ELECTRON-NUCLEAR DOUBLE RESONANCE STUDIES OF
FREE RADICALS TRAPPED IN IRRADIATED SINGLE CRYSTALS
OF CYTOSINE MONOHYDRATE AND CAFFEINE
HYDROCHLORIDE DIHYDRATE

by

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We accept this thesis as conforming to the
required standard.

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ABSTRACT

The technique of electron-nuclear double resonance spectroscopy (ENDOR) has been used to determine the identity and structure of radicals trapped in x-irradiated single crystals of cytosine monohydrate and caffeine hydrochloride dihydrate. The radical studied in cytosine monohydrate had previously been observed by several workers using electron paramagnetic resonance (EPR) but its identity was in question. All intramolecular and several intermolecular proton hyperfine couplings were analyzed in detail. The experimentally determined coupling parameters were compared with ones obtained by theoretical means for several possible radical species. The agreement between the observed and calculated parameters fit best for the radical formed by net addition of a hydrogen atom to O(2) of cytosine, with the hydroxyl proton lying in the nodal plane of the π-system. The appearance of twice the number of certain ENDOR lines than expected from the crystal symmetry was interpreted by postulating that the radical was stabilized in two different conformations at 77°K. The assignment of the hyperfine couplings to specific protons was supported by an ENDOR study of a partially deuterated cytosine monohydrate crystal, grown from heavy water.

Three different radical species were identified in caffeine hydrochloride dihydrate x-irradiated at room temperature. The EPR spectrum of this system, which had not previously been reported, was too complex to be analyzed. Furthermore, the EPR spectra of two of the radicals
studied by ENDOR could not be distinguished in the overall EPR lineshape. One of these radicals had a lifetime of only a few hours and was tentatively identified as the methyl radical, primarily on the basis of the observed isotropic proton hyperfine coupling constant of -62.65 MHz. The other radical was indefinitely stable and was identified as that formed by net abstraction of a hydrogen atom from N(9) of the caffeine moiety. This radical is equivalent to that which would be produced by the loss of an electron from a neutral caffeine molecule, the caffeine cation; as such, it is the first reported cation radical in a purine derivative.

The radical which dominated the EPR spectrum was identified as that resulting from net addition of a hydrogen atom to C(8) of the protonated caffeine molecule. The identification of this radical was based on the analysis of four different proton hyperfine couplings. Nitrogen (\(1^4\)N) hyperfine and quadrupole coupling tensors were also obtained from the ENDOR spectra and were attributed to N(7). The observation of \(1^4\)N-ENDOR lines, which had not previously been reported in any pyrimidine or purine derivative, provided a second, independent estimate of the unpaired spin density centred on N(7). An indirect second-order effect, giving rise to a non-crossing phenomenon, was observed between the methylene protons, which were also found to be non-equivalent. The structure of this radical was found to agree with those determined previously by EPR and by molecular orbital calculations for the analogous species in other purine derivatives.
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CHAPTER ONE

INTRODUCTION

An important characteristic of high-energy radiation is that it produces ionization, and subsequent chemical change, in its passage through matter. This provides a distinction between radiation chemistry and photochemistry. The boundary is not well defined, but is usually assumed to be at an energy of about 30 eV, corresponding to a wavelength of 40 nm in the vacuum ultraviolet. In photochemistry, the study of the chemical effects of light, the energy absorbed by a molecule is quantized. A quantum of light undergoes a resonance interaction with a molecule to excite an electron from one particular orbital to another, the light energy being completely absorbed in the process. In radiation chemistry, not all of the radiation energy need be transferred at an interaction and the process is not selective. The ionizing photon and the displaced electron are often both capable of producing further ionization, so one incident photon can affect many molecules.

Much of the current interest in radiation chemistry is centred on the fact that living tissue is not exempt from the effects, both good and bad, of high-energy radiation. For example, blood-producing cells in the bone marrow are particularly sensitive to X- or γ-irradiation, so
that exposure of these cells to radiation increases the statistical probability of contracting leukemia. Genetic mutations can be caused by radiation damage to the chromosomes in reproductive organs. However, because radiation can lead to cell death, it has been used to arrest the progress of certain types of cancer.\textsuperscript{1-4}

All the information needed to define a cell's structure and function is contained in the deoxyribonucleic acid (DNA) of its chromosomes. The DNA is the only component of the cell which is not freely replaceable so any change to the DNA which leads to faulty transcription of the genetic information must be a potential cause of cellular malfunction or inherited abnormality. As a result, knowledge of the radiation chemistry of DNA and its constituents is very important to the radio-biologist, who seeks to understand the effects of radiation on living organisms.

A deoxyribonucleic acid is a long-chain polymer formed from a series of nucleotides. Each nucleotide is formed from three components: D-2-deoxyribose, phosphoric acid, and a pyrimidine or purine base. The most common pyrimidine and purine bases found in DNA are shown in Fig. 1, along with their respective parent compounds. From data obtained by Wilkins and coworkers\textsuperscript{5}, Watson\textsuperscript{6} and Crick\textsuperscript{7} proposed that DNA is composed of two complementary chains of polynucleotides in a double-helical structure. The two chains of the helix are held together by hydrogen bonds between adjacent pyrimidine and purine bases, one from each chain. The chains are complementary in terms of the appropriate base pairing, adenine to thymine and guanine to cytosine. The aim of this research has been to gain further insight into the radiation damage of these pyrimidine and purine bases.
Fig. 1. The structure of some common pyrimidine and purine bases.
When radiation causes an electron to be ejected from one of these bases, it becomes paramagnetic, since the loss of the electron requires one of the remaining electrons to be unpaired. Furthermore, the ejected electron can be captured by an undamaged molecule, which then also becomes paramagnetic. All subsequent reactions of these paramagnetic molecules, or free radicals, must involve formation of a new radical species, unless two radicals combine to yield a diamagnetic species.

About 1955, Gordy was the first to recognize the potential of electron paramagnetic resonance (EPR) spectroscopy for studying radiation damage of molecules of biological interest. The first observation of an EPR spectrum was reported by E. Zavoisky in 1945. Initially only paramagnetic transition metal complexes were studied because the sensitivity of the instrumentation was very low. By the mid 1950's however, EPR spectra of organic free radicals trapped in polycrystalline solids had been observed. In 1956, Uebersfeld and Erb showed that it was possible, by high-energy irradiation, to form a radical inside a single crystal and for it to be trapped in a specific orientation.

The EPR spectrum of such an oriented radical yields more information about its structure than can be obtained from spectra of the radical in solution or in a polycrystalline solid. The magnetic moment of the unpaired electron is coupled to the magnetic moments of nuclei in its vicinity. This coupling is responsible for the hyperfine structure of an EPR spectrum and is the source of most of the structural information obtainable from the spectrum. One component of this coupling is analogous to the classical dipole-dipole interaction and is direction-dependent, or
anisotropic. The rotational motion of a radical in solution averages out the anisotropic component so that only an isotropic coupling remains. In polycrystalline solids, the radicals are oriented at random with respect to the magnetic field so the anisotropic contribution to the coupling leads to a blurring of the spectrum. In spite of this, much has been learned from the study of free radicals in irradiated, polycrystalline DNA and its constituents$^{11}$. Single crystal studies have not been performed on DNA simply because crystals of sufficient size cannot be grown.

With care however, single crystals of nucleic acid constituents can be grown. In 1965 Gordy$^{12}$ was the first to use a single crystal to study the nucleic acid constituent thymidine. Since then a large number of papers on nucleic acid constituents and their analogs have appeared and have recently been reviewed$^{13}$. The EPR technique has been successful in identifying the major radicals formed in most of these crystals on the basis of the largest hyperfine interactions. However, detailed information on the electronic structure of the radicals could not always be obtained because important intramolecular hyperfine splittings were buried within the EPR linewidth. Furthermore, more than one radical was often formed in these crystals and their spectra could not always be distinguished. As a result, the electron-nuclear double resonance (ENDOR) technique is increasingly being employed to study radicals in these systems.

The first successful ENDOR experiment was carried out by Feher$^{14,15}$ and was concerned with resonances of defects in silicon. The technique
was not applied to single crystals of nucleic acid constituents until 1973, when Hampton and Alexander\textsuperscript{16} studied the nucleoside cytidine and Box and coworkers studied the pyrimidine derivatives 1-methyl-cytosine\textsuperscript{17} and barbituric acid\textsuperscript{18}. One of the main applications of the ENDOR technique is resolution enhancement. A classic example of this is Holton, Blum and Slichter's study of the F-centre in lithium fluoride\textsuperscript{19}. Although the EPR spectrum showed one broad structureless line, the ENDOR technique allowed them to resolve the hyperfine splittings out to the seventh coordination sphere. Furthermore, the ENDOR spectrum is inherently simpler than its parent EPR spectrum, whose hyperfine structure has approximately $2^n$ lines for $n$ interacting protons. The ENDOR spectrum contains only two lines for each group of protons with a particular hyperfine coupling. Finally, using ENDOR one can separate different radical species by selective saturation of different EPR lines.

In the work presented here, the ENDOR technique will be used to study several free radicals trapped in single crystals of pyrimidine and purine bases. A brief introduction to the theory of ENDOR will be given in order to show how the structure of a radical can be determined from spin Hamiltonian parameters. This theory will then be used to interpret the ENDOR spectra of a radical trapped in cytosine monohydrate. This radical had been observed previously\textsuperscript{20} but the nature of the radical was uncertain\textsuperscript{21}. The remainder of this thesis will deal with an ENDOR study of caffeine hydrochloride dihydrate. Caffeine is a purine derivative and, prior to the commencement of this study, there had not been an ENDOR study on a purine reported in the literature.
CHAPTER TWO

THEORY OF EPR AND ENDOR

2.1 Introduction

Both EPR and ENDOR, being spectroscopic techniques, involve the observation of transitions between energy levels of some molecular system. The systems being studied in this work were organic free radicals trapped in crystal lattices. The interpretation of EPR and ENDOR spectra, and hence determination of the nature of the free radical, therefore requires a theoretical model of the radical's electronic structure in order to account for the energy levels and transitions between them. The treatment that follows will be brief as it has already been discussed in greater detail in several excellent articles²²-²⁶ and books²⁷-³¹.

The most important requirement for a molecular system to produce an EPR spectrum is that it must possess one or more unpaired electrons. We will restrict our discussion to systems having only one unpaired electron as these were the only systems studied experimentally. The electron, because it possesses both spin and charge, has associated with it a magnetic moment \( \mu \) which, by the Wigner-Eckhart theorem of quantum mechanics³², is proportional to its spin \( \mathbf{S} \). If a paramagnetic molecule, which by virtue of its magnetic moment has a degenerate ground
state, is placed in a steady magnetic field, the degeneracy is lifted and the levels undergo a Zeeman splitting. When this system is also subjected to a high frequency electro-magnetic field, transitions may be induced between Zeeman levels when they have the appropriate energy separations. The consequent absorption of energy shows a series of maxima as the static magnetic field is varied, corresponding to the separation between the energy levels.

For the general case of a crystal containing paramagnetic molecules, one should determine the energy levels by solving the Schrödinger equation for the electrons and nuclei in the entire crystal, in the presence of a static magnetic field $H$. This, however, is not possible at present and one is forced to simplify this many-body problem. Although the EPR experiment detects changes in the bulk magnetic susceptibility of the crystal, a great simplification occurs if the individual free radicals are considered to be independent and noninteracting. This is a very good approximation in the irradiated crystals we have studied. Thus we can now discuss the spectral properties of a crystal on a molecular level. Another useful approximation can be made because the nuclei are very massive compared to the electrons. This is the Born-Oppenheimer approximation\textsuperscript{33}, which states that the total wave function of a molecule can be separated into an electronic and a nuclear part. Thus the electronic wave function can be obtained with respect to fixed positions of the nuclei.

The total Hamiltonian for the molecule may now be divided into two parts:
\[ \mathcal{H} = \mathcal{H}_E + \mathcal{H}_M, \]

where \( \mathcal{H}_E \) describes the electrostatic and kinetic terms \(^{34}\),

\[ \mathcal{H}_E = -\frac{\hbar^2}{2m} \sum_i \nu^2_i + e^2 \left[ - \sum_i \sum_n \frac{Z_n}{r_{ni}} + \sum_{ij} \frac{1}{r_{ij}} \right] \]

\[ + \sum_{n<k} \frac{Z_n Z_k}{R_{nk}} \]  

and \( \mathcal{H}_M \) describes the magnetic interaction.

To describe the effects of these magnetic interactions we shall adopt the formalism of a spin Hamiltonian, first introduced by Pryce and Abragam \(^{35,36}\), whereby an EPR (ENDOR) spectrum is analyzed in terms of transitions between energy levels which are eigenfunctions of a Hamiltonian containing only spin operators. In the spin Hamiltonian, the spin operators are parameterized and describe the interactions of electron spin momenta, nuclear spin momenta and magnetic fields, both internal and external to the spin system. The eigenvalues of the spin Hamiltonian are energies measured relative to the energy of the molecule in the absence of any magnetic interactions.

2.2 The Spin Hamiltonian

2.2.1 The Electronic Zeeman Interaction

Classically, the energy of a magnetic moment \( \mu \) in a field \( H \) is given by:

\[ E = -\mu \cdot H \]  

(2-3)
and for an electron

\[ \mu = -g_e \beta S \]  

(2-4)

where \( g_e \approx 2 \), \( \beta \) is the Bohr magneton and \( S \) is the spin angular momentum operator representing the effective spin. The Hamiltonian describing the electronic Zeeman interaction is then:

\[ H_S = g_e \beta S \cdot H \]  

(2-5)

If the magnetic field direction is taken to define the \( z \) axis, the projection of \( S \) along \( H \) will be \( S_z \) and the energy of this system is then

\[ E_S = g_e \beta H_z m_S \]  

(2-6)

where \( m_S \) is the magnetic quantum number representing the value of \( S_z \).

The spin magnetic moment for an \( S = \frac{1}{2} \) system can thus be aligned either parallel or anti-parallel to the magnetic field. This gives rise to two states of differing energy with

\[ E(\uparrow \frac{1}{2}) = (\pm \frac{1}{2}) g_e \beta H_z \]  

(2-7)

Transitions can be induced between the levels by irradiating at the frequency given by the resonance condition:
\[ \Delta E = h\nu = g_e \beta H_z \quad (2-8) \]

Our spectrometer operates with \( \nu \approx 9.3 \text{ GHz} \) and the resonance condition occurs at about 3300 gauss.

In atoms and molecules, there is usually another contribution to the magnetic moment from electronic orbital motion. This contribution, from spin-orbit coupling, can only arise from non-spherical orbitals and introduces an orientation dependence into the Hamiltonian. Because \( g \) is a measure of the effective magnetic moment associated with an angular momentum \( S \), this orientation dependence is included in the Hamiltonian as an anisotropy in the \( g \) factor. A more general expression for the electronic Zeeman interaction is:

\[ \mathcal{H} = \beta H \cdot g \cdot S \quad (2-9) \]

where \( g \) is a symmetric tensor. The expression \( \mathcal{H} \cdot g \cdot S \) written in full becomes:

\[
\mathcal{H} = \begin{pmatrix} H_x, H_y, H_z \end{pmatrix} \left( \begin{array}{ccc} g_{xx} & g_{xy} & g_{xz} \\ g_{yx} & g_{yy} & g_{yz} \\ g_{zx} & g_{zy} & g_{zz} \end{array} \right) \begin{pmatrix} s_x \\ s_y \\ s_z \end{pmatrix} \quad (2-10)
\]

in terms of some convenient Cartesian axis system. A suitable reference frame can always be chosen to diagonalize the \( g \) tensor which is then represented by its principal values: \( g_{xx}', g_{yy}', g_{zz}' \).

In the organic free radicals we have studied, the orbital angular momentum is strongly quenched so the \( g \)-tensor is close to the free electron
value $g_e$. Furthermore, as will be shown later, the g-value does not enter the equations for ENDOR transitions below second-order. Therefore, in the following discussion we will restrict ourselves to an isotropic g-factor.

2.2.2 The Nuclear Zeeman Interaction

If a nucleus in the free radical also possesses a spin, an expression analogous to (2-4) can be written as:

$$\mu_N = g_N \beta_N I$$  \hspace{1cm} (2-11)

where $g_N$ is the nuclear g factor, $\beta_N$ is the nuclear magneton and $I$ is the nuclear spin angular momentum vector. The Hamiltonian which represents the electronic and nuclear Zeeman contributions is given by:

$$\mathbf{H}_z = g \beta \mathbf{S} \cdot \mathbf{H} - \sum_{i=1}^{K} g_N \beta_N \mathbf{H} \cdot \mathbf{I}_i$$  \hspace{1cm} (2-12)

where the summation is over all nuclei with $I \neq 0$. The anisotropy in the nuclear g-factor has been neglected as it is very small.

2.2.3 The Hyperfine Interaction

If a free radical contains at least one nucleus with $I \neq 0$, the unpaired electron will experience a local magnetic field due to this nucleus. The magnitude of this local field is determined by the electronic structure of radical, the magnetic moment of the nucleus and the orienta-
tion of the nuclear spin in the total field which it experiences. Since
the nuclear spin is quantized along this total field, the total field
experienced by the electron depends on the spin state of the nucleus.
Thus as the spectrometer's magnetic field is swept, the resonance con­
dition will be met as many times as there are nuclear spin states and
the spectrum will contain (2I+1) lines.

The classical interaction energy between two magnetic moments \( \mu_e \)
and \( \mu_N \) is given by

\[
E = r^{-3}(\mu_e \cdot \mu_N) - 3r^{-5}(\mu_e \cdot r)(\mu_N \cdot r)
\]  

(2-13)

where \( r \) is the radius vector from \( \mu_e \) to \( \mu_N \), with \( r \) as its magnitude.
The quantum mechanical version of (2-13) is obtained by the substitutions

\[
\mu_e = -gBS \quad \text{and} \quad \mu_N = g_N^e I
\]  

(2-14)

This yields the dipolar interaction Hamiltonian

\[
\mathcal{H}_{\text{Dip}} = -gSg_N \beta_N \left[ r^{-3}(I \cdot S) - 3r^{-5}(I \cdot r)(S \cdot r) \right]
\]  

(2-15)

Expression (2-15) must be averaged over the entire probability distribution
\( |\psi(r)|^2 \) of the electron. The integral over the spatial coordinates will
be of the form:

\[
\int \int \int \psi^*(r, \theta, \phi) \mathcal{H}_{\text{Dip}} \psi(r, \theta, \phi) \, dr \, d\theta \, d\phi
\]  

(2-16)
Unless the wave function goes to zero very rapidly at \( r=0 \), the integrand becomes infinite at this point in analogy to the break down of the point-dipole treatment for classical magnets. The integral only becomes tractable if the unpaired electron is in an orbital with a node at the nucleus (\( \ell > 0 \)). Expanding the scalar products in (2-15) in a Cartesian coordinate system with the nucleus at the origin we obtain:

\[
\mathbf{H}_{\text{dip}} = -g\beta g_N \beta_N \left\{ r^{-5} \left[ (r^2-3x^2)I_x S_x + (r^2-3y^2)I_y S_y \right. \right.
\]
\[
+ \left. (r^2-3z^2)I_z S_z \right\} - 3xyr^{-5}(I_x S_y + I_y S_x)
\]
\[
- 3yzr^{-5}(I_y S_z + I_z S_y) - 3xsr^{-5}(I_x S_z + I_z S_x) \tag{2-17}
\]

where \( r = (x^2 + y^2 + z^2)^{1/2} \).

The integration in (2-16) can now be carried out term by term.

It is clear that the spin Hamiltonian for dipolar coupling can be written in tensorial form:

\[
\mathbf{H}_{\text{Dip}} = \mathbf{S} \cdot \mathbf{T} \cdot \mathbf{I} \tag{2-18}
\]

Furthermore, addition of the first three terms of \( \mathbf{T} \), the anisotropic hyperfine tensor, shows that the trace of the tensor is zero. This means that dipolar coupling does not contribute to the resonance line positions in the spectrum of a radical undergoing rapid tumbling in a highly fluid solution. A second consequence of the traceless tensor is that the dipolar coupling vanishes when the electron cloud is spherical, as for an electron in an atomic s orbital.
The magnetic moments of the electron and nucleus are also coupled via the Fermi contact interaction\textsuperscript{37}, representing the energy of the nuclear moment in the magnetic field produced at the nucleus by electric currents associated with the spinning electron. It has the form

\[ \mathcal{H}_{\text{iso}} = \frac{8\pi}{3} g\beta g_N \beta_N \delta(r) S \cdot I = a S \cdot I \]  \hspace{1cm} (2-19)

where \( a \) is the isotropic hyperfine coupling constant and the \( \delta \) function imposes the condition that \( r = 0 \) in the integral over the electron coordinates. As a result the constant \( a \) is proportional to the squared amplitude of the electronic wave function.

\[ a = \frac{8\pi}{3} g\beta g_N \beta_N \left| \Psi(0) \right|^2 \]  \hspace{1cm} (2-20)

The contact interaction can only occur when the electron has a finite probability density at the nucleus. Consequently, for the interaction to be present, the electron must have some s-orbital character, or the nucleus must be within an orbital centred on another nucleus.

The Hamiltonian for the overall hyperfine coupling is:

\[ \mathcal{H}_{\text{Hyp}} = \mathcal{H}_{\text{iso}} + \mathcal{H}_{\text{Dip}} = a S \cdot I + S \cdot T \cdot I = S \cdot A \cdot I \]  \hspace{1cm} (2-21)

The evaluation of this term is of dominant importance in this thesis as it is very useful in determining the electron distribution in a radical.
2.2.4 The Quadrupole Interaction

Nuclei with I \geq 1 have quadrupole moments because their charge distributions can deviate from spherical symmetry. The symmetry axis, z, of the distribution is also the axis of the spin and the magnetic moment. The nuclear quadrupole moment Q, the magnitude of which is a measure of the deviation of the charge distribution from spherical symmetry, is defined by

\[ eQ = \int \rho_N(3z^2 - r^2) dV \]  \hspace{1cm} (2-22)

where \( \rho_N \) is the distribution function of the nuclear charge, z is the z-coordinate of the charge element a distance from the origin and the integral is evaluated over the volume of the nucleus. This moment interacts with an inhomogenous electric field, which is often found in molecules due to the non-spherical distribution of their electrons. The energy of the interaction depends on the magnitude of the quadrupole moment and the gradient of the electric field, and can be written:

\[ E_Q = \frac{1}{6} \sum_{\alpha, \beta} V_{\alpha\beta} Q_{\alpha\beta} \]  \hspace{1cm} (2-23)

where \( V \) is the potential produced by the electrons, \( \alpha, \beta = x, y, z \),

\[ V_{\alpha\beta} = \left. \frac{\partial^2 V}{\partial \alpha \partial \beta} \right|_{r=0} \]  and

\[ Q_{\alpha\beta} = \int (3\alpha \beta - \delta_{\alpha\beta} r^2) \rho_N d\tau . \]  \hspace{1cm} (2-24)
Equation (2-23) can be converted to the quantum mechanical expression by replacing \( Q_{\alpha\beta} \) with \( \hat{Q}_{\alpha\beta} \), the quadrupole operator.

\[
\hat{Q}_{\alpha\beta} = e \sum_k^{\text{nuclei}} (3\alpha_k^\beta r_k^2 - \delta_{\alpha\beta} r_k^2).
\]  

(2-25)

The Wigner-Eckhart theorem implies that the quadrupole interaction can be expressed in terms of the nuclear spin instead of the charge distribution. The matrix elements of \( \hat{Q}_{\alpha\beta} \) can then be shown to be

\[
Q_{\alpha\beta} = \frac{eQ}{I(2I-1)} \left\{ \frac{3}{2}(I_\alpha I_\beta + I_\beta I_\alpha) - \delta_{\alpha\beta} I^2 \right\}
\]  

(2-26)

and the Hamiltonian is:

\[
\mathbf{H}_Q = \frac{eQ}{6I(2I-1)} \sum_{\alpha,\beta} V_{\alpha\beta} \left\{ \frac{3}{2}(I_\alpha I_\beta + I_\beta I_\alpha) - \delta_{\alpha\beta} I^2 \right\}.
\]  

(2-27)

As can be seen from (2-27), this can be expressed more compactly as a tensor coupling of the nuclear spin with itself:

\[
\mathbf{H}_Q = I \cdot \mathbf{P} \cdot I
\]  

(2-28)

The potential \( V \), in general, satisfies Laplace's equation \((\nabla^2 V = 0)\) so the field gradient tensor is traceless. As a result the quadrupole coupling tensor is also traceless.

The quadrupole interaction affects the orientation of the nuclear spin, whose axis is colinear with that of the quadrupole moment in the absence of any external fields. Therefore the energies of the hyperfine
levels are determined by the quadrupole interaction as well as the magnetic Zeeman and hyperfine interactions. The total Hamiltonian we need to describe a free radical in a solid is:

\[ H = g_\mu H \cdot S + \sum_{i} (S \cdot A_i \cdot I_i + I_i \cdot P_i \cdot I_i - g_N \beta_N H \cdot I_i) \]  (2-29)

where we make the assumption that the nuclei are independent of each other.

2.3 The EPR Spectrum

In the following sections we will show how EPR and ENDOR spectra can be described in terms of their respective Hamiltonians. A simple example will first be demonstrated in order to illustrate the basic principles of both EPR and ENDOR. A more detailed description of how tensor parameters are obtained from ENDOR spectra will appear in later sections.

2.3.1 Energy Levels for a $S = \frac{1}{2}$, $I = \frac{1}{2}$ System.

The sample problem we shall solve involves the Hamiltonian for a free radical tumbling rapidly in solution and containing only one nucleus of spin $I = \frac{1}{2}$, all other nuclei having $I = 0$. The spin Hamiltonian is:

\[ H = g_\mu H \cdot S - g_N \beta_N H \cdot S + a S \cdot I \]  (2-30)
The energy levels are determined by solving the equation:

$$ H \Phi = E \Phi $$

(2-31)

This equation is usually solved with the aid of perturbation theory, in which the operator $H$ is separated into two distinct parts, $H_0$ and $H_1$. $H_0$ is the main component of the Hamiltonian and $H_1$ is treated as a small perturbation. The spin wave functions are then expressed in terms of linear combinations of the basis functions $\phi_n$ which are chosen to be eigenfunctions of $H_0$. The eigenvalues of $H_0$ are the unperturbed energies $\epsilon_n$. The perturbation $H_1$ yields modified wave functions and energies of the form

$$ \gamma_n = \phi_n - \sum_{m \neq n} \frac{\langle m | H_1 | n \rangle}{\epsilon_m - \epsilon_n} \phi_m $$

(2-32)

$$ E_n = \epsilon_n + \langle n | H_1 | n \rangle - \sum_{m \neq n} \frac{\langle m | H_1 | n \rangle \langle n | H_1 | m \rangle}{\epsilon_m - \epsilon_n} $$

(2-33)

The two terms on the right-hand side of (2-33) are the first-order and second-order corrections, respectively, and $\langle n | H_1 | n \rangle$ and $\langle m | H_1 | n \rangle$ are the matrix elements of $H_1$. An example of this matrix appears in expression (2-43).

For the electron, there are two allowed components of the spin along any direction. In an applied magnetic the electron spin is quantized along the field direction, $z$. The two possible spin functions will be denoted by $|\alpha\rangle$ and $|\beta\rangle$ with quantum numbers $M_s = \frac{1}{2}$ and $-\frac{1}{2}$, respectively.
respectively. This can be stated as:

\[ S_z |\alpha_e\rangle = \frac{1}{2} |\alpha_e\rangle \]
\[ S_z |\beta_e\rangle = -\frac{1}{2} |\beta_e\rangle \quad (2-34) \]

It is also convenient to introduce two new operators and the raising and lowering operators:

\[ S^+ = S_x + iS_y \]
\[ S^- = S_x - iS_y \quad (2-35) \]

These operators have the following properties

\[ S^+ |\alpha_e\rangle = 0 \]
\[ S^+ |\beta_e\rangle = |\alpha_e\rangle \text{ so that } \langle\alpha_e | S^+ |\beta_e\rangle = 1 \]
\[ S^- |\beta_e\rangle = 0 \]
\[ S^- |\alpha_e\rangle = |\beta_e\rangle \text{ so that } |\beta_e | S^- |\alpha_e\rangle = 1 \]

Operators completely analogous to \( S_z \), \( S^+ \), and \( S^- \) also exist for the nuclear spin.

Each orientation of the electron spin can be associated with either of the two nuclear spin orientations so the basis functions are chosen as products of the electron and nuclear spin functions:

\[ \phi_1 = |\alpha_e \alpha_N\rangle, \phi_2 = |\alpha_e \beta_N\rangle, \phi_3 = |\beta_e \alpha_N\rangle, \phi_4 = |\beta_e \beta_N\rangle \quad (2-37) \]
These functions are all eigenfunctions of $\mathbf{H}_0$ and the matrix of $\mathbf{H}_0$ is diagonal. For example,

$$
\mathbf{H}_0 |\alpha e_N^> = g\beta H_S z |\alpha e^> - g_N^\beta H^z |\alpha e^> = \frac{1}{2}g\beta H |\alpha e_N^> + \frac{1}{2}g N^\beta H |\alpha e_N^>
$$

(2-38)

and $\varepsilon_N = \frac{1}{2} g\beta H + \frac{1}{2} g N^\beta H$ .

(2-39)

The perturbation $\mathbf{H}_1$, the hyperfine interaction, is now added to the Hamiltonian so that

$$
\mathbf{H} = \mathbf{H}_0 + \mathbf{H}_1 = g\beta H_S z - g_N^\beta N H_I^z + a S^z I .
$$

(2-40)

Expanded in terms of the Cartesian coordinates, this becomes

$$
\mathbf{H} = g\beta H_S z - g_N^\beta N H_I^z + a (S_x x + S_y y + S_z z).
$$

(2-41)

or, using equation (2-36),

$$
\mathbf{H} = g\beta H_S z - g_N^\beta N H_I^z + a S^z I + \frac{a}{2} (S^+ I^- + I^+ S^-).
$$

(2-42)

Using the zeroth-order wave functions, the matrix of $\mathbf{H}$ is:

\[
\begin{array}{cccc}
|\alpha e_N^> & |\alpha e^> & |\beta e_N^> & |\beta e^> \\
|\alpha e_N^> & \frac{1}{2}Z_e - \frac{1}{2}Z_n + \frac{1}{4}a & 0 & 0 \\
|\alpha e^> & 0 & \frac{1}{2}Z_e + Z_n - \frac{1}{4}a & \frac{1}{2}a \\
|\beta e_N^> & \frac{1}{2}a & \frac{1}{2}Z_e - \frac{1}{2}Z_n - \frac{1}{4}a & 0 \\
|\beta e^> & 0 & 0 & -\frac{1}{2}Z_e + \frac{1}{2}Z_n + \frac{1}{4}a
\end{array}
\]

(2-43)
where $Z_e = g\beta H$ and $Z_n = g_N\beta_{N} H$. The eigenvalues and eigenfunctions of $H$ can be determined by diagonalization of the above matrix. The procedure is straightforward but tedious. The same result is obtained using the perturbation formulae (2-32) and (2-33), where the second-order terms involve the off-diagonal elements of the above matrix. The eigenvalues and eigenfunctions are:

$$
\begin{align*}
\varepsilon_1 &= \frac{1}{2}g\beta H - \frac{1}{2}g_N\beta_{N} H + \frac{1}{2}a \\
\varepsilon_2 &= \frac{1}{2}g\beta H + \frac{1}{2}g_N\beta_{N} H - \frac{1}{2}a + \frac{a^2}{4(g\beta H + g_N\beta_{N} H)} \\
\varepsilon_3 &= -\frac{1}{2}g\beta H - \frac{1}{2}g_N\beta_{N} H - \frac{1}{2}a - \frac{a^2}{4(g\beta H + g_N\beta_{N} H)} \\
\varepsilon_4 &= -\frac{1}{2}g\beta H + \frac{1}{2}g_N\beta_{N} H + \frac{1}{2}a \\
\end{align*}
$$

(2-44)

For many free radicals, the hyperfine coupling energy is much less than the electron Zeeman energy so the second-order corrections approach zero and may be omitted from the above equations. The energy levels listed above are shown in Fig. 2, in terms of successive splittings by the electron Zeeman, nuclear Zeeman, and hyperfine fields.

### 2.3.2 EPR Selection Rules

The experimental arrangement of an EPR spectrometer is such that an oscillating magnetic field, produced by microwave radiation, is applied
Fig. 2. Spin energy levels of an $S = \frac{1}{2}$, $I = \frac{1}{2}$ system, with first-order EPR transitions.
perpendicular to the steady field \( H_z \), in the \( x \) direction. If the strength of the oscillating field is \( 2H_1 \cos \omega t \), the resulting time-dependent perturbation on the atom is:

\[
V(t) = 2(\beta g_1 S_x - \beta g_n N_1 I_x) \cos \omega t = 2V \cos \omega t
\]  

(2-45)

The transition probability from state \( n \) to state \( m \) is equal to

\[
P_{nm} = \frac{2\pi}{\hbar^2} |\langle n | V | m \rangle|^2 \delta(\omega_{mn} - \omega)
\]  

(2-46)

where \( \omega_{mn} = E_m - E_n \) expressed in frequency units. As the electron resonance transitions are caused by the effect of \( H_1 \) on the electron spins, the nuclear spin operator \( I_x \) can be omitted from (2-45). The transition probability is therefore:

\[
P_{nm} = \frac{2\pi}{\hbar^2} g^2 \beta^2 H_1^2 |\langle n | S_x | m \rangle|^2 \delta(\omega_{mn} - \omega)
\]  

(2-47)

Evaluation of this expression for the unmodified spin wave functions yields the first-order EPR selection rules:

\[
\Delta m_s = \pm 1 ; \quad \Delta m_I = 0
\]  

(2-48)

These transitions are marked by the single arrows in Fig. 2. The frequencies of the transitions are:
\[ h\nu_a = (\frac{1}{2}g\beta H - \frac{1}{2}g_N \beta_N H + \frac{1}{2}a) - (\frac{1}{2}g\beta H - \frac{1}{2}g_N \beta_N H - \frac{1}{2}a) \]
\[ = g\beta H + \frac{1}{2}a \]  
(2-49)

\[ h\nu_b = (\frac{1}{2}g\beta H + \frac{1}{2}g_N \beta_N H - \frac{1}{2}a) - (\frac{1}{2}g\beta H + \frac{1}{2}g_N \beta_N H + \frac{1}{2}a) \]
\[ = g\beta H - \frac{1}{2}a \]  
(2-50)

The first-order EPR spectrum therefore consists of two lines separated by \( a \), the hyperfine coupling constant. The transition probabilities for the two lines are equal and hence the lines have equal intensities. Furthermore, the line positions are insensitive to the sign of "a" so the sign of the hyperfine coupling must be determined by other methods.

If the second-order energy corrections are included in (2-49) and (2-50), the separation between the resonance lines remains the same, but they are no longer centred about \( g\beta H \). Their intensities are reduced slightly through small changes in the spin wave functions. The transition \( \alpha e_N \leftrightarrow \beta e_N \), which is strictly forbidden to first-order, also becomes weakly allowed if \( H_1 \) is polarized parallel to the static field.

2.3.3 Thermal Equilibrium and Spin Relaxation.

The EPR spectrometer's detection system is sensitive to the net absorption of microwave energy by the spin system. This requires that there be a population difference between the upper and lower electronic Zeeman levels. The spin populations of the two levels are equal in the absence of a magnetic field, since they are degenerate. Because EPR spectra are observed, there must be some mechanism through which
the spin system returns to thermal equilibrium after application of a magnetic field. This mechanism is termed spin-lattice relaxation and involves interactions between the electrons and their environments which cause the spin orientation to change, with the excess energy being transferred to other degrees of freedom.

The spin system is coupled to the thermal motions of the lattice, which is a general term for the spin's environment and is not restricted to solids. For example, in a liquid the thermal tumbling produces randomly fluctuating magnetic fields through the motion of electron and nuclear spins in the solute and solvent molecules. The random field at a particular electron spin will, in general, contain a component at the resonance frequency that can induce transitions between the levels. The efficiency of this relaxation depends on the molecular structure and physical state of the spin's environment and can be specified by a parameter with the dimensions of time, the spin-lattice relaxation time \( T_1 \). This parameter characterizes the rate at which the bulk magnetization will approach its thermal equilibrium value through spontaneous, non-radiative transitions.

The microwave-induced transitions discussed earlier have equal transition probabilities in either direction. As a result, it can be shown that application of the resonant microwave field results in exponential decay of the population difference (in the absence of spin-lattice relaxation). However, because the lattice is at thermal equilibrium, the probabilities of spontaneous spin transitions up and down are not equal. Combination of these two competing effects leads to an
expression for the rate \( \frac{dE}{dt} \) of absorption of microwave energy:

\[
\frac{dE}{dt} = n_0 (E_m - E_n) \frac{P}{(1 + 2PT_1^2)}
\]

(2-51)

where \( n_0 \) is the population difference at thermal equilibrium and \( P \) is the transition probability in Equation (2-47). The probability \( P \) is directly proportional to the square of the alternating magnetic field, \( H_1^2 \). As long as \( 2PT_1 << 1 \), the power absorbed by the spin system can be increased by increasing the incident microwave power. However, once \( P \sim \frac{1}{2T_1^2} \), the power absorbed levels off despite an increase in \( P \). The effect is called saturation. In an EPR experiment one normally operates with low microwave power to avoid saturation. The ENDOR experiment however requires at least partial saturation of an EPR transition to produce a spectrum.

The transition probability equation (2-47) implies that absorption will only occur at precisely the resonance frequency and the spectral lines will be infinitely narrow. This lineshape is not experimentally observed because of mechanisms which broaden the \( \delta \)-function to a finite linewidth. Spin relaxation, which restores thermal equilibrium, endows the spin states with a finite lifetime. In accordance with the Heisenberg Uncertainty Principle, this results in an uncertainty in the energy of the spin states and hence transitions can occur over a range of frequencies centred about the resonance frequency described earlier. If the spin-lattice relaxation were very efficient, corresponding to a very short \( T_1 \), it is possible that the linewidth could become so large that the resonance could not be detected.
Linewidths are also determined by a different type of relaxation which does not involve the exchange of energy between the spin system and the lattice. The energy levels are modulated by effects such as rapidly recurring conformational changes of the radical or fields produced by unpaired electrons in neighbouring radicals. Because the energy levels are no longer sharp, a band of energy exists over which transitions can occur. These processes are termed transverse relaxation, and are characterized by the transverse relaxation time, \( T_2 \).

2.3.4 Hyperfine Interaction With More Than One Nucleus

Since, to first order, the nuclear Zeeman interaction does not affect EPR line positions, it will not be included in the following discussion. The first-order spin Hamiltonian for an electron coupled to more than one nucleus can be written as:

\[
\mathcal{H} = g\beta H B Z S + \sum_{i} a_{i} I^{(i)} Z S_{Z}
\]  

with eigenvalues

\[
E = g\beta H m_{Z} s + \sum_{i} a_{i} m_{I}^{(i)} m_{s}
\]

It can be shown that the resulting number of \( \Delta m_{Z} = 1, \Delta m_{I} = 0 \) transitions is:

\[
n = \prod_{i} (2I^{(i)} + 1)
\]
For example, there are $2^x$ transitions in the spectrum of an unpaired electron interacting with $x$ protons. This number is reduced if several of the nuclei have identical hyperfine couplings, either by coincidence or by symmetry. For the $k$ equivalent nuclei, it is convenient to specify the total resultant nuclear spin quantum number $m_F$:

$$ m_F = \sum_{k} m_{i}^{(k)} k^m_I $$  (2-55)

The hyperfine levels now have degeneracies equal to the number of combinations of the $k m_{i}$'s which result in the particular $m_F$. These degeneracies are directly reflected in the line intensities and, for $I = \frac{1}{2}$, are just the coefficients in the binomial expansion of $(1 + x)^k$.

The EPR spectrum of a radical in which the unpaired electron is coupled to many nuclei often contains so many lines that many of them overlap and the spectrum cannot be analyzed. In the solid state there is a further complication as a result of the anisotropic hyperfine interaction. The line positions will vary with the magnetic field orientation and unless the lines are well separated, their angular variations cannot be followed. These are two of the major reasons why the ENDOR technique is so useful, as will be discussed in the following sections.

2.4 The ENDOR Experiment

2.4.1 A Simple Description of the ENDOR Experiment.

In order to describe the basic principles of the ENDOR experiment, we shall use the example of a single unpaired electron interacting with
a single proton through an isotropic hyperfine interaction. Thus the Hamiltonian is:

\[ \mathcal{H} = g_N N S - g_N N H - I + a S I \]  

(2-56)

where "a" is expressed in frequency units. To first order, the energy levels for this system are:

\[ E(m_s, m_I) = \nu_e m_s - \nu_N m_I + am_s m_I \]  

(2-57)

where \( \nu_e = g_N H \) and \( \nu_N = g_N B H / h \). These energy levels are shown in Fig. 3 which is drawn with the assumption that \( a/2 < \nu_N \). This assumption is valid for many of the weak proton couplings of special interest to the ENDOR spectroscopist.

The relative populations of the levels at thermal equilibrium are given by the Boltzmann distribution. Because the nuclear Zeeman energy is much smaller than that of the electron, the population differences between the levels \( |m_s, 1/2\> \) and \( |m_s, -1/2\> \) can be ignored. For levels with different \( m_s \), the ratio of the number of spins in the lower state, \( N_a \), to those in the upper state, \( N_b \), is given by:

\[ \frac{n_a}{n_b} = \exp(gN H/kT) \]  

(2-58)

where \( k \) is the Boltzmann constant. At the temperature used in these
Fig. 3. The ENDOR experiment. (a) Thermal equilibrium populations of the energy levels. (b) Idealized populations after saturation of the EPR transition.
experiments, the electron Zeeman energy is small compared to kT, and the exponential in Equation (2-58) can be expanded with only the first-order term in $g\beta H/kT$ retained. The thermal equilibrium populations can then be calculated and are shown in Fig. 3, where $\xi = g\beta H/kT$.

If sufficient microwave power is applied to one of the EPR transitions, say the $|-\frac{1}{2}, \frac{1}{2}>$ to $|\frac{1}{2}, \frac{1}{2}>$ transition, the populations of the two levels are equalized. Furthermore, if the electron and nuclear spins relax through completely independent mechanisms, the saturation of the electron resonance does not effect the other spin populations. The idealized populations after saturation of the electron resonance are also shown in Fig. 3.

In the ENDOR experiment, a radio frequency field is also applied to the spin system while continuing to saturate the EPR transition and monitor its absorption intensity. As the radio frequency is varied, it will match the separation of the $|\frac{1}{2}, \frac{1}{2}>$ and $|\frac{1}{2}, -\frac{1}{2}>$ levels and induce transitions between them when:

$$v_{rf} = v_N - \frac{1}{2}a$$  \hspace{1cm} (2-59)

The net effect of this is to remove spins from the $|\frac{1}{2}, \frac{1}{2}>$ level, thereby restoring a population difference between the $|-\frac{1}{2}, \frac{1}{2}>$ and $\frac{1}{2}, \frac{1}{2}>$ levels. The EPR transition is no longer saturated and there is a resulting increase in microwave power absorption. Thus the ENDOR spectrum is a display of the enhancement of a partially saturated EPR absorption as a function of the radio frequency. The response has a linewidth more nearly
that of the narrower nuclear resonance absorption than the electron resonance absorption, which is being monitored.

As the radio frequency is further increased, the separation between the $|{-\frac{1}{2}, \frac{1}{2}}\rangle$ and $|{-\frac{1}{2}, -\frac{1}{2}}\rangle$ levels will also be matched when:

$$\nu_{rf} = \nu_{N} + \frac{1}{2}a$$

(2-60)

In this case, there are initially more spins in the $|{-\frac{1}{2}, -\frac{1}{2}}\rangle$ level than in the $|{-\frac{1}{2}, \frac{1}{2}}\rangle$. The application of a rf field results in an induced net emission of spins to the $|{-\frac{1}{2}, \frac{1}{2}}\rangle$ level, restoring a population difference between $|{-\frac{1}{2}, \frac{1}{2}}\rangle$ and $|{\frac{1}{2}, \frac{1}{2}}\rangle$, and consequently enhancing the EPR absorption. The complete ENDOR spectrum for this system consists of two lines centred about $\nu_{N}$ and separated by the hyperfine coupling constant. If $\frac{a}{2} > \nu_{N}$, then the ENDOR transition frequencies are the absolute value of expressions (2-59) and (2-60). The spectrum then consists of two lines centred about $\frac{a}{2}$ and separated by $2\nu_{N}$. Because of the symmetry of expressions (2-59) and (2-60), the sign of the hyperfine coupling constant cannot be determined from the ENDOR spectrum.

2.4.2 The Anisotropic Hyperfine Interaction.

The EPR and ENDOR line positions of free radicals trapped in single crystals often display an orientation-dependent hyperfine splitting resulting from the dipolar contribution to the hyperfine interaction. In this section, we will present an analytical solution for the energy levels of the spin Hamiltonian:
in order that the elements of $A$ can be obtained from the orientation dependence of the ENDOR line positions.

In the preceding section we have seen that, to first order, the ENDOR transition frequencies are independent of the electron Zeeman energy. Therefore, we need only concern ourselves with the quantization of the nuclear spin. For many of the spin systems studied by ENDOR, the hyperfine splitting is comparable to the nuclear Zeeman splitting and the nuclear spin is quantized along the resultant of these terms:

$$(S \cdot A - g_N \beta_N H) \cdot I$$

which to first order is:

$$(m_S h \cdot A - g_N \beta_N H) \cdot I = K \cdot I$$

where $h$ is a unit vector along the external field direction. The energy levels are given by $m_I |K|:

$$m_I |K| = (m_S h \cdot A^2 \cdot h - 2m_S g_N \beta_N H h \cdot A \cdot h + (g_N \beta_N H)^2)^{\frac{1}{2}} m_I$$

where $H = H_h$.

The nuclear transitions are induced by an oscillating rf field $H_2$ in the $y$-direction if the static field is along $z$. The first-order ENDOR
selection rules can be determined in a manner analogous to that shown for EPR in Sec. 2.3.2 using the time-dependent perturbation:

$$\mathcal{H}(t) = (g_B H_x S_x - g_N H_N H_x) \cos \omega t \quad .$$  \hspace{1cm} (2-65)

The ENDOR selection rules are thus found to be:

$$\Delta m_s = 0, \Delta m_I = \pm 1 \quad .$$  \hspace{1cm} (2-66)

The ENDOR transition frequencies are then:

$$v_{\pm}^2 = m_s^2 h_A^2 h_A + 2m_s m_N h_A h + \frac{g^2 \beta^2 H^2}{N} \quad .$$  \hspace{1cm} (2-67)

and if both transitions (for the $I = \frac{1}{2}$ case) are observed the hyperfine tensor can be determined using the equation:

$$v_+^2 - v_-^2 = 2v_p h_A h \quad .$$  \hspace{1cm} (2-68)

where the subscript signs refer to the sign of $m_s$.

If for example, a crystal is mounted in a spectrometer such that the magnetic field is rotated in the yz plane, equation (2-68) becomes:

$$v_+^2 - v_-^2 = 2v_p \sin^2 \theta (A_{yy}) + 4v_p \sin \theta \cos \theta (A_{yz}) + 2v_p \cos^2 (A_{zz}) \quad .$$  \hspace{1cm} (2-69)

where $\theta$ is the angle between the z-axis and the field direction. These
three elements of $A$ can be determined by measuring $v^2_+ - v^2_-$ for a minimum of three values of $\theta$ in this plane. The remaining elements can be obtained by remounting the crystal and rotating the magnetic field in the $zx$ and $xy$ planes.

When the hyperfine splitting is approximately 50 MHz or larger, the second-order corrections to the energy levels becomes significant. It is usually convenient to calculate the elements of $A$ to first order and then to refine these values using second-order perturbation theory.

2.4.3 The Quadrupole Interaction

The $^{14}$N nucleus has spin $I = 1$ and an appreciable quadrupole moment. The quadrupole term in the spin Hamiltonian was written in Equation (2-28) as a tensorial coupling of the nuclear spin with itself. In the principal axis system of the quadrupole tensor this becomes:

$$\mathcal{H} = P_{XX} I^2_X + P_{YY} I^2_Y + P_{ZZ} I^2_Z$$

(2-70)

For an axially symmetric quadrupole coupling, the interaction can be described using one parameter $P$:

$$\mathcal{H} = P[I_{ZZ}^2 - \frac{1}{3}I(I+1)]$$

(2-71)

where $P = \frac{3P_{ZZ}}{2}$. The first-order spin Hamiltonian containing an isotropic hyperfine coupling $a > 2v_N$:
\[ H = g\beta H S_z - g_N e H I_z + a S_z I_z + P[I^2 - \frac{1}{3}I(I+1)] \] (2-72)

is sufficient to illustrate the effect of the quadrupole term on the $^{14}$N ENDOR spectrum. The eigenvalues of this Hamiltonian:

\[ E_{m_s,m_I} = \nu m_s - \nu N m_I + a m_s m_I + P[m_I^2 - \frac{1}{3}I(I+1)] \] (2-73)

are shown in Fig. 4, with $a$ and $P$ both assumed to be positive. In the absence of a quadrupole interaction, the hyperfine levels in each $m_s$ manifold are equally spaced. Thus there is only one ENDOR transition frequency in each manifold, with the frequency given by:

\[ \nu_{rf} = |\nu_s \pm \frac{a}{2}| \] (2-74)

and the complete spectrum consists of only two lines.

The quadrupole interaction lifts the degeneracy of the ENDOR frequencies within each $m_s$ manifold, resulting in four different transition frequencies:

\[ \Delta 1 = \frac{3}{2}a + \nu_N - P \]
\[ \Delta 2 = \frac{3}{2}a + \nu_N + P \]
\[ \Delta 3 = \frac{3}{2}a - \nu_N - P \]
\[ \Delta 4 = \frac{3}{2}a - \nu_N + P \] (2-75)

Furthermore, the appearance of the ENDOR spectrum will now depend on the EPR transition being saturated. If the $|\frac{1}{2},0> to |\frac{1}{2},0>$ transition is saturated than all four ENDOR transitions can be observed. Saturation
Fig. 4. Energy levels for an $S = \frac{1}{2}$, $I = 1$ system.
of either of the other two EPR transitions results in only two lines in the ENDOR spectrum.

2.5 ENDOR Spectrum for an Electron Coupled to More Than One Nucleus

In Sec. 2.3.4, we introduced the Hamiltonian for an electron coupled to more than one nucleus and discussed the resulting expansion of the number of energy levels, which complicated analysis of the EPR spectrum. If internuclear interactions are neglected, the first-order energy levels are:

\[ E = m_s v_e - \nu (\sum_{m_I} (m_I) + \sum_{a_i m} (m_I)) \]

(2-76)

Applying the ENDOR selection rules:

\[ \Delta m_s = 0, \Delta m_I = \pm 1, \Delta m_{i \neq i} = 0 \]

(2-77)

where the spin of only one nucleus changes, the ENDOR transitions are described by:

\[ \nu_{rf} = |\nu_N (i) \pm \nu I_i| \]

(2-78)

Thus, the ENDOR spectrum consists of a pair of lines for each interacting, non-equivalent nucleus and can be analyzed in terms of an electron interacting with each nucleus separately. Equivalent nuclei only contribute a pair of lines to the ENDOR spectrum, although their intensity depends directly on the number of nuclei. The ENDOR spectrum is therefore often
much simpler than its parent EPR spectrum, which for $x$ nuclei may contain up to $2^x$ lines while the ENDOR spectrum consists of no more than $2^x$ lines.

2.6 Determination of Spin Hamiltonian Parameters

The procedures outlined in previous sections are very useful for explaining the gross features of an ENDOR spectrum. Many of the assumptions made in the discussion, however, are not valid for radicals trapped in a crystal. Analytical expressions for the ENDOR frequencies of oriented radicals are often difficult to obtain and cumbersome to use. In practice, the spin Hamiltonian parameters are best obtained by comparing the observed frequencies to those calculated by exact, computer diagonalization of the matrix, $\langle \Phi_a | \mathcal{H} | \Phi_b \rangle$, for the Hamiltonian:

$$\mathcal{H} = g_B \cdot S - g_N \beta_N H \cdot I + S \cdot A \cdot I + I \cdot P \cdot I$$

(2-76)

with appropriate spin functions and trial values of the parameters. The parameters are then adjusted by the method of least-squares until the calculated frequencies match the observed ones.

In this work we have used a computer program written by Dickinson and Hebden based on this approach. This program calculates the EPR or ENDOR transition frequencies for any specified spin Hamiltonian and performs the least-squares refinement of the Hamiltonian parameters using a multidimensional extended Newton-Raphson technique. The nuclear spin $I$ and initial values of the spectral parameters used as input for this program are obtained from the observed spectra by the first-order techniques described earlier.
CHAPTER THREE

EXPERIMENTAL METHODS

3.1 Introduction.

This chapter introduces the procedures and equipment used in these experiments. It will deal only with operation of the spectrometer and general treatment of the ENDOR data. Details on crystal preparation will be discussed in the chapters dealing with the individual samples.

3.2 The ENDOR Spectrometer.

The ENDOR spectrometer used in these experiments has been built around an X-band EPR spectrometer and is essentially the same as previously described by Dalai. There have been some modifications, though, and a revised block diagram of the spectrometer appears in Fig. 5. The spectrometer can be operated in either homodyne or superheterodyne mode but our experiments were performed solely in the superheterodyne mode, using an intermediate frequency of 30 MHz.

The source of microwave power was a Varian V-153C klystron with a Hewlett-Packard 716B power supply. The microwave frequency was stabilized by phase-locking to a Microwave Systems model MOS1 frequency stabilizer. Some of the microwave power was coupled out of the main waveguide with a 3dB directional coupler and fed into a balanced modulator
Fig. 5. Block diagram of the ENDOR spectrometer.
for the generation of sidebands. The sidebands were generated using Microwave Associates Varactor diodes type IN460A driven by 10 mA of current each at 30 MHz, which was obtained for convenience by multiplying a 10 MHz signal from the stabilizer. The balanced modulator was tuned such that the output power at the carrier frequency, \( v_0 \), was minimum while the power in the two sidebands was maximum. This output was passed through a high-Q transmission cavity (Model 585-BS2 of PRD Electronics Inc.), whereby one sideband is selected and used as a local oscillator for superheterodyne detection at a balanced detector using two IN23G diodes.

The main branch of microwave power at the carrier frequency was led through three 20dB attenuators to the cavity through a magic T bridge. Two different cavities were used, although both were rectangular and operated in a TE\(_{011}\) mode. The crystal could be mounted at the position of maximum microwave magnetic field on the bottom plate of one cavity or the narrow vertical side of the other. The reflected power from the cavity was led through the third arm of the magic T to the balanced detector mentioned above.

The detected 30 MHz intermediate frequency was first amplified by a LEL model IF31BP I.F. amplifier with a gain of 450 at a 3dB bandwidth of 8 MHz. After detection by a IN34 crystal, the output was processed by a PAR model 122 lock-in amplifier, which could operate at any frequency between 1.5 Hz and 150 KHz. The output of the lock-in amplifier was then recorded on an x-y recorder.

The steady Zeeman magnetic field was provided by a Varian 9" pole-face, rotatable electromagnet with a Mark II Fieldial power supply unit.
A signal proportional to the magnetic field was available from the power supply to drive the x-axis of the recorder. For EPR work, variable, audio-frequency magnetic field modulation was provided by modulation coils wound on the magnet pole-pieces. The coils were driven by the reference frequency output of the lock-in.

For ENDOR work, a second radio frequency was introduced at the sample with a three-turn coil of regular copper wire surrounding the sample inside the cavity. The r.f. source was a modified Marconi 1066B signal generator, which has a frequency range of 6 MHz to 240 MHz and can be frequency modulated to a depth of 5 to 100 kHz. The signal output was amplified by an ENI model 320 L r.f. power amplifier and led to the ENDOR loop by means of a coaxial cable. No serious attempt was made to match the loop to the amplifier over the range of frequencies used in this work. Thus the r.f. current in the ENDOR loop varied with change of frequency. However, this was not important because f.m. detection was used. The r.f. frequency was monitored by a Hewlett-Packard 5246L counter. Its digital output was converted by a Hewlett-Packard 508A digital-analog converter and used to drive the x-axis of the recorder. The counter also triggered a home-built digital event marker which produced a calibration pip in the spectrum once per 1 MHz interval.

The spectrometer was equipped with a glass double dewar system designed to cool the cavity to 4.2°K with liquid helium in the inner dewar and liquid nitrogen in the outer dewar. Our experiments were performed with liquid nitrogen in both dewars. This resulted in little
evaporation from the inner dewar provided the outer dewar was kept full. The outer dewar could also be refilled without destabilizing the spectrometer. The cavity was immersed in liquid nitrogen although care was taken to prevent it from entering the cavity.

3.3 The ENDOR Technique
3.3.1 Crystal Alignment

The directions of the anisotropic hyperfine tensors are of much greater value if they can be related to the orientation of the molecules in the crystal, as determined by x-ray crystal structure analysis. The methods used for identification of the crystal axes will be outlined in the chapters dealing with the individual compounds examined. The crystals were aligned in the cavity so that the magnetic field vector could be rotated in each of three orthogonal planes defined with respect to the crystal axes. The crystals under investigation were all monoclinic so, once ENDOR signals were observed, the alignment could be checked by studying the site splitting in the spectrum.

The site splitting arises because, in monoclinic crystals, radicals can occupy two magnetically inequivalent sites. Radicals occupying such sites are chemically identical but have different orientations in the crystal. If these different orientations are represented by two vectors, the radicals will have different spectra if the magnetic field makes unequal angles with the vectors. The difference between the line positions of the two radicals in the spectrum is termed the site splitting. Because the site splitting reflects the crystal symmetry and if there
is appreciable hyperfine anisotropy, the site splitting serves as a very
good indication of the degree of precision with which the crystal is
mounted.

In a monoclinic crystal, the sites are equivalent, and hence the
site splitting is zero, when the magnetic field vector is parallel with
or perpendicular to the b axis of the crystal. Using this information,
we found that we could align the crystal within 1° of the desired orienta-
tion. Because the crystal could not be re-aligned without disassembling
the cavity, it was very difficult to reduce this error.

3.3.2 Operation of the ENDOR Spectrometer

The spectrometer was allowed to stabilize for at least an hour with
the cavity maintained at 77°K. The EPR spectrum was then recorded and
a particular point on the EPR lineshape was selected for ENDOR study.
Because there was more than one radical present in the lineshape, the
point to be saturated was chosen more to isolate the radicals than to
obtain maximum ENDOR intensity.

The microwave power level for maximum EPR signal was determined
and the EPR transition was partially saturated by increasing the microwave
power above this level. The magnetic field modulation was then switched
off and the spectrometer was adjusted for higher sensitivity by increasing
the lock-in amplifier and recorder gains over two hundred-fold and
increasing the time constant to 3 seconds. The r.f. was then swept by
driving the tuning shaft of the signal generator by a slow, reversible,
synchronous motor, such that the sweep rate was not faster than 200 kHz
per minute. The r.f. was initially swept from 10-70 MHz in six separate
10 MHz scans and the spectrum was recorded at about 250 kHz/cm. The
frequency modulation used for detection was between 3000 Hz and 4000 Hz,
with a depth between 40 kHz and 60 kHz depending on the ENDOR linewidth.

Once the entire ENDOR spectrum had been observed and its intensity
optimized with the lock-in phase and microwave power level, the magnet
was rotated so that the angular variation of the spectrum could be
recorded. The magnet was rotated in increments of no more than 5° in
frequency ranges with no spectral overlap, or 2.5° near the free proton
frequency and when spectral lines were crossing. The magnet was rotated
through as much of a 180° arc as possible. Unfortunately, the spectro­
meter arrangement reduced the EPR or ENDOR transition probabilities to
zero at certain magnetic field orientations. For example, the microwave
field was parallel to the steady field at magnet orientations of 0° and
180°. This resulted in a total arc of about 60° in which no ENDOR
signals were observed because the EPR transition probabilities were too
small. At 90°, the r.f. field was parallel to the steady field and the
ENDOR transition probabilities approached zero. As a result, ENDOR
spectra could only be observed through a total arc of about 90°. This
was usually more than sufficient to determine the hyperfine tensors.
For those couplings where it was not, the crystal was re-aligned so that
the spectra for more orientations could be observed.

3.3.3 Data Reduction

The spectral line positions were determined by quadratic regression
analysis using the calibration marks in the spectrum. The limiting factor
in this procedure was the assignment of the line positions. In most cases, the line position could be taken as the centre of the line, where it crossed the baseline, and measured to an accuracy of 10 kHz. For overlapping lines which were asymmetric or did not cross the baseline, the line position was taken as the point of maximum slope. The errors in these measurements were considerably larger and could range up to 50 kHz.

The line positions were then plotted as a function of magnetic field orientation for each of the three orthogonal planes. For graphical presentation, the free proton frequency was set equal to 14.30 MHz and the observed proton transition frequencies were adjusted accordingly.

In each plane, resonances thought to belong to the same hyperfine coupling were fitted with a least-squares fitting procedure to the first-order expression:

\[ v^2 = a \sin^2 \theta + b \sin \theta \cos \theta + c \cos^2 \theta \]  

(3-1)

where \( v \) is the resonance frequency, \( \theta \) is the corresponding angle between a given axis and the magnetic field, and \( a \), \( b \), \( c \) are the fitting parameters. A programmable desk calculator (Monroe Model 1656) was used for this, as described in an earlier study. This procedure was especially useful in the free proton region where there were many overlapping lines. A few resonances thought to belong to the same coupling were fitted and used to predict the line positions at other orientations. The coupling was then refitted including any newly-found resonances. This procedure was then repeated until the angular variation in the plane was determined.
In most cases, the curve belonging to the same hyperfine coupling in each of the three planes was readily identified by comparing the curves in different planes at coincident axes. Experimentally observed points belonging to the same coupling were then fitted to the spin Hamiltonian with the least-squares adjustment program LSF, mentioned earlier, using an IBM 370/168 computer.
CHAPTER FOUR

INTERPRETATION OF SPIN HAMILTONIAN PARAMETERS

4.1 Introduction.

In Chapter Two, we dealt briefly with the relationship between the spin Hamiltonian parameters and the electronic structure of the radical. The hyperfine coupling tensor is the only parameter used for radical identification in this work. In this chapter, we shall present the methods used to deduce the radicals' electronic structure from the observed hyperfine tensors.

4.2 Coupling Tensors for $\alpha$-Protons.

In the radicals we have studied, the unpaired electron has occupied a molecular $\pi$ orbital delocalized over the carbon, nitrogen and oxygen skeleton of the molecule. This orbital, formed by overlap of the 2pz atomic orbitals, has a node in the molecular plane, which contains the ring hydrogen atoms. A proton adjacent to an atom possessing appreciable spin density in a p-type orbital and lying in the node of that orbital is conventionally called an $\alpha$-proton.
4.2.1 The Isotropic Hyperfine Coupling.

Because the $\alpha$-proton is in the node of the unpaired electron orbital, it should have zero isotropic coupling. In fact, the proton was experimentally observed to have a non-zero coupling which was directly proportional to the $2p$ spin population on the adjacent ring atom, as calculated by simple Hückel theory, for several aromatic hydrocarbons $^{43}$. This problem was first discussed, independently and simultaneously, by Jarrett $^{44}$, Weissman $^{45}$, Bersohn $^{46}$, and McConnell $^{47}$ in terms of correlation of the spins of the electrons in the C-H bond and the $\pi$-electrons in the ring. This correlation, which can be explained in terms of either valence bond or molecular orbital theory, results in a slight polarization of the electron spins in the C-H $\sigma$ bond. If the odd electron has spin $\alpha$, an excess $\beta$ spin is induced in the hydrogen $1s$ orbital and the hyperfine coupling constant is negative.

The extent to which the C-H $\sigma$ electrons are polarized is directly proportional to the $\pi$-electron spin density, $\rho_\pi$, representing the fractional probability of finding the unpaired electron in the carbon $2p_z$ orbital. This proportionality is usually expressed in terms of McConnell's relation $^{47}$:

$$a_H = Q_{CH}^H \rho_\pi$$  \hspace{1cm} (4-1)

where $Q_{CH}^H$ is the proportionality constant for a proton in a C-H fragment and has a value of about -63 MHz. The determination of the isotropic hyperfine coupling constant for an $\alpha$-proton is thus a very useful method
for deducing the electronic structure of a \( \pi \)-radical. McConnell's relation is valid for the N-H fragments we have observed as well, with an appropriate change in \( Q \). The assignment of a total, \( \alpha \)-proton hyperfine tensor to a specific \( X-H \) fragment in the radical is best made on the basis of the directions of the anisotropic hyperfine tensor.

4.2.2 The Anisotropic Hyperfine Tensor.

Experimental determination of \( \alpha \)-proton, anisotropic hyperfine tensors in several radicals has shown that the principal values of the tensors have approximately the form: \( t, 0, -t \), with the principal value \( "t" \) directed along the \( X-H \) bond direction, principal value \( "0" \) directed parallel to the axis of the \( p \) orbital and the principal value of \( "-t" \) directed perpendicular to the \( X-H \) bond, in the molecular plane.

These observations were first qualitatively explained in terms of the electronic structure of a \( \pi \)-radical by Ghosh and Whiffen. They examined the case of an isolated \( C-H \) fragment, with the unpaired electron in a carbon 2p orbital. The local symmetry of the problem required the three principal axes to lie along the directions experimentally observed for an \( \alpha \)-proton. Using the dipolar Hamiltonian given in expression (2-15), they determined the relative sizes and signs of the three principal values given by:

\[
-g_N B_N \langle (1 - 3 \cos^2 \theta) r^{-3} \rangle_{ave}.
\] (4-2)

where \( r \) is the distance from the electron to the nucleus, \( \theta \) is the angle between the radius vector and a principal axis of the coupling tensor,
and \( < >_{\text{ave}} \) signifies an average over the spatial distribution of the unpaired electron. The angular function \((1-3 \cos^2\theta)\) has a node at \(\theta_o \approx 55^\circ\) dividing space into two parts: in one part \((1-3 \cos^2\theta)\) is positive and in the other negative. The principal values of the tensor therefore depend on the orientation of the p orbital with respect to \(\theta_o\) in each of the three principal planes and are in qualitative agreement with experiment.

The principal values were calculated analytically by McConnell and Strathdee\(^{50}\) as soon as the importance of the above treatment was realized. Evaluation of the dipolar integrals given in expression (2-16) was complicated by the fact that the electron wave function was not centred on the proton, but could be performed in terms of polar coordinates centred on the carbon (or nitrogen) nucleus. The integrals were evaluated over Slater-type orbitals, with the assumption that the spin distribution was adequately represented by 2s- and 2p- atomic orbitals on the central atom and 1s atomic orbitals centred on the protons. The numerical results they obtained were in excellent agreement with values obtained for the radical in malonic acid\(^{27}\).

McConnell and Strathdee's calculation is not restricted to the anisotropic tensor of an \(\alpha\)-proton but can be applied to the interaction of the unpaired electron with any proton. However, the form of the tensor depends on the relative orientations of the unpaired electron's orbital and the proton and the distance between them. At sufficiently large distances, this treatment reduces to the expected point-dipole formula and the principal values of the tensor have axial symmetry.
In radicals which exhibit delocalization of the unpaired electron, the total anisotropic hyperfine tensor of a proton will not, in general, bear a simple relationship to the crystal structure. The assignment of the tensor to a particular proton is therefore made by comparison of its directions to theoretical predictions. The total tensor can be expressed as the sum of the interactions of the proton with the fraction of unpaired electron density localized in the 2s- and 2p- orbitals of each carbon, nitrogen or oxygen atom. We have used a program, provided by John Park and modified by us to account for different radical geometries, based on this approach. The interaction matrices of the proton with each C, N, or O atom were calculated separately using the McConnell-Strathdee equations, as corrected by Pitzer et al. and compiled by Barfield. These matrices were then added together to obtain the total interaction matrix. Diagonalization of this matrix gave the principal values and directions of the total tensor.

4.3 Isotropic Coupling Tensors for β-Protons.

Protons in free radicals are labelled by Greek letters depending on the number of bonds between them and the centre of spin density. For example, the methyl protons in the ethyl radical are two bonds distant from the unpaired electron and are called β-protons. The most notable feature of isotropic hyperfine splittings from β-protons is that they are at least equal to, or larger than the splittings expected for an α-proton replacing the substituent.
There has been considerable controversy in the literature over the mechanism of this coupling. A molecular orbital description involving hyperconjugation has frequently been invoked to explain $\beta$-proton couplings. Alternatively, simple valence bond theory has been used to describe methyl proton couplings, with the spin density at the protons arising through spin polarization. However, recent calculations are tending away from the spin polarization mechanism so we shall only discuss hyperconjugation.

Heller and McConnell found experimentally that the $\beta$-proton splitting is approximately equal to:

$$a_H(\theta) = Q_B(\theta)\rho$$

in analogy to the McConnell relation for $\alpha$-protons, where:

$$Q_B(\theta) = B_0 + B_2\cos^2\theta$$

In Equation (4-4), $\theta$ is the dihedral angle between the $\alpha$-carbon 2pz orbital and the plane containing the $\beta$-proton C-H bond. This $\cos^2\theta$ dependence can be explained by hyperconjugation. In order to qualitatively describe this mechanism, we shall use the example of the ethyl radical, $\text{CH}_2\text{CH}_3$.

The three hydrogen 1s orbitals $\phi_1$, $\phi_2$, $\phi_3$ can be combined to form a new basis set of orthogonal group orbitals:
\[ \gamma_1 = \frac{1}{\sqrt{3}} (\phi_1 + \phi_2 + \phi_3) \]  
\[ \gamma_2 = \frac{1}{\sqrt{6}} (2\phi_1 - \phi_2 - \phi_3) \]  
\[ \gamma_3 = \frac{1}{\sqrt{2}} (\phi_2 - \phi_3) \]

where \( \theta = 0^\circ \) for the \( \phi_1 \) proton. The wave function \( \gamma_2 \) has the correct
symmetry to interact with the \( 2p_z \) orbitals on the carbon atoms, where \( z \)
is perpendicular to the \( \text{H}_2\text{C}-\text{C'} \) plane. Three \( \pi \)-orbitals can be constructed
from \( \gamma_2 \) and the carbon \( 2p_z \) orbitals, and the odd electron can be thought
of as occupying an orbital of the form:

\[ \psi = ap + bp' + c\gamma_2 \]

where \( p \) and \( p' \) are the \( 2p_z \) orbitals on \( c \) and \( c' \), respectively.

This is an extreme approach to the electronic structure of the
radical but it provides a mechanism whereby the electron can penetrate
into the hydrogen \( 1s \) orbitals. Furthermore, this treatment predicts
positive spin density at the methyl proton, resulting in a positive coupling
constant, as can be confirmed by experiment. If the methyl group is
rotating rapidly with respect to the EPR time scale then an average
coupling given by

\[ \langle Q_B \rangle = \frac{\int 2\pi (B_o + B_2 \cos^2 \theta) d\theta}{\int 2\pi d\theta} = B_o + \frac{1}{2}B_2 \]

is observed for each proton.
4.4 Hyperfine Coupling for Ring Nitrogen Atoms

4.4.1 The Isotropic $N^{14}$ Hyperfine Coupling.

The theory of isotropic $N^{14}$ splittings is more complicated than for protons, although no new principles are introduced. The spin-polarization mechanism introduced in Section 4.2.1 that produces spin density at an $\alpha$-proton also produces spin density at the nucleus of the atom to which that proton is attached. The expression for this spin density produced at the ring atom yields a McConnell relation completely analogous to Equation (4-1).

This simple relation can fail if there is appreciable spin density on adjacent ring atoms. The unpaired $\pi$-electron density on the adjacent atoms, $X_1$ and $X_2$, polarizes the C-$X_1 \sigma$ bonds and induces negative spin density in the nitrogen 2s orbital. In such cases, the splittings conform to the equation:

$$ a_N = Q_N + \sum_i Q_{NX_i} \rho_{X_i} $$

However, the nuclear moment of $N^{14}$ is relatively small and the polarization produced by spin density on adjacent atoms can often be neglected. Thus the $N^{14}$ splitting is roughly proportional to the odd electron density in the nitrogen 2$p_z$ orbital, with a $Q$ value of about $75 \text{ MHz}$.

4.4.2 The Anisotropic $N^{14}$ Hyperfine Tensor.

Theoretical calculation of this tensor is analogous to the procedure outlined in Section 4.2.2 for $\alpha$-protons. The calculation is actually
more facile in this case because the electron wave function is centred on the nitrogen nucleus. The tensor possesses axial symmetry and the diagonal hyperfine tensor has the form: \((-B_0, -B_0, 2B_0\)), where:

\[
B_0 = \frac{2}{5} g g_N \beta N <r^{-3}>
\]  

(4-9)

The direction of the principal value \(2B_0\) is parallel to the nitrogen \(2p_z\) orbital.

4.5 Spin Density Calculations

Many of the early EPR experiments on aromatic \(\pi\)-radicals were performed in order to obtain molecular parameters with which to test molecular orbital theory. Such MO calculations have been advanced to the level that, at least for \(\pi\)-radicals, they can be used to support the assignment of hyperfine couplings in the radicals.

Because of the large size of the molecules we have studied, several simplifying approximations must be introduced into the calculation of self-consistent-field molecular orbitals (SCF-MO) using the linear combination of atomic orbitals approach (LCAO). In a series of papers Pople and co-workers\(^{60-64}\) developed an approximate SCF-MO theory based first on a complete neglect of differential overlap (CNDO) of all valence orbitals and then an intermediate neglect of differential overlap (INDO). Pople\(^{64}\) defines CNDO as the neglect of electron-interaction integrals for "the product of two different atomic orbitals \(\phi_{\mu}(1)\phi_{\nu}(1)\) associated with electron 1". An important consequence is that certain one-centre integrals are neglected, so that the \(\sigma-\pi\) correlation observed in isotropic
hyperfine couplings is not properly treated at this level of approximation.

The INDO approximation is less severe in that overlap contributions to the electron repulsion terms are retained in the one-centre integrals. As a result the INDO method takes more realistic account of electron correlation and the INDO spin densities can assume negative values. We have therefore used the INDO approximation to calculate the spin densities for various radicals in order to support our radical assignments, the computer programme being provided by Pople and collaborators\textsuperscript{65}. 
CHAPTER FIVE

ENDOR STUDY OF A STABLE RADICAL IN CYTOSINE MONOHYDRATE

5.1 Introduction

Because cytosine is one of the two most common pyrimidine bases found in DNA, its derivatives have been the subject of several radiation damage studies employing EPR spectroscopy$^{13,66}$. One of the most thoroughly investigated derivatives is the irradiated single crystal of cytosine monohydrate, in which at least six different radicals have been observed. Herak and Galogaza$^{67}$ have determined the radicals formed at low temperature to be the cation and, tentatively, the anion radicals. These are believed to be the primary products formed by irradiation. Of the four radicals stable at room temperature, the radical formed by the addition of a hydrogen atom to C-5 has been identified beyond any doubt$^{21,68}$. Herak$^{69}$ has assigned another of the observed radicals to a sulfur-containing impurity. The remaining two species have been assigned to the neutral oxidation$^{68}$ and reduction$^{20}$ products. The oxidation product is produced by net hydrogen atom abstraction from N$_3$. The reduction product had been assigned as a radical formed by net hydrogen addition to the carbonyl oxygen on the basis of a single proton coupling, apparently of the $\beta$-proton type. This assignment was made
without reference to the crystal structure. Furthermore, the principal values of the hyperfine coupling tensor were not in agreement with values obtained for similar radicals in alloxan\textsuperscript{70} and alloxantin\textsuperscript{71}. We have therefore undertaken a re-examination of this radical using the higher resolution of the ENDOR technique. We will show that on the basis of all intramolecular proton couplings (and some intermolecular ones) the nature of the radical is completely different.

5.2 Experimental

Single crystals of cytosine monohydrate were grown from aqueous solution by slow cooling near room temperature. During the cooling procedure, seed crystals were slowly rotated in the solution. Crystals with dimensions of $8 \times 3 \times 1$ mm were obtained and used in our ENDOR studies. The crystals were elongated along the $b$ axis and exhibited well-developed faces. The crystal morphology is shown in Fig. 6, along with the crystallographic and reference axes. Similar, partially deuterated crystals were obtained by the same procedure from heavy water solution.

The crystal structure of cytosine monohydrate has been determined by Jeffrey and Kinoshita\textsuperscript{72} and, more recently, by McClure and Craven\textsuperscript{73}. The atomic positions are essentially the same in both of these works, although the C(6)-H(6) bond length is reported to be 0.1 Å longer in the latter paper. The crystal is monoclinic, the unit cell dimensions being $a = 7.783(2)$, $b = 9.825(2)$, $c = 7.688(2)$ Å, $\beta = 99^\circ 34(1)$ and the space group $P2_1/c$. For ENDOR measurements we have used the reference coordinate system of Herak and Galogaza\textsuperscript{67} and the atomic numbering system of McClure
Fig. 6. Crystal Morphology of cytosine monohydrate, with crystallographic and reference axes.
and Craven. There are four molecules in the unit cell, only two of which are magnetically distinct.

The crystals were irradiated with X-rays at room temperature using a Machlett type OEG-60 X-ray tube operating at 40 kV, 20 mA. Good ENDOR signals were obtained after crystal irradiation times of about 24 h. ENDOR measurements were made at 77°K as described in Chapter Three. Spectra were recorded at intervals of 2.5° as the magnet was rotated about each of the reference axes, the frequency being scanned from 10-35 MHz. Furthermore, the crystal's well-developed 102 face was parallel to the molecular plane. The crystal was therefore mounted so that spectra could be recorded as the magnetic field was rotated in the molecular plane.

The characteristic doublet reported by Dertinger was the dominant feature of the EPR spectrum as is shown in Fig. 7. The double arrows mark lines belonging to the well-known C5-hydrogen addition radical. By selectively saturating various positions throughout the EPR lineshape, we established that at least two other radicals contributed to the overall EPR pattern. These radicals have been well-characterized by EPR spectroscopy so their ENDOR spectra have not been analyzed. A typical ENDOR spectrum of the doublet radical (radical I), obtained by saturating a point in the doublet where no other radicals were present, is shown in Fig. 8.

Careful, selective saturation of the EPR lineshape was also useful in determining the particular site to which an ENDOR line belonged. This was especially useful in studying cytosine monohydrate because, in
Fig. 7. EPR spectrum of irradiated cytosine monohydrate. $H_0 \parallel a^*$. 
Fig. 8. A typical ENDOR spectrum of Radical I. $H_0$ is parallel to the $(0.7071, 0.0000, 0.7071)$ direction in the crystal.
that crystal, the magnetically distinct sites differ only by a rotation in the molecular plane. As a result, it was very difficult to assign a coupling to a particular site solely on the basis of the directions of its tensor. If site splitting is well resolved in the EPR spectrum, then one should only observe ENDOR lines belonging to the site whose EPR line is being saturated. However, in cytosine monohydrate, the site splitting was buried within the EPR linewidth. By selective saturation at several orientations it was possible to unambiguously assign transitions to a particular site for all intermolecular couplings. For example, Fig. 9 shows spectra at an orientation demonstrating that the transition of coupling B belongs to the same site as that of A, while transition C belongs to the other site.

5.3 Results

The angular variation of the proton ENDOR frequencies of radical I (in cytosine monohydrate) is shown in Figs. 10 and 12 in the three orthogonal planes. Figure 10 presents the largest couplings, of which only the $v$-branch of the ENDOR spectrum was recorded. Figure 11 presents the resonances of the small couplings, for which both $v+$ and $v-$ were measured. These angular variations were determined using the methods outlined in Sect. 3.3.3. The solid curves represent the theoretical variations determined by the programme FIELDS using tensor parameters obtained from the programme LSF described earlier.

In most cases, the curve belonging to the same proton coupling in each of the three planes was readily identified by comparing the
Fig. 9. An example of a selective saturation study used to assign an ENDOR line to a particular crystallographic site. (a) EPR spectrum of radical I showing the saturation points used. (b) ENDOR spectrum for saturation point 1. (c) ENDOR spectrum for saturation.
Fig. 10. Angular variation of the high-frequency ENDOR resonances for couplings A and A'.

FREQUENCY, MHz

CRYSTAL ORIENTATION
Fig. 11. Angular dependence of the ENDOR frequencies around the free proton resonance frequency $\nu_0$, for the "small" couplings.
Fig. 12.
Angular variation of the ENDOR frequencies around $\omega_0$ in the crystal grown from D2O.
curves in different planes at coincident axes (the reference axes). Couplings \( D_1 \) and \( F \) (see Fig. 11) were nearly coincident at the \( b \) and \( c \) axes so the correct tensors could only be deduced by fitting the various possible solutions to the proposed structure of the radical. The diagonalized forms of the hyperfine tensors calculated by LSF are given in Table 1. Approximately fifty observed transitions were used to fit each tensor and the rms error was typically less than 10 kHz, equal to the linewidth of the recorder pen. This table includes tensors obtained for both cytosine monohydrate and the partially deuterated sample. The small couplings from the deuterated crystal, in which only the hydrogen atoms bonded to carbon are not replaced by deuterium, are shown in Fig. 12. The only large couplings belonging to radical I in this crystal are \( A \) and \( A' \), as in Fig. 10.

5.4 Assignment of the Couplings and Radical Identification

The assignment of the tensors to specific protons is shown in Table 2. The proton numbering scheme used in this table is shown in Fig. 13. We attempted to relate the direction of the largest anisotropic principal value of each tensor to various bond directions in the undamaged crystal. From such a comparison it is obvious that the largest couplings, here denoted by \( A \) and \( A' \), are associated with the \( C(6)-H \) bond. This assignment is further justified by the observation of these couplings in the deuterated sample, where a hydrogen bonded to oxygen or nitrogen would be replaced by deuterium. This explains the discrepancy between the hyperfine tensors obtained by Dertinger and
Table 1. Principal elements of the hyperfine interaction tensors for radical I.*

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<tr>
<th>Coupling (MHz)</th>
<th>Tensor Isotropic</th>
<th>Anisotropic</th>
<th>Direction Cosines</th>
<th>Polar angles</th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>-13.30</td>
<td>15.25</td>
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<tr>
<td></td>
<td>0.5075; 0.0467; 0.8604</td>
<td>0.7893; 0.3753; -0.4859</td>
<td>0.3456; -0.9257; -0.1536</td>
<td>31; 5</td>
</tr>
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<td>-12.65</td>
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<td></td>
<td>0.5001; 0.1440; 0.8659</td>
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<td>0.2723; -0.9518; -0.1414</td>
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<td>31; 5</td>
</tr>
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<td>31; -3</td>
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*The values are given in MHz for the principal elements of the hyperfine interaction tensors.
<table>
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<tr>
<th>Tensor</th>
<th>Coupling (MHz)</th>
<th>Isotropic Cosines</th>
<th>Anisotropic Cosines</th>
<th>Direction Cosines</th>
<th>Polar angles</th>
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* θ is polar angle from c; ϕ is defined from a toward b. Direction cosines (a,m,n) refer to one molecule in the unit cell. The other molecule is characterized by (-1,m,-n).
Table 2. Comparison of calculated and observed dipolar tensors

<table>
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<th>Directions for maximum elements</th>
<th>Assignment</th>
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<td>-1.27</td>
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<td>Principal Observed</td>
<td>Values (MHz)</td>
<td>Directions for maximum elements*</td>
</tr>
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<td></td>
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<tr>
<td>D2</td>
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<td>-4.07</td>
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<td></td>
<td>-1.02</td>
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<tr>
<td>G</td>
<td>3.67</td>
<td>5.6</td>
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<td>-1.74</td>
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</tr>
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<td>-1.67</td>
<td>-5.0</td>
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<td></td>
<td>-1.56</td>
<td>-0.5</td>
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<td>E</td>
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<td>2.8</td>
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<td>-1.14</td>
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<td></td>
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<tr>
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<td>2.1</td>
<td>50</td>
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<td></td>
<td>-2.96</td>
<td>-1.1</td>
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<tr>
<td></td>
<td>-0.58</td>
<td>-1.0</td>
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</table>

* θ is the polar angle from c; ϕ is from a toward b

* Roman numerals refer to different molecules in the unit cell. The radical is centred on molecule I.
Fig. 13. The spin densities (2p_z) used in the calculation of the dipolar parts of the coupling tensors.
other workers for >C-OH radicals. The misassignment of the origin of this coupling resulted from the site splitting not being resolved in the EPR spectrum.

The direct comparison of the small coupling tensors to bond directions is fruitless because, as the experimental results show, the unpaired spin is largely delocalized over the pyrimidine ring. We therefore fitted the observed coupling tensor parameters to the principal values and directions calculated with the use of molecular orbital theory. There were several possible radicals which would have a pronounced H-6 proton coupling. All of them are either ionized or neutral products of the reduction of the cytosine ring. The simplest ones are shown in Fig. 14, together with the spin densities predicted by the INDO MO approximation using the computer program written by Pople and Beveridge. The spin densities were obtained by squaring the coefficients of the highest occupied molecular orbital. This procedure does not make full use of the INDO approximation as the assumption is made that only the odd electron orbital contributes to the total spin density. Thus the effects of spin polarization are ignored. However, we have found that the INDO programme frequently over-estimates the contribution of spin polarization to the spin density. Table 3 lists the spin densities calculated for radical I using both the odd-electron orbital approach and the total spin density matrix. It will be shown later that the odd-electron orbital method agrees more closely with the experimental results. In all calculations we used atomic coordinates of the undamaged molecule, except for the atoms where a proposed addition or
Fig. 14. Theoretical distribution of unpaired spin density for several possible radical structures.
Table 3. The spin density distribution (2p₂) for radical I.

<table>
<thead>
<tr>
<th>Atom</th>
<th>C₂</th>
<th>C₄</th>
<th>C₅</th>
<th>C₆</th>
<th>N₁</th>
<th>N₃</th>
<th>N₄</th>
<th>O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square of the odd electron orbital</td>
<td>0.11</td>
<td>0.32</td>
<td>0.01</td>
<td>0.23</td>
<td>0.14</td>
<td>0.05</td>
<td>0.10</td>
<td>0.03</td>
</tr>
<tr>
<td>Spin density from the spin density matrix</td>
<td>0.16</td>
<td>0.43</td>
<td>-0.21</td>
<td>0.40</td>
<td>0.17</td>
<td>-0.00</td>
<td>0.09</td>
<td>0.03</td>
</tr>
<tr>
<td>Experiment</td>
<td>0.00</td>
<td>0.35</td>
<td>0.09</td>
<td>0.03</td>
<td>0.03</td>
<td></td>
<td></td>
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</tbody>
</table>
replacement took place. The calculations were performed only as an estimate; no attempt was made to optimize the results by varying the atomic positions.

The anion radical could be safely ruled out as a possibility in the present study for the following reasons. (a) The anion is not expected to be stable at room temperature\textsuperscript{67}. (b) The observed coupling attributed to H-6 is significantly smaller than that in cytidine 3'-phosphate at 4.2K\textsuperscript{76} which is more likely to belong to the anion. (c) In the present study we observe more intramolecular proton couplings than could be accounted for without an extra hydrogen added to the cytosine ring. It is more difficult to distinguish between the remaining three possibilities. The analysis which follows will show that we deal with radical I.

The assignment of the small couplings, hence the radical identification, was done mostly from the comparison of the observed magnitudes and directions of the anisotropic coupling tensors with the theoretical predictions. The theoretical estimate of the dipolar parts of the tensors was made using the McConnell-Strathdee equations, modified and compiled by Barfield\textsuperscript{53}. The calculation included contributions from the 2s and 2p\textsubscript{z} orbitals of the carbon, nitrogen and oxygen atoms in the cytosine molecule. The arrangement of these atoms was assumed to be the same as in the undamaged molecule. The 2p\textsubscript{z} spin densities used were based upon the INDO calculations (Table 3) or the values derived from the experimental results, where applicable.
The densities on the carbon and nitrogen atoms were derived from the couplings of the respective hydrogen atoms in α positions, using the McConnell constant Q of -73.7 MHz and -87 MHz for carbon\textsuperscript{77} and nitrogen\textsuperscript{78}, respectively. The comparison of the calculated and the observed dipolar tensors for radical I is shown in Table 2.

The largest couplings, denoted by A and A', are assigned to the C(6)-H proton. These two tensors are very similar to nature and are believed to originate from two slightly different conformations of radical I. This phenomenon will be discussed later in more detail. Tensor A exhibits a typical α-proton character and the direction of its largest dipolar value fits well to the direction of the C(6)-H bond. The direction of the anisotropic component closest to zero is within a few degrees of being perpendicular to the molecular plane. It should be noted that in the undamaged crystal the molecule is not exactly planar; it is buckled such that C-5 is out of the best-fit plane containing the heavier atoms. We also do not expect exact ring planarity in the radical.

Interactions B and B' result from an exchangeable proton and we have assigned them to the proton attached to N-1. The tensor consists of two component interactions. It contains a typical α-proton coupling, with a spin density of about 0.09 on the bonding nitrogen. The second contribution arises from the dipolar interaction of the proton with the remaining spin density, primarily that on C-4 and C-6. The direction of the positive anisotropic component is within 4° of the N(1)-H(1) direction in the undamaged molecule, and within 10° of the direction pre-
dicted by the calculation. Again, two slightly different tensors are believed to originate from two different conformations.

Interaction C has been assigned to the proton that becomes bonded to the carbonyl oxygen upon radical formation. It, like interactions B and B', is made up from the α-type coupling to the spin density on oxygen and the dipolar interaction with the remaining spin density in the rest of the molecule. The tensor directions indicate that this hydroxyl proton lies in the nodal plane of the π-electron system. This is in agreement with the structure of the C-OH radical observed by Henn and Whiffen\textsuperscript{79} in irradiated lithium glycollate monohydrate.

Interactions D\textsubscript{1} and D\textsubscript{2} have been assigned to the two amino protons, H-4 and H-4', respectively. This assignment would have been routine, except for the 1.48 MHz difference in their isotropic parts. If both protons were in the nodal plane of the π-system, they would have the same isotropic hyperfine couplings and essentially the same anisotropic components, differing only in their principal directions. The angle between their most positive anisotropic values is experimentally found to be 67° and is in excellent agreement with the angle of 65° predicted by the dipolar tensor calculation for these two protons (in their crystallographic positions). The dipolar contribution to the total hyperfine tensor thus arises from 0.03 spin density on N-4 and about 0.32 on C-4. If there had been no spin density on C-4, the angle determined above would have given the H-N-H bond angle directly\textsuperscript{80}. The observed difference in the isotropic couplings of the two amino protons can only be accounted for if H-4' is not in the nodal plane of the
\(\pi\)-system. If, as a result of radical formation, it has moved into a region of nonzero \(\pi\)-electron density, there will be a positive contribution to its isotropic coupling. This assumption is further justified by its observed anisotropic coupling tensor, whose principal value closest to zero is 20° from that observed in all the other intramolecular couplings. This value, in a planar molecule, should be perpendicular to the molecular plane. Thus H-4' cannot be in the plane described by the other couplings, although it is close enough to maintain the negative isotropic component characteristic of an \(\alpha\)-type interaction.

Interactions G and G' have been assigned to the last proton to be accounted for in the proposed radical, H-5, bonded to C-5. Both these tensors are essentially dipolar in character, indicating that there is almost zero spin density on C-5. The tensors were observed in both deuterated and undeuterated crystals but could only be identified in all three planes in the deuterated crystal. Along with A and A', they were the only intramolecular couplings observed in the deuterated crystal. The difference in direction of the positive anisotropic values of G and G' was 7°.

All intramolecular couplings in the radical have now been assigned. The remaining tensors represent intermolecular interactions. They are rather more difficult to assign since the spin density in the radical is spread over several centres. The directions of a tensor will be the vector sum resulting from these spin densities. Interactions E and F represent couplings to protons in the crystal layer above or below the one with the radical. Both tensors possess significant, positive isotropic
components, indicating that the proton is in a region of positive spin
density. The direction of the largest anisotropic component of both
tensors is perpendicular to the molecular plane, parallel to the $2p_z$
orbital axis. Neither coupling was observed in the deuterated crystal
and hence must represent interactions with exchangeable protons. The
dipolar calculation implies that the interactions are with IV H-4' or
II H-1, where the Roman numerals indicate neighbouring molecules in
the unit cell, according to the notation of Jeffrey and Kinoshita. The
latter proton lies above the centre of the radical and should
experience less spin density than IV H-4' which lies above the edge of
the ring. Thus IV H-4' should be represented by coupling E, which has
a larger isotropic component than F. A coupling with IV H-4 was not
observed but if its isotropic component was smaller it may have been
lost in the maze of lines around $v_o$.

The last interaction identified, H, was present in the deuterated
sample. Comparison with the estimated dipolar tensor implies that this
tensor represents a coupling to IV H-5, which is nonexchangeable. The
small isotropic component arises because this proton does not lie
directly above the ring.

Besides the above couplings a number of additional small couplings
were observable at least in one of the planes. The experimental data
were either incomplete or unreliable, so that their identification was
not seriously attempted.
5.5 Discussion

The proton couplings designated by A, B, and G in the previous chapter were easily assigned to H-6, H-1, and H-5, respectively. However, these couplings were not sufficient to determine the structure of the radical involved; they would fit equally well to all neutral radicals in Fig. 14. The radical could only be identified through the observation and analysis of all the remaining intramolecular couplings. The spin densities inferred by these couplings were compared to those obtained from the MO calculations (see Fig. 14). In all cases, the spin density at C-5 was quite large and negative, indicative of appreciable spin polarization. This was in contradiction with experiment, which showed negligible spin density at C-5. As a result, we ignored the effects of spin polarization and obtained the listed spin densities by squaring the coefficients of the odd electron orbital. This approach predicted negligible spin density at C-5 for all likely radicals. The calculations could be improved by choosing different atomic positions. The fact is though, that we have no way of knowing the exact atomic positions in a radical. Changes in the geometry will change hybridization and thereby change the spin densities. However, the calculations are sufficient to eliminate all possible radical structures except radical I.

Radical II and other radicals with a hydrogen atom added to N-3 have been eliminated on the basis of the following arguments. The MO calculations show that any such radical would have large spin density on N-3, and that $\rho_N(3) > \rho_N(1)$. This is in contradiction with the observations regardless which of the remaining couplings is assigned to
the N(3)-H proton. Furthermore, theoretical dipolar tensors, calculated using the McConnell-Strathdee equations for a N(3)-H proton interacting with a spin density of about 0.12 on N-3 and about 0.40 on C-4, clearly did not match any of the observed tensors. Various deaminated radicals were considered only because the MO calculations showed larger spin density on N-4 than could be accounted for. All such combinations were ruled out both because of disagreement between the observed and proposed number of protons and because of the fact that even in such radicals, like radical III, the theoretical spin density on the substituent was essentially the same as on the original amino group.

The number, principal values, and principal directions of intramolecular proton couplings were all consistent with radical I. However, the fit between the observed and predicted coupling parameters (Tables 2 and 3) was in some cases only fair. The MO calculation predicted smaller spin density on C-6 than the value derived from experiment. This discrepancy could easily be corrected by choosing different atomic positions in the C(5)-C(6) region. The dipolar tensors describing the small couplings are very sensitive to errors in the assumed spin densities on the neighbouring atoms. As a result the agreement between theory and experiment for these couplings is worse than for the larger couplings. The tensor directions in Table 2 suggest that the spin density on C-6, although derived from the experiment, was over-estimated and hence that the McConnell constant $Q_{C(6)}$ we used was slightly too large. The analysis we have described was sufficient to identify the observed species as radical I so we did not attempt to improve our theoretical calculations.
There were so many factors influencing these calculations that such attempts would be fruitless.

It has been shown earlier that the primary radicals formed in cytosine monohydrate at 77°K are the cation and anion radicals\(^6^7\). In the undamaged crystal there are three protons, instead of the usual two, hydrogen bonded to the carbonyl oxygen. This atom is involved in hydrogen bonds with the amino nitrogen of the adjacent cytosine molecule in the same plane, and to two water molecules, one above and one below the molecular plane. The direction of the O(2)-H coupling tensor rules out proton transfer from the adjacent amino group. Thus it is reasonable to assume that radical I is formed from the anion by acquisition of a proton from one of the water molecules hydrogen bonded to O-2. From our analysis it seems plausible that the sulfur-containing radical\(^6^9\) found in this crystal is the analogous radical formed in the 2-thiocytosine impurities by net addition of a hydrogen atom to S-2. The very large g-value and much smaller coupling of H-6 in the latter radical comes from different electronic properties of S and O.

The appearance of twice the number of certain ENDOR lines (couplings A and A', B and B', G and G') than expected from the crystal symmetry was the subject of much consideration. In a crystal belonging to the space group P2\(_1\)/c, with four molecules in the unit cell, each molecule is related to another through a centre of symmetry. Since these molecules are magnetically equivalent, one should in general see resonances from two sites only in the ENDOR spectra. We considered several possibilities which could explain the additional splittings observed. Misalignment of
the crystal could not give rise to this phenomenon. Twinning of the crystal was also ruled out by checking at least ten crystals; all of them gave the same doubling of the lines with approximately equal intensities. The splitting could result from there being a different crystal structure at 77°K, where the ENDOR measurements were made, from that determined at room temperature. If so, the crystal at 77°K should have a lower symmetry from that at room temperature. The loss of the centre of symmetry would make two chemically inequivalent molecules, rather than one, present in a general position in the unit cell.

An alternate interpretation is possible if one assumes that the splitting is an intrinsic property of the radical formed by irradiation. In the undamaged molecule, C(5) and C(6) are significantly displaced from the least-squares plane through the other cytosine atoms (excluding hydrogen). The protons attached to these two display the largest extra splittings, which are proposed to be manifestations of two different conformations of radical I. Horsfield et al. observed the same phenomenon in single crystals of glutamic acid and postulated several reasons for the effect. They could not obtain sufficient experimental evidence to establish the mechanism producing the splitting. Kwiram re-investigated this system using the more informative ENDOR technique. On the basis of the weakly-coupled proton interactions, he postulated that the splitting resulted from two different conformations of the same radical species, differing in the orientation of the carbonyl group relative to the skeleton of the radical.
Since the undamaged cytosine molecule is not strictly planar, hydrogen addition from above or below the ring would result in different conformations of the radical. Stabilization of the resulting trapped radicals within the framework of the crystal produces the largest difference between the conformations in the C(5)-C(6) region. Furthermore, the proton attached to O-2 in the radical must upset the hydrogen bonding scheme, inducing conformational changes to stabilize the radical. A dramatic example of protonation inducing conformational change was reported recently by Parthasarathy et al. They observed a marked conformational change in N-6 (N-glycylcarbonyl)adenosine produced by protonation at N(1). This change results in a new hydrogen bond, which stabilizes the molecule. It is likely therefore that radical I is stabilized in two conformations. An attempt was made to interpret couplings C and D as belonging to the same hydrogen atom in two different conformations. Neither could such an assignment fit to radical I nor could we find another radical structure that would be consistent with the other observed tensors.

Radicals formed by hydrogen addition to a carbonyl oxygen have been observed in several other pyrimidine compounds. Uracil derivatives, including thymine, also contain oxygen in a second carbonyl group at C-4. This oxygen was found to be the only one adding hydrogen in these compounds. This addition was found to occur in thymine monohydrate, thymidine, 5-bromouracil, 5-nitro-6-methyluracil, 5-bromodeoxyuridine and 5-iododeoxyuridine. However, this work is the first correct report of a radical generated by hydrogen addition to the C-2 carbonyl oxygen.
Elliot has used EPR spectroscopy to investigate radiation damage in a related crystal, 1-methyl cytosine. He identified the well-known radical formed by addition of hydrogen to C-5 of the cytosine ring but was unable to identify a second species produced in lesser concentration. Rustgi and Box used the ENDOR technique to characterize both species and have identified the second species as that formed by hydrogen addition at the C-6 position. They did not observe a radical corresponding to radical I in this system. Since methylation should not seriously affect the pyrimidine skeleton, the water of crystallization present in cytosine monohydrate, but not in 1-methyl cytosine, is an important factor in the production or stabilization of radical I. Huttermann, Schmidt and Weymann have investigated the influence of the water of crystallization on radical yields in a recent EPR and ENDOR study of barbituric acid, another pyrimidine derivative. They found that the yield of one of the radicals (produced by net hydrogen abstraction from C-5) was three times greater in the dihydrate than in the anhydrous crystals. The observation that the analog of radical I is not detected in 1-methyl cytosine can be explained in two ways. Radical I may be stabilized in cytosine monohydrate through involvement in hydrogen bonds with adjacent water molecules. Without these hydrogen bonds, the radical cannot be trapped and hence is not observed in 1-methyl cytosine. Alternatively, the water molecules serve as the source of hydrogen for radical I. This hypothesis is supported by the principal directions of the O(2)-H proton hyperfine coupling tensor, which indicate that, in the undamaged crystal, water protons are closest to the O(2)-H direction.
Since the anion has been identified as a primary species, a likely mechanism for the production of radical I is protonation of the radical anion, involving proton transfer across a water-O(2) hydrogen bond.

Subsequent to the completion of this work, a preliminary report of a new EPR study of cytosine monohydrate appeared in the literature. Westhof, Flossmann, and Muller determined the values of the largest hyperfine coupling tensor and assigned the tensor to the C(6)-H proton. Their values are in agreement with ours when the lower resolution of EPR spectroscopy is considered. They, too, have assigned this radical to that produced by hydrogen-addition to O-2.

It is interesting to note that the identified radical is structurally identical to the radical proposed by Dertinger on the basis of an EPR study. Dertinger's assignment was based however on the wrong observation that the prominent proton hyperfine came from a β-proton (C(2)-OH). The situation is quite different and more complex, as demonstrated by the present ENDOR analysis. The complexity is brought about by various factors. First, there are several radicals with overlapping spectra present at room temperature. The radical exhibits large delocalization of the unpaired spin, which produces a large number of coupling nuclei. Molecular orbital calculations were of limited help, because they gave essentially similar spin density distributions for the different radicals considered. Finally, the extra splitting for some proton couplings introduced an additional complexity in the ENDOR spectra. As a result, the cytosine monohydrate system proved very complex even for ENDOR analysis.
CHAPTER SIX

RADIATION DAMAGE IN CAFFEINE HYDROCHLORIDE DIHYDRATE

6.1 Introduction

Although there have been many EPR investigations of free radicals trapped in solid nucleic acid constituents, most of them have been concerned with pyrimidines. Similar studies on purine derivatives were hampered by low radical concentrations or by the difficulty in obtaining suitably sized single crystals. The pioneer work on radiation damage in purines was performed by Shields and Gordy, who observed a triplet caused by two equally coupling protons in guanosine and deoxyguanosine. This triplet was later interpreted by Gordy, Pruden, and Snipes as arising from a radical produced by net hydrogen addition to C-8. Herak and Gordy proved this interpretation by studying the EPR spectra of radicals produced by subjecting guanine and its derivatives to gaseous hydrogen atoms. These authors also demonstrated the importance of this radical in nucleic acids by observing the triplet in ribonucleic acid (RNA) bombarded with hydrogen atoms.

Even though this early work on powders served to identify the hydrogen-addition radical, it did not provide detailed information about the radical's electronic structure. Such information could only be obtained from analysis of an irradiated single crystal. The first such
EPR study was reported by Alexander and Gordy. Because single crystals of purines were very difficult to grow, they co-crystallized guanine with hydrochloric acid and obtained single crystals of guanine hydrochloride dihydrate. They, in effect, performed an acid-base neutralization and the resulting salt crystallized easily. Dertinger also concluded that the C-8 hydrogen-addition radical was formed in adenosine hydrochloride, deoxyadenosine monohydrate and deoxyguanosine hydrochloride. This radical has also been reported in adenine hydrochloride, adenine dihydrochloride, and 9-methyladenine.

Details of the structure of the hydrogen-addition radical in adenine derivatives were difficult to obtain. Site splitting and large linewidths prevented analysis of hyperfine coupling details in many orientations. Furthermore, in these crystals, the N(3)-C(2) and N(7)-C(8) bonds are nearly parallel. In order to distinguish between the two possible sites of hydrogen addition, previous authors have had to rely on comparisons with theoretical calculations. In an effort to obtain more information about the electronic structure of this radical, we have undertaken an ENDOR investigation of another purine derivative, caffeine hydrochloride dihydrate (CHD), with the caffeine ring protonated as shown in Fig. 15.

We chose to study caffeine for a number of reasons. If the hydrogen-addition radical detected in other purines was also produced in CHD, it could only arise from hydrogen addition to C-8. Thus CHD served as an excellent model system for determining the electronic structure of this radical in purines. There have also not been any reports of EPR studies on radiation damage in solid caffeine derivatives. This was
Fig. 15. Protonated caffeine molecule.
particularly surprising in light of the extensive use of caffeine in radiation damage studies on biological systems. Caffeine has been found to enhance the frequency of radiation-induced chromosomal aberrations in a variety of plant and animal tissues ranging from the yeast, *Schizosaccharomyces pombe*[^99], to human lymphocytes[^100,101]. Early work in this field has been reviewed by Adler[^102]. This enhancement of radiation damage by caffeine could lead to new methods for the treatment of cancers. If caffeine could be selectively introduced into cancer tissue, radiation damage and subsequent cell death would be enhanced in this tissue. Thus the dosage required to destroy a cancer could be reduced, thereby reducing radiation damage to healthy tissue.

The mechanism of caffeine's action is not yet fully understood, but appears to depend on the system[^100], as caffeine has no synergistic effect with ionization radiation in hamster cells and root tips of broad beans[^103]. However, in cases where this synergism is observed, it is clear that caffeine inhibits the post-irradiation repair of damaged DNA. Brogger[^100] has proposed a model for the effect of caffeine in irradiated human lymphocytes based on its inhibition of post-replication DNA repair. Gaps are formed in newly synthesized DNA during replication on a damaged template. These gaps can initiate an exchange process between DNA duplexes, which result in chromosomal aberrations. These gaps can be filled by de novo synthesis of DNA[^104]. Caffeine inhibits the gap-filling process, causing an accumulation of gaps and thereby increasing the aberration frequency.
The only reported EPR investigation of caffeine involved short-lived free radicals produced in aqueous solution\textsuperscript{105}. Nicolau used flow techniques to observe C-8 adducts with \textsuperscript{·}OH, \textsuperscript{·}NH\textsubscript{2}, and alcohol radicals. These radicals were produced by chemical means; radiation damage was not involved in this study. The spectra of caffeine reacted with \textsuperscript{·}OH and \textsuperscript{·}NH\textsubscript{2} were both attributed to C-8 adducts, with the unpaired spin centred at N-9. Spectra of the 2-propanol adduct showed that the two imidazole nitrogens had equivalent splittings. The equivalence of the N7 and N9 splittings is surprising, since N7 is substituted.

Prior to the commencement of this work, there had been no reports of ENDOR experiments on solid purine derivatives in the literature. However, since then, Box and Budzinski\textsuperscript{106} have reported an ENDOR analysis of the primary reduction product resulting from X-irradiation of adenine dihydrochloride at 4.2°K. Identification of this product was based upon determination of the C(8)-H proton hyperfine tensor. Our work is the first ENDOR analysis of the C-8 hydrogen addition radical in a purine. We have also observed \textsuperscript{14}N-ENDOR from this radical, the first such observation in any pyrimidine or purine. Two other radicals have also been observed in CHD irradiated at room temperature. One of these was assigned to that resulting from net hydrogen abstraction from N(9) while the other could only be tentatively assigned as the methyl radical.

6.2 Experimental

Single crystals of caffeine hydrochloride dihydrate were readily obtained from solution of caffeine in approximately 6N hydrochloric
acid by slow evaporation near room temperature. The crystals were elongated along the b axis and exhibited well-developed faces. The crystal morphology is shown in Fig. 16, along with the crystallographic and reference axes. In this figure, the b axis is perpendicular to the page.

The crystal structure of caffeine hydrochloride dihydrate had not been reported and hence was performed by Mercer and Trotter\textsuperscript{107} of this department to aid our ENDOR analysis. The crystal is monoclinic, the unit cell dimensions being $a = 12.391\,\text{Å}$, $b = 6.522\,\text{Å}$, $c = 17.167\,\text{Å}$, $\beta = 118.8^\circ$ and the space group $P2_1/c$. There are four molecules in the unit cell, only two of which are magnetically distinct.

The crystals were found to decompose rapidly in air and hence were stored in a coating of Dow-Corning high vacuum grease. The crystals were irradiated with x-rays at room temperature using a Machlett type OEG-60 x-ray tube operating at 40 kV, 25 mA. This irradiation polymerized the grease, which could then be easily peeled away from the crystal. The crystal was then protected with a new coating of grease. Thus, EPR spectra of the sample could only contain radicals produced in the crystal, not in the grease. Good EPR signals were obtained after crystal irradiation times of 20 min although for ENDOR measurements the crystals were irradiated for about 12 h.

An EPR study of CHD was performed at room temperature using a Varian E-3 spectrometer. Spectra were recorded at intervals of 5° as the crystal was rotated about each of the reference axes. The spectra were very complicated and showed considerable anisotropy, as is shown
Fig. 16. End view of the crystal morphology of caffeine hydrochloride dihydrate, indicating the crystallographic and reference axes.
in Fig. 17. The two spectra in this figure were recorded at an interval of $10^\circ$ in the a*c plane. In most orientations, the hyperfine lines were not well resolved so no attempt was made to interpret the EPR spectra.

ENDOR analysis of the CHD crystal was performed at $77^\circ$K as described in Chapter Three. Spectra were recorded at intervals of $2.5^\circ$ as the magnet was rotated about each of the reference axes, the frequency being scanned from 10-50 MHz. By selectively saturating various positions throughout the EPR lineshape, we established that at least two other radicals contributed to the overall EPR pattern. A typical ENDOR spectrum containing resonances from all three radicals is shown in Fig. 18. The intense line near 30 MHz has been assigned to the C-8 hydrogen adduct; its intensity relative to the lines in the free proton region is shown in Fig. 19. The nature of this coupling will be dealt with in a later section. We also used selective saturation to determine whether a resonance occurred at the higher or lower frequency, $\nu>$ or $\nu<$, given by the equation:

$$\nu_{rf} = \left| g_N \beta_N H \pm \frac{A}{2} \right|$$  \hspace{1cm} (6-1)

The frequency, $\nu>$, increases as the value of $H$ is increased, while $\nu<$ decreases for $\left| \frac{A}{2} \right| > g_N \beta_N H$. Thus we could determine the hyperfine coupling tensors for all resonances at frequencies higher than $\nu_p$ by observing only one of the pair of transitions given by Eq. (6-1). This was required since we did not observe any transitions above 50 MHz,
Fig. 17. Sample EPR spectra of irradiated CHD. (a) $\mathbf{H}_0$ parallel to $(-0.7660, 0.0000, -0.6428)$. (b) $\mathbf{H}_0$ parallel to $(-0.8660, 0.0000, -0.5000)$. 
Fig. 18. The complete ENDOR spectrum for $H_o$ parallel to (0.9397; 0.0000; -0.3420) of irradiated ChD.
Fig. 19. The relative intensity of the ENDOR line of coupling D with respect to the free proton region.
simply as a result of our experimental conditions. Other experimental considerations will be discussed in later sections dealing with the three different radicals.

6.3 The Abstraction Radical (Radical V)

Two of the ENDOR lines shown in Fig. 18 belong to a radical which could not be observed in the EPR spectrum. Figure 20 illustrates a saturation study demonstrating that these two lines do not belong to the dominant radical. The angular variation of the proton ENDOR frequencies of radical V in the three reference planes is shown in Fig. 21, in which only $v_>$ is shown. The two couplings are the only ones in this radical with absolute values greater than 3 MHz. The solid curves again represent the theoretical variations determined by the programme FIELDS (see Sec. 5.3) using tensor parameters obtained from the programme LSF described earlier. The diagonalized forms of the hyperfine tensors calculated by LSF are given in Table 4, along with bond directions in the undamaged crystal corresponding to these tensors.

Both of these tensors have been assigned to methyl group protons. The nature of the tensors indicates that the three protons in each methyl group are equivalent. This requires that the methyl group be rotating freely about the N-C axis at 77°K. This phenomenon has previously been studied in irradiated succinic acid by Read and Whiffen\textsuperscript{108}, using the ENDOR technique. Box, Budzinski and Potter\textsuperscript{109} have also used ENDOR to investigate methyl group rotation at 4.2°K in irradiated
Fig. 20. A selective saturation study used to identify the ENDOR lines associated with radical V. (a) The EPR spectrum. (b) The ENDOR spectrum at saturation point 1. (c) The ENDOR spectrum at saturation point 2.
Fig. 21. Angular variation of the high-frequency ENDOR lines of radical V.
Table 4. Principal elements of the proton hyperfine tensors for radical V.

<table>
<thead>
<tr>
<th>Tensor</th>
<th>Hyperfine Coupling (MHz)</th>
<th>Isotropic</th>
<th>Anisotropic</th>
<th>Direction Cosines w.r.t. a*bc</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-1.04</td>
<td>-0.3809; 0.6222; -0.6839</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.32</td>
<td>-2.12</td>
<td>-0.8905; -0.4459; 0.0903</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.16</td>
<td>-0.2488; 0.6434; 0.7239</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N&lt;sub&gt;7&lt;/sub&gt;-C&lt;sub&gt;14&lt;/sub&gt; direction</td>
<td>-0.3066; 0.7186; 0.6242</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.66</td>
<td>-0.4314; 0.3414; -0.8351</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>5.34</td>
<td>-1.09</td>
<td>-0.7992; -0.5741; 0.1781</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.75</td>
<td>-0.4186; 0.7442; 0.5205</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N&lt;sub&gt;3&lt;/sub&gt;-C&lt;sub&gt;12&lt;/sub&gt; direction</td>
<td>-0.5206; 0.7882; 0.3282</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
thymidine. However, in that study, the methyl group was found to exist in discreet rotational states.

In Sec. 2.2.3 we developed expressions for the principal directions of the anisotropic hyperfine tensor. For an -NCH fragment fixed in the crystal, the largest positive anisotropic value, t, points approximately along the NH direction, as is shown in Fig. 22. Determination of this direction involves an integration over the spatial coordinates of the nitrogen p orbital, so the direction illustrated is only an approximation. The discussion that follows, however, also applies to the true tensor. The principal direction can be described in terms of components perpendicular and parallel to the N-C bond direction. If the C-H group rotates about the N-C bond (See Fig. 22), the perpendicular components will be averaged to zero. The parallel component, however, will not be affected. Therefore the largest positive anisotropic value of a proton tensor for a rotating methyl group should lie along the rotation axis, the N-C direction. Similar arguments demonstrate that the remaining principal values are directed (a) parallel to the long axis of the nitrogen p orbital and (b) perpendicular to both the N-C bond and the nitrogen p orbital. Both tensors for radical V fit this model. Tensor A has been assigned to the methyl group attached to N-7. Its largest positive anisotropic value is directed within 8° of the N(7)-C(14) direction. Similarly, tensor B has been assigned to the methyl group attached to N-3. These assignments demonstrate the usefulness of the ENDOR technique. First of all, these couplings could not even be observed in the EPR spectrum. Even if they had been
Fig. 22  Direction of the largest, positive principal value of the proton tensor for an isolated ·NCH fragment.
detected, the couplings would have appeared to be isotropic and therefore
could not have been assigned to specific methyl groups.

In order to infer spin densities in the nitrogen p orbitals from
the observed isotropic proton couplings, we must choose an appropriate
value for the McConnell constant discussed in Sec. 4.3. The McConnell
constant $Q_{\mathrm{CH}_3}$ for a rotating methyl group in a $\cdot$C-CH$_3$ fragment is
generally accepted to be:

$$Q_{\mathrm{CH}_3} = B_0 + \frac{1}{2} B_2 = 9 \text{ MHz} + \frac{1}{2}(122 \text{ MHz}) = 70 \text{ MHz} \quad (6-2)$$

We could not find a value of $Q_{\mathrm{CH}_3}$ for the analogous $\cdot$N-CH$_3$ fragment,
but it is not expected to differ appreciably from the value for $\cdot$C-CH$_3$.
We conducted a literature survey of EPR studies radicals containing
$\cdot$N-CH$_3$ fragments and used the reported hyperfine coupling constants to
determine $Q_{\mathrm{CH}_3}$. The results are shown in Table 5. The spin density
in the nitrogen p orbital, $\rho_{\pi}^N$, of these radicals was inferred from the
observed isotropic nitrogen splitting, $a_S^N$ using the relation:

$$\rho_{\pi}^N = \frac{a_S^N}{Q_S^N} \quad (6-3)$$

where $Q_S^N = 73.6 \text{ MHz}$. The value of $Q_S^N$ was obtained from the nitrogen
hyperfine tensor determined by ENDOR analysis of the C-8 hydrogen-addition radical as discussed in Sec. 6.5. Table 5 indicates that
$Q_{\mathrm{CH}_3}$ for nitrogen is indeed close to that for carbon. We have there-
fore used $Q_{\mathrm{CH}_3} = 71 \text{ MHz}$ to obtain $\rho_{\pi}^N$ from the proton couplings in
Table 5. Hyperfine splittings and values of $Q_{\text{CH}_3}$ for $\cdot N-\text{CH}_3$ fragments.

<table>
<thead>
<tr>
<th>Radical</th>
<th>Nitrogen Hyperfine Coupling $a_N$ (MHz)</th>
<th>Methyl Proton Hyperfine Coupling $a_H$ (MHz)</th>
<th>Spin Density $\rho_N^\pi$</th>
<th>$Q_{\text{CH}_3}$ (MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wurster's blue cation radical</td>
<td>19.6</td>
<td>18.9</td>
<td>0.266</td>
<td>71.1</td>
</tr>
<tr>
<td>Methyl viologen cation radical</td>
<td>11.9</td>
<td>11.2</td>
<td>0.162</td>
<td>69.1</td>
</tr>
<tr>
<td>Ninhydrin-dimethyl amine</td>
<td>24.7</td>
<td>24.7</td>
<td>0.336</td>
<td>73.5</td>
</tr>
</tbody>
</table>

Average 71.2

a) Reference 110.  b) Reference 111.  c) Reference 112.
radical V. Tensor A indicates that there is a spin density of 0.272 in the N(7) $\rho_\pi$ orbital, while tensor B indicates that $\rho_\pi^N = 0.075$ for the N(3) $\rho_\pi$ orbital.

Tensors A and B have both been assigned to protons belonging to methyl groups attached to nitrogen atoms in the purine skeleton. Their principal directions have also indicated that the purine ring was not appreciably altered by radical formation. We therefore performed INDO-MO calculations for possible radical structures resulting from changes in the substituents on the purine ring. The two most likely structures would be produced by (a) net hydrogen abstraction from N(9) and by (b) homolytic cleavage of the N(1)-C(10) bond. The spin densities obtained from the INDO-MO computer programme, using the odd-electron orbital approach discussed in Sec. 5.4, are listed in Table 6 for these radicals. The atomic positions used in these calculations were those of the undamaged molecule as obtained from the X-ray crystal structure determination. No attempt was made to optimize the results by varying the atomic positions since these calculations were sufficient to identify the radical.

It is clear from Table 6 that radical V does not result from methyl group abstraction from N(1). This possibility was considered because another of the radicals observed in CHD had been tentatively assigned as the methyl radical, resulting from homolytic cleavage of an N-CH$_3$ bond. This process may still occur but the purine radical thus formed must react further until a non-radical species is stabilized. Methyl abstraction radicals have not previously been observed in either
Table 6.  Spin densities calculated for hydrogen-abstraction and methyl-abstraction radicals in caffeine hydrochloride dihydrate.

<table>
<thead>
<tr>
<th></th>
<th>N1</th>
<th>C2</th>
<th>N3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>N7</th>
<th>N9</th>
<th>O11</th>
<th>O13</th>
<th>C8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrogen-abstraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from N(9)</td>
<td>0.011</td>
<td>0.003</td>
<td>0.165</td>
<td>0.151</td>
<td>0.168</td>
<td>0.001</td>
<td>0.192</td>
<td>0.026</td>
<td>0.071</td>
<td>0.050</td>
<td>0.089</td>
</tr>
<tr>
<td><strong>Methyl-abstraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from N(1)</td>
<td>0.000</td>
<td>0.013</td>
<td>0.325</td>
<td>0.039</td>
<td>0.214</td>
<td>0.009</td>
<td>0.061</td>
<td>0.016</td>
<td>0.090</td>
<td>0.072</td>
<td>0.086</td>
</tr>
<tr>
<td><strong>Experimental</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>spin densities</td>
<td>0.075</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.262</td>
</tr>
</tbody>
</table>
pyrimidines or purines containing methyl groups. Instead, the methyl groups have been found to detach atomic hydrogen upon irradiation, yielding α-dihydro carbon radicals. This type of radical was clearly not formed in caffeine hydrochloride dihydrate under our experimental conditions. It has however been observed in methyl groups bound to the C(5) atom of 5-methylcytosine\textsuperscript{113}, 6-azathymine\textsuperscript{114}, and thymidine\textsuperscript{12}. Hydrogen abstraction from a N-methyl group was observed by Schmidt and Snipes\textsuperscript{97} in the purine 9-methyladenine and by Herak and McDowell\textsuperscript{115}, using the ENDOR technique, in 1-methyl uracil.

The spin densities calculated for the N(9)-hydrogen abstraction radical, as shown in Table 6, are consistent with the experimental spin densities. Changes of less than 0.09 in the calculated spin density at N3, N7, and C8 would reproduce the experimental results. The agreement is good in relation to the level of approximation inherent in the calculations. This assignment is further justified by the fact that this type of radical has previously been observed in pyrimidine derivatives. Hydrogen abstraction from N1 has been reported for cytosine monohydrate\textsuperscript{68}, thymine monohydrate\textsuperscript{116}, anhydrous thymine\textsuperscript{116}, and orotic acid\textsuperscript{117}. Box and Budzinski\textsuperscript{18} used the ENDOR technique to analyze this radical in irradiated barbituric acid dihydrate at 4.2°C. These radicals are analogous to the N(9)-hydrogen abstraction radical we have observed in CHD in that N(1)-nitrogen atoms in pyrimidines form the glycosidic link with the sugar moiety in nucleotides while the N(9)-nitrogen atom in purines form this linkage. Thus hydrogen abstraction occurs at the atom performing the same biochemical role in pyrimidines and purines.
An ionic mechanism has previously been proposed for the formation of hydrogen addition radicals in purines. Observations of the N(9)-hydrogen abstraction radical in CHD supports the ionic mechanism for radiation damage in purines. The caffeine moiety in undamaged CHD is protonated at N(9) and is therefore a cation. If irradiation were to cause an electron to be ejected from this molecule, a doubly-charged species would result. This species should be unstable at room temperature and would undergo further reactions. Loss of the proton at N(9) would restore its charge balance with its anionic environment (neighbouring chloride ions). Furthermore, the proton at N(9) is the most labile substituent on the purine ring and therefore the most likely one to be involved in a reaction to stabilize the radical. No matter how radical V is formed, the isolated radical itself is just a caffeine cation. The cation has been stabilized in irradiated CHD by its anionic environment of chloride ions. Although we can only speculate as to the mechanism of formation of radical V (the radical could also be formed by homolytic cleavage of the N(9)-H bond), its very existence demonstrates that cation radicals are formed in purines. Such radicals have not been previously reported in purine derivatives. Together with previous reports on anionic radicals formed and studied at low temperatures, this study demonstrates the importance of ionic species in the radiation damage of purines.
6.4 The Methyl Radical

The weak ENDOR transition occurring at 45.73 MHz in Fig. 18 was not associated with either of the other two radicals analyzed in irradiated CHD. The proton coupling giving rise to this transition was almost overlooked because its intensity was so low and because it could not be detected within a few days after irradiation. The absorption line was first thought to be merely an artifact of the rf detection system as it occurred near one such artifact at about 47 MHz, which was much more intense. When a new crystal was irradiated in order to re-investigate the $^{14}$N-hyperfine coupling of the C(8)-hydrogen addition radical, this absorption line was observed again. A more careful analysis of this ENDOR response demonstrated that it was indeed associated with a proton hyperfine coupling, as it showed a small site splitting and the appropriate magnetic field dependence (see Eq. 6-1). Its field dependence further indicated that it was the high frequency transition.

Because the radical was not indefinitely stable, a new crystal was freshly irradiated just prior to studying the coupling’s angular variation in each of the three orthogonal planes. The angular variation of this proton coupling is shown in Fig. 23, which was obtained by the methods described in Sec. 5.3. The diagonalized form of each of the two solutions for the proton hyperfine tensor calculated using LSF is given in Table 7. The two solutions arise because one cannot, on the basis of the angular variation plot alone, associate the curves corresponding to the same site on passing from the a*b to the bc plane. The
Fig. 23. Angular variation of the high-frequency ENDOR line of the methyl radical.
Table 7. Principal elements of the methyl radical proton hyperfine tensor.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Hyperfine Coupling (MHz)</th>
<th>Isotropic</th>
<th>Anisotropic</th>
<th>Direction cosines w.r.t. a*bc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (physical solution)</td>
<td>-62.65</td>
<td>1.48</td>
<td>0.3493; 0.9350; -0.0615</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>-62.62</td>
<td>4.04</td>
<td>0.4555; 0.5061; -0.7324</td>
<td></td>
</tr>
<tr>
<td>N_1-C_10 bond direction</td>
<td></td>
<td>0.4779, -0.5489; 0.6858</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
physically meaningful solution can often be determined by comparing the
tensor directions to directions consistent with the proposed radical
obtained from the crystal structure. This procedure was sufficient to
determine the physical solution for all the tensors of both the other
radicals analyzed in CHD. Unfortunately neither solution for this tensor
bore any distinctive relationship to the purine moiety in the undamaged
crystal, so additional experiments were required to remove the ambiguity.

As was suggested by Morton\textsuperscript{118} for EPR, this question can be solved by
measuring transitions in supplemental directions not in the three ortho-
gonal planes. Only the physical solution will, in general, account for
the observed site splitting in a supplemental plane. For example, if
the static magnetic field was parallel to the (0.8585; 0.3420; -0.3822)
direction, solutions I and II would entail site splittings of 0.44 MHz
and 1.04 MHz, respectively. The experimentally observed site splitting
for this orientation was 0.42 MHz, indicating that solution I was the
physically meaningful one. The ambiguity in the signs of the direction
cosines arose from the difficulty in assigning the tensor to a definite
crystallographic site and will be discussed later.

No other coupling, greater than 3 MHz, which could have been assigned
to this radical was detected. Since the observed coupling had such a
low intensity ENDOR spectrum, it is possible that other couplings were
present, but were below the detection limit of the spectrometer. The
observed coupling could not be assigned to any radical involving the
purine ring. We have therefore assumed that this coupling was the only
intramolecular proton coupling associated with the radical and have
attributed it to the three equivalent protons of a rapidly rotating methyl radical, \( \cdot \text{CH}_3 \). The four-line EPR spectrum characteristic of a hyperfine coupling to three equivalent protons could not be seen in the total EPR spectrum of CHD irradiated at room temperature, since the spectrum of the dominant radical completely masked that of the other radicals. We therefore attempted to observe the EPR spectrum of CHD irradiated and studied at 77°K.

This experiment was performed by mounting the crystal on a copper rod which was then immersed in liquid nitrogen and x-irradiated for two hours. The copper rod was then quickly inserted into another copper rod which was itself cooled to approximately 77°K by conduction through a cylindrical copper can filled with liquid nitrogen. This apparatus was placed in a glass Dewar and inserted into the Varian E-3 EPR cavity. The resulting, very intense EPR spectrum could not be attributed to the methyl radical, but its intensity was sufficient to mask the spectrum of \( \cdot \text{CH}_3 \). Further experiments showed that this intense spectrum resulted from radicals produced in the Dow-Corning High Vacuum Grease used to bind the crystal to the copper rod. We repeated the experiment with several different binders, using the smallest amount of binder possible, but in all cases radicals produced in the binder masked any produced in the crystal. The one coupling detected in the ENDOR spectrum was thus the only experimental information with which we could identify the radical. This coupling is in agreement with those determined previously for methyl radicals produced in other systems, as we shall now attempt to show.
The methyl radical has previously been observed in irradiated crystals of alkali metal acetates. Rogers and Kispert\textsuperscript{119} reported the first EPR study of oriented methyl radicals in sodium acetate trihydrate. They determined both the $^1\text{H}$ and $^{13}\text{C}$ hyperfine coupling tensors and observed satellite lines arising from simultaneous spin flips\textsuperscript{120} of nearby protons. Tolles et al.\textsuperscript{121} have observed CH$_3$ in zinc acetate dihydrate irradiated at 77°K and found that it converts quantitatively into CH$_2$CO$_2^-$ at higher temperatures. The first ENDOR analysis of an oriented methyl radical was recently reported by Toriyama, Nunome and Iwasaki\textsuperscript{122}, who studied the radical in lithium acetate dihydrate. They found a deviation from axial symmetry for the proton hyperfine tensor which was explained in terms of a hindered oscillation about an axis in the radical plane, in addition to rapid rotation about the C$_3$ axis.

The isotropic hyperfine coupling constant provided the first clue to the identity of the radical we observed. Its magnitude of -62.65 MHz is consistent with the value of -64.57 MHz reported by Fessenden and Schuler\textsuperscript{123} for CH$_3$ in the liquid phase. The slightly less negative value we have observed can be explained in terms of a slightly bent structure of the radical or by spin delocalization over the neighbouring molecules in the host crystal. Our value is in agreement with the values of -62 MHz and -62.5 MHz reported for zinc acetate dihydrate\textsuperscript{121} and sodium acetate trihydrate\textsuperscript{119}, respectively, and closer to the liquid phase value than that of -59.30 MHz reported for lithium acetate dihydrate\textsuperscript{122}. A hyperfine coupling of the magnitude we observed for three equivalent protons should have produced a second-order splitting\textsuperscript{108} in
the ENDOR spectrum similar to that observed for \( \text{CH}_3 \) in \( \text{CH}_3\text{COOLi} \cdot 2\text{H}_2\text{O} \). Toriyama et al.\(^{122}\) derived theoretical expressions, correct to second-order, for the ENDOR frequencies and transition probabilities of the methyl radical protons, which were shown to depend on the particular EPR line being saturated. Although the EPR spectrum of the methyl radical could not be distinguished in our case, the saturation point we chose in the total spectrum corresponded approximately to the expected position of the \( M_J = +\frac{1}{2} \) hyperfine line of \( \text{CH}_3 \), where \( J = \sum_{i=1}^{3} I_i \) the sum of the total nuclear spin angular momenta, and \( M_J \) is a component of \( J \). At this saturation point, Toriyama et al.\(^{122}\) observed a second-order splitting in the ENDOR spectrum of 0.18 MHz separating two absorption lines with an intensity ratio of approximately 2:1. This splitting, which we will discuss in more detail in a later section for the more general case of two non-equivalent couplings, would have provided the most satisfactory confirmation of the presence of the methyl radical. We could not detect this splitting so our assignment is only tentative. However, the observed ENDOR line was so weak that the weaker line, even if present, was below the detection limit of the spectrometer. We have assumed this to be the case because we cannot visualize any other radical structure to account for the observed coupling.

The existence of only one proton hyperfine tensor for the methyl radical indicates that the three methyl protons are equivalent. This equivalence can only occur if the methyl radical undergoes rapid re-orientation about its \( C_3 \) axis. The principal elements and directions
Principal elements and directions of the dipolar proton tensor for the methyl radical. (a) Fixed C-H$_1$ fragment. (b) Rapid rotation about C$_3$ axis. (c) Additional hindered oscillation about X (⊥C$_3$ axis).

Fig. 24.
of the dipolar tensor, discussed previously in Sec. 4.2.2, for one proton, \( H_1 \), of a methyl radical in the rigid state, are shown in Fig. 24a. The principal value perpendicular to the radical plane, \( B_3 \), is not expected to be exactly zero and depends on the precise geometry of the radical. For example, Lee and Box\textsuperscript{124} found a value for \( B_3 \) of +2.49 MHz for CH\(_2\)(OD)CH(ND\(_3\)) in irradiated serine-d\(_4\). In our study of irradiated cytosine monohydrate (Chapter Five), we found this value to be -2.09 MHz for the C(6)-H proton, with a spin density of 0.35 in the C(6) p-orbital. Normalized to unit spin density in the carbon p-orbital, this value becomes \( B_3 = -5.97 \text{ MHz} \). Our analysis of that radical suggested that the C(6)-H bond was displaced slightly out of the plane containing the other cytosine atoms. We will later show that the \( B_3 \) value for the cytosine radical is in agreement with that of the methyl radical in CHD.

Rapid rotation about the C\(_3\) symmetry axis of the methyl radical completely averages the tensor elements in the radical plane, as shown in Fig. 24b. The principal elements in this plane must each be equal to \(-\frac{B_3}{2}\), as the dipolar tensor is traceless. The observed anisotropic hyperfine tensor (Table 7), however, did not exhibit axial symmetry.

Toriyama et al.\textsuperscript{122} analyzed this deviation from axial symmetry in terms of a hindered oscillation about an axis perpendicular to the C\(_3\) axis. The effect of this oscillation is to partially average the principal values of the tensor parallel to the C\(_3\) axis and perpendicular to both this and the oscillation axis, as shown in Fig. 24c. For a fixed value of \( \theta \), the deviation from the equilibrium position in the
plane perpendicular to the oscillation axis, the tensor elements are:

\[
\begin{align*}
A_{xx} &= A_{XX} = -\frac{B_3}{2} \\
A_{xy} &= A_{xz} = 0 \\
A_{yy} &= A_{yy}\cos^2\theta + A_{zz}\sin^2\theta = (A_{yy} - A_{zz})\cos^2\theta + A_{zz} \\
A_{yz} &= (A_{yy} - A_{zz})\sin\theta\cos\theta \\
A_{zz} &= (A_{zz} - A_{yy})\cos^2\theta + A_{yy} = \left(\frac{3}{2}\cos^2\theta - \frac{1}{2}\right)B_3
\end{align*}
\]  
(6-4) (6-5) (6-6) (6-7) (6-8)

The averaged principal values are therefore:

\[
\begin{align*}
\langle A_{xx} \rangle &= -\frac{B_3}{2} \\
\langle A_{yy} \rangle &= B_3(1 - \frac{3}{2}\langle\cos^2\theta\rangle) \\
\langle A_{zz} \rangle &= B_3\left(\frac{3}{2}\langle\cos^2\theta\rangle - \frac{1}{2}\right)
\end{align*}
\]  
(6-9) (6-10) (6-11)

with principal directions corresponding to \( \theta = 0^\circ \) in Fig. 24c. Applying these expressions to the observed dipolar tensor, we obtained the following values for \( B_3 \) and \( \langle\cos^2\theta\rangle \):

\[
\begin{align*}
B_3 &= -2\langle A_{xx} \rangle = -6.30 \text{ MHz} \\
\langle\cos^2\theta\rangle &= 0.824
\end{align*}
\]  
(6-12) (6-13)

The preceding analysis has shown that the \( C_3 \) axis of the radical has the direction cosines, \((0.8256; \pm0.2761; 0.4921)\), obtained for the principal value, \(-4.64 \text{ MHz} \) (Table 7), of the dipolar tensor. These direction cosines were then compared to directions obtained from the
crystal structure in an effort to assign the tensor to a definite crystallographic site and to thereby identify the source of the methyl radical.

Toriyama et al.\textsuperscript{122} found that in irradiated CH\textsubscript{3}COOLi·2H\textsubscript{2}O the C\textsubscript{3} symmetry axis did not change upon radical formation; that is, the unpaired electron orbital of the methyl radical was parallel to the broken C-CH\textsubscript{3} bond. Furthermore, the methyl carbon was only slightly displaced from its position in the undamaged molecule.

The C\textsubscript{3} axis of the methyl radical in irradiated CHD was not, however, parallel to any N-CH\textsubscript{3} bond in either magnetically distinct caffeine moiety. As a result, we could not resolve the ambiguity in the signs of the direction cosines of the dipolar tensor without making major assumptions concerning the nature of the trapping site.

If we assume that formation of the radical involved minimal change in the environment of the methyl group and that the source of the methyl radical was the CH\textsubscript{3} group which had the N-CH\textsubscript{3} bond direction closest to either of the two possible directions of the radicals' C\textsubscript{3} axis, then the methyl group attached to N(1) is the source of the radical. If these assumptions are correct, then the principal directions of the dipolar tensor have the direction cosines shown in Table 7 without the signs in parenthesis. The projection of the C\textsubscript{3} axis is then within 3° of the N(1)-CH\textsubscript{3} bond in the molecular plane, but the axis itself is tilted out of the molecular plane by 25°.

It is interesting to note that although there are three different methyl groups attached to the purine ring, only one type of methyl
radical was detected. Radical formation is therefore a selective process. If the N-\text{CH}_3 bond is directly broken by the ionizing radiation, then the N(1)-\text{CH}_3 bond must be more susceptible to this cleavage than the other N-methyl bonds. The resulting purine radical must then react further until it becomes diamagnetic, since the methyl radical was clearly in the doublet state. Alternatively, the radical could result from release of a methyl group from the purine ring following the formation of an unstable radical in the caffeine moiety. The one methyl group is more labile than the others and its release results in a diamagnetic purine derivative.

The value for $B_3$ of $-6.30$ MHz for the methyl radical is in agreement with that of $-5.97$ MHz found for the C(6)-H proton in irradiated cytosine monohydrate. This suggests that \text{CH}_3 trapped in this crystal is not exactly planar, but is slightly bent. This is consistent with the observed isotropic hyperfine coupling, which is slightly less negative than the value found in solution$^{123}$. It is unfortunate that the ENDOR response of this radical was too weak to detect the second-order splitting and that its EPR spectrum could not be resolved. Without these observations the assignment can only be tentative, although our results are completely consistent with it.

6.5 The C(8)-Hydrogen Addition Radical

6.5.1 Experimental Results

The ENDOR absorption lines in Fig. 18, which have been marked by letters, were all associated with the radical that dominated the EPR
spectrum (Radical VI). The weaker lines, marked by primed letters, exhibited essentially the same angular variation as did their unprimed counterparts. These lines appeared with similar relative intensities for each of the five crystals examined and for any saturation point in the EPR spectrum. We have, therefore, concluded, that Radical VI is trapped in two slightly different conformations in irradiated CHD, as in the case of the hydrogen-addition radical in irradiated cytosine monohydrate (Sec. 5.5). Because analysis of both conformations, which differ marginally in the spin density distribution and orientation of the radical, provided very little additional information about the electronic structure of the radical, we will henceforth only discuss the hyperfine interactions of the dominant conformation. Several weak ENDOR lines also appeared in Fig. 18, which were not analyzed because they could not be detected in all three planes. Careful, selective saturation studies indicated that these lines were not associated with any of the three radicals we have analyzed. The relatively strong ENDOR response occurring at a slightly lower frequency than that of coupling C was found to be an artifact of the r.f. detection system.

The angular variations of the proton and nitrogen ENDOR frequencies of radical VI are shown in Fig. 25 and Fig. 26. The proton frequencies shown in Fig. 25 were found to represent the low frequency transitions, \( \nu< \), of the E and F proton hyperfine couplings, while those in Fig. 26 represented the high frequency transitions, \( \nu> \), of the C and D couplings. The solid curves in Fig. 26 represent the theoretical variations determined by the programme FIELDS\(^{74,75}\) using tensor parameters obtained from
Fig. 25. Angular variation of the methylene proton ENDOR frequencies of radical VI.
Fig. 26. Angular variation of the nitrogen and "weakly-coupled" proton ENDOR frequencies of radical VI.
the programme LSF\textsuperscript{39,40}. The ENDOR lines were too weak to be detected below 10 MHz so the angular variations below this frequency are not shown. The significance of the solid and dotted curves in Fig. 25 will be discussed in Section 6.5.5.

6.5.2 Spin Density Calculations

One of the original reasons for performing this ENDOR investigation was to study the C(8)-hydrogen addition radical, if it were indeed produced in this system, as a model for this radical in other systems. We therefore first performed spin density calculations for the expected radical to determine whether it could serve as such. The spin densities predicted by the INDO-MO approximation using the computer programme written by Pople and Beveridge\textsuperscript{65} are listed in Table 8, together with spin densities (>8%) obtained by Pullman and Mantione\textsuperscript{125} using the Hückel-MO method for the same radical in guanine and adenine. The atomic positions used in our calculations were obtained initially from the positions in the undamaged molecule, which were then altered in the region of C(8) to account for the extra hydrogen and the change in hybridization of C(8). The agreement between the spin density distributions for this radical in these two compounds indicated that radical VI in irradiated CHD could indeed serve as a useful model.

6.5.3 Assignment of the C and D Proton Hyperfine Couplings.

The diagonalized forms of the C and D hyperfine tensors, calculated by the programme LSF, are given in Table 9, along with bond directions
Table 8. Theoretical distribution of unpaired spin density in hydrogen addition radical of caffeine, guanine and adenine.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spin Density on Position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N1  C2  N3  C4  C5  C6  N7  C8  N9  O11  O13</td>
</tr>
<tr>
<td>caffeine</td>
<td>-0.03 -0.02 0.04 0.04 0.16 -0.10 0.47 0.09 0.04 0.19</td>
</tr>
<tr>
<td>guanine a</td>
<td>0.14 0.15 0.39 0.08</td>
</tr>
<tr>
<td>adenine a</td>
<td>0.11 0.11 0.11 0.43</td>
</tr>
</tbody>
</table>

a) Reference 125.
Table 9. Principal elements of two proton hyperfine tensors of radical VI.

<table>
<thead>
<tr>
<th>Tensor</th>
<th>Isotropic</th>
<th>Anisotropic</th>
<th>Direction Cosines (w.r.t. a*bc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-2.42</td>
<td>0.8694; 0.4846; -0.0967</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>-8.72</td>
<td>-6.87; 0.1024; 0.0153; 0.9946</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.30</td>
<td>-0.4834; 0.8746; 0.0364</td>
<td></td>
</tr>
<tr>
<td>Theoretical N(9)-H</td>
<td>-2.77</td>
<td>0.8535; 0.4968; -0.1573</td>
<td></td>
</tr>
<tr>
<td>dipolar tensor</td>
<td>-6.03</td>
<td>0.1722; 0.0159; 0.9849</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.80</td>
<td>-0.4917; 0.8678; 0.0719</td>
<td></td>
</tr>
<tr>
<td>N(9)-H bond direction</td>
<td>-0.4770</td>
<td>0.8765; 0.0646</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.60</td>
<td>0.3940; -0.5767; 0.7157</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>30.91</td>
<td>-2.48; -0.8797; -0.4623; 0.1118</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.09</td>
<td>-0.2662; 0.6737; 0.6893</td>
<td></td>
</tr>
<tr>
<td>N(7)-C(14) direction</td>
<td>-0.3066</td>
<td>0.7186; 0.6242</td>
<td></td>
</tr>
</tbody>
</table>
in the undamaged crystal corresponding to these tensors. Tensor C exhibits typical α-proton character and has been assigned to the N(9)-H proton. The direction of its largest positive dipolar principal value was within 2° of the N(9)-H bond direction. The spin density in the nitrogen p orbital, \( \rho_{\text{N}} \), was inferred from the isotropic proton coupling using a McConnell constant \( Q \) of \(-87\) MHz\(^{78}\) and was thus found to be \( 0.10 \). In order to obtain a second, independent estimate of this spin density we reconstructed theoretically the dipolar tensor using the McConnell-Strathdee method discussed in Sec. 4.2.2. The calculation included contributions from the 2s and 2p\(_z\) orbitals of the carbon, nitrogen, and oxygen atoms in the caffeine molecule. The atomic positions were assumed to be those used in the INDO-MO calculations and the spin densities used were obtained from these calculations (Table 8). The theoretical N(9)-H dipolar tensor is shown in Table 9 and is in excellent agreement with the experimental tensor, thereby implying that \( \rho_{\text{N}} = 0.09 \).

The relatively small anisotropy of tensor D indicates that it represents a β-proton coupling. Furthermore, the direction if its largest positive dipolar principal value is within 5° of the N(7)-C(14) bond direction. We have therefore assigned this tensor to the three equivalent protons of a rapidly rotating methyl group attached to N(7). (See Sec. 6.3 for a more complete description of the tensor expected for a rotating methyl group.) The ENDOR enhancement associated with this hyperfine interaction was considerably greater than that of any other coupling in this radical. Direct comparison of the intensity of different ENDOR lines is generally not profitable because both the
ENDOR enhancement\(^{126}\) and the impedance of the r.f. coil change with the r.f. frequency. The processes governing ENDOR enhancements are very complex\(^{126}\) and are not yet fully understood. The ENDOR intensity of this coupling, however, is clearly anomalous. It is possible that the rotation of the methyl group introduces spin-rotational coupling which allows new or enhanced relaxation mechanisms.

The spin density in the N(7) p orbital was inferred from the isotropic proton coupling using a McConnell constant, \(Q_{\text{CH}_3}\), of 71 MHz (see Sec. 6.3) and was thus found to be 0.44. This is in excellent agreement with the value of 0.47 determined in the INDO-MO calculation. We were also able to obtain an independent estimate of this spin density from the nitrogen hyperfine tensor discussed in the next section.

6.5.4 The \(^{14}\text{N}\) Hyperfine and Quadrupole Interactions

Although the \(^{14}\text{N}\) nucleus is present in many biologically important compounds, its ENDOR spectrum has been detected in only a few of these compounds. This is due in part to its small nuclear magnetic moment and to its rapid nuclear spin-lattice relaxation. Box, Freund and Budzinski\(^{127}\) reported \(^{14}\text{N}\) hyperfine constants obtained by ENDOR analysis of x-irradiated valine. Rist and Hyde\(^{128}\) observed \(^{14}\text{N}\)-ENDOR signals from a study of Cu-8-hydroxyquinolate doped in crystals of pthalamine and 8-hydroxyquinoline. More recently, Rustgi and Box\(^{129}\) have reported the \(^{14}\text{N}\) hyperfine and quadrupole coupling tensors of the inorganic species, NO\(_2\) and NO\(_3^-\), formed in x-irradiated glycylglycine-HNO\(_3\).
Nelson, Atwater and Gordy\textsuperscript{130} obtained the $^{14}\text{N}$ hyperfine and quadrupole coupling tensors from an ENDOR analysis of the CH$_3$C(NO$_2$)C(CH$_3$)$_3$NO\textsuperscript{-} radical formed in $\gamma$-irradiated dimethylglyoxime crystals.

The two ENDOR lines marked by the letter N in Fig. 18 were found to be very anisotropic and have been assigned to a $^{14}\text{N}$ nucleus in radical VI. The selection saturation study illustrated in Fig. 27 showed that these lines arose from transitions between different energy levels within the same $M_S$ manifold (see Sec. 2.4.3). The small site splitting (0.29 MHz) of the ENDOR lines shown in Fig. 27 resulted from a slight misalignment of the crystal and the large anisotropy of this hyperfine interaction. The ENDOR lines corresponding to transitions within the other $M_S$ manifold were masked by proton resonances in this orientation. The saturation study was also used to determine the relative signs of the hyperfine and quadrupole tensors, according to the method outlined by Cook\textsuperscript{131}. The energy level diagram shown in Fig. 4 is drawn for the case in which $a$ and $P$ were both positive. If the low field EPR line, corresponding to the $|\pm \frac{1}{2}, \frac{1}{2}\rangle$ to $|\pm \frac{3}{2}, \frac{1}{2}\rangle$ transition, is saturated then the two observed ENDOR transitions have frequencies given by:

\[
\Delta 1 = |\frac{a}{2}| + |\nu_N| - |P| \\
\Delta 4 = |\frac{a}{2}| - |\nu_N| + |P|
\]

(6-14)

If, however, the relative sign of $a$ and $P$ is reversed, saturation of the same EPR line would give rise to two ENDOR transitions with frequencies:
Fig. 27. A selective saturation study used to determine the relative signs of A and P for $^{14}$N. (a) The EPR spectrum for $H_0$ parallel to $a^*$. (b) $^{14}$N ENDOR lines for saturation point 1. (c) $^{14}$N ENDOR lines for saturation point 2.
\[ \Delta 1 = \frac{a}{2} + |v_N| + |p| \]
\[ \Delta 4 = \frac{a}{2} - |v_N| - |p| \]  

(6-15)

It should be noted that the expressions given in the reference\textsuperscript{131} for the ENDOR frequencies are incorrect in that the sign preceding $|v_N|$ is wrong.

The diagonalized forms of the $^{14}$N hyperfine and quadrupole tensors which were calculated using the computer programme, LSF, described in Sec. 2.6, are given in Table 10. The absolute signs of the principal elements of the hyperfine tensor were assigned on the basis of theoretical predictions (see Sec. 4.4). The signs of the quadrupole tensor elements were then determined from a selective saturation study. The orientation we chose for this study was nearly parallel to the direction of the most positive principal element of the hyperfine tensor, which was colinear with $X_3$ of the quadrupole tensor. The results of our fitting procedure indicated that the $^{14}$N-ENDOR lines shown in both Fig. 18 and Fig. 26 arose from transitions within the $M_S = +\frac{1}{2}$ manifold. When a low-field EPR line was saturated, only the higher-frequency line was observed in the ENDOR spectrum, as shown in Fig. 27. This ENDOR line must therefore correspond to $\Delta 4$ in Eq. 6-14 and $X_3$ must be positive.

A rigorous interpretation of the nuclear quadrupole coupling constants would be very difficult because of the complex dependence of the field gradient at the nitrogen nucleus on all of the extranuclear charges. Reasonable results have previously been obtained\textsuperscript{132}, however,
Table 10. Principal elements of the $^{14}$N hyperfine and quadrupole tensors.

<table>
<thead>
<tr>
<th>Hyperfine Coupling (MHz)</th>
<th>Isotropic</th>
<th>Anisotropic</th>
<th>Direction cosines in a*bc system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-19.56</td>
<td>-0.4829; 0.8741; -0.0513</td>
<td></td>
</tr>
<tr>
<td>30.91</td>
<td>-20.67</td>
<td>0.0753; 0.0998; 0.9922</td>
<td></td>
</tr>
<tr>
<td>40.22</td>
<td></td>
<td>0.8724; 0.4753; -0.1140</td>
<td></td>
</tr>
</tbody>
</table>

Quadrupole Tensor (MHz)

- $\chi_1 = -2.30$  
  -0.2538; 0.6667; 0.7008

- $\chi_2 = 0.76$  
  0.5661; -0.4851; 0.6665

- $\chi_3 = 1.53$  
  0.7843; 0.5659; -0.2543

$N(7)$-$C(14)$ bond direction -0.3066; 0.7186; 0.6242
using a very approximate method developed by Townes and Daily\textsuperscript{133-135} and later modified and extended by Gordy\textsuperscript{136-138}. In its simplest form, this theory attributes the field gradient to an unequal filling of the p orbitals in the valence shell of the atom containing the coupling nucleus; all other charges are ignored. The principal frame of the observed quadrupole tensor is nearly that defined by the N(7)-C(14) bond direction and the molecular plane. This is consistent with the \textsuperscript{14}N-hyperfine tensor, which was also assigned to N(7) for reasons to be discussed later. If we define an axis system (x,y,z) such that x is parallel to the N(7)-C(14) bond, z is perpendicular to the molecular plane and y is perpendicular to both x and z, then the principal elements of the theoretical coupling tensor are given by\textsuperscript{139}:

\begin{align*}
\chi_{1\mathrm{TD}} &= [n_x - \frac{1}{2}(n_y + n_z)]eQq_{210} \\
\chi_{2\mathrm{TD}} &= [n_y - \frac{1}{2}(n_x + n_z)]eQq_{210} \\
\chi_{3\mathrm{TD}} &= [n_z - \frac{1}{2}(n_x + n_y)]eQq_{210}
\end{align*}

(6-16)

(6-17)

(6-18)

where e is the proton charge, Q is the electric quadrupole moment of the nucleus, $n_x$, $n_y$ and $n_z$ are the occupation numbers of the 2p-orbitals and $q_{210}$ represents the field gradient due to an electron in an atomic 2p-orbital. There is some uncertainty in the value of $eQq_{210}$ for nitrogen. In this case, $eQq_{210}$ was assumed to be -10 MHz, as suggested by Gordy and Cook\textsuperscript{139}. The occupation numbers of the 2p-orbitals were obtained directly from the INDO-MO computer programme\textsuperscript{65} as an inter-
mediate output during the spin density calculation and were found to be:

\[ n_x = 1.15; \ n_y = 1.18; \ n_z = 1.42 \]  \hspace{1cm} (6-19)

The total valence shell population was found to be 5 so there is no formal charge associated with the nitrogen atom. The theoretical quadrupole tensor obtained using Eq. (6-16) - (6-18) is shown in Table 11, along with the experimental tensor.

It is clear from Table 11 that the theoretical tensor does not agree with the experimental results. It was first thought that the transitions responsible for the observed ENDOR lines had been incorrectly assigned. This is unlikely though, since the least-squares fitting programme converged for this assignment with an rms error of 0.02 MHz for fifty-five input transitions. This programme did not converge for any other assignment of the transitions consistent with the results of the saturation study. The disagreement between the tensors must therefore result from assumptions that were inherent in the field-gradient calculation and were not valid. One of these was the assumption that contributions by electronic and nuclear charges of atoms other than those of the coupling nucleus could be neglected. The radical is trapped in an ionic lattice (the chloride ion closest to N(7) is only 3.8 Å distant) so there will be a contribution to the total field-gradient at N(7) from the ions in the crystal. Calculation of this contribution would in theory involve a summation over all the ions, both positive and negative, of the lattice. Such a calculation would
Table 11. Experimental and theoretical principal elements of the $^{14}$N-quadrupole tensor.

**Experimental Quadrupole**

<table>
<thead>
<tr>
<th>Tensor (MHz)</th>
<th>Direction Cosines in a*bc System</th>
<th>Direction in caffeine moiety</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\chi_1 = -2.30$</td>
<td>-0.2538; 0.6667; 0.7008</td>
<td>$\parallel$ to N(7)-C(14)</td>
</tr>
<tr>
<td>$\chi_2 = 0.76$</td>
<td>0.5661; -0.4851; 0.6665</td>
<td>$\perp$ to $\chi_1^{TD}$ and $\chi_3^{TD}$</td>
</tr>
<tr>
<td>$\chi_3 = 1.53$</td>
<td>0.7843; 0.5659; -0.2543</td>
<td>$\perp$ to plane of molecule</td>
</tr>
</tbody>
</table>

**Theoretical Quadrupole**

<table>
<thead>
<tr>
<th>Tensor (MHz)</th>
<th>Direction Cosines in a*bc System</th>
<th>Direction in caffeine moiety</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\chi_1^{TD} = 1.50$</td>
<td>-0.3066; 0.7186; 0.6242</td>
<td>$\parallel$ to N(7)-C(14)</td>
</tr>
<tr>
<td>$\chi_2^{TD} = 1.05$</td>
<td>0.3786; -0.5096; 0.7726</td>
<td>$\perp$ to $\chi_1^{TD}$ and $\chi_3^{TD}$</td>
</tr>
<tr>
<td>$\chi_3^{TD} = -2.55$</td>
<td>0.8724; 0.4753; -0.1140</td>
<td>$\perp$ to plane of molecule</td>
</tr>
</tbody>
</table>
be complicated by the fact that the lattice is altered as a result of radical formation. Furthermore, the cation is a large molecule (caffeine-H\(^+\)), so cations close to the radical could not be replaced by point charges for this calculation.

A second assumption made in our simple, Townes-Dailey calculation was that the electrons in closed subvalence shells of the coupling nucleus could be neglected as a result of their spherical symmetry. This assumption is not valid if the combined field-gradients of the valence electrons and the external ions are large enough to polarize the closed shell electrons. These electrons would therefore lose their spherical symmetry and contribute to the total field-gradient at the nucleus. This effect has previously been observed and is referred to as Sternheimer shielding\(^{140}\), after R. M. Sternheimer who has made some of the most important contributions to the understanding of this phenomenon. The most dramatic examples of this shielding occur when a closed-shell ion is located in an ionic lattice at a point where the field-gradients are non-vanishing\(^{141}\). In such cases, the field-gradient induced by the polarization of the originally spherically symmetric shell can be as much as two orders of magnitude greater than that due to the ionic lattice. The actual field-gradient \(V_{zz}\) is usually expressed by:

\[
V_{zz} = V_{zz}^0 (1 - \gamma_{\infty})
\]  

(6-20)

where \(V_{zz}^0\) is the field-gradient from the ionic lattice and \(\gamma_{\infty}\) is the
shielding factor. A value of \( \gamma_\infty \) has not been reported for a nitrogen atom in a molecular environment and its calculation is not within the scope of this thesis. Furthermore, theoretical reconstruction of the quadrupole tensor would not provide any additional information concerning the nature of the hydrogen-addition radical in biological systems.

The \(^1\)H-\(N\)-hyperfine tensor was found to be almost axially symmetric, with the direction of the most positive principal value only 0.2° from the perpendicular to the molecular plane. The slight deviation from axial symmetry can be explained in terms of a puckering of the imidazole ring and is consistent with the observation that the methylene protons attached to C(8) were not equivalent (Sec. 6.5.5). Because the deviation is very small, we will henceforth assume the tensor has axial symmetry, with \( A_\parallel = 71.13 \text{ MHz} \) and \( A_\perp = \frac{1}{2}(11.35 + 10.24) \text{ MHz} = 10.80 \text{ MHz} \). The anisotropic part of the tensor has the form (Sec. 4.4.2): 

\[
\Delta B = \alpha B_0, \quad 2B_0, \quad -B_0, 
\]

where \( B_0 \) is related to the unpaired spin density in the p\(^\pi\)-orbital of the nitrogen atom. The theoretical value of \( B_0 \) for a spin density of unity in the p-orbital is 47.9 MHz. The spin density of the p\(^\pi\) orbital is therefore \( \frac{20.12}{47.9} = 0.420 \). This value is very close to the value of 0.44 obtained for N(7) from the isotropic part of the methyl proton coupling (Sec. 6.5.3), thereby confirming the assignment of the nitrogen tensor to N(7). The isotropic coupling of 30.91 MHz results from s-orbital spin density on \(^1\)H via spin polarization by the \( \pi \)-electron spin density on the nitrogen atom. Unit spin density in the nitrogen p\(^\pi\)-orbital would yield a value for the McConnell constant, \( Q_\Sigma^N \), of
30.91 MHz/0.420 = 73.6 MHz, in agreement with values found by Carrington and Santos-Viega\textsuperscript{59} for several nitrogen-containing heterocyclic radicals. This value is significantly higher, however, than the values of 60.0 MHz and 59.1 MHz reported for this radical in adenine dihydrochloride\textsuperscript{98} and in guanine hydrochloride dihydrate\textsuperscript{93}, respectively. Because the π-spin density distribution is essentially the same in all three radicals (Sec. 6.5.6), the difference in the values of $Q_N^S$ must result from a change in the s-orbital population of the nitrogen. This change can arise from differences in the bonding to the substituent attached to N(7), which is the CH$_3$ group in the caffeine radical and nominally H$^+$ in the others. The dative bond to the proton in the adenine and guanine radicals would clearly tend to draw s-electron density away from the nitrogen, thereby reducing the value of $Q_N^S$.

6.5.5 The Methylene Protons

The ENDOR lines marked by the letters E and F in Fig. 18 have been assigned to the two slightly non-equivalent, methylene protons attached to C(8). The angular variation of these lines in the three reference planes is shown in Fig. 28. In the bc plane, the experimental curves show a marked deviation from the theoretical curves, indicated by the dotted lines, which were obtained by treating each electron-proton interaction independently. This phenomenon has previously been observed by Gloux\textsuperscript{143} in an ENDOR study of the hydrogen-addition radical in γ-irradiated 1,2,4-triazole. Gloux used second-order perturbation theory to account for the observed non-crossing phenomenon. His analysis
Fig. 28. Angular variation of the methylene proton ENDOR frequencies of radical VI.
indicated that the electron-proton interactions of the methylene group
could not be treated independently because there was a repulsion between
the two intermediate energy levels within an $M_s$ manifold (Fig. 29)
which became significant when the energy difference between these levels
was small.

In order to illustrate the nature of this repulsion, we will use
the example of two protons with isotropic hyperfine couplings, $a_1$ and
$a_2$, which are large enough that the nuclear Zeeman interactions can be
neglected. The spin Hamiltonian for this system is:

$$\mathcal{H} = g \beta H \cdot S + a_1 S \cdot I_1 + a_2 S \cdot I_1$$  \hspace{1cm} (6-21)

$$\mathcal{H} = g \beta H \cdot S + a_1 S_z I_{1z} + a_2 S_z I_{2z}$$
$$+ \frac{1}{2} a_1 (S^+ I_{11}^+ + S^- I_{11}^-) + \frac{1}{2} a_2 (S^+ I_{22}^+ + S^- I_{22}^-)$$  \hspace{1cm} (6-22)

The first-order energy levels are shown in Fig. 29, where the spin
states are labelled by $|M_s, M_{I1}, M_{I2}>$. These spin states are not,
however, eigenfunctions of the spin Hamiltonian. If the two protons
are treated independently, the eigenfunctions can be obtained using
the methods outlined in Sec. 2.3.1 and, correct to second-order, are
given by:

$$\Upsilon_1 = |\frac{1}{2}, \frac{1}{2}, \frac{1}{2}>$$  \hspace{1cm} (6-23a)
$$\Upsilon_2 = |\frac{1}{2}, \frac{1}{2}, -\frac{1}{2}> + \frac{a_2}{2 \mu_e} |\frac{1}{2}, \frac{1}{2}, \frac{1}{2}>$$  \hspace{1cm} (6-23b)
$$\Upsilon_3 = |\frac{1}{2}, -\frac{1}{2}, \frac{1}{2}> + \frac{a_1}{2 \mu_e} |\frac{1}{2}, -\frac{1}{2}, \frac{1}{2}>$$  \hspace{1cm} (6-23c)
Fig. 29. Partial energy level diagram for two protons coupled to one electron.
\[ \mathcal{Y}_4 = \left| \pm_1, -\pm_2, -\pm_3 \right> + \frac{a_1}{2v} \left| -\pm_1, \pm_2, -\pm_3 \right> + \frac{a_2}{2v} \left| -\pm_1, -\pm_2, \pm_3 \right> \quad (6-23d) \]

It is clear from these equations that there is a matrix element between \( \mathcal{Y}_2 \) and \( \mathcal{Y}_3 \):

\[ \langle \mathcal{Y}_2 | \mathcal{Y}_3 \rangle = \frac{a_2}{2v} \left| -\pm_1, \pm_2, \pm_3 \right> \left| -\pm_1, \pm_2, \pm_3 \right> = \frac{a_1a_2}{4v} \quad (6-24) \]

which represents an indirect coupling of the nuclear spins as a result of their mutual coupling with the electron spin. This off-diagonal matrix element is responsible for the repulsion between \( \mathcal{Y}_2 \) and \( \mathcal{Y}_3 \). The diagonal elements of the energy matrix relevant to \( \mathcal{Y}_2 \) and \( \mathcal{Y}_3 \) are:

\[ \langle \pm_1, \pm_2, \pm_3 | \mathcal{H} | \pm_1, \pm_2, \pm_3 \rangle = \frac{1}{2} (a_1 - a_2) + \frac{a_2}{4v} \quad (6-25a) \]

\[ \langle -\pm_1, \pm_2, -\pm_3 | \mathcal{H} | -\pm_1, \pm_2, -\pm_3 \rangle = -\frac{1}{2} (a_1 - a_2) + \frac{a_1}{4v} \quad (6-25b) \]

The effect of the indirect coupling on \( \mathcal{Y}_2 \) and \( \mathcal{Y}_3 \) can then be determined by a diagonalization of the energy matrix. This effect only becomes important when the energy difference, \( \Delta = a_1 - a_2 \), between \( \mathcal{Y}_2 \) and \( \mathcal{Y}_3 \) is small, as is clearly shown in Fig. 28. The presence of anisotropic hyperfine interactions complicates the expressions for the energies of the spin states, with the result that the repulsion is orientation-dependent. The source of the repulsion is still the indirect coupling of the nuclear spins; the anisotropic hyperfine interactions merely change the energy difference between \( \mathcal{Y}_2 \) and \( \mathcal{Y}_3 \).
The preceding discussion has indicated that there are two conditions that must be met for this repulsion to be observed in an ENDOR spectrum. First, the hyperfine couplings must be large enough (greater than 50 MHz) to mix the first-order spin states. Secondly, the couplings must be approximately equal, so that $\gamma_2$ and $\gamma_3$ have similar energies. These conditions were met for the methyl radical discussed in Sec. 6.4, which had an isotropic hyperfine coupling of -62.65 MHz for three equivalent protons. In the case of the C(8)-hydrogen-addition radical of CHD, these conditions were only met in the be plane. In the other planes the hyperfine couplings were sufficiently different that the two protons could be treated independently. Lamotte and Gloux\textsuperscript{144} have used the ENDOR technique to study the radical analogous to radical VI formed in the imidazole crystal. In this case, however, the hyperfine couplings of the two methylene protons were sufficiently different that the indirect second-order effect could not be observed.

The solid curves in Fig. 28 represent the theoretical variations determined by the programme FIELDS\textsuperscript{74,75} using the tensor parameters obtained from the programme LSF\textsuperscript{39,40}. In both calculations, the hyperfine tensors of the two methylene protons were treated simultaneously in order to account for the indirect coupling of their nuclear spins. The agreement between the experimental and theoretical transition frequencies is very good. The diagonalized forms of the hyperfine tensors calculated by LSF are given in Table 12. Also included in this table are the theoretical dipolar tensors obtained by the methods outlined in Sec. 6.5.3. The principal values and directions of the theoretical tensors
Table 12. Principal elements of the methylene proton hyperfine tensors of Radical VI.

<table>
<thead>
<tr>
<th>Tensor</th>
<th>Isotropic</th>
<th>Anisotropic</th>
<th>Direction cosines (w.r.t. a*bc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>102.29</td>
<td>-2.42</td>
<td>6.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8576; 0.3369; 0.3885</td>
</tr>
<tr>
<td>Theoretical C(8)-H(1) dipolar tensor</td>
<td>-4.19</td>
<td>-2.92</td>
<td>7.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8916; 0.3643; 0.2689</td>
</tr>
<tr>
<td>F</td>
<td>108.76</td>
<td>-2.21</td>
<td>6.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-0.0166; 0.9445; 0.3281</td>
</tr>
<tr>
<td>Theoretical C(8)-H(2) dipolar tensor</td>
<td>-4.21</td>
<td>-2.95</td>
<td>7.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.6503; 0.5241; -0.5499</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-0.0524; 0.7530; 0.6560</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.7579; -0.3978; 0.5171</td>
</tr>
</tbody>
</table>
are consistent with the experimental tensors, but suggest that the spin density in the \( N(7) \) \( \pi \)-orbital of 0.47 obtained from the INDO-MO calculation (Table 8) is slightly over-estimated.

It is difficult to obtain information about the structure of radical VI from the isotropic hyperfine couplings of the methylene protons. In Sec. 4.3 we showed a semi-empirical relationship between the isotropic proton coupling and the spin density in the \( \pi \)-orbital of an atom \( \beta \) to the protons (Eq. 4-3 and 4-4). In this case, however, the formula becomes more complicated as there are two centres of spin density \( \beta \) to these protons. The observed couplings are too large to be accounted for by a summation of separate McConnell relations for the two nitrogen atoms:

\[
a_H \neq Q_\beta \rho_1 + Q_\beta \rho_2 \tag{6-26}
\]

where \( \rho_1 \) and \( \rho_2 \) refer to the \( \pi \)-spin densities on \( N(9) \) and \( N(7) \) respectively. Bersohn\(^{145}\) has derived expressions for the \( \beta \) couplings in semiquinone ions. Whiffen\(^{146}\) showed that his results explained the unexpectedly large \( \beta \) couplings in the cyclohexadienyl radical\(^{51,147,148}\) and that \( \rho(=c^2) \) in Eq. 4-3 would be replaced not by \( \rho_1 + \rho_2 = (c_1^2 + c_2^2) \) but by \( (c_1 + c_2)^2 \).

The local symmetry of the \( N(7)\text{-CH}_2\text{-N}(9) \) fragment of radical VI indicates that \( Q_\beta(\theta) \) should be very similar for \( N(7) \) and \( N(9) \). The hyperfine coupling can therefore be written as:
\[ a_H(\theta) = Q_8(\theta)(c_1 + c_2)^2 = (B_Q + B_2\cos^2\theta)(c_1 + c_2)^2 \quad (6-27) \]

We were unable to use this formula to obtain a value of the dihedral angle, \( \theta \), because we could not find values of \( B_Q \) and \( B_2 \) for spin density centred on nitrogen atoms. These constants are not expected to differ appreciably from those of carbon radicals. Unfortunately, even for carbon radicals, \( B_2 \) values have been reported ranging from 48 MHz to 151 MHz\(^{118, 148} \). We were, however, able to obtain a rough estimate of the difference in the dihedral angles of the two methylene protons by the following method. The \( \pi \)-orbital coefficients were obtained by taking the square-root of the experimentally determined spin densities. The results of the INDO-MO calculation indicated that both of these coefficients were positive. A value for \( \theta \) of 30° was obtained by assuming that the methylene protons were equivalent and that \( C(8) \) was \( sp^3 \)-hybridized. Using the isotropic coupling of tensor E and assuming that \( B_Q = 9 \text{ MHz}^{80} \), we calculated a value for \( B_2 \) from Eq. 6-27 of 140 MHz. This value, which is within the range reported for carbon radicals, was then used to obtain a value for the dihedral angle of the other proton (tensor F) of \( \theta = 27^\circ \). Thus there is only a difference of 3° between the dihedral angles of the two protons.

6.5.6 Discussion

We have determined the hyperfine tensors for one nitrogen and four different proton couplings. All of these couplings resulted from unpaired spin density centred on atoms in the five-membered imidazole ring. There
Fig. 30. INDO-MO spin density distribution for radical VI.
were no proton couplings, greater than 3 MHz, associated with the two methyl groups attached to the pyrimidine ring. The unpaired spin density in the pπ-orbital of either N(3) or N(1) therefore cannot exceed 3 MHz/71 MHz = 0.04. This observation is in excellent agreement with the INDO-MO calculation for this radical, as is shown in Fig. 30. Our experimentally determined spin densities of 0.09 - 0.10 and 0.42 - 0.44 for N(9) and N(7), respectively, are also in agreement with this calculation. Our results are also consistent with the spin density distribution determined for this radical in adenine dihydrochloride98 and guanine hydrochloride dihydrate93 by EPR, when the enhanced resolution of ENDOR spectroscopy is considered.
CHAPTER SEVEN

CONCLUSIONS

These studies have shown that the superior resolution of the ENDOR technique with respect to EPR is very useful in the identification of radicals trapped in x-irradiated single crystals of pyrimidine and purine derivatives. For example, we were able to analyze all six intramolecular proton hyperfine couplings of the O(2)-hydrogen addition radical in cytosine monohydrate. The EPR spectrum, however, only exhibited a partially-resolved doublet splitting produced by the largest coupling.

Another advantage of the ENDOR technique demonstrated by this work is the often much lower spectral density of the ENDOR spectrum, compared with the parent EPR spectrum, as was discussed in Sec. 2.5. The one nitrogen and four proton hyperfine coupling tensors, determined by ENDOR analysis for the C(8)-hydrogen addition radical in irradiated caffeine hydrochloride dihydrate, are responsible for 192 lines in the EPR spectrum, including site splitting. The resulting EPR spectrum was too complex to be analyzed.

In both systems we have studied, the overall EPR lineshape contained resonances from more than one radical. By saturating different
points on the EPR lineshape of CHD, we were able to identify two radicals whose EPR spectra could not be distinguished in the total EPR lineshape. The selective saturation technique was also used to assign the hyperfine coupling tensors determined for radical I in cytosine monohydrate to a specific crystallographic site. This procedure was necessary because the magnetically inequivalent sites were coplanar and, as a result, all possible intramolecular tensor solutions had one principal direction in common. Finally, this technique was used to determine the relative signs of the nitrogen hyperfine and quadrupole couplings of radical VI in CHD.

Nitrogen ENDOR lines have not previously been reported for any pyrimidine or purine derivative. Lamotte and Gloux also did not observe $^{14}$N-ENDOR from the radical analogous to radical VI formed in the imidazole crystal. This is not particularly surprising since detection of $^{14}$N by ENDOR is often hampered by the rapidity of its spin-lattice relaxation and the small size of its nuclear magnetic moment. Our results suggest that further experimentation may lead to information concerning the mechanism of ENDOR enhancement in these systems, which is not yet fully understood. Our observation of $^{14}$N-ENDOR lines from N(7) of radical VI might be related to the anomalous intensity of the proton ENDOR line associated with the rapidly-rotating methyl group attached to this nitrogen atom. This hypothesis could be tested by an ENDOR analysis of the analogous radical known to be formed in guanine hydrochloride dihydrate. In this radical, the methyl group attached to N(7) is replaced by a hydrogen atom. If $^{14}$N-ENDOR cannot
be detected for this radical, then its detection in the case of radical VI could be related to relaxation effects introduced by the methyl radical. Similarly, if the N(7)-H proton ENDOR lines do not exhibit a much higher intensity than the other proton couplings, then the anomalous intensity of the methyl proton coupling of radical VI was also associated with relaxation effects introduced by the presence of the methyl group.

It is difficult to correlate radicals trapped in single crystals with radiation damage in living organisms as a result of the far greater complexity of the living system. However, radicals which can be trapped in single crystals, can also be formed in the living organism, although their lifetimes are too short to allow their analysis by conventional ENDOR techniques. The identification of the caffeine cation, which was formed by net abstraction of a hydrogen atom from N(9) of the protonated caffeine molecule, supports the ionic mechanism which has been proposed for the radiation damage to DNA. According to this proposal, mutagenesis can be initiated by the ejection of an electron from a pyrimidine or purine base caused by the base's interaction with ionizing radiation. Genetic mutations produced by ultraviolet radiation have also been attributed to reactions involving these bases\(^\text{149}\). The action spectrum producing mutations corresponds to the absorption spectra of these bases, which all have an absorption maximum near 260 nm.

The O(2)-hydrogen addition radical identified in cytosine monohydrate could be of special biological importance, as the oxygen atom is involved in the Watson-Crick hydrogen bonding responsible for the
double-stranded helix of DNA. If, for example, this radical were formed on the single-strand DNA template during replication, there would be a break in the newly-formed complementary strand opposite the cytosine moiety. The cytosine molecule could not form three hydrogen bonds to the complementary purine base, guanine, and therefore would not be recognized. If not correctly repaired, the resulting chromosomal aberration could lead to genetic mutation or cell death.

The ENDOR technique may also prove to be useful in the study of the hydrogen bonding between the strands of the DNA double-helix. The crystal structure has been determined for more than twenty purine-pyrimidine co-crystals, primarily by Sobell and co-workers\textsuperscript{149}. Several of these co-crystals, 9-ethyl-2-aminopurine:1-methyl-5-bromouracil for example, exhibit Watson-Crick type base pairing. Our results have shown that, using the ENDOR technique, one should be able to identify and characterize the radicals trapped in these crystals. The high resolution of the ENDOR technique should allow the observation of the hyperfine couplings of the protons involved in the Watson-Crick bonding. Theoretical interpretation of these hyperfine couplings would yield valuable information concerning the nature of these hydrogen bonds, which play a vital role in living organisms.
BIBLIOGRAPHY

43. For example, see E. DeBoer and S.I. Weissman, J. Am. Chem. Soc., 80, 4549 (1958).
54. For example, see J.P. Colpa and E. DeBoer, Mol. Phys. 7, 333 (1964).
100. A. Brogger, Mutation Res. 23, 353 (1974).
107. A. Mercer and J. Trotter, to be published.
140. R.M. Sternheimer, Phys. Rev. 80, 102 (1950); 84, 244 (1951); 86, 316 (1952); 95, 736 (1954); 105, 158 (1957); 130, 1423 (1963); 132, 1637 (1963); 146, 140 (1966).