uncooked bacon before and after heat treatment measured at 93 K; (*a*) uncooked bacon sample; (*b*) sample (*a*) after being kept at 50 °C for 30 min, [the signal response for this sample was 0.4 that for sample (*a*)]

follows. In uncooked cured meat denaturation of the haemoprotein is limited, and the pigment thus consists of a mixture of nitrosylprotohaem (pentaco-ordinate) and nitrosylmyoglobin (hexaco-ordinate). On heating the sample the denaturation process is completed with the conversion of nitrosylmyoglobin into nitrosylprotohaem, so that the e.s.r. spectrum now resembles those of the cooked cured meats referred to earlier.

EXPERIMENTAL

Electronic spectra were recorded using a Pye Unicam SP8000, and are calibrated (holmium glass); i.r. spectra were recorded on a Perkin Elmer 257 instrument. E.s.r. spectra were recorded at the temperatures stated (generally 77—98 K) using a Decca X3 spectrometer. Solid samples were examined in capped silica tubes, i.d. 3 mm. Moist cured meat samples were examined as plugs (20 mm × 3 mm) in similar tubes. Solutions were prepared by distilling the required amount of degassed solvent onto the sample under vacuum, and then sealing the system. The parameters for certain spectra are collected in the Tables.

Nitrosyliron(II)*–protoporphyrin* (*Nitrosylprotohaem*).—The reaction was carried out under argon using a Schlenck tube. Protohaemin (200 mg) was dissolved in a mixture of chloroform (50 ml) and pyridine (3 ml) and flushed with argon (20 min). Nitric oxide in a stream of argon was then passed through the solution for 20 min, and methanol (degassed, 50 ml) was added ($\lambda_{max.}$ 478, 544 infl., and 568 nm). The solution was evaporated, and the residue was taken up in chloroform (5 ml) and treated with petroleum (50 ml). The precipitate was removed at the centrifuge, washed with petroleum, and dried to give nitrosylprotohaem as a red solid (192 mg, 90%), which could not be recrystallised without decomposition (Found: C, 59.05; H, 6.05; N, 9.7. $C_{34}H_{32}FeN_5O_5 \cdot 2.5H_2O$ requires C, 59.05; H, 5.4; N, 10.15%); ν(CsI) 3 460br, 1 710, 1 659s, 1 550, 1 396, 1 233, 1 088, 950, and 838 cm^{-1}. The e.s.r. spectrum (acetone, 93 K) resembled that shown in Figure 1, and had g_3 = 2.009, and a_3 = 1.62 mT.

Nitrosylprotohaem from Nitrosylhaemoglobin.—Oxyhaemoglobin (3 g, moist, from ox blood) was dissolved in phosphate buffer (6 ml, pH 7, 2M) and converted to deoxyhaemoglobin by six cycles of evacuation and equilibration with nitrogen in a Thunberg tube. Equilibration with nitric oxide gave a bright red solution of nitrosylhaemoglobin, which was flushed with nitrogen.

The remaining manipulations were carried out without interruption. The solution was carefully poured into acetone, and the precipitate was removed. The supernatant was concentrated (rotary evaporator) and the (largely aqueous) concentrate was extracted with chloroform ($\lambda_{max.}$ 478, 542 infl., and 569 nm). The chloroform solution was concentrated and the nitrosylprotohaem was precipitated with petroleum as before. The yield was low (*ca.* 2 mg). The e.s.r. spectrum (acetone, 77 K) was typical of a pentaco-ordinate nitrosylhaem (g_3 = 2.010 and a_3 = 1.68 mT).

Extraction of Nitrosylprotohaem from Cured Meat Samples.—The following procedure is typical (all solvents were nitrogen-flushed). The moist cured meat (ham slices, 50 g) was minced twice, placed in a conical flask under nitrogen, and extracted with acetone (2 × 100 ml, containing cysteine, 0.05 g; each extraction 15 min with occasional shaking). The acetone extracts were filtered through a pad of silica gel and anhydrous sodium sulphate, concentrated under reduced pressure until the onset of severe frothing, and then poured into water (200 ml). Extraction with dichloromethane (2 × 80 ml) gave a cherry-red solution of crude nitrosylprotohaem, $\lambda_{max.}$ (CH_2Cl_2) 480, 539 infl., and 566 nm. The e.s.r. spectrum (90 K, after removing dichloromethane and re-dissolving the residue in acetone) was of the type shown in Figure 1, with g_3 = 2.010 and a_3 = 1.68 mT.

The meat residue after extraction was washed with acetone (3 × 100 ml), and dried *in vacuo* at room temperature. The major feature of the e.s.r. spectrum of the solid at 93 K was a line at g_r = 2.027, together with what appeared to be a residual g_3 signal (2.008). The spectrum was not changed by the presence of acetone.

Nitrosylprotohaem could be extracted from samples of bacon and corned beef under similar conditions. When the extraction procedure was applied to fresh pork and fresh beef, virtually no extraction of haem pigment occurred.

E.S.R. Spectra in Various Solvents.—A sample of nitrosylprotohaem was extracted from ham: e.s.r. spectra in a variety of solvents were recorded. The results are shown in Table 4.

Spectroscopic Observations on Meat Samples.—(a) *E.s.r. spectra.* Moist meat samples were examined as plugs (20 mm × 3 mm) in capped silica tubes. All the cured meats examined gave nitrosylhaem spectra (Table 3 and Figure 3).

Samples of fresh meat were vacuum-dried at room temperature and examined at 90—98 K. Both pork (g = 2.005) and beef (g = 2.004) gave single lines. Further experiments with the pork sample showed that the signal diminished when the sample was treated with sodium dithionite, and that the species responsible for the signal

was not readily extracted by acetone (e.s.r. checks on extract negative; signal still present in residual sample).

(b) *Electronic spectra.* A thin slice of cooked cured meat was pressed firmly onto the inner transparent face of a 1-cm cuvette, and the visible spectrum was observed. Broad peaks were found with $\lambda_{max.}$ as follows: ham 414, 477 infl., 535, and 565 infl. nm; bacon 418, 482 infl., 535, and 566 infl. nm. The spectrum was changed little on filling the cell with carbon tetrachloride. On adding 1-methylimidazole to the cell contents the spectrum gradually changed to $\lambda_{max.}$ 418, 528, and 558 nm.

TABLE 4

E.s.r. spectra of nitrosylprotohaem in various solvents at *ca.* 93 K

Solvent	g_3	a_3/mT	g_s [a]	Spectral type [b]
Toluene	2.003	1.63		5
Benzene	2.001	1.69		5
Benzene–cyclohexanone (1 : 1)	2.001	1.65		5
Cyclohexanone	2.009	1.64		5
Dimethylformamide	2.009	1.66		5
Piperidine	2.003		1.982	6
Acetone–1-methylimidazole	2.003		1.981	6
Pyridine	2.003		1.986	6

[a] g_s Refers to the minimum of the broad high-field feature characteristic of the hexaco-ordinate nitrosylhaem system. [b] 5 = Pentaco-ordinate nitrosylhaem; 6 = hexaco-ordinate nitrosylhaem, where the second axial ligand is nitrogeneous. These spectral types are illustrated in Figures 1 and 2, respectively

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