

## A Theoretical Model of the Intensity of the Near-infrared Porphyrin-to-iron Charge-transfer Transitions in Low-spin Iron(III) Haemoproteins. A Correlation between the Intensity of the Magnetic Circular Dichroism Bands and the Rhombic Distortion Parameter of Iron

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A theoretical model is presented of the source of the intensity of the porphyrin-to-iron charge-transfer (c.t.) band of low-spin iron(III) haems in proteins and model complexes. The intensity mechanism involves intensity borrowing *via* the 'transfer term' from the intense visible and Soret bands of the haem spectrum. The experimental evidence suggests that about 5% of the intensity is borrowed. The intensity of the magnetic circular dichroism (m.c.d.) spectrum of the c.t. bands at 4.2 K depends upon the ground-state  $g_z$  value and upon the product  $ab$  where  $a$  and  $b$  are the coefficients of the iron(III) ion  $d$ -orbitals  $3d_{xz}$  and  $3d_{yz}$ , respectively, in the ground-state Kramers doublet. This leads to a dependence of the m.c.d.  $C$ -term intensity upon the rhombic distortion,  $V$ , experienced by the iron(III) ion. If  $V$  is zero then  $a = b$ , and the hole in the  $t_{2g}$   $d$  subshell is equally divided between the two orbitals. The c.t. band is  $x,y$ -polarized and the m.c.d. intensity a maximum value. As  $V$  tends to a large value either  $a$  or  $b$  drops to zero, and the hole becomes localized in one of the  $d_{xz,yz}$  orbitals. This results in a c.t. transition which is predominantly  $x$ - or  $y$ -polarized. The m.c.d. intensity drops to zero since a pair of perpendicularly polarized transitions is needed for the absorption of circularly polarized light. Experimental evidence drawn from measurements on a wide range of low-spin iron(III) haemoproteins has been used to substantiate the model. This study shows that the intensity of the near-i.r. m.c.d. transition can be a useful qualitative indicator of the magnitude of the rhombic crystal-field component at the iron(III) ion. This in turn allows some conclusions to be drawn about the relative orientation of the axial ligands to iron(III) in bis(histidine) co-ordinated haems.

The optical spectra of low-spin iron(III) haemoproteins, containing protohaem IX [(3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropionato)iron(III)], show in addition to the porphyrin-based transitions, porphyrin-to-iron(III) charge-transfer (c.t.) transitions in the near-i.r. wavelength region  $\approx 1\ 000$ – $2\ 000$  nm. The c.t. bands can be most readily located by magnetic circular dichroism (m.c.d.) spectroscopy.<sup>1</sup> The energy of the c.t. transition is related to the crystal field generated at the iron(III) ion by the axial ligands of the haem, and depends upon the energy of the hole in the  $t_{2g}$  sub-shell of the iron(III) ion  $d$  orbitals. The e.p.r.  $g$  values of low-spin iron(III) haem are also governed by the crystal-field parameters of the same sub-shell. Hence the energy of the c.t. transition is strongly correlated with the ground-state  $g$  values. These two sets of data namely, the e.p.r. and the near-i.r. m.c.d. spectra, provide a method for diagnosis of axial haem ligation states of low-spin iron(III) protohaem IX in proteins. The intensity of the m.c.d. signal due to the near-i.r. c.t. transition varies over an order of magnitude as the sixth axial ligand is changed. For example, at 4.2 K and 5 T the value of  $\Delta\epsilon$ , the differential absorption coefficient for left and right circularly polarized light, varies from  $25\ \text{dm}^3\ \text{mol}^{-1}\ \text{cm}^{-1}$  for the HS<sup>-</sup> derivative of metmyoglobin to  $550\ \text{dm}^3\ \text{mol}^{-1}\ \text{cm}^{-1}$  in the case of cyanometmyoglobin. In contrast the molar absorption coefficients for absorption in unpolarized light remain approximately constant at room temperature being  $200$ – $300\ \text{dm}^3\ \text{mol}^{-1}\ \text{cm}^{-1}$  for a number of compounds.

We have shown in an earlier report that the intensity of the m.c.d. of the near-i.r. c.t. band varies substantially for a series of methyl-substituted bis(imidazole)iron(III) complexes of 2,3,7,8,12,13,17,18-octaethylporphyrin ( $\text{H}_2\text{oep}$ ) in  $\text{CH}_2\text{Cl}_2$ .<sup>2</sup>

When the imidazole group carries a methyl substituent in the 2 position the intensity of the m.c.d. band is high and this complex also shows in the e.p.r. spectrum a value of  $g_z$  of 3.53. Recently a crystallographic analysis<sup>3</sup> of low-spin bis(2-methylimidazole)(5,10,15,20-tetraphenylporphyrinato)iron(III) perchlorate reveals an unusual, mutually perpendicular alignment of the two axial 2-methylimidazole ligands. This arrangement solves certain stereochemical problems associated with the formation of the low-spin iron(III) complex with sterically hindered axial ligands. Perpendicular alignment of two imidazole ligands yields an e.p.r. spectrum for the crystalline solid at  $g = 3.56$ .<sup>4</sup> In dimethylformamide (dmf) solution the same complex exhibits a dominant  $g_z$  signal at 3.41 with a ramp-shaped or folded line shape. Salerno<sup>5</sup> first associated this unusual line shape with a low value of the rhombic parameter at the iron(III) ion. A perpendicular orientation of the imidazole planes is expected to generate approximate  $S_4$  axial character at the metal ion, yielding a crystal field of low or zero rhombic distortion.

These model complexes provide a means of exploring the spectroscopic and redox properties of bis(histidine) haem complexes as a function of axial ligand orientation. Such factors are expected to be important in the control of redox properties of cytochromes. Therefore the ability to identify unambiguously the histidine orientation in proteins is of importance. Although a high  $g_{\text{max}}$  value with folded line shape may be diagnostic of this conformation there is the possibility of confusion given that some other ligation states, such as methionine-histidine, can also generate high  $g_{\text{max}}$  values.<sup>6</sup> For example, cytochrome  $c_4$ , *Azotobacter vinelandii*, contains two covalently bound haems which give two sets of low-spin iron(III) haem e.p.r. signals, namely, 3.20, 2.10 (1.17), and 3.64. The near-i.r. m.c.d. c.t. bands

lie at an energy which unambiguously identifies the axial ligand type of both haems as histidine-methionine. Hence there is a need to have secure axial ligand identification plus a range of spectroscopic methods which are sensitive to axial ligand orientation.

In this paper we show that the intensity of the near-i.r. m.c.d. c.t. band is correlated with  $V$ , the rhombic crystal-field parameter, which can be determined from an analysis of the three components of the ground-state  $g$  tensor. A theoretical model has been developed which relates the absorption and m.c.d. intensity of the near-i.r. c.t. transition to the coefficients of the ground-state wavefunction. This shows that, while the absorption intensity depends upon the sum of the in-plane,  $x$ - and  $y$ -polarized transition moments, the m.c.d. intensity depends upon the imbalance between the  $x$ - and  $y$ -polarized transition moments. The rhombic distortion experienced by the iron(III) ion controls the relative intensities of the  $x$ - and  $y$ -polarized components. Hence an intense m.c.d. signal corresponds to a low rhombic distortion with  $x$  and  $y$  components of the transition moment which are approximately equal in intensity. A low m.c.d. intensity by contrast implies a large rhombic distortion with one or other of the  $x$  and  $y$  transition-moment components approaching zero intensity. This observation can assist in the evaluation of the rhombic crystal-field parameter and, moreover, can lead to conclusions about the relative orientations of axial ligands of the iron(III) haem.

### Experimental and Results

The e.p.r. and near-i.r. m.c.d. spectra were recorded as described.<sup>1</sup> Table 1 collects the data from 25 different low-spin iron(III) haemoproteins and some derivatives made by addition of an exogenous sixth ligand. The e.p.r.  $g$  values are given. The coefficients of the ground-state Kramers doublet, calculated using the formulas given by Taylor,<sup>7</sup> enable the axial ( $\Delta/\lambda$ ) and rhombic ( $V/\lambda$ ) parameters to be obtained. The definition of these parameters are given in ref. 1. The m.c.d. intensity at 4.2 K and 5 T of the peak maximum of the near-i.r. c.t. band is given as  $\Delta\epsilon = \epsilon_L - \epsilon_R$ , where  $\epsilon_L$  and  $\epsilon_R$  are the molar absorption coefficients for left and right circularly polarized light, respectively. The peak wavelengths and energies of these derivatives, denoted by the same numbering system, are given in Table 1.<sup>1</sup> At 4.2 K and 5 T the m.c.d. intensity is not directly proportional to  $B/T$  because the sample is approaching magnetic saturation. Since we shall describe a theoretical model of the m.c.d. intensities using formulas<sup>8</sup> which are valid only when the measurements are carried out under conditions of the so-called linear limit we need to correct the experimental  $\Delta\epsilon$  values to obtain values that obey the linear limit. This can be done knowing the  $g_z$  value of each sample and using the fact that the near-i.r. c.t. transitions are in-plane polarized.<sup>9</sup> We use a program written to simulate m.c.d. magnetization curves in the non-linear limit.<sup>10</sup> The value of  $\Delta\epsilon$  calculated at 4.2 K and 5 T in the linear limit is denoted  $\Delta\epsilon^*$ . This is the experimental parameter of interest throughout this work. Table 1 shows that the difference between  $\Delta\epsilon$  and  $\Delta\epsilon^*$  becomes larger the higher is the value of  $g_z$ . This is expected since the larger the Zeeman splitting the greater is the degree of saturation of the m.c.d. signal at 4.2 K and 5 T.

A plot of  $\Delta\epsilon^*$  against  $V/\lambda$  and against  $\Delta/\lambda$  for all the derivatives listed in Table 1 is shown in Figure 1. There is a systematic variation of the m.c.d. peak intensity with the rhombic crystal-field parameter  $V$ , such that the smaller the value of  $V$  the more intense is the m.c.d. spectrum of the near-i.r. c.t. band. However,  $\Delta\epsilon^*$  does not vary systematically with  $\Delta$ , the axial distortion parameter. We justify this later with the theoretical model. Since  $\Delta\epsilon^*$  appears to be inversely correlated with  $V$  a plot is given, Figure 2, of the variation of  $\Delta\epsilon^*$  against  $\lambda/V$ . The general trend is evident.

We have not measured  $\epsilon$ , the absorption coefficient, in unpolarized radiation, of the c.t. bands of the protein samples. In order to do this it is necessary to have protein samples well exchanged in  $D_2O$  so that the partially obscuring vibrational overtones are removed from the spectrum. This is difficult to accomplish in many cases and we have not routinely attempted it. There are values of  $\epsilon$  recorded at room temperature, for some derivatives, see Figure 3. There are no  $\epsilon$  values reported for samples at 4.2 K. Line sharpening of the band on cooling the sample to liquid-helium temperature will undoubtedly lead to significant changes in  $\epsilon_{\max}$ . We therefore estimate  $\epsilon$  from our theoretical model. This is in the spirit of this account in which we seek to show that the measurement of the m.c.d. spectrum alone in the near-i.r. region can assist in axial ligand assignment. The near-i.r. absorption spectra have been recorded at room temperature for the set of bis(imidazole) complexes of iron(III) octaethylporphyrin whose near-i.r. m.c.d. have been reported.<sup>2</sup> These are shown in Figure 3.

### Theoretical Model of the Charge-transfer Intensity

In low-spin iron(III) haems the porphyrin-to-iron c.t. bands lying in the near-i.r. spectral region have an assignment which has been well established by plane-polarized single-crystal spectroscopy<sup>9</sup> and by the early analysis of the m.c.d. spectra.<sup>11,12</sup> Taking  $C_{4v}$  as the point group which approximates the haem symmetry, the iron(III) ground-state configuration is  $e^3$  giving a  ${}^2E$  state. The two excited c.t. configurations are ( $e^4a_1^1$ ) and ( $e^4a_2^1$ ) which give rise to states of  ${}^2A_1$  and  ${}^2A_2$  symmetries. Hence there are expected to be two c.t. transitions  ${}^2E \rightarrow {}^2A_1, {}^2A_2$   $x,y$ -polarized. This has been verified by polarized, single-crystal spectra.<sup>9</sup> It is generally supposed that both transitions lie within the total envelope of the near-i.r. spectrum.<sup>12</sup> It has been proposed that the most intense lowest-energy peak belongs to one transition and that the weaker peak (sometimes a poorly resolved shoulder) lying to higher energy is the second. However, this cannot easily be established. It is possible that the whole band envelope contains only one electronic transition and that the features to higher energy are vibronic components of a single electronic state.<sup>13</sup> In this case the second c.t. transition must lie either at longer wavelengths or at higher energies obscured by the intense visible-region transitions. In this account we do not choose between these possibilities. Our arguments apply equally well either to a single c.t. transition or to the sum of the two.

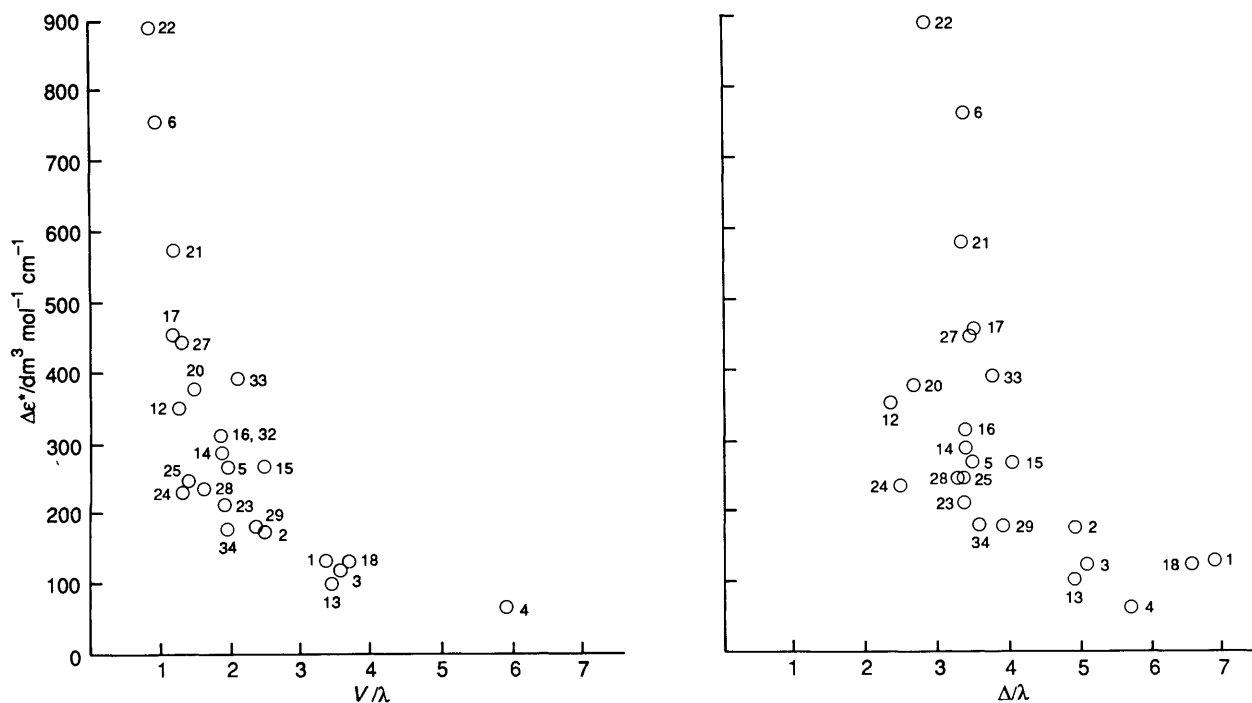
The co-ordinate frame,  $x,y,z$ , is centred at the iron with  $z$  normal to the haem plane and  $x,y$  running through the pyrrole nitrogen atoms of the porphyrin ring thereby defining a right-handed co-ordinate system. The out-of-plane orbitals are  $d_{yz}, d_{xz}$ , and  $d_{xy}$  is in the plane. This axis system is taken to define both the principal axes of the  $g$  tensor and of the electric dipole transition-moment tensor. The largest-magnitude  $g$  value is assigned to the haem normal. Although the orientation of the  $g$  tensor relative to the haem axes is not known for all the compounds in Table 1, single-crystal e.p.r. spectroscopy has been carried out on a wide range of proteins, including azidometmyoglobin,<sup>14</sup> cyanometmyoglobin,<sup>15</sup> and horse-heart cytochrome  $c$ .<sup>16</sup> Other studies include bis(imidazole)<sup>17</sup> and bis(thiolate) complexes of low-spin iron(III) haem.<sup>18</sup> All show that the largest-magnitude  $g$  value falls within a cone of approximately  $15^\circ$  from the haem normal. Exceptions to this rule are partially reduced haem groups such as chlorins.<sup>7</sup>

It has been common in analysing the e.p.r. spectra of low-spin iron(III) haems to adopt the convention of a 'proper co-ordinate system' such that values of  $|V/\Delta|$  are always less than or equal to  $\frac{2}{3}$ .<sup>7</sup> In some of the cases given in Table 1, for example (3), (4), and (13),  $|V/\Delta| > \frac{2}{3}$  and hence the convention of being a 'proper axis system' is not followed in all cases.

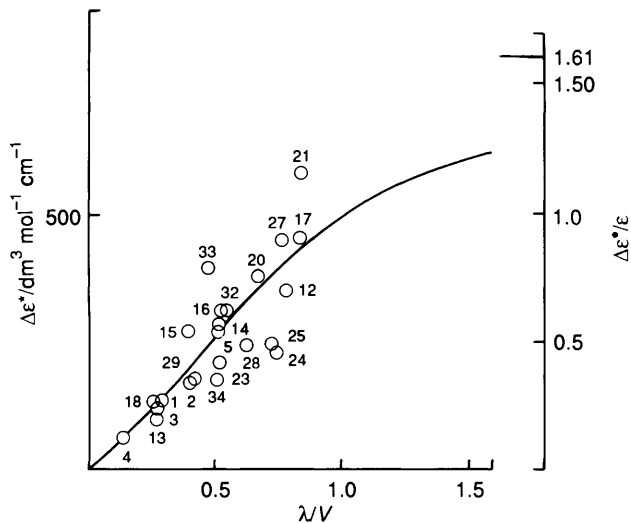
Table 1. E.p.r. g-values and m.c.d. intensities of charge-transfer bands of low-spin ferric haemoproteins

Derivative <sup>a</sup>	E.p.r. g values			Coefficients of ground-state Kramers doublet <sup>b</sup>			$V/\lambda^c$	$\Delta/\lambda^d$	$(a^2 + b^2)$	$\Delta\epsilon^e/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$	$\Delta\epsilon^{*f}/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$
	$g_z$	$g_y$	$g_x$	a	b	c					
(1) Metmyoglobin-OH <sup>-</sup>	2.59	2.17	1.88	0.986	0.152	0.066	3.35	6.88	0.996	102	131
(2) -N <sub>3</sub> <sup>-</sup>	2.79	2.21	1.72	0.975	0.204	0.093	2.46	4.94	0.991	134	175
(3) -SH <sup>-</sup>	2.56	2.24	1.84	0.985	0.150	0.085	3.54	5.09	0.992	95	122
(4) -SC <sub>3</sub> H <sub>7</sub> <sup>-</sup>	2.37	2.24	1.93	0.994	0.088	0.062	5.92	5.71	0.996	52	651
(5) -4-methylimidazole	2.92	2.25	1.54	0.958	0.254	0.130	1.94	3.47	0.983	203	271
(6) -CN <sup>-</sup>	3.45	1.89	0.93	0.895	0.417	0.158	0.94	3.36	0.975	550	757
(12) Leghaemoglobin-nicotinate	3.16	2.21	(1.07) <sup>g</sup>	0.915	0.355	0.193	1.26	2.35	0.962	261	351
(13) -OH <sup>-</sup>	2.54	2.24	1.84	0.986	0.149	0.074	3.44	4.91	0.995	79	101
(14) -imidazole, pD 6.4	3.03	2.29	1.50	0.956	0.262	0.134	1.87	3.40	0.982	215	288
(15) -imidazole, pD 8.4	2.82	2.29	1.69	0.973	0.205	0.108	2.48	4.05	0.988	205	269
(16) -1-methylimidazole	2.98	2.26	1.50	0.955	0.264	0.135	1.86	3.38	0.982	236	314
(17) -n-butylamine	3.38	2.05	[1.4] <sup>h</sup>	0.919	0.367	0.147	1.17	3.50	0.978	330	454
(18) -phenol	2.65	2.24	1.86	0.988	0.139	0.066	3.68	6.56	0.996	100	129
(20) Cytochrome c (horse heart) pD 6.6	3.07	2.23	1.26	0.932	0.318	0.176	1.47	2.67	0.969	282	377
(21) pD 11.0	3.33	2.05	[1.13] <sup>h</sup>	0.917	0.368	0.153	1.17	3.37	0.976	420	575
(22) Cytochrome c (horse heart)-CN <sup>-</sup>	3.47	1.85	(0.73) <sup>g</sup>	0.875	0.448	0.182	0.82	2.86	0.967	645	887
(23) Cytochrome c-imidazole	2.96	2.26	1.51	0.956	0.261	0.135	1.88	3.37	0.982	160	212
(24) Cytochrome c (tuna)	3.11	2.20	1.15	0.922	0.342	0.183	1.33	2.49	0.966	176	236
(25) Cytochrome c ( <i>Camidala Krusei</i> )	3.18	2.11	1.26	0.930	0.336	0.148	1.35	3.34	0.977	182	246
(27) Cytochrome c <sub>551</sub> ( <i>Pseudomonas aeruginosa</i> )	3.20	2.06	(1.23) <sup>g</sup>	0.933	0.335	0.133	1.28	3.45	0.982	330	446
(28) Cytochrome b <sub>562</sub> ( <i>Escherichia coli</i> ) pD 6.9	3.03	2.18	1.40	0.944	0.297	0.142	1.60	3.33	0.980	180	240
(29) pD 10.5	2.79	2.26	1.67	0.971	0.214	0.112	2.37	3.92	0.987	135	177
(32) Cytochrome c <sub>3</sub> (Norway) pD 6.5	3.02	2.25	1.50	0.955	0.266	0.131	1.83	3.56	0.982	234	313
(33) Cytochrome c <sub>3</sub> ( <i>Desulphovibrio vulgaris</i> ) pD 6.5	2.96	2.29	1.59	0.964	0.238	0.120	2.09	3.76	0.982	206	392
(34) Lactoperoxidase-CN	2.96	2.26	1.54	0.959	0.254	0.128	1.94	3.57	0.984	134	178

<sup>a</sup> Numbering of derivatives corresponds to that used in Table 1 of ref. 1. <sup>b</sup> a, b, and c are the coefficients of the d orbitals  $d_{xy}$ ,  $d_{xz}$ ,  $d_{yz}$  respectively, in the ground-state wavefunction. See ref. 7 for definition. <sup>c</sup> The rhombic distortion V is defined by the energy separation, in units of  $\lambda$ , the spin-orbit coupling constant, between  $d_{yz}$  and  $d_{xz}$ . The axial distortion  $\Delta$ , in units of  $\lambda$ , is the energy separation between the centroid of  $d_{xy}$ ,  $d_{xz}$  and the  $d_{yz}$  orbital. <sup>e</sup> M.c.d. peak intensity at 4.2 K and 5 T.  $\Delta\epsilon = \epsilon_L - \epsilon_R$  where  $\epsilon_L, \epsilon_R$  are the absorption coefficients for left and right circularly polarized light. <sup>f</sup>  $\Delta\epsilon^*$  is the value of  $\Delta\epsilon$  at 4.2 K, 5 T when the m.c.d. signal is linear in B/T. See text for method of calculation. <sup>g</sup> Value of g estimated from the relationship  $\Sigma g_i^2 = 16.00$ . <sup>h</sup> Experimental location of g value is uncertain owing to a low transition probability or to the possibility of confusion with other signals.



**Figure 1.** The peak intensities  $\Delta\epsilon^*$ , of the near-i.r. c.t. bands of low-spin iron(III) haemoproteins measured by m.c.d. spectroscopy plotted *versus* the rhombic,  $V$ , and the axial,  $\Delta$ , distortion parameters of the iron(III) ion;  $V$  and  $\Delta$  have been determined from the ground-state  $g$  values using the formulas of Taylor<sup>7</sup> and are expressed in units of  $\lambda$ , the spin-orbit coupling constant. The numbers identifying the compounds correspond to those used in Table 1



**Figure 2.** Plot of  $\Delta\epsilon^*$  against  $\lambda/V$ . The solid line is the theoretical curve (Figure 4) of  $\lambda/V$  vs.  $\Delta\epsilon^*/\epsilon$  obtained from equations (11) and (12) assuming  $c = 0$  and  $\epsilon = 500 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$

The choice of co-ordinate convention implies nothing about the orientation of the  $g$  tensor relative to the molecular frame. This can only be determined from a single-crystal experiment.

For non-zero c.t. intensity to arise for a transition between orbitals on one centre to those on a second centre, mixing between the two orbital sets must take place. This mechanism gives rise to the so-called 'transfer term' intensity of c.t. bands.<sup>19</sup> For the haem system considered here the  $e(\pi^*)$  porphyrin orbitals can mix with the  $e(xz,yz)$   $d$  orbitals of the metal under the action of interelectronic repulsion. Thus the  $a_1, a_2 \rightarrow e(xz,yz)$  c.t. transition borrows intensity from the  $a_1, a_2 \rightarrow e(\pi^*)$  transitions of the porphyrin ring which are

responsible for the visible and Soret bands of haem spectra. Using first-order perturbation theory we write equations (1) and (2) where  $yz'$  and  $xz'$  are the perturbed  $d$ -orbital functions,

$$|yz'\rangle = (1 - \alpha^2)^{1/2}|yz\rangle + \alpha|y\rangle \quad (1)$$

$$|xz'\rangle = (1 - \beta^2)^{1/2}|xz\rangle + \beta|x\rangle \quad (2)$$

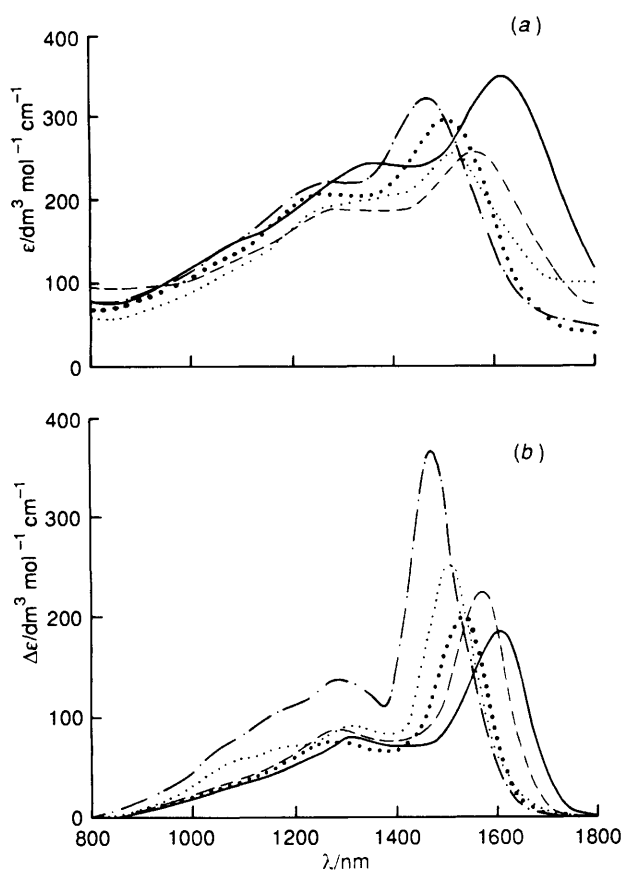
$xz, yz$  are the zero-order functions, and  $x, y$  the components of the porphyrin  $\pi^*$  orbitals;  $\alpha$  and  $\beta$  are the first-order mixing coefficients which have the form  $\alpha = \langle y|e^2/r_{12}|yz\rangle/\Delta E$  where  $\Delta E = E(yz) - E(y)$ . We make the simplifying assumption  $\alpha = \beta$ , assuming that there is equal mixing between the  $x$  and  $y$  components of the orbitals. Rhombic distortions will remove the degeneracy of the  $x, y$  porphyrin and  $xz, yz$   $d$  orbitals and in this case  $\alpha$  may not equal  $\beta$ , but we take this as an approximation.

The ground-state wavefunctions of the Kramers doublets can be written, following Taylor,<sup>7</sup> as  $|+\rangle = a|yz^+\rangle - ib|xz^+\rangle - c|xy^-\rangle$  and  $|-\rangle = -a|yz^-\rangle - ib|xz^-\rangle - c|xy^+\rangle$ , where  $xz^+$  denotes the  $d_{xz}$  orbital with  $\alpha$  spin, and  $a, b$ , and  $c$  are real numbers. Taylor<sup>7</sup> gives simple expressions enabling the principal  $g$  values to be expressed in terms of  $a, b$ , and  $c$  alone. These coefficients are normalized so that  $a^2 + b^2 + c^2 = 1$ .

After taking into account molecular-orbital formation between the  $e(xz,yz)$   $d$  orbitals and the  $x, y$  components of the porphyrin  $\pi^*$  orbitals according to equations (1) and (2), the ground-state wavefunctions of the Kramers doublet can be rewritten as in equations (3) and (4).

$$|+\rangle = a(1 - \alpha^2)^{1/2}|yz^+\rangle + \alpha a|y^+\rangle - ib(1 - \beta^2)^{1/2}|xz^+\rangle - ib\beta|x^+\rangle - c|xy^-\rangle \quad (3)$$

$$|-\rangle = -a(1 - \alpha^2)^{1/2}|yz^-\rangle - \alpha a|y^-\rangle - ib(1 - \beta^2)^{1/2}|xz^-\rangle - ib\beta|x^-\rangle - c|xy^+\rangle \quad (4)$$



**Figure 3.** Absorption spectra, measured at room temperature, (a), and m.c.d. spectra, recorded at 4.2 K and 5 T, (b), of bis(imidazole) complexes of low-spin iron(III) octaethylporphyrinate in  $\text{CH}_2\text{Cl}_2$ - $(\text{C}_2\text{H}_5)_2\text{O}$  (2:1 v/v). The  $\epsilon$  values are corrected for the presence of some high-spin haem in the case of the 1,2-dimethylimidazole and 2-methylimidazole complexes;  $\Delta\epsilon$  values have been corrected for contraction in the solvent on cooling to 4.2 K. The correction factor is 0.67. —, 1-Methylimidazole; ---, imidazole; ····, 4-methylimidazole; ····, 1,2-dimethylimidazole; ·-·-·, 2-methylimidazole

We assume that  $\alpha$  (and  $\beta$ ) are sufficiently small that they will have no appreciable effect on first-order perturbations within the  $t_{2g}$   $d$  sub-shell. The perturbations are spin-orbit coupling, rhombic and axial crystal-field distortions;  $\alpha$  (and  $\beta$ ) will have only a second-order effect on these properties giving a contribution proportional to  $1/\Delta E^2$ . Under this assumption the ground-state  $g$  values are independent of the magnitude of  $\alpha$  (or  $\beta$ ) to first order and the expressions of Taylor<sup>7</sup> can be used to calculate  $\Delta$  and  $V$  from the observed  $g$  values. However,  $\alpha$  (and  $\beta$ ) play a crucial role in the intensity mechanism of the c.t. transitions and cannot be ignored in the calculation of their intensities.

The two c.t. transitions each have a pair of components arising from the Kramers doublet, as follows,  $|+\rangle \rightarrow |A_1^+\rangle$ ,  $|A_2^+\rangle$  and  $|-\rangle \rightarrow |A_1^-\rangle$ ,  $|A_2^-\rangle$ . The intensity of one of the c.t. transitions, say to the excited state  $A_1$ , can be written in terms of the transition dipole moment  $D_0$  [equation (5)] where

$$D_0 = \sum_{i=x,y} | \langle + | m_i | A_1^+ \rangle |^2 + \sum_{i=x,y} | \langle - | m_i | A_1^- \rangle |^2 \quad (5)$$

$m_x, m_y$  are the electric dipole moment operators in the  $x, y$  plane. The component of intensity in the  $z$  direction is zero. The

transition dipole moment of each c.t. transition,  $D_{c.t.}$ , can be written in terms of one-electron matrix elements by substituting the expression for the ground-state Kramers doublets, equations (3) and (4), into equation (5) with the result shown in equation (6);  $|m^2|$  is the product,  $\bar{m}m$ , of the one-electron matrix

$$D_{c.t.} = \frac{1}{3} \alpha^2 (a^2 + b^2) |m^2| \quad (6)$$

elements, where  $m = \langle a_1 | m_x | x \rangle = \langle a_1 | m_y | y \rangle$  and  $\bar{m}$  is the complex conjugate of  $m$ .

Hence, the absorption intensity of the c.t. transition depends upon  $\alpha^2$ , the coefficient which mixes the first excited porphyrin state into the ground state, and upon the sum of the squares of the coefficients  $a$  and  $b$  of the  $d_{xz}, d_{yz}$  orbitals in the ground state;  $a$  and  $b$  are obtained from the experimental  $g$  values and hence  $(a^2 + b^2)$  calculated. Table 1 shows that  $(a^2 + b^2)$  varies less than 4% between values of 0.962 and 0.996. Since  $a^2 + b^2 + c^2 = 1$  the contribution of the  $d_{xy}$  orbital, given by  $c^2$ , to the intensity variation is negligible. The intensity of the c.t. band is controlled by  $\alpha^2$ , the extent to which intensity is borrowed from the porphyrin  $\pi-\pi^*$  transitions. The dipole strength of the latter,  $D_{\text{por}}$ , is given by  $(|m^2|)/3$ . Hence the ratio of the intensities of the porphyrin bands to that of the near-i.r. c.t. band will give an estimate of  $\alpha^2$ , viz.,  $D_{\text{por}}/D_{c.t.} = |m^2|/\alpha^2 |m^2| (a^2 + b^2)$  and hence  $\alpha^2 = D_{c.t.}/D_{\text{por}} = \epsilon_{c.t.}/\epsilon_{\text{por}}$ . The porphyrin transition which will contribute most to the c.t. band intensity is likely to be the  $\alpha$  band since this lies closest in energy to the c.t. band. Taking a value of  $\epsilon_{\text{por}}$  to be  $6 \times 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  and of  $\epsilon_{c.t.}$  as  $300 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ ,  $\alpha^2$  is  $\approx 5\%$ .

The m.c.d. intensity at 4.2 K is dominated by the contribution from the  $C$  terms.<sup>8</sup> Calculation of the  $C$ -term magnitude is readily carried through. Using the expressions given by Piepho and Schatz,<sup>8</sup> equations 4.6.14 and 4.6.15, the magnitude of the  $C$  term for the transition  $|\pm\rangle \rightarrow |A_1^\pm\rangle$  can be calculated by substituting equations (3) and (4) into the m.c.d. equations. The resulting expression is (7) where  $g_z$  is the ground-state  $g$  value.

$$C_{c.t.} = \frac{1}{3} g_z a b \alpha^2 |m^2| \quad (7)$$

The ratio of  $C_{c.t.}$  to  $D_{c.t.}$  yields a quantity independent of  $\alpha^2$  and  $|m^2|$  and dependent only upon parameters uniquely determined from the e.p.r. spectrum [equation (8)];  $g_z$  can be

$$\frac{C_{c.t.}}{D_{c.t.}} = g_z \cdot \frac{ab}{(a^2 + b^2)} \quad (8)$$

written in terms of  $a, b$ , and  $c$  using the formula of Taylor.<sup>7</sup> Thus we obtain equation (9).

$$\frac{C_{c.t.}}{D_{c.t.}} = \frac{2ab[(a+b)^2 - c^2]}{a^2 + b^2} \quad (9)$$

When the  $C$  term is the only contribution to the m.c.d. spectrum the relationship between the theoretical parameters  $C_{c.t.}$  and  $D_{c.t.}$  and the experimental parameters  $\Delta\epsilon^*$  and  $\epsilon$  is given by equation (10), where  $\mu_B$  is the Bohr magneton,  $B$  the

$$\frac{\Delta\epsilon^*}{\epsilon} = \frac{C_{c.t.}}{D_{c.t.}} \cdot \frac{\mu_B B}{kT} \quad (10)$$

magnetic field intensity,  $k$  is Boltzmann's constant, and  $T$  is the absolute temperature. In the limit of zero rhombic distortion  $a = b$  and  $d_{xz}, d_{yz}$  are degenerate orbitals. Then we obtain  $C_{c.t.}/D_{c.t.} = +g_z/2$  and  $\Delta\epsilon^*/\epsilon = g_z \mu_B B / 2kT$ . This agrees with the result first deduced by Cheng *et al.*<sup>11</sup>

The important result established by this analysis is evident

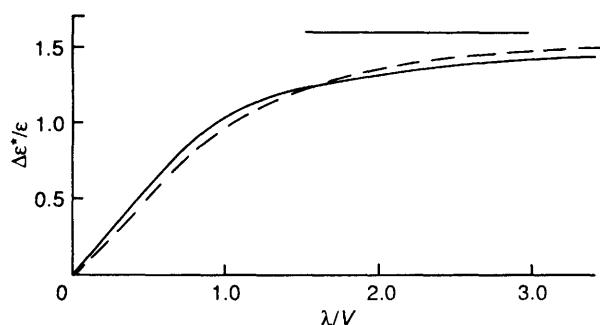


Figure 4. A plot of the ratio  $\Delta\epsilon^*/\epsilon$  against  $\lambda/V$ . The values plotted have been calculated according to equations (11) and (12) from values of  $a$ ,  $b$ , and  $c$ , the molecular orbital coefficients of the  $d_{xz}$ ,  $d_{yz}$ , and  $d_{xy}$  orbitals of  $\text{Fe}^{\text{III}}$  which contribute to the ground-state Kramers doublet. The two curves correspond to values of  $c$  selected to be zero (---) and 0.2 (—), respectively. The asymptotic limit of the plot when  $c = 0$  is 1.61

from equations (7) and (8). The intensity of the m.c.d. signal depends upon the product  $ab, g_{xz}$  and  $\alpha^2|m|^2$ . The ratio  $C_{c.t.}/D_{c.t.}$  depends upon  $ab/(a^2 + b^2)$ . Table 1 shows that the denominator ( $a^2 + b^2$ ) varies little over the range of compounds studied here. Therefore it is the product  $ab$  which controls the m.c.d. intensity;  $a$  and  $b$  are the coefficients of the  $d$  orbitals  $d_{yz}$  and  $d_{xz}$ , respectively of the hole in the  $t_{2g}$  subshell. When  $a = b$  the hole (or electron) resides equally in  $d_{xz}$  and  $d_{yz}$ . The c.t. transition is  $x,y$ -polarized and the m.c.d. intensity is a maximum. This occurs when  $x \equiv y$ , and the rhombic field is zero. As the rhombic distortion increases  $a$  or  $b$  will tend to zero and the hole becomes localized in either the  $d_{yz}$  or the  $d_{xz}$  orbital, respectively. The c.t. transition becomes purely  $y$ - or  $x$ -polarized in the limit. The m.c.d.  $C$ -term intensity goes to zero in this limit. For a transition to have a non-zero m.c.d. intensity it must possess two electric dipole transitions orthogonally polarized to one another and perpendicular to the applied magnetic field. This is the condition for the absorption of circularly polarized radiation. The m.c.d. signal of a linearly polarized transition is zero. Thus the intensity of the m.c.d. signal is sensitive to the imbalance between the intensities of the  $x$ - and  $y$ -polarized components of the near-i.r. c.t. band. This in turn is a function of the rhombic crystal field experienced by the low-spin iron(III) ion of the haem.

The rhombic distortion parameter  $V$  can be obtained from the e.p.r.  $g$  values and is related to the coefficients  $a$ ,  $b$ , and  $c$  of the ground state. Taylor<sup>7</sup> gives the expression (11). Equations

$$\frac{V}{\lambda} = \frac{a+c}{2b} - \frac{b+c}{2a} \quad (11)$$

$$\frac{\Delta\epsilon^*}{\epsilon} = \left\{ \frac{2ab[(a+b)^2 - c^2]}{a^2 + b^2} \right\} \frac{\mu_B B}{kT} \quad (12)$$

(9) and (10) can be written as (12). Hence  $\Delta\epsilon^*/\epsilon$  is related to  $V$  but the relationship between them is algebraically complicated. Therefore a graphical display of the relationship is given in Figure 4. Two examples are plotted, one with  $c$ , the coefficient of  $d_{xy}$ , equal to zero, and the other with  $c = 0.2$ . The maximum value of  $c$  for any of the haems considered here is 0.193, Table 1. For these two extreme cases of  $c$  values the curves are little different. The graph shows that  $\Delta\epsilon^*/\epsilon$  varies almost linearly with  $\lambda/V$  between zero and  $\approx 0.8$ . As  $\lambda/V$  approaches infinity ( $V \rightarrow 0$ )  $\Delta\epsilon^*/\epsilon$  tends to a limit. This limit is the purely axial case when  $a = b$ . The limiting value of  $\Delta\epsilon^*/\epsilon$  depends upon  $c$ . As  $c$  increases the m.c.d. intensity decreases, but the change is too small to be of experimental significance. Therefore plots of experimental values of  $\Delta\epsilon^*/\epsilon$

against  $\lambda/V$  should lie within the two lines plotted in Figure 4.

The experimental data points available to test this curve are given in Table 1. However, the curve can be tested only over the range of  $\lambda/V$  between 0 and  $\approx 1.0$ . In order to determine values of  $V$  and  $\Delta$ , measurements of the three components of the  $g$  tensor are required. When  $V/\lambda$  falls below  $\approx 1.0$  the system is sufficiently close to axiality for the  $g_x$  and  $g_y$  values to become difficult or impossible to detect. In the limit that  $V/\lambda$  tends to zero and  $\Delta$  is large the  $g$  values become  $g_{\parallel} = 4.0$  and  $g_{\perp} = 0$ . Since the intensity of the e.p.r. signal is directly proportional to  $(g_{\perp})^2$  it also becomes zero. Figure 2 shows the correlation between  $\Delta\epsilon^*$  and  $\lambda/V$  in the range of  $\lambda/V$  between 0 and 1.0.

The sign of the m.c.d.  $C$  terms of the c.t. transition depends upon the absolute sign of the  $g$ -tensor component normal to the haem plane, equation (7), since the optical transitions are polarized in the  $x,y$  plane. The fact that all 24 compounds in Table 1 give  $C$  terms of the same sign implies that all have positive  $g_z$  values.

We next turn to consider the value of the absorption coefficient,  $\epsilon$ , of the near-i.r. c.t. of samples held at 4.2 K. This parameter is required for evaluation of  $C_{c.t.}/D_{c.t.}$ . However, there are no  $\epsilon$  values at 4.2 K available. Some values have been recorded at room temperature. During the course of this work the room-temperature absorption spectra of a set of bis(imidazole) complexes of iron(III) octaethylporphyrin ( $\text{H}_2\text{oep}$ ) have been recorded, Figure 3. The complexes were prepared in a mixed solvent of  $\text{CH}_2\text{Cl}_2$  and diethyl ether (2:1 v/v) in order to form a good optical glass at low temperature. The m.c.d. spectra at 4.2 K and 5 T have also been measured, Figure 3, and previously reported in brief.<sup>2</sup> The sterically encumbered imidazoles, 2-methyl- and 1,2-dimethyl-imidazole, form mixed high-, low-spin complexes at room temperature. The absorption spectra have been used to estimate the low-spin component at 85 and 64% respectively. The intensities of the absorption spectra have been corrected to 100% low-spin content.

The  $\epsilon$  values lie within a relatively small range, Figure 3. The cyanide adduct of haemoglobin is the most intense because of the narrower linewidth of the band. On cooling haemoproteins to 4.2 K band sharpening will undoubtedly occur leading to an increase in the  $\epsilon$  value. It is the value of  $\epsilon$  at 4.2 K, measured under the same conditions as for the m.c.d. spectra, which is required. Since these data have not been recorded we proceed as follows.

We seek the best fit of  $\Delta\epsilon^*/\epsilon$  by the theoretical expression for  $V/\lambda$ , equation (11), and  $\Delta\epsilon^*/\epsilon$ , equation (12). Figure 2 plots the values of  $\Delta\epsilon^*$ , determined from the low-temperature m.c.d. spectra and given in Table 1, against  $\lambda/V$  obtained from the e.p.r.  $g$  values. All the points from Table 1 are plotted except for those of cyanometmyoglobin, and cytochrome  $c$ -CN, horse heart (6) and (22). The  $\Delta\epsilon$  (and  $\Delta\epsilon^*$ ) values of these two derivatives are exceptionally high in all the correlations sought here. This is undoubtedly because the linewidth of the near-i.r. c.t. band is considerably narrower than for the other derivatives. These examples provide exceptions in our procedure which can be recognized by the width of the transition.

Figure 2 also shows the theoretically derived curve, plotted using equations (11) and (12) for a reasonable best fit. The curve shown assumes that  $\epsilon = 500 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  for all the derivatives plotted. This value represents an intensification of the near-i.r. c.t. band at 4.2 K compared with the room-temperature values quoted in Table 2 and likely arises from band narrowing on cooling. Although there is considerable scatter in the plot especially as  $\lambda/V$  approaches 1.0 there is a positive correlation.

At the limit of complete axiality  $\Delta\epsilon^*/\epsilon$  is predicted by expressions (11) and (12) to approach a value of 1.61. Taking a value of  $\epsilon$  of  $\approx 500 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  the maximum value of  $\Delta\epsilon^*$  is

**Table 2.** E.p.r. and near-i.r. m.c.d. parameters of low-spin iron(III) haems in proteins and models with high  $g_{\max}$  values

	M.c.d. parameters of near-i.r. c.t. band			Ref.
	$g_z^a$	$\lambda^b/\text{nm}$	$\Delta\epsilon^c/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$	
Bis(histidine) co-ordination				
cytochrome $c^*$ ( <i>M. methylotrophus</i> )	3.65 <sup>d</sup>	1 560	400	<i>e</i>
cytochrome $b_1$ ( <i>P. aeruginosa</i> )	3.77 <sup>d</sup>	1 545	380	22
Bis(imidazole)				
bis(2-methylimidazole)iron(III) octaethylporphyrinate	3.53	1 475	360	2
Methionine-histidine co-ordination				
cytochrome $c_4$ ( <i>A. vinelandii</i> )	3.64 <sup>d</sup>	1 900		6
Lysine-histidine co-ordination				
cytochrome $f$ (rape, charlock)	3.51 <sup>f</sup>	1 520	675	23
Bis(amine) co-ordination				
bis(n-butylamine)iron(III) octaethylporphyrinate	3.69 <sup>f</sup>	1 320	300	2

<sup>a</sup> Maximum  $g$  value detected by X-band e.p.r. spectroscopy. No other components of the  $g$  tensor have been detected. <sup>b</sup> Wavelength of peak in m.c.d. spectrum. <sup>c</sup> Determined at 4.2 K and 5 T. The  $\Delta\epsilon^*$  values can be estimated by multiplying  $\Delta\epsilon$  values by 1.35. <sup>d</sup> This  $g_{\max}$  signal has a folded line shape with a sharp cut-off on the low-field edge. <sup>e</sup> M. J. Berry, S. J. George, A. J. Thomson, H. J. Santos, and D. L. Turner, *Biochem. J.*, in the press. <sup>f</sup> This  $g_{\max}$  value does *not* have a folded line shape but is close to Gaussian.

predicted to be  $800 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ . Assuming a set of axial  $g$  values to be  $g_{\parallel} = 4.0$ ,  $g_{\perp} = 0$ , then a maximum value of  $\Delta\epsilon$ , at 4.2 K and 5 T, of  $560 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  is predicted.

## Discussion

Using a collection of 24 haemoproteins which have histidine as one axial ligand and another of variable type we have demonstrated an empirical correlation between the intensity of the m.c.d. peak of the iron-to-porphyrin c.t. band and the inverse of the rhombic distortion parameter,  $V$ . The near-i.r. m.c.d. band varies in intensity at 4.2 K and 5 T between 50 and over  $500 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  for compounds with  $V$  between 5.92  $\lambda$  and 0.04  $\lambda$ , respectively. The energy splitting,  $V$ , between the  $3d_{xz}$  and  $3d_{yz}$  orbitals of the iron(III) ion, can be calculated from knowledge of the three  $g$  values of the low-spin  $d^5 g$  tensor.

A theoretical model of the near-i.r. c.t. band intensity has been established which confirms the empirical correlation and shows the origin of the wide intensity variation. The source of the c.t. intensity is proposed to be the mixing between the  $d_{xz,yz}$  orbitals of iron(III) ion and the lowest empty  $\pi^*$  porphyrin orbitals,  $e_{xy}$ , under the influence of interelectronic repulsion. This leads, in effect, to the c.t. intensity being stolen from the visible and Soret transitions of the porphyrin ring. The lower energy of the visible-region transitions would at first sight lead one to propose them as the major intensity source. However, the Soret transition is more intense by a factor of 5–10 than the visible-region transition although there is a greater energy gap to the near-i.r. c.t. state. The absorption coefficient,  $\epsilon$ , of the near-i.r. c.t. band depends upon both the mixing coefficient,  $\alpha^2$ , and the sum of the squares of the coefficients of the  $d_{xz,yz}$  orbitals,  $(a^2 + b^2)$ . The latter term varies less than 4% throughout the set of compounds studied here. Unfortunately, there is limited information about the  $\epsilon$  values of the near-i.r. c.t. band at room temperature and no data at 4.2 K. Measurement of this parameter is hampered by the vibrational overtones from solvent and protein backbone which partially obscure this spectral region. Careful and thorough deuteration overcomes this problem but this has not been carried out for many proteins. This was not routinely done in the present work since the data have been collected over a number of years for a variety of reasons. By contrast the m.c.d. spectrum is not obscured by the vibrational overtones because the m.c.d. spectra of vibrational transitions are several orders of magnitude weaker than those of electronic transitions of paramagnetic species.<sup>20</sup> This is the strength of the method for

detection of low-energy electronic states and hence its utility in axial ligand assignments. A study of the absorption coefficient variation of the near-i.r. c.t. band for a set of low-spin iron(III) haemoproteins would provide information about the constancy or otherwise of  $\alpha^2$ . However, we estimate that  $\alpha^2$  is about 5%. It would be of interest to attempt to justify this coefficient theoretically.

The theoretical model enables the ratio of the m.c.d. intensity to the absorption intensity,  $\Delta\epsilon^*/\epsilon$ , to be calculated from values of  $a$ ,  $b$ , and  $c$ , only. These are coefficients of the  $d$  orbitals,  $3d_{yz,xz,xy}$ , respectively, in the iron(III) ground state and are calculated from the three components of the ground-state  $g$  tensor. The rhombic distortion parameter,  $V$ , is also completely determined by these parameters. The correlation calculated between  $\Delta\epsilon^*/\epsilon$  and  $\lambda/V$  is given in Figure 2.

The reason for this correlation is clear. M.c.d. intensity depends upon the imbalance between the  $x$ - and  $y$ -polarized intensities of the near-i.r. c.t. band whereas the absorption in unpolarized radiation depends upon the sum of these two intensities. When  $V$  is zero,  $3d_{xz}$  and  $3d_{yz}$  are degenerate and the hole is located 50% in each orbital. The near-i.r. c.t. transition is equally  $x$ - and  $y$ -polarized. However, as  $V$  increases, the hole becomes localized in the highest-energy  $d$  orbital,  $3d_{yz}$  and the near-i.r. c.t. band becomes predominantly  $y$ -polarized. When the transition is completely  $y$ -polarized the m.c.d. intensity will be zero. Non-zero m.c.d. intensity requires absorption of circularly polarized light which in turn necessitates two components of intensity polarized perpendicular to one another and to the direction of the applied magnetic field.

Hence, intense m.c.d. transitions are associated with small value of  $V$  and *vice versa*. The theoretical curve, Figure 4, shows that there is an almost linear relationship between  $\Delta\epsilon^*/\epsilon$  and  $\lambda/V$  when the latter is between 0 and 1.0. Above this  $\Delta\epsilon^*/\epsilon$  tends asymptotically to a value independent of  $V$ . The theoretical model predicts a maximum value of the ratio  $\Delta\epsilon^*/\epsilon$  of 1.61.

Experimental points are available to fit the model only when all three components of the ground-state  $g$  tensor can be measured because only in that way can a value of  $V$  be obtained. As  $V$  decreases, the  $g$  values tend towards the axial limit, namely,  $g_{\parallel} = 4.0$ ,  $g_{\perp} = 0$ . Since e.p.r. intensity is proportional to  $(g_{\perp})^2$  the  $g$  values become difficult to detect. Even when  $g_z$  can be detected,  $g_x$  and  $g_y$  are usually too broad to be observed. Hence the data are not available to test the theoretical expressions above the region where  $\lambda/V > \approx 1.0$ .

This region is of interest precisely because for derivatives of

haemoproteins which have these parameters the e.p.r. technique does not permit the estimation of  $V$ . Fitting of Mössbauer spectra has been used to obtain the electric field gradient at the nucleus of  $^{57}\text{Fe}$  and to allow conclusions to be drawn about the magnitude of  $V$ .<sup>4</sup>

In order to obtain an estimate of  $V$  from the optical spectra it is necessary to measure both  $\Delta\epsilon^*$ , the peak intensity of the m.c.d. spectrum of the near-i.r. c.t. band, and  $\epsilon$ , the absorption coefficient of the same band under the same conditions of temperature. In this way the ratio  $\Delta\epsilon^*/\epsilon$  can be obtained and fitted by the curve in Figure 4. However, since it will not always be possible to obtain both parameters the magnitude of  $\Delta\epsilon^*$  (or  $\Delta\epsilon$ ) alone will indicate whether the protein has a low value of  $V/\lambda$ , that is less than 1.0. At values of  $V/\lambda$  less than  $\approx 0.5 \Delta\epsilon^*/\epsilon$  approaches 1.61, the limiting value, asymmetrically. Therefore  $\Delta\epsilon^*/\epsilon$  is rather insensitive to variations in  $V$  as the axial limit is approached. Hence m.c.d. spectroscopy does not provide a method for accurate determination of  $V$  at low values of the latter. Its utility resides in indicating whether the haem co-ordination is approaching the axial limit.

In conclusion we collect together in Table 2 all the low-spin iron(III) haems in proteins and as models which exhibit  $g_z$  values of 3.5 and greater and have an established axial co-ordination state. The near-i.r. m.c.d. spectra have been studied in this laboratory. Where the  $\Delta\epsilon$  values are known they bear out the analysis of this paper in showing that high  $g_{\text{max}}$  values go along with high  $\Delta\epsilon$  values implying low rhombic distortions. The examples of bis(histidine) co-ordination include the membrane-bound cytochrome  $b_1$  found in the formate dehydrogenase-nitrate reductase electron-transfer chain of *Pseudomonas aeruginosa* when it is grown in nitrate.<sup>21</sup> The e.p.r. spectrum has one of the highest  $g_z$  values known at 3.77 with a folded line shape. The  $\Delta\epsilon$  value at 4.2 K and 5 T is  $380 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  in accordance with the high value expected for a haem with low rhombicity. Cytochrome  $c''$ , from *Methylophilus methylotrophus*, is a soluble protein ( $M_r = 15\,000$ ) which is diagnosed as bis(histidine) in the oxidized state by the wavelength of the near-i.r. c.t. band at 1 560 nm. The e.p.r. spectrum has a single ramp-shaped peak at  $g_z = 3.65$ . Consistent with this is the  $\Delta\epsilon$  value of the near-i.r. c.t. band of  $400 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  at 4.2 K and 5 T.<sup>22</sup> The model compound bis(2-methylimidazole) iron(III) octaethylporphyrinate has a  $g_z$  at 3.53 with a folded line shape and, it can be assumed, has a similar ligand orientation to that found in the tetraphenylporphyrinate analogue,<sup>3</sup> namely, a near-perpendicular orientation of the two sterically hindered axial ligands. This gives rise to the near-i.r. m.c.d. band at 1 475 nm with a  $\Delta\epsilon$  value of  $360 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  at 4.2 K and 5 T.

Cytochrome  $c_4$  (*Azotobacter vinelandii*) is a two-domain protein containing two covalently bound haem groups.<sup>6</sup> The near-i.r. m.c.d. spectrum indicates that both haems have methionine-histidine co-ordination. One of the haems has a set of  $g$  values 3.20, 2.10, (1.17) typical of, say, cytochrome  $c_{551}$ . The other haem yields only one detectable  $g$  value at 3.64 with a folded line shape. The intensity of the near-i.r. c.t. band could not be determined since the contribution from each haem cannot be disentangled.

Other co-ordination types, namely lysine-histidine (cytochrome  $f$ ) and bis(amine) (not yet identified in a protein) can also generate high  $g_{\text{max}}$  values, see Table 2. However, in these two cases the e.p.r. line shapes are closer to Gaussian showing that  $g_{\text{max}}$  has not approached the axial limit.<sup>5</sup> The intensity of the near-i.r. c.t. band in the m.c.d. is very high. For cytochrome  $f$  it is  $675 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  at 4.2 and 5 T.<sup>23</sup> This is above the limiting value predicted from our theoretical model taking an  $\epsilon$  value of  $500 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ . However, inspection of the spectrum shows that the linewidth is narrow not unlike that of metmyoglobin cyanide,<sup>13</sup> for example. Hence our analysis can-

not be performed without a simultaneous measurement of the  $\epsilon$  value at 4.2 K. However, there seems little doubt on the basis of this evidence that lysine-histidine co-ordination can generate a field of low rhombicity and high axiality. This is rather surprising. Bis(amine) co-ordination can also generate a field of low rhombicity. The data, Table 2, suggest that this is so. More model compounds and proteins of well defined axial ligation state are required before further tests of the methodology can be made.

Table 2 shows that assignment of the axial ligation state of the haem group in protein is by no means straightforward especially when the  $g_z$  value rises above 3.50. A combination of spectroscopic signatures is required. The near-i.r. m.c.d. spectra are a powerful asset in unravelling ligand assignments and in describing the symmetry of the distortion field at low-spin iron(III) haem sites.

### Acknowledgements

We thank the S.E.R.C. and Royal Society for grants in support of this work. We also wish to acknowledge our collaborators who have supplied protein samples for study. Their names can be found in the references cited.

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