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Magnetization curves of haemoproteins measured by low-temperature magnetic-circular-dichroism spectroscopy

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The magnetic-circular-dichroism (m.c.d.) spectra of metmyoglobin cyanide and oxidized horse heart cytochrome *c* were measured in the region of the Soret band over a range of temperatures from 1.5 to 50 K and in fields from 0 to 5 T. A similar study has been made with reduced bovine heart cytochrome *c* oxidase, which contains one high-spin ferrous haem, namely a_3. M.c.d. magnetization curves are presented. It is shown that the two oxidized haemoproteins give magnetization curves characteristic of an isolated Kramer's ground state with spin $S = \frac{1}{2}$. These curves contrast with the magnetization curve of the high-spin ferrous haem with spin $S = 2$. The electronic ground state of the latter compound contains zero-field components that are thermally accessible over the temperature range of the experiment. Hence the magnetization curves are a complex nested set. The magnetization curves of the $S = \frac{1}{2}$ proteins were analysed and it is shown that it is possible to make estimates of the ground-state *g*-factors even in the presence of rhombic anisotropy, provided that some knowledge of the polarizations of the electronic transitions is available. The striking difference between the m.c.d. magnetization curves of a simple $S = \frac{1}{2}$ paramagnet and a magnetically complex ground state should prove extremely useful when m.c.d. spectroscopy is used to probe the magnetic properties of metal centres in proteins, and should have wide application beyond the field of haemoproteins.

M.c.d. spectroscopy can be used as an optical probe of the paramagnetism of metal centres in metalloproteins because paramagnetic species invariably give temperature-dependent m.c.d. spectra. We have previously reported the temperature-dependence of the m.c.d. spectra of haems in myoglobin (Springall *et al.*, 1976), in cytochrome *c* oxidase (Thomson *et al.*, 1977*a*), in the nitrite reductase of *Pseudomonas aeroginosa* (Walsh *et al.*, 1979) and also of iron–sulphur centres in rubredoxin (Rivoal *et al.*, 1977), and of the ferredoxins from spinach, *Spirulina maxima* and adrenodoxin (Thomson *et al.*, 1977*b*). An optical probe of paramagnetism can be especially valuable for the study of complex metalloproteins that contain more than one metal centre. Since the m.c.d. spectrum of a paramagnet at low temperatures is often very intense, it will become apparent even in the presence of other chromophores whose absorption bands may overlie it. Quantitative analysis of the form of the temperature-dependence should enable ground-state *g*-factors to be determined, and hence the presence of any magnetic couplings between metal centres to be detected. In this respect the technique is complementary to others that measure magnetic properties such as e.p.r. spectroscopy, Mössbauer spectroscopy and magnetic susceptibility. However, the m.c.d. experiment has certain advantages over each of these. For example, it is still useful for even-electron paramagnets, where zero-field splittings usually make the signal undetectable by e.p.r. It is not limited to iron compounds, as is Mössbauer spectroscopy, nor does it require isotopic enrichment. Since it is an optical probe m.c.d. spectroscopy is able to examine the magnetic properties of individual centres via their absorption bands, whereas magnetic susceptibility measures the sum of the magnetic properties of all centres present and of all impurities. Against these advantages can be set the disadvantage that it does not have the precision in determining *g*-factors, especially their anisotropies.

The strong temperature-dependence of m.c.d. signals from paramagnetic molecules is a consequence of the Boltzmann population distribution among the Zeeman-split sublevels, which result in

Abbreviations used: m.c.d., magnetic circular dichroism, e.p.r., electron paramagnetic resonance.

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differential absorption of left-minus-right-circularly polarized light (Schatz & McCaffery, 1969; Stephens, 1976). In the circumstance that $g\beta B/kT \ll 1$, where g is the ground-state g-factor, β is the Bohr magneton, B is the magnetic flux density, k is Boltzmann's constant and T is the absolute temperature, the general expression for $\Delta\varepsilon$ ($\varepsilon_L - \varepsilon_R$, the difference in absorption coefficient for the left- and right-circularly polarized light) can validly be expanded to the first power in B/T and the temperature-dependence of $\Delta\varepsilon$ is then directly proportioned to B/T. In this case the m.c.d. signal is obeying Curie's law and its magnitude is expressed by the so-called C term (Schatz & McCaffery, 1969). However, when $g\beta B/kT \geqslant 1$, $\Delta\varepsilon$ becomes non-linear as a function of B/T and eventually will be independent of B and T. Then the system is fully magnetized and the m.c.d. signal is said to be saturated. At this point there is Boltzmann population only of the lowest Zeeman sublevel. Very few experiments have been performed in this ultra-low-temperature and high-field region. Such measurements require the use of temperatures as low as 1.5K, with extremely accurate control of temperature. This necessitates immersion of the sample in a liquid-helium bath that can be pumped. Fields up to 5T, and preferably beyond, are also necessary. However, the information content is high, as we demonstrate in the present paper.

By studying the temperature-dependence of an m.c.d. spectrum in the Curie-law region, it is in principle possible to obtain the ground-state g-value by measuring the ratio of C/D, where C is determined from the slope of the temperature-dependence of the m.c.d. spectrum and D, the dipole strength, is determined from the absorption. However, in practice this will often not be possible, especially for metalloproteins. For example, the absorption band of the component being studied may be obscured by those of other metal centres or chromophores. In addition, it is often necessary to know parameters of the excited state. For example, the magnitude of C/D for the Soret band of a series of metmyoglobin derivatives depends not only on the percentage of low-spin form present, and on the ground-state low-spin g-factors, but also on the magnitude of spin–orbit coupling in the excited state (Thomson *et al.*, 1977*a*). It is quite erroneous to equate the magnitude of the m.c.d. signal with the percentage of one spin state (Vickery *et al.*, 1976). However, it turns out that estimates of g-factors can be made from the m.c.d. spectrum if it is measured down to its saturation limit. In an important paper Schatz *et al.* (1978) have developed theoretical expressions to treat the temperature-dependence of an m.c.d. spectrum to its saturation limit for the case of an 'isolated' Kramer's doublet, i.e. where no zero-field components become thermally populated over the temperature range of the experiment. No experimental results were compared with the theory in that paper.

It is the purpose of the present paper to present m.c.d. data on the low-spin ferric haemoproteins cytochrome *c* and metmyoglobin cyanide over the temperature range 1.5–50K and fields in the range 0–5.3T, and to show that estimates can be made of ground-state g-factors. These two proteins provide examples of systems with an isolated Kramer's doublet ground state with effective spin of $\frac{1}{2}$. The g-factors are rhombic, a case not considered explicitly by Schatz *et al.* (1978), although this is of greatest interest for metalloproteins.

We also show m.c.d. magnetization curves for reduced cytochrome *c* oxidase, which contains one high-spin ferrous haem, namely a_3, with a ground-state spin $S = 2$ (Thomson *et al.*, 1977*a*; Tweedle *et al.*, 1978). Splitting of this state by a rhombic environment removes all degeneracy in zero field, since it is a non-Kramer's ion. The form of the magnetization curves are, as expected, quite distinct from those of a paramagnet with an isolated Kramer's ground state. Although theoretical expressions are not yet available to analyse the curves, nevertheless their distinctive properties are readily recognized. Hence the m.c.d. saturation properties provide an important criterion for distinguishing between odd- and even-electron paramagnets.

Materials and methods

All chemicals used were of analytical-reagent grade, except for $Na_2S_2O_4$, which was laboratory grade, and were obtained from BDH Chemicals, Poole, Dorset, U.K. Freeze-dried horse heart cytochrome *c* and myoglobin were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K., and BDH Chemicals respectively. O_2-free N_2 gas was supplied by the British Oxygen Co., London S.W.19, U.K., and further purified for anaerobic work by passage over a B.A.S.F. copper catalyst at 140°C and through a dithionite wash-bottle. Samples of cytochrome *c* oxidase were supplied by Dr. C. Greenwood's group, School of Biological Sciences, University of East Anglia, Norwich, U.K.

Myoglobin and cytochrome *c* were dissolved in 0.1M-potassium phosphate buffer, pH 7.4, and treated with $K_3Fe(CN)_6$ to ensure complete oxidation. Before use the $K_3Fe(CN)_6$ was removed by using a Sephadex G-25 column. The cyanide derivative of metmyoglobin was prepared by addition of a 100-fold stoicheiometric excess of neutralized KCN solution. The concentrations of oxidized cytochrome *c* and metmyoglobin cyanide were assessed by using the published absorption coefficients (Margoliash & Schejter, 1966; Smith & Williams, 1970). Oxidized resting cytochrome *c*

oxidase in 0.1 M-potassium phosphate buffer, pH 7.4, containing 1% Tween 80 was reduced anaerobically by the addition of excess of $Na_2S_2O_4$. Complete reduction, as assessed by no further change in the visible-absorption spectrum, was achieved after approx. 1 h. The concentration of the resulting reduced cytochrome *c* oxidase sample was established per enzyme functional unit by using $\Delta\varepsilon_{630}$ (reduced−oxidized)−$\Delta\varepsilon_{630}$ (reduced−oxidized) = 27 $mM^{-1} \cdot cm^{-1}$ (Nicholls, 1978). To enable good optical-quality glasses to be formed on rapid freezing, 50% (v/v) ethanediol was added to all samples.

Absorption spectra were recorded on either a Cary 14 or Pye Unicam SP.8-200 spectrophotometer. M.c.d. spectra were recorded with either a JASCO J-500D spectropolarimeter or a Cary 61 dichrograph, fitted with an Oxford Instruments S.M.4 superconducting solenoid, capable of generating magnetic fields up to 5.3 T. Samples were placed in specially constructed open or anaerobic cells of measured path length and frozen rapidly in liquid helium. Any depolarization of the light-beam by the resultant glass was assessed and corrected for by measuring the natural c.d. of a sample of D-tris(ethylenediammine)cobalt(III) chloride placed after the magnet, with and without the sample in position, in the absence of an applied magnetic field. The magnetic fields experienced by the sample, for the applied currents used in this work, were measured by placing a Hall probe in the sample position. Sample temperatures in the range 1.5–4.2 K were obtained by having the sample immersed in a bath of pumped liquid helium, and measured by using a carbon/glass resistor, calibrated over the range 1.5–300 K by Cryogenic Calibrations, Pitchcott, Aylesbury, Bucks., U.K. Temperatures were held constant by applying a constant reduced pressure above the liquid helium by using an Oxford Instruments manustat. Sample temperatures in the range 4.2–200 K were achieved by having the sample in a stream of cold helium gas and measured by using a cryogenic linear temperature sensor (C.L.T.S.). The C.L.T.S. device and a heater on the sample block were connected to an Oxford Instruments D.T.C.2 temperature controller, which maintained constant temperatures in this range.

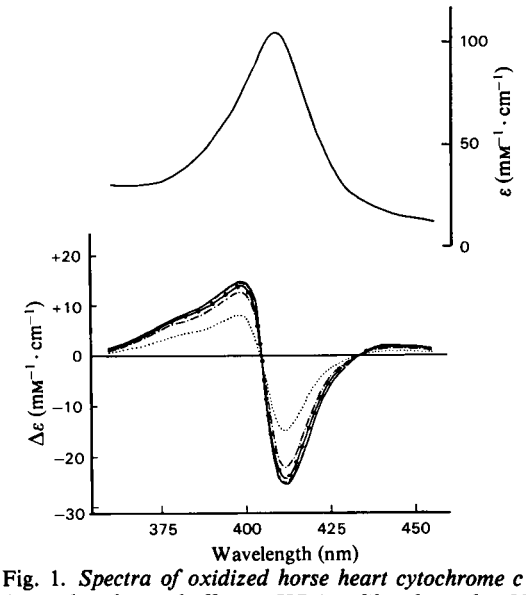

Fig. 1. *Spectra of oxidized horse heart cytochrome c in 0.1 M-phosphate buffer, pH 7.4, diluted with 50% ethanediol to give a final concentration of 21.3 μM*

Upper spectrum shows room-temperature absorption in a 1 mm-path-length cell. Lower spectra show 1.68 K m.c.d. at magnetic fields of 1.05 T (·····), 2.22 T (–·–·–), 3.19 T (–·–·–·), 4.24 T (– – – –) and 5.28 T (——), in a cell of path length 1.38 mm.

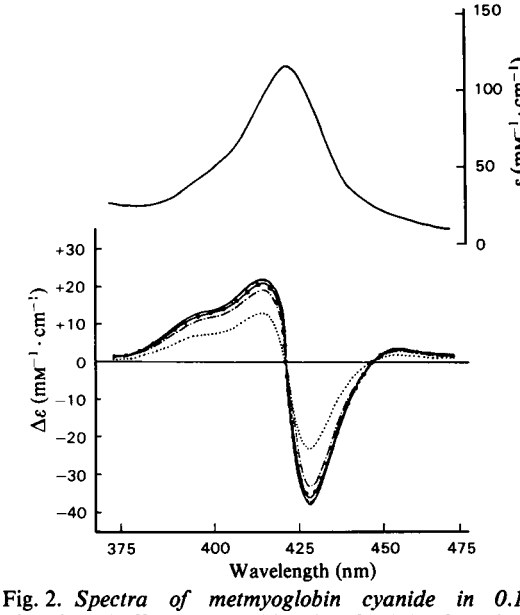

Fig. 2. *Spectra of metmyoglobin cyanide in 0.1 M-phosphate buffer, pH 7.4, diluted with 50% ethanediol to give a final concentration of 16.4 μM*

Upper spectrum shows room-temperature absorption in a 1 mm-path-length cell. Lower spectra show 1.53 K m.c.d. at magnetic fields of 1.05 T (·····), 2.22 T (–·–·–), 3.19 T (–·–·–·), 4.24 T (– – – –) and 5.28 T (——), in a cell of path length 1.33 mm.

Results and discussion

S = ½ *systems*

The room-temperature absorption, together with the low-temperature m.c.d. spectra, of oxidized cytochrome *c* and metmyoglobin cyanide are shown in Figs. 1 and 2 respectively. As illustrated in Figs. 1 and 2, the m.c.d. spectra were measured as a function of magnetic field in the range 0–5.3 T at several temperatures in the range 1.5–50 K. The field- and

temperature-dependences of the m.c.d. spectra of oxidized cytochrome *c* and metmyoglobin cyanide are summarized in Figs. 3 and 4 respectively. These plots are of $\Delta\varepsilon$, measured as the distance between the m.c.d. peaks and troughs at 398 and 411 nm in cytochrome *c* and at 414 and 427 nm in metmyoglobin cyanide, against $\beta B/2kT$. The curves in Figs. 3 and 4 constitute magnetization curves for these two proteins. There are several important features to be noted. First, for both haemoproteins all points fall on a single smooth curve whatever the field and temperature at which the m.c.d. spectrum was measured. Secondly, the curves can be characterized by two parameters, the slope of the linear part of the curve at low values of $\beta B/2kT$ and the asymptotic value at high values. The experimental curves of Figs. 3 and 4 conform to the picture expected, namely a linear part at high temperature and a saturation limit at low temperature and high magnetic field.

Analysis of curves. Schatz *et al.* (1978) have produced algebraic expressions for the field- and temperature-dependences of the m.c.d. spectra of systems with axial symmetry. In order that the expressions are valid for anisotropic systems in rigid solutions, it is necessary to express the ground-state Zeeman splitting and the transition dipole moments as functions of the angles of the principal *g*-tensor axes to the applied field. Since the molecules are randomly oriented, the resultant expression must be averaged over all orientations by integration. Because of the complexity of the resulting expressions, the integration cannot be accomplished in closed form, so that either numerical integration must be performed or various approximations invoked. We have extended the expressions of Schatz *et al.* (1978) to cover the case of a rhombic *g*-tensor. The details of this calculation are given in the Appendix. We show theoretical magnetization curves, evaluated from our expressions, for various cases of interest.

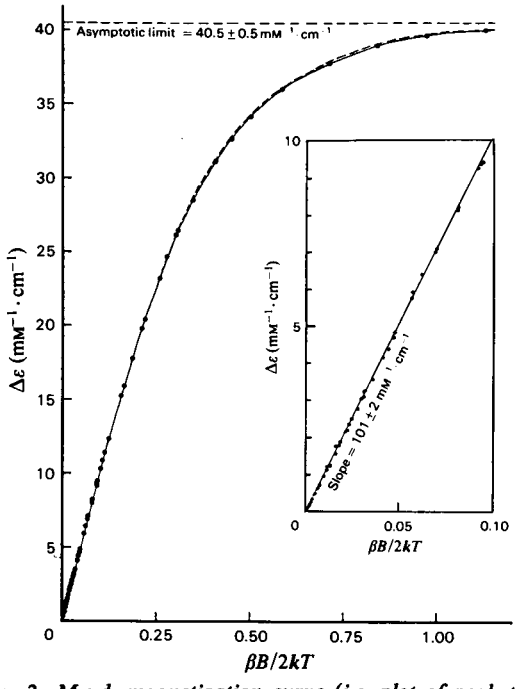

Fig. 3. *M.c.d. magnetization curve (i.e. plot of peak to trough for the Soret band, $\Delta\varepsilon$, versus $\beta B/2kT$) for oxidized horse heart cytochrome c*

The broken line represents the theoretical magnetization curve, normalized to the experimental saturation limit, by using the e.p.r.-determined rhombic *g*-values of cytochrome *c* (Mailer & Taylor, 1972), for an *x*–*y*-polarized transition. Inset shows detail of linear portion at low values of $\beta B/2kT$. Magnetic fields were in the range 0–5.3 T. Temperatures were 1.54, 4.22, 16 and 42 K.

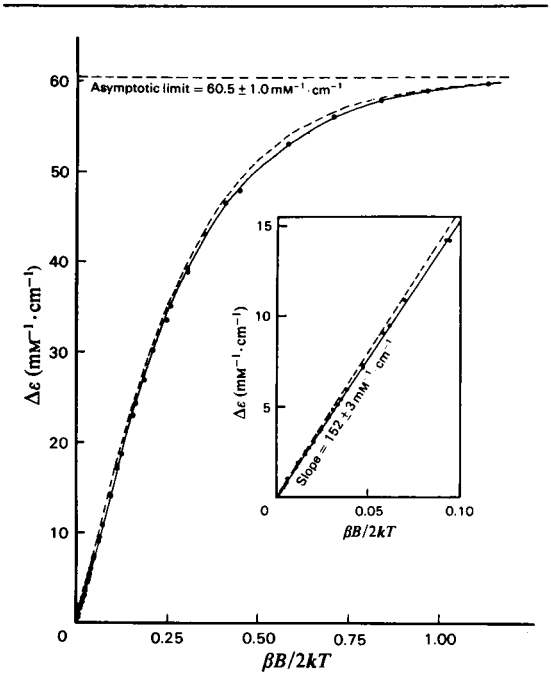

Fig. 4. *M.c.d. magnetization curve (i.e. plot of peak to trough for the Soret band, $\Delta\varepsilon$, versus $\beta B/2kT$) for metmyoglobin cyanide*

The broken line represents the theoretical magnetization curve, normalized to the experimental saturation limit, by using the e.p.r. determined rhombic *g*-values of metmyoglobin cyanide (Hori, 1971), for an *x*–*y*-polarized transition. Inset shows detail of linear portion at low values of $\beta B/2kT$. Magnetic fields were in the range 0–5.3 T. Temperatures were 1.54, 4.22, 17 and 44 K.

A considerable simplification results if the polarizations of the electronic transitions under consideration are known. For this reason we chose to study the Soret-band region of the haem spectrum, since there is evidence from polarized crystal spectra of metmyoglobin cyanide (Eaton & Hochstrasser, 1968) that the transitions giving rise to this band are predominantly x–y-polarized, i.e. in the plane of the haem ring. Fig. 5 shows two plots of magnetization curves that use our expression for the rhombic g-tensor. First, the effects of a constant value of $g_{av.}\left[=\frac{1}{\sqrt{3}}\sqrt{(g_x^2+g_y^2+g_z^2)}\right]$ with various anisotropy ratios, g_z/g_x and g_z/g_y, are illustrated. It can be seen that the slope in the linear limit and the asymptotic limit vary systematically, both increasing as the anisotropy increases. Secondly, we show the effect on the magnetization curves of keeping

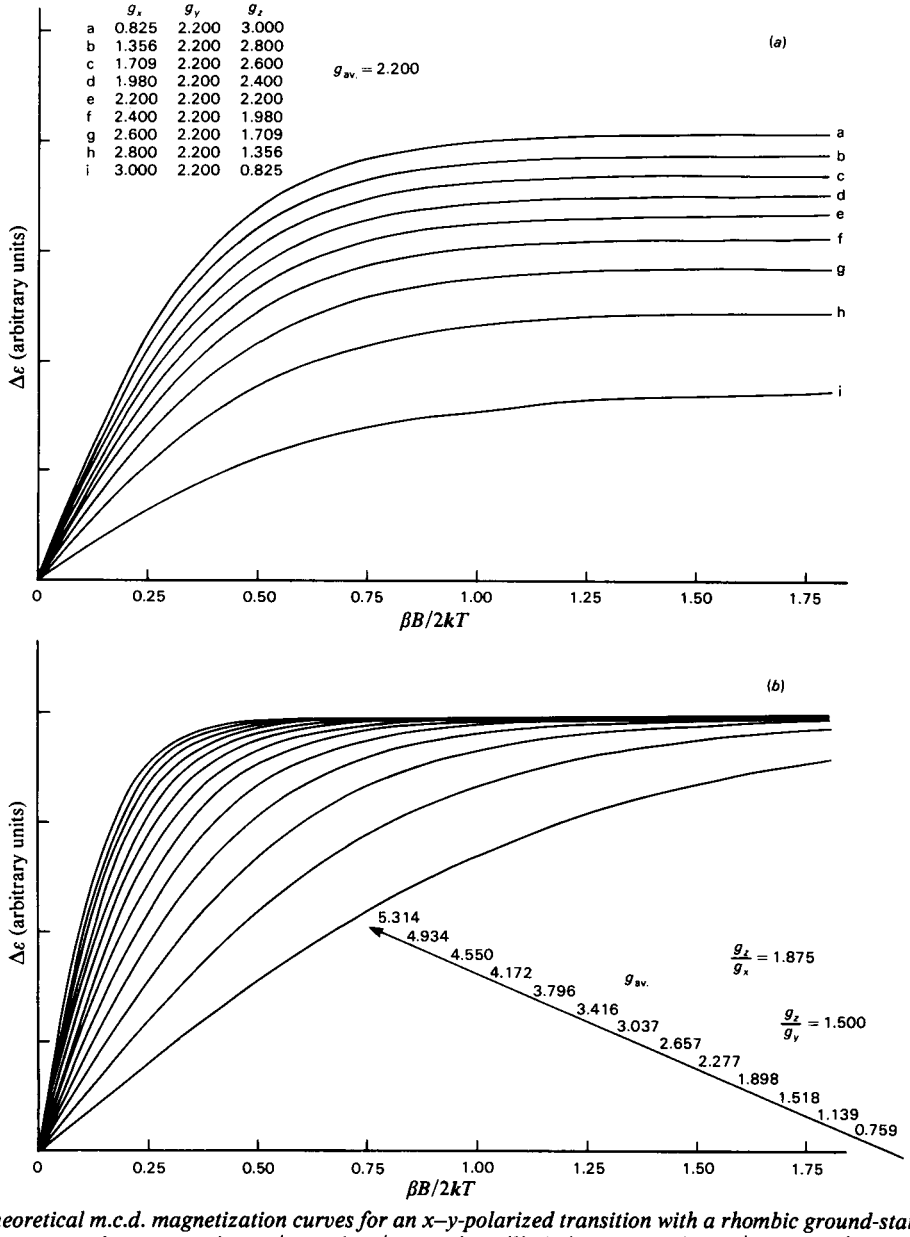

Fig. 5. *Theoretical m.c.d. magnetization curves for an x–y-polarized transition with a rhombic ground-state g-tensor* (*a*) $g_{av.}$ constant; anisotropy ratios g_z/g_x and g_z/g_y varying. (*b*) Anisotropy ratios g_z/g_x and g_z/g_y constant; $g_{av.}$ varying.

constant anisotropy ratios, g_z/g_x and g_z/g_y, but of varying the $g_{av.}$ value from 0.759 to 5.314. In this case the saturation limit remains constant but the initial slope steepens as $g_{av.}$ increases.

When analysing experimental magnetization curves to obtain estimates of ground-state g-factors it is in principle possible to carry out a least-squares fitting of our theoretical expression to the experimental curve. However, it is also possible to obtain an estimate of g_z by assuming that the system approximates to an axial one and by using the slope of the magnetization curve at low values of $\beta B/kT$ and the asymptotic value at high $\beta B/2kT$.

In the linear limit for an x–y-polarized transition from a ground state with an axial g-tensor, the slope of a plot of $\Delta\varepsilon$ against $\beta B/2kT$ is given by $\frac{1}{3}Kg_{\parallel}m_{x,y}^2$, where K is a proportionality constant and $m_{x,y}$ is the transition dipole moment. The asymptotic limiting value depends on the anisotropy, $\sigma = g_{\parallel}/g_{\perp}$, as shown in Fig. 5(*a*). When $\sigma = 1$, the isotropic limit, $\Delta\varepsilon$ at saturation is given by $\frac{1}{3}Km_{x,y}^2$. The expression that gives the relationship between σ and the saturation limit is derived by Schatz *et al.* (1978, eqn. 22b). The value of $\Delta\varepsilon$ at saturation can be written $Km_{x,y}^2/D$, where D is a parameter dependent on σ, the anisotropy,

$$\frac{1}{D} = \frac{\cosh\alpha}{\sinh^3\alpha}\cdot\left[\frac{\sinh 2\alpha}{4} - \frac{\alpha}{2}\right]$$

and $\sigma = \cosh\alpha$. Fig. 6 gives a plot of the dependence of D on σ.

Hence it is readily shown that the ratio of the slope at low $\beta B/2kT$ to the asymptotic value at high $\beta B/2kT$, which is given by the intercept, I, is related to $g_{\parallel}$ as follows: $g_{\parallel} = 3/D\cdot I$. When the system is isotropic, $\sigma = 1$ and $D = 3.0$, then $g_{\parallel} = 1/I$, but in the limit of complete anisotropy $g_{\parallel} \neq 0$ and $g_{\perp} = 0$, $\sigma \to \infty$ and $D \to 2.0$. Hence $g_{\parallel} = 3/2I$.

The application of these simple formulae to the curves of Fig. 5 is given in Table 1. There it is shown that it is possible to determine the g_z value of the theoretically constructed curves with rather good precision by making the approximation that the system is axial and by equating $g_{\perp}$ to

$$\frac{1}{\sqrt{2}}\sqrt{(g_x^2 + g_y^2)}$$

and $g_{\parallel}$ to g_z. Note that this also assumes that the electronic transitions are completely x–y-polarized. The agreement between the input value of g_z and the value of $g_{\parallel}$ determined from the intercept is always within 3%.

The results of analysing the experimental curves of oxidized horse heart cytochrome *c* and metmyoglobin cyanide in this manner are given in Table 1. The values of $g_{\parallel}$ obtained cover a range of values owing to the estimated uncertainty in determining the initial slope and asymptotic limiting value of the magnetization curves. Numerous data points at low $\beta B/2kT$ values were obtained to minimize the error in determining the initial slope, which is the principal source of error in determining the value of the intercept, I (see Figs. 3 and 4). For cytochrome *c* the true g_z value of 3.06 is covered by the range of values of $g_{\parallel}$, i.e. 2.97 ± 0.10, whereas for metmyoglobin cyanide the range of values of $g_{\parallel}$, i.e. 3.22 ± 0.12, represents an underestimate of the true g_z value of 3.45. Similarly, on normalizing to the experimental saturation limit, theoretical magnetization curves for an x–y-polarized transition, obtained by using the e.p.r.-determined rhombic g-values, give better agreement for cytochrome *c* than for metmyoglobin cyanide (see Figs. 3 and 4).

Since the underlying assumption in the above analysis is that the electronic transition is completely x–y-polarized, it is of interest to investigate the effect of adding a small z-polarized component. This can be done by using the expressions of Schatz *et al.* (1978, eqn. 22b), and involves the addition of an extra term. $\Delta\varepsilon$ at saturation becomes:

$$\frac{\Delta\varepsilon}{K} = m_{x,y}\cdot m_z\cdot E + m_{x,y}^2/D$$

where

$$E = \frac{\sqrt{2}}{\sinh^3\alpha}\left[\frac{\sinh 2\alpha}{4} - \frac{\alpha}{2} - \alpha\sinh^2\alpha\right]$$

and α and D are as defined earlier. For example, if 5% of the intensity of the Soret band of metmyoglobin cyanide were z-polarized, the estimated $g_{\parallel}$ value is 3.30 ± 0.12. The upper value of $g_{\parallel}$ now comes close to the experimental value of g_z, which is 3.45. Table 1 shows the effect on the estimated $g_{\parallel}$ values for both metmyoglobin cyanide and cytochrome *c* of allowing 5% and 10% of the Soret-band intensity to become z-polarized. From such con-

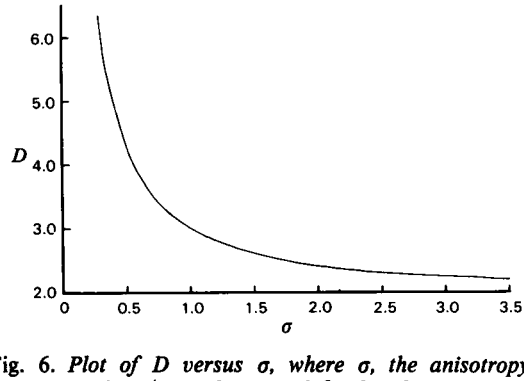

Fig. 6. *Plot of D versus σ, where σ, the anisotropy, equals $g_{\parallel}/g_{\perp}$ and D is as defined in the text*

Table 1. *Comparison of values of g_z with $g_{\parallel}$ for systems with rhombic g-tensors, where $g_{\parallel}$ is obtained from the asymptotic limiting value and the initial slope of both theoretical and experimental m.c.d. magnetization curves*

	g_x	g_y	g_z	$g_{av.}$*	σ†	D	I‡	$g_{\parallel}$§ 0% z-polarization	$g_{\parallel}$§ 5% z-polarization	$g_{\parallel}$§ 10% z-polarization
Fig. 5(*a*)	3.000	2.200	0.825	2.20	0.314	5.718	0.646	0.81	—	—
	2.800	2.200	1.356	2.20	0.539	4.050	0.550	1.35	—	—
	2.600	2.200	1.709	2.20	0.710	3.499	0.505	1.70	—	—
	2.400	2.200	1.980	2.20	0.860	3.195	0.476	1.97	—	—
	2.200	2.200	2.200	2.20	1.000	3.000	0.456	2.19	—	—
	1.980	2.200	2.400	2.20	1.147	2.847	0.441	2.39	—	—
	1.709	2.200	2.600	2.20	1.320	2.713	0.428	2.58	—	—
	1.356	2.200	2.280	2.20	1.532	2.592	0.418	2.77	—	—
	0.825	2.200	3.000	2.20	1.806	2.480	0.411	2.94	—	—
Fig. 5(*b*)	0.533	0.667	1.000	0.759	1.656	2.536	1.185	1.00	—	—
	0.800	1.000	1.500	1.139	1.656	2.536	0.791	1.50	—	—
	1.067	1.333	2.000	1.518	1.656	2.536	0.594	1.99	—	—
	1.333	1.667	2.500	1.898	1.656	2.536	0.476	2.49	—	—
	1.600	2.000	3.000	2.277	1.656	2.536	0.397	2.98	—	—
	1.867	2.333	3.500	2.657	1.656	2.536	0.341	3.47	—	—
	2.133	2.667	4.000	3.037	1.656	2.536	0.299	3.96	—	—
	2.400	3.000	4.500	3.416	1.656	2.536	0.267	4.43	—	—
	2.667	3.333	5.000	3.796	1.656	2.536	0.241	4.91	—	—
	2.933	3.667	5.500	4.172	1.656	2.536	0.219	5.40	—	—
	3.200	4.000	6.000	4.555	1.656	2.536	0.202	5.86	—	—
	3.467	4.333	6.500	4.934	1.656	2.536	0.187	6.33	—	—
	3.733	4.667	7.000	5.314	1.656	2.536	0.174	6.80	—	—
Metmyoglobin cyanide										
Theory	0.93	1.89	3.45‖	2.334	2.316	2.346	0.375	3.41	—	—
Expt.	0.93	1.89	3.45	2.334	2.316	2.346	0.398±0.014	3.22±0.12	3.30±0.12	3.37±0.12
Cytochrome *c*										
Theory	1.24	2.24	3.06¶	2.304	1.690	2.522	0.394	3.02	—	—
Expt.	1.24	2.24	3.06	2.304	1.690	2.522	0.401±0.013	2.97±0.10	3.03±0.10	3.07±0.10

* $g_{av.} = \sqrt{\frac{1}{3}(g_x^2 + g_y^2 + g_z^2)}$.
† $\sigma = \sqrt{2}g_z/\sqrt{(g_x^2 + g_y^2)}$.
‡ I = intercept on abscissa axis.
§ $g_{\parallel}$, calculated from $3/D \cdot I$.
‖ Experimental e.p.r. g-values (Hori, 1971).
¶ Experimental e.p.r. g-values (Mailer & Taylor, 1972).

siderations the percentage of Soret-band intensity that can be attributed to a z-polarized transition can be estimated as 5–20% for metmyoglobin cyanide and 0–15% for cytochrome *c*. These conclusions must be considered in the light of the polarized single-crystal spectra of these haemoproteins, which have been measured in careful work by Eaton & Hochstrasser (1967, 1968). For metmyoglobin cyanide, examination of the errors on the polarization ratios suggests that no more than 5% of the intensity of the Soret band could be z-polarized. No errors are quoted in the case of cytochrome *c*.

Clearly m.c.d. saturation studies on a wider range of derivatives and bands of different polarizations are required to explore the reliability of determining g-factors precisely in this way. When the polarizations of the electronic transitions are unknown, then a fitting procedure is required with the ratio of the z- to x–y-polarization being left as an adjustable parameter along with the g-factors.

S = 2 system

The room-temperature absorption and low-temperature m.c.d. spectra of reduced cytochrome *c* oxidase are shown in Fig. 7. The magnetization curves for this protein derivative are given in Fig. 8. The ordinate is the value of $\Delta\varepsilon$ measured from the m.c.d. peak at 446nm to the trough at 436nm. Twenty-five measurements have been made, namely five temperatures between 1.54 and 23K each at five fields, 0–5.3T. Fig. 8 shows that the magnetization curves are strongly field-dependent. The points do not fall on a single curve, as in Figs. 3 and 4, but rather they define a surface in a three-dimensional

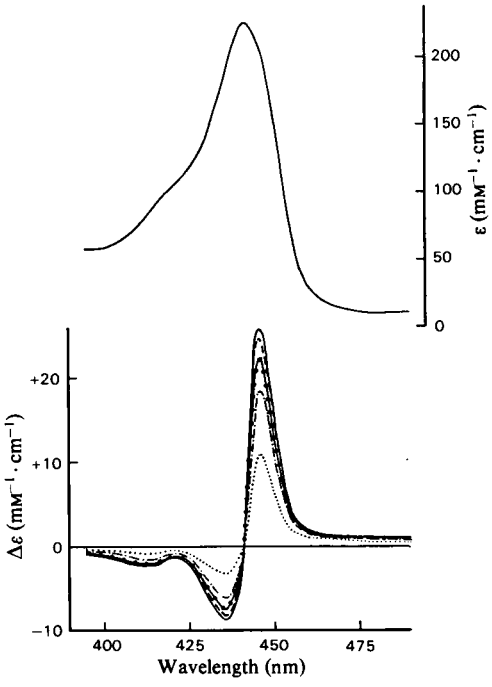

Fig. 7. *Spectra of anaerobically dithionite-reduced cytochrome c oxidase in 0.1 M-phosphate buffer, pH 7.4, containing 1% Tween 80, diluted with degassed 50% ethanediol to give a final concentration of 3.2 μM*

Upper spectrum shows room-temperature absorption in a 1 cm-path-length cell. Lower spectra show 1.53 K m.c.d. at magnetic fields of 1.05 T (·····), 2.22 T (–·–·–), 3.19 T (–··–··–), 4.24 T (– – – –) and 5.28 T (——) in an anaerobic cell of path length 1.29 mm.

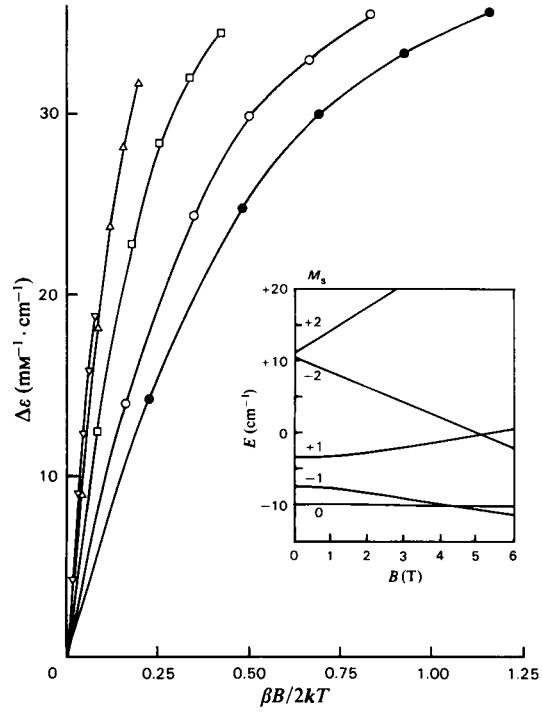

Fig. 8. *M.c.d. magnetization curve (i.e. plot of peak to trough of the Soret band, Δε, versus βB/2kT) for reduced cytochrome c oxidase*

Temperature: 1.54 K (●); 2.12 K (○); 4.22 K (□); 9 K (△); 32 K (▽). Inset shows a calculation of the zero-field splitting and magnetic-field-dependency of the high-spin ($S = 2$) ferrous state of myoglobin (Rudowicz, 1977).

space with co-ordinates of $\Delta\varepsilon$, B and $1/T$. The reason for this is the zero-field splitting of the $S=2$ ground state in an environment of less than cubic symmetry. Fig. 8. shows a calculation of the zero-field splitting and magnetic-field-dependency of the high-spin ferrous state of myoglobin, which also has $S=2$ (Rudowicz, 1977). The zero-field splitting parameters for this state of myoglobin and that of high-spin ferrous haem a_3 in reduced cytochrome *c* oxidase are probably rather similar (Tweedle *et al.*, 1978). However, we use the diagram only for illustrative purposes. It can be seen that the temperature-dependence of the m.c.d. signals at a field of 1 T will be different from that at 5 T.

Complex magnetization curves result either from ground states that have zero-field components that are thermally populated over the temperature range of the experiment or when the magnetic field is sufficiently strong to bring Zeeman sublevels low enough in energy to be thermally accessible. Hence they may arise for magnetic ground states in a crystal field of less than cubic symmetry other than those that are isolated Kramer's doublets.

The complete analysis of such a set of curves as shown in Fig. 8 has not yet been carried out. The theoretical expressions will be extremely cumbersome, since they will need to take into account the field-induced mixing of the zero-field components, and will also need to be averaged over all angles.

Conclusions

With the aid of measurements on magnetically well-characterized haemoproteins, we have demonstrated that m.c.d. magnetization curves plotted over a range of temperatures from 1.5 to 50 K and of fields from 0 to 5 T can successfully be used to distinguish paramagnets with isolated Kramer's ground states ($S = \frac{1}{2}$) from others with complex ground states containing zero-field components that are thermally accessible. We have illustrated the differences between an even ($S = 2$)- and odd ($S = \frac{1}{2}$)-electron system. Furthermore it is possible to

make estimates of the ground-state g-factors of $S=\frac{1}{2}$ paramagnets even in the presence of rhombic anisotropy, provided that some knowledge of the polarizations of the electronic transitions is available. It is important to note that the method of comparing the ratios of the asymptotic limiting value to the initial slope provides a parameter that is independent of the protein concentration and also of the depolarization properties of the low-temperature glass. Similarly the technique will be independent of impurities provided that they do not have absorption bands at the wavelengths at which the m.c.d. is being measured. Thus the technique should prove useful for the probing of magnetic states that are not amenable to study by other techniques. We have recently applied the method to study the magnetic state of the e.p.r.-undetectable haem centre in cytochrome *c* oxidase (A. J. Thomson, M. K. Johnson, C. Greenwood & P. E. Gooding, unpublished work).

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APPENDIX

In this Appendix the expressions of Schatz *et al.* (1978) for the field- and temperature-dependence of an m.c.d. signal are extended to cover the case of a molecule with a rhombic set of ground-state g-factors. We follow exactly the development of Schatz *et al.* (1978), using wherever possible the same notation. The case we consider is of an odd-electron system with a Kramer's doublet ground state and an allowed electronic transition, $\frac{1}{2}\rightarrow\frac{1}{2}$, polarized in the x–y-plane.

We can write down the Zeeman matrix, analogous to Table 3 of Schatz *et al.* (1978), and this is given as Table 1 in this Appendix. By diagonalizing this matrix, the energies of the two ground-state Zeeman components are obtained in terms of the principal g-values and the Euler angles, θ and ϕ. These energies, $E_{\pm\frac{1}{2}}$, are given by:

$$E_{\pm\frac{1}{2}} = (l^2\cdot g_x{}^2 + m^2\cdot g_y{}^2 + n^2\cdot g_z{}^2)^{\frac{1}{2}}/2 = \Gamma/2$$

where $\Gamma = (l^2\cdot g_x{}^2 + m^2\cdot g_y{}^2 + n^2\cdot g_z{}^2)^{\frac{1}{2}}$.

The wavefunctions of the ground state are also functions of the g-factors and the same angles. They can be obtained from the matrix (Table 1 in this Appendix) and can be written:

$$\psi_{+\frac{1}{2}} = C_1|\tfrac{1}{2}\rangle + C_2|-\tfrac{1}{2}\rangle$$

where the coefficients C_1 and C_2 are given by:

$$C_2/C_1 = (\Gamma - n\cdot g_z)/(l\cdot g_x + i\cdot m\cdot g_y)$$
$$|C_1|^2 = (\Gamma + n\cdot g_z)/2\Gamma$$

Table 1. *Matrix of the Zeeman Hamiltonian* $\mathscr{H} = -\mu_Z\cdot B_Z$

Matrix is in units of $\beta\cdot B$. $\mu_Z = -\beta(L_Z + 2S_Z)$, where L_Z and S_Z are the orbital and spin angular momentum operators in the space-fixed co-ordinates, X, Y and Z.

	$+\frac{1}{2}$	$-\frac{1}{2}$
$+\frac{1}{2}$	$n\cdot g_z/2$	$(l\cdot g_x + i\cdot m\cdot g_y)/2$
$-\frac{1}{2}$	$(l\cdot g_x - i\cdot m\cdot g_y)/2$	$-n\cdot g_z/2$

$g_z/2 = \langle\frac{1}{2}|\mu_z|\frac{1}{2}\rangle$

$g_x/2 = \langle+\frac{1}{2}|\mu_x|-\frac{1}{2}\rangle = \langle-\frac{1}{2}|\mu_x|+\frac{1}{2}\rangle$

$i\cdot g_y/2 = \langle\frac{1}{2}|\mu_y|-\frac{1}{2}\rangle = -\langle-\frac{1}{2}|\mu_y|+\frac{1}{2}\rangle$

$l = \sin\theta\cdot\sin\phi \qquad m = -\sin\theta\cdot\cos\phi \qquad n = \cos\theta$

Substituting these results into eqns. (9), (10) and (11) of Schatz *et al.* (1978), the following final expression is obtained for the m.c.d. of an *x*–*y*-polarized transition:

$$\frac{\Delta\varepsilon}{K} = -\frac{m_{x,y}{}^2}{4\pi} \int_{\phi=0}^{2\pi} \int_{\theta=0}^{\pi} \frac{\cos^2\theta \cdot \sin\theta \cdot g_z}{\Gamma} \cdot \tanh\left(\frac{\beta B \cdot \Gamma}{2kT}\right) \mathrm{d}\theta \mathrm{d}\phi$$

This expression cannot be evaluated in closed form. Evaluation is best accomplished on a computer numerically. This expression will reduce to that given by Schatz *et al.* (1978) as the second term in eqn. (17) if the following identities are made:

$$g_z \equiv g_\parallel \text{ and } g_x = g_y \equiv g_\perp$$

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