ELECTRON PARAMAGNETIC RESONANCE STUDY OF CYTOCHROME C SOLUTIONS AND SINGLE CRYSTALS

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in the Department of Physics

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
August, 1971
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Date Oct 15/1971
ABSTRACT

Electron paramagnetic resonance (EPR) signals from tuna ferricytochrome c solutions were obtained between 4.2°K and 77°K, with g-values $g_1 = 1.25$, $g_2 = 2.25$, $g_3 = 3.05$. The $g_3$ line is 380 gauss wide between 4.2°K and 50°K with Gaussian shape, but has become 700 gauss wide with Lorentzian shape at 77°K. The temperature independent shape and width are best explained by a distribution of rhombic crystal field potentials (r.m.s. deviation = 11%). The Lorentzian shape arises from a short ($10^{-8}$ sec.) spin-lattice relaxation time.

EPR spectra from horse heart ferricytochrome c single crystals were analysed to obtain the orientation of the g-axes relative to the crystallographic axes. The $g_3$-axis was 76° from the crystal c-axis, close to the heme normal (71.5° to c-axis) determined from the 3-dimensional X-ray structure by Dickerson. The other 2 g-axes lay approximately along the N-Fe-N directions in the heme ring.

An amended version of Eisenberger and Pershan's theory was used to explain the angular variation of the broad lines (300-2000 gauss) seen in the crystals—best fit was obtained with the distribution of ligand fields from the solution study plus a 1.5° variation in g-axis orientation.
The undifferentiated absorption line shapes observed at 4.2°K in both solutions and single crystals were explained by the Portis theory of rapid adiabatic passage in solids. This theory was tested with a model system of charred dextrose, and found to be valid. Using the theory the relaxation time (τ) of the cytochrome c system was found to be, from the phase lag of the EPR signal relative to the magnetic field modulation, \(3.8 \times 10^{-6}\) sec. at 4.2°K. τ was obtained between 4.2°K and 18°K from the rapid passage signals, and between 50°K and 70°K from the linewidth of the spectra. The temperature dependence of τ below 20°K could arise from a combination of a \(^9\)Raman spin-lattice relaxation process with a temperature independent spin-spin relaxation time of order \(10^{-8}\) seconds (which might arise from dipolar interactions between neighboring iron atoms).
PROLOGUE

THE THIRD DERIVATIVE

Turning and tumbling in the lattice,
The iron atom cannot bear the cold;
Things fall apart; the crystal cannot hold;
Disorder is loosed upon the world,
The blood-red block is loosed, and everywhere
The symmetry of solid state is drowned;
The best lack of all cohesion, while the worst
Are full of passionate intensity.
Surely some revelation is at hand;

After Dickerson et al. (1967)
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ACKNOWLEDGEMENTS

Many people have helped in the completion of this work. My especial thanks go to:

Dr. C.P.S. Taylor, my supervisor, whose clear scientific thinking and excellent experimental ability was a constant example to follow. It has been a pleasure to work under him.

My wife, Kathleen, who not only did the diagrams and equations but also gave constant support and encouragement.

Dr. M.R. Roach and the members of the U.W.O. Biophysics Department who made their "Honorary Visiting Graduate Student" welcome.

Drs. J.M. Bolton, P.W. Whippey and M.D. Owen who acted as an unofficial "examining committee" here at U.W.O.

The North Atlantic Treaty Organization and the Medical Research Council for financial support.
CHAPTER 1.0
INTRODUCTION

1.1 PREFACE

The aims of this chapter are, firstly, to describe the main uses of electron paramagnetic resonance (EPR) in biochemistry (section 1.2). Then, to review the EPR work done on proteins containing transition metal ions, with emphasis on cytochrome c (section 1.3). Finally, we give a brief introduction to those physical and chemical aspects of EPR which the remainder of the thesis will treat in detail (section 1.4).

1.2 EPR IN BIOCHEMISTRY

The basic property of EPR is its ability to detect unpaired electron spins in a compound. The presence of such unpaired spins in biological systems arise from various causes and these are detailed below. EPR is useful in the following cases where unpaired spins are found:
(i) in biological molecules which have been exposed to radiation, where they arise mainly from broken bonds.
(ii) in enzyme-substrate reactions, associated with the
intermediates (usually unstable) which are formed in the reaction process.

(iii) in those molecules which contain transition metal ions, in which they are associated with the electrons around the metal ion.

The work carried out in this thesis studies the behaviour of cytochrome $c$, a protein containing iron in a heme group, and therefore falls into category (iii). We shall not discuss any EPR studies outside of this category, as considerable information is available on (i) and (ii) in several recent books (Poole, 1967; Alger, 1968; and especially Ingram, 1969).

1.3 EPR IN TRANSITION METAL COMPOUNDS

1.3.1 Introduction

The only transition metals that have been studied by EPR in biological systems are cobalt, vanadium, molybdenum, manganese, copper, zinc, and iron. Very little work has been done on the first two. Cobalt is found in vitamin B$_{12}$ but only gives signals after irradiation (Hogenkamp et al., 1963). Vanadium EPR spectra have been obtained in a methanol extract from the mushroom Amanita muscaria by Musso (unpublished results quoted in Beinert and Palmer, 1965). The valence state of the metal is unknown.

Molybdenum has been extensively studied due to its presence in xanthine oxidase, particularly by Bray (1964)
and collaborators. These workers have used the appearance of the molybdenum signal to monitor the reaction kinetics of the enzyme. The manganese ion has also been used to probe enzyme action by its effect on the spin-lattice relaxation time of water protons in enzyme-metal-substrate complexes (Cohn, 1970). Copper gives EPR signals also, and the binding of this ion has been examined in a wide range of proteins (e.g., Malmstrom and Vanngard, 1960; Peisach et al., 1967). The mode of binding of copper ions into insulin single crystals has been studied by Brill and Venable (1964) and Venable (1965). (The latter reference, Venable's Ph.D. Thesis, provided much experimental guidance on crystal mounting and data analysis in the early stages of this project, although the methods finally used to obtain the results of chapter 4 differed from his).

The only class of proteins which have been studied both in (frozen) solution and as single crystals are those containing iron atoms in the form of heme. Non-heme iron proteins (San Pietro, 1965) have been examined by EPR, but only in frozen solution—see for example Palmer et al. (1967).

1.3.2 EPR of heme proteins, including cytochrome c

This section gives a historical survey of EPR studies of heme proteins.
The first published results were those of Ingram and Bennett (1955) on pastes of met-hemoglobin (met-Hb), met-myoglobin (met-Mb), hemoglobin fluoride (HbF) and myoglobin fluoride (MbF). These proteins were in the high spin form \((S = 5/2)\) with \(g\)-values of 5.95. The same paper also gave results for two low spin \((S = 1/2)\) compounds, Hb-azide and Mb-azide, which had \(g\)-values spread between 2.2 and 2.8. This work was extended to single crystals of Mb and met-Hb two years later (Bennett et al., 1957). In this classic paper these workers obtained the orientation of the principal \(g\)-axes in the molecule relative to the crystallographic axes. A study of single crystals was also reported on the azide complex (Gibson and Ingram, 1957). The data obtained over the period 1955-57 was reviewed by Gibson et al. (1958).

Gordy and Rexroad (1961) published the first EPR spectra of cytochrome \(c\) at a temperature of 4.2°K. These were obtained on a commercial powder sample of horse heart cytochrome, and gave the \(g\)-values 5.9, 2.8, 2.2 and 2.0. The first and last of these presumably arise from high spin denatured cytochrome \(c\) and/or from Hb or Mb impurities. The \(g = 2.8\) and 2.2 resonances had unusual shapes—that of unddifferentiated absorption lines—despite a first derivative display being used. It is possible that these workers observed fast passage dispersion signals similar to those in chapters 3 and 6 of this thesis. Taylor (unpublished results,
1961) observed an EPR derivative absorption spectrum at 77°K in tuna cytochrome c which had g-values of 3.05 and 2.24 in agreement with results published seven years later (vide infra). Taylor's results were in fact, the main stimulus for the work on cytochrome c presented in this thesis. Gordy and Rexroad (1961) also published powder EPR spectra of a cytochrome c-nitric oxide complex—they observed a triplet with splitting of 18 gauss which they interpreted as due to the nitrogen atom of the nitric oxide.

Bennett et al. (1961) published the heme plane orientations of crystals of Mb types B, C, D and F (from the muscles of diving mammals) determined from their EPR spectra, and compared the results with measurements on the optical dichroism of these crystals (Bragg and Pippard, 1953; Perutz, 1953). Due to the system of double bonds in the planar heme ring, the electric vector of polarised light should absorb more strongly when it lies parallel to the plane than when perpendicular. The observed dichroism agreed well with that calculated from the EPR determination of the heme plane orientation.

Ehrenberg (1962) unsuccessfully attempted to obtain EPR spectra from ferricytochrome c solutions at 77°K, but did report low spin spectra from several myoglobin complexes (Mb-OH, - NH₃, etc.) whose g-values agreed with those given in the earlier work (Gibson et al., 1958).
In 1964 (Stryer et al., 1964) was published the X-ray structure of Mb-azide and the correlation between principal g-axis directions and the molecular orientation was made four years later (Helcké et al., 1968). We shall discuss the Helcké paper more fully in chapter 4, for there are significant differences between their results on azide and ours on cytochrome c.

Yonetani and Schleyer (1968) also reported failing to detect EPR signals from cytochrome c at 77°K, in contrast to the results of Rein et al. (1968) who reported g-values of 3.0, 2.26 and 2.0 for solutions of native cytochrome c. Salmeen and Palmer (1968) obtained g-values of 3.06, 2.24 and 1.24 at 20°K. The small peak at g = 1.24 was not detected by Rein et al. probably because this is one of the lowest g-values reported in heme proteins and they did not cover a wide enough range of magnetic field. Rein et al. (1968) also published g-value data for cytochrome c at extremes of pH (alkaline pH - g values = 2.73, 2.14 and 1.77; at acid pH-g-values of 6.3 and 2.1), for ferricytochrome cyanide (3.04, 2.3, 2.01), for ferricytochrome azide (2.77, 2.27 and 1.85) and for ferricytochrome fluoride (6.1, 1.97). Yonetani and Schleyer (1968) also reported cytochrome c EPR spectra at extremes of pH that were similar to those of Rein et al.

These publications on solutions of native cytochrome c and derivatives—just at the time we obtained our preliminary results at 4.2°K—caused a change in emphasis of
this thesis project from solutions to single crystals. We wished to obtain the orientations of the g-axes, an explanation for the broad linewidths seen (in solution) and the line shapes seen at low temperatures.

Eisenberger and Pershan (1967) published the first line width study of proteins on single crystals of met-Hb and met-Hb-azide. They postulated that the lines seen in met-Hb were broad because the principal g-axes were not strictly parallel from one heme group to another. For the Hb-azide, they demonstrated that the linewidths could be largely explained by the combination of this g-axis misorientation with a variation in the principal g-values from one molecule to another. (This latter effect can arise from an unusually wide distribution of the rhombic potential of the ligand field around its mean value). Helcke et al. (1968) postulated that the widths they observed in another low spin complex, met-Mb-azide, were to be explained solely by the misorientation mechanism. We will discuss these results in considerable detail in chapter 5, where we will show that both the "Eisenberger" and "Helcké" theories published must be corrected slightly and extended before they can explain our linewidth results satisfactorily.

Kon (1969) studied the EPR of nitric oxide cytochrome c and presented good evidence, from the nitrogen hyperfine splitting observed, that the NO radical replaces the sulphur atom of methionine-80 in the native protein.
Kon was unable to duplicate the results of Gordy and Rexroad (1961); the nitrogen splittings he observed being 24 gauss and 6.8 gauss. Other examples of hyperfine structure from atoms bound to the central Fe$^{3+}$ ion are those of Kotani and Morimoto (1967) on the splitting produced by the fluoride atom in a myoglobin fluoride crystal. Scholes (1969) obtained the first EPR spectrum of the hyperfine lines from the four pyrrole nitrogens of the heme plane, by co-crystallisation of hemin with perylene. An EPR single crystal study of hemoglobin-NO was published by Chien (1969) in which he obtained the orientation of the bound NO radical.

Recently EPR spectra of cytochrome c at 77°K have been published by Morton and Bohan (1971) who examined the spectra of hyophilised powders and solutions of cytochrome c, and indicated that the molecule is considerably distorted on lyophilization. Their results are consistent with the unpublished work of Taylor mentioned above.

The observations on lyophilization also agree with an EPR study of Yonetani and Schleyer (1967) on myoglobin and cytochrome c peroxidase who demonstrated that these proteins were highly susceptible to changes in state, such as freezing, thawing and drying. Our own published work on solutions (Mailer and Taylor, 1971) is reported in detail in chapter 3.

There has been one EPR study of single crystals of cytochrome c reported—this was a preliminary note on the
heme plane orientation in bonito cytochrome $c$ by Hori and Morimoto (1970). These workers, however, made no mention either of line width changes or of the fast passage effects we report in the major sections of this thesis (chapters 3, 5, 6 and 7) since they obtained all their results at one temperature--20.3°K--the boiling point of liquid hydrogen. The bonito cytochrome has a different unit cell from horse and tuna cytochrome and detailed X-ray studies have not been reported on it.

1.4 BIOLOGICAL AND CHEMICAL PROPERTIES OF CYTOCHROME C

1.4.1 Introduction

We survey very briefly the biological properties of cytochrome $c$ (section 1.4.2) and then describe the chemistry of the ferric ion (section 1.4.3) and of the paramagnetic centre of the molecule (section 1.4.4).

1.4.2 Cytochrome $c$

Cytochrome $c$ is a low molecular weight (12,500) protein widely distributed in the animal and plant kingdom. This iron-porphyrin-containing protein is found in the terminal chain in the mitochondria of all aerobic organisms. Cytochrome $c$ transfers electrons between two cytochrome complexes--cytochrome reductase and cytochrome oxidase, the iron atom cycling between the ferric ($\text{Fe}^{3+}$) and the ferrous ($\text{Fe}^{2+}$) valence state. The properties of one of these states,
the ferric, can be studied by EPR as in this state there is an unpaired electron present. Cytochrome c is unique among the cytochromes in that it can be easily extracted from tissues without appreciable denaturation (e.g., Margoliash and Walasek, 1967). In consequence, it has been very widely studied (see the review of Margoliash and Schejter, 1966).

Since cytochrome c can transfer electrons, the electronic structure of the active centre (the heme group) is of particular interest, and EPR is an ideal technique to investigate this since it is specific for the electron interaction at the Fe atom, the active centre.

1.4.3 Chemistry of the Fe$^{3+}$ free ion

The ferric iron (Fe$^{3+}$) ion has 23 electrons of which 18 reside in closed shells; the five remaining have the orbital configuration (3d)$^5$. These 5 electrons are each characterized by an orbital angular momentum quantum number $l$ (equal to 2) and spin quantum number $s(=\frac{1}{2})$. There are thus altogether $(2l + 1)$ orbital states and $(2s + 1)$ spin states. The total number of allowed states available to the electrons being $(2l + 1)(2s + 1) = \text{equal to 10 for this system.}$

In the Russell-Saunders coupling scheme, the electrons are considered to be coupled to form a system with total orbital angular momentum $L(=\sum l_i)$ and total spin angular momentum $S(\sum s_i)$. There are several possible combinations of $L$ and $S$ to form the total angular momentum, $J$. 
These, when electrostatic interactions are taken into account, give rise to a series of states with different energies. Typical energy separations are $10^4\text{cm}^{-1}$ or more (see figure 1.1). Consequently in EPR we only consider the properties of the lowest state—EPR energies being of order $1\text{ cm}^{-1}$.

The ground state of $(3d)^5$ is $6s$ corresponding to a state with 5 electrons, each of spin $+1/2$ and zero orbital angular momentum ($J=5/2$; $L=0$; $S=5/2$). The $2S+1$ states are degenerate in the absence of a magnetic field, but in a magnetic field, $H_o$, the energy of each state is given by $g_L \beta H_o M_J$. $M_J$ is called the magnetic quantum number and takes integral or half integral values of $J$—from $+J$ to $-J$. $\beta$ is the Bohr magneton and $g_L$ is the Landé $g$-factor given by:

$$g_L = 1 + \frac{J(J+1) - L(L+1) + S(S+1)}{2J(J+1)}$$

For the free ion $g_L = 2$ (figure 1.1a).

If one performs an EPR experiment by inducing transitions between the split levels with r.f. energy of frequency $\nu$ (energy $\hbar \nu$), then under the selection rule $\Delta M_J = \pm 1$ we have resonance if:

$$\hbar \nu = g_L \beta H_o \left[(M_J + 1) - M_J\right]$$

$$= g_L \beta H_o = 2 \beta H_o$$
FIGURE 1.1

Electronic energy levels of the Fe$^{3+}$ ion.

(a) Splitting of ground state of a free Fe$^{3+}$ ion in an applied magnetic field.

(b) Splitting of the ground state of an Fe$^{3+}$ ion bound into cytochrome c in an applied magnetic field.

For explanation of symbols see text (section 1.4.4).
The image illustrates the electronic states of a free ion in various fields.

(a) Levels of a free ion:
- $^6S$
- $^{3/2}$
- $^{1/2}$
- $^{-1/2}$
- $^{-3/2}$
- $^{-5/2}$

$\hbar \nu = 2 \beta H$

(b) Energy levels under different fields:
- $e_g$
- $t_{2g}$

$\Delta$, $V$, $D$

$\langle x \rangle$, $\langle y \rangle$

$\hbar \nu = g \beta H$

Fields:
- Free Ion
- Cubic Field
- Axial Field
- Rhombic Field
- Spin Orbit Field (Kramer’s Doublet)
- Magnetic Field
In fact we are not concerned with the free ion but with a complex and must take account of the ion's environment. The iron atom of all heme proteins is co-ordinated to the nitrogen atom of 4 pyrrole groups—forming the planar heme ring (figure 1.2). In cytochrome c, there is further co-ordination to the S-nitrogen atom of the histidine amino-acid in the protein backbone and to the sulphur atom of methionine-80 (by convention these are labelled positions 5 and 6 respectively), see figure 1.2.

1.4.4 Chemistry of the Fe$^{3+}$ in cytochrome c

The six neighbours (or ligands) form an octahedral arrangement around the iron; their electrostatic interaction with the central ion will remove, partially or completely, the degeneracy of the iron d-orbitals. The pattern and magnitude of the splitting of these orbitals determines the magnetic properties of the molecule.

The study of this type of interaction is called Ligand Field Theory and a wide literature exists on this subject. Ballhausen (1962) and Griffith (1961) are standard texts; Griffith (1956, 1957, 1958, 1964, 1965) and Kotani (1961, 1964) have done much to provide a coherent theoretical frame work that covers most of the experimental data on hemoproteins. Harris-Loew (1970) and Weissbluth (1966) have provided recent reviews. The Weissbluth review is the best single source for the physicist wishing to understand the
FIGURE 1.2

Diagram of the atoms co-ordinated to Fe$^{3+}$ in cytochrome c. For explanation, see text (section 1.4.3).
chemistry of heme compounds; he develops the theory in a leisurely fashion (explaining the many different systems of notation used by others) and concentrates on the EPR, magnetic susceptibility and Mossbauer properties of hemoglobin.

The octahedrally co-ordinated ligands produce a cubic environment about the iron—the symmetry elements of this arrangement being the same as those of a cube. Group theoretical techniques are used to describe how the degeneracy of the d-orbitals will be lowered by this environment.

Under cubic symmetry the five 3d-orbitals segregate into two sets—one three-fold and the other two-fold spatially degenerate. For electronegative ligands (N, S), the three-fold degenerate set (in group theory notation—the \( t_{2g} \) set) is lower in energy than the other (the \( e_g \) set). The energy gap between the \( t_{2g} \) and \( e_g \) sets largely determines the magnetic properties of the complex.

Two limiting cases can be distinguished:

(i) high spin—when the energy gap is much smaller than the electron pairing energy (pairing energy being the result of electrostatic and exchange interactions which tend to align the electrons with the same value of spin) then the five electrons distribute themselves over all orbitals \( (t_{2g}^3 e_g^2) \) to yield maximum spin \( (S = 5/2) \).

(ii) low spin—when the splitting energy is greater than the pairing energy the five electrons enter the \( t_{2g} \) levels, have the configuration \( t_{2g}^5 \), and minimum spin
Magnetic susceptibility measurements in ferricytochrome c (Tasaki et al., 1967) have shown that it is a low spin complex of spin 1/2, and therefore has the five d-electrons in the six (3 spatial x 2 spin) \( t_{2g} \) orbitals.

The total capacity of the \( t_{2g} \) orbitals is 6 electrons, and hence the 5 electron system behaves as a single positive hole. This enables us to deal with a simple \((3d)^1\) hole system, rather than the complex \((3d)^5\) system.

The spin Hamiltonian concept is used to deal with the EPR of this system. The ground state is assumed to be a doublet with an effective spin, \( S \), of 1/2 and the energies of the levels in an applied field \( H_0 \) are \( g_B H M_S \) (\( M_S = \pm 1/2 \)). The g-factor is no longer the Landé g-factor, \( g_L \), but is a parameter to be determined in the EPR experiment from the values of \( g \) and \( H \) that give resonance. Generally \( g \) will behave like a symmetric tensor, and, with respect to an axis system suitably oriented in the molecule, can be characterized by at most 3 values.

It is found for cytochrome c that there are 3 g-values (Salmeen and Palmer, 1968) all different from the free spin value of 2. The hole therefore does not behave as a free spin; a contribution from orbital angular momentum is present (i.e., there exists spin-orbit coupling). The g-values all different imply that the environment around the iron has symmetry lower than cubic, i.e., must contain axial
and rhombic elements which remove the degeneracy of the $t_{2g}$ orbitals (figure 1.1b). In the absence of spin-orbit coupling the $t_{2g}$ orbitals would form a closed shell except for a hole in $|d_{zx}\rangle$ (figure 1.1b). Spin-orbit coupling mixes these states so that the $|d_{zx}\rangle$ hole is distributed over all the orbitals, resulting in a set of three Kramers doublets, each a linear combination of the electronic wavefunctions (see Appendix 1). The lowest energy doublet is the only one appreciably occupied at low temperatures—application of a magnetic field to this doublet can account for the EPR spectra observed. One can work back from the observed g-values to obtain the splitting of the $t_{2g}$ orbitals. A rhombicity ($V/D$) of 0.55 and tetragonality (or axiality) ($D/\lambda$) of 2.64 account for the g-values of 3.06, 2.24 and 1.25 observed for cytochrome $c$ (Salmeen and Palmer, 1968; Blumberg, 1968). ($\lambda$ is the spin-orbit coupling constant).

Apart from our discussion in chapter 4, we do not carry out an analysis of the g-values in terms of energy levels, since our main intent is to determine the relationship between the directions of the principal g-axes and the molecular axes, and to study the dependence of line width on orientation, temperature and instrumental conditions in our EPR experiments.
CHAPTER 2.0
EXPERIMENTAL APPARATUS

2.1 INTRODUCTION

The aim of this chapter is to describe the apparatus used to obtain the EPR results.

We begin with a listing of the hemoprotein properties that are relevant to EPR spectroscopy, and what limits these impose on the spectrometer (section 2.2). After this we describe the EPR apparatus, with emphasis on those features that require special comment (section 2.3). We then give the details of the low temperature apparatus (section 2.4) and of the temperature measurement system (section 2.5). Finally, we present the method of mounting samples (section 2.6).

2.2 HEMOPROTEIN PROPERTIES RELEVANT TO EPR

2.2.1 Concentration

The small amount of iron in most hemoproteins (0.5% by weight in cytochrome c) means that the number of unpaired spins is low. For cytochrome c there is only one iron atom per molecule, so that a one molar solution contains
$6 \times 10^{23}$ spins per litre. Typical concentrations of hemoproteins used are 10 millimolar, which in an EPR sample volume of 0.2 ml. is about $10^{18}$ spins. For single crystals the molecular concentration is an order of magnitude higher, but the sample volume is very much less—cytochrome c tuna crystals have a volume of about $2 \times 10^{-5}$ ml. (needles 2 mm x 0.1 mm x 0.1 mm) containing approximately $10^{15}$ spins.

2.2.2 Solvent

Native proteins survive only in aqueous solution, and at room temperature the liquid causes heavy damping of the microwave power. It is possible to overcome this problem by using very small samples, but this reduces the number of spins considerably. Freezing of the sample will also solve the problem and is one reason for operating at temperatures below the freezing point of water.

2.2.3 Linewidth

The main reason for operating at low temperatures is to narrow the EPR lines so that the absorption signals can be seen above the noise background. In low spin hemoproteins this condition is very necessary for the electron spins interact strongly (via spin–orbit coupling) with thermal lattice vibrations giving lines hundreds to thousands of gauss wide at temperatures of 77°K and above. Very few workers have been able to observe cytochrome c lines at 77°K, although in other low spin hemoproteins EPR can easily be seen at this
temperature (e.g. Ehrenberg, 1962). This implies we have to work at or near liquid helium temperature (4.2°K).

EPR studies of other low spin hemoproteins (e.g., hemoglobin azide—Eisenberger and Pershan, 1967) have shown that even at liquid helium temperatures the lines remain broad, being several hundreds of gauss wide.

The area of an absorption line is proportional to the number of spins contributing to the line, and is constant for a constant number of spins. For a first derivative spectrum, the area of the (integrated) absorption line is approximately equal to the signal height \( \times (\text{the peak-to-peak linewidth})^2 \)—Poole, 1967, p. 551. Therefore the signal height is inversely proportional to the square of the linewidth. For a derivative linewidth of 100 gauss it can be calculated that the system must be able to detect \( 10^{11} \) spins or less.

2.2.4 Saturation

Unfortunately a long spin-lattice relaxation time limits the rate at which the upper spin level can lose energy to the lattice. Application of too much power to the spin system causes the individual absorption lines to broaden and to decrease in height—this effect is called dynamic saturation (Abragam and Bleaney, 1970). Working at very low microwave powers—\( 10^{-5} \) to \( 10^{-6} \) watts—should overcome this problem.
2.2.5 **Dispersion**

During the course of this work it became necessary to examine the dispersion signal from cytochrome c, and this required modification of the conventional automatic frequency control system, as described below (section 2.3.4).

2.2.6 **Summary**

The requirements, then, of the spectrometer are:

- An ability to detect $10^{11}$ unpaired spins (or fewer) in a one gauss line both by dispersion and by absorption, at an incident microwave power of $10^{-5}$ watts or less, with sample temperatures between 4.2°K and 77°K.

2.3 **THE EPR SPECTROMETER**

2.3.1 **Basic design – Henning (1961) spectrometer**

The basic design of the spectrometer follows that of Henning (1961) and Faulkner (1962) and is a homodyne balanced mixer system.

The main advantages of this type of spectrometer are:

(i) the signal detection system is operated independent of the microwave power incident on the paramagnetic sample;
(ii) the spectrometer can be tuned to detect either the absorption or dispersion component of an EPR signal, without requiring that an EPR signal be present;
(iii) balanced mixer detection minimises klystron noise;
(iv) only a single klystron is used.
The block diagram of the complete spectrometer is shown in figure 2.1, and the details of the microwave section in figure 2.2. The mode of operation is now described, following Henning (1961).

The sample is placed in a cavity which forms one of the side arms (2) of a microwave bridge. The other arm (1) contains a variable load which can balance the bridge. The klystron power is fed into the bridge through arm (E). When the magnetic field is swept through resonance the reflection coefficient of the cavity is altered and an out of balance signal appears in arm (H), which is fed into one arm of the balanced mixer detector via a 60 db. isolator. The bias arm of the mixer (E) is connected to the main waveguide via a directional coupler, and provides bias power to the mixer crystals; the amplitude and phase of the bias can be adjusted by means of an attenuator and a phase shifter.

The antiphase audio frequency signals from both mixer crystals are fed into a balanced transformer connected to a conventional electronic detection system of preamplifier, phase sensitive detector and chart recorder.

The magnetic field is modulated at an audio frequency, which is also supplied to the phase sensitive detector. When the d.c. field is swept slowly through the resonance line the first harmonic of the modulated signal is detected, filtered and displayed.
FIGURE 2.1

Block diagram of 24 GHz spectrometer.
Power Supply

Klystron

AFC

AFC Pre Amplifier

Microwave Circuit

Power Meter

Chart Recorder

Signal Detector

Phase Sensitive Detector

Coil Drive Amplifier

D.C. Magnet Coils

A.C. Modulation Coils

Varian Magnet Supply

X-Drive
FIGURE 2.2

Schematic diagram of microwave section of the EPR spectrometer.
### TABLE I

Dependence of EPR signal height on microwave frequency for various types of samples

*(after Alger, 1968, p. 98)*

<table>
<thead>
<tr>
<th>Case</th>
<th>Limited Sample</th>
<th>Dielectric loss</th>
<th>Saturation</th>
<th>$Q$</th>
<th>$(p_c)^{3/2}$</th>
<th>$\chi''$</th>
<th>Filling Factors</th>
<th>Total Frequency Dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>$\omega^{-1/2}$</td>
<td>--</td>
<td>$\omega$</td>
<td>$\omega^3$</td>
<td>$\omega^{7/2}$</td>
</tr>
<tr>
<td>2</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>$\omega^{-1/2}$</td>
<td>--</td>
<td>$\omega$</td>
<td>--</td>
<td>$\omega^{1/2}$</td>
</tr>
<tr>
<td>3</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>$\omega^{-1/2}$</td>
<td>$\omega^{-3/4}$</td>
<td>$\omega$</td>
<td>$\omega^3$</td>
<td>$\omega^{11/4}$</td>
</tr>
<tr>
<td>4</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>$\omega^{-1/2}$</td>
<td>$\omega^{-3/4}$</td>
<td>$\omega$</td>
<td>--</td>
<td>$\omega^{-5/2}$</td>
</tr>
<tr>
<td>5</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>$\omega^{-1/2}$</td>
<td>--</td>
<td>$\omega$</td>
<td>$(\epsilon'')^{-1/3}$ $\omega^{1/3}$ $\omega^{1/2}$</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>$\omega^{-1/2}$</td>
<td>$\omega^{-3/4}$</td>
<td>$\omega$</td>
<td>$(\epsilon'')^{-1/3}$ $\omega^{1/3}$ $\omega^{-1/4}$</td>
<td></td>
</tr>
</tbody>
</table>
The audio frequency used by Henning was 30 Hz, and with this system he estimated he could detect $2 \times 10^{13}$ spins of solid DPPH (linewidth 2 gauss) with unity signal to noise ratio at a power of $10^{-3}$ watt. This is about an order of magnitude larger than the theoretical minimum value of about $10^{12}$ spins at this power level at room temperature. At liquid helium temperatures this would be improved by a factor of 150 due to an increased spin population difference (in the ratios $T_{\text{room}}/T_{\text{helium}} = 300/4.2 = 60X$) and to improved coupling and higher cavity $Q(2-3X)$.

Small protein crystals frozen in a minimal amount of solution have very little dielectric loss. If the sample does not saturate at high microwave power levels, then we have case I of table I, and if it does we have case 3. The former case gives an 11 fold increase in signal for a 2 fold increase in microwave frequency, and the latter a 7 fold increase for the same change in frequency.

In solutions where the sample size is limited by the cavity dimensions and by the radio frequency field distribution, there is little to be gained by increasing the microwave operating frequency (cases 2 and 4 of table I).

Since our main aim was to observe EPR from single crystals of cytochrome c, we chose a frequency of 24-25 GHz for operation. This is approximately two and a half times greater than the more commonly used X-band frequency of 9 GHz, and leads to a 25 times increase in signal for case 1.
The only drawback of this increased frequency is that the magnet used must be capable of producing higher fields to obtain the same range of g-values at 25 GHz as at 9 GHz.

2.3.3 Modification (ii) - increased magnetic field modulation frequency

We operate with a high magnetic field modulation frequency in order to reduce the noise appearing at the input of the phase sensitive detector.

The silicon diodes used to detect the modulated microwave power from the cavity add noise to the EPR signal. The amount of noise added depends upon the power incident on the diode and the frequency of the magnetic field modulation (which becomes the signal modulation frequency).

The most important source is "1/f" noise (so called because of its frequency dependence), as it dominates the noise power spectrum at all frequencies below about 100 kHz. Above this frequency, only frequency independent thermal and shot noise remain.

By using a variable frequency phase sensitive detector, and a wide band power amplifier, we can modulate the magnetic field at frequencies from 100 Hz to 100 kHz. The "1/f" noise is clearly seen at the lower frequencies. Since we detect voltages (proportional to the square root of the power) the noise, \( N \), varies with the square root of the frequency and therefore \( N(100 \text{ kHz}) = 1.7 \times 10^{-2} N(30 \text{ Hz}) \).
The large range of detection frequencies is necessary for some of the rapid adiabatic passage experiments on cytochrome c in chapter 6.

2.3.4 Modification (iii) – improvements to AFC circuits*

The fast passage experiments also required that we operate the spectrometer in the dispersion mode. Tuning the detection system for dispersion is done by changing the phase of the power in the bias arm by 90° from that used for absorption detection (Henning, 1961). In order to maintain a low signal to noise ratio in this mode of operation the automatic frequency control system (AFC) had to be modified.

Wilhmshurst (1967), in a recent book, has given a description of the requirements that an AFC system of an EPR spectrometer must fulfil.

A spectrometer operated in the dispersion mode is inherently noisier than one operated in the absorption mode. The phenomenon of dispersion results in a shift of the frequency of the cavity as the field moves through the magnetic resonance. In the dispersion mode the instrument is sensitive to this frequency change, in the absorption mode it is not (providing the difference between the klystron and the cavity resonance frequencies is small). In the dispersion mode the

*This section was written in collaboration with Dr. C. P. S. Taylor, who designed and built the final version of the AFC.
spectrometer converts the frequency modulation noise of the klystron to amplitude noise in the output. In addition, any noise or drift in the natural resonant frequency of the cavity will appear as output noise.

Stabilizing the klystron to an external cavity will reduce the klystron noise but not that caused by random drift in the resonant frequency of the experimental cavity. If the AFC is locked on the experimental cavity resonance, the AFC will act to compensate for the shift of frequency caused by dispersion and thus reduce the dispersion signal. However, this effect can be nullified by modulating the magnetic field, and hence the true dispersion signal, at a higher frequency than the AFC carrier. At the higher frequency the loop gain of the AFC is zero and the dispersion signal is not reduced. In such an arrangement the klystron and the cavity track each other over the frequency band pass of the AFC.

An important point must be made about an AFC that derives its error signal from the mismatch of klystron and cavity resonance frequencies (class O type AFC—Wilmshurst, 1967). To our knowledge it has not been made before. The true control point in the loop is not at the resonant cavity but at the input to the DC amplifier that provides the correction voltage for the klystron reflector (see figure 2.3). The feedback loop operates in such a fashion as to reduce the voltage at the input of the DC operational amplifier to zero. This means that if the offset voltage of the amplifier
FIGURE 2.3

Block diagram of automatic frequency control (AFC).
Reflector Voltage

EM II 38V Klystron

D.C. Correction Voltage

Integrator

Pre Amplifier

II kHz Tuned Amplifier

II kHz Phase Detector

EPR Cavity

11 kHz Oscillator

11 kHz to Klystron

P.S.
drifts, then the frequency difference between the klystron and the resonant cavity must drift off in the opposite sense so that the signal coming from the phase-sensitive detector to the amplifier will just oppose the change in the offset voltage and maintain the amplifier input at zero. The basic conclusion is that drifts at the amplifier input, of whatever sort, will result in compensatory displacements of the klystron frequency from the cavity resonance.

In similar fashion noise arising in the DC amplifier will be reflected onto the klystron as FM noise. In the absorption mode such noise is discriminated against, but the dispersion mode efficiently transfers it to the output of the spectrometer.

If the gain of the DC amplifier is kept at unity in the frequency region where it exercises proportional control only, then the noise from any high grade operational amplifier is only a few microvolts, and will not cause trouble. However, operational amplifiers differ in the stability of their input offset currents and voltages. Those not specially stabilized suffer variations of mV/°C, and mV over 24 hr, and nA for the current, while chopper stabilized operational amplifiers suffer changes in the order of µV and pA. Clearly only the latter class are suitable in an AFC for a dispersion mode instrument.

The AFC used initially was based on the proportional controller of George and Teaney (1960). This circuit
modulated the klystron reflector at 11 kHz producing frequency modulated microwave power incident on the cavity. Performance was improved by adding an integrator into the feedback loop (Berry and Benton, 1965), which provides integral plus proportional control, and reduces the steady state error to zero (class I AFC). Our circuitry went through several versions; the final and most successful one used integrated circuits for amplification, and a very high quality chopper stabilised operational amplifier (Philbrick/Nexus 1701) as integrator. The AFC circuit is schematized in figure 2.3.

Experimentally we found that this AFC added no noise to the spectrometer output, and as it operates at about 11 kHz it had no effect on dispersion signals modulated at higher than that frequency; and because of the bandwidth of the integrator, no effect below that frequency until 100 to 200 Hz. Its stabilizing effect on the EPR dispersion signal was evident and satisfactory.

2.3.5 Performance of the EPR Spectrometer

Hyde (1961) states that "of all the measurements one can make with EPR equipment, the determination of absolute spin concentrations is the most difficult."

We have sidestepped this problem of direct measurement by using a secondary standard—Cr$^{3+}$ ions in MgO. This is calibrated against gaseous oxygen lines by the
manufacturers (Strand Labs. Inc., Cambridge, Mass., USA). They claim that the effective spin concentrations are accurate to within 5%.

The chromium ion, in the MgO host lattice, gives a narrow line, one gauss wide, with g-value 1.97. Our sample is in powder form sealed into a 2 mm o.d. quartz tube, containing an effective spin concentration of $2 \times 10^{14}$ spins per centimetre of tube. At room temperature this standard does not show saturation below 10 mW incident power, but below 77°K it saturates at microwatt power levels. As a result, our sensitivity measurements were carried out at room temperature.

A typical measurement of signal height (converted to effective number of spins) over a wide range of cavity power is shown in figure 2.4 (O). These were obtained with 100 kHz field modulation of 1 gauss amplitude. For comparison, the theoretical sensitivity, which was calculated using a treatment similar to that given in Poole (1967, chapter 14), is also presented in figure 2.4.

Our measured sensitivity at room temperature is within a factor of 2 of the theoretical one. The 150x improvement estimated in going from room to helium temperature gives a final sensitivity of between $10^{10}$ and $10^{11}$ spins at $10^{-5}$ watts of microwave power incident—our design goal.

It should be mentioned, however, that the theoretical minimum number of detectable spins may be somewhat less
FIGURE 2.4

Measured and calculated curves of minimum detectable number of spins as a function of microwave power, at room temperature.
# of Spins Detectable with S/N of Unity and Linewidth of 1 Gauss

- **Power incident on Cavity - Watts**
  - $10^{-2}$
  - $10^{-1}$
  - $10^{0}$
  - $10^{1}$
  - $10^{2}$

- **Calculated Measured on Cr^3+ Standard**
than that given, because of measurement uncertainties in the values of such quantities as the Q of the cavity, the filling factor of the sample and the noise contribution from the detection diodes. Both Poole (1967) and Alger (1968) have lengthy discussions on this subject.

2.4 **LOW TEMPERATURE APPARATUS**

2.4.1 **Introduction**

The purpose of the low temperature system is to enable any temperature between 4.2°K (liquid helium boiling point) and 77°K (liquid nitrogen b.p.) to be maintained in the EPR sample.

The dewar that encloses the EPR cavity must be able to fit inside the 2.25 inch magnet gap, and contain an EPR cavity of about 1.6 inches diameter. These inner and outer diameter restrictions mean that we must use a dewar with a single vacuum space between room and liquid helium temperature. We briefly consider the sources of heat leak into this type of dewar and describe its construction in the next three sections.

The sources of heat leak are:

(i) radiation across the single vacuum space (section 2.4.2);

(ii) conduction through the residual gas in the single vacuum space (section 2.4.3);

(iii) conduction down the waveguide and other connections to the EPR cavity (section 2.4.4).
2.4.2 **Thermal Radiation**

Thermal radiation from the 'hot' outer wall across the vacuum space is large enough that a radiation shield cooled to 77°K must be inserted into the vacuum space. (In the more common two dewar systems, an outer dewar containing liquid nitrogen surrounds an inner dewar holding liquid helium. Radiation into the helium dewar only comes from the vacuum wall cooled to 77°K).

Various single wall dewars have been suggested, e.g. Kroon (1966) or Frindt and Stuart (1968), which differ only in the ways by which the heat shield is connected to the nitrogen bath. Most designs have complicated glass/metal seals which are difficult to fabricate. Our glass blower* circumvented this sealing problem by shrinking one wall of the nitrogen container onto a copper heat shield—as shown in figure 2.5.

A lower nitrogen bath is placed around the portion of the dewar tail which projects below the pole pieces of the magnet to make sure that the heat shield is cooled as close to 77°K as possible along its whole length.

A pre-cooling period of several hours with liquid nitrogen was found to be essential for successful helium transfer.

---

*Mr. R. Eberhardt of the Dept. of Physics, University of Windsor, Ont.
FIGURE 2.5

Schematic diagram of single vacuum space dewar containing copper heat shield.
2.4.3 Conduction through residual gas

In order to reduce thermal conduction through the residual gas, the dewar vacuum space had to be pumped to less than $10^{-6}$ torr (1 torr = 1 mm mercury pressure).

For most of the experiments in this thesis, the pumping system was a 2" diameter Veeco oil diffusion pump, with a Veeco rotary oil pump for backing. When the diffusion pump cold trap was cooled with liquid nitrogen, the required pressure could be reached. However, since this system was designed for other experiments that did not require such low pressures, the attainment of less than $10^{-6}$ mm was never certain, as frequent unsuccessful transfers showed. This situation was relieved by the purchase of our own pumping system (from Edwards High Vacuum, Canada, Ltd., Oakville, Ontario). This system could easily attain $10^{-6}$ torr when cooled with liquid nitrogen. If one kept pumping while transferring liquid helium, the dewar pressure dropped to $4.10^{-7}$ torr due to the combined effect of the diffusion pump and the cryopumping effect on the cold helium.

Gaseous heat conduction is therefore negligible.

2.4.4 Conduction down EPR cavity connections

The amount of heat conducted into the EPR cavity—via the stainless steel waveguide and other connections—was

*Pumping system of Dr. P. W. Whippey, Dept. of Physics, University of Western Ontario, London, Ontario, to whom our thanks.*
estimated to cause a boil off of 0.5 to 1.0 litre per hour of liquid helium.

Another source of heat in the system is the joule heating effect in the magnetic field modulation coils fixed to the cavity. This heating was seldom a problem because the EPR signals observed when liquid helium was in the dewar were those of fast passage, and one of the features of this signal is that the maximum signal height is obtained at a field modulation of 1 gauss. At this modulation amplitude the joule heating was negligible.

2.4.5 Performance of the cryogenic system

Under the conditions mentioned above, viz. good dewar vacuum, several hours precooling and low magnetic field modulation, the helium from a single transfer—about one litre—took from one to two hours to boil down to the level of EPR cavity. Once below the cavity, boil off slowed down as the major source of heat input was removed from the liquid. However, sufficient cold gas was evolved (by residual heat radiation) that the cavity and sample could be maintained at 4.2°K for a further 30 minutes.

The best performance used 5 litres of liquid helium in 2 transfers to cool the EPR cavity from 77°K to 4.2°K and maintain the helium level above the cavity for 5 hours.

The warm up time from 4.2°K to 77°K was approximately 3 hours. The initial temperature risk was 1°K per
**TABLE II**

from White (1968) p. 371

Thermoelectric potential difference $E$ with respect to $0^\circ K$ and thermopower $dE/dT$ (after Powell, Bunch, and Corruccini, 1961)

<table>
<thead>
<tr>
<th>$T$ ($^\circ K$)</th>
<th>Constantan versus Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E$ ($\mu V$)</td>
</tr>
<tr>
<td>1</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>0.66</td>
</tr>
<tr>
<td>3</td>
<td>1.48</td>
</tr>
<tr>
<td>4</td>
<td>2.62</td>
</tr>
<tr>
<td>5</td>
<td>4.07</td>
</tr>
<tr>
<td>6</td>
<td>5.83</td>
</tr>
<tr>
<td>7</td>
<td>7.90</td>
</tr>
<tr>
<td>8</td>
<td>10.26</td>
</tr>
<tr>
<td>9</td>
<td>12.92</td>
</tr>
<tr>
<td>10</td>
<td>15.88</td>
</tr>
<tr>
<td>12</td>
<td>22.64</td>
</tr>
<tr>
<td>14</td>
<td>30.50</td>
</tr>
<tr>
<td>16</td>
<td>39.43</td>
</tr>
<tr>
<td>18</td>
<td>49.40</td>
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<tr>
<td>20</td>
<td>60.40</td>
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<tr>
<td>22</td>
<td>72.42</td>
</tr>
<tr>
<td>24</td>
<td>85.43</td>
</tr>
<tr>
<td>26</td>
<td>99.43</td>
</tr>
<tr>
<td>28</td>
<td>114.4</td>
</tr>
<tr>
<td>30</td>
<td>130.3</td>
</tr>
<tr>
<td>32</td>
<td>147.1</td>
</tr>
<tr>
<td>34</td>
<td>164.7</td>
</tr>
<tr>
<td>T (°K)</td>
<td>Constantan versus Cu</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------</td>
</tr>
<tr>
<td></td>
<td>E (μV)</td>
</tr>
<tr>
<td>36</td>
<td>183.3</td>
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<tr>
<td>38</td>
<td>202.7</td>
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<tr>
<td>40</td>
<td>222.9</td>
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<td>45</td>
<td>276.8</td>
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<tr>
<td>50</td>
<td>335.6</td>
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<tr>
<td>55</td>
<td>398.8</td>
</tr>
<tr>
<td>60</td>
<td>466.2</td>
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<td>65</td>
<td>537.5</td>
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<tr>
<td>70</td>
<td>612.7</td>
</tr>
<tr>
<td>75</td>
<td>691.2</td>
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<tr>
<td>80</td>
<td>773.0</td>
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<tr>
<td>85</td>
<td>858.1</td>
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<td>90</td>
<td>946.7</td>
</tr>
<tr>
<td>100</td>
<td>1133.7</td>
</tr>
<tr>
<td>120</td>
<td>1546.4</td>
</tr>
<tr>
<td>140</td>
<td>2009.5</td>
</tr>
<tr>
<td>160</td>
<td>2522.7</td>
</tr>
<tr>
<td>180</td>
<td>3083.1</td>
</tr>
<tr>
<td>200</td>
<td>3688.6</td>
</tr>
<tr>
<td>250</td>
<td>5388.0</td>
</tr>
<tr>
<td>300</td>
<td>7330.2</td>
</tr>
</tbody>
</table>
minute from 4.2° to 10 degrees, slowing to 0.5°K by 20°K. By 70°K the rate had slowed to 0.2°K per minute.

The rapid initial rise made EPR measurements difficult for the bridge balance and cavity resonant frequency were changing. Attempts were made to maintain the temperature just above 4.2°K by a very slow transfer of liquid helium for the storage dewar. These were not very successful as the flow of liquid could not be regulated sufficiently closely. The temperature simply fell to 4.2°K and remained there. However, above 20°K it was possible to stabilise the temperature to within ±1°K by such a slow transfer of helium.

2.5 TEMPERATURE MEASUREMENT

The temperature in the EPR cavity was monitored by a thermocouple mounted in the wall as in figure 2.6. This thermocouple was constructed of copper and constantan wire (C-V Instruments Co., Wallingford, Conn. #3006).

The calibration of the thermocouple was made by using the data published in White (1968) for a Cu-constantan thermocouple, in table II.

With one junction in liquid helium and the other in liquid nitrogen, the voltage difference was less than 2μV from the 720μV calculated from table II. We have therefore assumed that this scale is correct for our thermocouple.

We sought the answer to the question—is the sample temperature the same as the thermocouple temperature?—by
FIGURE 2.6

Schematic diagram of EPR cavity showing method of mounting thermocouple.
making another thermocouple and mounting it inside a sample holder.

At temperatures between 4.2°K and 77°K the temperature difference between the two thermocouples (wall and tube) was not greater than about 0.1°K, even when the temperature was changing at 1°K/minute. (This is a result of the low specific heat of metals at low temperatures and the high thermal conductivity of helium gas; these two facts ensure that thermal equilibrium is attained very rapidly throughout the apparatus).

We therefore concluded that the sample temperature is given by the wall thermocouple readings between 4.2 and 77°K, to within 0.1°K.

2.6 SAMPLE MOUNTING

2.6.1 Introduction

In the next sections we describe the sample changing apparatus (2.6.2), the arrangements for mounting solutions (2.6.3) and for mounting crystals (2.6.4).

2.6.2 Sample changing system

In order to conserve liquid helium, we wanted to be able to change samples while the EPR cavity was at 4.2°K. The main problems to be overcome were:

(1) how to prevent the entry of air into the apparatus, which could freeze and prevent further samples being mounted;
to have sample holders rugged enough to survive cycling between room and liquid helium temperatures.

The first problem was solved by using the air lock sample changing system of Estle and Walters (1961). The apparatus is shown in figure 2.7. These authors gave a sequence of operations including evacuation of the air lock to ensure that no air leaked into the dewar. However, our experience was, that provided one keeps the knurled cap tight when inserting the sample rod, and ensuring that the plug valve was closed when there was no tube in the cavity, there was no problem of blockage due to frozen air.

The second problem is more difficult as we could not use teflon or nylon sample tubes, due to their EPR signals at $g = 4.3$ and $g = 2$. These were strong enough to obscure the cytochrome $c$ EPR lines. Even quartz tubing of very high purity (Spectrosil, Thermal American Fused Quartz, New Jersey, USA) often gave large signals at 4.2°K. However, by searching through the batch of quartz tubing, portions could be found that gave a reduced EPR signal, and these were used as often as possible.

We had a number of problems with sample tube breakage, especially upon withdrawal, both from thermal stresses due to large temperature gradients, and from mechanical forces on the tube. Careful annealing of the tubing helped reduce the first cause of breakage. Mounting the quartz tube on the end of a short nylon rod instead of directly to the
FIGURE 2.7

stainless steel tube used for sample changing helped reduce breakages from the second cause.

Even with these precautions, breakages did occur which caused constant attrition of our "signal free" quartz sample tubes.

2.6.3 Mounting cytochrome c solutions

The cytochrome c solutions were mounted in a Spectrosil quartz tube—3 mm o.d., 2 mm i.d.—glued to a nylon rod which was screwed into a 1/8" o.d. stainless steel tube. Figure 2.8 shows the arrangement.

The glue used was a 50:50 mixture by volume of Ivory soap and glycerine (Alger, 1968, p. 241). This produces a waxy, soapy solid that forms good seals down to 4.2°K. This glue worked well, although very occasionally the sample was left behind on withdrawal of the rod because the bond across the small area of contact was not strong enough.

2.6.4 Mounting cytochrome c single crystals

The apparatus is shown in figure 2.9. The sample tube is a 3 mm o.d., 2 mm i.d. Spectrosil quartz tube 5 cm. long, closed off in the middle. The crystal was placed in the top half of the tube as shown. By suitable tilting of the tube, and teasing with a fine hair, the cytochrome crystal could be oriented more or less as desired. The angles of the crystal faces relative to the vertical could be measured with
FIGURE 2.8

Mounting system for solution samples.
Stainless Steel Tube
Fiduciary Marker
Knurled Cap
Nylon Rod
Glue
Quartz Tube (Solution)
EPR Cavity
FIGURE 2.9

Mounting system for single crystals.
protractor and straight edge when viewed in a binocular microscope.

For maximum signal, the crystal must be positioned in the region of largest microwave magnetic field in the cavity. The crystal was inserted to a position 7.5 mm ± 1 mm below the top of the EPR cavity. This was the region of maximum (>90%) r.f. field strength along the central axis.

When the sample was rotated using the knob-pointer-protractor system shown, figure 2.9, slight deviations from perfect straightness in the stainless steel tube caused the sample to move several millimeters laterally. This displacement was reduced to less than 2 mm by having the extra length of sample tube extend into a teflon collet in the cavity base, thereby keeping the cytochrome c crystal in the maximum region of the axial r.f. field.

In practice the system worked well, with no problem on insertion; however, as mentioned, breakage often occurred on withdrawal.
CHAPTER 3.0

EPR OF CYTOCHROME C IN SOLUTION

3.1 INTRODUCTION

In this chapter we present the results of an EPR study of tuna cytochrome c in solution, between a temperature of 4.2°K and 77°K. The aim is to explain the signals seen in terms of current theories of the EPR of heme compounds.

We give a brief general introduction (3.2) then sections on the physical properties of the signals seen (3.3). After this special attention is focussed on the linewidth variation as a function of temperature (3.4) and our conclusions are drawn in section 3.5.

3.2 THEORY

The theory of EPR signals from low spin ferric heme compounds, such as cytochrome c, has been worked out by Griffith (1957) and Kotani (1964). There are recent reviews by Harris-Loew (1970) and Weissbluth (1966). This theory

*The results and much of the substance of this chapter have been published: C. Mailer and C. P. S. Taylor, Can. J. Biochem. 49, 695-699, (1971).
predicts three g-values arising from axial and rhombic distortions of the octahedral environment of the heme iron. Poole (1967) and Kneubühl (1961) have shown how, in solution, the EPR absorption is distributed over these g-values. For delta function line shapes the absorption spectrum is as shown in figure 3.1 a; for lines of finite width the spectrum becomes as in figure 3.1 b and in the usual derivative display obtained from the EPR detection system, the spectrum of figure 3.1 b appears as in figure 3.1 c.

The peaks in the derivative spectrum at the extremes of the distribution are good approximations to the undifferentiated absorption line that would be seen in a single crystal at the same magnetic field value. This is because the solution line shape as a function of magnetic field, F(H), is obtained by summing the line shape of an individual electron spin absorption, f(H_0 - H), over a distribution, g(H_0). The function g(H_0) takes account of the variation in the resonance position (H_0) and in the transition probability arising from the random orientation of the molecules with respect to the magnetic field (Bleaney, 1960). Thus we have

\[ F(H) = \int_{H_3}^{H_1} f(H_0 - H) g(H_0) \, dH_0 \]

however, near the ends of the distribution g(H_0) is approximately constant, with the result that
FIGURE 3.1

EPR spectra from randomly oriented molecules possessing 3 g-values.

(a) Theoretical spectrum for the case where the individual absorption lines are infinitesimally narrow. This curve thus represents the function \( g(H_0) \).

(b) The absorption spectrum for real molecules with finite absorption line-width. This curve is modified from a passage effect dispersion spectrum of ferricytochrome \( c \) at 5°K by artificially narrowing the line-width near \( g_1 \) so as to produce a more pronounced shoulder. The g-values obtained from the actual experimental trace were \( g_1 = 1.26 \), \( g_2 = 2.25 \), \( g_3 = 3.06 \) to within about 5%.

(c) A hand sketched derivative of curve (b). The usual output of EPR spectrometers is a derivative such as this. In the regions of \( g_1 \) and \( g_3 \) the curve has the shape of individual absorption lines, as discussed in the text.
\[ f(H) = g(H_3) \int_{H_3}^{H_1} f(H_0 - H) \cdot dH \]

hence \[ \frac{dF(H)}{dH} = g(H_3) \cdot f(H_3 - H) \]

so that the derivative spectrum is proportional to the actual absorption line at \( H_3 \). We make use of this in our analysis of line widths.

### 3.3 RESULTS

#### 3.3.1 g-values

Above 20°K, we obtained g-values of 3.05 and 2.24, similar to the results of Salmeen and Palmer (1968) on beef heart cytochrome c. The derivative of the absorption spectrum was as predicted in figure 3.1 c, but the \( g = 1.25 \) line seen by Salmeen and Palmer was not discernible as the signal-to-noise ratio was too low. The presence of a \( g = 1.25 \) line could be inferred from spectra run at 4.2°K where saturation and fast passage effects gave large signals. In order to determine the properties of the cytochrome c paramagnetic resonance we confined our attention to the \( g = 3.05 \) low field absorption line, where the line width was narrowest.

#### 3.3.2 Signals obtained above 20°K

At temperatures above 20°K no saturation effects were seen and we shall consider this 'high' temperature region first.
At 22°K we varied the microwave power from 30 microwatts to 10 milliwatts, and plotted the height of the $g = 3.05$ line versus microwave power in figure 3.2. The straight line obtained of slope $1/2$ shows that height is proportional to the square root of power, the expected behavior for an unsaturated EPR absorption (Abragam and Bleaney, 1970). To avoid saturation line broadening we did not exceed 10 milliwatts of microwave power, incident on the cavity.

The power absorbed by the sample is proportional to the difference of population in the two energy levels between which the resonance takes place. In a system in equilibrium this population difference is given by the Boltzmann equation. For $\hbar \nu$ very much less than $kT$ (here $\hbar \nu = 0.7 \text{ cm}^{-1}$) the population difference is proportional to the power absorbed and the number of absorbers, we expect a graph of power absorbed by the line versus $1/T$ should be a straight line passing through the origin. To test this, the temperature of the sample was varied from 22°K to 77°K and the variation of area of the $g = 3$ peak determined—figure 3.3 shows the results. The area was taken to be proportional to line height $X$ half width. The experimental points lie near an appropriate straight line, the major error in the value of the area being the measurement of the line width. The results summarized in figures 3.2 and 3.3 established that the EPR line results from a two-level system. (It is implicit in
FIGURE 3.2

Height of the absorption line at $g = 3.05$ of a frozen solution of tuna cytochrome $c$ at $22^\circ$K as a function of the microwave power incident on the sample.
FIGURE 3.3

Area of the absorption line at $g = 3.05$ of a frozen solution of tuna cytochrome $c$ as a function of the inverse temperature. (The area is proportional to the difference in electronic population of the two levels between which the resonance occurs.)
the theories of Griffith (1957) and Kotani (1964) that the EPR signals arise from a particular two-level system called a Kramers doublet.)

3.3.3 Signals obtained below 20°K

In the region below 20°K, the shape of the EPR spectrum changed from the derivative form (figure 3.1 c) to a line shape that resembled the undifferentiated line shape of figure 3.1 b. With 1 mW of incident power the amplitude of this broad line increased with decreasing temperature, until at 4.2°K the g = 3 region had increased in amplitude approximately 150 fold. The same line shape, with lower amplitude was recorded with as little as 10 μW of power.

To decide whether this unexpected result was an instrumental fault of the K-band spectrometer, this experiment was repeated on the X-band equipment of Dr. C. Schwerdtfeger (U.B.C. Physics Department); the results are shown in figure 3.4 for temperatures of 4.2°K and 1.7°K. The 4.2°K result, similar to that of Salmeen and Palmer at 20°K, shows that our spectrometer was operating differently. However, the eventual appearance of the anomalous signal at the lower temperature confirmed that the effect was not solely an artifact of our apparatus.

Weger (1960) systematically examined how the microwave power level, magnetic field modulation frequency and magnetic field sweep rate affect the EPR signals obtained
FIGURE 3.4

EPR spectrum of frozen solution of ferricytochrome c at X-band (9 GHz). Magnetic field modulation frequency is 400 Hz.

(a) $T = 4.2^\circ K$, showing absorption derivative.

(b) $T = 1.7^\circ K$, showing fast passage dispersion.
$T = 4.2^\circ K$

(a)

$T = 1.7^\circ K$

(b)

Field - Kilogauss
from a spectrometer. This study suggested to us that the behavior of the signal in the K-band spectrometer, and at low temperatures at X-band arises from a combination of dynamic saturation and fast passage effects due to a long spin-lattice relaxation time, and that what is observed is a dispersion signal and not absorption. Similar effects have been reported in alkali halides by Hyde (1960) and were used by him to estimate the value of spin-lattice and spin-spin relaxation times. The explanation of the different results on the two spectrometers is therefore as follows:

At 20°K, the spin system is not saturated and the expected derivative absorption signal is seen. At 4.2°K the spin system is saturated, the absorption line is broadened and the dispersion signal appears. Normally, a large proportion of any dispersion signal is removed by the automatic frequency control (AFC) system. In the X-band experiment (figure 3.4) this is what happened, and only the absorption derivative signal was seen. In the K-band apparatus, however, the bandwidth of the AFC system is too small, and the dispersion signal is not removed. At 1.7°K the dispersion signal was sufficiently large that the X-band AFC could not remove it all.

To test this hypothesis, we varied the sample temperature between 4.2°K and 20°K and observed the signals at constant microwave power—figure 3.5 gives the results.
FIGURE 3.5

EPR spectra of frozen solutions of tuna ferricytochrome c at two different temperatures:

(a) $T = 20^\circ\text{K}$ — absorption derivative spectrum.

(b) $T = 4.2^\circ\text{K}$ — dispersion fast passage spectrum.
\[ P_{\text{cav}} = 1 \text{ mW} \]

Field - Kilogauss

20 K

4.2 K

impurity

5 10 15
With the temperature fixed at 18.9°K microwave power levels of 10 and 1 mW gave the spectra of figure 3.6. Therefore, either lowering the temperature (to increase the relaxation time) or increasing the microwave power (to increase the saturation) gives the anomalous signal, thus supporting the hypothesis.

We have put the appearance of these fast passage signals to good use, as the results of the single crystal studies show. (Chapter 4, 5, and 6)

3.3.4 **Shapes of solution EPR spectrum**

As mentioned in the Theory section, both Poole (1967) and Kneubuhl (1961) have attempted to explain the broad EPR spectra seen in solution.

The Poole treatment—originally derived in 1958 (Kohin and Poole, 1958) and quoted in his recent book (1967)—calculates the lineshape by assuming a random distribution of orientations of the molecules with respect to the magnetic field, then averaging the resonance magnetic field over all orientations assuming 3 unequal g-values $g_1$, $g_2$, $g_3$ ($g_3 > g_2 > g_1$).

His treatment was applied to systems whose g-values are very close together (e.g. for carbazyl, $g_3 - g_1$ is approximately 0.06) and neglects the variation in transition probability that becomes important when the g-values are far apart (Bleaney, 1960).
FIGURE 3.6

EPR spectra of frozen solutions of tuna ferricytochrome c at two microwave power levels:

(a) Power incident on cavity = 10 milliwatts - showing fast passage signal.

(b) Power incident on cavity = 1 milliwatt - showing absorption derivative signal.
$T = 18.9^\circ K$

$P_{\text{cav}} = 10 \text{ mW}$

Field - Kilogauss

$P_{\text{cav}} = 1 \text{ mW}$

Field - Kilogauss
TABLE III

Comparison of line heights at g-extremes

\[ g \text{-value } g_1 = 1.25 \quad g_2 = 2.25 \quad g_3 = 3.06 \]

<table>
<thead>
<tr>
<th>(height at ( g_3 ))</th>
<th>Kneubühl</th>
<th>Poole</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.7</td>
<td>13.3</td>
<td>5.5</td>
</tr>
<tr>
<td>(height at ( g_1 ))</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Kneubühl (1961) included this variation of probability in his calculations (which are considerably more complex than Poole's), the general effect being to raise the intensity of the low g-value (high field) region of the spectrum.

Doing this for cytochrome c gives the results shown in table III.

This agreement with the more general Kneubühl theory is encouraging, since it supports our implicit assumption that we are dealing with a single molecular species with the three principal g-values quoted. However, we shall see how this assumption must be modified when we consider our line width results in the next section.

3.4 DISCUSSION OF LINESHAPES

3.4.1 Explanation of lineshapes below 50°K

The line width at g = 3.05 is plotted as a function of temperature in figure 3.7 which shows that the line width has a constant value of about 380 gauss below 50°K but broadens to a value of 700 gauss at 77°K. The usual interpretation for such behavior is that at lower temperatures spin-spin relaxation has taken over from spin-lattice relaxation as the dominant energy removal process. However, if this were so the line shape would remain Lorentzian—the appropriate shape for a spin system relaxing with a single time constant.
FIGURE 3.7

Width of the absorption line at $g = 3.05$ of a frozen solution of tuna cytochrome $c$ as a function of absolute temperature.
TABLE IV

Lineshapes as a function of temperature

From Theory (Poole, 1967)

<table>
<thead>
<tr>
<th>$\frac{\Delta H_h}{\Delta H_2}$</th>
<th>$\frac{h}{h_{\text{max}}}$ (Gaussian)</th>
<th>$\frac{h}{h_{\text{max}}}$ (Lorentzian)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.84</td>
<td>0.80</td>
</tr>
<tr>
<td>1.5</td>
<td>0.21</td>
<td>0.30</td>
</tr>
<tr>
<td>2.0</td>
<td>0.06</td>
<td>0.20</td>
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</table>

From Figure 3.8

<table>
<thead>
<tr>
<th>$\frac{\Delta H_h}{\Delta H_2}$</th>
<th>$\frac{h}{h_{\text{max}}}$ (Gaussian) $T=20^\circ K$</th>
<th>$\frac{h}{h_{\text{max}}}$ (Mixed) $T=30.5^\circ K$</th>
<th>$\frac{h}{h_{\text{max}}}$ (Lorentzian) $T=62.5^\circ K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.88</td>
<td>0.81</td>
<td>-</td>
</tr>
<tr>
<td>1.5</td>
<td>0.18</td>
<td>0.26</td>
<td>0.31</td>
</tr>
<tr>
<td>2.0</td>
<td>0.05</td>
<td>0.17</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Gaussian Mixed Lorentzian
In fact, the line shape changed from Lorentzian at 77°K to Gaussian at the lowest temperature studied, 20°K, with intermediate Voigt line shapes at temperatures between. (Voigt line shapes are a super-position of Lorentzian and Gaussian lines of approximately equal width often used in radioastronomy (see Posener, 1959).) The line shape data are shown in figure 3.8 and in table IV for temperatures of 20°K, 30.5°K and 62.5°K.

The Lorentzian line described above should have a width independent of the microwave frequency used to observe the EPR signal. This explains the finding of Morton (1971) in a study of a horse heart cytochrome c solution at 77°K, at a frequency of 9 GHz, that the line width for the g = 3 line was 700 gauss. In contrast to the Lorentzian the Gaussian line shape implies that the absorption at 20°K is made up of a number of narrow lines, randomly distributed about the g-value of 3.06. One source of such a distribution is unresolved hyperfine structure, but the evidence is against this. Hyperfine broadening is independent of the microwave frequency used, but the X-band (9GHz) results (Salmeen and Palmer, 1968) at 20°K show that the g = 3 line is reduced in width to 135 gauss from the K-band (24 GHz) value of 380 gauss. The ratio of the two line widths (2.8) is similar to the ratio of the microwave frequencies (2.7). Calculations by Dr. B. R. Sreenathan, a member of our laboratory, suggest that with the five nitrogen neighbors of the iron atom in
FIGURE 3.8

(a) Examples of high temperature tuna cytochrome c absorption lines.

(b) Notation used in Table IV.
cytochrome c, the total splitting would be 60-100 gauss. Finally, recent work by Scholes (1969) who co-crystallized isolated heme groups (hemin) in perylene, showed that the hyperfine interaction of four nitrogens was about 50 gauss. We conclude that the hyperfine splitting is therefore obscured by another broadening process.

3.4.2 Explanation of lineshapes above 50°K

A possible explanation is that the broadening represents a distribution of g-values arising from variations in the ligand field potential from molecule to molecule. Eisenberger and Pershan (1967) postulated this to explain the angular variation in linewdths which they observed in single crystals of myoglobin azide, another low spin ferric compound.

We have applied their theory to our results and find that a ±11% variation (not ±6% as published) in the rhombic potential is required to give a g-value distribution that could explain the 380 gauss line seen. The same variation in axial potential would cause a linewdth of only 40 gauss. Eisenberger and Pershan (1967) required a rhombic variation of ±4.5% to explain their observations. The line widths at the other g-values can be calculated on this basis and agree reasonably with measured values. The g₂ line—which has extra broadening due to the g-value distribution (see figure 3.1 b ) had a calculated width of 1050 gauss as compared to the measured width (peak-to-peak of derivative)
of 1150 gauss. The $g_1$ line was calculated to be 2800 gauss wide but the signal-to-noise ratio in the spectrum was not high enough to permit a reliable estimate of the width. Extrapolating from the width of Salmeen and Palmer's $g_1$ line suggests that at 24 GHz the $g_1$ line should be approximately 2500 gauss wide.

A theoretical treatment by Blumberg (1968) gives graphs of $g$-values as functions of axial and rhombic potential. These curves show that the desired $g$-value distribution would result from a variation in rhombic potential similar to that calculated.

This hypothesis can be checked by a single crystal study of the line width variation with the orientation of the magnetic field. This study has been made and is presented in chapter 5.

3.5 CONCLUSIONS

We have shown that the microwave power and temperature dependence of the EPR spectrum obtained from tuna ferricytochrome $c$ fit the theory for spin 1/2 Kramers doublet split by a magnetic field.

At temperatures below $20^\circ$K the EPR signal has the form of an undifferentiated absorption line. This arises from a combination of dynamic saturation and rapid passage effects which we discuss more fully in chapter 6 and 7.
Above 20°K, we have made use of the observation that the peaks seen at the extremes of the derivative EPR spectrum obtained in solution are good approximations to an undifferentiated absorption line to obtain the line shape as a function of temperature.

The Gaussian line of constant width below 50°K can be explained as arising from a small variation (± 11%) in the rhombic symmetry of the heme iron environment. At higher temperatures (≥ 77°K) the line shape becomes Lorentzian and appears to be determined by the electron spin-lattice relaxation time.
CHAPTER 4.0
SINGLE CRYSTAL EPR - ORIENTATION OF G-AXES

4.1 INTRODUCTION

The aim of this chapter is to describe the results of an EPR study of single crystals of horse heart ferricytochrome c.

In sections 4.2 and 4.3 we give brief descriptions of the cytochrome c crystals, and the measurement technique. Then we present the method of analysis of data (section 4.4), followed by the results themselves (section 4.5). We discuss, in section 4.6, the errors in g-axis orientations obtained, and compare the results with the three dimensional X-ray structure analysis on ferricytochrome c.

4.2 CRYSTAL PROPERTIES

4.2.1 Crystal Preparations

The horse heart ferricytochrome c crystals were a gift from Dr. R. E. Dickerson and Dr. E. Margoliash, who in collaboration, carried out the three dimensional X-ray analysis of cytochrome c to 2.8Å resolution (Dickerson et al., 1971).
The crystals were prepared from horse hearts using the method of Margoliash and Walasek (1967).

Briefly, the cytochrome c was extracted from horse hearts with aluminum sulphate, then purified by column chromatography, and crystallised from nearly saturated ammonium sulphate solution at pH 6 to 7.

4.2.2 Crystal data

The crystals are needles of rectangular cross section. X-ray crystallographic results (Dickerson et al., 1971) have shown that the space group is P43. Only faces of the {100}, {101}, and {111} classes are well developed. We have labelled the mutually perpendicular axes as shown in figure 4.1.

4.3 Technique of Measurement

4.3.1 Crystal mounting

The crystals were mounted into 3 mm o.d., 2 mm i.d. quartz tubes as described in the Experimental section (2.4).

We found that the mother liquor present in the tube contributed a broad background EPR solution signal which tended to obscure the single crystal line positions. This difficulty was overcome by suspending the crystals in a nearly saturated ammonium sulphate solution before mounting. For the horse heart crystals no concentration of ammonium sulphate was found in which the crystals did not eventually dissolve. However, in 90% saturated solution the dissolution took about 2 hours, long enough to get the crystals mounted and frozen.
FIGURE 4.1

The labelling of the a, b, and c axes in a ferricytochrome c single crystal.
4.3.2 EPR Measurements

The crystals were rotated about a vertical axis in the centre of the cylindrical microwave cavity, and EPR spectra were run at 10° intervals over 360°.

The microwave power incident upon the cavity was approximately 10 mW and the magnetic field modulation was at a frequency of 100 kHz and an amplitude of 1 gauss.

The samples were immersed in liquid helium at 4.2°K. At this temperature and modulation frequency the conditions for fast passage are fulfilled (chapter 6) and the signals had the shape of undifferentiated "absorption" lines (figure 4.2).

4.4 METHOD OF ANALYSIS

4.4.1 Introduction

This section deals with the methods used to analyse the data obtained from the experimental spectrum, in order to find the orientation of the principal g-axes relative to the crystallographic axes.

We give, in section 4.4.2, a description of how the experimental g-values obtained from rotation of the crystal about an axis perpendicular to the magnetic field give the directions of the g-axes relative to the rotation axis. By comparison of the results of two or more orientations of the crystal we show that the angles between the g-axes and crystal axes can be obtained (section 4.4.3).
FIGURE 4.2

EPR spectrum from a single crystal of horse heart ferricytochrome c at 4.2°K.

Experimental conditions:

(i) Microwave power = 10 mW
(ii) Modulation frequency = 100 KHz
(iii) Modulation amplitude = 1 gauss
cytochrome c lines

impurity

Magnetic Field - kilogauss
Finally in section 4.4.4 the stereographic projection technique used to display the results is described.

4.4.2 Theory of q-value variation

The direction of principal g-values of a molecule in a crystal can be obtained from measurement of the g-value variation in planes which are defined relative to a known set of axes in the crystal.

If the magnetic field has direction cosines \( l_1, l_2, l_3 \) relative to these axes, the square of the corresponding g-value is given by (Pryce, 1950):

\[
g^2 = \sum_{i,j=1}^{3} A_{ij} l_i l_j \quad (A_{ij} = A_{ji}) \quad (4.1)
\]

where the \( A_{ij} \) depend upon the choice of reference axes.

From equation (4.1) Schonland (1959) has shown that the g-value variation in the magnetic field plane perpendicular to the axis of rotation is:

\[
g^2 = A + B \cos 2 \psi + C \sin 2 \psi \quad (4.2)
\]

where \( \psi \) is the angle of rotation from an arbitrary zero and \( A, B \) and \( C \) are constants which are functions of the direction cosines of the plane of measurement relative to the reference axes.

We fit our data to equation (4.2) by a least squares computer routine, then, using the values of \( A, B \) and \( C \) so obtained, compute the g-value variation as a function
of $\psi$. From this we get the maximum ($g_+$) and minimum ($g_-$) g-values and the angles at which they occur ($\psi_+, \psi_-$).

From $g_+$ and $g_-$ we can obtain the direction cosines of the principal g-axes relative to the axis of rotation, using the following relations (Schonland, 1959):

$$g_+^2 + g_-^2 = g_1^2 (1-l^2) + g_2^2 (1-m^2) + g_3^2 (1-n^2)$$  \hspace{1cm} (4.3a)

$$g_+^2 - g_-^2 = g_2^2 \cdot g_3^2 \cdot l^2 + g_1^2 \cdot g_3^2 \cdot m^2 + g_1^2 \cdot g_2^2 \cdot n^2$$  \hspace{1cm} (4.3b)

$$l = l^2 + m^2 + n^2$$  \hspace{1cm} (4.3c)

where $l$ is direction cosine of $g_1$ relative to the axis of rotation

$m$ is direction cosine of $g_2$ relative to the axis of rotation

$n$ is direction cosine of $g_3$ relative to the axis of rotation.

The 3 g-axes are chosen to form a right handed triad as shown in figure 4.3.

The directions of the g-axes relative to $\psi_+$ are easily calculated. If there is more than one set of g-axes the angular relationship between the sets can be found from the comparison of the $\psi_+$.

4.4.3 Direction of g-axes relative to crystal axes

In section 4.4.2 we showed how to obtain the principal g-axis directions relative to the rotation axis for a particular orientation. We now wish to show how, by comparing the results from rotation of the crystal about two different
Co-ordinate system used to describe the positions of principal g-axes relative to the laboratory magnetic field direction.
axes, the g-axes directions relative to the crystal axes can be obtained. Rotation of the cytochrome c crystal about its c-axis will give the g-value variation in the ab plane (Orientation I). Similarly, rotation about the b-axis will give the corresponding variation in the ac plane (Orientation II).

If the magnetic field is directed along the crystal a or b axes, the four heme groups in the unit cell only produce two lines. This is because each member of the pair of hemes related by a 180° rotation is equally inclined to the magnetic field, and therefore has the same g-value. As the a-axis is common to Orientation I and Orientation II there will be a point on each g-value variation curve where identical g-values occur.

Once the point common to both is known, the angles the g-axes make with any other axis can be determined.

The above analysis of the data is made easier if some method of displaying the three dimensional information in two dimensions is used. We give a description of such a method in the next section.

4.4.4 Stereographic projection and the Wulff net

The orientation of the principal g-axes relative to the magnetic field plane and/or the crystallographic axes can be best shown by using stereographic projections (Bennett et al., 1957; Phillips, 1962). The method used to represent
three dimensional space in two dimensions is given in figure 4.4. A sphere is imagined to enclose the crystal, and any directions of importance are extrapolated to intersect the surface of the sphere. These points on the spherical surface are projected onto the diametral plane by joining them to the south pole (P) of the sphere, and noting where these lines cut the plane.

In figure 4.4a, for a cytochrome c crystal at the centre of the sphere placed with its c-(001) axis vertical, the a(100) and b(010) axes intersect the spherical surface on the equatorial circle, as indicated in 4.4b. A general point \( X(\theta, \psi) \) is also shown, with its projection \( X' \).

In order to plot out results conveniently, we use a Wulff net. This is shown in figure 4.5, and is a representation of "lines of latitude and longitude" on the surface of the sphere at 2° intervals, projected onto the diametral plane.

4.5 RESULTS

4.5.1 Introduction

We give the results for two orientations of the horse heart ferricytochrome c crystals. In Orientation I the horse heart ferricytochrome c crystal is rotated with its c-axis vertical (figure 4.1). For Orientation II the b-axis is vertical.
FIGURE 4.4

Stereographic projection of cytochrome c crystal

(a) Method of projection from spherical surface to the diametral plane.

(b) The resulting stereogram.
FIGURE 4.5

The Wulff net

Projections of 'lines of latitude and longitude' on the surface of a sphere at 2° intervals, projected onto the diametral plane.
TABLE V

Orientation I

Results of least squares fit to equation (4.2)

<table>
<thead>
<tr>
<th>Line 1</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( g_+^2 = 8.97 )</td>
<td>( \psi_+ = 101.0 )</td>
<td>( A = 6.38 )</td>
</tr>
<tr>
<td></td>
<td>( g_-^2 = 3.79 )</td>
<td>( \psi_- = 11.0 )</td>
<td>( B = -2.40 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( C = 0.98 )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Line 2</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( g_+^2 = 8.99 )</td>
<td>( \psi_+ = 10.25 )</td>
<td>( A = 6.44 )</td>
</tr>
<tr>
<td></td>
<td>( g_-^2 = 3.89 )</td>
<td>( \psi_- = 100.25 )</td>
<td>( B = 2.39 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( C = 0.89 )</td>
</tr>
</tbody>
</table>
4.5.2 Orientation I - c-axis vertical

The cytochrome c was oriented with its long c-axis not more than 2° from the vertical.

The c-axis is a four-fold symmetry axis and since the pairs of heme planes in the unit cell related by 180° rotation are equally inclined to the magnetic field only two lines are seen (one from each pair), instead of the four there would be in an arbitrary orientation.

The magnetic field of the maxima of the "absorption" lines were obtained and, knowing the microwave frequency, the g-values were calculated. As the line width varied strongly with g (400 gauss at g \textfrac{1}{2} 3 to 1000 gauss at g \textfrac{1}{2} 2) there was considerable line overlap. In addition, at 4.2°K the quartz tube signal also showed a fast passage signal, resulting in a broad absorption line shape centred on g=2. This and the broad cytochrome c lines, caused difficulty in estimating the true position of the line maxima (see section 4.6 for discussion of errors).

For this orientation, however, there were only two lines, so it was possible to follow the motion of each line over a rotation of 120°. The g-values and rotation angles, at least 18 data points for each line, were fitted by at least squares computer program to the theoretically expected dependence of g upon angle – equation (4.2).

The computed results are given in table V; the data points and best fitted curves are plotted in figure 4.6.
FIGURE 4.6

g-value as a function of angle of field rotation for Orientation I.

The data points are shown, together with the curves giving the best least squares fit to these data points using equation (4.2).
Orientation I

c-axis vertical

\[
\psi - \text{Angle of Rotation in Degrees (Arbitrary zero)}
\]
### TABLE VI

**Orientation I**

Direction cosines and angles of principal g-axes relative to rotation axis

**Line 1**

<table>
<thead>
<tr>
<th>g-axis</th>
<th>Direction Cosine</th>
<th>Angle°-°</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_1(1) = 1.25$</td>
<td>$l_1 = \pm 0.777$</td>
<td>$\alpha_1 = \pm 38.9$</td>
</tr>
<tr>
<td>$g_2(1) = 2.25$</td>
<td>$m_1 = \pm 0.575$</td>
<td>$\beta_1 = \pm 54.9$</td>
</tr>
<tr>
<td>$g_3(1) = 3.06$</td>
<td>$n_1 = \pm 0.256$</td>
<td>$\gamma_1 = \pm 75.2$</td>
</tr>
</tbody>
</table>

**Line 2**

<table>
<thead>
<tr>
<th>g-axis</th>
<th>Direction Cosine</th>
<th>Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_1(2) = 1.25$</td>
<td>$l_2 = \pm 0.796$</td>
<td>$\alpha_2 = \pm 37.2$</td>
</tr>
<tr>
<td>$g_2(2) = 2.25$</td>
<td>$m_2 = \pm 0.553$</td>
<td>$\beta_2 = \pm 56.4$</td>
</tr>
<tr>
<td>$g_3(2) = 3.06$</td>
<td>$n_2 = \pm 0.247$</td>
<td>$\gamma_2 = \pm 75.7$</td>
</tr>
</tbody>
</table>

*+ here symbolise that the angle the g-axis makes with the rotation axis can be e.g. $0° \pm 38.9$ or $180° \pm 38.9$. 
In table VI we present the direction cosines and angles of the 3 principal $g$-axes of each set, relative to the axis of rotation (the normal to the magnetic field plane).

We now wish to determine the angular positions of the two sets of axes in relation to each other.

The $g$-value maximum will coincide with the largest principle $g$-value direction ($g_3$) only when this axis happens to lie in the plane of rotation. For any other (known) orientation of the $g$-axes we can calculate the angles between the maximum $g$-value and the projection of the $g$-axes in the plane of rotation.

For orientation I, the differences between $\Psi_+$ and the $g_3$-axis projection are small ($\approx 2^\circ$); the projections are shown in figure 4.7. The projections of the two $g_3$ axes are almost $90^\circ$ apart:

$$
\Psi_{g_3(1)} = 98.8^\circ \\
\Psi_{g_3(2)} = 8.1^\circ \\
\text{difference} = 90.7^\circ
$$

and show the 4 fold symmetry predicted by the X-ray and optical measurements (section 4.2.2).

Because of the four-fold symmetry about the c-axis we do not know the directions of the a and b crystal axes relative to the $g$-axes. However we do know that wherever, say, the a-axis is when the magnetic field is along this axis at most two lines will be seen, for which $g^2$ lies between 8.98 and 3.79 (table V). If now we mount a crystal with its
FIGURE 4.7

Stereogram of Orientation I

**Notation:**

I(1) are the projections of the principal g-values of line 1 \((I = 1, 2, 3)\).

I(2) are the projections of the principal g-values of line 2 \((I = 1, 2, 3)\).

\(\psi_{+}(K)\) is the rotation angle at which line \(K\) \((K = 1, 2)\) has its maximum g-value.

\(\psi_{3}(K)\) is the rotation angle at which principal g-value \(g_{3}\) lies for line \(K\) \((K = 1, 2)\).
### TABLE VII

**Orientation II**

Results of least squares fit to equation (4.2)

<table>
<thead>
<tr>
<th>Line</th>
<th>$g_+^2$</th>
<th>$g_-^2$</th>
<th>$\psi_+$</th>
<th>$\psi_-$</th>
<th>$A$</th>
<th>$B$</th>
<th>$C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.40</td>
<td>1.85</td>
<td>45°</td>
<td>135°</td>
<td>3.62</td>
<td>0.06</td>
<td>1.77</td>
</tr>
<tr>
<td>2</td>
<td>5.29</td>
<td>2.02</td>
<td>110°</td>
<td>20°</td>
<td>3.65</td>
<td>-1.21</td>
<td>-1.10</td>
</tr>
<tr>
<td>3</td>
<td>8.18</td>
<td>3.79</td>
<td>75°</td>
<td>165°</td>
<td>5.98</td>
<td>-1.96</td>
<td>0.99</td>
</tr>
<tr>
<td>4</td>
<td>8.15</td>
<td>3.11</td>
<td>80°</td>
<td>170°</td>
<td>5.63</td>
<td>-2.42</td>
<td>0.72</td>
</tr>
<tr>
<td>Line 1</td>
<td>g-axis</td>
<td>Direction Cosine</td>
<td>Angle* - °</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>------------------</td>
<td>------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$g_1(1) = 1.25$</td>
<td>$l_1 = \pm 0.201$</td>
<td>$\alpha_1 = \pm 78.4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$g_2(1) = 2.25$</td>
<td>$m_1 = \pm 0.268$</td>
<td>$\beta_1 = \pm 74.4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$g_3(1) = 3.06$</td>
<td>$n_1 = \pm 0.942$</td>
<td>$\gamma_1 = \pm 19.6$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Line 2</th>
<th>g-axis</th>
<th>Direction Cosine</th>
<th>Angle* - °</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_1(2) = 1.25$</td>
<td>$l_2 = \pm 0.249$</td>
<td>$\alpha_2 = \pm 75.5$</td>
<td></td>
</tr>
<tr>
<td>$g_2(2) = 2.25$</td>
<td>$m_2 = \pm 0.214$</td>
<td>$\beta_2 = \pm 77.6$</td>
<td></td>
</tr>
<tr>
<td>$g_3(2) = 3.06$</td>
<td>$n_2 = \pm 0.944$</td>
<td>$\gamma_2 = \pm 19.23$</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Line 3</th>
<th>g-axis</th>
<th>Direction Cosine</th>
<th>Angle* - °</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_1(3)$</td>
<td>$l_3 = \pm 0.735$</td>
<td>$\alpha_3 = \pm 48.7$</td>
<td></td>
</tr>
<tr>
<td>$g_2(3)$</td>
<td>$m_3 = \pm 0.513$</td>
<td>$\beta_3 = \pm 59.1$</td>
<td></td>
</tr>
<tr>
<td>$g_3(3)$</td>
<td>$n_3 = \pm 0.443$</td>
<td>$\gamma_3 = \pm 63.7$</td>
<td></td>
</tr>
</tbody>
</table>

* ± here symbolise that the angle the g-axis makes with the rotation axis can be e.g. 0° ± 78.4 or 180° ± 78.4.
Table VIII continued

<table>
<thead>
<tr>
<th>g-axis</th>
<th>Direction Cosine</th>
<th>Angle° ± °</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g_1(4) )</td>
<td>( l_4 = ± 0.611 )</td>
<td>( \alpha_4 = ± 52.3 )</td>
</tr>
<tr>
<td>( g_2(4) )</td>
<td>( m_4 = ± 0.632 )</td>
<td>( \beta_4 = ± 50.7 )</td>
</tr>
<tr>
<td>( g_3(4) )</td>
<td>( n_4 = ± 0.476 )</td>
<td>( \gamma_4 = ± 61.6 )</td>
</tr>
</tbody>
</table>
b-axis vertical, then the horizontal magnetic field plane includes both the a and c axes. In a general direction four lines will be seen but only two lines will be observed when the field is along the a-axis. The pair of lines whose $g^2$ values lie between the limits mentioned (8.98, 3.79) will correspond to the a axis direction.

4.5.3 **Orientation II - b-axis vertical**

The cytochrome c crystal was oriented with its broad face as close to horizontal as possible, and the normal to this face, taken as the b-axis, was measured to be $5^\circ \pm 1^\circ$ from the vertical. As before, spectra was taken over as many points as possible in a 360° range.

At first sight only three lines were seen; however, closer examination showed that there were in fact four lines: one pair clearly separate and another pair largely overlapping. The position of the resonance maxima were measured and the results of the line assignments tested by the least squares fitting program. The best fits to the data gave the computed curves shown in figure 4.8. The computed g-maxima, g-minima, $\Psi$, and the parameters defining the theoretical curves are given in table VII, and table VIII presents the appropriate direction cosines and angles of the principal g-axes for the 4 lines. After computing the angular positions of the g-axes relative to the g-maxima, we plot the projections in figure 4.9.
FIGURE 4.8

g-value as a function of angle of rotation for Orientation II. The data points are shown, together with the curves giving the best least squares fit to equation (4.2).
Orientation II

b-axis vertical

\( \psi \) - Angle of Rotation in Degrees (Arbitrary zero)
FIGURE 4.9

Stereogram of Orientation II

Notation:

$\mathbf{I}(1)$ are the projections of the principal $g$-values of line 1, $(I = 1, 2, 3)$.

$\mathbf{I}(2)$ are the projections of the principal $g$-values of line 2, $(I = 1, 2, 3)$.

$\mathbf{I}(3)$ are the projections of the principal $g$-values of line 3, $(I = 1, 2, 3)$.

$\mathbf{I}(4)$ are the projections of the principal $g$-values of line 4, $(I = 1, 2, 3)$.

$\Psi$ is the angle of rotation.
4.5.4 Crystallographic axes

We now determine the directions of the crystallographic axis relative to the principal g-axes.

As mentioned above, with the b-axis vertical, the a-axis will lie along one of the directions where four lines become two. From figure 4.8 this occurs at the experimental rotation angles of:

<table>
<thead>
<tr>
<th>( \psi )</th>
<th>320°</th>
<th>348°</th>
<th>77°</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g^2 )</td>
<td>4.6</td>
<td>3.25</td>
<td>8.15</td>
</tr>
<tr>
<td>( g^2 )</td>
<td>1.85</td>
<td>3.0</td>
<td>4.3</td>
</tr>
</tbody>
</table>

\( g^2 \) limits from Orientation I

The a-axis therefore lies at \( \psi = 77° \) and we add this onto the projection diagram of figure 4.9. The c-axis is also shown, being 90° from the a-axis.

Now that we have the g-values when the magnetic field is directed along the a-axis in orientation II, we can find the corresponding position in orientation I. In figure 4.6, \( g^2 \) values of 8.15 and 4.30 lie in the angular ranges 24°-34°, 77°-83°, and at multiples of 90° from these. Because of the four fold symmetry of this orientation we do not know which of these corresponds to the a-axis. This ambiguity cannot be resolved by EPR and we must look to the three dimensional structure determined by X-ray diffraction (Dickerson et al., 1971) to decide which choice is the correct one, and which is the mirror image. This will be discussed in the next section.
4.6 DISCUSSION OF ORIENTATION RESULTS

4.6.1 We discuss in this possible errors (section 4.6.2) and then compare our results for the orientation of the g-axes in the crystal in the light of the 3-dimensional X-ray results for the same crystal (section 4.6.3).

4.6.2 Experimental errors

We introduce our discussion of the experimental errors in the measurement of the orientations of the g-axes by asking the following question: Can we transform one of the projection diagrams—either figure 4.6 or 4.9—into the other by a 90° rotation about the a-axis? Any discrepancies between the rotated version and the experimental one can then be examined to see if they fall within the experimental error.

Because of the four fold symmetry of orientation I, we could choose any one of four directions for the a-axis (27-35, 77-83, 117-125; 167-173) and rotate by 90° about this axis. Figure 4.10 is the complete stereographic projection for orientation I, showing the four sets of g-axes. The best fit of figure 4.9 to figure 4.10 was given by rotating the molecule with c-axis vertical 90° counter clockwise about the 117° direction, as shown in figure 4.11. The points without letters are those measured in orientation II.

The discrepancies can be summarized by saying the g^3 axes projections differ by 4°-10° and the g^1, g^2 axis
FIGURE 4.10

Full stereogram of Orientation I

Notation:

AI (I = 1, 2, 3) are the projections of the 3 principal g-axes of the molecule that gives line 2 of figure 4.7.

BI (I = 1, 2, 3) are the projections of the 3 principal g-axes of the molecule that gives line 1 of figure 4.7.

The CI and DI are the projections of the principal g-axes of the molecules related by a 180° rotation about the c-axis to AI and BI respectively.
Orientation I

- a-axis
  - $\psi = 117^\circ$

- b-axis
  - $\psi = 180^\circ$

Points:
- A1
- A2
- A3
- B1
- B2
- B3
- C1
- C2
- C3
- D1
- D2
- D3
FIGURE 4.11

Comparison of Orientations I and II

A 90° counter-clockwise rotation of the stereographic projection of Orientation I about the a-axis (figure 4.10) moves the directions of the g-axes determined in Orientation I for comparison with those from Orientation II.

The alphanumeric symbols (AI, etc.) correspond to the rotated axes of Orientation I. The other axes are those of Orientation II (figure 4.9).
projections differ by 5°-20°. The disagreement is greatest in lines 1 and 2 of figure 4.11a. To decide whether this disagreement is significant we must discuss the experimental errors involved in obtaining the results.

The sources of errors are:

(i) measurement of the principal g-values ($g_1$, $g_2$ and $g_3$)
(ii) reading the magnetic field value at the line centre
(iii) setting the angle of rotation
(iv) orientation of the crystal in the sample tube.

We now discuss these in turn:

(1) The principal g-values were obtained from measurements on the mother liquor of the horse heart crystals. Only two values could be obtained, $g_2 = 2.25$ and $g_3 = 3.06$, as the solution was so dilute (1mM) that the high field line was not visible. We took $g_1$ to be 1.25, consistent with the results of Salmeen and Palmer on beef heart cytochrome c.

The effect of uncertainties in the principal g-values is reflected in the calculated angles of the g-axes relative to the axis of rotation.

When the c-axis is vertical, for example, and $g_1 = 1.25$, $g_2 = 2.25$ and $g_3$ is as shown below (using the rotation of figure 4.3):
Variations in $g_1$ and $g_2$ have lesser effect; with $g_2 = 2.24$, and $g_3 = 3.06$ we have for variations in $g_1$:

<table>
<thead>
<tr>
<th>$g_1$</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.20</td>
<td>38.3°</td>
<td>55.6°</td>
<td>75.3°</td>
</tr>
<tr>
<td>1.25</td>
<td>38.9</td>
<td>54.9</td>
<td>75.2</td>
</tr>
<tr>
<td>1.30</td>
<td>39.7</td>
<td>54.0</td>
<td>75.0</td>
</tr>
</tbody>
</table>

with similar results for $g_2$ varying from 2.2 to 2.3. When the b-axis is vertical, the errors are of the same magnitude.

For $g_3$, we find a value of $3.06 \pm 0.02$ and this, with the other principal values of $g_2 = 2.25 \pm 0.025$ and $g_1 = 1.25 \pm .05$ gives a possible error in angles with respect to the rotation axis of

- $\pm 1°$ for $\alpha, \beta$ (angles of $g_1$, $g_2$ axes)
- $\pm 2°$ for $\gamma$ (angle of $g_3$ axes)

from this cause.
TABLE IX

Errors in least squares fit to equation (4.2)

<table>
<thead>
<tr>
<th>Orientation I (c-axis vertical)</th>
<th>Error sum of squares</th>
<th>Residual error in $g^2$</th>
<th>Number of data points used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 1</td>
<td>0.18</td>
<td>0.09</td>
<td>22</td>
</tr>
<tr>
<td>Line 2</td>
<td>0.34</td>
<td>0.14</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Orientation II (b-axis vertical)</th>
<th>Error sum of squares</th>
<th>Residual error in $g^2$</th>
<th>Number of data points used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 1</td>
<td>0.05</td>
<td>0.05</td>
<td>20</td>
</tr>
<tr>
<td>Line 2</td>
<td>0.07</td>
<td>0.07</td>
<td>17</td>
</tr>
<tr>
<td>Line 3</td>
<td>0.33</td>
<td>0.14</td>
<td>20</td>
</tr>
<tr>
<td>Line 4</td>
<td>0.23</td>
<td>0.18</td>
<td>11</td>
</tr>
</tbody>
</table>
(ii) The determination of the position of the line centre was difficult because of the very wide lines. The uncertainty is 10-20 gauss for the narrowest lines to 100 to 250 for the widest lines. This is reflected in the goodness of fit of the computed curves, as shown in table IX.

For the c-axis vertical, the fitting errors in $g^2$ would lead to an error of $\pm 2-3^\circ$ in the angle between $g_3$ and the vertical. However, as the highest $g$-values measured were also the most precise, most of this possible error will lie in the minimum $g$-value, where it produces an uncertainty in the $g_1$ and $g_2$ directions of $\pm 5^\circ$. Comparison of the angles from the two sets of results (table VI) shows that they agree well within these limits.

With the b-axis vertical, the residual error in lines 1 and 2 is less than any other because we could measure $g$-values over a wide range of angles. For lines 3 and 4, which were largely overlapping, the residual error is higher.

This source of error gives:

for orientation I $\pm 5^\circ$ for $a,\beta$ in line 1 and 2
$\pm 1^\circ$ for $\gamma$

orientation II

$\pm 1^\circ$ for $a,\beta,\gamma$ in line 1 and 2
$\pm 3^\circ$ for $a,\beta,\gamma$ in line 3 and 4
(iii) The rotation angle could be set to within 0.5°; rotations of the sample were made in one direction to avoid backlash. We also rotated the magnet, in order to change the angle between the crystal and the d.c. field. No difference in results was obtained with the two rotation methods. A combination of sample rotation and magnet rotation was quite useful, with the magnet rotation being used as a vernier for small angles.

This error is so small that it can be neglected in comparison with (i) and (ii).

The sum of the errors from these three independent causes gave the error in the $g_1$ and $g_2$ axis as $\pm 4.5°$ for both orientations, with the $g_3$ axis having a possible error of $\pm 2.4°$.

Even allowing for these errors, there is still a discrepancy between the rotated c-axis results and the experimental b-axis vertical results.

(iv) We must therefore postulate that our measurements of the orientation of the crystal in the sample tube were in error.

For the c-axis vertical case our estimated error was not more than 2° in the direction of the long axis of the crystal from the vertical. The experimental g-values and particularly the directions of the $g_3$-axes show that indeed the crystal was aligned vertically within 0.5°. Otherwise
the $g_3$-axes angles would not have been so nearly equal (i.e. 75.2°, 75.7°).

For the b-axis vertical case, the setting up errors were greater, the b-axis being estimated as 5° off the vertical, while the c-axis was within 2° of horizontal.

As figure 4.11 shows, the greatest discrepancy between the rotated projection and the observed one was for lines 1 and 2. We attempted to remove the differences for line 1 by a small rotation of the projection of the orientation II angles. It was found that an extra rotation of 10° counter clockwise about an axis at $\psi = 99°$ did make the projections of line 1 overlap within the experimental error and improved the projections of lines 2, 3, and 4.

We therefore assume that before freezing the crystal moved in the tube by a few degrees, tipping the b-axis about 10° from the vertical towards the bc plane.

We have given this fairly lengthy discussion of the possible errors to satisfy ourselves that we have obtained the orientation of the $g$-axes as precisely as possible.

We should point out that in fact, the complete orientation picture could have been obtained from Orientation I only—thanks to the four fold symmetry about the c-axis. It is encouraging that the results of Orientation II, despite the difficulties of analysis and mounting, are consistent with those of Orientation I.
**TABLE X**

Principal g-axis directions relative to the crystal axes

(a) Our co-ordinate system - abc

<table>
<thead>
<tr>
<th>g-axis \ crystal axis</th>
<th>a</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_1$</td>
<td>126</td>
<td>91</td>
<td>37</td>
</tr>
<tr>
<td>$g_2$</td>
<td>43</td>
<td>113</td>
<td>56</td>
</tr>
<tr>
<td>$g_3$</td>
<td>72</td>
<td>24</td>
<td>76</td>
</tr>
</tbody>
</table>

(b) Dickerson et al. (1971) co-ordinate system - xyz

<table>
<thead>
<tr>
<th>g-axis \ crystal axis</th>
<th>x</th>
<th>y</th>
<th>z</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_1$</td>
<td>68</td>
<td>118</td>
<td>143</td>
</tr>
<tr>
<td>$g_2$</td>
<td>96</td>
<td>33</td>
<td>122</td>
</tr>
<tr>
<td>$g_3$</td>
<td>24</td>
<td>72</td>
<td>76</td>
</tr>
</tbody>
</table>

(c) Heme plane directions relative to crystal axes

<table>
<thead>
<tr>
<th>Axis</th>
<th>x</th>
<th>y</th>
<th>z</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_2$-Fe-N$_4$</td>
<td>86</td>
<td>46</td>
<td>135</td>
</tr>
<tr>
<td>$N_1$-Fe-N$_3$</td>
<td>68</td>
<td>133</td>
<td>129</td>
</tr>
<tr>
<td>$S$-Fe-N$_{18}$</td>
<td>22</td>
<td>75</td>
<td>71.5</td>
</tr>
</tbody>
</table>

*These directions are estimated from the stereo pictures presented in Dickerson et al. (1971).*
To sum up, then, we have determined the orientations of the $g_1$ and $g_2$ axes to within 5° to 10° and of the $g_3$-axis to within 2° to 4°. In table Xa we present the angles between the crystallographic axes and the $g$-axes explicitly. We chose the set of $g$-axes which, in our system of notation, put the $g_3$-axes in the positive octant of the abc co-ordinate system of figure 4.1.

In chapter 5, it will be shown that a study of the EPR linewidth as a function of crystal rotation also serves as a check on the results presented here.

4.6.3 Comparison of our results with optical and X-ray data

Before the publication of the recent paper by Dickerson et al. (1971), the only other data available on the orientation of the heme relative to crystal axes was that from the electronic spectra of single crystals of cytochrome $c$ in polarised light (Kabat, 1967; Eaton and Hochstrasser, 1967). These workers could only obtain the direction of the heme normal relative to the crystal c-axis. Their data, the X-ray determined orientation, and our results are:

<table>
<thead>
<tr>
<th>Angle of heme normal to c-axis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eaton and Hochstrasser</td>
</tr>
<tr>
<td>Kabat</td>
</tr>
<tr>
<td>This work ($g_3$)</td>
</tr>
<tr>
<td>Dickerson et al.</td>
</tr>
</tbody>
</table>
We can therefore take our $g_3$-axis as being directed closely along the heme normal, with the other two axes lying in the heme plane.

We now compare the direction of the $g_1$ and $g_2$ axes with those of the pyrrole nitrogen-iron-pyrrole nitrogen (N-Fe-N) axes in the porphyrin ring.

Firstly we convert our abc co-ordinate system into the xyz system used by Dickerson et al. (1971) to describe their X-ray structure results. The relation between the axes are: $+a = +y$; $+b = +x$; $+c = -z$.

Table Xb shows the angles of the EPR $g$-axes in the xyz system, and table Xc gives the directions of various axes in the heme ring as estimated from the published stereographic X-ray pictures.

The $g_1$ and $g_2$ axes lie close to the N-Fe-N axes directions as indicated in figure 4.12, and are coplanar with the heme ring. We labelled the nitrogen atoms co-ordinated to the iron as shown; the pyrrole ring containing $N_1$ is that ring which is bound, via phenylalanine-46, to the amino acid back bone.

4.6.4 Comparison of our results with myoglobin azide and cyanide complexes

The only other low spin protein complexes for which EPR and X-ray structure data are available are met myoglobin
FIGURE 4.12

Projection of g-axes onto heme plane.
$g_1 = 1.25$

$g_2 = 2.25$

Plane of Histidine - 18
azide* (Helcke et al., 1968) and myoglobin cyanide (W. E. Blumberg, personal communication). Both of these compounds have their largest g-value axis aligned approximately along the heme normal. In the azide the deviation is 9°, and in the cyanide it is 13°.

Helcke et al. (1968) speculate that the first nitrogen in the azide molecule is prevented from exactly replacing the oxygen usually bound to the protein. They estimate that the nitrogen atom would only need to be displaced by 0.4 Å to explain this observed discrepancy in angle; such a small displacement would not be visible on the X-ray structure (Stryer et al., 1964). Obviously the same argument is applicable to the cyanide compound, although one might expect the discrepancy to be less, as the cyanide is less bulky than the azide.

In both myoglobin azide and myoglobin cyanide the other two g-axes are aligned parallel and perpendicular to the projection of the histidine-18 imidazole ring on the plane of the heme. This projection is approximately the same in all three molecules, passing through methene bridges on opposite sides of the ring bisecting adjacent nitrogens—figure 4.12.

---

*Hemoglobin azide has also been studied by EPR (Gibson and Ingram, 1957) and its structure has been determined by X-ray diffraction (Perutz and Mathews, 1966). However, the directions of g-axes have not been published.
In the azide it is the lowest $g$-value ($g_1$) that is parallel to the histidine plane, while in the cyanide it is the middle $g$-value ($g_2$). Also, the cyanide complex is different from cytochrome $c$ and the azide in that its $g_1$-axis is approximately 13° out of the heme plane, pointing towards the histidine imidazole.

In both these myoglobin compounds it is the position of the histidine imidazole ring which determines the direction of the $g$-axes, presumably through the action of the $\pi$-orbitals of the $\delta$-nitrogen atom bound to the iron. Blumberg (personal communication) suggests "the fact that $g_1$ and $g_2$ have been interchanged (in the cyanide) as compared to the azide case, is probably indicative that the cyanide ion is participating in back donation of electron density, while the azide ion is not".

However, in cytochrome $c$ we cannot assume that the rhombic field which splits the orbitals to make $g_1$ not equal to $g_2$ arises from the histidine imidazole, because the $g$-axes are almost equally inclined to the plane of the histidine. Also, the amount of rhombic splitting by this imidazole ring has been calculated by Kotani (1964) to be 60 cm$^{-1}$--far less than the amount required to give the observed $g$-values--$\approx 900$ cm$^{-1}$ for the azide, 500 cm$^{-1}$ for cytochrome $c$ (Harris, 1970) and $\approx 400$ cm$^{-1}$ for the cyanide (Blumberg, personal communication).
Mizuhashi (1969) has attempted to explain the anisotropy of g-values in hemoglobin azide by calculating the contribution to the rhombic field from the azide ion and from the Jahn-Teller distortion of the heme ring. However, he did not discuss the orientation of the g-axes in his treatment. He concluded that the large anisotropy seen was due to the combined action of the dynamical Jahn-Teller interaction and the rhombic fields of azide and imidazole.

We have extended Mizuhashi's treatment to cytochrome c to attempt to explain the g-value anisotropy.

4.6.5 Jahn-Teller effect in cytochrome c

The Jahn-Teller theorem is stated as follows: "A nonlinear polyatomic molecule in an orbital state with orbital degeneracy will be unstable in its symmetrical equilibrium position with respect to distortions which destroy those elements of symmetry responsible for the degeneracy" (Jahn and Teller, 1937).

In practice, this means such a degeneracy occurs whenever there is a doubly degenerate pair of orbitals with an odd number of electrons in them. The odd electron has a choice between either of the degenerate pair of orbitals and the molecule distorts in such a way that the degeneracy is removed.

If the stabilisation energy gained by the distortion is very much greater than the zero point energy of the
<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eisenberger and Pershan (1967)</td>
<td>0.885</td>
<td>0.174</td>
<td>0.431</td>
</tr>
<tr>
<td>Harris (1970)</td>
<td>0.885</td>
<td>0.174</td>
<td>-0.431</td>
</tr>
<tr>
<td>Mizuhashi (1969)</td>
<td>0.426</td>
<td>-0.885</td>
<td>0.174</td>
</tr>
</tbody>
</table>

(The coefficients are for cytochrome c)
normal mode of the appropriate vibration which distorts the molecule, then the molecule adopts the distorted configuration, and we have the static Jahn-Teller effect. If the stabilisation energy is of the same order, or less than, the zero point energy of the vibration then there is coupling between the electronic and nuclear motions to form a 'vibronic' state. The molecule vibrates between its various distorted forms. This is called the dynamic Jahn-Teller effect.

We now ask—is it the static or dynamic Jahn-Teller effect that is operating in cytochrome $c$? Following the treatment of Mizuhashi (1969) with our $g$-values,* we find that energy gained by Jahn-Teller interaction, neglecting nuclear motion, is approximately $225 \text{ cm}^{-1}$. The zero point energy of the vibration associated with the distortion ($B_{2g}$) is approximately $300 \text{ cm}^{-1}$. The static Jahn-Teller effect is therefore not operating here. We must therefore solve the Hamiltonian taking into account the kinetic energy of the vibration. Mizuhashi carried out this calculation and obtained a series of curves of $g$-value as a function of rhombic field and of the coupling between the electronic and nuclear systems. A reproduction of his results for the ground doublet is given in figure 4.13.

*The notation used by Mizuhashi differs from ours—that of Eisenberger and Pershan (1967) and from that of Harris (1970) who published a review on the chemistry of low spin ferric heme compounds. The relationship between the various conventions is shown in table XI.
FIGURE 4.13

g-values as a function of rhombic field and Jahn-Teller coupling (after Mizuhashi, 1969).
For our g-values (3.06, 2.25, 1.25) a value of rhombic field strength of 150 cm⁻¹ and a coupling of $\gamma = 0.7$ fit best. ($\gamma$ is defined to be the square root of the ratio of the energy gained by the J.T. distortion to the zero point energy of the distorting vibration.)

These results show that the dynamic Jahn-Teller effect can 'amplify' a small rhombic splitting to produce the difference in g-values observed. If there is no rhombic splitting then $g_1 = g_2$ and the anisotropy of g-values does not occur for any vibronic state.

Mizuhashi attempted to calculate $\gamma$. He assumed that the t₂g electrons were subject to a vibrating potential field from the neighbouring porphyrin nitrogens. Numerical evaluation, using Hartree-Fock wave functions for the Fe³ t₂g orbitals, gave $\gamma = 0.3$—in reasonable agreement with his value of 0.8 for hemoglobin azide and ours of 0.7 for cytochrome c.

The directions of the g-axes will be a function of both the orientations of the imidazole and any other ligands and of the mode of vibration of the ring. The $B_{1g}$ and $B_{2g}$ modes vibrate as:

- $B_{1g}$
- $B_{2g}$
Since according to Mizuhashi the $B_{2g}$ mode combined with the rhombic contributions from the imidazole and azide operate to give the $g$-axes with orientations parallel and perpendicular to the histidine imidazole as seen in myoglobin azide, in a similar fashion the $B_{2g}$ mode with the imidazole rhombic field give the results found for myoglobin cyanide. We suggest that in cytochrome $c$ the $B_{1g}$ mode (in the N-Fe-N directions) with the histidine rhombic field gives the picture found by us—the $g$-axes lying between the imidazole and N-Fe-N projections on the heme plane.

4.7 SUMMARY

We have presented the methods of analysis used to derive the directions of the principal $g$-axes, relative to the crystallographic axes, from the EPR spectra of horse heart ferricytochrome $c$.

The results show that the direction of the largest principal $g$-value ($g_3 = 3.06$) lies within $5^\circ$ of the heme normal determined from X-ray diffraction measurements and other data. The other two $g$-axes lie in the heme plane, approximately $15^\circ$ from the N-Fe-N directions in the porphyrin ring.

Mizuhashi's theory, using the Jahn-Teller effect to account for the $g$-values seen in hemoglobin azide, was applied to cytochrome $c$. From this theory a rhombic distortion of $150 \text{ cm}^{-1}$ and dynamic J.T. coupling coefficient ($\gamma$)
of 0.7 were needed to explain the g-values (3.06, 2.25, 1.25) observed experimentally. These are in reasonable agreement with a 60 cm⁻¹ rhombicity contribution, from the imidazole of histidine-18 co-ordinated to the fifth position of the iron; and with a γ of 0.3, predicted from a vibrating point charge model of the 4 nitrogens in the porphyrin ring system co-ordinated to the iron.
CHAPTER 5.0
SINGLE CRYSTAL EPR -
LINEWIDTH VARIATION AS A FUNCTION OF G-VALUE

5.1 INTRODUCTION

In this chapter we present our results, and the theory to explain them, of the changes in EPR line width produced by rotation of single crystals of ferricytochrome c, relative to the d.c. magnetic field direction.

Materials and methods are treated in section 5.2. This is followed by a section on the causes of line broadening (section 5.3), then on the theory applicable to our system (section 5.4). The results and discussion follow (sections 5.5 and 5.6) and our conclusions are drawn in section 5.7.

5.2 MATERIALS AND METHODS

The materials and methods used are largely described in chapter 4.

Measurement of the widths of any of the lines is difficult for several reasons. At low fields, close to the maximum g-values, where the lines are relatively narrow, they overlap with the others present, and with an impurity
resonance close to \( g = 2 \). This makes determination of the line shape difficult. At higher fields, where the lines are wider, the lowered signal-to-noise ratio and an uncertain baseline lead to larger uncertainties in the measurements.

5.3 SOURCES OF LINE BROADENING

5.3.1 Minor contributions to line width

Many of the usual causes of line broadening can be ruled out immediately as their contributions are too small:

(i) The simplest one—that of relaxation time broadening—is not the cause, for the line shape seen is Gaussian, not Lorentzian, and the width is independent of temperature between 4.2 and 50°K. (Above this temperature, the line shape does become Lorentzian—chapter 3).

(ii) Dipolar and exchange interactions between iron atoms are not important here since the smallest possible distance between nearest neighbors is approximately 30 Å. Dipolar interactions between water protons and the iron atom could contribute to the linewidth. However, ENDOR (electron-nuclear double resonance) on myoglobin azide (Eisenberger and Pershan, 1967) showed that the hyperfine coupling to the iron was only of the order of 0.5 MHz—equivalent to a linewidth contribution of about 10 gauss.

(iii) Because of the presence of five nitrogen atoms, each with nuclear spin of \( 1 \), it might be expected that there would be hyperfine broadening from this source. Indeed,
Scholes (1969) has shown that in hemin co-crystallized with perylene, there is a hyperfine interaction sufficient to produce a line of about 50 gauss at X-band (9GHz). Since this interaction is frequency independent it would therefore produce a similar linewidth at our frequency, 24 GHz. The interaction with the nitrogen is, therefore, too small to account for the broad linewidths observed.

5.3.2 Major causes of line broadening

The mechanical softness and fragility of protein crystals, together with their high content of water of crystallisation (50% in cytochrome c, Dickerson, 1967) suggest that some degree of disorder might exist in them. As will be shown, this is capable of causing very large line broadening. Basically, the spectral line seen is the sum of a series of lines whose effective g-values are spread about a mean value (the centre of the measured spectrum).

We know that (Pryce, 1950):

\[
g^2 = \sum_{ij} A_{ij} \cdot \ell_i \cdot \ell_j \quad (A_{ij} = A_{ji})
\]

then, formally:

\[
\Delta g^2 = \sum (\Delta A_{ij}) \cdot \ell_i \cdot \ell_j + \sum A_{ij} \cdot \Delta (\ell_i \cdot \ell_j)
\]

The first term represents the effect on the spectrum of variation in g-tensor (A), i.e. a variation in the principal g-values. This is an internal molecular property and arises from small changes in the ligand field symmetry from molecule
to molecule. The second term represents a broadening of the spectrum from a change in orientation of the molecules with respect to the magnetic field. It is well known that, in general, crystals are not truly single but consist of many small crystallites, each with slightly different orientation. The g-tensor is the same in each crystallite. The broadening is a minimum along the directions of the principal g-axes of the crystal, where this misorientation effect vanishes to first order.

We now give the theory to calculate the line width contributions from these two causes.

5.4 THEORY OF LINE BROADENING

5.4.1 Introduction

If the effective g-value is a function of several parameters—i, j, k say,—then independent small random variations of these parameters $\Delta i, \Delta j, \Delta k$, produce the following change in $g$:

$$
\Delta g = \frac{\partial g}{\partial i} \Delta i + \frac{\partial g}{\partial j} \Delta j + \frac{\partial g}{\partial k} \Delta k
$$

(5.2)

The total effect of these independent and random variations will be given by the root mean square value of $\Delta g$, defined as $\langle \Delta g^2 \rangle^{1/2}$:

$$
\langle \Delta g \rangle^{1/2} = \sum_i \left[ \langle \left( \frac{\partial g}{\partial i} \right)^2 \rangle \langle (\Delta i)^2 \rangle \right]^{1/2} + \sum_{i \neq j} \langle \frac{\partial g}{\partial i} \rangle \langle \frac{\partial g}{\partial i} \rangle \langle \Delta i \rangle \langle \Delta j \rangle
$$
For independent variables, the second term is zero, and
\[ \langle \Delta g^2 \rangle^{\frac{1}{2}} = \sum \langle (\frac{\partial g}{\partial i})^2 \rangle^{\frac{1}{2}} \cdot \langle (\Delta i)^2 \rangle^{\frac{1}{2}} \]  
(5.3)

\( \langle \Delta g^2 \rangle \) is a measure of the width of the resonance line, while the \( \langle \Delta i^2 \rangle \) measure the uncertainty in the values of \( i \).

We now wish to obtain analytical expressions for the partial derivatives \( \frac{\partial g}{\partial i} \), and to relate these to the linewidths observed.

5.4.2 Evaluation of partial derivatives—linewidth due to misorientation of molecules

When the d.c. magnetic field is at a position with direction cosines \( \xi, \eta, \zeta \) relative to the molecular g-axes—\( g_1, g_2, g_3 \)—the g-value in that direction is given by
\[ g^2 = g_1^2 \xi^2 + g_2^2 \eta^2 + g_3^2 \zeta^2 \]

In polar coordinates we have
\[ g^2 = g_1^2 \sin^2 \theta \cos^2 \phi + g_2^2 \sin^2 \theta \sin^2 \phi + g_3^2 \cos^2 \theta \]

whence
\[ \frac{\partial g}{\partial \theta} = \frac{\sin 2\theta}{2g} \left[ (g_2^2 - g_1^2) \sin^2 \phi - (g_3^2 - g_1^2) \right] \]

If we call \( \Delta \theta \) the r.m.s. deviation in \( \theta \), then the r.m.s. value of \( g, \Delta g \), is
\[ \overline{\Delta g} = \left| \frac{\partial g}{\partial \theta} \right| \Delta \theta \]

Using \( h \nu = g \beta H \) the resulting r.m.s. value of the line width, \( \Delta H_\theta \), due to the variation in \( \theta \) is given by
\[ \Delta H_{\theta} = \frac{\hbar \nu}{\beta} \cdot \frac{\Delta g}{g^2} \]

\[ = \frac{\hbar \nu}{\beta} \left\{ \sin 2\theta \left[ \left( g_2^2 - g_1^2 \right) \sin^2 \phi - \left( g_3^2 - g_1^2 \right) \right] \Delta \theta \right\} \]

We must also consider the broadening produced by a variation in \( \phi \), where \( \phi \) is the angle of the d.c. field in the \( g_1 \) \( g_2 \) plane relative to the \( g_1 \) axis, as shown in figure 5.1b. One can obtain an expression similar to (5.4) for the line width contribution:

\[ \Delta H_{\phi} = \frac{\hbar \nu}{\beta} \left\{ \sin 2\phi \sin^2 \theta \left( g_2^2 - g_1^2 \right) \Delta \phi \right\} \]

where \( \Delta \phi \) is the r.m.s. deviation in \( \phi \).

Necké et al. (1968) used (5.4) and (5.5) to calculate the line widths in met-myoglobin azide. In order to fit their data they chose \( 2 \Delta \theta = \Delta \phi \) and added the two contributions linearly.

This procedure, to us, seems incorrect, for the variations in \( \theta \) and \( \phi \) are independent and should be combined by squaring and adding \( \Delta H_{\theta} \) and \( \Delta H_{\phi} \), then taking the square root to obtain the total linewidth. In addition, the use of \( \Delta \phi \) as the measure of the random variation is wrong.

Around any direction \( \theta, \phi \) --see figure 5.1--there are a number, \( N(\omega) \), of crystallites whose directions are displaced by an angular distance, \( \alpha \). Randomness means that:
Notation used for linewidth calculations

(a) Cone of solid angle, $\alpha$, in g-axis co-ordinate system.

(b) Definition of element of area in polar co-ordinates for a general point $(\theta, \phi)$ in the g-axis co-ordinate system.
\[ \Delta u = \sin \theta \cdot \Delta \phi \]
(i) \( N(\alpha) \) is a Gaussian function of

\[
N(\alpha) \propto \exp\left(-\frac{1}{2} \left(\frac{\alpha}{\sigma}\right)^2\right)
\]

where \( \sigma \) is the r.m.s. value of \( \alpha \)

(ii) \( N(\alpha) \) is independent of direction around \( \theta, \phi \)

On the unit sphere, \( \alpha = ds \), an element of length on the surface and \( (ds)^2 = (d\theta)^2 + \sin^2 \theta \cdot (d\phi)^2 \equiv (d\theta)^2 + (du)^2 \)

Clearly, also

\[
\sigma^2 = \langle \alpha^2 \rangle = \langle ds^2 \rangle = \langle d\theta^2 \rangle + \langle du^2 \rangle
\]

and \( \langle d\theta^2 \rangle = \langle du^2 \rangle = \frac{1}{2} \sigma^2 \)

The correct form for equation (5.5) is thus

\[
\Delta H_u = \frac{h\nu}{\beta} \left| \sin 2\phi \cdot \sin \theta \cdot (g_2^2 - g_1^2) \right| \Delta u
\]

and the total line width, \( \Delta H_{\text{mosaic}} \) is

\[
\Delta H_{\text{mosaic}} = \left[ (\Delta H_\theta)^2 + (\Delta H_u)^2 \right]^{\frac{1}{2}}
\]

Equations (5.4), (5.6) and (5.7) have been used to obtain our theoretical curves.

5.4.3 Evaluation of partial derivatives - g-value distribution due to ligand field variations

Here we calculate the contribution to the line widths of a distribution of g-values arising from a variation in ligand fields from molecule to molecule.

The magnitude of the splitting of the ground state spin doublet (and therefore the g-values) at any d.c. field
value is a function of the rhombic (V) and axial (D) potentials. We wish to calculate \( \frac{\partial g}{\partial V} \) and \( \frac{\partial g}{\partial D} \) to obtain \( \Delta H_V \) and \( \Delta H_D \), the line broadening produced by the random distributions of V and D about their mean value.

As before, we start from:

\[
g^2 = g_1^2 \sin^2 \theta \cos^2 \phi + g_2^2 \sin^2 \theta \sin^2 \phi + g_3^2 \cos^2 \theta
\]

and by differentiation

\[
\frac{\partial g}{\partial V} = \frac{1}{g^2} \left( g_1 g_1 \sin^2 \theta \cos^2 \phi + g_2 g_2 \sin^2 \theta \sin^2 \phi + g_3 g_3 \cos^2 \theta \right)
\]

where \( g_i' = \frac{\partial g_i}{\partial V} \) and \( i = 1, 2, 3 \).

There is a similar expression for D with

\[
g_i'' = \frac{\partial g_i}{\partial D}
\]

The derivation of the explicit formulae leading to \( g_i' \) and \( g_i'' \) are given in Appendix I. The complete expression for rhombic variation in terms of the line width, is

\[
\Delta H_V = \frac{h \nu}{\beta} \cdot \frac{1}{g} \left[ \frac{\partial g}{\partial V} \right] \Delta V 
\]

(5.8)

where \( \Delta V \) is the r.m.s. variation in rhombic potential. The expression for \( \Delta H_D \), the axial linewidth, is similar with \( \Delta D \) the variation in axial potential. We can add these expressions and obtain

\[
\Delta H_{\text{symmetry}} = \left[ \left( \Delta H_V \right)^2 + \left( \Delta H_D \right)^2 \right]^{\frac{1}{2}}
\]

(5.9)
We now combine (5.7) and (5.9) to obtain the total linewidth, $\overline{\Delta H}_{\text{total}}$, 

$$\overline{\Delta H}_{\text{total}} = \left[ (\Delta H_{\text{mosaic}})^2 + (\Delta H_{\text{sym}})^2 + (\Delta H_I)^2 \right]^{\frac{1}{2}} \quad (5.10)$$

$\Delta H_I$ is an isotropic contribution from the minor causes of section 5.3.1.

5.4.4 Transformation from laboratory system to $g$-axes coordinate system

The expressions given above for the various contributions to the line width use a coordinate system based on the 3 $g$-axes (figure 5.1). Our measurements, however, are made by a rotation of the cytochrome $c$ crystal about an axis perpendicular to the magnetic field direction (figure 5.2), in the right handed co-ordinate system $(X,Y,Z)$. In our crystal orientation study (chapter 4) we obtained the angles between the $g$-axes and the $X,Y,Z$ laboratory system.

We use these angles, and standard formulae for rotation of axes (Handbook of Mathematical Tables, 2nd edition, supplement to Handbook of Chemistry, Chemical Rubber Company, p. 574) to obtain $\theta$ and $\phi$ in the $g$-axis system for any given $\psi$ in the laboratory system.

If the direction cosines of $g_1$, $g_2$ and $g_3$ relative to $X$ and $Y$ are $(e_x, e_y)$, $(m_x, m_y)$ and $(n_x, n_y)$ respectively, we obtain

$$\theta = \arccos(n_x \cos \psi + n_y \sin \psi)$$

$$\phi = \arctan \left[ \frac{(m_x + m_y \tan \psi)}{(e_x + e_y \tan \psi)} \right]$$
FIGURE 5.2

Co-ordinate system used to describe the positions of the principal g-axes relative to the laboratory magnetic field direction (same as figure 4.3).
We make use of these relations in our computer programs to calculate the theoretical line width variation on rotation.

5.5 RESULTS

5.5.1 Introduction

We give the data required to compute the theoretical linewidth variation in section 5.5.2. In sections 5.5.3, 5.5.4 are presented the results of the line widths measured in the same two orientations used in chapter 4 (viz., c-axis vertical and b-axis vertical).

5.5.2 Data for linewidth calculations

In order to calculate the various contributions made by the mechanisms mentioned above we require the following information about the system:

(i) the g-values along the magnetic axes—$g_1$, $g_2$, $g_3$—to substitute into the formulae. These can be obtained from EPR of the mother liquor from which the crystals were grown.

(ii) an estimate of the 'background' isotropic contributions from the causes mentioned in section 5.3.1.

(iii) the linewidth at the low field extremum of the solution spectrum in order to calculate the magnitudes of the variations in rhombic and axial potentials $(\Delta V$ and $\Delta D$).

We use an extreme g-value because, when the d.c. magnetic field is along a g-axis the misorientation contribution vanishes to first order. We choose the $g_3$-axis
because there the linewidth is narrowest, and the signal is greatest.

(iv) an estimate of the magnitudes of the variations in orientation \((\Delta \theta, \Delta \nu)\).

We give our values of these data below:

(i) Only two g-values could be obtained from the mother liquor; \(g_2 = 2.25\) and \(g_3 = 3.06\); we took \(g_1\) to be 1.25.

(ii) We assume that the \(\Delta H_I\), the isotropic contribution is about 50 gauss, from our considerations in section 5.3.1. Most of this contribution is unresolved hyperfine structure. This will not contribute very much to the overall line width for at no angle of the magnetic field relative to the g-axes does \(\Delta H_I\) become the most important contributor to \((\Delta H)_{\text{total}}\).

(iii) The linewidth at \(g_3 = 3.06\) was found to be 400 \(\pm\) 20 gauss.

For the d.c. magnetic field along the \(g_3\) direction, the angle \(\theta\) is zero and \(\phi\) is 90°. This makes \((\Delta H)_{\text{mosaic}}\) equal to zero and therefore:

\[
\overline{\Delta H_{\text{total}}} = \left[ \left( \overline{\Delta H_V} \right)^2 + \left( \overline{\Delta H_D} \right)^2 + \left( \overline{\Delta H_I} \right)^2 \right]^{1/2}
\]

at \(\theta = 0°, \phi = 90°\)

\[
\overline{\Delta H_V} = \frac{h\nu}{\beta} \frac{1}{g_3^3} \left[ g_3 \cdot g_3^* \right] \cdot \overline{\Delta V}
\]

and

\[
\overline{\Delta H_D} = \frac{h\nu}{\beta} \frac{1}{g_3^3} \left[ g_3 \cdot g_3^* \right] \cdot \overline{\Delta D}
\]
TABLE XII

Data for theoretical calculation of linewidths

<table>
<thead>
<tr>
<th>g-values</th>
<th>( g_1 = 1.25 )</th>
<th>( g_2 = 2.25 )</th>
<th>( g_3 = 3.06 )</th>
</tr>
</thead>
</table>

Partial derivatives

<table>
<thead>
<tr>
<th>Rhombic</th>
<th>( g_1' = -0.522 \lambda^{-1} )</th>
<th>( g_2' = 0.487 \lambda^{-1} )</th>
<th>( g_3' = 0.485 \lambda^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axial</td>
<td>( g_1'' = 0.138 \lambda^{-1} )</td>
<td>( g_2'' = 0.154 \lambda^{-1} )</td>
<td>( g_3'' = 0.059 \lambda^{-1} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Where \( \lambda \) is the spin-orbit coupling constant (see Appendix I)

R.m.s. deviations

<table>
<thead>
<tr>
<th>Mosaic</th>
<th>( \Delta \theta = \Delta u = 0.027 ) radians</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symmetry</td>
<td>( \Delta V = \Delta D = 0.22 \lambda )</td>
</tr>
</tbody>
</table>
If we now assume that $\Delta V$ is approximately equal to $\Delta D$, then the magnitudes of $\Delta H_V$ and $\Delta H_D$ depend upon the size of $g''_3$ and $g''_3$. For our system $g''_3$ is approximately ten times greater than $g''_3$; therefore along this $g$-axis the axial linewidth contribution can be neglected relative to the rhombic, so:

$$\Delta H_{\text{total}} = \left[ (\Delta H'_V)^2 + (\Delta H'_H)^2 \right]^{\frac{1}{2}} \quad (5.11)$$

For a $\Delta H$ total of 400 gauss and $\Delta H'_H$ of 50 gauss we obtain $\Delta H'_V$ equal to 397 gauss. This gives $\Delta V = 0.22\lambda$. This is similar to that for myoglobin azide ($\Delta V = 0.11\lambda$), (Eisenberger and Pershan, 1967), where $\lambda$ is the spin-orbit coupling constant.

(iv) An estimate of the molecular misorientation can be obtained from the X-ray crystallographic data. The diffraction patterns of proteins fade out at spots corresponding to lattice spacings of order 1 to 2 Å.

If, for a spherical protein molecule of diameter 50 Å, there exists an uncertainty of its orientation relative to its neighbours of $\pm 1.5$ Å, at the periphery this is equivalent to an angular uncertainty in position of about $\pm 1.5^\circ$ (or $\pm 0.027$ radians). Therefore we take $\Delta u$ equal to $\Delta \theta$ with a value of $0.027$ radians.

These data we summarised in table XII.
5.5.3 Linewidth variations in a crystal with b-axis vertical

This orientation corresponds to orientation II of chapter 4. We give the results of the variation in linewidth on rotation of the crystal for lines 1 and 2. These lines were chosen because they are well separated due to the range of g-values ($g^2$ varies from 5.40 to 1.85) making line width measurements easier.

Figure 5.3 presents the data points, together with the theoretical curves computed from the parameters given in table XII. In figure 5.4 is shown the individual contributions to the total linewidth from the axial, rhombic, mosaic and isotropic effects for line 2.

The agreement is fair, considering the uncertainties in line width measurement, estimated to be $\pm$ 50 gauss for widths less than 1000 gauss, $\pm$ 200 gauss for the higher values. As mentioned before, the errors are caused by the presence of other (overlapping) lines, low signal-to-noise ratio at high fields and the presence of the quartz impurity at $g = 2$.

From figure 5.4, it can be seen that the angular variations of the three important contributions all have roughly the same form, i.e., the maximum and minimum linewidth values of all three occur at $\psi \approx 20^\circ$ and $\psi \approx 110^\circ$ respectively. Hence this orientation is not very useful for
FIGURE 5.3

Variation in width of line 1 and 2 of Orientation II as crystal is rotated relative to d.c. magnetic field. The theoretical curve is obtained from equation (5.10).
Rotation Angle of Crystal

Linewidth - Gauss

$g_{\min}^{(2)}$, $g_{\max}^{(1)}$, $g_{\max}^{(2)}$, $g_{\min}^{(1)}$

$\psi$ - Rotation Angle of Crystal
FIGURE 5.4

Theoretical variation in width of line 2 of Orientation II showing individual contributions.
deciding upon the relative values of $\Delta \theta, \Delta u, \Delta v, \Delta D$. The main reason for this is $\theta$ does not change by more than 40° in a 180° rotation of the crystal.

A better orientation would be one in which $\theta$ and $\phi$ both vary widely. A crystal oriented with its c-axis approximately vertical will give 100° variation in $\theta$ and $\sim 180°$ variation in $\phi$ on a 180° rotation of the crystal.

5.5.4 Line width variation for crystal with c-axis vertical

This corresponds to orientation I of chapter 4. The data for the linewidth variation of one line is shown in figure 5.5, with the calculated curve. The individual contributions are presented in figure 5.6.

Once again the agreement between calculated and experimental results is reasonable, in that there is a large region of roughly constant linewidth over a 90° rotation (30-120°). An impurity resonance around $g = 2$ gave a wide line which made measurement of the linewidths near $g_{\text{min}}$ impossible, and led to the large uncertainties shown by the error bars.

5.6 DISCUSSION

5.6.1 Value of parameters

The values of $\Delta \theta, \Delta u, \Delta v,$ and $\Delta D$ were the same for both orientations.
FIGURE 5.5

Variation in width of line 1 of Orientation I as crystal is rotated relative to d.c. magnetic field. The theoretical curve is obtained from equation (5.10).
Orientation I

Line I

Data: ● 0 - 180°
△ 180 - 360°

Theory

ψ - Angle of Rotation in Degrees (Arbitrary zero)
FIGURE 5.6

Theoretical variation in width of line 1 of Orientation I showing individual contributions.
As $\Delta V$ and $\Delta D$ are fixed by the g-values in solution, the only adjustable parameters are $\Delta \theta$ and $\Delta u$. We tried to get better fits to the data by varying these systematically, but no improvement was obtained over the values given. This merely reflects that uncertainties in the linewidths measured are too large to permit more precise values of these parameters to be obtained.

The only other work done on the linewidth variations as a function of orientation is that of Eisenberger and Pershan (1967) and Helcke et al. (1968) on acid-met-Hb and met-Mb respectively.

As indicated earlier, the theory we used is essentially that of Eisenberger and Pershan (1967), but we have extended it to include the contributions from a distribution of axial potential.

These authors calculated the linewidth variation due to misorientation ($\Delta \theta$) and to variations in rhombic potential ($\Delta V$). They had problems in obtaining a wide enough range of data to test their theory, because of lines overlapping, or being too broad to be visible, or being split. This resulted in their being able to fit the theory for rotation of the crystal in the $g_2$-$g_3$ plane (the yz plane in their notation)—corresponding to varying $\theta$ from 0 to 90° with $\phi = 90°$.

Since $\phi$ was constant they were unable to test the contribution from $\Delta u (= \sin \theta \cdot \Delta \phi)$ although this can contribute
as much to the line width as $\Delta \theta$.

The wider range of g-values in our system (3.06, 2.25, 1.25) as compared with theirs (1.72, 2.22, 2.80) avoided some of the problems of line overlap by separating lines better.

The other published work, that of Helcké et al. (1968), does not explain the observed line widths nearly as well as Eisenberger and Pershan (1967). Helcké neglected any contribution from rhombic or axial variations in potential, using a purely misorientational model. They used as parameters $\Delta \theta$ and $\Delta \phi$, finding that values of .027 radians (1.5°) and .072 radians (4°) were required to fit their data. Our results (figures 5.4 and 5.6), and those of Eisenberger and Pershan, show the contributions from axial and rhombic variations in potential are just as great as the misorientational one. Helcké et al. added the individual contributions linearly ($\Delta H_{\text{tot}} = \Delta H_\theta + \Delta H_\phi$), a procedure we consider incorrect (section 5.4.2). Also, $\Delta u = \sin \theta \cdot \Delta \phi$ is the correct variable to describe the physical situation, especially as no special conditions, apart from randomness, need be imposed in our calculation.

We mention here that the model of the system obtained in chapter 4 is checked by a linewidth study such as this. If one assigns the g-axis projections to an incorrect quadrant, then the calculated g-values and linewidths for a given value of the magnetic field direction do not
agree with the experimentally observed linewidth and g-value at that field orientation. This was extremely useful in the early stages when we were testing various projections.

5.6.2 Physical reasons for the presence of distortions and misorientation

We have already alluded to the softness and fragility of protein crystals as a possible reason for the orientation of the molecules relative to each other to vary. This possibility is supported by the fact that X-ray diffraction pictures fade out at 1 to 2 Å corresponding to an uncertainty in position of 1 to 2°. This is much greater than in inorganic crystals where it is about 0.2° (Stout and Jensen, 1969).

The structure of proteins as deduced from X-ray diffraction analysis shows the presence of substantial amounts of water of crystallisation (up to 50% by volume in cytochrome c). This means that protein crystals are not so much "crystals" as "ordered polyelectrolyte gels" (Dickerson, 1967). It is therefore reasonable that the molecular packing in the crystal would be loose, leading to the misorientation required by our results.

The ligand field distributions ($\Delta V, \Delta D$) present in frozen crystals and solutions of proteins could be another manifestation of the non-rigid structure of the protein molecule—due to the dynamic Jahn-Teller effect.
As discussed in chapter 4, this mechanism shows that the low frequency (infra-red-breathing) vibrations of the heme ring can couple with the iron electronic d-orbitals, lowering the ligand field symmetry.

We suggest that there is a variation in vibration frequencies from molecule to molecule, having a range of values about some mean frequency. Such a distribution could arise from slight changes in the covalent bonding of the heme group into the amino acid chain, caused by changes in the atomic positions from molecule to molecule. The vibrating heme group would be 'weighed down' to a greater or lesser extent, thereby giving a spread of frequencies.

We can also ask: what changes can occur in the environment of the heme when the molecule is frozen? Two effects might be expected—distortions due to the presence of ice crystals and changes in the volume of the ice/protein matrix as a function of temperature.

Yonetani and Schleyer (1967) carried out an investigation on the effects of physical state—drying, repeated freezing and thawing—on the EPR signals from ferrimyoglobin and cytochrome c peroxidase crystals. They showed that signals were highly susceptible to such changes in state. The orientations became random or the EPR signals disappeared. They suggest that irreversible changes may have occurred even at the initial freezing step. This is supported by the work of Low et al. (1966) who showed that the mosaic character of
insulin crystals was irreversibly enhanced on cooling to 
-150°C. It is difficult, however, to put a numerical esti-
mate on such changes to test whether they explain our data.

Some idea of the effect of volume changes on the 
frozen solid can be obtained from studies of proteins at 
high pressures. Grenoble et al. (1968) reported changes in 
the Mossbauer spectra of hemin when up to 100 kilobars 
pressure was applied. They attributed these changes to dis-
tortions of the orbitals—the most likely of which was a 
spreading of the 3d orbitals. This would alter the shielding 
of the inner orbitals, thereby changing the interaction of 
the s-orbitals with the iron-57 nucleus. Any d-orbital 
variations could be expected to affect the EPR signals, but 
it is difficult to estimate how large this effect might be.

5.7 CONCLUSIONS

We have shown in this chapter how the EPR line-
widths of single crystals of ferricytochrome can be 
reasonably explained by a combination of crystallite mis-
orientation and molecular distortion.

The misorientation theory proposed by Helcké et 
al. was shown to be inadequate, due to a conceptual error, 
and was corrected. In addition, we extended the theory of 
Eisenberger and Pershan--derived for rhombic potential 
variations--to axial potentials.
The best theoretical fit to the data resulted from a mean angular distribution of 1.5° and a rhombic potential variation of about 11% (axial and isotropic contributions being small in comparison). Some physical causes for these effects were given.
CHAPTER 6.0

CYTOCHROME C EPR - LINESHAPES AND RAPID PASSAGE

6.1 INTRODUCTION

The aim of this chapter is to explain the shapes of the EPR lines seen in frozen single crystals of ferricytochrome c, using the theory developed by Portis (1955).

In section 6.2 we present the behavior of the signals obtained from a ferricytochrome c crystal, discuss how it differs from 'normal' slow passage EPR, and suggest that rapid passage may be the cause. Following this we describe the basic theory of rapid passage in a single spin packet (section 6.3) and generalise this for a distribution of packets (section 6.4) by using the theory of Portis (1955).

Section 6.5 describes a test of the theory using a model system. In section 6.6 the cytochrome c results are presented and explained. Also given in this section are data on the relaxation time and spin packet linewidth of cytochrome c at 4.2°K, determined from the fast passage results.
6.2 THE NATURE OF THE PROBLEM

6.2.1 Results to be explained

A study of one of the EPR lines from a single crystal of horse heart ferricytochrome c at 4.2°C gave the following set of results.

(i) no detectable absorption derivative signal.
(ii) a large signal when the detector was tuned to observe dispersion, with the shape of an undifferentiated Gaussian absorption line (figure 6.1).
(iii) as the microwave power incident on the crystal was increased, the signal of (ii) first increased in amplitude, then levelled off (figure 6.2).
(iv) the signal of (ii) lagged the modulation field in phase by 157°, as compared with a standard (DPPH) when the modulation frequency was 100 kHz.
(v) the signal of (ii) had a maximum amplitude at magnetic field modulation of one gauss, although the linewidth is 400 gauss (figure 6.3).

6.2.2 Discussion

The absence of the expected slow passage EPR absorption signal derivative (of the form shown in the inset to figure 6.1) suggests that we are satisfying the saturation condition:

\[ \gamma H_1 \tau \gg 1 \]  

(6.1)
FIGURE 6.1

EPR dispersion line from a single crystal of horse heart cytochrome c at 4.2°K.

Inset: EPR absorption derivative spectrum of charred dextrose standard.
FIGURE 6.2

Microwave power dependence of cytochrome c single crystal dispersion signal height.
FIGURE 6.3

Magnetic field modulation dependence of cytochrome c single crystal dispersion signal height.
where $\gamma$ is the gyromagnetic ratio of the electron
$H_1$ is the amplitude of the microwave r-f field in gauss
$T$ is the mean relaxation time $= \sqrt{T_1 \cdot T_2}$
$T_1$ is the spin-lattice relaxation time
$T_2$ is the spin-spin relaxation time.

If this were the case then a dispersion signal should be detectable, since dispersion does not show the same saturation behaviour as absorption. Dispersion tends to a limit at high microwave powers (Aragam, 1961) and this is seen here (figure 6.2).

From our work on line shapes in chapter 3 we know this dispersion is Gaussian, but we would expect it to have the derivative shape shown:

where $D/A = 3.5$, Poole (1967, p. 531). The negative side (A) of the dispersion signal would be easily visible in figure 6.1 if it were present.

In addition, absorption and dispersion signals are usually in phase with the magnetic field modulation, not lagging as observed.

Finally, the magnetic field modulation amplitude required to produce the maximum signal height is very much less than expected. Normally maximum signal height is not
attained in an EPR line until the modulation amplitude is of the same order as the line width (Poole, 1967, p. 406)—here a line 400 gauss wide has its maximum height at one gauss modulation.

We therefore, have a dispersion signal of unusual shape (arising from a Gaussian distribution of lines) whose height is very much greater than common EPR operating conditions would predict.

6.2.3 Rapid adiabatic passage

Bloch (1946) examined the equation of motion of a spin system with magnetisation, \( \overline{M} \), placed in a magnetic field, \( \overline{H} \) whose value can be swept. The equation is

\[
\frac{d \overline{M}}{dt} = \gamma \overline{M} \times \overline{H}
\]  

(6.2)

He obtained two solutions for (6.2); one in which the field is varied sufficiently slowly that all times steady state conditions occur, the other when the field is altered so quickly that relaxation processes do not have time to act. The first solution gives the more usual 'slow passage' EPR while the second gives rise to 'rapid adiabatic passage' signals.

It is these latter which appear in cytochrome \( c \), by virtue of the long relaxation time of the paramagnetic centre of the molecule.
In the next section (6.3) we describe rapid passage in a single spin system, and extend the treatment to a distribution of spins in section 6.4.

6.3 RAPID ADIABATIC PASSAGE IN A SINGLE SPIN PACKET

(Bloch, 1946)

Rapid adiabatic passage required a special solution of the equation of motion of a spin system:

\[
\frac{d\vec{M}}{dt} = \gamma \cdot \vec{M} \times \vec{H} \quad (6.2)
\]

We assume the spins are subject to a steady magnetic field, \( \vec{H} \), along the z-axis of a right handed coordinate system, together with an rf field \( \vec{H}_1 \), precessing with angular velocity \( \vec{\omega} \) in the x-y plane. The special solution describes the forced precession of the magnetisation with the same velocity as that of the applied rf field. The components of \( \vec{M} \) are:

\[
\begin{align*}
M_y &= M_0 \cdot \sin \theta \cdot \sin \omega t \quad (6.3a) \\
M_x &= M_0 \cdot \sin \theta \cdot \cos \omega t \quad (6.3b) \\
M_z &= M_0 \cdot \cos \theta \quad (6.3c)
\end{align*}
\]

These satisfy the equation of motion (6.2) provided

\[
\tan \theta = \frac{\vec{H}_1}{(\vec{H} - \vec{H}')} \quad (6.4)
\]

where

\[
\vec{H}' = -\vec{\omega} / \gamma
\]
If we view the solution from a coordinate system rotating with angular velocity about $\vec{H}$, we see that for $\vec{H} - \vec{H}^* \gg \vec{H}_1$, i.e. above resonance, $\theta \sim 0^\circ$

$\vec{H} - \vec{H}^* \sim \vec{H}_1$ at resonance, $\theta \sim \pi/2$

$\vec{H} - \vec{H}^* \ll \vec{H}_1$ below resonance $\theta \sim \pi$

Therefore, above resonance the magnetisation is almost parallel to $\vec{H}$. If $\vec{H}$ is decreased slowly, the angle between $\vec{M}$ and $\vec{H}$ increases, attaining $\pi/2$ at resonance and eventually reaching a limiting value of $\pi$ far below resonance, where $\vec{M}$ is antiparallel to $\vec{H}$. This solution is strictly valid only when $\vec{H}$ is constant, but also holds if $\vec{H}$ is varied 'slowly'. A change in $\vec{H}$ is called 'slow' if the magnetisation, $\vec{M}$, is close at all times to $\vec{H}_{\text{eff}}$ (the vector sum of $\vec{H}_1$ and $\vec{H} - \vec{H}^*$). This leads to the adiabatic condition:

$$\frac{dH}{dt} \ll \gamma H_1^2$$  \hspace{1cm} (6.5)

where $\frac{dH}{dt}$ is the maximum rate of change of $H$ in gauss per second.

From the description given above of the behavior of $\vec{M}$ it is implied that the sweep through resonance must occur in a sufficiently short time that relaxation processes cannot restore $\vec{M}$ parallel to $\vec{H}$ (the equilibrium position of $\vec{M}$). This requires that:

$$\frac{dH}{dt} > \frac{H_1}{\tau}$$  \hspace{1cm} (6.6)
In addition the slowest precession time must be faster than the relaxation time so that we also require:

$$\gamma H_1 > 1/\tau$$

(6.7)

Combining these 'rapid' conditions, (6.6) and (6.7), with (6.5) gives the requirements for rapid adiabatic passage

$$\gamma H_1^2 > \frac{dH}{dt} > \frac{H_1}{\tau}$$

(6.8)

The saturation condition for slow passage—$$\gamma H_1 \tau > 1$$—is seen from (6.8) as a necessary, but not sufficient, condition for rapid adiabatic passage to occur.

Having introduced the conditions under which rapid adiabatic passage can arise in a single spin packet, we now show how the solutions of the equations of motion must be modified when there is a distribution of spin packets.

6.4 RAPID ADIABATIC PASSAGE IN A DISTRIBUTION OF SPIN PACKETS

6.4.1 The treatment follows that of Portis (1955), and Weger (1960). The papers by Hyde (1960) on fast passage in irradiated LiF, and by Bugai (1963) on rapid passage at high modulation frequencies, should be mentioned also. They provide good descriptions, in non-mathematical terms, of the behavior of distributions of spin systems.
6.4.2 Portis (1955) begins with the solutions of

\[
\frac{d\vec{M}}{dt} = \gamma (\vec{M} \times \vec{H}) + \frac{(\vec{M}_0 - \vec{M})}{\tau}
\]

where \( M_0 = X_0 H \) is the equilibrium value of the spin magnetisation. The solutions are, in terms of the rf susceptibilities

\[
\chi'(\omega) = \frac{1}{2} \chi_0 \omega \tau \frac{(\omega_0 - \omega) \tau}{1 + (\gamma H_1 \tau)^2 + (\omega_0 - \omega)^2 \tau^2} \quad (6.10a)
\]

\[
\chi''(\omega) = \frac{1}{2} \chi_0 \omega \tau \frac{1}{1 + (\gamma H_1 \tau)^2 + (\omega_0 - \omega)^2 \tau^2} \quad (6.10b)
\]

which, provided \( \gamma H_1 \tau >> | \), give:

\[
\chi'(\omega) = \frac{1}{2} \chi_0 \omega \frac{1}{\gamma H_1} \left[ 1 + \left( \frac{\omega_0 - \omega}{\gamma H_1} \right)^2 \right]^{-\frac{1}{2}} \quad (6.11a)
\]

\[
\chi''(\omega) = 0 \quad (6.11b)
\]

We note that the dispersion is at a maximum at the line centre when \( \omega = \omega_0 \). Portis then carried out the integration of the equations (6.10a) and (6.10b) over a distribution of resonant frequencies, \( h(\omega - \omega_0) \), for an expression of the form

\[
\chi'(\omega) = \frac{1}{2} \chi_0 \omega \tau \int_0^\infty \frac{(\omega - \omega') \tau \cdot h(\omega - \omega_0)}{1 + (\gamma H_1 \tau)^2 + (\omega - \omega')^2 \tau^2} d(\omega \tau)
\]
To correspond to the usual conditions of resonance, we take \( \omega \) constant, and apply a swept d.c. field:

\[
H'(t) = \frac{dH}{dt} + H_m \cos(\omega_m t)
\]

where \( \cos(\omega_m t) \) is the magnetic field modulation of frequency \( \omega_m \).

\( H_m \) is the magnetic field modulation amplitude

\( \frac{dH}{dt} \) is the constant rate of sweeping the mean magnetic field \( H_0 \).

As we are working in terms of the frequency, \( H'(t) \), is converted to \( \omega'(t) \) by

\[
\omega'(t) = \gamma H'(t) = \gamma \frac{dH}{dt} + \gamma H_m \cos(\omega_m t)
\]

Portis developed the integrals by repeated integration by parts, and the results were a series of terms consisting of only those expressions periodic in \( \omega_m \). We give here the first two terms of this series:

\[
\chi_0'(\omega) = \chi_0 \gamma H_m \cos \omega_m t \cdot \frac{d}{d\omega_0} \left\{ \int_0^\infty \frac{\omega' d\omega}{h(\omega'-\omega_0)(\omega'^2-\omega_0^2)} \right\} (6.12)
\]

\[
\chi_1'(\omega) = \frac{\pi}{4} \chi_0 \omega \epsilon \cos \phi_1 \cdot \sin(\omega_m t - \phi_1) \cdot h(\omega-\omega_0)
\]

\[
- \frac{\pi}{4} \chi_0 \omega \mu \cos(\omega_m t) \cdot \gamma H_m \cdot \frac{d}{d\omega_0} [h(\omega-\omega_0)] (6.13)
\]

where

\[
\epsilon = \frac{(\omega_m H_m \tau)}{H_1}
\]

\[
\mu = \tau \left( \frac{dH}{dt} \right) / H_1
\]

\[
\phi_1 = \tan^{-1}(\omega_m \tau)
\]
Convergence requires that $\mu$ and $\cos \phi_1$ be less than unity. For a slow sweep rate of the d.c. field $\mu < 1$ is easy to fulfil. For $\cos \phi_1$ less than unity $H_m$ must be less than $H_1$ whenever $\omega_m \tau \gg 1$; this restriction on $H_m$ is not necessary when $\omega_m \tau \lesssim 1$, and $H_m$ can then exceed $H_1$.

6.4.3 Three special cases can now be considered.

(i) When the relaxation time, $\tau$, is very short but $\gamma H_1 \tau$ is still greater than unity then

$$\omega_m \tau < \frac{H_1}{H_m} < 1$$

and expression (6.12) is the dominant contribution to the susceptibility, and so

$$\chi'(\omega) = \chi_0 \gamma H_0 \cos(\omega_m \tau) \frac{\partial}{\partial \omega} \left\{ \int_0^\infty \omega' h(\omega') \frac{\omega' d\omega}{(\omega'^2 - \omega^2)} \right\}$$

(6.14)

which has the shape of a derivative of dispersion and is in phase with the magnetic field modulation.

(ii) As the mean relaxation time in the system increases, then we obtain

$$\frac{H_1}{H_m} < \omega_m \tau < 1$$

and now the first term in (6.13) is the dominant one so that

$$\chi'(\omega_0) = \frac{\pi}{4} \chi_0 \frac{H_m}{H_1} \omega_m \tau \cdot \sin \omega_m \tau \cdot h(\omega - \omega_0)$$

(6.15)
Here $\phi_1$ is small, so that $\cos \phi_1 \sim 1$. The signal shape is that of the absorption envelope, $h'(\omega - \omega_0)$ and lags the modulation field by $90^\circ$.

(iii) For $H_l/H_m \leq 1 < \omega_m \tau$ resulting from an even longer relaxation time than in (ii), the first term of $\chi_1'(\omega)$ is still dominant and

$$\chi'(\omega) = \frac{\pi}{4} \chi_0 \omega \frac{H_m}{H_l} \omega_m \tau \cos \phi_1 \sin (\omega_m t - \phi_1) h(\omega - \omega_0)$$

Here $\omega_m \tau > 1$ and $\cos \phi_1 \to 0$ (since $\phi_1 \to 90^\circ$), and the product $\omega_m \tau \cos \phi_1 \to 1$ so that:

$$\chi'(\omega) \sim -\frac{\pi}{4} \chi_0 \omega \frac{H_m}{H_l} \cos (\omega_m t) h(\omega - \omega_0) \quad (6.16)$$

This is the envelope of the distribution of lines but $180^\circ$ out of phase with the modulation field. At intermediate values of $\omega_m \tau$, the phase of the signal will vary between these two limits ($90^\circ - 180^\circ$) with little change in magnitude, since $\omega_m \tau \cos \phi_1$ remains approximately constant as $\omega_m$ or $\tau$ vary.

6.4.4 At high modulation fields where $H_m > H_l$, the series expansion method used above breaks down. Portis solved the equations of motion making certain approximations about the arguments of integrals used, and obtained the results that, for $H_m > H_l$: 
\[ \chi' H_1 \alpha = -H_1 \ln \left[ \frac{2H_m}{H_1} \right] \cos \omega_m t \cdot h(\omega - \omega_0) \quad \omega_m \tau \gg 1 \] (6.17)

\[ \chi' H_1 \alpha = -H_1 \ln \left[ \frac{2H_m \omega_m \tau}{H_1} \right] \sin \omega_m t \cdot h(\omega - \omega_0) \quad \omega_m \tau \ll 1 \] (6.18)

giving a signal which increases only slowly as \( H_m \) increases.

6.4.5 We can summarize the results of the calculations so far, as follows:

(i) for a mean relaxation time, \( \tau \), shorter than all other times except \( 1/(\gamma H_1) \) we obtain the dispersion derivative, identical with that in slow passage EPR. The signal amplitude we detect is proportional to \( \chi' H_1 \) and therefore to \( H_1^2 \).

(ii) as \( \tau \) increases we get a change over to rapid passage when the dispersion signal has the appearance of an absorption curve with an amplitude proportional to \( H_m^2 \), and whose phase lags behind the modulation frequency. For large \( H_m \), the signal height becomes logarithmic with \( H_m \). Also in this case \( \chi' H_1 \) is independent of \( H_1 \).

6.5 TEST OF PORTIS EQUATIONS WITH MODEL SYSTEM

6.5.1 Introduction

In order to test these results (expression (6.14)-(6.18)) we studied rapid passage in a model system, using
charred dextrose (Pastor and Hoskins, 1960). This is ideal for our purposes as a wide range of relaxation times can be obtained by suitable choice of charring temperature.

6.5.2 Materials

Heating the dextrose to 200°C breaks down the carbon bonds, giving free radicals. For chars formed below 300°C the density of the radicals is low ($5 \times 10^{17} / \text{cm}^3$) and they have a number of different local environments. Under these conditions EPR shows that the resulting line has Gaussian shape—implying inhomogeneous broadening—and the system has a long relaxation time. At higher temperatures, up to 565°C, the spin density increases, the line becomes narrower and changes shape to Lorentzian, and the relaxation time shortens. Above 565°C the dextrose becomes largely graphite and the spin density rapidly falls.

We used dextrose heated to approximately 250°C for this study.

6.5.3 Methods and Results

The rapid passage properties we particularly wished to examine were:

(i) The effect of varying $H_1$.

With $\omega_m$ set to $2 \pi \times 100$ kHz and $H_m = 1$ gauss the incident microwave power was varied from one microwatt to ten milliwatts.
At the very lowest power, where $H_\perp$ is approximately 0.001 gauss, the condition $\gamma H_\perp T < 1$ was attained and slow passage absorption and dispersion derivatives were seen (figure 6.4a shows absorption).

As the power was increased the signal changed from a derivative to an absorption shape approximately 170° out of phase with the magnetic field modulation (figure 6.4b) whose amplitude increased then levelled off (figure 6.5). This is in agreement with the behaviour predicted from equation (6.16)—the signal height becomes independent of $H_\perp$ when all the conditions for rapid adiabatic passage are fulfilled.

(ii) The effect of varying the magnetic field modulation amplitude, $H_m$.

At a modulation frequency, $\omega_m$, of $2\pi \times 100$ kHz, and a power level of 10 mW, the modulation amplitude was varied from 0 to 5 gauss. The resulting variation in signal height is shown in figure 6.6, and shows the behaviour predicted by equations (6.16) and (6.17) viz., a linear increase of signal with increased $H_m$, levelling off at high $H_m$. It should be noted that the linear portion of the curve extends up to $H_m = 0.5$ gauss. This is about five times greater than $H_\perp$ and shows that equation (6.16) holds for $H_m > H_\perp$ when $\omega_m T$ is greater than 1. This is a consequence of the convergence conditions mentioned in the discussion following equation (6.13) above. It is particularly useful that this occurs,
FIGURE 6.4

EPR spectra from charred dextrose test sample.

(a) slow passage absorption derivative

(b) rapid passage dispersion
Slow Passage Absorption

\[ P_{\text{cav}} = 1 \mu W \]

Fast Passage Dispersion

\[ P_{\text{cav}} = 10 \text{ mW} \]

Modulation Frequency
\[ = 100 \text{ kHz} \]

\[ H_m = 1 \text{ gauss} \]
FIGURE 6.5

Microwave power dependence of charred dextrose dispersion signal height.
FIGURE 6.6

Magnetic field modulation amplitude dependence of charred dextrose dispersion signal height.
Signal Height - $X' H_I$ - Arbitrary Units

Modulation Amplitude - $H_m$ - Gauss

$f_m = 100 \text{ kHz}$
since signals can be measured at large \( H_m \), with corresponding increase in signal amplitude, making accurate phase measurements easier. (The signal drops at high modulation amplitudes because the conditions are then such as to produce non-adiabatic fast passage).

(iii) The effect of varying \( \omega_m \), the magnetic field modulation frequency.

At a microwave power level of 10 mW and modulation amplitude of 0.5 gauss, the signal lag was measured over the range of frequencies, 5 kHz to 100 kHz.

The phases were obtained by a nulling method—making the signal zero by tuning the P.A.R. HR-8 phase sensitive detector 90° out of phase with the signal.

The 'zero' of phase was obtained from the signal at the lowest powers where fast passage effects do not occur. The phase lags obtained, and the corresponding \( \omega_m \tau \) are shown below and in figure 6.7.

<table>
<thead>
<tr>
<th>( \omega_m/2\pi )</th>
<th>phase lag relative to zero</th>
<th>( \omega_m \tau ) (from definition of ( \phi_1 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 10^5 )</td>
<td>170.5 ± 5°</td>
<td>6.0</td>
</tr>
<tr>
<td>( 5 \times 10^4 )</td>
<td>158.2 ± 5°</td>
<td>4.8</td>
</tr>
<tr>
<td>( 3 \times 10^4 )</td>
<td>153.5 ± 5°</td>
<td>3.4</td>
</tr>
<tr>
<td>( 10^4 )</td>
<td>133.0 ± 5°</td>
<td>0.9</td>
</tr>
<tr>
<td>( 5 \times 10^3 )</td>
<td>130.0 ± 5°</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Magnetic field modulation frequency dependence of phase lag of charred dextrose dispersion signal at room temperature.

The measured lag, $\phi_1$, is related to the ordinate $-\omega_m\tau$ by $\phi_1 = \tan^{-1}(\omega_m\tau)$

See text, section 6.5.3.
6.5.4 Discussion

Feher (1959) and Bugai (1963) both show that the onset of the plateau in signal height as a function of $H_m$ signifies that the modulation amplitude is greater than the individual linewidth of the spin packets that make up the inhomogeneously broadened line. (These authors use a semi-classical treatment for the case of $\omega_m T >> 1$ and $H_m > H_1$). Their derivation requires that $H_m$ sweep through a large number of spin packets).

Consequently we can see from figure 6.6 that the spin packet width is approximately one gauss, equivalent to a spin-spin relaxation time, $T_2$ of $5 \times 10^{-8}$ sec. in agreement with the calculations of $T_2$ ($\sim 10^{-8}$ sec) made by Pastor and Hoskins (1960).

The P.A.R. HR-8 phase sensitive detector used in these experiments has a possible error in phase setting of $5^\circ$ on the 50 kHz - 100 kHz range and this accounts for the large error bars at the highest frequencies. Any error is exaggerated also because of the rapid increase in the tangent function close to $90^\circ$. At lower frequencies $5^\circ$ of error has much less effect. We used the value of $\omega_m T$ obtained at 10 kHz, to reduce the effect of this possible error. Hence,

$$\omega_m T = 0.9$$

so

$$\tau = 0.9 / (2 \times 10^4) = 1.5 \times 10^{-5} \text{ sec.}$$

and

$$\gamma H_1 \tau = (1.7 \times 10^7) \times (10^{-1}) \times 9.5 \times 10^{-5}$$

$$= 23.5 \text{ at } 10 \text{ mW.}$$
At 1 microwatt, $H_1 = 10^{-3}$ gauss, and $\gamma H_1 \tau \sim 0.24$ implying that we should be out of the fast passage conditions, as observed.

During the investigation at low $\omega_m$, it was noticed that a true null could not be obtained—figure 6.8a shows the trace. This is an absorption derivative signal in phase with the magnetic field modulation, although the spectrometer is tuned to dispersion. It arises from the second term in equation (6.13), which is a function of the sweep rate of the d.c. magnetic field, $dH/dt$. Its phase is changed by 180° on reversal of the magnetic field sweep direction as required by the Portis theory (second term of equation 6.13). This term becomes appreciable at the lower frequencies because the rate of sweeping field by a.c. modulation—$\omega_m H_m$—becomes closer to the rate of the d.c. field sweep—$dH/dt$.

The presence of this signal is further proof of the validity of the Portis equations.

6.6 RESULTS FOR CYTOCHROME C

6.6.1 Introduction

We are now able to discuss the cytochrome C results presented in section 6.2.

To recapitulate: we obtained no detectable absorption derivative, but instead an absorption line of Gaussian shape, lagging the magnetic field modulation. This signal also levelled off at low modulation amplitudes and high microwave powers.
FIGURE 6.8

EPR dispersion spectra of charred dextrose for a special case of the Portis theory.

(a) The larger passage signal is phased out—leaving the smaller 'absorption derivative' signal arising from second term of equation (6.13).

(b) Both passage signals.
(a) 

\[ f_m = 10 \text{ kHz} \]

\[ \text{P.A.R. Phase} = 180 + 80^\circ \]

(b) 

\[ \text{P.A.R. Phase} = 270 + 80^\circ \]
6.6.2 Discussion of results of section 6.2

We suggest that the lack of detectable absorption derivative is because $\gamma H_1 \tau > 1$. Equation (6.11) shows that under this condition $\chi'(\omega)$ is very small. Because the line lags the modulating field by 157° at a modulation frequency of 100 kHz, the relation $\omega_m \tau > 1$ holds at this frequency (equations 6.15 and 6.16).

Since in the spectrometer we detect $\chi' H_1$, equations (6.15) and (6.16) also predict that the signal seen will be independent of $H_1$ (and therefore of the microwave power) and this is observed at high microwave powers of 10 mW (figure 6.2).

The variation of the signal height with modulation amplitude, $H_m$, follows directly from the Portis equations.

6.6.3 Information derived from data assuming Portis theory is correct

We can calculate:

(i) the value of the mean relaxation time, $\tau$

(ii) an approximate value of the linewidth of the individual spin packets that make up the inhomogeneously broadened line.

(i) Evaluation of the mean relaxation time - $\tau$

The magnetic field modulation frequency was varied between 10 kHz and 100 kHz and $\omega_m \tau$ obtained from the measured phase lag as in the dextrose experiments. Since the signals from the single crystals were not visible at very
low powers, the zero of phase was obtained from measurements on a DPPH crystal. This material did not show passage effects at 4.2°K and high powers (10 mW) so that a 'zero phase'-setting could be obtained. The results are given in figure 6.9, and indicate the value of $\tau$ to be $3.8 \times 10^{-6}$ seconds. The corresponding value of $\gamma H_1 \tau$ is:

$$\gamma H_1 \tau = (1.7 \times 10^7) \times (10^{-1}) \times (3.8 \times 10^{-6})$$

which implies that we will be out of the saturation region if we reduce $H_1$ to $10^{-2}$ gauss or less (equivalent to reducing the microwave power from 10 mW to less than 100 $\mu$W). In fact, fast passage is seen down to powers of less than 10 $\mu$W which suggests that the value of $\tau$ estimated is too short. It is difficult to account for this discrepancy especially in view of the reasonable dextrose results. It is possible that the use of a different sample holder and sample for the 'zero phase' measurement is the cause, even although the large signal given by the DPPH sample resulted in a very accurate null being obtained.

Portis (1955) mentions that, unless the time of passage through an individual resonance line is short compared with both the spin-lattice relaxation time, and the spin diffusion time, then deviations from his fast passage theory are to be expected.

Spin diffusion means that when only some of the spins in a system are resonating, the energy is transferred
FIGURE 6.9

Magnetic field modulation frequency dependence of phase lag of cytochrome $c$ single crystal dispersion signal at 4.2°K.

The measured lag, $\phi$, is related to the ordinate $\omega_m \tau$ by $\phi = \tan^{-1}(\omega_m \tau)$.
Cytochrome c

Modulation Frequency $\frac{\omega_m}{2\pi} \quad \text{kHz}$

Phase Uncertainty

$\omega_m \tau$ - radians
slowly via spin-spin interaction from the resonant spins to the remainder. Sweeping an inhomogeneously broadened line with a linear d.c. field \((\text{d}H/\text{d}t)\) on which is superimposed a varying a.c. magnetic field \((\omega_m H_m)\), modulation produces resonance only in a region \(2 H_m\) wide. In our experiments \(2 H_m \approx 2\) gauss and as the inhomogeneous line-width is \(\sim 400\) gauss, spin diffusion could be taking place, limiting the overall relaxation time to \(3.8 \times 10^{-6}\) seconds.

(ii) Estimation of spin packet width.

As already mentioned (section 6.5.4), Feher (1959) and Bugai (1963) both show that the onset of the plateau in signal height as a function of modulation amplitude, \(H_m\), (figure 6.3) signifies that \(H_m\) is greater than the linewidths of the individual spin packets which make up the inhomogeneously broadened line. From figure 6.3 we can estimate the packet width \((\Delta H_2)\) to be approximately one gauss. The relaxation time corresponding to this line width is about \(5 \times 10^{-8}\) seconds using the formula (Alger, 1968, p. 45):

\[
\tau = \frac{h}{g\beta \Delta H_2} \text{ sec.}
\]

We shall postpone any considerations of the causes of this relaxation time until section 7.6.1 where we shall discuss the causes of all the relaxation times observed in more detail.
6.7 CONCLUSIONS

In this chapter we have presented our EPR results on a single crystal of cytochrome c, namely: no detectable absorption derivative, but instead an absorption line of Gaussian shape, 157° out of phase with the magnetic field modulation. The signal height levelled off at high microwave powers and at low modulation amplitudes (at much lower amplitudes than the width of the line would imply).

We suggested that rapid adiabatic passage might be the cause and then outlined the conditions under which passage effects could be seen giving a brief description of Portis' theory for such effects in inhomogeneously broadened lines. The theory was tested by studying the rapid passage behaviour of a model system of charred dextrose. The dextrose experimental results were successfully explained on the basis of Portis' treatment. Thus encouraged, we discussed our cytochrome c results in the light of his theory.

Finally the overall relaxation time of the system and the spin packet linewidths were estimated for the temperature of 4.2°K, and found to be \(3.8 \times 10^{-6}\) seconds and \(\Delta H_{1/2} \approx 1\) gauss.
CHAPTER 7.0
RELAXATION TIME STUDY OF CYTOCHROME C

7.1 INTRODUCTION

The experiments on cytochrome c in the previous chapter were carried out at 4.2°K. The fast passage effects observed remain at temperatures up to approximately 20°K, where the overall relaxation time (τ) becomes sufficiently short that the usual slow passage conditions prevail.

By use of the Portis (1955) theory presented in the previous chapter we calculate the magnitude of the fast passage signal height as a function of T (section 7.2). From the experimental results we obtain the variation in signal height as the temperature is altered and we combine them with the theory of section 7.2 to produce a graph of relaxation time as a function of temperature in the range 4.2°K to 20°K (section 7.3).

Above 50°K, the relaxation time can be determined directly from the experimental curves (section 7.4). After a brief review of relaxation time theories (section 7.5), we discuss the experimental data in the light of them (section 7.6).
7.2 **CALCULATION OF RAPID PASSAGE SIGNAL AS A FUNCTION OF RELAXATION TIME**

From equation (6.13) in the previous chapter we have:

\[
\chi' H_1 = \frac{\pi}{4} \chi_o \omega_m H_m \cos \phi_1 \cdot \omega_m \tau \cdot \sin(\omega_m t - \phi) \cdot h(\omega - \omega_0) \tag{7.1}
\]

at the output of the phase sensitive detector this becomes

\[
|\chi' H_1| = \text{const} \cdot \omega_m \tau \cdot \cos \phi_1 \quad \text{at the line centre (7.2)}
\]

where \( \phi_1 \equiv \tan^{-1} \omega_m \tau \)

The signal phase changes with variation in \( \tau \) due to the \( \sin(\omega_m t - \phi_1) \) term in (7.1).

There is not time in the experiment to find the optimum phase of the signal at each point during the warming of the sample, as the temperature changes too rapidly. Consequently, we multiply the theoretical signal by \( \cos(\phi_{\text{sig}} - \phi_{\text{ref}}) \) where \( \phi_{\text{sig}} \) is the calculated \( \phi_1 \) for a given \( \omega_m \tau \), and \( \phi_{\text{ref}} \) is the angle corresponding to the starting value of \( \omega_m \tau \).

The final expression is

\[
(\chi' H_1)_{\text{calc}} = \text{const} \cdot \omega_m \tau \cdot \cos \phi_{\text{sig}} \cdot \cos(\phi_{\text{sig}} - \phi_{\text{ref}}) \tag{7.3}
\]

This equation is plotted for a range of \( \omega_m \tau \) in figure 7.1.
Rapid passage signal height as a function of relaxation time. The curve is a plot of equation (7.3) derived from the Portis equations for rapid adiabatic passage.
7.3 RELAXATION TIME FROM FAST PASSAGE BETWEEN 4.2° AND 20°K

The temperature rose in the cavity once the helium had boiled off, and the signal from a single crystal line was obtained as a function of temperature.

The signal drops in intensity not only from change in relaxation time but also from the decrease in spin population difference between the two energy levels. Since the temperature of each signal is known, the latter effect can be allowed for, and the results of several runs are shown in figure 7.2 normalized to the population difference at 4.2°K. The signals from various crystals varied due to differences in crystal size and position of resonance line. All lines were in the region $g = 2.5$ to 2.9.

For each height there correspond values of $\tau$ and temperature which we plot in figure 7.3. The graph is drawn as $(1/\tau)$ versus temperature on a log-log scale. The solid line on the graph is that of a $\tau^7$ dependence of $\frac{1}{\tau}$.

7.4 RELAXATION TIMES FROM EPR LINEWIDTHS ABOVE 20°K

7.4.1 Introduction

Above 20°K conventional slow passage signals are present, detected as derivatives of the absorption. As already mentioned in the linewidth chapters, when the temperature is greater than 50°K the Gaussian lineshape produced by a distribution of spin packets becomes broadened. This is
FIGURE 7.2

Rapid passage signal height as a function of absolute temperature.
FIGURE 7.3

Plot of inverse relaxation time versus temperature between 4.2°K and 20°K. The data points are taken from figures 7.1 and 7.2 (see text).
### TABLE XIII

**Summation of Lorentzian lines with a Gaussian distribution**

Gaussian Width - 300 gauss

<table>
<thead>
<tr>
<th>Lorentzian Linewidth (Gauss)</th>
<th>Total Linewidth $\Delta H_{1/2}$ (Gauss)</th>
<th>Peak-to-Peak Linewidth $\Delta H_{ptp}$ (Gauss)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>312</td>
<td>260</td>
</tr>
<tr>
<td>100</td>
<td>340</td>
<td>280</td>
</tr>
<tr>
<td>200</td>
<td>400</td>
<td>320</td>
</tr>
<tr>
<td>300</td>
<td>480</td>
<td>350</td>
</tr>
<tr>
<td>400</td>
<td>560</td>
<td>380</td>
</tr>
<tr>
<td>500</td>
<td>640</td>
<td>430</td>
</tr>
</tbody>
</table>
due to the individual spin packets that make up the distribution having linewidths of the same order of magnitude as the width of the distribution itself.

7.4.2 Analysis

Below 20°K the fast passage condition gave an 'absorption' lineshape from which the width at half height could be obtained directly. Above 20°K we have conditions of slow passage through the line yielding the conventional derivative display. The linewidth measured in the derivative display is the peak-to-peak separation ($\Delta H_{\text{ptp}}$) and this does not coincide with the width at half height of the absorption curve ($\Delta H_{2\text{}}$).

For a Lorentzian line,

$$\Delta H_{2\text{}} = 1.73 \cdot \Delta H_{\text{ptp}}$$

and for a Gaussian line

$$\Delta H_{2\text{}} = 1.17 \cdot \Delta H_{\text{ptp}}$$

If the lineshape is a combination of the two, the multiplying factor lies between these two values.

We wrote a computer program to sum the contributions from a Gaussian distribution of Lorentzian lines. Table XIII opposite shows the results for a Gaussian of 300 gauss width, onto which have been folded Lorentzians of various widths. The peak-to-peak linewidths are those at the points of maximum slope and these are used for comparison with the experimental results.
The lineshape is approximately Lorentzian. This is reasonable since the wide wings of the Lorentzian shape add to broaden the distribution even when the individual Lorentzian linewidths are small. By measuring the peak-to-peak derivative linewidths as the temperature increases and comparing them with the calculated widths, one can obtain the width of the Lorentzians as a function of temperature.

7.4.3 Results

The data from one crystal which had particularly narrow lines (300 gauss at 4.2°K) are plotted in figure 7.4. The open circles are the Lorentzian linewidths which fitted the experimentally observed derivative widths (assuming a 300 gauss distribution), together with the total observed width of the absorption lineshape from the combination of Lorentzian and Gaussian (Θ) all as a function of temperature.

From the Lorentzians we obtain the inverse relaxation time (1/τ) in seconds, corresponding to their linewidth in gauss, from (Alger, 1968, p. 45):

\[
\frac{1}{\tau} = \frac{\text{linewidth in gauss}}{5.6 \times 10^{-8}} \text{ seconds} \quad (7.4)
\]

Doing this, we obtain:
FIGURE 7.4

Individual contributions to the overall linewidth from a Gaussian distribution 300 gauss wide and Lorentzian lines between 30°K and 77°K in a cytochrome c single crystal (see text).
<table>
<thead>
<tr>
<th>$T^\circ K$</th>
<th>Lorentzian Width</th>
<th>Inverse Relaxation Time ($1/T$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>50</td>
<td>$8.9 \cdot 10^8$</td>
</tr>
<tr>
<td>53</td>
<td>100</td>
<td>$1.8 \cdot 10^9$</td>
</tr>
<tr>
<td>57.5</td>
<td>190</td>
<td>$3.4 \cdot 10^9$</td>
</tr>
<tr>
<td>62</td>
<td>205</td>
<td>$3.7 \cdot 10^9$</td>
</tr>
<tr>
<td>65</td>
<td>300</td>
<td>$5.4 \cdot 10^9$</td>
</tr>
<tr>
<td>68</td>
<td>400</td>
<td>$7.1 \cdot 10^9$</td>
</tr>
</tbody>
</table>

We use these relaxation times in section 7.6.3 to decide between two theoretical models of the variation of relaxation time with temperature.

7.5 REVIEW OF THEORIES OF RELAXATION TIMES

The mean relaxation time of this system, $T$, is equal to $(T_1 T_2)^{1/2}$ where $T_1$ is the spin-lattice relaxation time and $T_2$ is the spin-spin relaxation time.

In general, the spin-spin relaxation time does not change rapidly with temperature, while the spin-lattice time does. As we wish to explain a dependence of relaxation time on temperature ($T$) of $T^7$, our attention will be focussed on the spin-lattice relaxation time.

The most direct interaction of the spin system is with the thermal electro-magnetic radiation bath—energy being lost from the system by downward magnetic resonance transitions induced by photons. However, the number of photons with suitable energies at the frequency and
temperature involved is too small to give relaxation times short enough to agree with experiment.

Waller (1932) proposed that, since the energy density of lattice vibrations (the phonon radiation bath) was of order $10^{15}$ times greater than the photon radiation bath, modulation of the spin-spin interaction by phonons could induce magnetic resonance transitions and thus provide a relaxation pathway for the spin system to lose energy.

He distinguished two processes:

(i) Direct interactions - A phonon of the same energy as the spin quantum required for a resonance transition is emitted, and this is accompanied by a 'down' transition in the spin system.

(ii) Raman interactions - A phonon of any frequency $\omega_p/2\pi$ interacts with a spin (up or down) causing a transition within the spin system, the phonon being scattered with a different frequency $(\omega_p/2\pi) + \nu$, where $\nu$ is the magnetic resonance frequency.

The Raman process dominates over the direct process at temperatures above a few degrees Kelvin. This is because at temperature, $T$, where $(h\nu/kT) \sim 1$ only a small proportion of the phonons present lie in the frequency range $\nu \pm \Delta \nu$ while there are large numbers of phonons with energies differing by $h\nu$.

However, Waller's theory of phonon modulation of the spin-spin interaction gave relaxation times many orders
of magnitude too long.

Kronig and Bouwkamp (1939) and Van Vleck (1940) postulated another relaxation mechanism that employs modulation by phonons and which gives much better quantitative agreement with experiments. They proposed that lattice vibrations modulated the ligand field. This produces a variable electric field which can interact with the electronic orbits, and, via spin-orbit coupling, with the electron spins.

The effectiveness of this mechanism is dependent on the strength of the spin-orbit coupling—-with small spin-orbit coupling, i.e. g-values close to the free spin value of 2.0023, the relaxation times will be long. In our cytochrome c system, whose g-values deviate markedly from 2, we expect the relaxation times given by this mechanism to be short, except when there are few phonons present.

We must also consider the Orbach process (1961): This mechanism requires the presence of a low lying state with energy, $\Delta$, a few tens of inverse centimetres above the magnetic states, to and from which transitions can be made.

In general the temperature dependence of the spin-lattice relaxation time can be written as

$$\frac{1}{T_1} = aT + bT^9 + c \cdot \exp(-\Delta/T)$$  \hspace{1cm} (7.5)

The first term comes from the one phonon direct process, the next arises from the Raman process, and the last from
the Orbach process.

At very high temperatures the Raman process goes over to a $T^2$ dependence for $1/T_1$, at temperatures above the Debye temperature of the sample ($T_D$), (Abragam and Bleaney, 1970, p. 572).

We now wish to discuss our measurements in the light of these theories.

7.6 DISCUSSION

7.6.1 The mean relaxation time of this horse heart cytochrome c system is approximately constant from 4.2 to 9°K, then starts to rise following an approximately $T^7$ dependence up to 20°K.

We suggest that the process which gives rise to this power temperature dependence is a combination of the $T^9$ Raman spin-lattice relaxation process and a temperature insensitive spin-spin relaxation process.

The evidence for this model is as follows:

(i) We have measured the widths of the spin-packets of the individual lines to be approximately one gauss. The simplest cause of a broadening of the natural linewidth to 1 gauss is dipolar spin-spin interaction between the electrons of neighboring iron atoms.

We know from X-ray data (Dickerson et al., 1971) that the heme iron atoms are approximately 25 Å apart in
cytochrome c. The induced field, $B$, due to six nearest neighbours (assuming roughly cubic packing) is:

$$B \approx \frac{6\beta}{4\pi r^3}$$

(7.6)

with $\beta$ = Bohr magneton ($1.16 \times 10^{-24}$ weber/metre)

$$r = 25 \times 10^{-10} \text{ metres}$$

This gives $B \approx 3$ gauss, supporting our hypothesis.

We also note that another source of broadening could be from protons close to the iron. Bozanic et al. (1969) showed by spin-echo experiments that there were relaxation times of $10^{-7}$ seconds or less in frozen ferri-myoglobin solutions at 4.2°K. They attributed these to a dipolar interaction of a proton with the iron. As ferri-myoglobin is in a high spin state, with a water molecule in the sixth coordination position, this interpretation is quite reasonable. However, this mechanism if present would be the major pathway for energy removal from the spin system, bypassing the spin-lattice relaxation process. The very small relaxation times predicted by this mechanism ($<10^{-7}$ sec) are so short that no fast passage effects would occur.

In cytochrome c, the heme iron is bound to five nitrogen atoms and a sulphur atom in a hydrophobic environment. In this structure protons are probably not close enough to the iron atom for the inverse cubic term in the dipolar interaction relation (7.6) to overcome the small proton dipole moment and produce the observed broadening.
Assuming a one gauss linewidth—equivalent to a spin-spin relaxation time \(T_2\) of \(5 \times 10^{-8}\) seconds—we can obtain \(1/T_1\) from using \(\tau = (T_1 T_2)^{1/2}\) and the data of figure 7.3.

Over the region 10 to 18°K, as shown in figure 7.5, the data can be fitted by either a \(T^9\) curve (Raman process) or by an exponential of the form \(\exp(-\Delta/T)\) (Orbach process) assuming \(\Delta = 166^\circ\)K \((\sim 115\text{ cm}^{-1})\).

In order to decide between these two possible processes we use the experimental results obtained at temperatures greater than 20°K (section 7.4).

7.6.2 The relaxation times in table XIII are plotted on figure 7.5 and have an approximate \(T^5\) variation over the range. Neither theoretical curve—\(T^9\) or \(\exp(-\Delta/T)\)—fit the high temperature data as they stand. However, theory predicts that the Raman \(T^9\) process goes over to a \(T^2\) dependence for \(1/T_1\) at temperatures above the Debye temperature \(T_D\) of the sample. In the intermediate region where \(T\sim T_D\) we might expect a dependence between \(T^9\) and \(T^2\). As the data show an approximate \(T^5\) variation, this suggests that we are in the transition region.

The exponential also levels off as the temperature increases but if the Orbach process is dominant, the spin lattice relaxation time would have to be an order of magnitude shorter than is measured for it to be the correct mechanism.
FIGURE 7.5

Plot of inverse spin-lattice relaxation time as a function of temperature from 4.2°K to 70°K.

The points (©) are estimated from fast passage data, while the points (△) are obtained from Lorentzian linewidth data. The temperature dependence of a Raman (T^9) process and an Orbach (exp - (166/T)) shown fit the data between 10°K and 20°K (see text).
7.6.3 On the basis of the foregoing considerations, we have obtained an estimate of the Debye temperature. Can we make any other estimate of this temperature?

As a general rule, at the Debye temperature the wavelength of the lattice vibrations is of the same order as the interionic separation (Rosenberg, 1965). If we knew the phonon velocity and the interionic spacing we could calculate the approximate Debye frequency and, hence, the Debye temperature.

Abragam and Bleaney (1970) quote values of approximately $5 \times 10^5$ cm/sec for the phonon velocity, i.e. the velocity of sound in paramagnetic crystals. In water, the velocity of sound is $1.5 - 2 \times 10^5$ cm/sec. Therefore, let us assume for our discussion that phonon velocity is approximately $3 \times 10^5$ cm/sec in cytochrome $c$.

Let us also assume that only the heaviest atoms ($\text{Fe}^{3+}$) determine the upper end of the phonon spectrum. The interionic separation of the $\text{Fe}^{3+}$ is about 25 Å in cytochrome $c$, so that:

$$\nu_D \sim \frac{3 \cdot 10^5}{2.5 \times 10^{-7}} = 1.2 \times 10^{12} \text{ Hz}$$

then $$\hbar \nu_D = 1.2 \times 10^{12} \times 3 \times 10^{-11} = 4.2 \text{ cm}^{-1}$$

using $$\hbar \nu_D \equiv kT_D$$

we obtain $$T_D \sim \frac{42}{0.7} = 60^\circ \text{ K}$$
which is of the correct order—unexpectedly close in view of the assumptions made. This value is low compared with the Debye temperatures measured for most elements (e.g., 400° for metallic iron, 2000° for diamond). However, independent evidence for a Debye temperature of order 100°K comes from Mossbauer work on frozen solutions containing iron compounds.

Dézsi et al. (1968) obtained $T_D \sim 100°K$ for Fe$^{2+}$ ions in frozen aqueous solution. By measurements on the size of the recoil free fraction at low temperatures, Gonser and Grant (1965) studied the temperature dependence of the recoil fraction in rat oxy-hemoglobin and obtained a Debye temperature of about 180°K. These authors suggest that the simple Debye model, which contains only accoustical vibrational modes, may not be sufficient to describe the phonon spectrum, and that optical modes should also be considered. These are characterised by a single vibrational frequency (or single effective temperature, $T_E$, the Einstein temperature). The same dependence of recoil fraction on temperature would be seen in hemoglobin with $T_E \sim 120°K$.

Gonser and Grant (1965) also attempted to explain an anisotropy in the quadrupole splitting of oxy-hemoglobin by the presence of an optical vibration mode with $T_E \sim 27°K$ vibrating in a direction perpendicular to the heme plane. However, the presence of this optical mode has been questioned...
by Lang (1970) who suggested that the asymmetry may be due to impurities in the preparation, since he has observed highly symmetric spectra in at least some samples of reduced rat hemoglobin.

7.7 CONCLUSION

We have shown that the temperature dependence of the overall relaxation time curve can be followed from 4.2°K to 20°K using rapid adiabatic passage techniques and from 50°K to 77°K by analysis of the signal linewidth.

We conclude that, between 10°K and 20°K the overall relaxation time is a combination of a temperature independent spin-spin relaxation time arising from dipolar interaction, combined with a Raman $T^9$ dependence of the spin lattice relaxation time. Above these temperatures the spin-lattice relaxation time is dominant.

The demonstration that the dependence of spin lattice relaxation time has become an approximate $T^5$ function by 50° to 60°K suggests that this might eventually become a $T^2$ function at higher temperatures. Such a dependence of spin lattice relaxation time on the square of the temperature is the behaviour produced by a Raman relaxation process at 'infinite temperatures'.

We have also given an estimate for the Debye temperature ($T_D$) of 60°K in cytochrome c and note that Mossbauer determinations of $T_D$ in aqueous and protein solutions containing iron atoms are consistent with this value.
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APPENDIX - A1

CALCULATION OF EPR LINE BROADENING DUE TO VARIATIONS
IN RHOMBIC AND AXIAL POTENTIAL

A1.1 INTRODUCTION

The method given by Eisenberger and Pershan (1967) will be used. These authors obtained expressions for the line widths in terms of the energy of the axial and rhombic splittings that gave rise to the low spin g-values. Their publication contained a number of errors, and so the calculation is rederived below. Some of the expressions have been found to have a simpler form than given by Eisenberger and Pershan.

The calculation starts with the energy matrix of the ground state Kramers doublet, from which the coefficients of the orbitals making up the doublet can be obtained. With these coefficients, the g-value equations can be found and the derivatives calculated.

A1.2 THE GROUND STATE KRAMERS DOUBLET

Low spin Fe$^{3+}$ has five 3d electrons in $t_{2g}$ orbitals. For a system quantised along the heme normal these orbitals
are $t_{2g}^0$, $t_{2g}^1$, $t_{2g}^{-1}$. In terms of the pure d-orbitals conventionally called $d_2$, $d_{-2}$, $d_1$, $d_{-1}$, $d_0$ (Ballhausen, 1962, p. 63) the $t_{2g}$ are:

\[
\begin{align*}
t_{2g}^0 &= \frac{1}{\sqrt{2}} \cdot (d_2 - d_{-2}) \equiv | \zeta \rangle \\
t_{2g}^1 &= d_1 \quad \equiv | +1 \rangle \\
t_{2g}^{-1} &= d_{-1} \quad \equiv | -1 \rangle
\end{align*}
\]

The real form of these orbitals, denoted by $|d_{xy}\rangle$, $|d_{yz}\rangle$, and $|d_{xz}\rangle$ are

\[
\begin{align*}
|d_{xy}\rangle &= \frac{1}{\sqrt{2}} \cdot (d_2 - d_{-2}) \equiv | \xi \rangle \\
|d_{yz}\rangle &= -\frac{i}{\sqrt{2}} \cdot (d_1 + d_{-1}) \equiv | \xi \rangle \\
|d_{xz}\rangle &= -\frac{i}{\sqrt{2}} \cdot (d_1 - d_{-1}) \equiv | \eta \rangle
\end{align*}
\]

In low spin ($S=1/2$) cytochrome $c$ the environment of the iron is predominantly cubic, but also contains axial and rhombic components. Under cubic ($O_h$) symmetry the $t_{2g}$
orbitals are degenerate, reduction of symmetry to \( D_{4h} \) by
the addition of an axial potential separates \( |d_{xy}\rangle \) from
\( |d_{yz}\rangle \) and \( |d_{xz}\rangle \). Further reduction of symmetry to \( D_{2h} \)
by a rhombic potential splits \( |d_{yz}\rangle \) and \( |d_{xz}\rangle \) lifting the
orbital degeneracy completely. Each orbital still has a
2-fold spin degeneracy—the two states are denoted by (+)
for spin up and (-) for spin down, e.g. \(|+1^+\rangle\) and \(|+1^-\rangle\).

Spin orbit coupling mixes these orbitals which
combine into three sets of Kramers doublets. Application of
a magnetic field splits these doublets, and an EPR experiment
will observe the transitions between the two levels of the
ground state. The behaviour of the five electrons in the
t\(_{2g}\) orbitals is the same as a hole in the \( t_{2g}^6 \) closed shell,
provided we invert the energy levels.

In this treatment we neglect orbital reduction
(see Stevens, 1953). From Griffith (1961, p. 354) the \( t_{2g} \)
orbitals (including spin) factorise under spin-orbit coupling
of \(-\lambda \mathbf{L} \cdot \mathbf{S}\) into the matrix

\[
\begin{array}{ccc}
|{-1}\rangle & |{-\xi^+}\rangle & |{-1^-}\rangle \\
\text{or} & \text{or} & \text{or}
\end{array}
\begin{array}{ccc}
|{1^+}\rangle & |{\xi^-}\rangle & |{-1^+}\rangle \\
\end{array}
\]

\[
\begin{array}{ccc}
|{-1}\rangle \text{ or } |{1^+}\rangle & \frac{\lambda}{2} & \frac{\lambda}{\sqrt{2}} & \frac{\nu}{2} \\
\end{array}
\begin{array}{ccc}
|{-\xi^+}\rangle \text{ or } |{\xi^-}\rangle & \frac{\lambda}{2} & D & 0 \\
\end{array}
\begin{array}{ccc}
|{1^-}\rangle \text{ or } |{-1^+}\rangle & \frac{\nu}{2} & 0 & -\frac{\lambda}{2} \\
\end{array}
\]

(Al.1)
where the crystal field energies corresponding to holes in the $|d_{zx}\rangle$, $|d_{yz}\rangle$ and $|d_{xy}\rangle$ orbitals are given by $-V/2$, $+V/2$ and $D$ respectively:

$$
\begin{array}{c}
\text{D} \\
\text{V/2} \\
\text{-V/2} \\
\text{d_{zx}} \\
\text{d_{xy}} \\
\text{d_{yz}} \\
\text{d_{xy}}
\end{array}
$$

The ground state Kramers doublet has the form

$$
\begin{align*}
\psi^+ &= A|l^+\rangle + B|\xi^+\rangle + C|-l^+\rangle \\
\psi^- &= A|-l^+\rangle - B|\xi^+\rangle + C|l^+\rangle
\end{align*}
$$

and the energy, $E$, of this ground state relative to the zero of (A1.2) can be found from the secular determinant of matrix (A1.1) giving:

$$
\begin{align*}
(0.5\lambda - E)A + (\lambda/\sqrt{2})B + (V/2)C &= 0 & (A1.4a) \\
(\lambda/\sqrt{2})A + (\Delta - E)B &= 0 & (A1.4b) \\
(V/2)A + (-0.5\lambda - E)C &= 0 & (A1.4c)
\end{align*}
$$

and the normalisation condition gives

$$
A^2 + B^2 + C^2 = 1
$$

(A1.5)
The ground doublet \( g \)-values are

\[
g_1 = 2 (\sqrt{2} A + B) \cdot (\sqrt{2} C - B) \quad (A1.6a)
\]

\[
g_2 = 2 (\sqrt{2} A + B) \cdot (\sqrt{2} C + B) \quad (A1.6b)
\]

\[
g_3 = 2 (2A^2 - E^2) \quad (A1.6c)
\]

We now wish to obtain the derivatives of these \( g \)-values with respect to the rhombic potential, \( V \), and the axial potential, \( D \).

We define \( g_i' = \frac{\partial g_i}{\partial V} \) and \( g_i'' = \frac{\partial g_i}{\partial D} \) with \( i = 1, 2, 3 \).

**A1.3 EVALUATION OF RHOMBIC CONTRIBUTION**

In order to obtain the \( g_i' \), we must obtain \( A', B', C' \) and \( E' \). From (A1.4b)

\[
B = \frac{\lambda A}{\sqrt{2}} \quad (A1.7)
\]

Differentiate with respect to \( V \), and

\[
B' = \frac{\partial B}{\partial V} = (\lambda / \sqrt{2}) \left[ A' (E - D)^{-1} - A (E - D)^{-2} E' \right]
\]

\[
= (\lambda / \sqrt{2}) A (E - D)^{-1} \left[ A'/A - E'(E - D) \right]
\]

\[
B' = B \left[ A'/A - E'(E - D)^{-1} \right] \quad (A1.8)
\]
similarly, from (A1.4c)

\[ C = \frac{V \cdot A}{2(0.5 \lambda + E)} \]

and differentiation plus simplification gives

\[ C' = C \left[ \frac{A'}{A} + \frac{1}{V} - E' \cdot (E - 0.5 \lambda)^{-1} \right] \tag{A1.9} \]

To obtain \( A' \), we use the normalisation condition; differentiating we get

\[ 2A \cdot A' + 2B \cdot B' + 2C \cdot C' = 0 \]

Substitution of (A1.7) and (A1.8) gives

\[ A' = A \left[ C^2 \cdot (E - 0.5 \lambda)^{-1} - B^2 \cdot (D - E)E' - (C^2 / V) \right] \tag{A1.10} \]

To obtain \( E' \), we differentiate (A1.4a), substitute for \( A' \), \( B' \) and \( C' \) and eventually produce

\[ E' = AC \tag{A1.11} \]
The analytical expressions for the $g_i'$ are

\[
g_1' = 2 \left[ (\sqrt{2}A + B)(\sqrt{2}C' - B') + (\sqrt{2}C - B)(\sqrt{2}A' + B') \right]
\]
\[
g_2' = 2 \left[ (\sqrt{2}A + B)(\sqrt{2}C + B') + (\sqrt{2}C + B)(\sqrt{2}A' + B') \right] \tag{A.12}
\]
\[
g_3' = 2 \left[ 4AA' - 2BB' \right]
\]

We substitute into these relations the values of $A'$, $B'$ and $C'$ obtained above. Finally, these $g_i'$ are put into the equation that gives the contribution to the linewidth from the distribution of rhombic potential.

\[
\Delta H_V = \frac{\hbar \nu}{\beta} \cdot \frac{1}{g_3} \left[ g_1' g_1' \sin^2 \theta \cdot \cos^2 \phi \right. \\
\left. + g_2' g_2' \sin^2 \theta \sin^2 \phi \\
\left. + g_3' g_3' \cos^2 \theta \right] \cdot \Delta V \tag{A.13}
\]
Al.4 EVALUATION OF AXIAL CONTRIBUTION

By exactly similar methods we extend this treatment to the axial case to obtain the $g_1''$.

The relationships are:

\[
A'' = A \left[ B^2 (1 - E'') \cdot (D - E)^{-1} + C'' \cdot E'' \cdot (E - 0.5\lambda)^{-1} \right]
\]

\[
B'' = B \left[ A''/A - (I - E'') \cdot (D - E)^{-1} \right]
\]

\[
C'' = C \left[ A''/A - E'' \cdot (E - 0.5\lambda)^{-1} \right]
\]

\[
E'' = \lambda \cdot AB \cdot \left[ \sqrt{2} (D - E)^{-1} \right]
\]

And by substitution:

\[
\Delta H'_D = \frac{h \nu}{\beta} \cdot \frac{1}{g^3} \left[ g_1 g_1'' \sin^2 \theta \cos^2 \phi + g_2 g_2'' \sin^2 \theta \sin^2 \phi \right.
\]

\[
+ g_3 g_3'' \cos^2 \theta \left] \cdot \Delta D \right.
\]