

# Electron Paramagnetic Resonance of Beef-Heart Ferricytochrome c\*

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We have observed an anisotropic EPR spectrum in a frozen solution of the low-spin ( $S = \frac{1}{2}$ ) hemoprotein ferricytochrome c. These results are interpreted in terms of the theory for the low-spin ferric ion in an octahedral crystal field which is subject to an (axial) distortion along the axis perpendicular to the heme plane and a (rhombic) distortion in the plane of the heme, and a spin-orbit constant which is less than that found in the free ion. This model also leads to calculated values for the effective magnetic moment which agree well with experiment.

## INTRODUCTION

Cytochrome c is a small hemoprotein of molecular weight 12 600 which plays an important part in biological electron transport. The prosthetic group which is responsible for the function of the protein is the iron-porphyrin called heme (Fig. 1). From magnetic-susceptibility<sup>1</sup> measurements it is known that oxidized ferricytochrome c is low-spin ferric ( $S = \frac{1}{2}$ ) and that reduced ferrocycytochrome c is diamagnetic.

One would expect to find an EPR spectrum similar to that observed in other low-spin ferric systems such as potassium ferricyanide<sup>2</sup> and the hemoprotein ferrihemoglobin azide.<sup>3</sup> Such spectra are characterized by a highly anisotropic  $g$  tensor with two principal values above the free electron value and one below. However, previous attempts<sup>4</sup> to observe the EPR spectrum of cytochrome c have failed to resolve any anisotropy and only a single broad resonance has been observed. The difficulty in resolving the spectrum presumably arises from the short spin-lattice relaxation time which is associated with the large orbital contribution to the magnetic moment.

We wish to report the resolution of an anisotropic  $g$  tensor in ferricytochrome c which was achieved by working at 20°K. We also describe a ligand field model which is consistent with the EPR  $g$  values and observed magnetic moment.

## EXPERIMENTAL

The EPR spectra were recorded at both K-band (35-GHz) and X-band (9-GHz) frequencies using a Varian V4500 spectrometer with 100-kHz field modulation. The Varian V4561 bridge was used at K band and at X band we employed a bridge of similar configuration constructed by Sands.

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<sup>1</sup> E. Boeri and A. Ehrenberg, *Biochem. Biophys. Acta* **12**, 273 (1953).

<sup>2</sup> J. M. Baker, B. Bleaney, and K. D. Bowers, *Proc. Phys. Soc. (London)* **B69**, 1205 (1956).

<sup>3</sup> J. F. Gibson, D. J. E. Ingram, and D. Schonland, *Discussions Faraday Soc.* **26**, 72 (1958).

<sup>4</sup> A. Ehrenberg, *Arkiv Kemi* **19**, 119 (1962).

Cryogenic cooling was achieved using a stream of helium gas obtained by boiling liquid helium. A 10- $\Omega$  electric heater and an evacuable transfer tube were designed to fit into and seal a helium Dewar. By using a Variac transformer to control the power dissipation in the heater one can obtain the constant flow of cold gas necessary to achieve the required working temperature. For operation at X band the gas was passed through the quartz variable-temperature Dewar which was mounted in the rectangular cavity in the normal way. Temperature was measured using a copper-constantan thermocouple inserted through the base of the Dewar. For K-band measurements the cooling gas was passed into an evacuated cylindrical jacket which surrounded the cavity; thus in this arrangement the entire cavity is refrigerated. The temperature was measured by mounting the thermocouple in a K-band sample tube and periodically placing it into the cavity. At these temperatures, the thermocouple error is estimated to be as large as  $\pm 2^\circ$ .

For  $g$ -value measurements we relied on the Fieldial to monitor the magnetic field and a Hewlett-Packard 532B frequency meter to determine the klystron frequency at X band and the Varian V4561 frequency meter at K band. Although the nominal accuracy of such measurements is only 0.2% it is our experience from repeated measurements on strong pitch that the accuracy is good to at least 0.1%.

Cytochrome c was prepared from beef hearts by the aluminum sulfate method of Margoliash<sup>5</sup>; this avoids contamination of the final product with polymers. By accepted spectroscopic criteria it was of very high purity and did not bind carbon monoxide.<sup>6</sup> Some of the results reported here have been confirmed on a sample of crystalline cytochrome c provided by Margoliash.

## RESULTS

Figure 2 shows the EPR spectrum of a frozen solution of ferricytochrome c at 20°K. It is a typical powder spectrum of a spin doublet with complete anisotropy in the  $g$  factor having high- and low-field extrema corresponding to  $g_x$  and  $g_z$  and a principal slope at  $g_y$ . The in-

<sup>5</sup> E. Margoliash (private communication).

<sup>6</sup> E. Margoliash and J. Lustgarten, *J. Biol. Chem.* **237**, 2297 (1962).

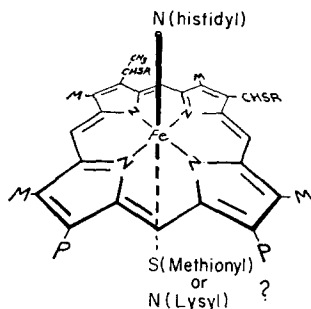


FIG. 1. Heme prosthetic group of cytochrome c. M, methyl; P, propionic acid; R,  $\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ .

tensity of the spectrum decreases very rapidly with increasing temperature and at 77°K only a very broad unresolved resonance is recorded. The measured  $g$  values were  $g_x = 1.24$ ,  $g_y = 2.24$ ,  $g_z = 3.06$ . Measurements at K band were used to confirm these assignments. The increased linewidth at the higher frequency resulted in a severe reduction in the signal-to-noise ratio and the quality of the spectra suffered accordingly. Nevertheless, identical values were obtained for  $g_x$  and  $g_y$ ; unfortunately the limitations of our magnet did not permit us to observe the high-field resonance ( $g_z$ ) at the K-band frequency.

When the cytochrome is reduced chemically, all magnetic resonance absorption disappears. This is expected since susceptibility measurements show that *ferroc*cytochrome c is diamagnetic.

### THEORETICAL

One can interpret the measured  $g$  values in terms of the ligand field theory for low-spin ferric ion. In order to explain the  $g$  values of potassium ferricyanide Bleaney and O'Brien<sup>7</sup> suggested that low-spin iron can be described by assuming a crystal field of arbitrary symmetry. More recently Harris<sup>8</sup> has carried out an extensive calculation on low-spin ferric ion and shown

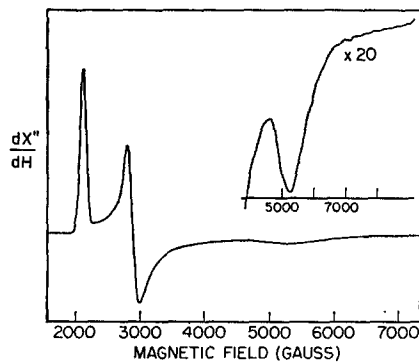


FIG. 2. EPR spectrum of a  $10^{-2}M$  solution of beef-heart cytochrome c in 0.1M phosphate pH7.6. Spectrum was recorded with a Varian V4500 spectrometer using 100-kHz field modulation; modulation amplitude, 6 G; microwave frequency, 9.22 GHz; temperature, 20°K.

<sup>7</sup> B. Bleaney and M. C. M. O'Brien, Proc. Phys. Soc. (London) **B69**, 1216 (1956).

<sup>8</sup> G. Harris, Theoret. Chim. Acta **5**, 379 (1966).

that the  $g$  values of the low-spin ferrihemoglobin azide can be explained by assuming an (axial) distortion of the octahedral ligand field along the axis perpendicular to the heme plane and a (rhombic) distortion in the plane of the heme. This theory can be extended to include cytochrome c.

In a strong crystal field of octahedral symmetry the ground state of the ferric ion is  ${}^2T_2(t_{2g}^5)$ . Harris<sup>8</sup> has shown that, for a discussion of magnetic properties one can, to a good approximation, ignore effects of the  $e_g$  orbitals when the strength of the octahedral crystal field is greater than some 27 000  $\text{cm}^{-1}$ . If the symmetry is exactly octahedral, then the  ${}^2T_2(t_{2g}^5)$  state is sixfold degenerate (orbital  $\times$  spin). Under the influence of axial and rhombic distortions together with spin-orbit coupling, the sixfold degeneracy is removed and three Kramers doublets result (Fig. 3). Each of the three resulting twofold degenerate states can be described as a linear combination of the  ${}^2T_2(t_{2g}^5)$  basis functions. In the complex representation<sup>9</sup> each state has the form,

$$\begin{aligned}\psi_i &= A_i {}^2T_2(1, -\frac{1}{2}) + B_i {}^2T_2(0, \frac{1}{2}) + C_i {}^2T_2(-1, -\frac{1}{2}), \\ \psi_i' &= A_i {}^2T_2(-1, +\frac{1}{2}) + B_i {}^2T_2(0, -\frac{1}{2}) + C_i {}^2T_2(1, +\frac{1}{2}),\end{aligned}\quad (1)$$

where  $\psi_i$  and  $\psi_i'$  are the partners of the twofold degenerate Kramers doublet.

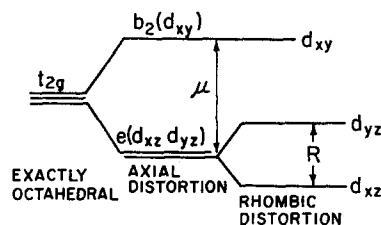


FIG. 3. Energy-level diagram for  $t_{2g}$  orbitals in one-hole formalism.

The energy of each state and the coefficients of Eq. (1) are found from the eigenvalues and eigenvectors of the perturbation matrix which describes the coupling of the basis functions. This matrix can be written in terms of three parameters which we assume to be the same order of magnitude:  $\mu$ , the axial distortion;  $R$ , the rhombic distortion; and  $\lambda$ , the spin-orbit coupling constant. The matrix has the form shown in Table I. Assuming that the Zeeman operator does not mix terms to first order, the  $g$  values are given by the following<sup>8</sup>:

$$\begin{aligned}g_x &= 2 | (\sqrt{2}A - B) (\sqrt{2}C - B) |, \\ g_y &= 2 | (-\sqrt{2}A + B) (\sqrt{2}C + B) |, \\ g_z &= 2 | 2A^2 - B^2 |.\end{aligned}\quad (2)$$

To obtain a fit to the measured  $g$  values, we systematically assigned values to the three parameters  $\mu$ ,  $R$ ,

<sup>9</sup> J. S. Griffith, *The Theory of Transition Metal Ions* (Cambridge University Press, New York, 1961), p. 160.

and  $\lambda$ , and calculated the corresponding  $g$  values. Matrix diagonalization was carried out by machine, using the Jacobi method. We covered the ranges  $\lambda=250$ – $420$   $\text{cm}^{-1}$ ,  $\mu=250$ – $1000$   $\text{cm}^{-1}$ , and  $R=250$ – $1000$   $\text{cm}^{-1}$ . Within these ranges of values for the parameters there is only one set, viz.,  $\lambda=340$   $\text{cm}^{-1}$ ,  $\mu=900$   $\text{cm}^{-1}$ , and  $R=500$   $\text{cm}^{-1}$ , which yields  $g_x=1.26$ ,  $g_y=2.24$ , and  $g_z=3.07$ , in good agreement with experiment. Inspection of the results of Harris<sup>8</sup> eliminated other possible ranges for the parameters. Table II gives the energy eigenvalues and the coefficients of the terms in the eigenfunctions which result from this set of parameters.

As a check we also calculated the effective magnetic moment using the expression given by Harris.<sup>8</sup> We calculate a value of  $2.35 \mu_B$  at  $300^\circ\text{K}$  (including second-order contributions from the first excited state),  $2.17 \mu_B$  at  $150^\circ\text{K}$ , and  $2.01 \mu_B$  at  $4^\circ\text{K}$ . Boeri and Ehrenberg<sup>1</sup> have measured the magnetic susceptibility of beef-heart cytochrome c and found a value for the effective magnetic moment of  $2.24 \mu_B$  which when corrected for temperature-independent orbital paramagnetism of their standard becomes  $2.36 \mu_B$ .<sup>10</sup> On the basis of preliminary measurements Tasaki *et al.*<sup>11</sup> report values of  $2.40 \mu_B$  at  $150^\circ\text{K}$  and  $2.05 \mu_B$  at  $4.2^\circ\text{K}$ . These workers also observe a temperature dependence of the effective

TABLE I. First-order perturbation matrix for coupling between  $t_{2g}$  basis states in complex representation in terms of parameters:  $\mu$ , axial field strength;  $R$ , rhombic field;  $\lambda$ , spin-orbit constant.

	${}^2T_2(1, -\frac{1}{2})$	${}^2T_2(-1, -\frac{1}{2})$	${}^2T_2(0, \frac{1}{2})$
${}^2T_2(1, -\frac{1}{2})$	$-\frac{1}{2}\lambda$	$\frac{1}{2}R$	$\lambda/\sqrt{2}$
${}^2T_2(-1, -\frac{1}{2})$	$\frac{1}{2}R$	$\frac{1}{2}\lambda$	0
${}^2T_2(0, \frac{1}{2})$	$\lambda/\sqrt{2}$	0	$\mu$

magnetic moment which suggests that the energy difference between the first excited state and the ground state is comparable to room temperature. Our calculations yield a value of  $625 \text{ cm}^{-1}$  for this energy difference which is compatible with experimental observation.

## DISCUSSION

The agreement between the calculated and measured  $g$  values and effective magnetic moment leads us to believe that this ligand field model is a good description of the energy levels of the  $t_{2g}$  orbitals in ferricytochrome c. We also observe that the value of the spin-orbit coupling constant of  $340 \text{ cm}^{-1}$  is reduced from the free-ion value of  $420 \text{ cm}^{-1}$ . A similar effect was observed by Bleaney and O'Brien<sup>7</sup> in the case of potassium ferricyanide and Owen<sup>12</sup> in the study of certain optical properties of hydrated iron group salts.

<sup>10</sup> A. Ehrenberg, *Svensk Kem. Tidskr.* **74**, 103 (1962).

<sup>11</sup> A. Tasaki, J. Otsuka, and M. Kotani, *Biochim. Biophys. Acta* **140**, 284 (1967).

<sup>12</sup> J. Owen, *Proc. Roy. Soc. (London)* **A227**, 183 (1955).

TABLE II. Eigenvalues ( $E_i$ ) and coefficients of basis functions [Eq. (1)] yielding observed  $g$  values.

	$A_i$	$B_i$	$C_i$	$E_i(\text{cm}^{-1})$
$\psi_1(\psi_1')$	0.88	-0.17	-0.43	-340
$\psi_2(\psi_2')$	0.41	-0.16	0.90	+284
$\psi_3(\psi_3')$	0.22	0.97	-0.07	+958

In the calculation of the  $g$  values, it was assumed that the spin-orbit interaction could be included in the Hamiltonian by the expression,

$$\mathcal{H}_{so} = \frac{1}{2}\alpha^2 \langle r^{-1} [\partial U(r) / \partial r] \rangle \sum_i \mathbf{l}_i \cdot \mathbf{S}_i = \lambda \mathbf{L} \cdot \mathbf{S}. \quad (3)$$

This expression is derived assuming that the electric field in which the electrons move is spherically symmetric and holds for a free atom or an ion in a crystal field. However, in a complex, several mechanisms are possible which lead to a reduction of the value of  $\lambda$  from the free atom.

Dunn<sup>13</sup> points out that the reduction in  $\lambda$  could be due to charge transfer from the ligands to the metal upon covalent bonding. The reduction is brought about by modifying the potential  $U(r)$ . Second, as suggested by Missetich and Buch,<sup>14</sup> it is necessary to include contributions to the spin-orbit interaction from the ligands. However, if the ligands are of low atomic number, as in our case, this may be a small effect. Third, it has been suggested by Marshall and Stuart<sup>15</sup> that the radial wavefunctions of the  $d$  orbitals may expand. This results in a lower expectation value,

$$\langle r^{-1} [\partial U(r) / \partial r] \rangle.$$

Further, this expansion means that the electron density is lower, hence the functional form of  $U(r)$  would be modified. It may turn out, however, that this effect

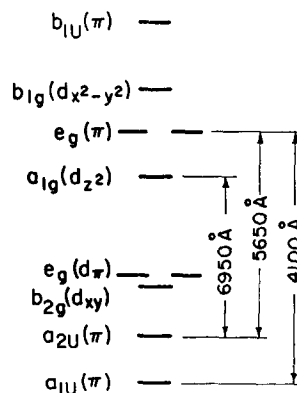


FIG. 4. Energy-level diagram for molecular orbitals calculated by Zerner and Gouterman.<sup>17</sup> Transitions shown measured by Eaton and Hochstrasser<sup>18</sup> for ferricytochrome c.

<sup>13</sup> T. M. Dunn, *J. Chem. Soc.* **1959**, 623.

<sup>14</sup> A. Missetich and T. Buch, *J. Chem. Phys.* **41**, 2524 (1964).

<sup>15</sup> W. Marshall and R. Stuart, *Phys. Rev.* **123**, 2048 (1961).

is negligible.<sup>16</sup> A fourth consideration, pointed out by Misetich and Buch,<sup>14</sup> is that the expression  $\lambda \mathbf{L} \cdot \mathbf{S}$  may not be valid in the case of covalency because it is implicit in (3) that electrons move in a central field of spherical symmetry. In covalent complexes it is not obvious that this spherical symmetry is preserved.

If one assumes that the effective field is spherically symmetric and the principal cause of the reduction in  $\lambda$  is a charge-transfer mechanism arising from covalency, then the analysis of Owen<sup>12</sup> is appropriate. Then, one can say that the ratio of the spin-orbit coupling constant of the complex to the spin-orbit coupling constant of the free ion is the fraction of time that the electron resides on the metal. In the case of ferricytochrome c we have  $\lambda_{\text{complex}}/\lambda_{\text{atom}} = 340/420 = 0.8$ , which corresponds to 20% delocalization. The recent extended Hückel molecular-orbital calculations of Zerner and Gouterman<sup>17</sup> indicate that the  $d_{xy}$  is about 98% metal and the  $d_{xz}$  and  $d_{yz}$  are about 80% metal. In our calculation these states are strongly mixed, so that the estimate of the covalency as measured by the reduction in the spin-orbit constant using the above argument is reasonable.

One further observation is interesting. The Zerner and Gouterman<sup>17</sup> calculations on low-spin iron-porphin establish an energy-level diagram which is shown in Fig. 4. The recent optical study of Eaton and Hochstrasser<sup>18</sup> fixes the  $a_{1u}(\pi) \rightarrow e_g(\pi)$  energy distance at about 24 400  $\text{cm}^{-1}$ . Assuming that in ferricytochrome c, the  $t_{2g}$  orbitals lie very close to the  $a_{2u}(\pi)$  and the  $d_z^2$  very close to the  $e_g(\pi)$  then, at most, the separation between the  $t_{2g}$  and the  $e_g$  orbitals is some 24 400  $\text{cm}^{-1}$ . However, if the assignment of the 6950-Å absorption band is correct, i.e.,  $a_{2u}(\pi) \rightarrow a_{1g}(d_z)$ , then the separation between the  $d_z^2$  and the  $t_{2g}$  set would be  $\sim 14\,000 \text{ cm}^{-1}$ . If this were the case, it would indicate that in the molecular complex, a low-spin ferric ion can occur with a much smaller separation between the  $t_{2g}$  and  $e_g$  orbitals than is called for by the purely ionic model of Harris. This can be understood in qualitative terms by saying that in the complex the interelectronic repulsion is reduced. Hence the ligand field required to produce low spin is smaller in the complex than in the free ion. This conclusion should be qualified by pointing out that the assignment of the 6950-Å band is not firmly established. Both Eaton and Hochstrasser<sup>18</sup> and Day *et al.*<sup>19</sup>

observe that this transition could equally well arise from a charge transfer from a neighboring polypeptide group to either the iron or the porphyrin. Furthermore, the results of the Zerner and Gouterman calculation should be employed cautiously when discussing transitions involving the metal orbitals because these calculations are designed to fit the  $\pi-\pi^*$  transitions in ferric porphyrin cyanide, and consequently may not predict very well the energy levels which we are discussing.

In another respect our EPR results are nicely consistent with the optical studies. The Zerner and Gouterman calculations assume that the symmetry of the complex is  $D_{4h}$ . Were this the case in ferricytochrome c, the  $d_{xz}$  and  $d_{yz}$  orbitals would be degenerate. However, as we have seen, the observed  $g$  tensor with  $g_x \neq g_y \neq g_z$  indicates that the symmetry must be lower than  $D_{4h}$ , thus removing the  $d_{xz}-d_{yz}$  degeneracy. It therefore follows that the lowest unfilled molecular orbital, which is  $e_g(\pi)$  in  $D_{4h}$  symmetry must be split and hence the Soret band would be expected to be split; this is in fact observed by Eaton and Hochstrasser.<sup>18</sup> The fact that the EPR spectrum shows rhombic symmetry indicates that the asymmetry in the placement or in the nature of the axial ligands is responsible for the removal of the  $e_g(\pi)$  degeneracy. It is unlikely that side-chain interaction of the porphyrin with the protein structure would produce very pronounced effects in the EPR spectrum except for possible second- or third-order effects. This is supported by measurements on a variety of vanadyl porphyrins which show that the EPR spectrum is insensitive to peripheral substitutions in the porphyrin ring structure.<sup>20</sup>

## CONCLUSIONS

We have succeeded in demonstrating the anisotropic EPR spectrum of ferricytochrome c. To a good approximation, the  $g$  values and effective magnetic moment can be accounted for by assuming an (axial) distortion of the octahedral ligand field along the axis perpendicular to the heme plane and a (rhombic) distortion in the plane of the heme together with a spin-orbit constant less than the free-ion value. This reduction in the spin-orbit constant can be regarded as a measure of covalency. This amount of covalency is consistent with the extended Hückel treatment of iron-porphin.

## ACKNOWLEDGMENT

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<sup>16</sup> J. Owen and J. H. M. Thornley, Rept. Progr. Phys. **29**, 675 (1966).

<sup>17</sup> M. Zerner and M. Gouterman, Theoret. Chim. Acta **6**, 363 (1966).

<sup>18</sup> W. A. Eaton and R. M. Hochstrasser, J. Chem. Phys. **46**, 2533 (1967).

<sup>19</sup> P. Day, D. W. Smith, and R. J. P. Williams, Biochem. **6**, 1563 (1967).

<sup>20</sup> E. M. Roberts and W. S. Koski, J. Chem. Phys. **34**, 591 (1961).