CARBON-13 NMR IN HAEMS AND HAEMOPROTEINS

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ABSTRACT

A survey is presented of the application of ^{13}C n.m.r. techniques for the investigation of structure–function relations in haemoproteins. Work in this field started soon after the introduction of commercial Fourier transform n.m.r. spectrometers approximately three years ago. The different approaches which have been described in the meantime include experiments which are in principle generally applicable for studies of the molecular conformations in proteins, e.g. measurements of chemical shifts and relaxation times of ^{13}C present in the polypeptide chains in natural abundance or in isotope-enriched groups introduced through chemical or biosynthetic reactions. In addition there are a variety of experiments designed for investigations of structural and functional features which are specific for haemoproteins. These include the n.m.r. observation of low molecular weight ^{13}C enriched haem iron ligands, e.g. ^{13}CO and $^{13}CN^{\ominus}$, ^{13}C n.m.r. studies of porphyrin biosynthesis, and investigations of the electronic states in the haem groups through observation of the ^{13}C n.m.r. of the porphyrin skeleton.

INTRODUCTION

Haemoproteins are involved in many vital processes in living organisms. Prominent among their biological functions are those of haemoglobin, which is the oxygen-transporting protein in the blood, myoglobin, which binds and stores oxygen in the muscles, various cytochromes, which act as electron-transferring oxidation reduction carriers, and of various enzymatically active haemoproteins which control diverse biochemical reactions. A haemoprotein molecule consists of one or several polypeptide chains, each of which is typically made up of 100 to 200 amino acid residues, and one or several haem groups. The latter are iron porphyrin complexes (*Figure 1*) which can be combined with the polypeptide moiety of the molecule through one or several covalent bonds and through a multitude of weaker interactions. The haem groups appear to be an integral part of the active centres in all the haemoproteins known to date.

From the viewpoint of n.m.r. it is of particular relevance that haemoproteins are rather large molecules. This may be illustrated by the molecular weights of 12500 for cytochrome c, 18000 for myoglobin, and 64000 for mammalian haemoglobins. Correspondingly the polypeptide chains contain several hundred to several thousand carbon atoms and protons per molecule.

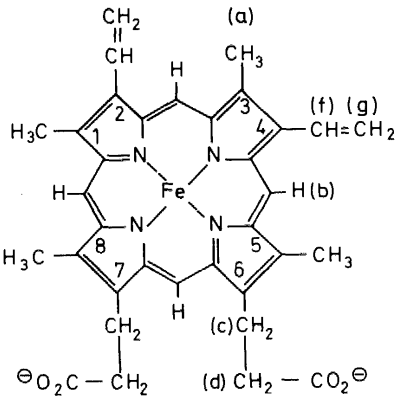


Figure 1. The iron protoporphyrin IX complex, 'protohaem IX', is the prosthetic group in a variety of haemoproteins, including haemoglobin, myoglobin, cytochromes b_2 and b_5, catalase, and peroxidase. In the haem group of cytochrome c, the 2,4-vinyl sidechains are replaced by $-CH(CH_3)-S-$, where the thioether groups form covalent links with the polypeptide chain. One of the axial coordination sites of the haem iron is always occupied by an amino acid sidechain of the protein. Depending on the biological role of the haemoprotein, the sixth coordination site is either occupied by an amino acid residue or is available for binding of low molecular weight ligands, e.g. O_2 in haemoglobin.

The numbers and letters given in the figure will be used for the resonance identifications. The carbon atoms 1 to 8 are usually referred to as β-carbons, the atoms b as 'meso-carbons'.

The haem group in *Figure 1*, on the other hand, contains 34 carbon atoms, and 30 protons. Yet because of the paramagnetism of the haem iron, the resonances of the relatively few nuclei of the haem group can be quite prominent in the n.m.r. spectra of haemoproteins (*Figure 2*).

Four different electronic configurations of the haem iron are commonly encountered. Three of these are paramagnetic, i.e. the high spin ferric [Fe(III), $S = \frac{5}{2}$], low spin ferric [Fe(III), $S = \frac{1}{2}$], and high spin ferrous [Fe(II), $S = 2$] forms, whereas low spin ferrous iron [Fe(II), $S = 0$] is diamagnetic. Many biochemical reactions of haemoproteins involve changes of the oxidation and spin states of the haem iron, e.g. the oxygenation of myoglobin and haemoglobin

$$Mb^{II}[Fe(II),\ S = 2] + O_2 \rightleftarrows Mb^{II}O_2\,(Fe^{2+},\ S = 0) \qquad (1)$$

and the electron transfer in cytochrome c

$$Cyt\ c^{III}\,(Fe^{3+},\ S = \tfrac{1}{2}) + e^{\ominus} \rightleftarrows Cyt\ c^{II}\,(Fe^{2+},\ S = 0) \qquad (2)$$

This paper deals mainly with low spin ferrous and low spin ferric haems and haemoproteins, in which the proton n.m.r. have also been extensively investigated[1].

With the introduction of commercial Fourier transform (FT) spectrometers about three years ago, ^{13}C n.m.r. of haemoproteins, and of macromolecules in general, became practicable on a larger scale. Several groups started work based on the observation of the natural abundance ^{13}C of the polypeptide

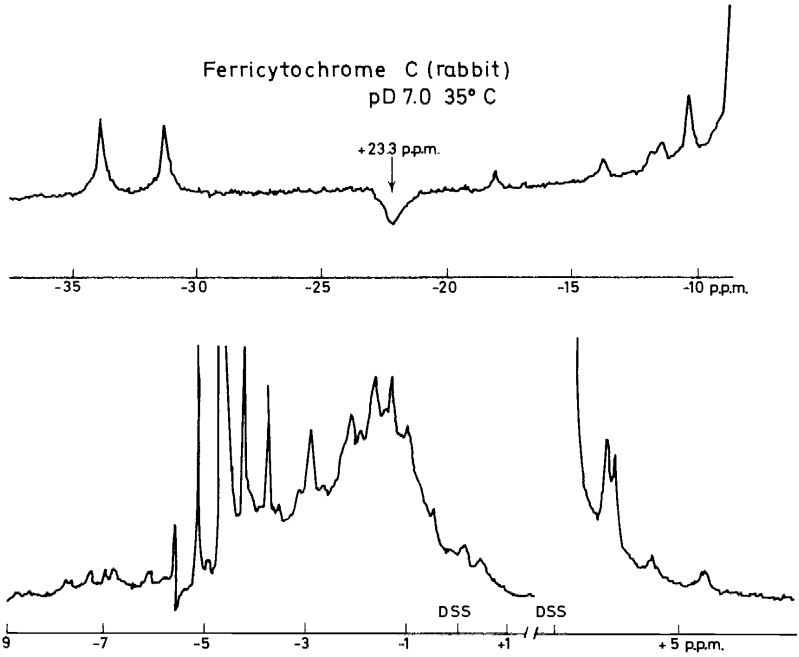


Figure 2. ^{1}H n.m.r. spectrum of the paramagnetic oxidized form of rabbit cytochrome c. The spectrum contains essentially all the 650 proton resonances of the polypeptide chain between 0 and − 10 p.p.m. The resonances outside this spectral region, which are represented with a greatly expanded vertical scale, correspond to 1 to 3 protons each. Many of these lines, in particular the two low field methyl resonances between − 32 and − 35 p.p.m., come from the haem group, and are largely shifted by hyperfine interactions with the unpaired electron. This spectrum is very similar to those of the cytochromes c from different species which had previously been described[1-3].

This spectrum was recorded on a Varian HR-220 spectrometer which operates with 10 kHz field modulation, where the first upfield sideband is routinely observed. The high field line at + 23.3 p.p.m. appears as an inversed resonance of the centre band. The five sharp lines between − 3.5 and − 6.0 p.p.m. correspond to the resonance of the residual water protons, and its first and second spinning sidebands.

chains of proteins and specifically also in haemoproteins, and of ^{13}C-enriched 'n.m.r. labels' which had been covalently linked with the polypeptide chains. These measurements promise to yield particularly interesting information on the dynamics of the molecular conformations in proteins[4]. Other experiments which are more specific for haemoproteins include the observation of ^{13}C-enriched low molecular weight ligand molecules, e.g. ^{13}CO and ^{13}CN$^{\ominus}$, in their reactions with the haem groups. ^{13}C n.m.r. is also employed for investigations of porphyrin biosynthesis. From studies of the ^{13}C resonances of the porphyrin ring (*Figure 1*) one can expect to gain more detailed insight into the electronic structure of the haem groups. This is of much interest for the continued research on the relations between electronic structures in the prosthetic groups and the biological roles of haemoproteins[1,3], and as

a basis for studies of the changes of the protein conformations during reactions of the types 1 and 2, which involve paramagnetic states of the haemoproteins[5, 6].

^{13}C NMR OF THE POLYPEPTIDE CHAINS IN HAEMOPROTEINS

Figure 3 shows a series of ^{13}C n.m.r. spectra of reduced cytochrome c [Fe(II), $S = 0$], and of the constituent components of this molecule. Even though, as has been convincingly demonstrated in lysozyme[7] and in the basic pancreatic trypsin inhibitor[8], rather sizeable ^{13}C shifts can arise as a consequence of the folding of the polypeptide chains in native protein molecules, the distribution of spectral intensity corresponds quite closely to that expected from the spectra of the component molecules[9–12].

The ^{13}C spectra of the diamagnetic forms of myoglobin and haemoglobin have been described by Conti and Paci[13] who pointed out that the protein resonances could be roughly accounted for as the sum of the lines of the constituent amino acids. Moon and Richards[14] proposed that from inspection of the ^{13}C spectra certain conclusions could be drawn on conformational differences between haemoglobins in the different oxidation and spin states of the haem iron. Even though some of these qualitative conclusions may well be valid, it should be pointed out that the authors did not consider the influence of the paramagnetism of the haem group on the chemical shifts and the nuclear relaxation times of the polypeptide resonances[5, 6].

Nigen *et al.* [15–17] described a series of measurements of the longitudinal ^{13}C spin relaxation times in myoglobins from different species. In these experiments, from which conclusions could be drawn on the segmental mobility of the polypeptide chain in different myoglobin preparations, either the natural abundance ^{13}C of the polypeptide chain was observed (corresponding to *Figure 3* B), or [2-^{13}C] bromoacetate was used to carboxymethylate certain amino acids in the proteins, with subsequent observation of the ^{13}C n.m.r. of these isotopically enriched marker groups.

^{13}C NMR OF HAEM LIGANDS

Mammalian haemoglobins consist of four subunits, two each of two types called α- and β-chains, each of which contains one polypeptide chain and one haem group. The sequence of reactions which lead to the fully oxygenated species $Hb^{II}(O_2)_4$ are interrelated by the well known cooperativity of ligand

$$Hb^{II} + O_2 \rightleftarrows Hb^{II}O_2 + O_2 \rightleftarrows Hb^{II}(O_2)_2 \overset{O_2}{\rightleftharpoons} Hb^{II}(O_2)_4 \qquad (3)$$

binding to haemoglobin, which has far reaching physiological consequences. Over the past forty years tremendous efforts have been made to unravel the structural basis of these subunit interactions in haemoglobin. Besides x-ray crystallography[18], high resolution ^1H n.m.r.[1, 19, 20] has been prominent among the many techniques applied to haemoglobin research. One of the specific questions which have emerged is whether the α- and β-subunits can be distinguished in their reactions with ligand molecules. Since the behaviour of CO and O_2 towards haemoglobin is in many respects very

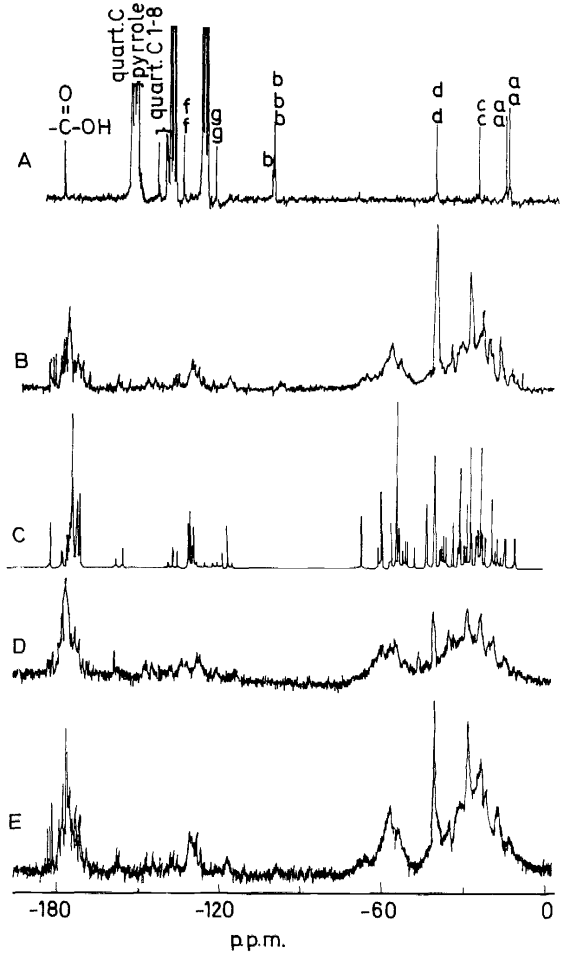


Figure 3. ^{13}C n.m.r. spectra of reduced diamagnetic horse cytochrome c and some of its constituent components. The spectra were recorded at 25.16 MHz on a Varian XL-100 FT spectrometer, sample size 12 mm, $T = 25°$.

A. Zn(II) protoporphyrin IX in d_5-pyridine, proton noise-decoupled[12]. For the resonance assignments see *Figure 1.* The solvent resonances are at -124, -137, and -150 p.p.m. from TMS.

B. Cyt c^{II}, *ca.* 20 per cent in D_2O, $pD = 7.0$, proton noise-decoupled.

C. Hypothetical spectrum for a random coil form of the polypeptide chain, computed as the sum of the amino acid resonances from the known amino acid composition of horse cytochrome c and the spectra for the individual amino acid residues[9–11].

D. Same as B, without proton irradiation.

E. Same as *B* and *D*, proton double resonance irradiation of the meso protons (*Figure 1*, b) at -10.6 p.p.m.[1].

similar, and ^{13}CO is readily available, n.m.r. observation of ^{13}CO in its reactions with haemoglobin seems in principle to be an ideal technique to tackle this problem.

Several different authors have already described experiments with ^{13}CO[13, 21–24], and from various recent oral presentations it can be expected that others will soon join in. So far the primary goal of these studies has been to assign the observed resonance lines. In principle the different authors chose very similar approaches to identify the resonances. The ^{13}CO lines of tetrameric haemoglobins were compared with those of monomeric haemoproteins in the carbon monoxide form, i.e. myoglobin, or isolated α- or β-chains of haemoglobin. All the different authors agree in several points, i.e. that the resonances of ^{13}CO bound to these proteins are at approximately − 206 p.p.m. from external TMS, a single line is observed in solutions of the monomeric proteins, and more than one line is present in tetrameric haemoglobins. The chemical shift difference between ^{13}CO bound respectively to the α- and β-chains seems to be of the order 0.5 p.p.m., with $^{13}CO(\beta)$ at higher field at approximately 206.5 p.p.m., and $^{13}CO(\alpha)$ at lower field at 207.0 p.p.m.[23, 24]. With these resonance assignments it would appear that the stage is now set for experiments such as comparison of the state of ligation in the different subunits of partially ligated haemoglobin, and measurements of the ligand exchange rates in the different subunits during reactions of the type[21]

$$Hb^{II}(^{13}CO)_4, O_2 \rightleftharpoons Hb^{II}(O_2)_4, {}^{13}CO \qquad (4)$$

Considering the rather limited resolution of the ^{13}CO resonances obtained at conventional field strengths[21–24], it seems quite likely that these experiments might benefit in the future from the application of higher fields in superconducting magnets.

A discrepancy between different authors, which can well serve to illustrate one of the typical potential pitfalls that one has to be aware of when working with proteins, arose concerning the interpretation of the ^{13}CO resonances in rabbit haemoglobin. Moon and Richards[21] reported that the $^{13}CO(\alpha)$ and $^{13}CO(\beta)$ resonances were much better separated in rabbit haemoglobin than in human haemoglobin, i.e. by approximately 3 p.p.m. Subsequently Matwiyoff *et al.*[22] pointed out that there exists a haemoglobin heterogeneity in rabbits which varies among individual animals. This heterogeneity involves a functionally different subunit whose abnormality is apparently reflected in a large downfield shift of the ^{13}CO resonance. These authors demonstrated the existence of three ^{13}CO resonances in rabbit blood, and suggest that the low field line of Moon and Richards comes from ^{13}CO bound to the abnormal haemoglobin subunits. In normal rabbit haemoglobin the relative chemical shift between $^{13}CO(\alpha)$ and $^{13}CO(\beta)$ would then also be of the order 0.5 p.p.m.

We have recently studied $^{13}CN^{\ominus}$ which binds preferentially to the ferric form of haems and haemoproteins. So far we have mainly been observing the n.m.r. of the free cyanide ligands present in equilibrium with the complexes formed with the haem iron. A typical experiment is represented in *Figure 4*.

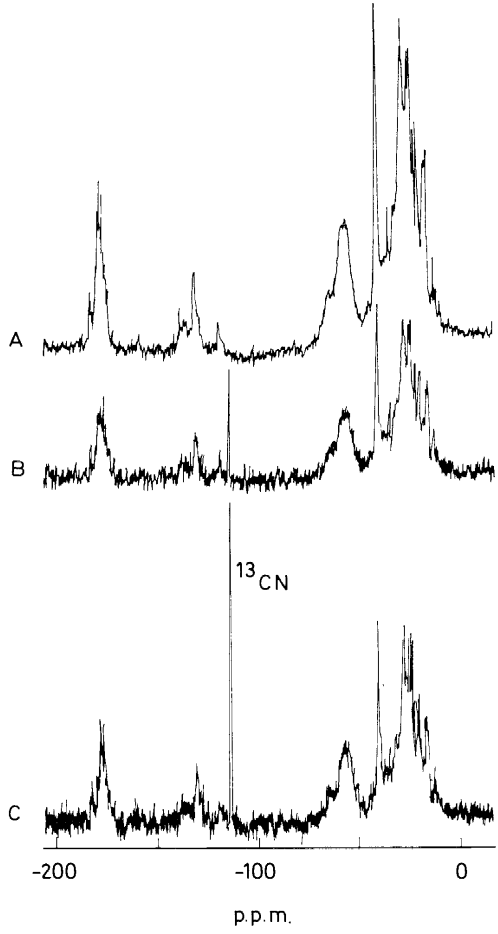


Figure 4. Investigation of the exchange of cyanide ion between cyanoferrimyoglobin and the aqueous medium.

A. FT ^{13}C n.m.r. spectrum of a *ca.* 20 per cent solution of sperm whale Mb^{III} (^{13}CN) in neutral D_2O. Excess $^{13}CN^{\ominus}$ had been removed by dialysis.

B. Same solution as *A* after addition of one equivalent of KCN, which had displaced part of the $^{13}CN^{\ominus}$ from the complex with Mb^{III}.

C. Same solution as *A* and *B*, after addition of six equivalents of KCN.

All the spectra were recorded with proton noise decoupling, $T = 25°$. The resonance of the $^{13}CN^{\ominus}$ bound in Mb^{III} (^{13}CN) is not seen in these spectra.

From this the rates of exchange of $CN^{\ominus}$ from different complexed species could be measured. For example it was found that exchange of $CN^{\ominus}$ between cyanoferrimyoglobin and the bulk of the solution is 'instantaneous' on the n.m.r. time scale, i.e. the half-time is shorter than approximately one minute, whereas the half-time for the corresponding reaction in cyanoferricytochrome c is of the order of several hours or days, depending on the conditions of the experiment.

^{13}C NMR FOR STUDIES OF PORPHYRIN BIOSYNTHESIS

Battersby *et al.*[25] have synthesized protoporphyrin IX dimethyl esters in which individual ones of the four meso carbon positions (b in *Figures 1* and *3*A) were enriched in ^{13}C. From this the resonances of the four structurally non-equivalent meso carbons could be individually assigned. Under the experimental conditions chosen by Battersby *et al.*, these resonances were spread out over the spectral region from -95.8 to -97.7, and could (in contrast to our *Figure 3*A) be individually resolved. These resonance assignments are now being used for detailed investigations of the enzyme-controlled incorporation of ^{13}C labelled porphobilinogen into protoporphyrin IX[26].

INVESTIGATION OF THE ELECTRONIC STRUCTURE IN THE HAEM GROUPS

Studies of the electronic states in the haem groups of haemoproteins and their significance for the biological roles of these proteins have for many years attracted a lot of interest, and for several years ^{1}H n.m.r. has been widely employed for investigations in this field[1]. The application of n.m.r. is particularly attractive for low spin ferric [Fe(III), $S = \frac{1}{2}$] haem compounds where the longitudinal electronic relaxation time is very short, and hence the line width of the nuclear resonances is very little affected by the interactions with the unpaired electron[1]. However, some ambiguities remained in the interpretation of the ^{1}H n.m.r. data[1, 27–30], and it is to be expected that a more detailed description of the electronic structure will result from the combined ^{1}H and ^{13}C data.

In addition to their relevance for the investigation of the relations between electronic structures and biological functions of haemoproteins, more reliable data on the electronic states will also enhance the potential of n.m.r. for investigations of the molecular conformations in paramagnetic haemoproteins[5, 6]. If, for example, high resolution n.m.r. is employed to investigate conformational changes during reactions of the type of equations 1 or 2, the local magnetic fields of the haem groups have to be known for both oxidation states, and this includes of course the fields produced by the unpaired electrons in the paramagnetic haems.

A brief outline of the analysis of ^{1}H and ^{13}C hyperfine shifts is probably the best way to illustrate some of the principal questions which arise in these studies. The nuclear resonance positions $\Delta\nu$ observed in paramagnetic species can be decomposed into two terms,

$$\Delta\nu = \Delta\nu_{\text{diam}} + \Delta\nu_{\text{hf}} \tag{5}$$

where $\Delta\nu_{\text{diam}}$ would be the resonance positions in the absence of the electronic paramagnetism, and $\Delta\nu_{\text{hf}}$ are the hyperfine shifts which arise from the interactions with the unpaired electrons. In the interpretation of $\Delta\nu_{\text{hf}}$ the following contributions have to be considered

$$\Delta\nu_{\text{hf}} = \Delta\nu_{\text{pc}}^{\text{M}} + \Delta\nu_{\text{pc}}^{\text{L}\sigma} + \Delta\nu_{\text{pc}}^{\text{L}\pi} + \Delta\nu_{\text{c}}^{\sigma} + \Delta\nu_{\text{c}}^{\pi} \tag{6}$$

$\Delta\nu_{\text{pc}}^{\text{M}}$ is the pseudocontact shift arising from dipole–dipole coupling with the electron spin localized on the central metal ion[31, 32], $\Delta\nu_{\text{pc}}^{\text{L}\sigma}$ and $\Delta\nu_{\text{pc}}^{\text{L}\pi}$ are the

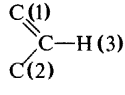
pseudocontact shifts from interactions with the electron spin delocalized to the ligands through the σ- and π-bonds, respectively[32], $\Delta\nu_c^\sigma$ the contact shift arising from Fermi contact coupling[32] with the electron spin delocalized to the ligand atoms through the σ-bond system, and $\Delta\nu_c^\pi$ the contact shifts from spin delocalization in the π-bond system.

When dealing with low spin ferric haems, it appears that equation 6 can be somewhat simplified by omitting $\Delta\nu_{pc}^{L\sigma}$ and $\Delta\nu_c^\sigma$ because only very small contributions to $\Delta\nu_{hf}$ should come from electron spin delocalization through the σ-bonds[1, 28, 30]. Furthermore it appears that $\Delta\nu_{pc}^{L\pi}$ does not contribute sizeably to $\Delta\nu_{hf}$ for protons[30]. At this point one then has to separate out the contributions to $\Delta\nu_{hf}$ from the different terms remaining in equation 6. Since for most of the molecules in question no complete description of the electronic **g**-tensor under the conditions of the n.m.r. experiments is available, one has to rely essentially exclusively on the n.m.r. data[1, 28]. It can reasonably be expected that the combined ^1H and ^{13}C hyperfine shifts will allow us to narrow down the limits of uncertainty in this step of the analysis[33].

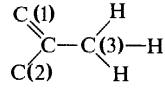
With $\Delta\nu_c^\pi$ determined through equation 6, one can then proceed to the computation of the contact interaction constants A^I. With certain simplifying approximations[1, 12, 33], $\Delta\nu_c^\pi$ can be given by[34]

$$\Delta\nu_c^\pi = -A^I \left|\frac{\gamma_e}{\gamma_I}\right| \frac{S(S+1)}{3kT} \tag{7}$$

where γ_e and γ_I are the gyromagnetic ratios of the electron and the nucleus, S is the electronic spin, k the Boltzmann constant, and T the absolute temperature. The contact interaction constants are related to the unpaired electron spin densities ρ_c^π localized on the aromatic carbon atoms by empirical factors Q^I. In the fragments **I** and **II**,



I



II

where finite spin density can be localized in the π-orbitals of the three aromatic carbon atoms, one has for the aromatic proton H(3) in **I**[35]

$$A^H = Q_{CH}^H \times \rho_C^\pi \tag{8}$$

where C denotes the carbon atom to which the proton is attached. A corresponding relation has been proposed for the methyl protons in **II**[36]

$$A^H = Q_{CCH_3}^H \times \rho_C^\pi \tag{9}$$

For an aromatic carbon atom the contact interaction constant A^C can be related to the spin densities ρ_C^π and $\rho_{X_i}^\pi$ centred on its π-orbital and on the π-orbitals of the three atoms X_i to which it is bonded[37]. For the central carbon atoms in the fragments **I** and **II** one has

$$A^C = S^C + \sum_{i=1}^{3} Q_{CX_i}^C \rho_C^\pi + \sum_{i=1}^{3} Q_{X_iC}^C \rho_{X_i}^\pi \tag{10}$$

where S^C accounts for the polarization of the 1s-electrons, the $Q^C_{CX_i}$s for the polarization of the 2s-electrons by the π-electron spin density on the observed carbon atom, and the $Q^C_{X_iC}$s for the polarization of the 2s-electrons by the π-electron spin densities on the neighbouring atoms. For the methyl carbon atom in **II**, one then obtains from equation 10

$$A^C = Q^C_{C'C} \times \rho^\pi_{C'} \tag{11}$$

where C′ denotes the aromatic carbon to which the methyl group is bound.

At this point the combination of ^1H and ^{13}C n.m.r. data enables one to check on the validity for haem groups of the different empirical factors Q^I, which had originally been obtained from studies of various organic radicals[35–37]. From this it should then also be possible to decide whether the markedly different ^1H contact shifts in isolated low spin ferric porphyrin complexes and in haemoproteins (compare e.g. *Figures 2* and *5*)) arise solely from modifications of the spin density distribution in the haems (ρ^π_c in equations 8 and 9), or if on the contrary the factors Q^I are also modified by the haem–polypeptide interactions. Finally, since some of the factors Q^I depend on the steric orientation of the ring substituents, the latter becomes in principle accessible for experimental investigation when ^1H and ^{13}C n.m.r. data are available[12].

The ^{13}C resonances have been identified in a number of iron porphyrin complexes. This is illustrated in *Figures 5* and *6* for iron(III) protoporphyrin IX dicyanide. Because the ^1H resonances are widely separated by the paramagnetism of the complex (*Figure 5*), the different types of ^{13}C resonances could be assigned in a single off-resonance double irradiation experiment (*Figure 6* B). More detailed resonance identifications resulted from

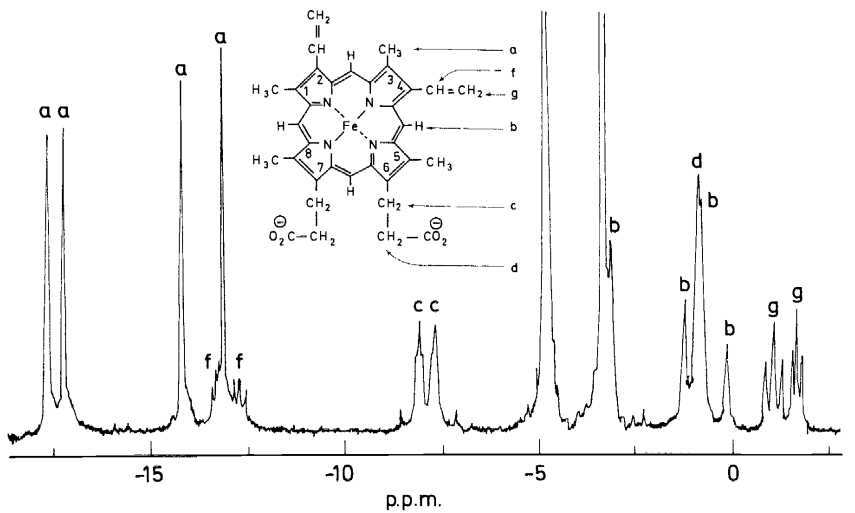


Figure 5. FT ^1H n.m.r. spectrum at 100 MHz of Fe(III) (protoporphyrin IX) $(CN)_2$ in CD_3OD, $T = 29°$. The structure of the complex, where the axial cyanide ligands have been omitted, and the resonance assignments[1] are also indicated.

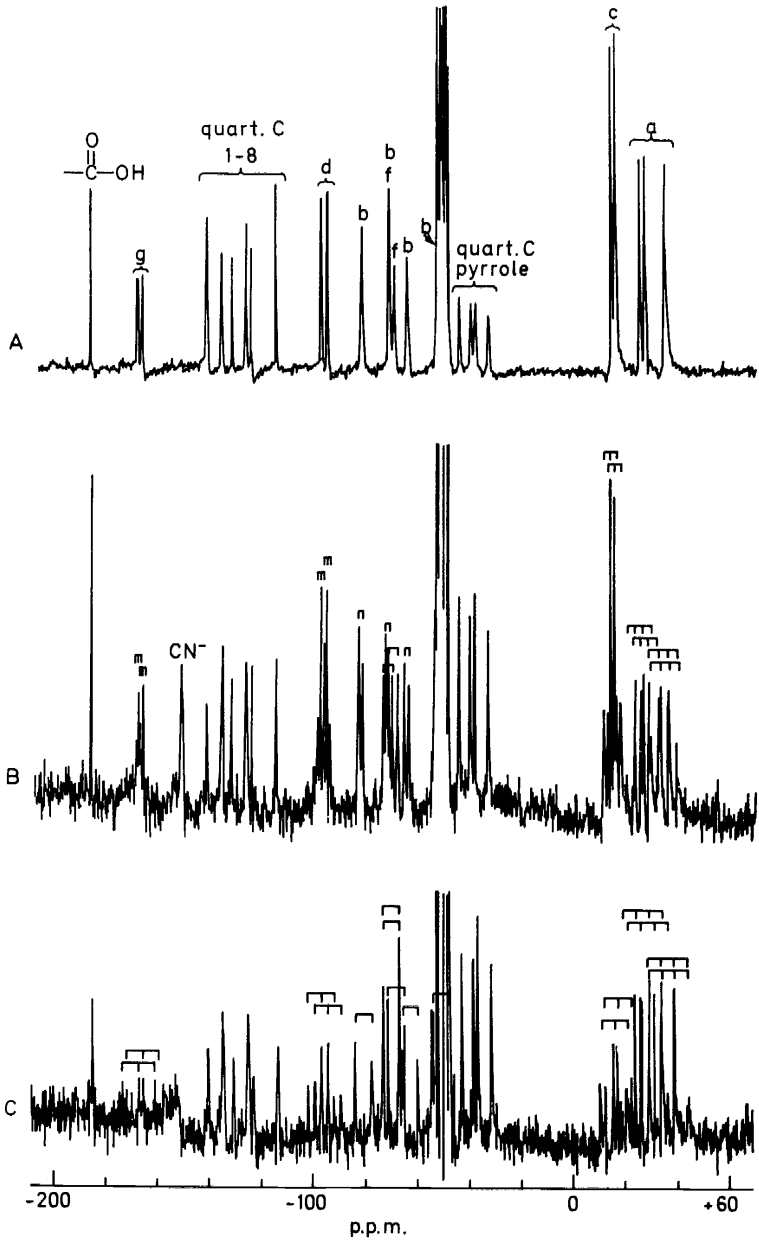


Figure 6. FT ^{13}C n.m.r. spectra at 25.14 MHz of Fe(III) (protoporphyrin IX) $(CN)_2$ in CD_3OD. The strong line at -49 p.p.m. comes from the solvent. $T = 29°$.

A. Proton noise decoupled.

B. Proton off-resonance irradiation at $+5$ p.p.m. (see *Figure 5*).

C. Without proton irradiation. The resonance assignments[12] are also indicated (see *Figures 1* and *5* for the nomenclature).

specific ^1H–^{13}C spin decoupling [6, 12]. The chemical shifts $\Delta\nu_{diam}$ (equation 5) were obtained from the corresponding diamagnetic Zn(II) complex (*Figure 3* A)[12]. ^{13}C hyperfine shifts have so far also been determined in the more highly symmetrical complexes Fe(III) (porphin) $(CN)_2$[33] and Fe(III) (tetraphenylporphin) $(CN)_2$[33], and in Fe(III) (deuteroporphyrin IX) $(CN)_2$[12]. A preliminary analysis of these data seems to imply that sizeable contributions to the hyperfine shifts of the ring carbon atoms come from $\Delta\nu_{pc}^{L\pi}$ (equation 6)[33] and that $Q_{CH_3}^H$ is noticeably smaller in the haems than in neutral aromatic radicals[1, 12, 27, 28]. We are currently working on a more detailed interpretation of the combined ^1H and ^{13}C hyperfine shifts in these complexes.

With the presently available equipment, individual ^{13}C resonances of the haem groups in haemoproteins can in principle be observed at natural abundance of ^{13}C. This is illustrated in *Figure 3*, where on the basis of comparing the spectra A to E the resonances of the haem meso carbon atoms b were identified at approximately −98 p.p.m. in the spectrum of ferrocytochrome c. Yet we are still looking for the hyperfine-shifted ^{13}C resonances of the haem group in ferricytochrome c and other paramagnetic haemoproteins.

CONCLUSION

This survey of ^{13}C n.m.r. studies of haems and haemoproteins was written very soon after work in this direction had actually started. It is to be expected that applications of ^{13}C n.m.r. for studies of this class of compounds will develop rapidly in the near future. It is the hope of the author that this article may help the reader to evaluate in what directions the further development is likely to go. From the present state of the art it would in particular appear almost certain that work with ^{13}C labelled compounds will be very fruitful in the continued endeavours, e.g. in further studies of small molecules in their reactions with haemoproteins, in biosynthetic studies, and in the investigation of the hyperfine-shifted haem carbon resonances.

ACKNOWLEDGEMENTS

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