An ESR Study of Radiation Effects in Metalloproteins

A Thesis presented for the degree of
Doctor of Philosophy
in the
Faculty of Science
of the
University of Leicester
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August 1984
Thesis
24.2.1985
To my parents, without whose encouragement, at all stages of my education, this thesis would not have been possible.
"Everything that I am now, all of me, so far, is in that"

D. H. LAWRENCE
STATEMENT

The accompanying thesis submitted for the degree of Ph.D. entitled "An ESR Study of Radiation Effects in Metalloproteins" is based on work conducted by the author in the Department of Chemistry at the University of Leicester mainly during the period between October 1978 and August 1981.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other university.

Signed: JH Osborne Date: August 1984
ACKNOWLEDGEMENTS

I should like to express my gratitude to Professor Symons for suggesting the field of study and for his guidance and "prodding" throughout the course of this research. Thanks are due also to Jan for her hospitality during many helpful days of discussion at Queniborough.

I am indebted to Briv and Lorraine for their unfailing attention and assistance in times of crisis and to all members of the departmental, technical and workshop staff for their help.

I should like to thank Nick Bartlett for his collaboration in the work presented in Chapter 4.

Finally, I wish to thank Vicky and Ann for the translation of my handwritten notes into a typed thesis.

Financial support from the Science Research Council is gratefully acknowledged.
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CHAPTER 1

PRINCIPLES OF ESR SPECTROSCOPY
1.1 INTRODUCTION

Electron spin resonance (ESR) spectroscopy is a technique which allows the detection and characterisation of molecules containing unpaired electrons.

ESR was discovered at the end of the Second World War almost simultaneously by Zavoisky [1] in Russia and Bleaney [2] in Oxford. The scope for this type of spectroscopy was subsequently demonstrated by various workers on readily available samples of lanthanide complexes and transition metals. Indeed, hyperfine structure was first observed by Penrose [3] in 1949 whilst studying the copper nucleus of a diluted Tutton salt.

In the early 1950's, it became evident that chemists were able to apply successfully the resonance technique to the detection and study of free radicals such as NO₂⁻ [4,5]. The suggestion that many enzyme reactions proceed via free-radical intermediates was made thirty years ago [6] and was strengthened by the work of Michaelis [7-9]. ESR has provided a useful method whereby these theories can be tested.

The existence of paramagnetic atoms in such enzymes as catalase supported the idea that one-electron intermediates play a vital mechanistic rôle in biological processes, especially in electron transport and oxidation-reduction reactions [9,10]. Many of the tissues' essential transition group "trace metals" are bound as prosthetic groups to the enzymes which catalyse these reactions. In such metalloproteins, where the mechanism involves transfer of single electrons, the ESR spectra of the unpaired electrons in the atomic orbitals of the metal component can provide structural information and help elucidate the mechanism of action of the particular protein.

Exposure of biological material to X- or γ-radiation can give informa-
tion on the general types of free radical and molecular breakdown produced in different samples, as well as the amount of radiation damage conferred on the specimen. The study of these breakdown processes, as a result of irradiation, is one of the important problems in modern physics and the work presented in this thesis aims to illustrate how ESR spectroscopy may be used in this type of study.

1.2 BASIC THEORY

Permanent magnetic dipoles occur whenever an atom or ion contains a partly filled electron shell. Such systems have a resultant angular momentum due partly to orbital motion and partly to the intrinsic spin of the electrons, with each of which is associated a magnetic dipole moment. ESR takes advantage of the fact that only paramagnetic entities can be made to interact with a suitable external magnetic field.

In the absence of an external magnetic field the unpaired electrons and implicit magnetic moments are randomly orientated and in the same energy state. If an external magnetic field is applied, the dipoles become aligned, either parallel to the field (the more stable condition) or antiparallel, thus lifting the degeneracy of the \( \pm \frac{1}{2} \) states of the unpaired electron (for \( S = \frac{1}{2} \)).

Resonance occurs when the quantum energy of an applied microwave field corresponds to the energy difference, \( \Delta E \), between the ground state and excited state, Figure 1.1. Energy transitions result in which the orientation of the electron spin will change. Particles thermally distributed between energy levels tend to prefer the more stable state and the resonance phenomenon therefore gives rise to a net absorption of energy from the field which is detected and amplified to provide the ESR signal. In practice, the radiation frequency is fixed and the field
FIGURE 1.1
Effect of an applied magnetic field, B, on the $M_S = \pm \frac{1}{2}$ levels of an unpaired electron. $B_r$ is the magnetic field strength at resonance.

The magnetic field is slowly varied until resonance absorption occurs.

The following discussion is not concerned with the rigorous mathematical theory of ESR explained in detail in references 11-14. A more general treatment of Electron Spin Resonance and its applications may be found in reference 15.

1.2.1 The $g$-value

The ESR $g$-value is the proportionality constant in the equation:

$$h\nu = g\mu_B B$$

(for a system with $S = \frac{1}{2}$ and $I = 0$), where

- $h =$ Planck's constant
- $\nu =$ fixed frequency of microwave radiation
- $\mu_B =$ Bohr magneton
- $B =$ magnetic field.
If $B$ is the magnetic field at the electron, then $g_e = 2.00232$. However, internal magnetic fields can add to or subtract from the external field. Therefore $B$ is best defined as the external magnetic field at resonance, and local magnetic fields accounted for by allowing the $g$-value to vary. Hence the $g$-value is a unique property of the molecule in which the unpaired electron is located and a valuable aid for identification purposes.

Deviations from $g_e$ are brought about by coupling of the electron spin and orbital angular momentum, when the atomic spin-orbit coupling constant $\lambda > 0$ (the atomic shell is less than half full) $g < g_e$, when $\lambda < 0$ (the shell is more than half full) $g > g_e$.

The $g$-value is generally anisotropic with three principal values along three orthogonal axes, the magnitude of the local field depending on the orientation of the molecule in the external magnetic field. For example, in a single crystal the magnetic field, and hence $g$-value, will vary as the crystal is rotated in the external field. This can make interpretation very difficult, but in many cases can provide important structural information such as the orientation of the four haem groups in a single crystal of haemoglobin [16]. In most biological systems the paramagnetic species are randomly orientated resulting in a spectrum which represents a superimposition of all possible $g$-values. This may give rise to a broad, structureless line, unless the principal $g$-values differ significantly such that individual values may be obtained, Figure 1.2.

In solution, paramagnetic entities may also exhibit a single isotropic $g$-value resulting from an averaging process. However, most biological systems have a large relative molecular mass and therefore slow tumbling
FIGURE 1.2
Idealised ESR spectra for randomly orientated species with g-anisotropy.
(A - absorption spectrum, B - first-derivative spectrum.)

\[ g_x \neq g_y \neq g_z \]

\[ g_y = g_z \]

\[ g_y = g_z \neq g_x \]

\[ g_x = g_y \neq g_z \]
rate. Thus they exhibit a solid-state type spectrum as discussed above.

1.2.2 Measurement of g-values

G-values may be measured directly using the equation $h\nu = g \mu_B B$ if the microwave frequency and resonant magnetic field are known accurately. The E-109 ESR spectrometer is equipped with a Hewlett-Packard 5246L frequency counter and Bruker B-H12E field probe for calibration procedures. However, a number of errors may be incurred and hence absolute measurements are infrequently employed. A more accurate method ($\pm 0.001$) involves using this equipment in conjunction with a standard for which the g-value is well known. In these laboratories DPPH ($\alpha,\alpha$-diphenyl-$\beta$-picrylhydrazyl) powder is used, $g = 2.0037 \pm 0.0002$ [17,18]. The unknown g-value, $g_x$, is then calculated using the expression:

$$g_x = \frac{g_s B_s}{B_x} - \frac{g_s B_s}{B_s - \Delta B} = g_s \left(1 + \frac{\Delta B}{B_s}\right)$$

where $g_s = g$ value of standard
$B_s = \text{resonant magnetic field for standard}$
$B_x = \text{resonant magnetic field for unknown}$.

1.2.3 Nuclear Hyperfine Coupling

The interaction between the spin of an unpaired electron and the magnetic moments of neighbouring nuclei leads to a phenomenon known as nuclear hyperfine coupling.

The spin of a nucleus is characterised by the quantum number $I$ (possible values of $I$ are 0, $\frac{1}{2}$, 1, $\frac{3}{2}$, 2 ...). The z component of angular momentum is restricted and is characterised by the magnetic quantum number $M_I$ which may take the values $-I$, $-I+1$, ..., $I-1$, $I$.

Hence for a nuclear spin, $I$, there are $(2I+1)$ possible nuclear spin states.

The local magnetic field generated by a nuclear magnetic moment adds
vectorially to the external magnetic field giving an effective field. Since there are \((2I+1)\) possible values of \(M_I\), \((2I+1)\) values of the external magnetic field will be observed at resonance. The resulting spectrum is split into a number of lines known as hyperfine splitting. Many species give characteristic splitting patterns which greatly enhances the value of ESR for identification purposes. An insight into the electronic structure can also be gained.

(i) \(M_I = \pm \frac{1}{2}\), the hydrogen atom

Since the proton has a spin \(I = \frac{1}{2}\), \(M_I\) has the two allowed values \(M_I = \pm \frac{1}{2}\). There will therefore be two possible values of the external magnetic field at which resonance may occur provided that the nuclear spin does not reorientate during the electron spin transition, Figure 1.3. This is the high field approximation and the following equation is valid:

\[
h\nu = g \mu_B (B + M_I A)
\]

where \(A\) is the hyperfine coupling constant.

Before the external magnetic field is applied the electron and nuclear spins are coupled leading to apparent "triplet" \((-1, 0, +1)\) and "singlet" \((0)\) states. These differ slightly in energy as illustrated in Figure 1.4. When the magnetic field is applied the \(\pm 1\) states diverge but the zero states are not affected by the applied field. As the field is increased the four states tend towards the high-field approximation of Figure 1.3. The effect of this "zero field splitting" is to shift both transitions to lower field, the shift being greatest for the low field component. This leads to larger observed \(A\) and \(g\)-values than predicted and must be corrected using the equation:

\[
A = \frac{2B_0(B_2-B_0)}{B_0(2I+1)-B_2} = \frac{2B_0(B_0-B_1)}{B_0(2I+1)-B_1}
\]
FIGURE 1.3
Divergence with field of the $M_S = \pm \frac{1}{2}$ levels when associated with a nucleus having $I = \frac{1}{2}$ (high-field approximation).

FIGURE 1.4
Energy levels as shown in figure but at low-field showing "singlet" and "triplet" states with resultant shift of transitions to lower field.
for any value of $I$, where

- $B_0 =$ resonance corresponding to the $g$-value
- $B_1 =$ low field transition
- $B_2 =$ high field transition

(ii) **One nucleus with $I > \frac{1}{2}$**

For the general case where $I = n$, there will be $2(2n+1)$ possible energy levels and transitions. Thus, for $^{14}$N with $I = 1$, three allowed transitions are expected (+1, 0, -1) and therefore three components observed in the ESR spectrum, Figure 1.5.

![Energy level diagram](image)

**FIGURE 1.5**

Energy level diagram for a system with $S = \frac{1}{2}$ and $I = 1$. Three lines of equal intensity are predicted in the ESR spectrum.

### 1.2.4 Anisotropic Hyperfine Coupling

Since electrons are not localised at one position in space, the effective local magnetic field must be an average of all possible electron positions.

When the electron has a finite
probability of being found at the nucleus (i.e. electrons in s-orbitals), an isotropic interaction arises. However, p, d, f ... orbitals have a node at the nucleus giving rise to anisotropic hyperfine coupling (orientation dependent). Like the g-value the anisotropic hyperfine tensor has three principal values.

(i) For electrons in a p-orbital

When the field is parallel to the axis of the orbital, the interaction has the value $2B$. When the field is perpendicular to this axis, the field at the electron is reversed and takes the value $-B$. Since anisotropic hyperfine splitting comprises an isotropic component ($A_{\text{iso}}$) and an anisotropic component ($B$), the values for $A_\parallel$ and $A_\perp$ become:

$$A_\parallel = A_{\text{iso}} + 2B \quad \text{and} \quad A_\perp = A_{\text{iso}} - B$$

Measurement of $A_\parallel$ and $A_\perp$ cannot directly give $A_{\text{iso}}$ and $2B$ since the signs of the coupling constant are unknown. Liquid phase studies can determine $|A_{\text{iso}}|$ thus fixing the relative signs of $A_\parallel$ and $A_\perp$.

The values of $A_{\text{iso}}$ and $2B$ can be used to determine the s or p-character, respectively, of an orbital near a given nucleus. Theoretical calculations give values of $A_{\text{iso}}^0$ and $2B^0$ for unit population. By comparison with experimental values, an estimate of spin density in that orbital can be obtained using the following equations:

$$a_s^2 = \frac{A_{\text{iso}}}{A^0} \quad \text{and} \quad a_p^2 = \frac{2B}{2B^0}$$

(ii) For electrons in d-orbitals

For the $d_{z^2}$ orbital, coupling resembles that in p-orbitals with $A_\parallel = +2B + A_{\text{iso}}^d$ and $A_\perp = -B + A_{\text{iso}}^d$. For the remainder, the change in orbital symmetry reverses the sign of the interaction, i.e. $A_\parallel = -2B$ and $A_\perp = +B + A_{\text{iso}}^d$.

As with p-orbitals, if the sign of the anisotropic coupling can be determined, then a choice can be made between an orbital of $d_{z^2}$ symmetry.
and one of the remainder, and an estimate of orbital population gained.

1.3 EXPERIMENTAL ASPECTS

1.3.1 The Spectrometer

The essential features of an ESR spectrometer are:

(i) a source of microwave radiation of constant frequency and variable amplitude
(ii) a means of applying the microwave power to a sample
(iii) a means of measuring the power absorbed from the microwave field
(iv) an homogeneous but variable magnetic field.

Most spectrometers employ radiation of frequency 9.3 GHz corresponding to the microwave X-band. Some spectrometers employ Q-band frequency of 35.0 GHz.

The usual source of monochromatic radiation is a Klystron oscillator and the polarised beam of microwaves is transmitted by means of a rectangular brass tube or waveguide. The intensity of the microwave beam (microwave power) can be varied with an attenuator, calibrated in milliwatts.

The sample is placed in a resonant cavity, the purpose of which is to concentrate energy onto the sample by means of multiple reflections of the travelling microwave from the two end walls.

The steady magnetic field is generally produced by an electromagnet perpendicular to the oscillating field of the microwave radiation.

Detection is performed by a semiconductor crystal detector which proportionately converts microwave power into direct current. As resonance is approached, the sample begins to absorb energy from the magnetic field causing a decrease in the reflected microwaves which may be seen by direct observation of the change in crystal current. The inherent noise is reduced by modulating the resonant signal at 100 kHz.
with two Helmholtz coils placed one on each side of the cavity. This signal is amplified and serves as the detector input. This is rectified and sent to a chart recorder or computer input. If the amplitude of the field modulation is kept small compared with the signal linewidth, the displayed signal will be a first derivative of the absorption signal (Fig. 1.6).

A block diagram showing the basic elements of the ESR spectrometer is shown in Figure 1.7.

All ESR spectra described were measured on a Varian E109 X-band spectrometer unless otherwise indicated. Q-band measurements were made using an instrument constructed in these laboratories and described in reference 19.

### 1.3.2 Sample preparation

Several methods of freezing samples were applied:

(i) drops of the solution at room temperature were rapidly frozen in liquid nitrogen to form small, spherical beads,

(ii) a pellet was prepared by placing approximately 1 cm$^3$ of sample in a 5mm internal diameter tube with one sealed end. This was frozen to 77 K and the pellet blown into liquid nitrogen after unsealing the tube,

(iii) the solution was directly frozen in a 3mm internal diameter silica or Suprasil tube by slowly lowering the tube into liquid nitrogen.

### 1.3.3 Sample handling

Samples to be studied at 77 K were transferred to a finger Dewar, constructed of fused silica and borosilicate glass, containing liquid nitrogen. The Dewar was then inserted directly into the spectrometer
FIGURE 1.6
Effect of superimposing a 100 kHz modulating field ($B_m < B_o$) during the traverse of an absorption peak and the resulting first derivative ESR signal.
FIGURE 1.7
Block diagram of the Varian E109 ESR spectrometer showing the basic elements. [Key is given overleaf]
Two methods were available for annealing samples to cause preferential loss of some radicals. The first consisted of decanting the liquid nitrogen from the Dewar, replacing the Dewar in the cavity and continuously monitoring the spectrum during warming. This permitted the anneal to be controlled to a certain extent, the sample being quenched in liquid nitrogen when a significant spectral change was observed. The second method involved removing the sample, contained in a tube, from the finger Dewar and inserting it into a brass cylinder, equilibrated at the required temperature by raising or lowering in liquid nitrogen. Temperature was monitored with a thermocouple attached to the cylinder at the sample insert. This method could only be used, however, when the required temperature was known.

For controlled annealing at temperatures of 90 K upwards a Variable Temperature Unit, constructed in these laboratories, was used. Gaseous nitrogen, cooled by passing through a heat exchanger immersed in liquid nitrogen, was pre-heated to a fixed temperature and blown through a vacuum-jacketed tube, containing the sample, held in the cavity.

Spectra were also measured at 4.2 K using the accessory described in
Figure 1.8. The whole accessory, without X-ray tube and power supply unit could be manipulated such that the continuous flow cryostat replaced the existing cavity in the spectrometer.

1.3.4 Sample irradiation

Samples were irradiated at 77 K in a Vickrad $^{60}$Co $\gamma$-source which supplied a dose rate of about 1.7 MRad h$^{-1}$. Samples were transferred to a Dewar flask containing liquid nitrogen and lowered into the source for the required period of time.

$\gamma$-Radiolysis of silica tubes was found to induce an ESR signal in the silica which could only be partially photobleached using a tungsten-filament light source. Irradiation of Suprasil tubes gave rise to a less intense signal which did not interfere with ESR signals from the sample.

Exposure of samples to 200 kV X-rays at 77 K was carried out by placing a Dewar flask containing the sample in liquid nitrogen in front of the beryllium window of an Andrex X-ray generator. The entire unit was sealed in a lead radiation shield. X-irradiation at 4.2 K was performed using the continuous flow system illustrated in Figure 1.8. Liquid helium was pumped through the cryostat, over the sample and returned to be recycled. Annealing from 4.2 K to 77 K was carried out by controlling the flow of coolant and the power to the electrical heater attached to the heat exchanger (analogous to the Variable Temperature Unit previously described).

Photolysis at 77 K was performed by placing the sample, in a finger Dewar containing liquid nitrogen, in the path of a visible light source with blue "$K2$ interference band filter" supplied by Balzers High Vacuum Ltd., focussed by a silica lens.
FIGURE 1.8
Simplified block diagram of continuous flow system for X-irradiation at 4 K.
1.4 SOME EXPERIMENTAL PROBLEMS

1.4.1 The Water Effect

One of the major disadvantages of biological sample study by ESR is the presence of water in the specimen. In its liquid phase, water has a large non-resonant absorption due to the interaction of its large dipole moment with the microwave electric field. As a result, large damping and a considerable reduction in sensitivity is observed when aqueous samples of the usual size are examined. This can be overcome by

(i) reducing sample size
(ii) working in the high radiofrequency region
(iii) freezing the specimen and making measurements at liquid nitrogen temperatures
(iv) freeze-drying the material before insertion into the cavity.

The generally adopted procedure involves freezing the sample and carrying out the analysis at 77 K, due to the inherent lack of sensitivity of the first two methods and possible structural damage caused during the freeze-drying process.

1.4.2 The Oxygen Effect

The presence of oxygen can affect experimental ESR results in several ways since the oxygen molecule is itself paramagnetic. As a result, it exhibits a broad ESR spectrum extending over several thousand gauss. This is not usually a problem since gaseous oxygen can be removed by circulating dry nitrogen gas through the waveguide (also preventing condensation of water vapour). Liquid oxygen is a far larger problem in experiments carried out at 77 K. Because the boiling point of liquid nitrogen is lower than that of liquid oxygen, liquid nitrogen rapidly condenses oxygen from the air. Liquid oxygen has an intense ESR absorption appearing as a large baseline drift over much of the region studied with biological samples. This is overcome to a large extent by frequent
replacement of liquid nitrogen or application of a vacuum to the finger Dewar in the cavity (this also prevents liquid nitrogen "bubbling").
REFERENCES

6. Haber F. and Willstätter R., Berichte, 64, 2844 (1931).
CHAPTER 2

SUPEROXIDE
2.1 INTRODUCTION

(a) Biological Significance of the Superoxide Ion

A minor product of the biological univalent reduction of molecular oxygen is the superoxide radical anion, $O_2^-$, which has a remarkably high self-reactivity. This results in a fleeting existence in aqueous solution, i.e. a dismutation reaction occurs:

$$\text{HO}_2^- + O_2^- \rightarrow \text{HO}_2^- + O_2$$

with a rate constant $k = 8.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ [1].

In contrast, the reaction:

$$H^+ + O_2^- + O_2^- \rightarrow \text{HO}_2^- + O_2$$

proceeds very slowly in the absence of catalysis, probably because of electrostatic repulsion between the radical anions. The reaction between two molecules of HO$_2^.$ (the weak acid to which $O_2^-$ is the conjugate base) is also slower than the reaction between HO$_2^.$ and O$_2^-$. As a consequence, the dismutation of these radicals is most rapid at pH 4.8, the pKa [2] for the equilibrium:

$$\text{HO}_2 \rightleftharpoons O_2^- + H^+$$

and falls an order of magnitude for each unit the pH is raised due to the progressive decline in the concentration of HO$_2^-$. At physiological pH, the rate constant is approximately $10^5 \text{ M}^{-1} \text{s}^{-1}$ [1].

The chemistry and biochemistry of O$_2^-$ is currently an active area for biomedical research since superoxide is both oxidant and reductant and hence can modify a variety of biologically significant molecules and may be an important intermediate in oxygen toxicity [3].

(b) Studies on the Solvation of Superoxide

It has been known for some time that the value of $g_\parallel$ for the superoxide ion is variable, apparently depending on the nature of the solvent and temperature [4-6]. A similar type of dependence has been illustrated
by studying the width of the ESR singlet assigned to trapped electron
$(e^-)$ centres in ethanol at 4.2 K and 77 K [7]. Following the observation
that the linewidth was greatly reduced at low temperatures, considerable
interest was kindled in this field since work carried out in these
laboratories several years earlier had suggested that the linewidth of
the 77 K species was mainly influenced by proton hyperfine coupling to
the alcohol OH group [8]. Subsequent optical work showed that trapped
electrons in alcohols give intense bands at 550 nm whilst $e^-$ centres in
ethers do not, although infrared bands at 1500 nm become apparent. Both
pieces of information were combined in the research of Hase et al. [9]
who measured the optical spectra at various low temperatures. They showed
that the infrared peak was present at 4.2 K for trapped electrons in an
ethanol medium, but decreased on warming to 77 K with concomitant
increase in the visible band. The effect was irreversible.

The work presented in this chapter deals with the solvation of super­
oxide ions in alcohols, determined by ESR measurements, analogous to the
work of Kevan et al. [10] on trapped electrons in alcoholic glasses.
Kevan's investigations [11] highlighted a difference in the properties of
trapped electrons in methanolic glasses and other alcoholic glasses.
They showed that the ESR linewidth and optical spectra for $e^-$ centres
formed in methanol at 4.2 K (and, in later work, even at 1.6 K [12]) were
comparable with those formed at 77 K and concluded that OH protons were
intimately involved in defining the centres.

The symmetry of superoxide ion solvates was also of interest in this
work since they are probably asymmetric in protic solvents [13,14],
whereas most anions are generally assumed to solvate symmetrically.
2.2 ELECTRONIC STRUCTURE AND ORIGIN OF THE SUPEROXIDE ESR SPECTRUM

The O-O bond in superoxide may be thought of as one sigma bond and one-half \( \pi \) bond; a molecular orbital scheme is shown in Figure 2.1.

\[
\begin{array}{cccc}
\pi_2^* & \gamma & \Delta \\
\sigma_3 & \sigma_1 \\
\pi_1 & \sigma_2^* \\
\end{array}
\]

\text{Oxygen} \quad \text{Superoxide}

\text{FIGURE 2.1}
Qualitative molecular orbital scheme for dioxygen and superoxide radical.

The superoxide ion is formed by adding one electron to one of the degenerate \( 2p_{\pi^*} \) orbitals \([15]\). The unequal occupancy of these orbitals causes a Jahn-Teller distortion leading to splitting of these levels.

The paramagnetism of superoxide allows study by the electron spin resonance technique. Angular momentum about the \( z \) axis is quenched because electrons in the filled \( \pi_x^* \) orbital prefer to lie in the plane containing the cations, leaving the half-filled orbital perpendicular to the plane (Fig. 2.2). The electrons can therefore only circulate by flow from \( \pi_x^* \) into \( \pi_y^* \). This leads to an induced magnetic field along the molecular axis (\( z,// \)) which adds to the applied field, causing resonance to shift to low-field and giving a correspondingly higher \( g \)-value. Thus the rôle of the environment is to lift the degeneracy of the \( \pi^*_x \) and \( \pi^*_y \) orbitals, causing the value of \( g_z \) to depend on the magnitude of the
asymmetric crystal field. In protic solvents, specific hydrogen-bonding takes over the role of the cations [13]. If \( g_\parallel \) becomes large, the value of \( g_\perp \) becomes controlled by orbital angular momentum about \( z \), giving a typical \( g_\perp \) value of 1.999 for \( g_\parallel = 2.175 \).

2.3 EXPERIMENTAL

(a) Materials

Solvents, of AnalaR grade, were used as supplied. Methanol and ethanol were obtained from British Drug Houses Ltd., Poole, England. Deuterated methanol (CD\(_3\)OD) was supplied by Nuclear Magnetic Resonance Ltd., High Wycombe, Bucks., deuterated ethanol (C\(_2\)D\(_5\)OD) by Koch Light Laboratories Ltd., Colnbrook, Bucks., and deuterated water (D\(_2\)O), 99.8 atom % D, was supplied by the Aldrich Chemical Company, Milwaukee, USA. Water was doubly distilled. Potassium superoxide was obtained from PCR Inc., Florida, USA, ground in a nitrogen atmosphere and stored in a dessicator until used.
(b) **Superoxide Radical Generation**

It has long been known that superoxide radicals are formed from hydrogen peroxide solutions in the presence of an oxidant such as permanganate or catalysts [5,6]. The radicals may also be formed in alkaline solutions of concentrated hydrogen peroxide in the absence of such oxidants and catalysts [16].

The superoxide radical is known to be stable in some aprotic solvents and may be generated in high concentration by dissolution of one of its salts in such solvents.

For protein work both methods of superoxide radical generation are unsatisfactory. The first involves the use of high pH which, in most cases, causes inactivation of the protein by modification of protein structure. High concentrations of hydrogen peroxide (which is toxic to cells) and most of the aprotic solvents suitable for the second method may also cause protein denaturation.

Therefore a method of producing superoxide, in situ, without the requirements of denaturing solvents or extremes of pH was necessary for subsequent work investigating the effect of superoxide on a particular class of enzymes. The requirement for this study of superoxide solvation was, as with $e^-_c$ centres, very low temperature radical generation, i.e. the previously described methods followed by freezing were unsuitable and also complicated by phase separation and ion-pair formation.

One possible method employs in situ photolysis or radiolysis followed by rapid freezing [17]. However, the method used in this work involved addition of electrons to dioxygen after freezing its solutions [13,14]. Electrons may be generated radiolytically or photolytically; X- or γ-radiolysis of the oxygen-saturated alcohols was employed. Preliminary
research used radiation from added tritiated water [14].

Solvents were saturated with oxygen gas by controlled bubbling through a capillary into 0.2 cm³ solvent contained in a thin-walled Suprasil tube, 3 mm internal diameter, length 8 cm. A flow rate of 10 cm³ min⁻¹ for ten minutes was used to ensure saturation. The period of bubbling was determined by fixing the oxygen flow to give a steady stream of gas through the solvent then removing the sample after a set time for analysis. This was repeated for increasing oxygen bubbling times. After removal from the bubbling system the samples were immediately frozen in liquid nitrogen. The ESR superoxide signal intensity was then monitored at 77 K after all samples had been exposed to the same dose of γ-radiation. Figure 2.3 shows the saturation curve for oxygen in methanol. All saturation experiments were performed at room temperature.

Traces of water (or D₂O) were added to some samples to prevent crystallisation and possible loss of oxygen during the freezing process.

In some cases it was necessary to remove all traces of oxygen. This was carried out using a repeated freeze-pump-thaw cycle under vacuum, followed by sealing the deoxygenated sample in the tube.

In order to determine the ESR spectrum of superoxide in methanol and ethanol without exposure to γ- or X- radiation, samples of the potassium salt were dissolved in the alcohol and frozen to 77 K as small spherical beads. The ESR spectrum was then recorded (Fig. 2.4).

2.4 RESULTS

(a) Ethanol glasses

The spectrum of glassy, oxygen-saturated ethanol, γ-irradiated at 77 K, was dominated by features from C₂H₅OH derived radicals such as CH₃CHOH which masked the superoxide parallel signal. Only on annealing to remove these radicals was the superoxide signal observed. As a result,
FIGURE 2.3
Oxygen saturation curve for methanol monitored by $O_2^-$ formation on exposure to $\gamma$-radiation. [Solubility of $O_2$ in ethanol is similar; a bubbling period of 10 minutes gives saturation in both alcohols.]
FIGURE 2.4
ESR Spectrum of potassium superoxide in methanol, \( g = 2.078 \) (ethanol, \( g = 2.081 \)).
subsequent samples were prepared with the fully deuterated solvent, 
$C_2D_5OD$, to reduce the spread of the central features such that $O_2^-$ was 
immediately detected at 77 K.

An oxygen-saturated sample of $C_2D_5OD$ was $\gamma$-irradiated at 77 K and 
used to select the required parameters for optimum $O_2^-$ signal during 
ESR monitoring at 4.2 K. Annealing this sample to approximately 90 K 
induced slight narrowing of the line and shift to $g$-values identical 
with those for superoxide formed in a frozen solution of potassium 
superoxide in $C_2D_5OD$.

An oxygen-saturated $C_2D_5OD$ glass $X$-irradiated at 4.2 K gave no low-
field ESR detectable signals at this temperature. On warming the sample 
to 40 K a broad, asymmetric feature with $g = 2.103$ became apparent and 
was not lost on recooling to 4.2 K. Further annealing caused narrowing 
of the feature with a simultaneous shift of $g$-value to higher field. At 
77 K the parallel feature was identical with that observed on $\gamma$-irradi-
ation of oxygen-saturated $C_2D_5OD$ at 77 K with indistinguishable 
characteristics on warming to 90 K (Figs. 2.5 and 2.6).

Oxygen-free samples gave no ESR observable features in the low-field 
region at 4.2 K or 77 K indicating that the signals described above were 
all oxygen derived. The central $g = 2$ region at 4.2 K was dominated at 
low powers by the narrow singlet from $e_t^-$ described by Smith and Pieroni 
[7]. This was greatly reduced in the presence of oxygen as the concen-
tration of superoxide anions was built up, indicating that the oxygen 
molecule is indeed a good electron capture centre.

(b) Methanol glasses

A sample of fully-oxygenated methanol was $\gamma$-irradiated at 77 K to 
pre-select ESR parameters for 4.2 K analysis, and was annealed above 
77 K. Narrowing of linewidth and $g_{\|}$ shift from 2.081 to 2.078 was
FIGURE 2.5
First derivative X-band ESR Spectra for C\textsubscript{2}D\textsubscript{15}OD, saturated with oxygen, after exposure to X-rays at 4.2 K (a) at 4.2 K, (b) at 40 K, (c) at 50 K, (d) at 60 K, (e) at 70 K, (f) at 90 K. In the absence of oxygen no features were obtained (a').
<table>
<thead>
<tr>
<th>Temperature</th>
<th>$g_{\parallel}$-values for superoxide</th>
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<th></th>
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<tr>
<td></td>
<td>Methanol</td>
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<tr>
<td>40 K</td>
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<td></td>
</tr>
<tr>
<td>50 K</td>
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<td></td>
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<tr>
<td>60 K</td>
<td>2.081</td>
<td>doublet 2.091 2.085</td>
<td></td>
</tr>
<tr>
<td>70 K</td>
<td></td>
<td>2.083</td>
<td></td>
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<tr>
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</tr>
<tr>
<td>90 K</td>
<td>2.078</td>
<td>2.081</td>
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</table>

FIGURE 2.6
Table of $g_{\parallel}$-values for superoxide generated by radiolysis in methanol and ethanol at different temperatures. [$g_{\parallel}$-values for potassium superoxide in the alcohols are included for comparison.]
FIGURE 2.7
First derivative X-band ESR spectra for CD₃OD, saturated with oxygen, after exposure to X-rays at 4.2 K (a) at 4.2 K, (b) at 60 K, (c) at 90 K. In the absence of oxygen no features were obtained (a').
observed.

Deuterated methanol, CD$_3$OD, was used for some aspects of the study since it reduced the spread and linewidth of solvent features, although the O$_2^-$ parallel signal was not masked by radiation induced radicals from methanol.

Preliminary studies on oxygen-saturated CD$_3$OD solutions X-irradiated at 4.2 K gave rise to an asymmetrical double peak at 4.2 K with a $g$-value of about 2.095. The low-field half of the doublet was irreversibly lost on warming. This was accompanied by a slight $g$-shift to higher field. At 77 K the signal was indistinguishable from the feature obtained by $\gamma$-irradiating a similar sample at 77 K. Similarly, on annealing to 90 K, further narrowing was observed culminating in an O$_2^-$ spectrum identical with that observed for rigid potassium superoxide in methanol.

In investigating the nature of the asymmetry, attention was focussed on the CH$_3$O$^-$ radical observed by Iwasaki in oxygen-free, polycrystalline samples of dry methanol [19]. A control sample of deoxygenated CH$_3$OH, dried over activated molecular sieve 4 A, was therefore studied at 4.2 K. Low-field signals from O$_2^-$ were not observed although a broad feature with $g_{\text{max}}=2.088$ was evident. The feature decayed at 30-40 K, a temperature coincident with sharpening of the superoxide signal in oxygenated samples. This was in full agreement with Iwasaki's CH$_3$O$^-$ radicals ($g_{\text{max}}=2.088$, $g_{\text{min}}=1.999$, $\Delta g=131$ G and $A(\text{H})=52$ G), indicating that in the oxygenated sample at 40 K, CH$_3$O$^-$/CD$_3$O$^-$ radicals had decayed thus resolving the parallel superoxide feature.

Previous experience [14] had illustrated that traces of water added to the alcohol ensured glass formation (a property important in this work). The effect on the spectrum was to greatly reduce the CH$_3$O$^-$ feature by broadening. Repeating analysis of the fully oxygenated glassy
sample at 4.2 K revealed the parallel features presented in Figure 2.7 and g-values tabulated in Figure 2.6, equivalent to those observed in the polycrystalline sample, but with less initial asymmetry.

All experiments were repeated several times to ensure reproducibility.

2.5 DISCUSSION

At low temperatures the OH groups of alcohols form two hydrogen bonds to neighbouring alcohol molecules (Fig. 2.8). Oxygen molecules cannot contribute to this hydrogen bonding, but superoxide anions will readily solvate by forming hydrogen bonds with OH groups in close proximity. However, in this work superoxide is generated after the alcohol molecules have established their hydrogen bonding system and solvation of O$_2^-$ must necessitate the initial breaking of one or both of the alcohol interactions before formation of its own. The results indicate that ethanol molecules do not reorientate at 4.2 K whereas methanol molecules do, thereby stabilising the anion centre. This may be explained by the relative size of the methanol and ethanol molecules. A requirement for superoxide becoming solvated in this way is that the parent oxygen molecule lies in close proximity to the hydrogen-bonded OH groups of the alcohol. In methanol, this is inevitable. In ethanol, however, the
ethyl groups are large enough to form hydrocarbon regions enclosing the parent oxygen molecules. On univalent reduction by γ- or X- radiation, the distance of the anions from the alcohol OH groups is such that there is an energy barrier to breaking the hydrogen bonds which the coulombic forces of the newly-formed anions cannot overcome. Thus movement towards becoming solvated is restricted and the superoxide is not ESR detectable. This suggests that the parent hydrogen bonds in ethanol must be broken in order for the molecules to reorientate before OH groups and anions may interact. In methanol, however, this would be unnecessary and solvation facilitated by formation of the anion stabilised transition state illustrated in Figure 2.9 with only a small energy requirement.

![Figure 2.9](image)

**FIGURE 2.9**  Possible methanol : $O_2^-$ transition state involving a split hydrogen bond.

Hence the solvated $O_2^-$ ESR signal is present even at 4.2 K.

The apparent g-shift may indicate a change in solvation number of superoxide. The $g=2.1$ feature may suggest one hydrogen bond, whilst two hydrogen bonds give rise to the $g=2.09$ feature. The bonds must lie in one plane in order to give a maximum $\Delta$ value (Fig. 2.1) and therefore a further two hydrogen bonds may give the fully solvated anion.

These results compare favourably with the earlier work of Symons et al. [18] which gave spectra similar to those of Figure 2.7 on addition of methanol to dimethylsulphoxide containing potassium superoxide.

One aim of this irradiation work on superoxide solvation was to study
the effect of different alcohols on superoxide and make a comparison with the \( e^- \) results discussed in section 2.1(b), since the nature of these centres is not well understood. It is apparent that a great similarity exists and therefore the explanation given may also be applied to trapped electron centres, provided the centres are strongly localised as references 20 and 21 suggest. If this assumption is incorrect, the analogy must break down.
REFERENCES

3.1 INTRODUCTION

Prior to the discovery of superoxide dismutase (SOD) activity [1], SOD had been isolated from human erythrocytes, brain and liver cells. These blue-green, copper-containing proteins were named erythrocuprein, cerebrocuprein and hepatocuprein respectively [2,3,4]. Examination proved erythrocuprein homologous to the bovine erythrocyte protein, E.C.1.15.1.1., first isolated by Mann and Keilin [5], on which the following study has been made.

The physiological function of these proteins is to catalyse the dismutation of superoxide as represented by the equation

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]  

hence their present name, superoxide dismutases. Since elucidation of its function, the enzyme has been isolated from practically all tissues.

Bovine superoxide dismutase has a relative molecular mass of approximately 31,500 and binds one atom of both copper and zinc in each of its two polypeptide sub-units (prokaryotic enzymes have a relative molecular mass of 40,000 and contain one atom of manganese in each sub-unit [7,8]). Steinman and co-workers [9] have shown that the two 151-residue sub-units are of identical amino acid sequence. X-ray crystallography data [10] has suggested that these would be equivalent in conformation in solution. The dominant structural feature of the enzyme is a large cylindrical, eight-stranded barrel of antiparallel \( \beta \)-pleated sheet, containing approximately 75 residues (Fig. 3.1). The interior of the \( \beta \) barrel is packed with hydrophobic side chains. The 4 chains to the outside of the sub-unit are quite regular and within hydrogen bonding distance of each other for 9 or 10 residues; the 4 chains internal to the sub-unit are more twisted and contribute 4 ligands to each metal and one end to a
FIGURE 3.1
Structure of a single sub-unit of the enzyme superoxide dismutase.

FIGURE 3.2
Schematic representation of a single monomer of superoxide dismutase illustrating the positions of metal ligands and the disulphide bridge. [O zinc ligands, X copper ligands, ◊ shared ligand]
disulphide bridge (Fig. 3.2). There is one α-helical section in the external loop of one or one-and-a-half turns, the remainder of the structure being made up of two long loops of non-repetitive structure beginning and ending in two adjacent chains of the internal β barrel. The first loop (residues 48-78) is partly held against the barrel by a disulphide bridge and participates in the hydrophobic side chain interactions which link the sub-units. The rest of the loop contributes 3 ligands to zinc and, like the second loop (residues 119-141), is very hydrophilic.

The two copper atoms of a dimer are 34 Å apart ruling out any possibility of direct interaction between active sites. The copper and zinc of a sub-unit are 6 Å apart linked by the imidazole ring of His 61. Zinc resides in an approximately tetrahedral site liganded by His 61, His 69, His 78 and Asp 81. The copper ligands are His 61, His 44, His 46 and His 118 in a distorted square planar arrangement with one side relatively open to solvent access. The imidazole ring of His 61 appears to reside in a position such that the proximal nitrogen has a straight bond to zinc and the distal nitrogen sits above the square planar arrangement of the other 3 nitrogen ligands to copper, in a position between planar and axial on the side accessible to solvent (Fig. 3.3).

![Metal binding sites of copper and zinc on superoxide dismutase.](image-url)
Chemical evidence suggesting that an unusual ligand was associated with the metal binding site came from its oxidation/reduction properties as a function of pH [11]. Using an hexacyanoferrate II/III redox buffer and constructing a Nernst-type plot, the mid-point potential was shown to decrease linearly at -0.06 V/pH between pH 5.5 and pH 8.7. This behaviour was described by the half-reaction

$$\text{BCu}^{2+} + e^- + H^+ \leftrightarrow H^+\text{BCu}^+$$

in which the proton is bound to a basic group on the reduced protein.

In an independent experiment, Fee and Dicorleto [11] demonstrated that one proton was bound for each electron taken up.

During these experiments it was noted that the mid-point potential became independent of pH below 5.5. It has since been shown [12] that a reversible transition involving Cu$^{2+}$ occurs between pH 4 and pH 3 which does not involve unfolding of the protein or dissociation of the dimer. The process is slow and changes in the ESR properties suggest that some of the rhombic character of the native protein is lost. (Below pH 3 more drastic changes occur involving loss of metals from the protein [13].)

The apparent pKa of the ligand had to be greater than 8.7 to account for the linear decrease in the mid-point potential up to this value. This can be associated with a bridging imidazolato anion and can be justified in terms of known pKa values, >14 for free [14] and 11.7 for metal bound imidazole [15,16]. At low pH, deviation from the expected relationship can be explained by protonation of the ligand prior to reduction. It has been argued [17] that Cu$^+$ has a co-ordination position open to solvent which is necessary for catalysis. Blumberg et al. [18] have found that only the copper has altered X-ray absorption properties on reduction.
Combining these observations leads to the conclusion that the copper-imidazole coordinate bond is broken on reduction, but cannot clarify the low pH situation (Fig. 3.4).

\[
\begin{align*}
\text{Zn}^{2+} & \text{N} \cdots \text{N} - \text{Cu}^{2+} \quad \text{e}^- + \text{H}^+ \rightarrow \text{Zn}^{2+} \text{N} \cdots \text{NH} - \text{Cu}^+ \\
\end{align*}
\]

FIGURE 3.4
Possible structural changes at the metal binding sites of superoxide dismutase on reduction (A) and at low pH (B) [17].

3.2 COPPER STRUCTURE IN PROTEINS AND THEIR ESR PARAMETERS

For many copper complexes, ligand atoms form an octahedral configuration around the central ion. As a result the five d-orbitals are split into two sets of different energy, the lower $t_{2g}$ set involving degenerate $d_{xy}$, $d_{xz}$, $d_{yz}$ orbitals and the upper $e_g$ set involving $d_{x^2-y^2}$ and $d_{z^2}$ orbitals. The lowest energy for a complex involving Cu$^{2+}$ is obtained with one "hole" in the $e_g$ set. However, this is unstable against distortion according to the Jahn-Teller theorem and, in protein-
bound Cu$^{2+}$, ligands will cause large deviations from octahedral symmetry. Distortion usually occurs so that two opposing ligands are further from the metal ion than the remaining four. A roughly tetragonal symmetry results with $d_{x^2-y^2}$ being highest in energy since the lobes are in closer proximity to the negative charge of the ligands than those of $d_{z^2}$.

Thus $d_{x^2-y^2}$ contains the unpaired electron and "hole" (Fig. 3.5).

FIGURE 3.5
Schematic representation of crystal field splitting showing deviation from octahedral symmetry caused by ligands in a protein-bound Cu$^{2+}$ system (not to scale).

This effect of crystal field dictates that the unpaired "hole" resides in an orbital well-separated in energy from other orbitals. Orbital motion now contributes to energy differences measured in ESR only if the "hole" can move from one orbital to another. As this is not possible, orbital contribution to magnetism is quenched and $g$-values observed for Cu$^{2+}$ are close to that of a free electron with spin-only motion, $g = 2.0023$.

Deviations, causing the unpaired "hole" to spend some time in other orbitals, results from spin-orbit coupling and so re-introduces some
orbital motion depending on the ratio of spin-orbit coupling to strength of crystal field. The relationship is given by

\[ g = 2.0023 \left(1 + \frac{\lambda}{\Delta_0}\right) \]

where \( \lambda \) is the spin-orbit coupling constant and \( \Delta_0 \) is a constant depending on the system and direction of the applied magnetic field. In order to acquire angular motion around the z axis, a "hole" in \( d_{x^2-y^2} \) may reside in \( d_{xy} \). In order to obtain angular motion around the axis perpendicular to z, \( d_{x^2-y^2} \) may "mix" with \( d_{xz} \) and \( d_{yz} \). However, this is less efficient than mixing with \( d_{xy} \) and so \( g_\parallel \) deviates more from 2.0023 than \( g_\perp \).

Spin-orbit coupling constants are obtained from optical spectra. The value for free gaseous Cu\(^{2+}\) is -830 K giving a \( g \)-value greater than 2.0023.

\( \Delta_0 \), the splitting between the \( t_{2g} \) and \( e_g \) sets of orbitals, is characteristic of the complex studied and is obtained from absorption spectra.

Observed \( g \)-values are always smaller than predicted, to some extent, as a result of spreading of the unpaired electron onto ligands. Because of this delocalisation and the effect of crystal field splitting, there is a connection between \( g \)-value and ligand atom such that the \( g \)-value resulting from a nitrogen ligand will be larger than from a sulphur ligand, but smaller than from an oxygen ligand.

Bovine superoxide dismutase exhibits the ESR spectra shown in Figure 3.6. The parallel feature is split into four lines of equal abundance, \( g_{uv}/g_{av} = 2.268, \lambda_{av} = 140 \text{ G} \), due to interaction of the unpaired electron of Cu(II) with its nuclear spin (\( I = \frac{3}{2} \)). At 9 GHz, \( \lambda_\perp \) is too small to be resolved, although resolution is brought about at 35 GHz. With reference to the plot of Figure 3.7 [18], the spectral values for the copper ion of
FIGURE 3.6
X- and Q-band ESR spectra of bovine superoxide dismutase.
FIGURE 3.7
Plot to show the relationship between $g_\|/g$ and $A_\|$ for some Cu(II) compounds with 4 nitrogen ligands. All model compounds lie within the dotted region with total charge on the metal ion, as illustrated, 2 have been chosen for comparison with SOD.

○ biuret complex at high pH
× Cu(imidazole)$_4$ complex
• SOD.
SOD lie just outside the region describing 4 nitrogen ligands per metal ion. The rhombic environment of SOD [19], however, effectively drives $g_\parallel$ and $A_\parallel$ to smaller values suggesting that the undistorted position would lie in the 4 N ligand region. The plot of Figure 3.8 [20], implies that the copper/nitrogen complex of SOD lies between a square planar and tetrahedral structure in agreement with the rhombic distortion quoted earlier. This is further confirmed by the lack of superhyperfine pattern due to the 4 nitrogen ligands. The nuclear spin of $^{14}$N is 1 and, therefore, each feature of a Cu(II) ESR spectrum, in which the copper ion is ligated to a single nitrogen atom, should be split into a triplet of signals of equal intensity. Where Cu(II) is ligated to 4 equivalent nitrogen atoms, the superhyperfine pattern should consist of 9 lines of relative intensity 1:4:10:16:19:16:10:4:1. Since this pattern is not observed, the nitrogen ligands in SOD cannot be all magnetically equivalent.

The ESR spectrum of the native protein is a very sensitive probe for changes in the environment of Cu(II) and can therefore be used extensively to study the site of catalysis.

### 3.3 THE EFFECT OF $\gamma$-RADIATION ON SOD

#### 3.3.1 Introduction

There are two current opinions concerning the mechanism of action of SOD. The first has been discussed to some extent in section 3.1 and involves a change in oxidation state of the copper ion. This view has wide support: Fielden et al. [21] imply a link between copper and zinc ions in each sub-unit whilst McAdam et al. [22] define the bridging histidine ligand as the source of protons for the process. The second view, proposed by Bielski [23], supports formation of a copper-oxygen
Figure 3.8
Plot of $A_\parallel$ against $\Delta g_\parallel$ for a range of $Cu^{2+}$ complexes.
complex:

\[ \text{Cu}^{II} + \text{O}_2^- \rightarrow \text{Cu}^{II} - \text{O}_2^- \]

\[ \text{Cu}^{II} - \text{O}_2^- + (2\text{H}^+) + \text{O}_2^- \rightarrow \text{Cu}^{II} + \text{H}_2\text{O}_2 + \text{O}_2^- \]

In this case the disproportionation of superoxide proceeds with no change in oxidation state of the metal ion. Research in support of this mechanism has been performed by Plonka et al. [24-26].

The primary task of this research was to provide more evidence in support of one or other of the present views.

3.3.2 Experimental

Samples of native SOD in aqueous solution were provided by Dr. H. A. O. Hill and freeze-dried for storage purposes in these laboratories.

All reagents used were of the highest quality supplied by BDH, May & Baker and Sigma, and further purified where necessary (section 3.4).

Samples of SOD were prepared to a concentration of 0.3 mM in 0.1 M potassium phosphate buffer at pH 7.4. Classification of samples was brought about by addition of ethane-1,2-diol to a concentration of 15%. All water was doubly distilled.

Reduced enzyme was prepared in two ways:

1. by the addition of potassium hexacyanoferrate(II), freshly prepared to 0.1 M concentration, and
2. by the addition of sufficient solid sodium dithionite to just bleach the colour of the protein.

Samples were oxygen-saturated or deoxygenated in Suprasil tubes and γ-irradiated as previously described.

3.3.3 Results and Discussion

The work of Plonka et al. involved exposure to γ-radiation of aqueous samples of SOD, frozen to 77 K, containing sodium formate as an \( \cdot \text{OH} \)
radical scavenger. No major change in intensity or g-value of the Cu(II) signal was reported after irradiation or during the annealing process. However, it is known that on freezing, phase separation occurs resulting in a polycrystalline sample. In order to eliminate this unknown factor, samples prepared in this laboratory incorporated a glass former to produce an homogeneous medium on cooling. Irradiation of these samples caused a loss of Cu(II) signal intensity, in contrast with the work of Plonka. The plot shown in Figure 3.9 shows the relationship between loss of Cu(II) signal intensity for the two parallel features at lowest field and time of exposure to γ-radiation. Increase in signal intensity due to ·OH radicals is also recorded to illustrate the simultaneous formation of radicals on irradiation.

Samples were also prepared in the absence of glass former and a very small relative loss of Cu(II) signal intensity was observed. This served to demonstrate the importance of studying an homogeneous sample and suggests that the results of Plonka may not be an accurate representation of the functional mechanism.

A well-defined parallel feature due to solvated superoxide anions was also observed in these samples (Fig. 3.10). Since it seemed possible to generate superoxide in situ in the presence of the enzyme, signals due to SOD and its substrate could be monitored during the annealing process.

On warming to 120 K to give loss of ·OH radicals, Plonka and his co-workers noted a feature with $q_{\parallel} = 2.039$ and $q_{\perp} = 2.008$. It appeared stable to about 200 K and was identified as the HO$_2^*$ radical. It was suggested that some type of stabilisation by coordination with Cu(II) must have occurred since HO$_2^*$ radicals in ice decay at the lower temperature of 160 K [27]. The proposed mechanism leads to a complex of the type ECu$^{2+} \cdots$ HO$_2$, HO$_2$ replacing the water molecule at the active site. This
FIGURE 3.9
Plot to show loss of Cu$^{II}$ signal intensity with time of exposure to γ-radiation.

△ $+^{1/2}_{29}$Cu$^{II}$; □ $+^{3/2}_{29}$Cu$^{II}$; ○ ·OH.
FIGURE 3.10
ESR spectrum of oxygen-saturated SOD after exposure to γ-radiation and anneal to loss of •OH radicals.
Figure 3.11
ESR spectrum of oxygen-saturated SOD after exposure to γ-radiation and anneal above 77 K to reveal ·O₂⁻ radical features.
implies that HO$_2^\cdot$ is the substrate rather than O$_2^\cdot$. Similarly, annealing homogeneous samples of SOD above 77 K in this laboratory revealed a parallel feature with $g_{\parallel} = 2.032$ and $g_{\perp} = 2.008$ (Fig. 3.11). We suggest that this feature is more likely to be due to RO$_2^\cdot$ radicals resulting from association with organic radicals as the system warms up and becomes more mobile

$$\text{R}^\cdot + \text{O}_2 \rightarrow \text{RO}_2^\cdot$$

The reasons for putting forward this view are based on several pieces of evidence, primarily the pK value for the reaction

$$\text{HO}_2^\cdot \leftrightarrow \text{H}^+ + \text{O}_2^-$$

being 4.5. HO$_2^\cdot$ should therefore not be present in samples buffered to pH 7.4. Secondly, HO$_2^\cdot$ is usually observed as a doublet with $\Delta g_{\parallel}$ in the order of 40-45 G due to proton splitting. In the presence of deuterated solvent a doublet due to HO$_2^\cdot$ radicals should be replaced by a single peak, DO$_2^\cdot$. No effect was observed. Although $g$-values are of the order expected for HO$_2^\cdot$ in various systems (Fig. 3.12), they are also in accordance with data for RO$_2^\cdot$ recorded in this laboratory, $g_{\parallel} = 2.034$ and $g_{\perp} = 2.004$.

<table>
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<th>System</th>
<th>$g_{\parallel}$-value</th>
<th>$^1\text{H}$ splitting</th>
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<tbody>
<tr>
<td>H$_2$O$_2$/H$_2$O</td>
<td>2.035 or 2.039</td>
<td>14.2 or 13.5</td>
</tr>
<tr>
<td>H$^\cdot$ + O$_2$</td>
<td>2.04</td>
<td>13.5</td>
</tr>
<tr>
<td>LiSO$_4$</td>
<td>2.033</td>
<td>12</td>
</tr>
<tr>
<td>CaSO$_4$</td>
<td>2.051</td>
<td>17</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>2.055</td>
<td>20</td>
</tr>
<tr>
<td>SOCl$_2$</td>
<td>2.035</td>
<td>17.2</td>
</tr>
</tbody>
</table>

**FIGURE 3.12**

Some values for HO$_2^\cdot$ radicals in various systems.

Furthermore, RO$_2^\cdot$ radicals have been shown to decay at approximately 200 K consistent with the temperature noted by Plonka for decay of the
enzyme-HO$_2$ complex.

Although closely monitored, growth of the feature in question was independent of loss of superoxide signal.

A summary of the effect of temperature on the samples under investigation is shown in Figure 3.13. With and without exposure to γ-radiation loss of the Cu(II) signal seems gradual and the Cu(II) centre appears not to be affected by irradiation in any way that may cause its rate of 'decay' to be altered. [Errors in plotting the intensity of Cu(II) signal after irradiation are necessarily larger because the reduced concentration of Cu(II) results in a smaller signal to noise ratio.] It might be expected that if some reaction between Cu(II) and the γ-induced O$_2^-$ or RO$_2^*$ had taken place, the 'decay' would be stabilised and show some increase or decrease inconsistent with the non-irradiated sample. However, the concentrations of Cu(II) and Cu(I) are approximately equal after irradiation. Since either form may react with O$_2^-$, no loss or gain of signal intensity can be expected.

The results emerging from this work demonstrated that the interaction of SOD with its substrate could not be followed under these conditions. Subsequent experiments were therefore performed using the enzyme in its chemically reduced form. The Cu(I) centre, being diamagnetic, was unaffected by γ-radiation and undetected by ESR. The product of irradiation was now an increased and well-resolved parallel feature due to O$_2^-$ with little or no residual Cu(II) signal from SOD reduced with hexacyanoferrate(II) and dithionite respectively.

Features for RO$_2^*$ radicals were observed during the annealing process and O$_2^-$ 'decayed' at approximately 180 K as in previous experiments. However, this loss was concomitant with growth of Cu(II) features of identical g- and A-values to the native protein (Fig. 3.14). These
FIGURE 3.13
Plot to determine the effect of temperature on concentration of Cu\textsuperscript{II} in SOD (before and after γ-irradiation at 77 K).

No γ

\[ \text{O}_2^- \text{ becomes apparent} \]

\[ \text{\textbullet OH anneals out} \]

\[ \gamma 77K \]

\[ \text{2hrs.} \]

\[ g \cdot \text{RO}_2 = 2.032 \]

\[ g \cdot \text{O}_2^- = 2.071 \]

\[ \text{\textbullet O}_2^- \text{ anneals out} \]

\[ \text{\textbullet O}_2^- \text{ max.} \]

\[ \text{\textbullet O}_2^- \text{ anneals out} \]

\[ \text{\textbullet O}_2^- \text{ present as shoulder} \]

Signal intensity of low-field parallel feature/arb. units

Temp./K

100 120 140 160 180 200

Data for control (no Cu\textsuperscript{II})
FIGURE 3.14
ESR spectra showing growth of Cu(II) signal intensity during anneal of oxygen saturated, γ-irradiated Cu(I) SOD.
1. After irradiation
2. After anneal to loss of \( \cdot \text{OH} \)
3. After anneal to loss of \( \text{O}_2^- \).
results were repeatable and suggested that formation of a long lived complex of the type \([\text{BCu(I)} \cdots \text{O}_2^-]\) or \([\text{ECu(I)} \cdots \text{HO}_2]\) was unlikely. Furthermore, a complex with \(\text{O}_2^-\) would be expected to give a well-defined spectrum with \(g_\parallel (\text{O}_2^-)\) moving closer to free spin and featuring a hyperfine pattern due to coupling with the copper nucleus. Control experiments in the absence of oxygen did not reveal the \(\text{Cu(II)}\) spectrum on warming. The reaction which may be occurring is
\[
\text{Cu(I)} + \text{O}_2^- \rightarrow \text{Cu(II)} + \text{O}_2^{2-}
\]

It can be concluded that the \(\text{Cu(II)}\) form of SOD must react in a similar fashion though undetected by ESR. This is based on our results of \(\gamma\)-irradiated native SOD in which no overall change in copper signal intensity was noted. In these glassy samples \(\text{Cu(II)}\) and \(\text{Cu(I)}\) are present in approximately equal proportions. If reaction of \(\text{Cu(I)}\) with \(\text{O}_2^-\) was the only process taking place, the net result would be an increase in \(\text{Cu(II)}\) signal on anneal. By virtue of this result, we must draw the conclusion stated above. Similarly, the formation of \([\text{BCu(II)} \cdots \text{O}_2^-]\) or \([\text{ECu(II)} \cdots \text{HO}_2]\) can be disregarded since these would have resulted in loss of \(\text{Cu(II)}\) signal intensity. No detectable ESR spectrum would be expected from these species as they would undoubtedly be diamagnetic [28].

The conclusions from these results can be summarised as follows:
1. Direct reduction of \(\text{ECu(II)}\) by 'dry' electrons has been observed in aqueous glassy samples of SOD.
2. Formation of solvated \(\text{O}_2^-\) has been detected in non-crystalline samples of the enzyme.
3. \(\text{RO}_2^*\) radicals have been formed on annealing as a result of interaction between oxygen and organic radicals formed by radiolysis. \(\text{HO}_2^*\) radicals reported by Plonka et al. may have been incorrectly assigned.
or formed in regions of ice in polycrystalline samples. Furthermore, the signal noted at about \( g = 2 \) may be due to \( \cdot \text{CO}_2^- \) in the systems studied by Plonka, a product of radiolysis of the electron scavenger, sodium formate.

4. Direct interaction of ECu(I) and \( \text{O}_2^- \) has been observed with resultant formation of ECu(II).

5. Reaction between ECu(II) and \( \text{O}_2^- \) has been implied without formation of intermediate complexes.

This work supports the popular view of a change in oxidation state during the dismutation reaction, but does not provide any conclusive evidence concerning the source of protons. However, previous work has suggested that superoxide ions are solvated at 77 K. If we assume that the bridging imidazolate of His 61 is the source of protons when the enzyme is in its reduced form, a mechanism can be proposed in which oxidation of the copper(I) centre occurs via the water molecules: protonation of \( \text{O}_2^- \) may occur causing an electron to be withdrawn from the copper centre and reforming the Cu-N\text{His}61 link.

3.4 THE EFFECT OF REDUCING AGENTS ON SOD

Section 3.3 was mainly concerned with the addition of "dry" electrons to the copper centre of superoxide dismutase and it therefore seemed an interesting extension of that work to study the reducing effects of solvated electrons as supplied by the reducing agents quoted in that section, namely potassium hexacyanoferrate(II) and sodium dithionite.

The most recently accepted mechanism of reduction of SOD [11] suggests that between pH 5 and 8.7, reduction of each copper(II) centre is accompanied by uptake of a proton from the medium, characterised by the
half reaction

$$\text{BCu}^{II} + e^- + H^+ \leftrightarrow \text{HECu}^I$$

The proton is probably bound by a ligand released from CuI with a pKa value greater than 9. This implies possible involvement of the bridging histidine ligand in which the NH group has the required pKa (Fig. 3.4). Hence it is suggested that all reducing agents react in a ratio of one molecule of reductant per atom of copper. The redox potential for the copper ions of SOD has been calculated to be +0.42 V [11] showing that it is well suited for effective involvement in a dismutation reaction involving alternate reduction and reoxidation, being intermediate between the $O_2^-/O_2$ couple, $E'_0 = -0.45 V$ and the $O_2/H_2O_2$ couple, $E'_0 = +0.98 V$ [29].

3.4.1 The potassium hexacyanoferrate II/III couple

The redox couple Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ has an $E'_0$ of +0.36 V [30] making it an ideal reagent for reduction of the copper moiety.

Addition of hexacyanoferrate(II) to solutions of SOD resulted in the reduction of Cu(II) to Cu(I) monitored by loss of copper ESR signal, confirming the work of Rotilio et al. [31]. The rate of electron exchange between the copper ions and Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ couple was slow. To determine the optimum conditions for reduction, a time dependence study was undertaken. Samples were prepared containing a slight excess of Fe(CN)$_6^{4-}$ and frozen to 77 K after being allowed to react at room temperature for varying lengths of time. The copper ESR signal was monitored. Equilibrium was found to be established after four minutes.

Titrations with hexacyanoferrate(II) were carried out to determine the concentration of reagent required for reduction of Cu(II). Titrant was added to samples of fixed volume using a Hamilton 10 μl syringe. The
samples were allowed to reach equilibrium and ESR spectra recorded. A plot showing reduction of Cu(II) signal intensity is shown in Figure 3.15. It is interesting to note that 12% of Cu(II) is non-reducible, in agreement with the 10-20% non-reducible copper observed by Fielden [21], and that Cu(II) is reduced at the expense of the Fe(CN)₆⁴⁻/Fe(CN)₆³⁻ equilibrium.

The titration illustrated here was performed in the presence of air and there was no evidence that the reduced enzyme was reoxidised by oxygen during the experimental time period. This was confirmed by introducing oxygen to the samples as described in previous sections and repeating the ESR spectra. No change in signal intensity was observed, consistent with the early reports of Mann and Keilin [5] which stated that the reduced protein is kinetically stable to oxygen.

Reoxidation titrations with hexacyanoferrate(III) were also performed on the reduced protein samples using a similar technique. The plot shown in Figure 3.15 shows a relatively slow reoxidation with only 50% recovery of Cu(II) signal. At this point the Fe(CN)₆⁴⁻/Fe(CN)₆³⁻ equilibrium is probably established and becomes the dominating mechanism.

ESR monitoring of these experiments highlighted a modification of the Cu(II) signal, the modification becoming more apparent with increasing addition of either reducing or oxidising agent (Figure 3.16). Our initial interpretation was that the modified signal, in which the original \( g_\| \)-value of 2.268 was retained but the perpendicular feature split into two components, was to follow the theory discussed by Fielden in reference 21. We suggested that perhaps the copper ions of each subunit were not identical as previously indicated, but slightly different, one with an axial and the other a non-axial ESR spectrum. Together, the two spectra superimposed would account for the observed rhombicity of the
FIGURE 3.15
Plot to show the effect of hexacyanoferrate II and III on ESR detectable Cu(II) in SOD (3.2 \times 10^{-4} \text{ M}).

Signal Intensity (arb. units)

\begin{align*}
50\% \text{ recovery} \\
\text{Fe(CN)}_6^{3-} \\
\text{Fe(CN)}_6^{4-}
\end{align*}

conc. / M \times 10^{-4}

0 1 3 5 7
FIGURE 3.16
ESR spectrum of SOD in the presence of hexacyanoferrate II/III.

native SOD ESR signal. Our results indicate that hexacyanoferrate(II) may, initially, selectively reduce the Cu(II) centre giving rise to the axial spectrum and hence allow the non-axial Cu(II) to predominate, the two-component perpendicular feature becoming exposed. Only when all axial Cu(II) is reduced, does the non-axial Cu(II) become "active" and available for reduction. A link between sub-units is implied. On re-oxidation, hexacyanoferrate(III) is only able to reoxidise non-axial Cu(II) before equilibrium between oxidising and reducing agent is established. This is summarised in Figure 3.17.

Dialysis experiments were carried out on the reduced protein in an attempt to remove hexacyanoferrate ions, and investigate the effect of removal on the ESR spectrum. 24-Hour dialysis against water had little effect on the modified Cu(II) signal. 24-Hour dialysis against phosphate buffer, pH 6.8, restored approximately 70% of the copper signal but enhanced the two-component perpendicular feature. This result conflicted
FIGURE 3.17
Schematic representation of reduction and reoxidation of the copper centre in the two sub-units of SOD.

with our proposed mechanism as a spectrum more closely related to that of the native enzyme had been expected.

The problem of the modified signal remained unresolved although the dialysis results now implied that some species had become bound to the enzyme as a result of addition of hexacyanoferrate(II). Fee and Diorleto [11] ruled out the possibility that Fe(CN)$_6^{4-}$ itself binds to the enzyme, so some impurity contained in the reagent may have caused the modification. Reduction of SOD with hexacyanoferrate(II), recrystallised from ethanol-water and stored in the dark did appear to reduce the two-component signal, suggesting that binding of trace amounts of cyanide ion may have been the problem.

Further work on cyanide binding is discussed in section 3.6.3.

It would have been interesting, had time permitted, to have made some activity measurements. Cyanide bound protein is inactive and had the modified samples described above shown reduced activity, these results would have served to confirm our speculation as to the nature of the modification.
3.4.2 Sodium Dithionite

In the absence of oxygen, sodium dithionite dissociates to give the radical anion \( \text{SO}_2^- \) which is the species responsible for reduction of Cu(II) to Cu(I). In preparation of samples of reduced enzyme, dithionite was added in solid form as described in section 3.3. However, for the purposes of the first part of this investigation into the effects of reducing agents, an oxygen-free solution of sodium dithionite was prepared for use as titrant against the protein. The results are illustrated in Figure 3.18 and show a linear reduction in Cu(II) signal intensity (reduction is complete in less than one minute after mixing).

Reoxidation with potassium hexacyanoferrate(III) is also shown on the same plot and results in approximately 70% recovery of Cu(II) ESR signal. The spectra observed during reoxidation were unexpected (Fig. 3.19).

Initially, the rhombicity of the native SOD spectrum seemed to have been removed suggesting that the symmetry of the copper centre had become more axial, \( g_\parallel = 2.268, A_\parallel = 155 \text{ G} \). Using the plot shown in Figure 3.8, the copper parameters fit more closely the square planar configuration. Hyperfine splitting with \( A_\perp = 14 \text{ G} \) became resolved on the copper perpendicular feature. This value of \( A_\perp \) is consistent with values for nitrogen. Even with the aid of a second derivative spectrum, it is difficult to determine whether there are 7 or 9 hyperfine lines resulting from 3 or 4 nitrogen atoms. Resolution of these lines implies either that nitrogen from the bridging histidine has been lost through cleavage of the Cu-N bond on reduction as previously described, or that some relaxation of this bond has occurred bringing it into the plane of the other nitrogen atoms of the 3 bound histidine residues. As more hexacyanoferrate(III) was introduced the spectrum regained its original rhombicity.

Having reduced the enzyme in the absence of oxygen so that no ESR
Plot to show the effect of dithionite reduction and subsequent hexacyanoferrate(III) oxidation on ESR detectable Cu(II) in SOD (3.2 x 10^{-4} M).
FIGURE 3.19
First and second derivative ESR spectra of dilithium-reduced ScP after subsequent reoxidation.
detectable Cu(II) remained, we were interested to observe whether SOD reduced in this way was the same as enzyme reduced with Fe(CN)$_6^{3-}$, i.e. whether or not this form of Cu$^+$SOD was stable to oxygen. This investigation was also necessary as work carried out in the previous section required reduced enzyme, in the presence of an oxygen-saturated medium, to be γ-irradiated.

Introducing oxygen into the dithionite reduced sample of protein resulted in restoration of approximately 85% of Cu(II) signal intensity, (Fig. 3.20), but with Cu(II) in the axial form described above.

![FIGURE 3.20](image)

Plot to show the effect of molecular oxygen on dithionite reduced SOD.
Since both oxidising agents resulted in this type of spectrum, it was suggested that alteration of the copper centre was brought about in the reduction process by dithionite itself, conflicting with earlier observations of Mann and Keilin [5].

3.4.3 Discussion

These results appeared to fit with our original scheme, if the difference between copper centres of each sub-unit was caused by accessibility of the active site. Reduction with Fe(CN)$_6^{4^-}$ resulted in a non-axial spectrum suggesting the axial site had access for large molecules such as this reducing agent. If the recent mechanism of reduction is correct, formation of axial Cu(I), with subsequent cleavage of the Cu-bridging histidine nitrogen ligand, may trigger a movement of the second Cu site, with limited access, to become "active" or more accessible and hence allowing further reduction to take place. The reverse process would seem to occur in reoxidation with Fe(CN)$_6^{3^-}$. Reduction with S$_2$O$_4^{2^-}$ could occur at an equal rate as this is a small radical anion which could be accommodated by both copper centres.

Though the scheme satisfies the results of reduction and reoxidation by Fe(CN)$_6^{3^-}$, it does not explain why oxygen should affect SOD differently when reduced by these two reagents.

It seems reasonable to assume that use of Fe(CN)$_6^{4^-}$ does give rise to the generally accepted reduction mechanism, that observed modification of the ESR spectrum was possibly due to binding of trace amounts of cyanide ion, and that reoxidation with molecular oxygen does not occur.

Reduction with dithionite must therefore follow a different route. It is possible that dithionite in solution may be forming sulphite ions by the reaction

$$S_2O_4^{2^-} + 4OH^- \rightarrow 2SO_3^{2^-} + 2H_2O + 2e^- \quad E^\circ = +1.12 \text{ V}.$$
If this is indeed occurring, then the dithionite is acting as a powerful reducing agent with respect to the copper centres, but is also supplying sulphite ions to the sample. Sulphite ions are known to attack disulphide bridges of proteins forming R-S-SO$_3$ and RS$^-$ [32]. As SOD possesses a single disulphide bridge per sub-unit, which holds in position a loop of protein containing three of the active site histidine residues, including the bridging histidine, it was decided that this little-studied aspect of the enzyme may be informative.

3.5 THE DISULPHIDE BRIDGE

3.5.1 Introduction

The disulphide bridge of superoxide dismutase occurs between cystine residues 55 and 144, in a very hydrophilic region of the protein. Its function appears to be structural as previously described and the link appears to be very stable. This has been demonstrated by its resistance to sodium dodecyl sulphate, a commonly-used reagent to break disulphide links, up to a concentration of 4-5%. Similarly, it is unaffected by dithiothreitol. Therefore, it was impossible to observe the effect on the Cu(II) ESR signal of attack at the -S-S- bond by the usual methods.

As early as 1955, Gordy uncovered many phenomena when using ESR to observe free radicals induced by ionising radiation in biological materials [33]. He noted that cystine-like "sulphur resonances" dominated the ESR spectra of many proteins even when these residues formed only a small part of the total structure. An intramolecular transfer of electrons to form the radical RSS$^+$ was suggested by Hadley and Gordy [34] when they established the presence of two inequivalent sulphur atoms. It was proposed that this ability to stabilise radiation energy at -S-S- links helped them to be more radio-resistant, thus avoiding protein deamination and decarboxylation.
Research in this laboratory suggested that an alternative species may be RS−SR₂, an adduct of RS• and R₂S or RSH molecules.

It therefore seemed possible that the disulphide bridge and the effect of reducing agents may be directly observed by ESR after exposure to γ-radiation. However, irradiation of SOD showed preferential electron-capture at the Cu(II) centres. In order to observe any sulphur spectra, it was necessary to either remove the metal ions to form the apoenzyme or reduce Cu(II) to Cu(I). Initial investigations were performed on the apoenzyme since it had already been shown (section 3.3) that annealing reduced enzyme preparations above 77 K caused regrowth of Cu(II). This regrowth may have obscured the features we were hoping to study.

3.5.2 Preparation of Apoenzyme [35]

Native enzyme (20g dm⁻³ concentration) was dissolved in 10⁻³ M EDTA⁻ containing 10⁻³ M sodium ethanoate buffer, pH 3.9. 0.2g of enzyme was the maximum used in any preparation. This solution was dialysed against the same EDTA/ethanoate solution for three days, the dialysate being replaced each day. The resulting protein solution was dialysed against a solution of molar sodium chloride containing 2 x 10⁻² M phosphate buffer, pH 7, for 24 hours. This was repeated 3 times using water as dialysate. The resulting apoenzyme solution was freeze dried for storage purposes.

3.5.3 Results and Discussion

The ESR spectrum for cystine in D₂O and CD₃OD (4:1 v/v) obtained after exposure to γ-radiation and annealing to remove signals from solvent radicals is shown in Figure 3.21. The observed feature is assigned to RSSR•⁺ anions and reveals a triplet pattern of proton hyperfine coupling, probably a result of interaction of the unpaired electron in the 3p-3p orbital on sulphur with two of the β protons of the anion.

† EDTA = ethylenediamine-tetra-acetic acid.
FIGURE 3.21
ESR spectrum of cystine in D₂O and CD₃OD (4:1 v/v) after γ-irradiation and annealing to remove solvent radical signals.
[RCH₂S⁻−SCH₂R]. It is characterised by its g-tensor components, $g_\perp = 2.019$
and $g_\parallel = 2.002$, and its proton hyperfine coupling constant of 8.5 G.

Exposure to γ-radiation of apoenzyme and apoenzyme in the presence of
hexacyanoferrate(II) also revealed features assigned to RSSR⁻ anions
(Figure 3.22). In the presence of dithionite, γ-irradiation formed
RSSR⁻ (Figure 3.23), and, on further anneal, features assignable to
species 'X', protonated RSSR⁻ (RSSRH) [36] (Figure 3.24).† These observa-
tions were readily repeatable with Cu(I) SOD formed by reduction
with the specified reducing agents.

We conclude from these results that the presence or absence of metal
ions in the active site has no effect on conformation of the protein,
but that the presence of $SO_2^-$ or $SO_3^{2-}$ may modify the protein in the
disulphide region. We suggest that under 'normal' conditions [apo SOD or
holoenzyme reduced with hexacyanoferrate(II)], γ-irradiation causes very
selective addition of mobile electrons to the disulphide bridge. This
does not result in bond cleavage and either access to the site is small
so that subsequent protonation is impossible, or there are insufficient
readily available protons for species 'X' to form before softening
temperature of the sample is attained. Confirmation that $SO_3^{2-}$ does not
cleave the $-S-S-$ bond to form an adduct was obtained by formation of
RSSR⁻ only on annealing a sample of apoenzyme + sodium sulphite.

In the Cu(II) holoenzyme, results showed a rapid decrease in Cu(II)
signal intensity on exposure to γ-radiation prior to growth of RSSR⁻
anions. The implication is that electrons selectively add to the metal
centres, but are then able to move within the protein in order to seek

† An interesting experimental observation was the marked change in
colour of frozen samples from purple, due to trapped electrons,
to yellow as features attributed to radicals containing sulphur
were detected.
FIGURE 3.22
ESR spectrum of apoenzyme, after γ-irradiation and anneal, showing signal due to RSSR\(^{−}\). Although the feature is broad, it is sufficiently distinctive to be assigned to RSSR\(^{−}\).
FIGURE 3.23
RSSR⁻ radical anion signals formed after γ-irradiation and anneal of apoenzyme in the presence of dithionite.
Features assigned to species 'X' after γ-irradiation and anneal of apoenzyme in the presence of dithionite.
out -S-S- bonds at which they are permanently trapped.

In the presence of dithionite, either access to RSSR\(^-\) ions is enlarged so that protonation is possible, or more protons become available in the region of the disulphide bridge. Since SOD has been shown to be resistant to the usual chemical reagents which cause -S-S- bond cleavage, it seems unlikely that SO\(_2^-\) or SO\(_3^{2-}\) is forming an adduct and thereby changing its structural function: access to the bridge is probably not becoming enlarged. The -S-S- bond lies buried in a hydrophilic region of protein and although it appears to be external to the bulk of a monomer, it lies in a region where the two monomers are held together by electrostatic interactions to form the active dimer unit. It is therefore our opinion that the presence of dithionite simply increases the availability of protons to the enzyme, possibly in the following way:

\[
\begin{align*}
S_2O_4^{2-} &\rightarrow 2SO_2^- \\
SO_2^- + H_2O &\rightarrow SO_3^{2-} + 2H^+ + e^- \\
RSSR^- + H^+ &\rightarrow RSSRH
\end{align*}
\]

Thus species 'X' can be formed during the annealing process.

### 3.6 EFFECT OF ANIONS

Structural studies of the enzyme SOD have demonstrated the presence of an open coordination position occupied by a rapidly exchanging water molecule on the protein bound Cu(II) centre. The importance of this position for catalysis of the dismutation reaction has already been emphasised. In addition to this primary function, it is also a potential anion binding site.

#### 3.6.1 pH dependence of SOD

Although this is not direct anion binding, pH changes affect the water molecule associated with the active site.
Samples of SOD were prepared in purified water containing 15% ethane-1,2-diol and adjusted above pH 7 by small additions of potassium hydroxide and below pH 7 with concentrated hydrochloric acid. pH measurements were made using a Pye-Dynacap meter equipped with a Pye-Ingold micro-electrode.

On raising the pH of aqueous SOD the Cu(II) ESR spectrum changed as depicted in Figure 3.25. At pH 11.4 $g_{||}$ and $A_{||}$ values were the same as in native SCD although the spectrum was becoming more axial in the $I$ feature. At pH 12.5 the sample took on a purple colouration and rhombicity of the spectrum was lost. A well-resolved set of nine hyperfine lines was apparent on the $g_{\perp}$ feature with splitting in the order of 15 G.

These results are in agreement with the work of Rotilio et al. [37] who suggest that raising pH relaxes the rhombic distortion of the copper site. This causes the appearance of nitrogen ligand hyperfine structure not previously apparent due to overlap of the $x$ and $y$ components. Relaxation may be brought about either by movement of the bridging histidine ligand into the plane of the other 3 nitrogen ligands or by cleavage of the same bond. Because the hyperfine pattern is evident only on the $Cu_{||}$ feature, this problem cannot be resolved: the value of $A_{\perp}$ for Cu can also be in the region of 15 G and overlap of copper hyperfine structure can produce a greater number of lines than would be expected on a single copper hyperfine line. It may therefore be incorrect to assign 9 superhyperfine lines as resulting from 4 nitrogen ligands. At high pH the water molecule will be ionised to OH$^-$.

Lowering the pH of the enzyme solution resulted in the spectra of Figure 3.26. At pH 2.85 some of the rhombic character of the spectrum again appeared to be lost. It has been suggested that this is due to protonation and hence cleavage of the bridging histidine ligand with
FIGURE 3.25
ESR spectra showing the effect of increasing pH on the Cu(II) centre.
1. pH 11.4
2. pH 12.1
3. pH 12.5
FIGURE 3.26
ESR spectra showing the effect of lowering pH on the Cu(II) centre.
1. pH 3.3
2. pH 2.85
3. pH 2.75
either the copper or zinc centre (Figure 3.4). This change is reversible and probably reflects a modification of the symmetry of the copper site which favours removal of the metal. We suggest that the low pH spectrum is a result of Zn-His 61 bond cleavage with subsequent protonation of His 61. This allows the loop of protein usually held against the β-barrel to be released producing a change in conformation of the active site. Rotilio [37] suggests that subsequent removal of zinc may expose the sulphhydryl group of cysteine-6 and possibly, by interchange with the disulphide bridge, cause an irreversible conformational change, thus explaining the irreversible nature of the zinc depleted enzyme. However, our results of section 3.5 imply no change of conformation in the apo-enzyme as a result of reaction at the disulphide bridge and so we cannot agree with this proposal. Nevertheless, cleavage of this bond does confirm the rôle of zinc as structural, preforming the copper site in its distorted environment, and is in agreement with the work of Pantoliano et al. [38]: the ESR spectrum of Cu_{II}E_{2} SOD (no metal ions bound at the zinc site) compares favourably with the low pH spectrum.

At pH values lower than 2.85, new copper features became apparent due to aqueous Cu(II) observed as a result of loss of metal ions from their protein bound sites.

3.6.2 Effect of sulphite ions

In earlier sections it has been proposed that, under given experimental conditions, the active component of dithionite is SO_{3}^{2−}, the sulphite ion. In the absence of protein-bound copper, it was believed that the ion attacked the disulphide bridge. Although this has been discounted, during the course of the work leading to this conclusion, sulphite ions were directly added to SOD. In the presence of holoenzyme the ESR spectrum of Figure 3.27 was observed. This is very similar to both the
FIGURE 3.27
ESR spectrum of native enzyme + sodium sulphite.
spectrum obtained on reoxidation of dithionite-reduced SOD and the high pH spectrum in which Cu(II) has an associated OH⁻ ion. Therefore we tentatively suggest that sulphite ions bind to Cu(II) in an axial position with displacement of the associated water molecule and relaxation of the bridging histidine ligand. This accounts for a hyperfine set of lines on the i feature due to 3 or 4 nitrogen ligands and also provides an explanation for the reoxidation of dithionite-reduced enzyme by molecular oxygen since anion-bound SOD is known to be oxidised rapidly in the presence of air.

However, reduction of native SOD with dithionite did not reveal the modified axial spectrum immediately. The spectrum was only revealed after complete reduction of ESR detectable Cu(II) and subsequent oxidation, suggesting that sulphite ions are bound primarily to Cu(I). In the SOD/dithionite system, SO₃²⁻ ions may be generated and bound in the following way:

1. $S_2O_4^{2-} \rightarrow 2SO_2^-$
2. $\text{Cu}^{II} + SO_2^- \rightarrow \text{Cu}^{I} + SO_2$
3. $SO_2 + H_2O \rightarrow SO_3^{2-} + 2H^+$ or
   $SO_2 + 2OH^- \rightarrow SO_3^{2-} + H_2O$
4. $\text{Cu}^{I} + SO_3^{2-} \rightarrow \text{Cu}^{I} - SO_3^{2-}$
5. $\text{Cu}^{I} - SO_3^{2-} [0] \rightarrow \text{Cu}^{II} - SO_3^{2-}$

Reoxidation by hexacyanoferrate(III) of dithionite-reduced protein (section 3.4.2) slowly returned the axial to the native ESR spectrum. This can now be explained by hexacyanoferrate(III) oxidation of sulphite to sulphate ions which may then be released back into solution.

3.6.3 Effect of cyanide ions

Cyanide binds strongly to Cu(II) in SOD and its effect on the ESR
spectrum is well documented [13,19,37,39,40,41]. Work in this laboratory with the reducing agent hexacyanoferrate(II) led to the suggestion that trace quantities of cyanide ion present as impurity may be bound to the copper site causing splitting of the $Cu_\perp$ feature. It was therefore decided to repeat work quoted in the literature.

Addition of potassium cyanide to SOD revealed distinct ESR spectra depending on the concentration of cyanide ions used in the experiment (Figure 3.28). At low concentrations the value of $A_\parallel$ for copper was approximately 140 G and closely resembled the parallel features for native SOD. However the $i$ feature was split into two components, the low-field component showing some resolution. At higher concentrations of CN$^-$ a very different spectrum was observed with $g_{av\parallel} = 2.22$ and $A_\parallel = 181$ G. The low field $i$ component was resolved and revealed at least 7 lines probably arising from an interaction between the unpaired electron and $^{14}N$ nuclei ($^{14}N_\parallel$ hyperfine coupling constant = 18 G).

The experiments were repeated with potassium cyanide enriched with $^{13}C$ and at low concentrations a poorly-resolved spectrum was observed (Figure 3.29), although splitting due to the $^{13}C$ nucleus seems large in the $Cu_\perp$ feature, but less than 20 G in the $Cu_\parallel$ feature. This implies a mixed $\sigma/\pi$ bond to copper via the carbon end suggesting that cyanide was bound off axis and the complex remained highly distorted. At higher concentrations a splitting of 47±3 G was observed in the parallel features showing that cyanide was now bound through its carbon end by a $\sigma$-bond. Since the spectrum was well resolved, cyanide must lie in a square planar arrangement with 3 magnetically very equivalent nitrogen atoms, the bridging imidazole nitrogen being bound out of this plane.

Beem et al. [40] suggest that initial attack by CN$^-$ may be axial to the plane of the 3 nitrogen ligands since the known ability of cyanide
FIGURE 3.28
ESR spectra showing the effect of increasing concentration of cyanide ion on the Cu(II) centre of SOD.
FIGURE 3.29
ESR spectra of Cu(II) SOD showing the effect of binding increasing concentrations of K\textsuperscript{13}CN.
ions to form π-bonds with planar Cu$^{2+}$ complexes may stabilise initial formation of the axial adduct. They suggest the second type of spectrum is a result of a subsequent rearrangement to form a planar complex. Rotilio [19] has demonstrated, by computer simulation of $^{63}$Cu$_2$Zn$_2$SOD, that resolution of the low field copper hyperfine feature involves interaction of the unpaired electron with 3 equivalent $^{14}$N atoms. Hafner and Coleman [41] have found that a single cyanide binds to each Cu$^{2+}$. Our work at pH 7.4, however, indicated that possibly more than one equivalent of cyanide was bound per copper ion and this would fit the information from the ESR spectra if one CN$^-$ was bound in the plane and one bound axially, since the latter would not give rise to any coupling with the unpaired electron. However, these results may be erroneous since incomplete binding is most probably occurring due to cyanide hydrolysis which becomes extensive at pH <10.

We must therefore confirm the mechanism described above suggesting that one cyanide ion binds per copper centre, through its carbon end, in a position out of the plane of the 3 nitrogen ligands. Rearrangement then follows. Cleavage of the bridging histidine ligand is not necessarily implied.
REFERENCES

CHAPTER 4

HAEMOGLOBIN
4.1 GENERAL INTRODUCTION

4.1.1 Early History

Haemoglobin is the vital protein of red blood cells. Its function is to transport oxygen from the lungs to the tissues and to aid the return of carbon dioxide. Blood leaving the lungs is red and blue on return. Until the seventeenth century, it was believed that circulation of these different forms was completely isolated. Harvey illustrated that it was "the same blood" by placing a venous sample in a dish and observing the colour change to scarlet. Lavoisier attempted to explain this observation when he recognised oxygen as a component of air; he suggested the colour change was due to oxygen uptake. It was later speculated that the red pigment in blood was protein in nature and from its colour change, which resembled the rusting of iron, contained at least one iron atom per molecule.

It was not until 1959 that the crystal structure was determined by Max Perutz.

4.1.2 Basic Structure

The haemoglobin molecule is made up of 4 polypeptide chains, 2α and 2β. The former contain 141 amino acid residues, the latter 146, in different sequences, but both with the same tertiary structure which forms the protein backbone of the molecule, or the globin, Fig. 4.1. Each polypeptide chain is folded to create a pocket in which a complex cyclic tetradeutate haem resides, Fig. 4.2.

X-ray studies of ferric iron porphyrins have determined iron nitrogen bond lengths of 2.061 Å for high-spin ferric compounds and 1.99 Å for low-spin ferric compounds [1,2]. In metal-free porphyrins, the distance from the centre of the ring to a nitrogen atom is 2.01 Å. Hence the
FIGURE 4.1
Diagram to show the conformation of the beta chain of haemoglobin and helix nomenclature.
FIGURE 4.2
Diagram to illustrate the structure of the tetradeutate haem unit.
relative positions in or above the plane of the porphyrin ring can be determined. In six-coordinated low-spin ferric porphyrins the iron atom lies within 0.05 Å of the planar nitrogens [2] but in five-coordinated high-spin ferric complexes the iron atom is forced to lie out of the plane by 0.38 to 0.47 Å. A comparison of atomic radii can give an insight into the configuration of ferrous iron porphyrins. The radius of low-spin ferrous iron is within 0.02 Å of low-spin ferric iron, thus an equivalent configuration is expected. High-spin ferrous iron, however, has an ionic radius 0.12 Å greater than high-spin ferric iron [3] thus creating an iron-nitrogen distance of 2.18 Å displacing the ferrous atom 0.83 Å from the porphyrin plane.

Of the haem derivatives known, all ferric forms are six-coordinated, the acid, met- and fluoride derivatives being high-spin, the remainder low-spin. Deoxyhaemoglobin is the five-coordinated high-spin ferrous complex and oxy- and carbonmonoxy- are six-coordinated low-spin ferrous derivatives.

A covalent bond extending from the iron atom to nitrogen, N₇, of the proximal histidine, F₈, attaches the haem group to the globin. There is no direct link between iron and globin on the distal side, although histidine, E₇, is in close proximity as shown in Fig. 4.3. The ligand comes to lie between the iron atom and this residue. Further removed from the haem centre are sixty atoms from the protein which are in Van der Waals contact with the porphyrin ring.

The quaternary structure of the tetramer depends only on coordination of the iron atom, not on valency or spin-state. All liganded forms have the same "relaxed" structure, the deoxy- "tense" form, however, is quite unique. The C-terminal residues of all 4 polypeptide chains in "liganded"
FIGURE 4.3
Arrangement of the proximal, F8, and distal, E7, histidine residues in oxyhaemoglobin.
haemoglobin derivatives are free to rotate, and the penultimate tyrosine residues spend only a short time in their bound positions between the F and H helices, hence the term "relaxed". The uniqueness of the deoxy-form arises from its locked or "tense" structure caused by two salt bridges per C-terminal residue; the α-carboxyl group of arginine 141α₁ is linked to the α-amino group of valine 1α₂ and its guanidinium group to aspartate 126α₂, and the α-carboxyl of histidine 146β₁ is linked to the ε-amino group of lysine 40α₂ and its imidazole to aspartate 94β₁. Pockets between the helices F and H contain tyrosine residues by Van der Waals contact and hydrogen bonding, formed between the tyrosine-OH groups and valine FG5 carbonyl groups.

Neither oxy- nor deoxyhaemoglobin contain any direct links between the two β chains, although a link is formed in the red cell by 2,3-diphosphoglycerate (DPG).

4.1.3 Haem-haem interaction

The distribution of oxygen among haemoglobin molecules in solution gives rise to a sigmoid curve, signifying that the first oxygen molecule is taken up by deoxyhaemoglobin with some difficulty, but oxygen affinity increases as more oxygen molecules are bound. This phenomenon suggests some communication between haem groups in each tetramer, known as haem-haem interaction. When a logarithmic plot of the curve is obtained, Fig. 4.4, it can be seen that the equilibrium curve begins with a straight line 45° to the axes because oxygen molecules are scarce, allowing the monomers to react independently since each haemoglobin tetramer has the chance of taking up only one oxygen molecule. As more oxygen is introduced the haem tetramers begin to interact until a 45° line is again obtained since one haem per molecule is likely to remain oxygen-free and must again react independently of the oxygenated haem.
FIGURE 4.4
Hill's graph for haemoglobin saturation by oxygen.

Y: fractional saturation of haemoglobin
$P_{O_2}$: partial pressure of oxygen
groups. The tangent to the maximum slope is known as Hill's coefficient with a normal value of about three; without haem-haem interaction this value is unity.

Haem-haem interaction ensures that affinity falls as successive oxygen molecules dissociate. This is essential since venous blood pressure is about 35 mm of mercury whereas the partial pressure of oxygen in the lungs is about 100 mm of mercury. If the curve was hyperbolic, less than 10% of the oxygen carried would be released to the tissues.

Several chemical factors in the red blood cell serve to shift the curve towards lower oxygen affinity. These are protons, carbon dioxide, chloride ions and 2,3-diphosphoglycerate (DPG).

4.1.4 The Bohr Effect

Protons lower the affinity of haemoglobin for oxygen, such that liberation of oxygen molecules causes haemoglobin to combine with two protons and vice versa. This reciprocal action is known as the Bohr effect. Its purpose is to render more soluble carbon dioxide released by respiring tissues, by the carbonic anhydrase catalysed combination with water to form bicarbonate ions. Deoxyhaemoglobin acts as a buffer shifting the equilibrium towards formation of bicarbonate. In the lungs the process is reversed.

The Bohr effect is only observed in those reactions involving the transition between tense and relaxed structures on combination of O₂, CO or NO with ferrous haemoglobin or oxidation of deoxy- to methaemoglobin. [The explanation lies in the ability of hydrogen ions to strengthen the salt bridges containing the tense deoxy-structure by increasing the fraction of molecules carrying positive charges on one α-amino and two imidazole groups.]
In the tense conformation the pKs of the α-amino groups are raised through linkage of the C-terminus of one α-chain with the N-terminus of its partner chain. In both β-chains, the imidazole of histidine 146β becomes linked to aspartate 94β producing a much larger increase in pK than the more distant interaction with its own carboxyl group in oxy haemoglobin.

4.1.5 Mechanism of the Conformational Change

The distance between haem groups, 25-37 Å, is too large for electromagnetic interactions to be an effective trigger of the change, suggesting a stereochemical mechanism. As the iron atom undergoes the transition from high-spin to low-spin, the construction of the haem group is such that the small change in atomic radius is amplified into a large movement of the proximal histidine, F8, relative to the porphyrin ring. This movement triggers changes in the tertiary structure in which helix F moves towards the centre of the molecule narrowing the pocket between it and helix H. As a result the penultimate tyrosine, HC2, is expelled and the C-terminal salt-links between subunits broken with release of Bohr protons, Fig. 4.5. As each link is broken one constraint holding haemoglobin in the tense conformation is removed and moves the equilibrium in favour of the relaxed structure. [However, although ligand entry to the α-haem pocket of deoxyhaemoglobin is unrestricted, the β-pocket is narrower and blocked by valine, Ell.]

It has therefore been suggested that the order of subunit oxidation is α1 α2 β1 β2: oxidation of the two α subunits ruptures four of the six salt-links between α subunits. The α1β2 and α2β1 contacts are then broken with concomitant conformational change to the oxy-form. This results in liberation of one mole of DPG per mole of haemoglobin, bound in the tense structure to both β subunits. The β chains are now strained
FIGURE 4.5
Diagrammatic sketch showing the change in tertiary structure of the haemoglobin subunits on reaction with ligands. Movement of the iron atom into the plane of the porphyrin ring causes a movement of helix F towards helix H, which expels tyrosine from its pocket between the two helices.
but the quaternary change has served to reduce the activation energy required for β tyrosine expulsion by breaking αβ salt-links. The expulsion of tyrosine is brought about by rupture of the links between the C-terminal histidines and aspartates of the same β chains releasing the last Bohr protons and causes helix E to move away from the haem, thus widening the haem pocket for reaction of Fe₃ with oxygen. This mechanism is summarised in Fig. 4.6.

4.1.6 ESR data

Haemoglobin and oxyhaemoglobin contain only ferrous iron which is not paramagnetic when covalently bonded. However, ferric compounds possess unpaired electrons in both covalent and ionic states and hence many oxidised derivatives of haemoglobin are available for study by ESR. Both methaemoglobin and haemoglobin labelled with an NO-group yield characteristic spectra [4]. A variety of these can be prepared by substitution and changes in the ESR spectra yield information about the haem site and its surroundings and the effects of oxygen bonding [5]. Much attention has also been focussed on cobalt oxyhaemoglobin since this contains an extra electron giving well-defined ESR spectra [6,7,8]. Similarly, model compounds have been studied [9], known as 'picket fence' complexes which have a cavity resembling the globin haem pocket on one side of a porphin plane.

It has been suggested [10] that the superoxide representation, \( \sqrt[4]{Fe^{+}(III)O_2^-} \) may be preferable to the normal oxygen complex representation \( \sqrt[4]{Fe(II)O_2} \). This is supported by infra-red [11] and Mössbauer [12] studies and polar solvent effects [13], although a theoretical study favours the \( \sqrt[4]{Fe(II)O_2} \) formation [14].

The aim of this ESR research has been to study the ferrous oxy-centre of haemoglobin which until recently had not been directly studied. In
Figure 4.6

Diagrammatic sketch of the allosteric mechanism of haemoglobin. [Smaller subunits represent α, larger subunits represent β. Clamps represent salt bridges. Unstable forms are shown with broken lines.]
1975, Symons [15] showed that low temperature $^{60}$Co $\gamma$-irradiation of oxyhaemoglobin suspensions gave rise to paramagnetic centres resembling those for low-spin ferric derivatives. These were initially assumed to be formed by electron loss since reduction of low-spin components on annealing was accompanied by formation of the normal high-spin ferric feature at $g = 6$. Further work in this laboratory [16] identified the low-field components as the $\alpha$ and $\beta$ subunit haem Fe-O$_2$ centres.

The addition of various electron carriers resulted in a decreased yield of $\alpha$ and $\beta$ centres and the loss in intensity of the $g = 6$ feature on exposing methaemoglobin to $^{60}$Co $\gamma$-rays. This suggested that the observed signals resulted more reasonably from electron gain at the Fe-O$_2$ centres than electron loss.

The involvement of oxygen in the $\alpha$ and $\beta$ centres was directly established using $^{17}$O.

The use of additives to encourage glass formation in haemoglobin samples increased the yield of $\alpha$ and $\beta$ centres with a greater effect on the $\beta$ centre, thus illustrating the importance of preventing phase separation. Later pH studies [16] suggested that crystalline haemoglobin has an 'effective' acid pH with equal $\alpha$ and $\beta$ yields. It is believed that the effect of glassifying agents is to neutralise this pH resulting in the observed yield of $\alpha$ and $\beta$ centres.

Annealing $\alpha$ and $\beta$ centres above 77 K gave rise to further features such that $\alpha + \gamma$, $\beta + \delta$ and $\delta + \gamma'$ with all signals finally giving the high-spin ferric $g = 6$ signal.

To explain the different $g$-values of the $\alpha$ and $\beta$ centres, Symons and Petersen suggested that hydrogen bonding to the distal imidazole (or a water molecule) differs in the $\alpha$ and $\beta$ subunits since solid state hydrogen bonding is often sensitive to steric factors. The changes on
annealing may be

\[
\frac{\text{Fe}^+_{(III)} - O_2(-)}{\text{HN}} \rightarrow \frac{\text{Fe}^{(III)} - O_2H + N^<}{(1)}
\]

\[
\frac{\text{Fe} - O_2H}{\rightarrow \text{Fe}^+ + \text{HO}_2^-}
\]

The electron affinity of the \( \beta \) centre would be increased if the degree of proton transfer in the hydrogen bond was greatest for \( \beta \), thus giving an explanation for the larger yield of \( \beta \) centres on irradiation. Since the \( g \)-values for \( \alpha \) and \( \delta \), \( \gamma \) and \( \gamma' \) are nearly equivalent, it has been proposed that the changes \( \alpha + \gamma \) and \( \delta \rightarrow \gamma' \) are the proton transfers of equation (1) with \( \gamma, \gamma' \rightarrow g = 6 \) as the process illustrated in equation (2); \( \beta \) may be a unique 'primary' centre formed by electron addition before structural modification of the \( \alpha \) and \( \beta \) species has occurred.

4.2 EXPERIMENTAL

Samples were prepared as described in the text of Section 4.3.

Phosphate buffer was prepared as described in Chapter 3.

The following procedure was carried out where stated on samples of powdered, commercial haemoglobin, supplied by the Sigma Chemical Co. Ltd., in order to remove methaemoglobin and denatured material.

Pre swollen microgranular cation exchanger CM52 carboxymethyl cellulose was obtained from Whatman. The ion exchanger was stirred into a volume of 0.1M pH6 mixed phosphate buffer (15-30 cm³ per dry gram). This was left to stand for 10 minutes and the supernatant liquid then decanted. The procedure was repeated until the filtrate attained buffer pH. The slurry was poured into a glass column of 2 cm length and 1 cm internal diameter and the effluent run to waste. Buffer was run through at a flow rate of 4 cm³ min⁻¹ until the height of the bed remained
constant. It was finally equilibrated with 0.01M pH 6 buffer. A solution of powdered haemoglobin (10 g dm\(^{-3}\)) in 0.01M pH 6 buffer was applied to the column, washed several times and the haemoglobin eluted with 0.1M pH 7.4 buffer.

4.3 RESULTS AND DISCUSSION

The initial aim of this research was to repeat and then extend the work of Petersen carried out in these laboratories several years earlier and discussed in Section 4.1.6.

For ease of storage the preliminary studies were attempted using powdered haemoglobin samples. After initial experimentation with various preparative techniques, it was decided to use aqueous samples of the powdered protein frozen in the form of small spherical beads where possible. The alternative, freezing samples in Suprasil tubes resulted in an ESR doublet due to H\(^+\) caused by irradiation of the tube. This interfered with primary \(\alpha(z)\) and \(\beta(y)\) ESR features.

A sample of powdered bovine haemoglobin prepared in phosphate buffer at pH 6.9 to a concentration of 50 g dm\(^{-3}\) haemoglobin gave rise, on irradiation, to a large \(g = 6\) feature due to methaemoglobin. \(\alpha\) and \(\beta\) features were not observed.

Subsequent samples, to which a small amount of solid sodium dithionite had been added, revealed large reduction of methaemoglobin observed visually as a colour change from brown to pink and spectrally by the absence of \(g = 6\) signal. A concomitant increase in \(\alpha\) and \(\beta\) yield was observed with an equal yield of both \(\alpha\) and \(\beta\) centres. Addition of a glassifying agent, in this case ethane-1,2-diol, to a concentration of 20% by volume in the final sample revealed centres identical with those observed by Petersen.
In an attempt to aid resolution of \( z \) and \( y \) features, the aqueous buffer system was replaced by phosphate prepared in deuterated water to an equivalent pH (although pD was not accounted for). A significant result arose from this as the \( \beta/\alpha \) signal intensity ratio increased from 1.45 in the water-based sample to 2.08 in the deuterated sample. This may imply greater involvement of a hydrogen bond at the \( \beta \) centre than at the \( \alpha \) centre or a replacement of \( \text{H}_2\text{O} \) by \( \text{D}_2\text{O} \) in the region of the haem pocket thus influencing oxygen affinity or electron capture by the iron-oxy unit. [This \( \text{D}_2\text{O} \) effect may be more prominent in samples prepared with powdered haemoglobin than whole blood since H/D-exchange may be more facile.]

Annealing samples of this type above 77 K revealed several features that had not been reported by Petersen. Initially a broadening of the \( \beta \)-centre was observed which became resolved in both \( z \) and \( y \) directions to form a new signal hereafter referred to as \( \beta' \). Further warming resulted in a decrease in \( \beta \) as \( \beta' \) became better resolved together with formation of the features \( \delta \) and \( \gamma \). A slight narrowing and increase in yield of \( \alpha \) centres accompanied the changes. \( \beta' \) then decayed completely with an approximately proportional increase in \( \gamma \) leaving a spectrum dominated by \( \gamma \) and an \( \alpha,\delta \) doublet, the latter finally being lost to the species assigned \( \theta \), Fig. 4.7.

Although no chemical structures can yet be attributed to the above mentioned features, several tentative conclusions may be drawn concerning the annealing process. The apparent initial narrowing of \( \alpha \) may be explained as increased resolution due to \( \beta \) decay. The apparent \( \alpha \) increase may be due to capture of trapped electrons released during the first annealing process. This latter explanation is supported by a sample colour change during this period also observed while \( \gamma \)-irradiated samples
FIGURE 4.7
ESR spectra showing features resolved at various stages of the annealing process of a glassy sample of powdered haemoglobin buffered to pH 6.9.
were photolysed by visible light when a similar process was thought to occur. The explanation implies that electron capture at the \( \alpha \) centre is less efficient than at the \( \beta \) centre in samples of this type. It also seems possible that \( \delta \) may not be a product of the annealing process, but may be present at 77 K and "hidden" beneath \( \alpha \), only becoming apparent with increased resolution of \( \alpha \).

The sequence of the annealing process as described may be summarised as follows:

\[
\begin{align*}
\beta &\rightarrow \beta' \rightarrow ? \rightarrow \gamma \rightarrow \delta \rightarrow \alpha \\
&\quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad 
\end{align*}
\]

These results were readily repeatable and revealed further information about resolution of \( \alpha \) and \( \delta \) features: as \( \delta \) became apparent and equal in intensity to \( \alpha \), loss of both signals occurred at the same rate within the same temperature range suggesting a very similar chemical structure.

Experiments repeated with powdered haemoglobin buffered to lower and higher pH were inconclusive and often unrepeatable as a possible result of partial denaturation of the protein. However, one common characteristic of all low pH samples (approximately pH 5-6) prepared from this material was the presence of the \( \gamma \) feature prior to anneal and absence of \( \theta \) after anneal, \( \gamma \) being the final product of \( \alpha \) and \( \delta \) decay. [\( \alpha \) and \( \beta \) signals were also obtained in equal yield.] If this is indeed "real", the results may have several explanations:

(i) at low pH, \( \beta \) chains have a lower affinity for oxygen,

(ii) the oxygen affinity of the \( \alpha \) chains may be greatly increased to the detriment of \( \beta \), e.g. by denaturation, loss of quaternary structure, with implied loss of haem-haem interaction,

(iii) \( \beta \) chains may alter in configuration such that they resemble \( \alpha \) in ESR terms (this seems unlikely and one would expect a significantly larger signal due to \( \alpha \) centres),
(w) since $\alpha$ appears to anneal to $\delta$, this may reveal the origin of $\delta$ as the $\alpha$ centre.

Results using samples prepared from powdered haemoglobin were inconclusive and the necessity for work on whole blood samples became apparent, despite the problems of supply and storage. Experiments described here after, unless otherwise indicated, were performed on the student's own blood, taken immediately prior to sample preparation without addition of anticoagulant. In fact, experiments were later performed on blood samples containing anticoagulant (heparin) and no obvious differences were observed.

The advantage of using whole blood samples immediately became apparent. Spectra were surprisingly "cleaner" suggesting that a large proportion of the powdered haemoglobin may have been denatured. This explained some of the unsatisfactory results obtained and it was noted that any subsequent work on samples from that source would necessitate a chemical procedure to remove inactive material before sample preparation. $\alpha$ and $\beta$ signals were obtained in much higher yield from whole blood meaning a larger signal-to-noise ratio and hence spectra with better definition of features.

The initial experiments were performed on the blood itself with no additions; a polycrystalline sample was therefore expected. Freezing to 77 K, followed by a two hour exposure to $\gamma$-radiation revealed $\alpha$ and $\beta$ signals of equal intensity. Later work involving varying the pH of the

Footnote: freezing to low temperatures and/or addition of ethane-1,2-diol to samples necessarily causes rupture of the red blood cell, so that haemoglobin studied was not restricted to the cell environment. This was shown to be the case by the use of sonication on whole blood samples. This technique is known to rupture the cell membrane and release its contents. Experimental results obtained were consistent with those described below.
haemoglobin environment in glassy samples established that equal intensity of \( \alpha \) and \( \beta \) signals occurred only at slightly acid pH, suggesting that haemoglobin in these samples may be residing in an acidic pocket resulting from the overall pH of its constituent amino acids.

Annealing the sample showed growth of \( \beta' \), \( \delta \) (as a small shoulder on \( \alpha \)) and \( \gamma \) as the \( \beta \) signal decreased in intensity. A further increase in \( \gamma \) was noted as \( \beta' \) decayed. Finally, \( \alpha \) and \( \delta \) were lost simultaneously with broadening of \( \gamma \) to become very asymmetric in both \( x \) and \( z \) sets of signals; \( \theta \) was not resolved but may have given rise to the asymmetry, although the \( y \) set of features did not indicate the presence of two species.

The previous anneal scheme may be slightly modified from these results as follows, although there now arises a discrepancy in the origin of \( \delta \), previously believed to emanate from the \( \alpha \) centre.

If this flow diagram is correct, an explanation needs to be found to determine why \( \beta \) might anneal to form two different, though structurally similar species. At this stage, two explanations, neither of which are wholly satisfactory, may be put forward.

[1] If the Fe-O\(_2\) centres are labelled \( \beta\beta\alpha\alpha \alpha \) where \( \beta \) is a \( \beta \) centre in the conformation of a \( \beta \) helix and \( \alpha \alpha \) an \( \alpha \) centre in an \( \alpha \) helical conformation, then the anneal process may invoke a conformational change of the \( \beta \) centres such that one takes on the conformation of an \( \alpha \) helix, \( \beta\alpha \), thus becoming more \( \alpha \)-like with a slight \( g \)-shift towards free spin (the feature \( \delta \)); the other may retain its \( \beta \)-like conformation although some slight change may be expected due to the \( \beta\alpha \) cross-over resulting in the \( g \)-shift to \( \beta' \). This can be summarised:
The flaw in this explanation lies in the relative intensities of \( \gamma \) and \( \theta \). One would expect a more intense signal for \( \theta \), approximately a 3:1 ratio of \( \theta:\gamma \). The observed yields do not comply with this.

[2] An alternative scheme implies hydrogen bonding. The difference in g-value between \( \alpha \) and \( \beta \) centres may result from a loose \( \beta \text{Fe}-\text{O}-\text{O}----\text{H} \) association making the \( \beta \text{Fe}^{II} \) centre appear more \( \text{Fe}^{III} \)-like than the \( \alpha \text{Fe}^{II} \) centre in which the association may not exist (it must be noted here, however, that myoglobin chains tend to have \( \alpha \)-character and the \( \text{Fe}-\text{O}_2 \) centres are known to be hydrogen bonded). If this is the case, then the sequence accounting for the anneal features of \( \beta \) chains may be as follows:

\[
\begin{align*}
\text{Fe}^{II} & \rightarrow \text{Fe}^{II} & \rightarrow \text{Fe}^{III} + \text{HO}_2^- \\
\beta & \rightarrow \beta' & \rightarrow \gamma
\end{align*}
\]

This assumes the source of the proton being the distal histidine which lies above the haem plane. Drawbacks of this scheme, though giving an identity to the features \( \beta, \beta' \) and \( \gamma \) and accounting for their respective g-values, are:

(i) it does not involve \( \delta \) although its origin is disputable, and

(ii) it suggests the sequence for \( \alpha \) must contain more steps as the initial association to hydrogen also has to be made. Results show more steps to the \( \beta \) annealing process.

Subsequent experiments in which whole blood was buffered to physiological pH and ethane-1,2-diol incorporated as glassifier revealed a \( \beta/\alpha \) signal ratio greater than 1 after exposure to \( \gamma \)-radiation. The sequence on

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warming above 77 K did not change.

At higher pH values (around 8.5) in which the β/α ratio remained greater than 1, two significant differences were observed

(i) both β' and α signals were lost on annealing with an increase in γ, δ was not lost concurrently with α,

(ii) features due to θ became apparent with loss of δ, and finally decayed with simultaneous growth in γ.

At lower pH values (around 6.0) the β/α signal ratio approached 1. This led to the suggestion made earlier that haemoglobin possesses its own slightly acidic functional pH indicated by the preliminary unbuffered experiments on polycrystalline samples. Results from these low pH experiments did not agree with those performed on powdered haemoglobin samples to the extent that γ appeared during the annealing process, not at 77 K, and was accompanied by a second feature at lower field strength, γ', the origin of which was not clear. All features were subsequently lost to γ'.

Since the course of these investigations now hinged on the sequence of the anneal process, several other simple experiments were performed to try to shed light on the exact nature of the process.

The first involved the effect of varying exposure time to γ radiation thereby studying the effect of an increasing proportion of Fe-O₂⁻ centres and O₂⁻ in the sample. Although the anneals of each sample provided no further information a graph showing relative yields of both α and β centres was drawn, Fig. 4.8. This revealed a peak intensity of both centres after approximately 7 hours exposure to γ radiation. However, since there was no effect on the anneal process and sufficient yield was obtained with exposure times of between 2 and 4 hours, it was considered more practical to use the shorter dose periods. It was noticed that yield was also dependent on age of the whole blood. 48 hours after
FIGURE 4.8
Graph to show the relative $\alpha$ and $\beta$ signal intensities with time of exposure to $\gamma$-radiation.
taking the sample, the proportion of oxyhaemoglobin was greatly reduced, being replaced by methaemoglobin.

The second set of experiments were to study the extent of oxygenation of haemoglobin. Unfortunately, these too provided no further information as we were unsuccessful in passing limited concentrations of oxygen into deoxygenated samples. Due to the co-operativity effect, one molecule of haemoglobin was completely oxygenated before the next became partly oxygenated. It had been hoped to be able to study a large proportion of monoxyhaemoglobin, dioxy and so on.

Experiments were not performed on separated α and β chains as the study described was concerned with the nature of the complete haemoglobin molecule.

At this time, access to a data processor was unavailable and manual integration of the spectra was very difficult due to overlap of features. However, several very carefully measured studies were made on samples of whole blood prepared as above, Fig. 4.9, and on powdered human haemoglobin samples prepared in a similar way after the procedure described in Section 4.2 for removal of inactive material, Fig. 4.10. These confirmed several features of the process:

1. γ and δ are formed simultaneously,
2. a signal, γ", results from decay of both α and δ,
3. γ' is present only in slightly acidic samples. If indeed the identity of γ' is low spin Fe^{III} (as suggested earlier), then H_{2}O_{2} is indicated as the species lost during deoxygenation of oxyhaemoglobin in these samples. This would explain why the feature is observed at low pH since the second proton will be more readily available. The feature may not be observed in samples at higher pH if the change occurs at a temperature higher than the low spin to high spin Fe^{III} transition.

To summarise:
FIGURE 4.9
ESR spectra showing annealing events of a glassy sample of buffered whole blood.
<table>
<thead>
<tr>
<th>Feature</th>
<th>g-value (z)</th>
<th>g-value (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>2.223</td>
<td>2.126</td>
</tr>
<tr>
<td>β</td>
<td>2.251</td>
<td>2.148</td>
</tr>
<tr>
<td>β'</td>
<td>2.263</td>
<td>2.159</td>
</tr>
<tr>
<td>γ</td>
<td>2.312</td>
<td>2.183</td>
</tr>
<tr>
<td>δ</td>
<td>2.237</td>
<td>2.136</td>
</tr>
<tr>
<td>γ″</td>
<td>2.322</td>
<td>2.192</td>
</tr>
</tbody>
</table>

**FIGURE 4.10**
Table showing g-values for features observed in spectra of powdered haemoglobin.

The numbers 1, 2, 3, 4, 5 indicate the order in which the changes take place as observed by ESR. Steps 2 and 3 appear to occur simultaneously.

In discussion of these results we should like to combine two explanations described earlier (pages 109 and 110) based on the mechanism of the equation

![Chemical equation](image)

Low spin or high spin

Using the earlier notation a simple scheme may be drawn up:
where
\[
\begin{align*}
\beta_1, \alpha_1 &= \beta, \alpha \text{ centres already hydrogen bonded} \\
\beta_2, \alpha_2 &= \beta, \alpha \text{ centres with loose association to a hydrogen atom} \\
\beta, \alpha &= \beta, \alpha \text{ centres in conformation of } \alpha, \beta \text{ haems respectively, now hydrogen bonded} \\
\gamma, \gamma'' &= \text{high spin Fe}^{III} \text{ in } \beta \text{ and } \alpha \text{ haem units} \\
\gamma' &= \text{low spin Fe}^{III}
\end{align*}
\]

Again this scheme omits several pieces of information:

(i) no reference is made to \(0\) which was observed on several occasions,

(ii) only one feature is assigned to low spin Fe\(^{III}\), whereas two are attributed to high spin Fe\(^{III}\).

The scheme also assumes that, though \(\beta_1\) and \(\beta_2\) and \(\alpha_1\) and \(\alpha_2\) are different species, their structures are so similar that the features initially described as \(\beta\) and \(\alpha\) are not resolved.

Although this explanation is held together by rather tenuous connections, from the work discussed in the text and the results obtained to the end of this particular research programme, it is very difficult to link the observations in a feasible manner. The explanation does, however, accommodate all Fe-O\(_2\) units being in the relaxed configuration as expected. It suggests that freezing to 77 K results in one of each type of chain being in the proximity of a proton, the other actually associated to form a hydrogen bond, a situation which may arise from swinging of the Fe-O\(_2\) bond into the correct position close to the distal histidine.

In conclusion, there is still a great deal of work to be performed on oxyhaemoglobin, although the aid of a data processor will be essential in finally establishing the annealing sequence and identity of the species described. However, the work completed is encouraging and it is hoped that ESR will eventually become a useful technique by which to study mechanisms within the haemoglobin unit.
REFERENCES


AN ESR STUDY OF RADIATION EFFECTS IN METALLOPROTEINS

J. M. OSBORNE

ABSTRACT

Glassy methanolic and ethanolic solutions of molecular oxygen were exposed to X-rays at 4.2 K. A marked inhibition of the formation of e$_{g}$ centres together with formation of O$_2^-$ was observed. The results suggest that the environment of O$_2$ in methanol is such that initial hydrogen-bond transfer to O$_2^-$ is facile even at 4.2 K. In ethanol, O$_2^-$ is too far from hydrogen bonded OH groups for this mechanism to operate.

Dilute aqueous glassy solutions of the enzyme superoxide dismutase (SOD) were exposed to γ-radiation. Reduction of the native Cu(II) centre to Cu(I) was observed. A reaction was established between this reduced form and O$_2^-$. Potassium hexacyanoferrate II was shown to be a suitable chemical reducing agent for SOD after purification, whereas sodium dithionite was shown to modify the copper centre.

Study of the disulphide bridge of reduced SOD revealed RSSR$^-$ radicals and, in some cases, species 'X', depending on the nature of the reducing agent. The pH dependence of SOD and the effect of some anions has been illustrated and serves to confirm documented results.

Dilute aqueous glassy solutions of powdered haemoglobin and diluted samples of whole blood were exposed to γ-radiation. α and β subunit haem Fe-O$_2$ centres were identified at 77 K and several spectral changes recorded during warming. Tentative identification of these species has been made.