Electron Spin Resonance of Haemoglobin and Myoglobin

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1 Introduction

The purpose of this review is to give sufficient detail of the past electron spin resonance (e.s.r.) work on haemoglobin and myoglobin to bring the general reader up to date (early 1983), and to present a critical appraisal of some of the unresolved problem areas which are areas of active research. It is assumed that the reader has a foundation in transition metal e.s.r. spectroscopy from experience with other systems or from any of the several introductory works on the technique. See, for example, references 1—3 or specific works on haemoglobin and myoglobin.[4, 5]

A. E.s.r. Parameters of Haems: An Overview. — The native form of deoxyhaemoglobin [Fe^{II}; d^6] is diamagnetic and hence can only be studied by e.s.r. spectroscopy if it has a paramagnetic ligand [*e.g.* NO or O_2^-). Also, oxyhaemoglobin gives no e.s.r. signals. However, good spectra can be obtained from the oxidized [Fe^{III}; d^5] forms, and these have been widely studied by e.s.r. spectroscopy.

For many purposes the porphyrin moiety may be regarded as an axially symmetric tetradentrate ligand for iron (Figure 1). In $^{Fe}Mb^+(H_2O)$ the five electrons are unpaired, as has been demonstrated by magnetic susceptibility studies, and thus form a ^6S state with manifolds of $S_z = \pm \frac{1}{2}, \pm \frac{3}{2}, \pm \frac{5}{2}$ where S_z

Abbreviations: ^{Fe}Hb or Hb, ferrous haemoglobin; ^{Fe}Mb or Mb, ferrous myoglobin; $^{Fe}Hb^+$, ferric (met) haemoglobin; $^{Fe}Mb^+$, ferric (met) myoglobin; ^{Co}Hb, cobaltous haemoglobin; $^{Co}HbO_2$, oxycobalt haemoglobin; ^{Co}Mb, cobaltous myoglobin; $^{Co}MbO_2$, oxycobaltomyoglobin; Hb_{Kansas} haemoglobin Kansas; Mb(Im), myoglobin with imidazole sixth ligand.

[1] H. M. Swartz, J. Bolton, and A. Borg, 'Biological Applications of Electron Spin Resonance', Academic Press, New York, 1972.
[2] A. Carrington and A. D. MacLachlan, 'Introduction to Magnetic Resonance', Harper and Row, New York, 1967.
[3] (*a*) M. C. R. Symons, 'Chemical and Biochemical Aspects of Electronic Spin Resonance Spectroscopy', Van Nostrand, New York, 1978. (*b*) P. Atkins and M. C. R. Symons, 'Structure of Inorganic Radicals', Elsevier, New York, 1968.
[4] M. Weissbluth, 'Haemoglobin', Springer Verlag, New York, 1974.
[5] E. Antonini and M. Brunori, 'Haemoglobin and Myoglobin in Their Reactions with Ligands', Elsevier, New York, 1971.

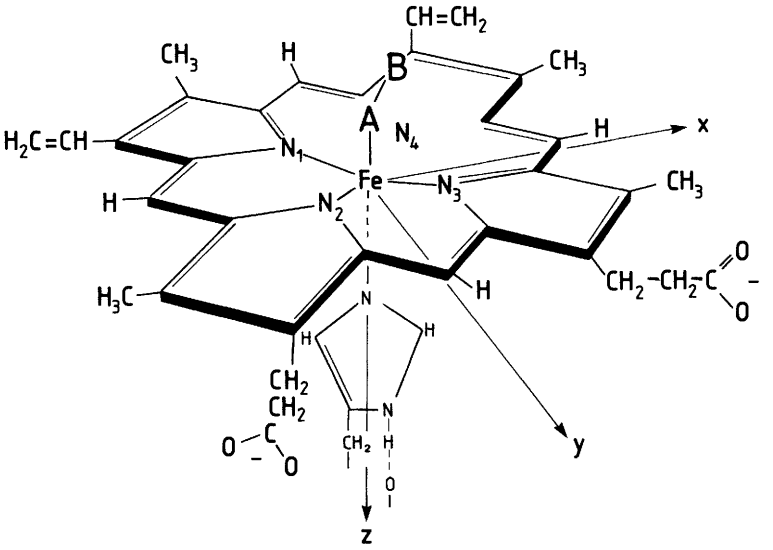


Figure 1 *Diagram of haem moiety showing axes chosen, angle of tilt of A—B ligand, and numbering of porphyrin nitrogen system*

is the component of magnetic spin in the direction of the magnetic field, z. This is the 'high spin' case. The apparent g-values which result are $g_{\parallel} \approx 2$ and $g_{\perp} \approx 6$. These are limiting positions for one of the many allowed transitions. This large anisotropy has provided very accurate determination of the orientation of the haem groups in haemoglobin single crystals. There is with Fe^{3+} the possibility of an intermediate spin, $S = \frac{3}{2}$ case, but this has been observed only in model haems.[6]

In Fe^{3+} with strongly bound ligands (*e.g.*, CN^-, N_3^-) the electronic structure changes drastically to ^2S with one unpaired electron. The g-values become 2.82, 2.20, 1.70 in $Hb^+N_3^-$. This is the 'low-spin' case.

Hyperfine splitting is not directly seen in the e.s.r. spectra of ferric haems although some is seen in ENDOR studies that we describe below. This is because of the fairly large linewidths and quite small splitting parameters of the low natural abundance (2.2%) ^{57}Fe, and low unpaired electron density on porphyrin and protein nuclei. For this reason much interest has been directed towards metal-replaced cases such as ^{Co}Hb which give an indication of electron density on the metal through the large hyperfine splitting by ^{59}Co. In order to detect superhyperfine splitting from ligand nuclei, it is necessary again to examine non-native species such as $^{Co}HbO_2$, HbNO, or HbO_2^- in order to gain some insight into the electronic structures and densities within the ligand–metal–porphyrin moiety (Figure 2).

[6] C. A. Reed, T. Mashiko, S. P. Bentley, M. E. Kastner, W. R. Scheidt, K. Spartalian, and G. Lang, *J. Am. Chem. Soc.*, 1979, **101**, 2948.

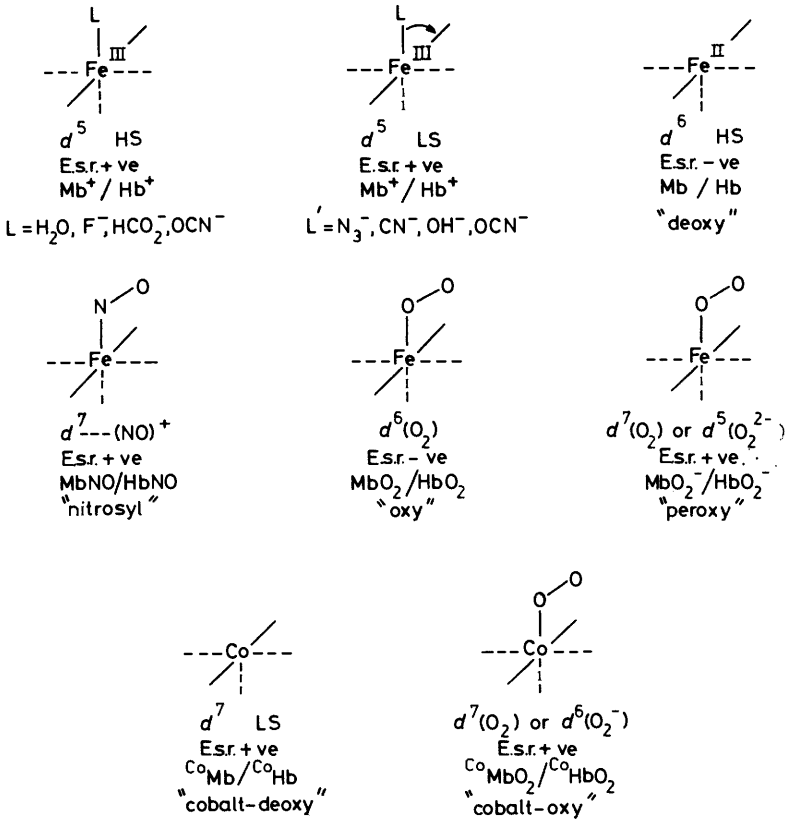


Figure 2 *Structures and abbreviations of species discussed.* L *are sixth ligands which prefer* Fe^{III} *high-spin* (HS) *and* L' *are ligands favouring low-spin* Fe^{III} (LS)

B. E.s.r. Measurements.—E.s.r. spectral measurements are most commonly made on randomly orientated samples, either in solution or as polycrystalline materials. Such samples which contain all possible orientations of the paramagnetic species, yield spectra which are the sum of all angle-dependent spectra. [See the appendix of reference (3b) for a detailed discussion.] In principle, this spectrum contains all the information about the e.s.r. parameters and indeed there are powerful computer programs for extracting such information from the 'envelope spectra.' However, in cases such as $^{Co}MbO_2$ where the *g*-tensor and ^{59}Co hyperfine tensor do not share principal axes, the envelope spectrum can give misleading parameters. Thus, there is an increase of information and accuracy in e.s.r. crystallographic results which also allow direct comparison of orientation of e.s.r. parameter tensors with atomic co-ordinates obtained from *X*-ray crystallography.

Because some spectra are complex and ambiguous it is often advantageous to

obtain spectra at several frequencies, usually 9 GHz (X-band) and 35 GHz (Q-band). Good hyperfine parameters can be defined since the separation between g features is proportional to field strength and hyperfine features are invariant with field strength; this greatly aids spectral interpretation.

C. Haem–Globin Complexes.—In this work haemoglobin refers to the tetrameric haem–globin complex which is the primary oxygen carrier from lung to tissue in most higher animals, but is also found in some molluscs and plants. Haemoglobin has four haems, each in a separate protein chain, all of which closely resemble myoglobin. In haemoglobin A, of adult humans, there are two each of two distinct protein sequences, labelled α- and β-chains. Each haem is firmly packed in a protein matrix of hydrophobic amino-acids and there is a covalent bond between the iron and the F8 imidazoyl ε-nitrogen. (F8 refers to the eighth residue of the F helix and is histidine at the 87th residue in α-chains, and at the 92nd in β-chains. The haem is ensconced in a pocket: on one side is the F8 histidine, on the other an open cavity where ligation can occur (Figure 1). Nearby, but not bonded to the iron, is the distal histidine (E7) which may influence ligand bonding in some cases. One of the intriguing questions in haemoglobin research has been the mechanism of change in oxygen affinity of haemoglobin depending on the oxygen pressure. That is, at low oxygen tension the haemoglobin has a low affinity for oxygen; with increasing addition of oxygen molecules to the haemoglobin tetramer the affinity for oxygen increases greatly. This phenomenon of allosterism (co-operativity, or 'haem–haem' interaction) requires that there be some linkage between the subunits so that bonding of some oxygen molecules changes the molecular geometry of the remaining haem moieties. The low affinity conformation corresponding to deoxyhaemoglobin has been labelled '*T*' for tense; the high affinity or oxygenated form '*R*,' for relaxed. The terms tense and relaxed can be related to the Fe–porphyrin distances as seen in *X*-ray crystallography of haemoglobin and model compounds. In the *T* form the Fe nucleus is as much as 0.75 Å out of the porphyrin ring towards the F8 histidine; in the *R* form the Fe atom is virtually in the ring. For further details on the physical properties of haemoglobin the reader is referred to an excellent monograph by Antonini and Brunori.[5] The most commonly accepted structural interpretation of *R* and *T* effects is that of Perutz, details of which can be found in his original paper.[7]

A number of abnormal haemoglobins have been found in humans. These are caused by genetic defects such that there is a substitution of an amino-acid in the ususal peptide chain. For example, Hb_{Kansas} has asparagine G4(102) in the β-chain replaced by threonine and is designated Hb_{Kansas}[AsnG4(102)$\beta \rightarrow$ Thr]. This is of interest, not because of a direct influence on the substitution of the haem environment, but because of the indirect influence caused by a change in *T* conformation.

[7] M. F. Perutz, *Nature*, 1970, **228**, 726.

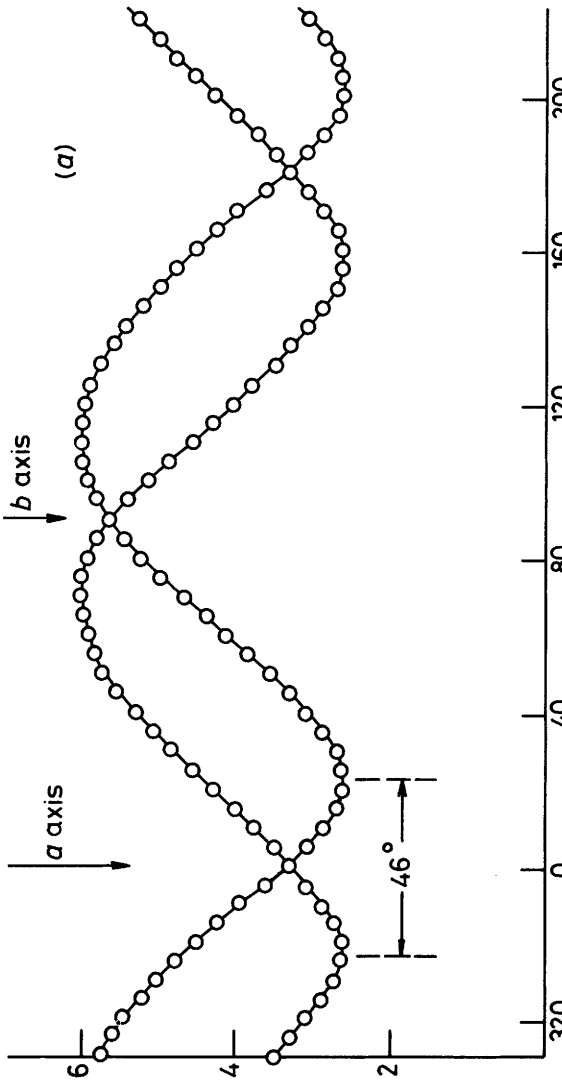


Figure 3 *Variation of g-value in the* ab *phase of a type-A crystal of metmyoglobin showing that* $g_{\parallel}$ *is* ca. $\pm 20°$ *from the* a *axis*

2 Monomeric Species: Ferric Forms

Myoglobin offers the simplest case for study of haem–globin complexes because it has no co-operative haem–haem interaction, and in the single crystals (type A)[8] there are only two inequivalent haem orientations. Hence it has been extensively studied and was one of the first haemproteins investigated by e.s.r. in the classic work from Ingram's laboratory.[9] We present myoglobin results first so that the reader may appreciate 'basic' haem–globin interactions before being introduced to the complexities which arise in the tetrameric haemoglobins.

A. High-spin Derivatives

Initial observations on aquo-metmyoglobins employed the large anisotropy of the g_{app}-tensor ($g_{\|} = 2.0, g_{\perp} = 6.0$) to determine the orientation of the normals of the haem plane with respect to the crystallographic unit-cell axes (Figure 3). This was done prior to the solving of the detailed atomic structure by *X*-ray crystallography and illustrates the power of e.s.r. crystallography. At that time the nature of the sixth ligand was not known but was later determined to be a single water molecule. In this case the axial symmetry of the porphyrin ligand field generates an axial g_{app}-tensor, as seen at 77 K. The g_{app}-value, once the principal values are known, is given by

$$g^2(\theta) = g_{\|}^2 \cos^2\theta + g_{\perp}^2 \sin^2\theta$$

where θ is the angle between the magnetic field direction and the symmetry axis of the paramagnetic system. The distortion of the ligand field from axial symmetry by the fifth ligand, an imidazoyl nitrogen, is not seen at 77 K, although it is observable in some haem proteins if the temperature of observation is lowered to near 1 K.[10] Although a small rhombicity is seen in crystal studies on aquo-metmyoglobin,[11, 12] frozen solution studies show only a reduction of linewidth from 75 to 36 G* as the temperature is lowered from 77 to 1.6 K.[10] The direction of the minimum g_{app}-value in the haem plane is found to correspond to the projected proximal imidazole plane. The rhombicity of the g_{app}-values arises because the *E* term is non-zero. The theory for such cases has been treated by Kotani *et al.*[13] and by Dowsing and Gibson[14] and others. Since variation of the g_{app}-value on magnetic field strength is small, there is a large error in the estimates of the values of *D* and *E*. The best estimates of *D* and *E* come from the high microwave frequency single crystal study of Slade and Ingram[15] where $D = 8.8 \pm 0.8\,\text{cm}^{-1}$ and $E = 0.2\,\text{cm}^{-1}$.

*G = 10^{-4} Tesla.

[8] J. C. Kendrew and R. G. Parrish, *Proc. R. Soc. London, Ser. A*, 1957, **283**, 305.
[9] J. E. Bennett, J. F. Gibson, and D. J. E. Ingram, *Proc. Chem. Soc. (London)*, 1957, **A240**, 67.
[10] J. Peisach, W. E. Blumberg, S. Ogawa, E. A. Rachmilewitz, and R. Oltzik, *J. Biol. Chem.*, 1971, **246**, 3342.
[11] J. F. Gibson, D. J. E. Ingram, and D. Schonland, *Discuss. Faraday Soc.*, 1958, **28**, 72.
[12] G. A. Helcke, D. J. E. Ingram, and E. F. Slade, *Proc. R. Soc. London, Ser. B.*, 1968, **169**, 275.
[13] M. Kotani, and H. Watari, in 'Magnetic Resonance in Biological Systems' ed. C. Franconi, New York, Gordon and Breach, 1971, p. 75.
[14] R. D. Dowsing, J. F. Gibson, M. Goodgame, and P. J. Hayward, *J. Chem. Soc. (A)*, 1969, 187.
[15] E. F. Slade and D. J. E. Ingram, *Proc. R. Soc. London, Ser. A*, 1969, **312**, 85.

Table 1 *E.s.r. parameters of aquo-metmyoglobin*

			Ref.
$g_{\parallel}$		2.0	7, 10, 16
$g_{\perp}$		6.0	7
		5.92	10
		5.98, 5.86 ± 0.01	16
D		$10\,cm^{-1}$	12
		4.4	13
E		0.02	13
N haem	$^{N}A_x$	20.5 ± 1.5 MHz	
4 equivalent	$^{N}A_y$	30.0 ± 2.5	14
	$^{N}A_z$	7.6 ± 0.02	
^{57}Fe	A_{xx}	−27.05 ± 0.01	14
	A_{zz}	−27.77 ± 0.2	
N_{His}	$^{N\alpha}A_z$	11.46 MHz	14
H_2O	^{H}A	6.02 ± 0.08	15
haem meso ^{1}H		0.79 ± 0.008	15

Because the directions of the anisotropy in the haem plane do not correspond to those for low-spin derivatives, it was concluded that a factor beyond the influence of fifth and six ligands, such as puckering of the porphyrin ring, was responsible for the anisotropy. There seems to be no final consensus on the size or molecular cause of the rhombic distortion of the g_{app}-tensor.

Even when linewidths of e.s.r. spectra are very much larger than any hyperfine splitting, it is possible to determine, in favourable cases, the hyperfine splitting parameters by strongly irradiating n.m.r. transitions and observing changes in the e.s.r. intensity. This is ENDOR or electron nuclear double resonance spectroscopy. By ENDOR it has been possible to determine the z or parallel components of the porphyrin nitrogen, and the proximal histidine nitrogen, hyperfine coupling.[16] The small ^{14}N quadrupolar splitting constants have also been obtained from ENDOR line spacings. Proton ENDOR reveals hyperfine coupling from haem-bound water protons and from the four *meso*-protons of the haems.[17] These small hyperfine couplings, as measured by ENDOR, agree accurately with the dipolar calculations based on the known co-ordinates in Mb^{+}, and thus are not a consequence of electron delocalization. The complete hyperfine tensor for ^{57}Fe has been determined.

An intriguing effect, seen in single crystals of myoglobin and its derivatives, is the dramatic change in linewidth with change in angle to the magnetic field. For example, as the magnetic field direction is rotated in the haem plane, the linewidth varies from 40 to 130 G. This was studied in great detail by early workers in the field[12, 18] who concluded that in aquo-metmyoglobin there was a slight random misorientation of molecular axes within the crystal, and because of the large g-anisotropy, a standard deviation of only 1.6° was sufficient to explain their data.

[16] G. Feher, R. A. Isaacson, C. P. Scholes, and R. Nagel, *Ann. N.Y. Acad. Sci.*, 1973, **222**, 86.
[17] C. F. Mulks, C. P. Scholes, L. C. Dickinson, and A. Lapidot, *J. Am. Chem. Soc.*, 1979, **101**, 1645.
[18] P. Eisenberger and P. S. Pershan, *J. Chem. Phys.*, 1967, **47**, 3327.

More recently, Calvo and Bemski[19] have offered a detailed model that purports to explain the variation in linewidth in the haem plane based on a distribution of positions of fifth and sixth ligands. Probably both of these factors contribute.

Fluoro-metmyoglobin. The water molecule co-ordinated to the haem iron can be replaced by a fluoride ion, although high concentrations are required for complete conversion ($pK_b = 1.9$). In the single crystal study the ^{19}F nucleus ($S = \frac{1}{2}$) causes a splitting of 43 G on $g_{||}$ and slightly anisotropic splitting of 23.5 and 21.5 G in the haem plane.[20] This change of sixth ligand to a charged electronegative species reduces the zero field splitting parameter, D, to 5 cm^{-1}. The ENDOR spectrum of fluoro-metmyoglobin is very similar to that of aquo-metmyoglobin except that the hyperfine splitting from the histidine nitrogen is reduced in the former case by 0.5 MHz. Curiously, the *meso*-protons of the porphyrin show a slight increase in hyperfine splitting. The Mb^+F^- complex has been shown in careful single crystal studies to have its haem axis of symmetry titled 5° from that of the $Mb^+(H_2O)$.[21]

$Mb^+(HCO_2^-)$ derivatives have been shown, in a single crystal study, to exist in two forms having distinguishable g_{app}-tensors, each with distinct orientation.[21] The observation of two species may be related to the observation of two species for MbNO and $^{Co}MbO_2$ derivatives.

$Mb^+(OCN^-)$ has been studied in single crystals. The relatively large rhombic splitting gave in-plane g-values of 5.82 and 6.11.[22] The large E term is thought to arise from tilt of the OCN^- with respect to the haem plane, but it is not clear why there are two forms. This species is in thermal equilibrium with a low-spin form (*vide infra*). OCN^- is isoelectronic with N_3^- which only forms low-spin form and is discussed below.

B. Ferric Forms: Low-spin Derivatives.—Addition of strong ligands such as CN^-, N_3^-, imidazole or OCN^- causes the energy levels of the iron atom to split to such a degree that the five electrons are confined to the three t_{2g} orbitals. Thus a single electron remains unpaired. The measured g-values are now true g-values, which are a function of the total ligand field. Most theoretical interpretations originate from that of Griffith,[23-25] who divided the ligand field into rhombic and tetragonal components. Hence the data have been divided into five types depending on the ratio of rhombic to tetragonal components as shown in Figure 4.[25]

Mb^+CN^- has been studied in single crystal forms by several workers who determined principal g-values of (0.93, 1.89, 3.45) with g_x making an angle of approximately 30° with the projection of the proximal imidazoyl group onto the haem plane, g_z lying within 10° of the haem normal of $Mb^+(H_2O)$ and g_y making

[19] R. Calvo and G. Bemski, *J. Chem. Phys.*, 1976, **64**, 2264.
[20] H. Morimoto and M. Kotani, *Biochem. Biophys. Acta*, 1966, **126**, 176.
[21] E. F. Slade and R. H. Farrow, *Biochem. Biophys. Acta*, 1972, **278**, 450.
[22] H. Hori, *Biochem. Biophys. Acta*, 1971, **251**, 227.
[23] (*a*) J. S. Griffith, *Nature (London)*, 1957, **180**, 30. (*b*) J. S. Griffith, 'The Theory of Transition Metal Ions', Cambridge University Press, Cambridge, 1961. (*c*) J. F. Gibson, D. J. E. Ingram, and D. S. Schonland, *Faraday Soc. Discuss.*, 1958, **24**, 72.
[24] G. M. H. Loew, *Biophys. J.* 1970, **10**, 196.
[25] W. E. Blumberg and J. Peisach, 'Probes of Enzymes and Haemproteins', ed. T. Yonetani and A. Mildvan, Academic Press, New York, 1976, p. 215.

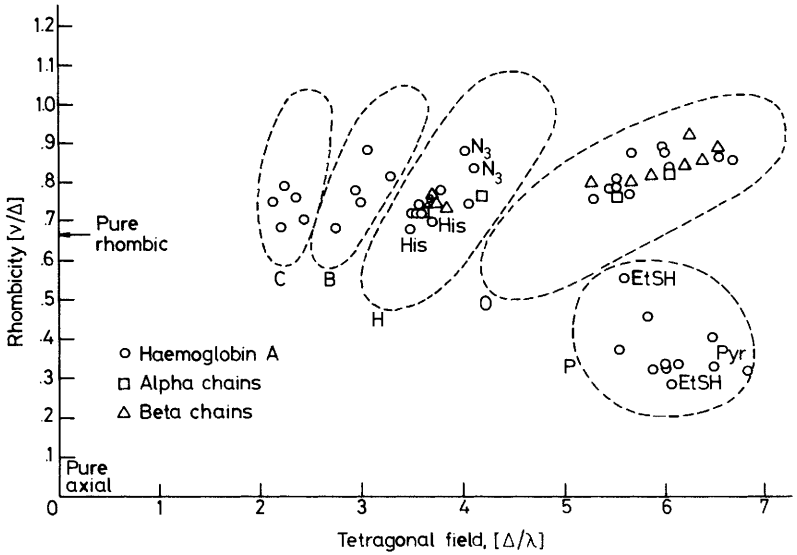


Figure 4 *Grouping diagram for many low-spin haem compounds. The five classes are divided according to relative values of V and Δ, computed from observed low-spin g-values*

an angle of 9° above the haem plane (Figure 5). Scholes has made a detailed ENDOR study of $Mb^+(^{13}CN^-)$ and deuteriated $Mb^+(^{12}CN^-)$[17] determining $|A_{zz}(^{13}C)| = 28.64 \pm 0.08$ MHz with evidence that the hyperfine splitting is nearly isotropic. This most probably means that spin polarization of the σ-electrons exceeds the expected dipolar coupling due to π-delocalization. Nitrogen ENDOR of $Mb^+(CN^-)$ has proven somewhat ambiguous in that the four lines detected can be interpreted as a single four-line multiplet from one type of nitrogen, or considering some weaker lines in the spectrum, there may be two inequivalent types of haem nitrogen. In $Mb^+(C^{15}N^-)$ additional resonances are seen indicating weak

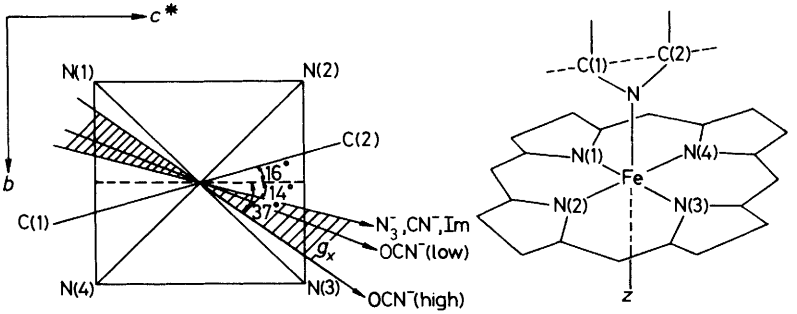


Figure 5 *Orientations of various low-spin haem derivative g-values with respect to the haem plane*

coupling of the ^{15}N to the iron electrons. No ^{57}Fe hyperfine splitting has been reported in $Mb^+(CN^-)$. All ^{14}N splittings seen by ENDOR are attributed to haem nitrogen atoms.[17]

$Mb^+N_3^-$ is perhaps the most thoroughly studied low-spin derivative of myoglobin.[12, 23a, 26] These single crystal studies substantially agree on (g_x, g_y, g_z) values of (1.72, 2.22, 2.80) but the reported angles from direction cosines, relating the directions of the principal values to the crystallographic (a, b, c^*) axes, differ by as much as 20°. There is agreement that the direction of g_z in $Mb^+N_3^-$ deviates about 7° from the direction of the haem normal. The g-tensors of CN^-, N_3^-, and Im complexes have very nearly identical orientations of the x, y, z axes.

The linewidth variation in the spectra of $Mb^+N_3^-$ with orientation has attracted a great deal of attention because of the possibility of observing variation of the strong ligand field.[12, 18] The minimal linewidth of 250 G is larger than that for $Mb^+(H_2O)$, indicating that such an influence is present. Eisenberger and Pershan[18] include terms for the variation of the rhombic distortion of the crystal field and form misalignment. They ignore distortions of the tetragonal field because these are expected to be much smaller. A $\pm 3.5\%$ variation in the rhombic distortion parameter and $\pm 2°$ in haem orientation satisfactorily explain the variation of linewidth in $Mb^+N_3^-$.

No hyperfine splitting was seen directly in the e.s.r. spectrum of $Mb^+N_3^-$, but ENDOR signals on $Mb^+N_3^-$ with ^{15}N-enriched haem have been studied in detail[17] at the g_x and g_y positions in the frozen glass. The nitrogen coupling constants were approximately equal along x and y. No azide ^{14}N splitting was seen, but two types of haem nitrogen were seen with $^{14}A_{zz} = 5.64$ and 6.14 MHz. The inequivalence could arise either from direct ligand electronic influence (either N_3^- or proximal imidazole nitrogen) or from porphyrin ring puckering. It is puzzling that this haem nitrogen inequivalence was observable in $Mb^+N_3^-$ and not in Mb^+CN^-. The azide derivative is known from X-ray crystallography to contain a linear ion bound to the haem at an angle of 11°. This bending probably arises primarily from steric reasons as is discussed below for MbNO and $^{Co}MbO_2$. This asymmetric influence of the azide ligand on the electronic structure of the porphyrin appears to be a source of inequivalence. One study attributes half of the g-anisotropy to electronic influences of N_3^- and the other to a dynamic Jahn–Teller effect.[27] The latter seems unlikely in the absence of any remaining degeneracy.

Single crystal studies of $Mb^+(OH^-)$ have been reported. $Mb^+(H_2O)$ and $Mb^+(HO^-)$ exist in equilibrium with a pK of 8.9. Two forms of $Mb^+(OH^-)$ (low-spin) are seen which interconvert at about the same pH.[23c] The principal g-values of Mb^+OH^- (1.85, 2.17, 2.55),[25] show a lower anisotropy than the CN^- or N_3^- derivatives, but theoretical considerations show that both rhombic and tetragonal fields are largest for this ligand. The interconversion of H_2O and OH^- forms has been studied in detail by other techniques but will not be treated here. No $Mb^+(OH^-)$ ENDOR data are available.

[26] J. F. Gibson and D. J. E. Ingram, *Nature (London)*, 1957, **180**, 29.
[27] S. Mizumashi, *J. Phys. Soc. Jpn.*, 1969, **26**, 468.

$Mb^+(Im)$ e.s.r. results have been reported for crystals of $Mb^+(H_2O)$ into which imidazole was diffused.[22] The spectra show that both high- and low-spin haems are present. The high-spin form is identical to $Mb^+(H_2O)$. The observed g-values for the second species in the crystal are (1.53, 2.26, 2.91) and the alignment of the tensor is nearly identical to that of $Mb^+N_3^-$.

$Mb^+(OCN^-)$, as mentioned above, has the ligand co-ordinated with the haem iron in such a way that there are high-spin and low-spin forms. In this case the high-spin form is quite different from $Mb^+(H_2O)$ as discussed above. The low-spin form shows a rather large g-anisotropy with g = (1.08, 2.02, 3.32) and an orientation of g_x in the haem plane intermediate between that of the high spin form and the N_3^- forms (see Figure 5). The z axis for $Mb^+(H_2O)$ makes angles of 39°, 123°, and 0° with the g_x, g_y, and g_z directions respectively in Mb^+OCN^-.[22]

Haemichromes. The protein conformation of myoglobin can be distorted from its native average structure by various chemical and mechanical agents. In the native ferrous case, this can result in an increase of oxidation to the inactive ferric state and attachment of an endogenous amino-acid side-chain as a sixth ligand to the iron. This has been extensively investigated for haemoglobins but not myoglobins. Some low-spin forms are seen in aged or poorly handled samples of myoglobin, so this possibility should be kept in mind.[10]

Sulphmyoglobin. A pathological physiological condition in which a single sulphur atom is added to the porphyrin ring of haemoglobin has been studied by preparing the simpler sulphmyogloblin. The ferric sulphmyoglobin gives g-values for the OH^-, N_3^-, and CN^- derivatives which differ from the corresponding native derivative.[28]

3 Monomeric Species: Ferrous Forms

The liganded ferrous forms of myoglobin are of keen interest because they more closely resemble native oxygenated myoglobin than do the ferric forms. The nitrosyl derivatives and the superoxide derivative that is formed by electron addition to MbO_2 at low temperature, however, are the only species to have an unpaired electron, which comes from the ligand. To what extent these are ferrous derivatives is discussed below.

A. MbNO

Most studies of nitrosyl haemproteins have been on frozen solution samples that yield spectra which are, in general, complicated by overlap and not unambiguous resolution. However, two types of spectra are seen for MbNO as discussed by two recent papers on the temperature dependence of the e.s.r. spectra. A frozen solution study by Morse and Chan[29] resolved the e.s.r. features into two components, one with a rhombic (type I) tensor (2.080, 1.998, 1.979) and the other with an axial (type II) g tensor (2.041, 1.983). A recent single crystal study by Hori *et al.*[30]

[28] J. A. Berzofsky, J. Peisach, and E. W. Blumberg, *J. Biol. Chem.*, 1971, **246**, 3367.
[29] R. H. Morse and S. I. Chan, *J. Biol. Chem.*, 1980, **255**, 7876.
[30] H. Hori, M. Ikeda-Saito, T. Yometani, *J. Biol. Chem.*, 1981, **256**, 7849.

resolved similar rhombic g-tensors at low temperature (2.076, 2.002, 1.979) as well as the complete hyperfine tensor (15, 17, 19 G) for one species. A second species, observed in MbNO could not be fully described.

Room temperature spectra gave not only a different g-tensor (2.050, 2.022, 1.993) and non-coincident $A(^{14}N)$-tensor (10.8, 14.6, 18.6) but the principal g-values (but not the A-values), had radically changed their direction! ^{14}N hyperfine splitting was seen along the direction of g_{min} in all cases. This has very important implications for changes of haem environment upon sample freezing. We consider herein only the room temperature data. This effect was not considered in earlier single crystal work on MbNO at 77 K.[29] ENDOR spectra on MbNO have been obtained[31] that yielded, in addition to the above ^{14}N splittings, ^{1}H splittings assignable to *meso*-protons of the porphyrin, $N_{\varepsilon 2}$ of the distal histidine, and the methyl protons of valine E11 on the distal side of the haem pocket. Replacement of the native iron with ^{57}Fe ($I = \frac{1}{2}$) gave a splitting of 5.5 G observed only along g_{min}.[32] For completeness, it is mentioned that a third type of nitrosyl-haem e.s.r. spectrum has been observed[33] and only recently assigned as a six co-ordinated complex with variant Fe—N distances and bond angles.[34] Among the many studies there have been warnings as to the use of e.s.r. spectra in distinguishing clearly between five and six co-ordinate nitrosyl haems and one cannot be too cautious about the ease of generation of denatured species.[35]

In solution at 26 °C MbNO shows an apparently axial e.s.r. spectrum with g_1, $g_2 = 2.026$, and $g_3 = 1.998$. The spectrum is not isotropic because of the slow tumbling time of the protein molecule. The spectrum is unchanged over a pH range of at least 6.3—9.4.[36]

Because of the convenience of describing directional results from single crystal studies by stereographic projections, we insert here a description of this depiction (Figure 6) so that the reader may easily grasp their content. The directional results from single crystal work on MbNO[37, 38] are shown in Figure 7. The data of reference 38 have been transformed to a co-ordinate system constructed from vectors connecting nitrogen atoms I and II, and connecting atoms I and III. The cross product of these vectors comes out of the plane of the paper and represents the haem normal on the distal side of the haem pocket. The co-ordinates used are Kendrew's.[39] No great stress is to be laid on the exact angles but we wish to discuss the general location of components.

It must be realized that for all myoglobin type A crystals, which are $P2_1$ monoclinic space group, there are two haems per unit cell. In the case of met-myoglobin where the g-tensor was axially symmetric, it was easy to assign the e.s.r.

[31] M. Höhn and J. Hüttermann, personal communication.
[32] L. C. Dickinson and J. C. W. Chien, *Biochem. Biophys. Res. Commun.*, 1973, **51**, 587.
[33] T. Yonetani, H. Yamamoto, J. E. Erman, J. S. Leigh, jun., and G. H. Reed, *J. Biol. Chem.*, 1972, **247**, 2447.
[34] T. Yoshimura, T. Ozak, Y. Shintani, and H. Watanabe, *Arch. Biochem. Biophys.*, 1979, **193**, 301.
[35] D. M. Scholler, M.-Y. R. Wang, and B. M. Hoffman, *J. Biol. Chem.*, 1979, **254**, 4072.
[36] E. Trittlevitz, H. Sick, and K. Gersonde, *Eur. J. Biochem.*, 1972, **31**, 578.
[37] L. C. Dickinson and J. C. W. Chien, *J. Am. Chem. Soc.*, 1971, **95**, 5036.
[38] H. Hori, M. Ikeda-Shito, and T. Yonetani, *J. Biol. Chem.*, 1981, **256**, 7849.
[39] J. E. Kendrew and H. C. Weston, personal communication.

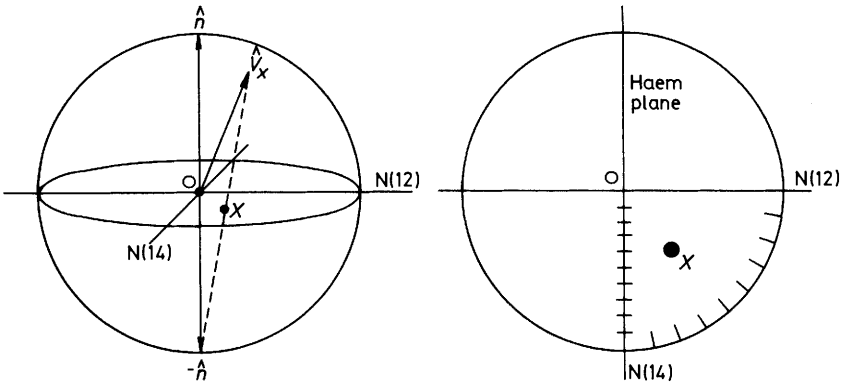


Figure 6 *Stereographic projection of principal directions of tensors are the best way to display all of the directional information from e.s.r. crystallography at a glance. The projection can be simply envisioned by beginning with a sphere of unit vector radius about an origin. We take the haem plane as the plane onto which we project the unit vectors of the principal directions.* $\hat{n}$ *is the normal to the haem plane. The projection point in the haem plane is then formed by 'sighting along' the line containing the tip of the vector,* $\hat{v}_x$*, to be projected and the point at the* $-\hat{n}$ *(or* $+\hat{n}$ *if* $\hat{v}_i$ *is below the plane). The point,* X*, at which the line strikes through the haem plane then represents the vector* V_x *in projection. If* V_x *is above the haem plane, we represent* X *by a solid symbol, if below, by an unfilled symbol. Stereographic projection distorts by spreading adjacent vectors near* n *and adjacent vectors near the haem plane, but is very useful for clearly combining relationships of vectors in a single diagram. The linear radial scale from the centre of the projection plane has values from* 0 *to* 1.0 *and are converted easily to angle values by the table below*

Linear value	*Angle to* n
0.0	0
0.1	5
0.2	12
0.3	18
0.4	27
0.5	36
0.6	47
0.7	57
0.8	67
0.9	78
1.0	90

$g = 2$ direction to a given haem normal from *X*-ray crystallographic results. However, this simplicity is lost once the *g*-tensor becomes rhombic and the rationale for assignment of one of the two sets of e.s.r. directions to a given haem becomes indirect.

In the NO case at room temperature, Hori *et al.*[38] assign the direction of the Fe—N(NO) bond to the minimum *g*-value, both because it is near free-spin ($g = 2.0$) and because it is close to a haem normal direction based on other e.s.r. and *X*-ray results. The assumption that the haem does not move significantly seems soundly based on a number of results on several myoglobin derivatives. However, based on our assignment of the electronic structure discussed below and variations expected in *g*-values with angle, the assignment of g_{min} as the Fe—N direction does

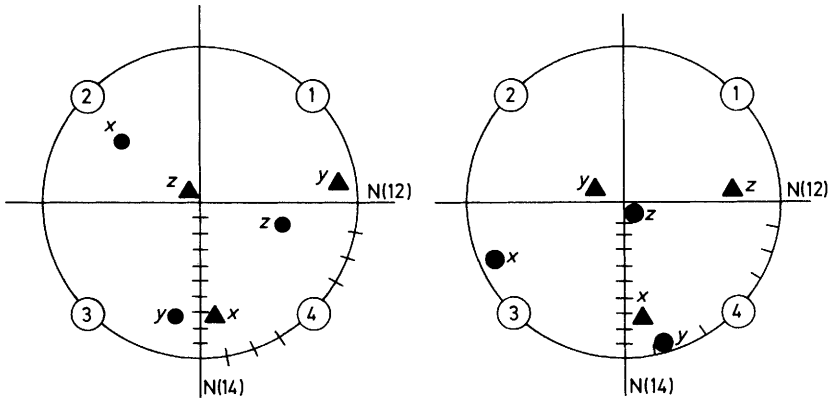


Figure 7 *Stereographic projection of principal directions for the g-tensor and A (^{15}N)-tensor of* MbNO *onto haem co-ordinates as described in text. Both projections are shown as there are two sites observed and there is no* a priori *way of selecting which one belongs to which haem.* ●. *A-tensor component direction;* ▲, *g-tensor component direction:* $A_x = 15.6$, $A_y = 21.4$; $A_z = 26.7$; $g_x = 2.050$, $g_y = 2.022$, $g_z = 1.993$

not seem on secure ground. Additionally, it should be pointed out that as g_{max} ($=g_x$) lies along the *b* (dyad) axis of the crystals, it remains the same in both haem co-ordinate systems of the crystal, but depending upon which set of e.s.r. tensors is assigned to which haem co-ordinate system, the directions of g_y and g_z are virtually reversed. While there are arguments for assigning a given e.s.r. tensor to one haem in the crystal, as presented in the cited papers, we wish to emphasize that these assignments are by no means final.

In the MbNO case, as mentioned above, there is no ambiguity in the assignment of g_{max} to (g_x) in the haem co-ordinate system, because of a coincidence of the direction of the *x* vector in that system. However, the assignment of *z* and *y* is open to question. These are the near free-spin *g*-values. As we argue below, the negative deviations from $g = 2.0023$ can arise from several terms so there can be no prediction as to whether g_{min} will occur perpendicular to the haem normal or perpendicular to the Fe—NO plane. The nitrogen hyperfine splitting shows a rather small anisotropy and cannot be resolved into axial dipolar tensors in a convincing way so that it does not contribute unambiguously to the assignment of a given tensor to a set of haem co-ordinates. Thus with MbNO the e.s.r. data in single crystals, except for the g_{max} assignment, can only be ambiguously related to the haem co-ordinates.

As a further *caveat* on the interpretation of single crystal e.s.r. data it must be realized that standard methods of diagonalization may be misleading in cases where the point group symmetry drops below orthorhombic. This situation has been recently reviewed[40] and it seems that off-diagonal elements in the *g*-tensor are insignificant, but that those for the *A*-tensor can be quite significant resulting in

[40] J. Pilbrow and M. R. Lowrey, *Rep. Prog. Phys.*, 1980, **43**, 432.

a rotation of the apparent principal axis of the metal hyperfine tensor with respect to those of the g-tensor. The electronic structural meaning of these off-diagonal elements in A cannot be simply interpreted. For cases where no axes of g and A are coincident, such as we observe here, the theory has not been derived. Thus one may expect that future analysis may greatly modify the directional data currently derived from e.s.r. for these haem proteins.

B. MbO_2^-

This species is of recent discovery. When oxymyoglobin was γ-irradiated in an aqueous glass at 77 K, electron capture occurred at the FeO_2 moiety.[41] This species is isoelectronic with $^{Co}MbO_2$, discussed below. Principal values of the g-tensor of the species formed (2.20, 2.11, 1.97) show smaller anisotropy than has been observed for normal low-spin Fe^{3+} forms, as is shown in Figure 8. A second signal, resolved in glycol–water glasses when the sample was annealed to temperatures above 77 K, had a larger anisotropy with g(2.295, 2.164, 1.942). The bonding in this complex requires the unpaired electron to be in a molecular orbital which includes the iron prominently and yet has sufficient density on the peroxy-ligand to explain the major splittings of 65 and 52 G for the ^{17}O atoms. The orientation of the g-tensor with respect to the haem axes (Figure 9) has been determined[41] and the results have been used to rationalize the bonding scheme (see below).

The MbO_2^- case offers a complicated electronic structure in that the spin-orbit coupling on the iron atom increases the g-anisotropy. The direction of g_{max} is expected to be near the normal of the haem plane. We have revised interpretation of the single crystal results given by Symons and Peterson,[41] and x is now believed to be about 30° off the haem normal. If our predictions are correct and g_{min} is along the 'hinge orbital' (π^*) direction then x is roughly the direction of the O—O tilt. The results can be compared with the recent X-ray diffraction study of Phillips, who found the dioxygen tilt in oxymyoglobin to be *ca.* 65° from the haem normal.[42]

C. Electronic Structure

For the normal ferric derivatives, high- or low-spin, there are no serious problems regarding electronic structure. In particular, e.s.r. studies clearly establish that the unpaired electrons are strongly confined to the metal atom, with relatively minor delocalization onto the porphin and histidine ligands. However, for the NO and O_2 derivatives there are several reasonable alternative structures, some with extensive delocalization onto these ligands.

The structural problems are far from being resolved, but it may help if we recall some results for electron addition to the nitroprusside ion, $[Fe(CN)_5NO]^{2-}$. Electron addition at 77 K gave a species in which the SOMO is largely confined to one of the π^* orbitals of NO. The relatively small value for g_z (along the NO bond) of

[41] M. C. R. Symons and R. L. Peterson, *Biochem. Biophys. Acta*, 1978, **535**, 241; M. C. R. Symons and R. L. Peterson, *Proc. R. Soc. London, Ser. B*, 1978, **201**, 285–300.
[42] S. E. V. Phillips, *J. Mol. Biol.*, 1980, **142**, 531.

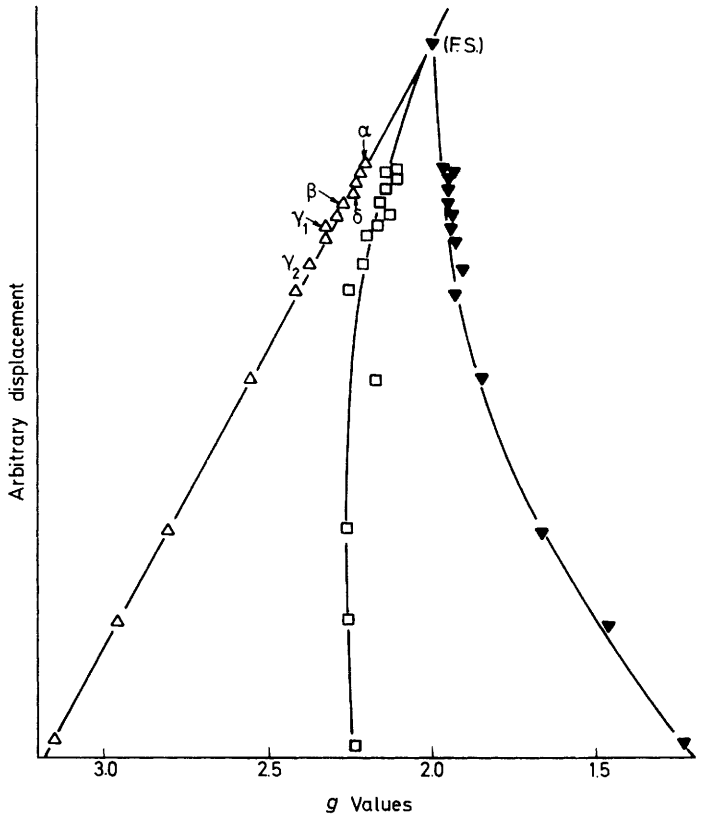


Figure 8 *Display of g-values for an arbitrarily selected range of low-spin ferric haem complexes* ($\triangle g_{max}$; $\square g_{int}$; $\blacktriangledown g_{min}$). *The trend in* g_{max} *values has been plotted linearly, and data for the various forms of* $(FeO_2)^-$ *derivatives* (α, β, γ) *discussed in the text have been included. This shows the* $(FeO_2)^-$ *derivatives, with extensive delocalization of the unpaired electron onto oxygen*

1.928 shows that the π-degeneracy is strongly lifted, there must have been a major bending distortion on electron addition.[43] On warming, this species was lost irreversibly, being replaced by a species having a $3d^1_{z^2}$ structure with major σ^* interaction with nitrogen. This was established by the form of the g-tensor components ($g_\perp \gg g_\parallel \approx$ free-spin) and of the ^{14}N hyperfine coupling ($A_\parallel > A_\perp \gg 0$). It seems clear that the axial cyanide ligand moves away as the electron moves onto the metal. In this case, there is less incentive for bending, and deviations from linearity are small.

E.s.r. results for MbNO and HbNO complexes can be used to distinguish between these alternatives.

[43] M. C. R. Symons, J. G. Wilkinson, and D. X. West, *Radiat. Phys. Chem.*, 1982, **19**, 309.

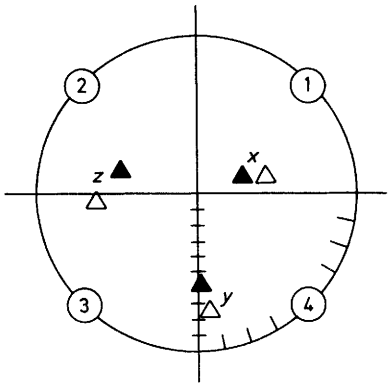
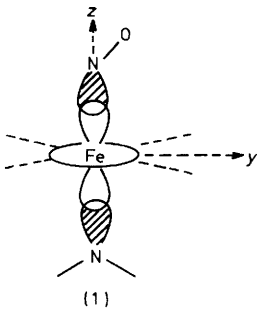


Figure 9 *Projection of the principal directions of the g-tensor of* MbO_2^- *onto the haem co-ordinate system* $x = g_{max}$; $y = g_{int}$; $z = g_{min}$. *Both possible projections of the e.s.r. crystal data are shown*

FeNO Derivatives. At least four different electronic structures have been proposed, attention being focused on the orbital of the unpaired electron (SOMO). We now outline these suggestions in their simplest form, and endeavour to select the most satisfactory in terms of the e.s.r. parameters.

Structure (1). *The* σ^* *model.* This places the electron into the $3d_{z^2}$ iron orbital, with slight delocalization onto the N(σ) orbital of N_ε and greater delocalization onto the NO(σ, π) orbital.[45]



(1)

Structure (2). *The* π^* *(*d_{yz}*) model.* In this case, π_y^* is placed below the two σ/π levels, and for this structure, the major contributor is thought to be d_{yz} (Fe).

Structure (3). *The* π^* (NO) *model.* This is the same π_y^* level as in (2), but the contribution from the π^* (NO) orbital is envisaged as being greater than that from the d_{yz} (Fe) orbital.[46,47]

Structure (4). *Doetschman's model.* In a detailed attempt to accommodate the g- and A-values for various nitrosyl derivatives, Doetschman[44] has proposed a level which involves an admixture of almost all possible levels, namely π_y^* (0.18), π_x^* (0.35), d_{xz} (0.24), d_{z^2} (0.23). In order to account for the large ^{14}N (NO) hyperfine coupling he invokes admixture of the σ-(NO) level in addition to all these. We assume that this implies that π_x^* is the (σ/π) level in our scheme. In that case, this orbital somewhat resembles an admixture of our $(\sigma/\pi)_1$ and π_y^* levels.

A preferred-level scheme. The reason for this proliferation of models is surely the fact that the e.s.r. results are remarkably complex, especially when directions for the various tensor components are taken into account. However, there are certain aspects of the results common to all examples which, in our view, point firmly towards structure (1), the σ^*-structure, as the correct one. The case for (1) is as follows:—

(a) The g-shifts are small. Almost all complexes with d_{xz}^1-type structures exhibit large g-shifts, including the $(FeO_2)^-$ species, which have two electrons more than the (FeNO) species.

(b) The large value of A_{iso} (^{14}N) is typical of a σ^*-structure,[43] and cannot, in our view, be accommodated by a π^*-structure. Indeed, the limit of a π^*-structure is NO itself, which has $A_{iso} \approx 7$ G—a factor of three less than the values for (FeNO) complexes, which have a far lower spin density on NO.

(c) The very small magnitude of the ^{14}N anisotropic hyperfine coupling for N_ϵ also firmly establishes a σ-interaction rather than a π-interaction. The small magnitude of A_{iso} (*ca.* 6 G) shows that delocalization onto this ligand is small, which probably indicates weak bonding. This, in turn, may explain why this bond is relatively readily broken in the $R \rightleftharpoons T$ change. Partial displacement of this ligand accords nicely with results for $Fe(CN)_5NO^{3-}$ discussed above.

(d) The form of the ^{57}Fe coupling also accords best with a $d_{z^2}^1$ configuration on iron. The maximum coupling of *ca.* 6 G lies close to the haem normal, as required for $d_{z^2}^1$. Its magnitude (*ca.* 6 G) requires considerable population of this level, comparison with results for CoMb indicating at least 50% spin-density therein.

(e) The negative g-shift. The fact that there is a small negative shift for one of the g-values has been taken as clear evidence against the σ^*-structure.[44] However, our scheme suggests that the σ^*- and π_y^*-levels are relatively close together, in which case a negative g-shift stems naturally from coupling between them. Thus the σ^*-model *requires* that there be one negatively shifted g-value, as observed.

Against structure (2) for the Fe(NO) derivatives (π^*:d_{yz}^1) is the small magnitude of Δg. Relatively large positive g-shifts are expected for this structure. The only way to explain the small shifts observed would be to postulate very low spin-density on iron: this leads us to structure (3) (π^*:NO).

If the unpaired electron is largely in a pure π^*-level on NO, the hyperfine

[44] D. C. Doetschman, S. A. Schwartz, and S. G. Utterback, *Chem. Phys.*, 1980, **49**, 1.

coupling to ^{14}N should tend towards that for NO itself, and the results should resemble those for the π^*-structure of the nitroprusside ion. In particular, small isotropic and a relatively large anisotropic coupling constants to ^{14}N are required, quite different from those observed. Again, this model would require negligible spin-density of N_ε. None of these expectations is fulfilled, so we neglect this model.

We conclude that the simple σ^*-structure (1) is quite satisfactory for the nitrosyl derivatives. In particular, it nicely explains why there is some tendency for the proximal histidine ligand to move away from iron for some of these complexes.

FeO_2^- Derivatives. Two possible structures can again be envisaged. The oxy-derivatives contain one electron more than the NO derivatives, and it is therefore tempting to suggest that the extra electron be placed in the d_{z^2} orbital to give the diamagnetic complex. However, this neglects the extra nuclear charge on the ligand, and does not explain the large degree of bending observed for HbO_2 and MbO_2. It seems to us possible that the d_{z^2} σ^*-orbital remains unoccupied for the oxy-derivatives, the extra two electrons being accommodated in one of the π^*-orbitals on oxygen, with some antibonding interaction with the iron orbitals.

Addition of a third electron once again presents us with a problem, since this could go into the d_{z^2} σ^*-orbital or into the other π^*-orbital (the 'hinge' orbital). The e.s.r. results strongly support the latter concept. Thus the form of the g-tensor components is remarkably similar to that for low-spin Fe^{III} complexes, except that the g-shifts are reduced. Also, the form of the ^{17}O hyperfine coupling is in accord with this π^*-structure, and rules out the σ^*-structure. The fact that when these unstable $(HbO_2)^-$ complexes are warmed to room temperature there is loss of peroxide, leaving an Fe^{III} derivative, also strongly supports this formulation.

The $(MbO_2)^-$ derivative discussed herein is isoelectronic with oxycobalt myoglobin, which has been extensively studied by e.s.r. spectroscopy, as outlined below. The results show that the SOMO is now largely confined to oxygen. Replacement of iron by cobalt must lower the d_{z^2} orbital so that there is now a high probability that the d_{z^2} σ^*-orbital will be doubly occupied. Indeed, the structure can be simply understood in terms of binding O_2 to the deoxy-cobalt derivative, which has its unpaired electron largely confined to the d_{z^2} orbital. On binding, one of the π^*-electrons of oxygen pairs with this electron, leaving the other in the 'hinge' π^*-orbital largely confined to oxygen.

In fact, there are several forms for these oxy-cobalt complexes, including a remarkable change in structure on cooling. It is just possible that there is an electronic switch in structure between the $(\pi_1^*)^2(\pi_2^*)^1$ structure envisaged for the $(MbO_2)^-$ complexes and the $(d_{z^2})^2(\pi_1^*)^1$ structure normally envisaged for the cobalt analogues.

4 Haemoglobin Derivatives: Ferric Forms

A. High Spin

The haemoglobin tetramer has received a great deal of attention using e.s.r. techniques because the e.s.r. parameters are sensitive to subtle electronic structural changes at the O_2 bonding site. Thus, for example, e.s.r. spectroscopy can be used

to detect conformation shifts between '*R*' and '*T*' states. One must appreciate the difficulties in partitioning the results between differences in *R* and *T* states, and differences between α- and β-chains.[45-47]

$Hb^+(H_2O)$. As with myoglobin the initial classical work resulted in the determination of the orientation of the haem planes relative to the crystallographic axes,[9] and relative to the then crudely pictured protein chain.[48] In the monoclinic horse methaemoglobin crystals there is by symmetry effectively one tetramer per unit cell, so that only four e.s.r. signals are observed. No differences can be observed between α- and β-chains in the single crystals. Early frozen-solution work with signals from isolated α- and β-chains in the ferric state led to observations of differences, later related to denaturation.[49] An observation has also been made that the ferric α-chain in frozen solution shows a splitting of the $g = 6$ feature which is removed upon binding of the α-chains to β-chains.[50] However, later studies show no such anisotropy.[51] An extended theoretical analysis has been made to understand the anisotropy of the fundamental linewidth/relaxation times of the Hb^+ e.s.r. signal. It was concluded that a major contribution to T_1 anisotropy arises from nitrogen spin-state mixing[52] of the electronic wave function at 4 K. No change was seen in the fundamental linewidth in Hb^+ single crystals upon replacement with D_2O, although a reduction in the minimal linewidths or second moment was expected. A 4° haem misalignment in both *a*- and β-haems can explain the angular dependence of the linewidths.[53] The linewidths of haemoglobin solutions have been found not to vary significantly with temperature.[54] The effects of pH on the intensity and form of the e.s.r. signals of ferric haemoglobins have been followed showing alkaline (pH 11), acid (pH 4—5), and heat-denatured (pH 3—4) forms, as well as the conversion from the aquo into the hydroxy form.[55]

ENDOR studies of ferric haemoglobin gave similar results to those for myoglobin.[16] Hb_m Hyde Park (β92His → Tyr) does not give a nitrogen histidine ENDOR signal. In $Hb_{Milwaukee}$ (β67Val → Glu) where the β-chains are ferric, a shift in ENDOR signals is seen with oxygenation of the α-chain,[16] however, hybrid haemoglobin $A(\alpha_2\beta_2^+)$ did *not* show the same shift. Other e.s.r. work with hybrids[56] gave evidence for α–β interaction in the $\alpha_2^+\beta_2$ hybrid only, although the method of β-chain preparation has been called into question.[57] The hyperfine

[45] S. K. Mun, J. C. Chang, and T. P. Das, *Proc. Natl. Acad. Sci. USA*, 1979, **76**, 4842.
[46] J. C. W. Chien, *J. Chem. Phys.*, 1969, **51**, 4220.
[47] E. Trittlevitz, K. Gersonde, and K. H. Winterhalter, *Eur. J. Biochem.*, 1975, **51**, 33.
[48] D. J. E. Ingram, J. F. Gibson, and M. F. Perutz, *Nature (London)*, 1956, **178**, 906.
[49] Y. Henry and R. Banerjee, *J. Mol. Biol.*, 1970, **50**, 99.
[50] J. Peisach, W. E. Blumberg, B. Wittenberg, J. Wittenberg, and L. Kampa, *Proc. Natl. Acad. Sci. USA*, 1969, **63**, 934.
[51] J. Peisach, W. E. Blumberg, S. Ogawa, E. A. Rachmilewitz, and R. Oltzik, *J. Biol. Chem.*, 1971, **246**, 3342.
[52] A. S. Brill, C.-I. Shyr, and T. C. Walker, *Mol. Phys.*, 1975, **29**, 437.
[53] A. S. Brill and D. A. Hampton, *Biophys. J.*, 1979, **25**, 313.
[54] T. Asakura, G. Reed, and J. S. Leigh, jun., *Biochemistry*, 1972, **11**, 334.
[55] T. C. Hollocher and T. M. Buckley, *J. Biol. Chem.*, 1966, **241**, 2976.
[56] R. Bannerjee, F. Stekowski, and Y. Henry, *J. Mol. Biol.*, 1973, **73**, 455.
[57] M. F. Perutz, E. J. Heidner, J. E. Ladner, J. G. Beethstone, C. Ho, and E. F. Slade, *Biochemistry*, 1974, **132**, 187.

splitting parameters for ^{57}Fe are about 2% smaller than in ferric myoglobin.[58] *Hb*$^{+}$*F*$^{-}$. $Hb^{+}F^{-}$ gives an histidine ENDOR splitting about 0.5 MHz lower than in $Hb^{+}(H_2O)$ indicating that there is a trans effect of fluoride through the haem plane.[16] The zero-field splitting in $Hb^{+}F^{-}$ is also substantially reduced compared to $Hb^{+}H_2O$.[15]

We conclude that no clear cut inequivalence of α- and β-chain structures is seen in high-spin ferric haemoglobin.

B. Low Spin

The OH^{-}, CN^{-}, and N_3^{-} derivatives of haemoglobin have been studied to some degree but none show significant interpretable differences from the corresponding myoglobin derivatives discussed above and none show any α–β inequivalence. Attention has been addressed to low-spin forms generated by protein denaturation.[51,55] ENDOR spectra for $Hb^{+}(^{13}CN^{-})$ show a 1.5 MHz (12%) smaller z component of the $A(^{13}C)$ in haemoglobin as compared with myoglobin. However, the splitting from *meso*-protons is 1.19 MHz in myoglobin cyanide and 1.34 in haemoglobin cyanide. The ENDOR of haemoglobin azide is also somewhat different from that of myoglobin azide with an unclear but possible indication of alpha–beta inequivalence in the former.[17]

5 Haemoglobin Derivatives: Ferrous Forms

A. Nitrosylhaemoglobin.—In many ways, for the e.s.r. spectroscopist this is the most important haemoglobin derivative since the spectra of HbαNO and HbβNO are quite distinct and the e.s.r. spectra are remarkably sensitive to the $R \rightarrow T$ shift in conformation of the tetramer. This haemoglobin derivative was curiously neglected until about 10 years ago following a paper on horse single-crystal HbNO[46] and model compounds[59] though there were earlier cursory reports on this derivative.[60–62]

Rein *et al.*[60] noted that the tetramer, human haemoglobin A, gave two distinct types of e.s.r. spectra (Figure 10) which were interconverted by addition of inositol hexaphosphate, IHP, an effector molecule known to promote the $R \rightarrow T$ transition. In the absence of IHP the rhombic spectrum has a feature at $g = 2.06$ and a number of less well resolved shoulders in the $g = 2.0$ region. The addition of IHP gives rise to a complicating change in the $g = 2.06$ region and a very characteristic three line hyperfine pattern centred at $g = 2.00$ split by 16.6 G. Work on separated subunits[61] showed that the HbNO α- and β-chains gave distinct e.s.r. spectra, the β-subunit spectrum being more symmetric than that of the α. At pH 7.4 the

[58] C. P. Scholes, R. A. Isaacson, T. Tohetani, and G. Feher, *Biochem. Biophys. Acta*, 1973, **322**, 457.
[59] (*a*) H. Kon, *J. Biol. Chem.*, 1968, **243**, 4350. (*b*) H. Kon and N. Katoaka, *Biochemistry*, 1969, **8**, 4757.
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[61] T. Shiga, K.-J. Hwang, and I. Tyuma, *J. Biol. Chem.*, 1968, **243**, 203.
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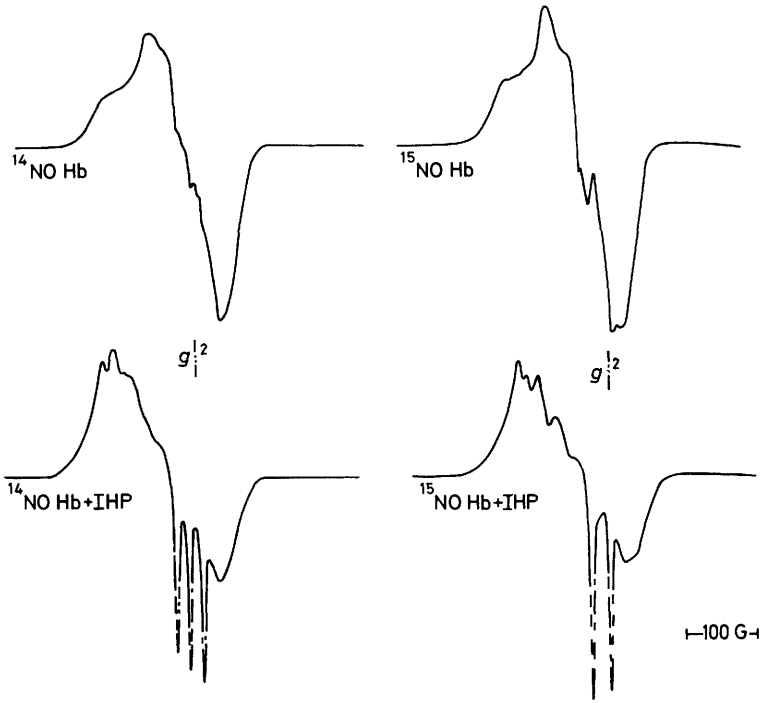

Figure 10 *E.s.r. spectra of* Hb_ANO *at pH* 6.3 *with and without* IHP

spectrum of the combined tetramer was that of the arithmetic sum of the spectra of the separated chains.

The e.s.r. spectra of a number of nitrosyl haemoprotein complexes have been grouped into two types based on $g_y <$ or $>$ 1.96,[63] and more recently a third type of haem–NO e.s.r. spectrum has been characterized, as discussed in the section on MbNO above. This work has been extended by Henry and Banerjee[64] to a study of HbNO chains and tetrameric hybrids of the type $\alpha_2(X)\beta_2(NO)$ or $\alpha_2(NO)\beta_2(X)$ where X means deoxy, oxy, carboxy, or ferric (H_2O, F^-, N_3^-, or CN^-). The e.s.r. spectrum of $\alpha_2(NO)\beta_2(X)$ was found to depend upon the spin state carried by the haem in the β-chain. For low-spin X the e.s.r. spectrum of $a_2(NO)\beta_2(X)$ was identical to that of the free chain, but for high-spin X the e.s.r. spectrum was modified. The $\alpha_2(X)\beta_2(NO)$ spectra were found to be invariant with X.

Further work by Gersonde and co-workers[65] on HbNO extended careful measurement with both ^{14}NO and ^{15}NO to several mutant haemoglobins. Although

[63] T. Yonetani, H. Yamamoto, J. E. Erman, J. S. Leigh, jun., and G. H. Reed, *J. Biol. Chem.*, 1972, **247**, 2447.
[64] Y. Henry and R. Banerjee, *J. Mol. Biol.*, 1973, **73**, 469.
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structural inequivalence is unambiguously demonstrated by the inequivalence of e.s.r. spectra of α- and β-nitrosyl derivatives, the two subunits have been shown by rapid-freeze e.s.r. techniques to react at the same rate with NO.[66] In contrast, a long time-scale NO titration technique showed that β-haems react in deoxyhaemoglobin four-fold faster than α-haems.[68]

Trout blood contains four distinct haemoglobin tetramers, which show a range of oxygen affinities and dependence of P_{50} on pH and temperature, thus offering a variety of examples of R–T shift with conditions. Component 4, with a strongly pH-dependent oxygen affinity, also shows a pH dependence of the e.s.r. spectrum of $Hb_{IV}NO$. Component 1, with pH-independent oxygen affinity, shows no change in the e.s.r. spectrum of $Hb_{I}NO$ with pH.[67]

A frozen-solution work on $Hb_{Kansas}NO$ suggested the presence of four spectral components.[69] Subsequent single-crystal work on $Hb_{Kansas}NO$, which is locked in the T state, showed two types of haem–NO moieties. The β-haems show a splitting from the $^{14}N_{\varepsilon}$ histidine atom, and the α-haems show no $^{14}N_{\varepsilon}$ histidine splitting.[70] This gave e.s.r. spectral evidence for five and six co-ordinated haems. It is interesting to note that infra-red spectroscopic evidence[71] had been previously used to infer such a change in structure. This structural difference was first deduced from frozen-solution e.s.r. by Szabo and Perutz.[72]

In marked contrast, a study of carp nitrosyl haemoglobin in the R and T states reveals only slight differences for these two forms in the e.s.r. parameters in comparison with human nitrosyl haemoglobin. The authors stress the ease of generation of denatured HbNO species[35] and the fact that this has a unique e.s.r. spectrum assigned to the ligand-off species.

A very recent single crystal e.s.r. study of $Hb_{A}NO$ by Doetschman and Utterback[73] reported the photolytic replacement of NO by O_2 and re-attachment kinetics of NO in subunits at 9 °C finding that the α-subunits exchange with O_2 faster than β-subunits. The crystal e.s.r. spectra of the tetramer should display hyperfine coupling to ^{14}NO and [^{14}N]histidine ligands for both α- and β-chains.

B. Peroxyhaemoglobin.—As was described above for γ-irradiated oxymyoglobin, electron-addition centres were first discovered in oxyhaemoglobin.[42] Because these centres are isoelectronic with oxycobalt haemoglobin they offer an exciting structural pair for comparison. Additionally, separate centres were found that are associated with α- and with β-chains. Further centres are formed upon annealing to higher temperature and upon using a different matrix, *i.e.*, glycol *versus* water–buffer only.

[66] R. Hille, G. Palmer, and J. S. Olson *J. Biol. Chem.*, 1977, **252**, 403.
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6 Metal-replaced Derivatives

The replacement of iron by other metal ions can yield species which are benefically studied by e.s.r. As with myoglobin, oxy- and deoxy-cobalt haemoglobins are both paramagnetic. ^{Co}Hb reversibly and co-operatively binds O_2 at a reduced affinity compared to Hb.[74, 75]

A. Cobalt Derivatives

^{Co}Hb. The first successful metal-replacement technique for cobalt was accomplished by Hoffman.[74] Subsequent work in other laboratories led to a single crystal study[76] of horse ^{Co}Hb which demonstrated that the haem normals differ in direction from those in methaemoglobin crystals by as much as 30°. Resolution in one plane was poor so that the complete tensor and orientation could not be determined. Frozen solution spectra give a large $A_{\|}(Co) = 76$ G, with ^{14}N splitting of 17 G. Proto-, meso-, and deutero-cobalt porphyrins were substituted into haemoglobin by Yonetani *et al.*[75] with slight changes resulting in the e.s.r. parameters. A table of e.s.r. properties for these species can be found in reference 75 for the oxy- and deoxy-derivatives. These workers observed a small but distinct narrowing of the e.s.r. lines when D_2O replaced H_2O in solutions of $^{Co}HbO_2$. This is taken as evidence that a water molecule is involved in the influence of the distal histidine upon oxygen bonding. This effect was not seen in *Glycera* haemoglobin which lacks a distal histidine.[77] Subsequent studies[78] of $^{17}O_2$-^{Co}Hb showed that there are two non-equivalent oxygen atoms which eliminated the Griffith π-bonding model for the Co—O—O bond. A very productive aspect of metal replacement has been the preparation of hybrid haemoglobins, that is, tetramers such as $\alpha_2(Co)\beta_2(Fe)$ or $\alpha_2(Fe)\beta_2(Co)$.[79] The iron-containing subunit is e.s.r. silent and thus the differences between Co in the α- and β-subunits can be observed by e.s.r. in the tetrameric state. The e.s.r. spectra of each oxygenated hybrid differ, but each is the same as the spectrum of the corresponding separated subunit.[80] The tetrameric ^{Co}Hb gives an e.s.r. spectrum which is the arithmetic sum of the spectra of the separated subunits. The main difference between the e.s.r. spectra of $^{Co}Hb_\alpha(O_2)$ and $^{Co}Hb_\beta(O_2)$ is that the former has sharper lines. The deoxy-separated chains give indistinguishable e.s.r. spectra. Thus the Co–Fe hybrids are not useful indicators of co-operative effects between subunits.

^{Co}Mb. As with the ferric forms, cobalt myoglobin offers a simpler case more amenable to single-crystal study than cobalt haemoglobin. ^{Co}Mb gives e.s.r. parameters shown in Table 2. The orientation of the g-tensor[81] was found to be identical

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[79] M. Ikeda-Saito, H. Yamamoto, and T. Yonetani, *J. Biol. Chem.*, 1977, **252**, 8639.
[80] M. Ikeda-Saito, H. Yamamoto, K. Imai, F. J. Kayme, and T. Yonetani, *J. Biol. Chem.*, 1977, **252**, 620.
[81] L. C. Dickinson and J. C. W. Chien, *Proc. Natl. Acad. Sci. USA*, 1972, **69**, 2783.

Table 2 *E.s.r. parameters for cobalt haemoglobin and myoglobin*

	$g_{1.1}$	$g_{1.2}$	g_{11}	A_1^{Co}	A_{11}^{Co}	a^N	
^{Co}Mb	2.33	2.32	2.02	6	79	17.5	7
$^{Co}MbO_2$	1.989	2.006	2.083	16.7, 5.95	9.3	—	7
^{Co}Hb	2.31	2.037		6.2	76	17.5	6, 12
$^{Co}MbO_2$	2.00	2.008		—		16	2

to that in $^{Fe}Mb^+(H_2O)$ with respect to the crystallographic axes. A very small anisotropy is observed in the haem plane g-value. Because ^{Co}Mb is an $S = \frac{1}{2}$ species there are no zero-field complications as in the case of iron species. Thus, this g-anisotropy must arise from ligand influence.

$^{Co}MbO_2$. The single crystal $^{Co}MbO_2$ study showed that the g-tensor and the cobalt hyperfine tensor do not share the same principal axes.[81] In fact, it was assumed that the cobalt hyperfine tensor had a principal plane in the porphyrin plane and that the g-tensor was orientated with the O—O axis along the major g-principal value direction. The latter assumption was proven correct in single crystal $^{Co}Mb^{17}O_2$ studies, but some corrections to the first assumption caused a revision of the estimated Co—O—O angle from 90° to 120°.[82] The complete ^{17}O hyperfine tensor was resolved for each inequivalent oxygen atom allowing spin-density calculations for both π-orbitals of the O_2 moiety.

The e.s.r. data for $^{Co}MbO_2$[74, 79, 81] show a markedly reduced ^{59}Co hyperfine interaction, typical of a superoxide derivative rather than a Co^{II} species. Thus, in terms of the π^* structure postulated for the isoelectronic $Mb(O_2^-)$ species discussed above, the spin density has shifted markedly from the metal to the di-oxygen ligand. This is expected on electronegativety grounds for the antibonding electron, and indeed, it establishes the antibonding character of the orbital. The ^{59}Co coupling can be interpreted in terms of a maximum of about 10% spin population of the cobalt d_{yz} orbital, but it may be less than this, since spin-polarization is expected to make a considerable contribution also.

Studies of $^{17}O_2$-enriched samples[82] confirm that the spin is largely localized on the two oxygen atoms. The data also confirm the π_y^* character of the orbital in good accord with our simple model.

One important aspect of this work[81] was the discovery of two forms of $^{Co}MbO_2$ at 77 K which differ markedly in their g- and A-tensor orientation with respect to the haem plane. It now seems that at room temperature there exists only one form, which clearly differs from both low-temperature forms.[83, 84] This result ties in well with the behaviour of MbNO outlined above. It is of great importance in that it implies a significant change in protein conformation upon freezing. Since many such studies are carried out at 77 K or below, the possibility of such changes must always be borne in mind.

As in all these studies, difficulties are experienced when analysing tensor direc-

[82] L. C. Dickinson and J. C. W. Chien, *Proc. Natl. Acad. Sci. USA*, 1980, **77**, 1235.
[83] H. Hori, M. Ikeda-Saito, and T. Yonetani, *J. Biol. Chem.*, 1982, **257**, 3636.
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tions. Ideally this should be done in the light of single-crystal *X*-ray results at the same temperature. The near room temperature e.s.r. results have indeed been compared with the $-30\,^{\circ}C$ *X*-ray data, but the placement of the near free spin *g*-value for $^{Co}MbO_2$ along the O—O axis leads to serious contradiction with all previous sense of the theory of bound π-ligands. Thus some error must be suspected. Our computation of the *g* and *A* principal directions in the porphyrin co-ordinate frame from the direction cosines in the references 83 and 84 do not lead to the directions reported in those references. Further, as discussed above there is a two-fold ambiguity in assigning a given tensor orientation to either of the two haems per unit cell. The two possibilities are depicted in Figure 11. The correspon-

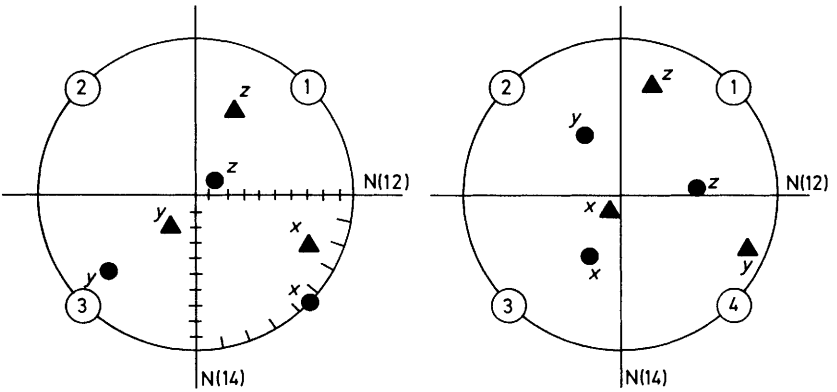

Figure 11 *Stereographic projections for principal g and A directions of* $^{Co}MbO_2$ *at room temperature for our assignment as discussed in the text which disagrees with that of the original authors.*[81,82] ▲, *A component direction,* ●, *g-component direction.* $A_x = 186$, $A_y = 11$, $A_z = 7.3$; $g_x = 2.056$, $g_y = 2.011$, $g_z = 2.003$. *Both possible projections are shown, although we favour the projection on the left*

dence of the O—O direction with $g = 2.00$ presents us with severe difficulties. Our expectations for an O_2^- ligand is that g_{max} should lie along the O—O bond direction, as with O_2^- itself. Indeed this argument was originally used to decide upon the Co—O—O bond angles and direction for the 77 K species.[81] However, it is inferred for the room temperature data that g_{min} (2.003) lies close to this direction! We reject this assignment on the grounds that O_2^- is a π-ligand and must have its largest *g*-value along, or nearly along, the O—O axis.

Our own calculations based on the angles given by Hori *et al.* yield similar results for the most reasonable assignment of the ^{Co}A tensor. That is, *z* is near the porphyrin normal, and *x* and *y* are near the porphyrin plane. Our calculations of the *g*-tensor orientation do not agree with those of Hori *et al.* in that $g_{max}(g_x)$ is found to lie near the *X*-ray crystallographic O(1)—O(2) direction, $g = 2.03$ is within about 18° of the porphyrin normal, and $g = 1.98$ is 43° from the porphyrin plane. This seems to remove a major objection to our general bonding scheme. The observation that the ^{17}O principal directions coincide with the *g*-tensor direction

also supports our scheme. The major splitting is along the hinge direction, or y direction in Figure 11a, thus supporting π^*-occupancy for $^{Co}MbO_2$.

When oxygenated $^{Co}MbO_2$ containing mesoporphyrin is photolysed at low temperature and observed at high microwave power, broad features appear in the e.s.r. spectrum at $g = 3.87$ and 1.9, attributable to an intermediate containing high-spin cobalt ($S = \frac{3}{2}$). This has been observed in ^{Co}Mb of several species.[75, 77, 83, 85]

γ-Radiolysis of Co^{III} myoglobin at 77 K has been shown to generate in high yield a six co-ordinate Co^{II} species which is not observable in room temperature Co^{II} solutions. This means that Co^{III} myoglobin has an endogenous strong sixth ligand, probably the distal histidine nitrogen.[86]

B. Manganese Derivatives

Manganese porphyrins substituted into haemoglobin and myoglobin give e.s.r. spectra which are somewhat sensitive to the environment of the prosthetic group and are thus of value as probes. Mn^{II} porphyrin complexes, like the Fe^{III} ones, have five unpaired electrons, so that observed g-factors are dependent upon zero-field splitting parameters. Unfortunately, ^{Mn}Hb and ^{Mn}Mb oxidise very rapidly to Mn^{III} and the oxygenated state cannot be observed. Frozen-solution spectra of ^{Mn}Hb at X-band show a prominent seven-line feature at $g = 5.9$ and a weaker sextet centred at $g = 2.0$. The hyperfine splitting is approximately $A_{\perp} = 0.0073\,cm^{-1}$, $A_{\parallel} = 0.011\,cm^{-1}$.[87] The hyperfine lines on the perpendicular region serve as an effective vernier on the g-rhombicity and this is used to estimate zero-field splitting. For ^{Mn}Hb, $D = 0.5$, $E = 0.0056\,cm^{-1}$; for ^{Mn}Mb the respective parameters are 0.56 and $< 0.0028\,cm^{-1}$. No single crystal e.s.r. work on ^{Mn}Hb or ^{Mn}Mb has been published, although $^{Mn}Hb^{+}$ has been shown to crystallize isomorphously with the native Hb^{+}.[88]

C. Zinc Derivatives

^{Zn}Hb and ^{Zn}Mb have been prepared and give upon photolysis an excited triplet state. The e.s.r. parameters of these species are sensitive to the environment of the zinc porphyrin and to the presence or absence of the vinyl side-chains on the porphyrin.[89]

D. Other Derivatives

Metalloporphyrins of copper, silver, nickel, chromium, ruthenium, and rhenium have been added to apohaemoglobin and apomyoglobin. The copper and silver(II) derivatives show hyperfine splitting in the e.s.r. spectrum. Nickel(II) surprisingly gives an e.s.r. spectrum indicating high symmetry and the chromium(III) complex

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gives spectra very different from those for model compounds, showing significant interaction with the protein ligands.[90]

7 Leghaemoglobin

This review has been concerned with mammalian haemoglobin. We conclude by making brief reference to some e.s.r. studies of a species of haemoglobin which has been extracted from the roots of soy bean and from cow pea plants. It differs from mammalian haemoglobin in its amino-acid sequence, but is very similar in the region of the haem group. It is normally described as leghaemoglobin. E.s.r. studies of the ferric form co-ordinated with F^-, OH^-, N_3^-, CN^-, and $MeCO_2^-$ have been reported, the results being similar to those for the corresponding mammalian haemoglobin derivatives.[91, 92] The nitrosyl derivative has also been studied,[92] as have the oxy- and deoxy-forms of the cobalt derivative.[93]

[90] T. S. Srivastava and T. Yonetani, *Fed. Proc.*, 1974, **33**, 1449.
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