Free Rad. Res., Vol. 24, No. 4, pp. 269–280
Reprints available directly from the publisher
Photocopying permitted by license only

An EPR Investigation of Human Methaemoglobin Oxidation by Hydrogen Peroxide: Methods to Quantify all Paramagnetic Species Observed in the Reaction

DIMITRI A. SVISTUNENKO[¶], RAKESH P. PATEL AND MICHAEL T. WILSON*,

Department of Biological and Chemical Sciences, Central Campus, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK

Accepted by Dr B Kalyamaraman

(Received July 10th, 1995; in revised form, August 14th, 1995)

The method of Electron Paramagnetic Resonance (EPR) spectroscopy was used to study the reaction of human methaemoglobin (metHb) with hydrogen peroxide. The samples for EPR measurements were rapidly frozen in liquid nitrogen at different times after H_2O_2 was added at 3- and 10-fold molar excess to 100 μM metHb in 50 mM phosphate buffer, pH 7.4, 37°C. Precautions were taken to remove all catalase from the haemoglobin preparation and no molecular oxygen evolution was detected during the reaction. On addition of H_2O_2 the EPR signals (– 196°C) of both high spin and low spin metHb rapidly decreased and free radicals were formed. The low temperature (– 196°C) EPR spectrum of the free radicals formed in the reaction has been deconvoluted into two individual EPR signals, one being an anisotropic signal ($g_{||} = 2.035$ and $g_{\perp} = 2.0053$), and the other an isotropic singlet ($g = 2.0042$, $\Delta H = 20$ G). The former signal was assigned to peroxyl radicals. As the kinetic behaviour of both peroxyl ($ROO^{\bullet}$) and non-peroxyl ($P^{\bullet}$) free radicals were similar, we concluded that $ROO^{\bullet}$ radicals are not formed from $P^{\bullet}$ radicals by addition of O_2. The time courses for both radicals showed a steady state during the time required for H_2O_2 to decompose. Once all peroxide was consumed, the radical decayed with a first order rate constant of 1.42×10^{-3} s^{-1} (1:3 molar ratio). The level of the steady state was higher and its duration shorter at lower initial concentration of H_2O_2. The formation of the rhombic Fe(III) non-haem centres with $g = 4.35$ was found. Their yield was proportional to the H_2O_2 concentration used and the centres were ascribed to haem degradation products. The reaction was also monitored by EPR spectroscopy at room temperature. The kinetics of the free radicals measured in the reaction mixture at room temperature was similar to that observed when the fast freezing method and EPR measurement at –196°C were used.

Key words: methaemoglobin, ferryl haemoglobin, hydrogen peroxide, protein radical, peroxyl radical, EPR spectra deconvolution

Abbreviations: Hb, haemoglobin; Mb, myoglobin; H_2O_2, hydrogen peroxide; EPR, Electron Paramagnetic Resonance; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DPPH, 2,2′-diphenyl-1-picrylhydrazyl

INTRODUCTION

The reaction of methaemoglobin (metHb, i.e. ferric haemoglobin) with hydrogen peroxide (H_2O_2)

[¶]Visiting from: ChimBio Department, Institute of Chemical Physics, Russian Academy of Sciences, Kosygina st. 4, Moscow, 117977 Russia
* Corresponding author: Fax: + 44 206 873598, Phone: + 44 206 872538, E-mail: wilsmt@essex.ac.uk

has been studied since 1900.[1] In the 1950's it was shown by electron paramagnetic resonance (EPR) spectroscopy that free radicals are produced when H_2O_2 is added to either ferric haemoglobin or myoglobin.[2] Kelso King and Winfield further characterised this two electron oxidation process and showed that it proceeded with the formation of a ferryl haem iron, Fe(IV), detected by optical spectroscopy, and a globin-associated free radical, detected by EPR specroscopy.[3] It has been shown in a number of EPR studies that free radicals are formed in the reaction between H_2O_2 and bovine metHb,[2,3] horse metHb,[5] human metHb,[6,7] legHb,[8] sperm whale metMb[5,9] and horse metMb.[10,11]

A similar radical was found in nitrite mediated oxidation of oxyhaemoglobin,[12–15] however, the mechanism proposed suggested that the radicals *were formed from a step involving the direct* interaction of ferric haemoglobin and hydrogen peroxide, both being produced during the oxidation.[13]

There is conflicting evidence as to the nature of the free radical(s) obtained. By comparative analysis of amino acid residues, Kelso King *et al.*[5] came to the conclusion that the free radicals observed in a frozen solution were tyrosine based. The same statement was made by McArthur and Davies, who simulated an EPR spectrum of a tyrosine derived phenoxyl radical and showed its similarity to the experimentally derived spectrum of metHb and H_2O_2 at room temperature.[4] This interpretation is further supported by findings that show tyrosine residues in Mb are critical for forming haem-protein cross links on interaction with H_2O_2,[16] and also play an important role in the autoreduction process of ferryl.[17] The position of this radical has been postulated to be on Tyr 151 in metMb[16,18] and on Tyr α-42 in bovine metHb.[4] However, it was shown by site directed mutagenesis that a radical was still produced when all tyrosine residues were removed from sperm whale Mb,[19] thus suggesting the presence of a number of possible radical centres in the globin.

It is generally thought that peroxyl radicals are produced in these reactions. The g = 2.03 EPR signal, the parallel component of peroxyl radical EPR signals,[20] was seen by Kelso King and Winfield as early as in 1963,[3] although at that time this signal had not been interpreted as such. It was in 1989 that the g = 2.03 band in metMb treated with H_2O_2 was assigned to peroxyl radicals for the first time.[21] Recently it was confirmed with O^{17}-labelled molecular oxygen, as well as by measurement of all three components of the peroxyl radical g-tensor, that peroxyl radicals are indeed formed with metMb.[22] For the reaction between methaemoglobin and hydrogen peroxide, the radical formed has been trapped by DMPO and the adduct was interpreted as that of a peroxyl radical.[4]

There appear to be some characteristics of the reaction which are the same, independent of the specific haemprotein used. For example, the amount of globin free radicals formed is never in the stoichiometry 1:1 with the protein. The ferric haem is transformed rapidly and almost completely to the Fe(IV) state, but the levels of free radicals formed are reported by different authors to be between 4–16% of the protein.[3,5,10,23] Such a low relative yield of one of the two oxidising equivalents is thought to be related to the rapid decay of the radical(s). However, a more stable free radical has been reported for metMb and H_2O_2, although in very low concentrations.[23]

We have shown elsewhere that very low concentrations of catalase contaminating a metHb preparation, dramatically effects the kinetic behaviour of the free radical(s) obtained.[24] The aim of this present investigation was to study the radicals obtained in the reaction between catalase free human metHb and H_2O_2 by low temperature (–196°C) and room temperature EPR spectroscopy. The results suggest that at least two protein based radicals are produced, a peroxyl and a nonperoxyl radical. We give radiospectroscopic parameters of these two kinds of radicals and the methods of their quantitation in terms of absolute concentrations. Furthermore it appears that the peroxyl radical is not directly derived from the observed nonperoxyl radical.

MATERIALS AND METHODS

Human Hb was prepared according to Antonini and Brunori,[25] further purified from contaminating catalase according to Bonaventura *et al.*,[26] using a DEAE Sephadex column pre-equilibrated with 5 mM phosphate buffer, pH 6.7. The preparations of metHb have been checked for catalase contamination by making sure that no oxygen was evolved during the reaction with H_2O_2. The level of catalase was found to be less than 10^{-11} M. The concentration of metHb is expressed in this paper in terms of haem iron concentration. Experiments were conducted in 50 mM sodium phosphate buffer (pH 7.4) at 37°C or 20°C. The EPR measurements were made at both liquid nitrogen (−196°C) and at room temperatures using a Varian E109 spectrometer equipped with an Archimedes 440 computer. A Marconi Instruments 20 GHz Microwave Counter 2440 was used for microwave frequency measurements. Modulation frequency was 100 kHz, and microwave power and external magnetic field levels were taken from spectrometer settings.

Frozen EPR samples were made and measured as described elsewhere.[24] Room temperature measurements were conducted using a Bruker flat cuvette. Hydrogen peroxide (0.080 ml of 20 mM stock solution) was injected into the cuvette containing 0.229 mM solution of metHb (0.7 ml) to give the final desired concentrations. After injection of peroxide, the reacting mixture was allowed to flow out of the cuvette, such that only its flat part remained filled. EPR spectra were recorded consecutively, each scan taking 65 seconds. Sodium phosphate buffer (pH 7.4, 50 mM) was used to measure a base line, which was subtracted from all experimental spectra.

The g-factors of the signals in the 4000 G field width spectra (Figure 1) were calculated by using the $g = 6$ and $g = 2.00$ signals as references. The g-factors in the free radical area (Figure 2) were determined using a DPPH standard. In the liquid phase experiment (Figure 7), a fresh ascorbic acid solution was used as a standard: the reference parameters of the ascorbic acid EPR doublet were taken as $g = 2.00518$ and $a^{H}_{C4} = 1.76$ G.[27]

The concentrations of paramagnetic species were determined by double integration of the EPR spectra, the degree of saturation being taken into account when necessary. Second integrals of EPR signals were considered proportional to concentration, and the reference was taken to a known concentration of metHb in a frozen sample after double integration of its EPR spectrum measured over a 4000 G range. The measured double integrals were expressed in units of concentration after normalisation to the same scan and integration range, using the relation: second integral $\propto 1/(\text{range of integration})^2$.

To quantify high spin and low spin metHb, the EPR signals of which overlap, the following formula was used:

$$\frac{[\text{High spin metHb}]}{[\text{Total metHb}]} = \frac{1.35\,(A_{g=6}/A_{g=2.25})}{1.35\,(A_{g=6}/A_{g=2.25}) + 1} \quad [1]$$

where $A_{g=6}$ – the amplitude of the high spin $g = 6$ signal and $A_{g=2.25}$ – that of the signal of the low spin form $g = 2.25$ (see Figure 1, C). This formula was empirically obtained from precisely determined values of the second integral of the overall spectrum measured over 4000 G scan range and of the parameters $A_{g=6}$ and $A_{g=2.25}$, for two different metHb samples which contained low spin and high spin forms in different proportions (Appendix A).

RESULTS AND DISCUSSION

Characterisation of the EPR signals obtained at −196°C

Figure 1 shows the EPR spectra of methaemoglobin before and after addition of hydrogen peroxide. Before the addition (Figure 1, A), the spectrum comprises mainly two EPR signals with $g = 6$ and $g = 2.25$. In order to increase the signal-to-noise ratio, an EPR spectrum of a high concentration of metHb (1 mM) has been recorded

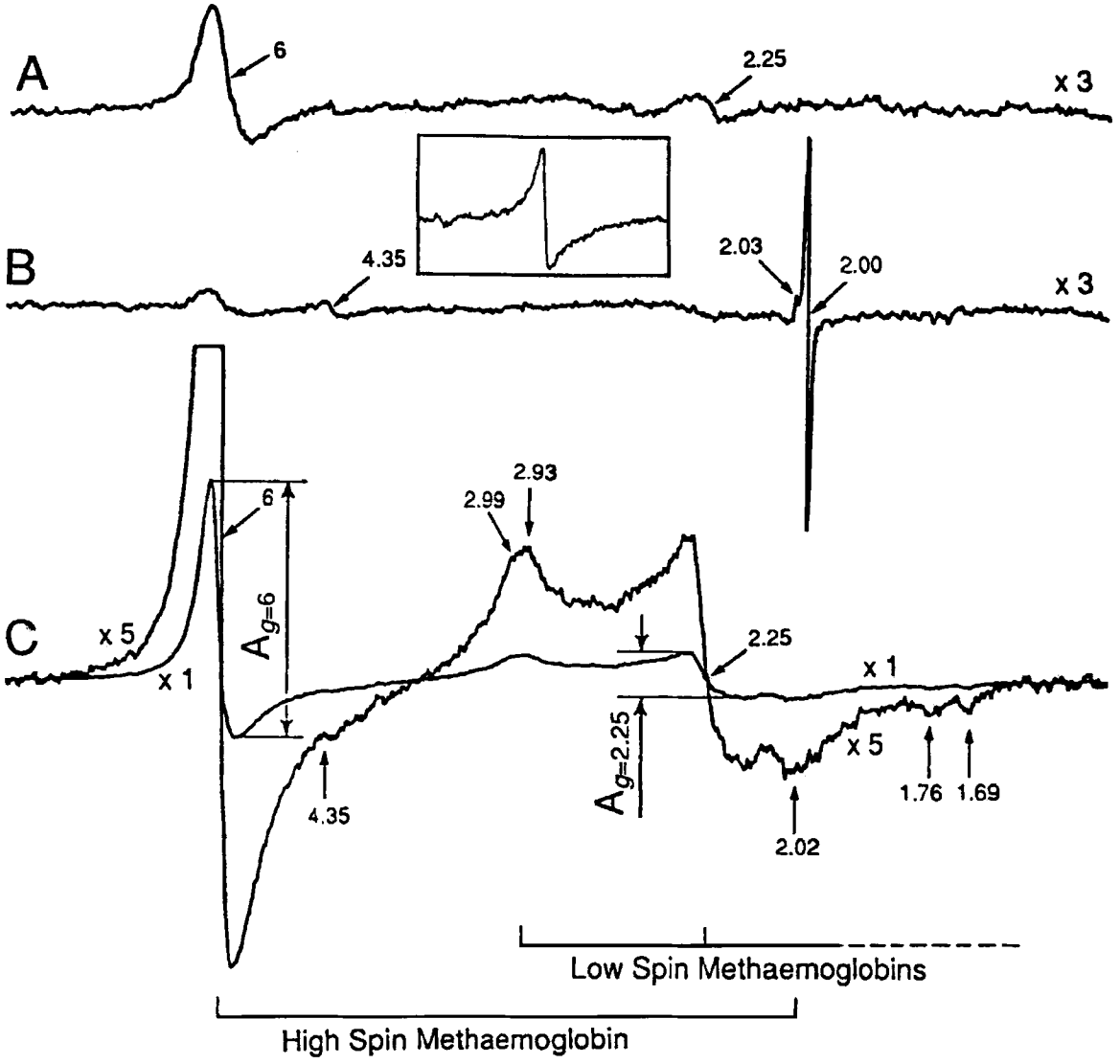

FIGURE 1 The EPR spectra of frozen metHb solutions. **A** – 100 μM metHb. **B** – 100 μM metHb + 1 mM H_2O_2 frozen 15 s after addition of hydrogen peroxide. **C** – 1 mM metHb; the way of measuring of the amplitudes of high spin ($A_g = 6$) and low spin ($A_g = 2.25$) EPR signals is shown. Experimental conditions: microwave frequency $\nu = 9.216$ GHz; microwave power $P_{MW} = 30$ mW; central field $H_0 = 2331$ G; sweep width $\Delta H = 4000$ G; sweep time ST = 180 s; time constant $\tau = 0.128$ s; amplitude of modulation M = 8 G; gain $GN = 2 \times 10^3$; number of scans NS = 4; temperature of measurement T = −196°C. The **g**-factors are indicated with arrows, and relative amplification of spectra – with the "**x**" signs. The **g**-factors 2.99, 2.93, 2.02, 1.76 and 1.69 indicate the positions of specific features of the spectrum and are given to aid the reader. *Inset* – the **g** = 4.35 signal recordered as a differential spectrum: a spectrum of metHb (100 μM) multiplied by a variable factor was subtracted from a spectrum of metHb (100 μM) + H_2O_2 (1 mM) taken 50 min after mixing, i.e. when the **g** = 4.35 signal had reached its maximum value and no longer changed. The factor was varied to minimise input of the **g** = 6 signal in the resultant spectrum. Experimental conditions for the spectrum in the *inset*: $\nu = 9.216$ GHz; $P_{MW} = 50$ mW; $H_0 = 1509$ G; $\Delta H = 1000$ G; ST = 65 s; $\tau = 0.128$ s; M = 8 G; $GN = 10^4$; NS = 32; T = −196°C.

(Figure 1, **C**). This spectrum clearly shows the EPR signals of two main forms of metHb: with high spin haem Fe(III) ions characterised by axial symmetry and two **g**-factors ($g_\perp = 6$ and $g_\parallel = 2$), and with low spin haem Fe(III) ions which has three direction anisotropy of **g**-factor – with two **g**-factors being greater and one being less than 2.00. There is an obvious mixture of different forms of

the low spin haemoglobin (haemichromes) with the g = 2.25 band being a characteristic g_2-factor of several forms of haemichromes.[28] We found by applying formula [1] that average amount of the high spin form in the control samples of metHb, i.e. before addition of H_2O_2, is ~ 87–91% of total metHb. Methaemoglobin, in absence of H_2O_2, did not exhibit any free radical EPR signal.

A few seconds after addition of H_2O_2, a free radical EPR signal at g = 2.00 with a shoulder at g = 2.03 appears, while the EPR signals of both high spin and low spin metHb decrease. An iron centre with an EPR absorption at g = 4.35, characteristic of non-haem rhombic Fe(III) centres, appears (Figure 1, B). The release of haem iron, presumably via haem degradation, opens up the possibility of Fenton reactions. The appearance of the g = 4.35 signal was H_2O_2 dependent and can be used as a measure of protein degradation. It should be noted that the rhombic Fe(III) centres at a very low concentration can also be detected in the control samples (Figure 1, A and C) illustrating a spontaneous haem degradation process. For the concentration of metHb used in most our experiments, 100 μM, the concentration of the product of spontaneous haem degradation was estimated to be 0–3 μM. Irrespective of the precise concentration of the rhombic Fe(III) centres in the control sample, it increased significantly on addition of H_2O_2. This effect was used to record the g = 4.35 signal at a higher signal-to-noise ratio and on a flat baseline (Figure 1, *Inset*), allowing signal integration and hence quantitation of the non-haem rhombic Fe(III) centres.

Free radical region of the EPR spectrum – decomposition into two individual signals

The EPR spectrum (– 196°C) of the free radicals which appear on addition of H_2O_2 to metHb is shown in more detail in Figure 2, A. The observed signal is seen to consist of at least two distinct radical species. This is supported by the fact that the intensity of the low field (g = 2.03) signal, relative to the main (g = 2.0051) signal, depended on the absolute concentrations of metHb and H_2O_2 and on the time of the reaction (Figure 2, B). The different proportions of the two EPR signals in spectra A and B were used to separate them – spectrum A was multiplied by a coefficient (0.55), chosen so that when spectrum B was subtracted from this product, the signal with a peak at g = 2.03 disappeared in the differential spectrum C. Spectrum C is a nearly isotropic singlet, with g = 2.0041 and a line width of 20 G (Figure 2, C and D). We assign this singlet to a protein radical ($P^\bullet$).

The g = 2.03 signal is reminiscent of the g_{II} component of peroxyl radicals. These radicals are well documented for such systems.[4,21,22,23] In order to test this conjecture and to obtain a pure line shape of the signal with this low field component, signal D was combined in different proportions, by varying a multiplying factor k, with spectrum E (Figure 2, F). With an appropriately chosen value of k (k = – 0.3 ± 0.1) a line shape is obtained (spectrum 1, Figure 2, F) which we identified as that of peroxyl radicals $ROO^\bullet$ on the basis of its g-factors (g_{II} = 2.035 and g = 2.0053) and also from the ratio of its component intensities (a/b_1 = 0.223).[19,20,23,29]

In Figure 2, F, we show five spectra constructed by varying the value of k. These spectra are, therefore, mixtures comprising a constant peroxyl radical $ROO^\bullet$ contribution with varying contributions of the radical $P^\bullet$. These mixtures of known composition of $ROO^\bullet$ and $P^\bullet$ radical signals can be used to construct a calibration curve (Figure 3) relating the composition to the relative amplitude parameter a/b, where a and b are defined in Figure 2, F. This was achieved by expressing the contribution of each signal as a percentage of the total, e.g. % peroxyl = 100 % × I_{ROO}/I_T, where I_{ROO} is the second integral of the peroxyl component (constant here) and I_T is the second integral of the total spectrum. The percentage of the peroxyl radical signal was approximated by a polynomial function, thus allowing the share of each signal to be calculated analytically from the measured parameter a/b (Figure 3). However to calculate the relative concentrations of the two

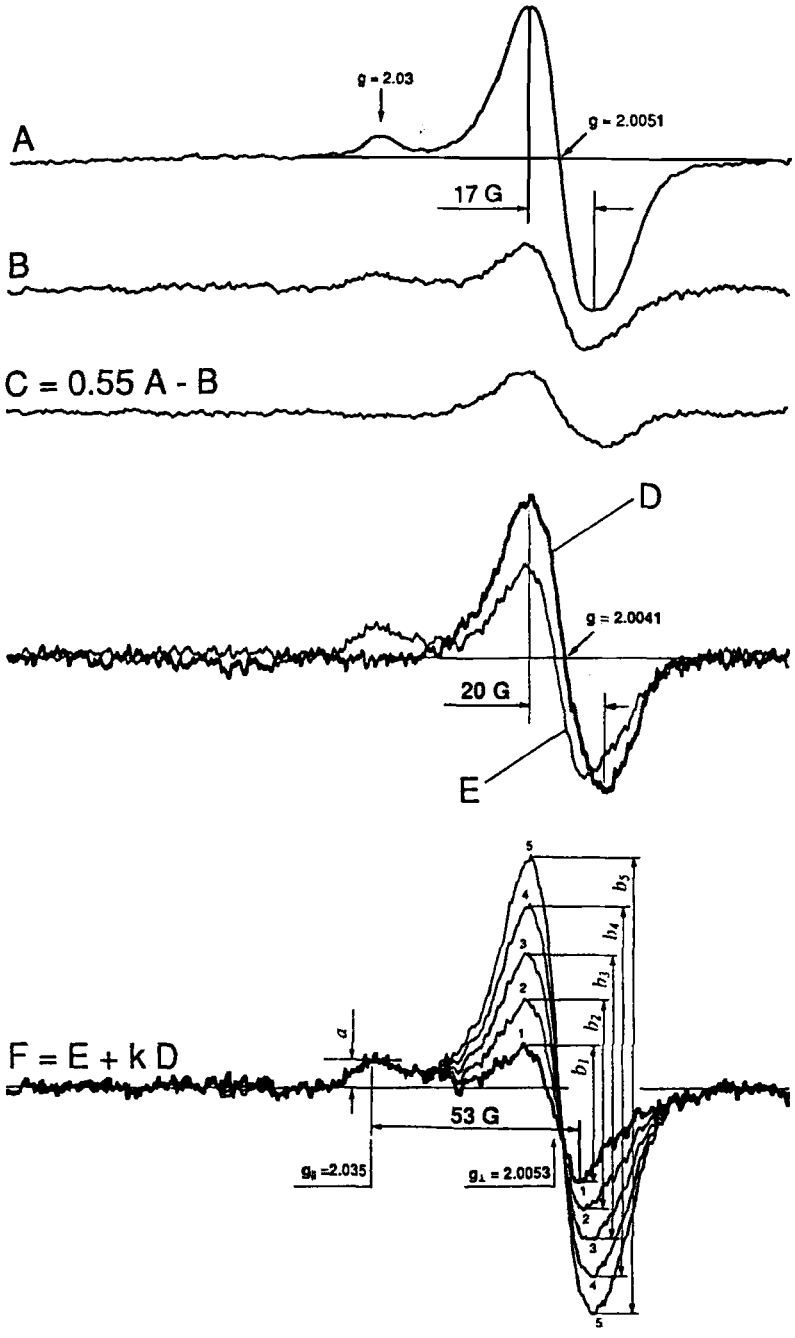

FIGURE 2 Analysis of the free radical region of the EPR spectrum obtained after addition of H_2O_2 to metHb. **A** – metHb (200 μM) + H_2O_2 (2 mM) frozen 1.1 min. after initiation of the reaction; **B** – metHb (100 μM) + H_2O_2 (1 mM) frozen 8 min. after initiation of the reaction; **C** = 0.55 × **A** – **B**; **D** – spectrum **C** enlarged by a factor of 3.9; **E** – spectrum **B** enlarged by a factor of 2; **F** – five sums of spectra **E** and **D**, **E** + **k** × D, where **k** varies: **k** = –0.3 (spectrum 1), **k** = 0 (2), **k** = 0.3 (3), **k** = 0.6 (4), **k** = 0.9 (5); every sum spectrum is characterised by two parameters: *a* and *b*. Experimental conditions: ν = 9.216 GHz; P_{MW} = 30 mW; H_0 = 3230 G; ΔH = 200 G; ST = 65 s; τ = 0.128 s; M = 3.2 G; GN = 5×10^3 (**A**) and GN = 10^4 (**B**); NS = 2; T = –196°C.

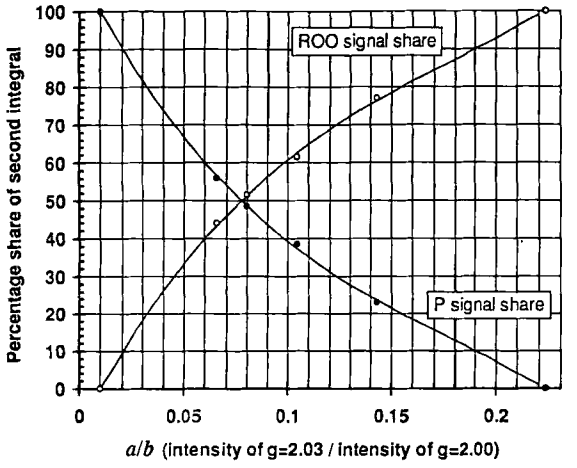

FIGURE 3 The relationship between the relative amplitude parameter *a/b* and the percentage share of the ROO$^{\bullet}$ and P$^{\bullet}$ radical signals in the second integral of the EPR spectrum. Continuous lines are polynomial approximations:
$f_{ROO}(a/b) = -10.18 + 1073(a/b) - 4467\,(a/b)^2 + 8388\,(a/b)^3$,
$f_P(a/b) = 100\% - f_{ROO}(a/b)$.

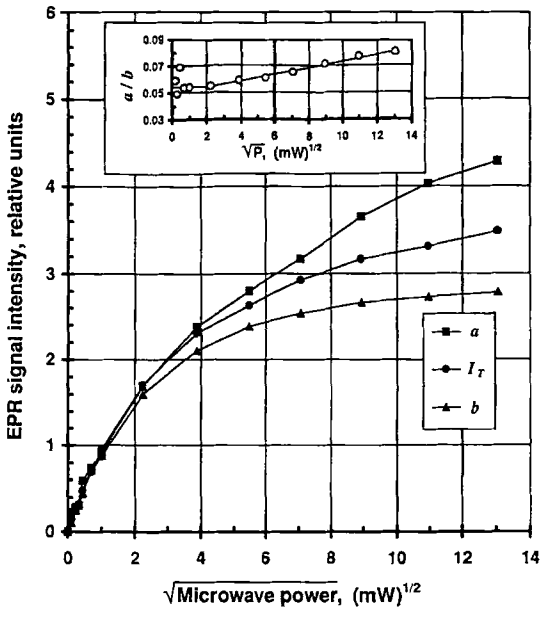

FIGURE 4 The dependencies of the free radical EPR signals on square root of microwave power (saturation curves): I_T – total spectrum second integral; *a* – height of the g = 2.035 band; *b* – amplitude of the central feature at g = 2.00 (*a* and *b* are defined as shown in Figure 2). The three curves are normalised to an equal initial slope. *Inset* – the increase of the *a/b* parameter with increasing microwave power. The sample for EPR measurements was frozen 45 s after addition of H_2O_2 (100 μM) to metHb (100 μM). Experimental conditions: ν = 9.216 GHz; H_0 = 3269 G; ΔH = 200 G; ST = 65 s; τ = 0.128 s; M = 4 G; GN = 1.25×10^4 (0–1 mW), GN = 6.3×10^3 (1–50 mW) and GN = 3.2×10^3 (50–170 mW); NS = 4; T = −196°C.

kinds of paramagnetic species on the basis of an experimental EPR spectrum, we have to know not only the relative values of the second integrals, but also the saturation curves for both signals, thus ensuring that saturation does not effect the estimate.

Saturation characteristics of the ROO$^{\bullet}$ and P$^{\bullet}$ radicals

The saturation characteristics of the EPR lines at g = 2.035 and g = 2.00 are different. Figure 4 shows three saturation curves obtained from a sample made by mixing 100 μM metHb with 100 μM H_2O_2. The second integral of the total spectrum I_T and the parameters *a* and *b* (defined in Figure 2, F) were measured at different microwave powers. These three curves are depicted after normalisation to equal initial slope. The fact that *a/b* changes with increasing microwave power (Figure 4, *Inset*) confirms that there is more than one free radical species within the sample.

Every point on the saturation curve I_T (Figure 4) is characterised by a specific value of the parameter *a/b*. Thus, for every point, the shares of second integral attributed to ROO$^{\bullet}$ and P$^{\bullet}$ signals can be determined using the calibration graph (Figure 3). Curve I_T (Figure 4) was therefore decomposed into the true saturation curves of ROO$^{\bullet}$ and P$^{\bullet}$ EPR signals (Figure 5). For comparison the initial slopes have been normalised. The saturation coefficients were calculated for the ROO$^{\bullet}$ and P$^{\bullet}$ signals at 30 mW, the microwave power used for all measurements. At this microwave power good signal-to-noise ratios were achieved, allowing easy measurement of the parameter *a/b*. For quantitation of the ROO$^{\bullet}$ and P$^{\bullet}$ radicals in concentration units, the relative proportion of the second integrals under non-saturating conditions was required. This was achieved as described in Appendix B.

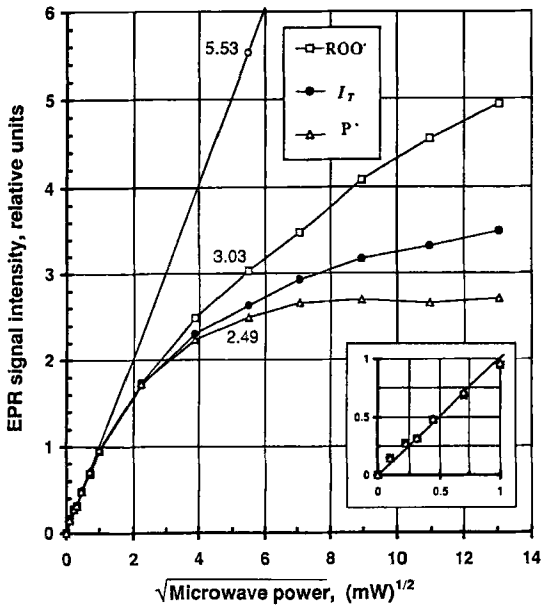

FIGURE 5 Saturation curves for ROO$^\bullet$ and P$^\bullet$ EPR signals, obtained with the use of the calibration graph (Figure 3) from the data on saturation of total free radical spectrum I_T measured by double integration and from the dependencies of a and b on microwave power (Figure 4). The straight line corresponds to an extrapolation of the linear part of the dependencies at low microwave power shown in more detail in the *Inset*. The saturation coefficients for the ROO$^\bullet$ and P$^\bullet$ signals when a spectrum is measured at 30 mW (5.48 mW$^{1/2}$) are: $S_{ROO} = 5.53/3.03 = 1.825$ and $S_P = 5.53/2.49 = 2.221$. Experimental conditions as stated in Figure 4.

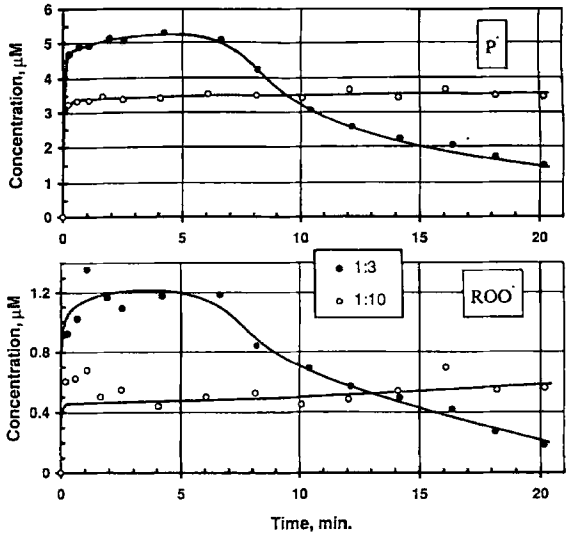

FIGURE 6 The time dependencies of the P$^\bullet$ and ROO$^\bullet$ radicals measured for 1:3 and 1:10 initial molar ratios of metHb:H_2O_2. The initial concentration of metHb was 100 μM. The reaction was conducted at 37°C, and the EPR measurements were performed at −196°C after fast freezing of the samples in liquid nitrogen. Each data point is the average of two EPR measurements of the duplicated samples.

Kinetics of the ROO$^\bullet$ and P$^\bullet$ radicals

Figure 6 shows the kinetic behaviour of peroxyl, ROO$^\bullet$, and non-peroxyl, P$^\bullet$, radicals. At either 1:3 or 1:10 molar ratios (metHb:H_2O_2) the progress curves for both radicals are the same. It can be calculated from Figure 6 that the peroxyl radicals constitute 19% of the total radical concentration when the Hb:H_2O_2 ratio is 1:3 and 13% when the ratio is 1:10 (with [metHb] = 100 μM).

The time courses for both radicals show that there is an apparent steady state during which the radical concentration remains constant. This steady state is shorter at lower H_2O_2 concentration. On exhaustion of H_2O_2 both radicals begin to decay (Figure 6, at 1:3 molar ratios of Hb:H_2O_2), suggesting that the apparent steady state represents a balance between radical production and decay. We have shown that this behaviour only occurs when catalase free haemoglobin samples are used.[24] A single exponential approximation for the decay of both P$^\bullet$ and ROO$^\bullet$ radicals after the steady state had ended (see Figure 6 for the 1:3 condition) gave the rate constant 1.42×10^{-3} s^{-1}. At a fixed protein concentration the rate constant for this decay process was independent of the initial H_2O_2 concentration in the range of peroxide excess 1:1–1:3 (at higher initial excess of H_2O_2 the rate constant for the decay of the radicals decreased, approximately 2 fold at the 1:10 ratio). It is believed that this decay, which occurs in the absence of H_2O_2, is an autoreduction process involving modification of amino acids and hence the haemprotein.[3] The rate constant of the radical decay did not change significantly (1.46×10^{-3} s^{-1}) when the metHb concentration was increased two-fold, indicating that the decay is a first order reaction

with respect to metHb, i.e. the autoreduction occurs via an intramolecular process. Haem-protein cross linking, which is seen with myoglobin,[30] or formation of substrate-haemoglobin cross links,[31] could be result of autoreduction reactions.

The synchronous time courses for $P^\bullet$ and $ROO^\bullet$ indicate that the latter is not derived from the former. There are at least two possible ways to explain the formation of the radicals:

a) On addition of H_2O_2 two different radicals $R^\bullet$ and $P^\bullet$ are produced. One of these reacts with oxygen yielding $ROO^\bullet$ and the other ($P^\bullet$) does not.
b) A common radical precursor ($R^\bullet$) is produced which gives rise to both $ROO^\bullet$ and $P^\bullet$. A mechanism by which this may occur, would involve a competition between oxygen addition giving rise to $ROO^\bullet$ and a transformation of $R^\bullet$ giving rise to the oxygen insensitive radical $P^\bullet$.

The approach to the steady state is rapid (within 15 s, Figure 6) and much faster than the autoreduction rate. Thus in order to sustain a steady state at a low concentration of radicals, as seen experimentally, we must conclude that, during the steady state period, there exists an H_2O_2 concentration dependent process which depletes radicals. This process must be much faster, at least in an excess of H_2O_2 over metHb, than the first order H_2O_2 independent autoreduction process. An increase in H_2O_2 will therefore increase both the rates of formation and the rate of decay of the radicals. Hence the balance between these opposing rates will determine the level of free radicals seen. This conclusion is strongly supported by the unexpected result that the steady state concentration of radicals decrease with increasing initial H_2O_2 concentration (Figure 6). Both the H_2O_2 concentration dependent rates of production and decay of the radicals are such that the decay process is favoured at high H_2O_2 concentration leading to lower radical concentration, yet longer steady state during which H_2O_2 is consumed. At low H_2O_2 concentration the decay process is less favoured and allows a greater build-up of radicals but can sustain only a short steady state.

A mechanism in which metHb is continuously regenerated through a cycle of transformations, is currently being studied with the use of computer simulation and will be reported elsewhere.

Room temperature EPR measurements

The steady state concentration of radicals was also observed by monitoring the reaction continuously by EPR spectroscopy at room temperature (Figure 7). The EPR signal acquired from the liquid phase sample (Figure 7, *Inset*) is in agreement with that reported earlier.[4,6,14] However, here, for the first time this signal is measured at its steady state concentration, and not using a continuous flow or fast scan technique. In spite of using a relatively low modulation amplitude (2 G) and a low time constant (0.128 s), which is appropriate to resolve ~2 G hyperfine structure at the scan rate of

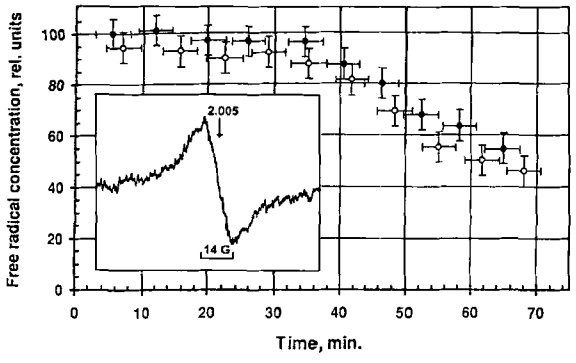

FIGURE 7 Time courses for free radicals produced in the reaction of metHb (205 μM) with H_2O_2 (1:10 initial excess), as measured by room temperature EPR spectroscopy. Each data point corresponds to a four scan EPR spectrum measured in consecutive order. X axis error bars correspond to the time of acquisition, Y axis error bars are defined by the noise level in the spectra. Open and closed symbols are two similar series. *Inset* – the sum of the five EPR spectra of the 'closed' series in the steady state part of the time course, i.e. a 20 scan spectrum accumulated during initial 36 min of the reaction. The g-factor and peak-to-trough width of the free radical EPR signal are indicated. Experimental conditions: $\nu = 9.454$ GHz; $P_{MW} = 130$ mW; $H_0 = 3354.3$ G; $\Delta H = 100$ G; ST = 65 s; $\tau = 0.128$ s; M = 2 G; $\nu_M = 100$ kHz; GN = 10^4; NS = 4; room temperature.

100 G/65 s = 1.54 G/s, no hyperfine structure was detected. In contrast to metMb which yields a radical with a resolved hyperfine structure in the liquid phase EPR spectrum,[11,18,32] metHb does not exhibit such a resolved spectrum under similar conditions. The reason for this may be in the fact that Hb molecule is approximately 4 times bigger than that of Mb and hence tumbles more slowly. Alternatively, the free radical sites in the α- and β-subunits of Hb may have slightly different EPR spectra which overlap blurring the hyperfine structure.

Acknowledgements

We thank BBSRC of the U.K. for a CASE award to R. Patel. The work of D. Svistunenko was partially supported by the Royal Society Fellowship Award and by the Wellcome Trust Travelling Research Fellowship.

References

1. R. Kobert (1900) *Arch. Gen. Physiol.* **82,** 603. Cited by: J.B. Fox, Jr., R.A. Nicholas, S.A. Ackerman and C.E. Swift (1974) A Multiple wavelength analysis of the reaction between hydrogen peroxide and metmyoglobin. *Biochemistry,* **13,** 5178–5186.
2. J.F. Gibson and D.J.E. Ingram (1956) Location of free electrons in porphin ring complexes. *Nature (London),* **178,** No. 4538, 871–872.
3. N. Kelso King and M.E. Winfield (1963) The mechanism of metmyoglobin oxidation. *Journal of Biological Chemistry,* **238,** 1520–1528.
4. K.M. McArthur and M.J. Davies (1993) Detection and reactions of the globin radical in haemoglobin. *Biochimica et Biophysica Acta,* **1202,** 173–181.
5. N. Kelso King, F.D. Looney and M.E. Winfield (1967) Anino acid free radicals in oxidised metmyoglobin *Biochimica et Biophysica Acta,* **133,** 65–82.
6. T. Shiga and K. Imaizumi (1975) Electron spin resonance study on peroxidase- and oxidase-reactions of horse radish peroxidase and methemoglobin. *Archives of Biochemistry and Biophysics,* **167,** 469–479.
7. A. Tomoda, K. Sugimoto, M. Suhara, M. Takeshita and Y. Yoneyama (1978) Haemichrome formation from haemoglobin subunits by hydrogen peroxide. *Biochemical Journal.,* **171,** 329–335.
8. M.J. Davies and A. Puppo (1993) Identification of the site of the globin-derived radical in leghaemoglobins. *Biochimica et Biophysica Acta,* **1202,** 182–188.
9. J.F. Gibson, D.J.E. Ingram and P. Nicholls (1958) Free radical produced in the reaction of metmyoglobin with hydrogen peroxide. *Nature,* **181,** 1398–1399.
10. T. Yonetani and H. Schleyer (1967) Studies on cytochrome *c* peroxidase. IX The reaction of ferrimyoglobin with hydroperoxides and a comparison of peroxide-induced compounds of ferrimyoglobin and cytochrome *c* peroxidase. *Journal of Biological Chemistry,* **242,** 1974–1979.
11. M.J. Davies (1991) Identification of a globin free radical in equine myoglobin treated with peroxides. *Biochimica et Biophysica Acta,* **1077,** 86–90.
12. F. Jung, G. Lassmann and B. Ebert (1981) Detection of paramagnetic species in the autocatalytic reaction between hemoglobin and nitrite. *Studia Biophysica,* **85,** 139–140.
13. H. Kosaka, K. Imaizumi and I. Tyuma (1982) Mechanism of autocatalytic oxidation of oxyhaemoglobin by nitrite. An intermediate detected by electron spin resonance. *Biochimica et Biophysica Acta,* **702,** 237–241.
14. B. Ebert, G. Lassmann and F. Jung (1983) Analysis of paramagnetic intermediates at the reaction of hemoglobin with nitrite using EPR. *Biomedica Biochimica Acta,* **42,** No. 11/12, S154–S158.
15. C. Spagnuolo, P. Rinelli, M. Coletta, E. Chiancone and F. Ascoli (1987) Oxidation reaction of human oxyhemoglobin with nitrite: a reexamination. *Biochimica et Biophysica Acta,* **911,** 59–65.
16. D. Tew and P.R. Ortiz de Montellano (1988). The myoglobin protein radical. Coupling of Tyr-103 to Tyr-151 in the H_2O_2-mediated cross-linking of sperm whale myoglobin. *The Journal of Biological Chemistry,* **263,** 17880–17886.
17. M. Uyeda and J. Peisach (1981) Ultraviolet difference spectroscopy of myoglobin: assignment of pK values of tyrosyl phenolic groups and the stability of the ferryl derivatives. *Biochemistry,* **20,** 2038–2035.
18. H. Miki, K. Harada, I. Yamazaki, M. Tamura and H. Watanabe (1989) Electron spin resonance spectrum of Tyr-151 free radical formed in reaction of sperm whale metmyoglobin with ethyl hydroperoxide and potassium irridate. *Archives of Biochemistry and Biophysics,* **275,** 354–362.
19. A. Wilks and P.R. Ortiz de Montellano (1992) Intramolecular translocation of the protein radical formed in the reaction of recombinant sperm whale myoglobin with H_2O_2. *The Journal of Biological Chemistry,* **267,** 8827–8833.
20. M.D. Sevilla, M. Champagne and D. Becker (1989) Study of lipid peroxyl radicals in urea clathrate crystals. Oxygen-17 couplings and rotational averaging. *The Journal of Physical Chemistry,* **93,** 2653–2658.
21. R.L. Petersen, M.C.R. Symons and F.A. Taiwo (1989) Application of radiation and electron spin resonance spectroscopy to the study of ferryl myoglobin. *Journal of the Chemical Society, Faraday Transactions 1,* **85,** 2435–2443.
22. D.J. Kelman, J.A. DeGray and R.P. Mason (1994) Reaction of myoglobin with hydrogen peroxide forms a peroxyl radical which oxidizes substrates. *Journal of Biological Chemistry,* **269,** 7458–7463.
23. C.E. Cooper, E.S.R. Green, C.A. Rice-Evans, M.J. Davies and J.M. Wrigglesworth (1994). A hydrogen-donating monohydroxamate scavenges ferryl myoglobin radicals. *Free Radical Research Communications,* **20,** 219–227.
24. R.P. Patel, D.A. Svistunenko, V.M. Darley-Usmar, M.C.R. Symons and M.T. Wilson (1996) Redox cycling of human metHb by H_2O_2 yields persistent ferryl iron and protein based radicals: effects of catalase. Submitted to *Free Radical Research,* **00,** 000–000.
25. E. Antonini and M. Brunori (1971) Haemoglobin and myoglobin in their reactions with ligands. North Holland publishing company. Amsterdam.
26. J. Bonaventura, W.A. Schroeder and Suen Fang (1972)

Human erythrocyte catalase: an improved method of isolation and a reevaluation of reported properties. *Archives of Biochemistry and Biophysics*, **150**, 606–617.
27. G.P. Laröff, R.W. Fessenden and R.H. Schuller (1972) The electron spin resonance spectra of radical intermediates in the oxidation of ascorbic acid and related substance. *Journal of American Chemical Society*, **94**, No. 26, 9062–9073.
28. W.E. Blumberg and J. Peisach (1971) A unified theory for low spin forms of all ferric heme proteins as studied by EPR. In: *Probes of structure and function of macromolecules and membranes*. Vol. 2. Eds.: B. Chance, T. Yonetani and A.S. Mildvan. Acad. Press, N.Y., pp 215–229.
29. Z. Joffe and B. Ranby (1973) ESR studies of UV-induced degradation of polyolefins. In: ESR application to polymer research. Eds.: P.-O. Kinell, B. Ranby and V. Runnstrom-Reio, Almqvist and Wilsell, Stockholm, p. 180.
30. C.E. Catalano, Y.S. Choe, P.R. Ortiz de Montellano (1989) Reactions of the protein radical in peroxide-treated myoglobin – formation of a heme-protein cross-link *Journal of Biological Chemistry*, **264**, 10534–10541.
31. P.P. Kelder, M.J.E. Fischer, N.J. deMol, L.H.M. Janssen (1991) Oxidation of chlorpromazine by methemoglobin in the presence of hydrogen peroxide. Formation of chlorpromazine radical cation and its covalent binding to methemoglobin. *Archives of Biochemistry and Biophysics*, **284**, 313–319.
32. K. Harada and I. Yamazaki (1987) Electron spin resonance spectra of free radicals formed in the reaction of metmyoglobins with ethylhydroperoxides. *The Journal of Biochemistry*, **101**, 283–286.

APPENDIX

A. Derivation of formula [1] – the relationship between relative concentration of the high spin form of metHb and the parameter $A_{g=6}/A_{g=2.25}$

The high spin metHb EPR signal has not been isolated from the low spin one. Instead, double integrals of the EPR spectra of two different metHb samples, which contained low and high spin forms in different proportions, have been accurately measured.

Two samples, 0.5 mM and 1.2 mM metHb, were frozen in liquid nitrogen. For each sample four identical EPR spectra were measured (scan width and integration range 4000 G). Baseline (the EPR spectrum of a blank sample) was measured at the same conditions four times as well (see Methods). So, 4 × 4 = 16 differential spectra for integration were obtained for each sample. After averaging, the mean values and their standard deviations were calculated for second integrals I of the EPR spectra of each sample:

$I_{1.2\ mM} = 1658 \pm 58$ (relative units of double integral)
$I_{0.5\ mM} = 662 \pm 46$ (relative units of double integral)

The signal amplitudes of g = 6 and g = 2.25 were measured for both samples as shown in Figure 1, C. After averaging over 16 spectra the mean values were calculated:

$A_{g=6;\ 1.2\ mM} = 2343$ $\quad A_{g=2.25;\ 1.2\ mM} = 186$
$A_{g=6;\ 0.5\ mM} = 909$ $\quad A_{g=2.25;\ 0.5\ mM} = 110$

(in relative units of signal amplitude).

The values $I_{1.2\ mM}$ and $I_{0.5\ mM}$ are the sums of second integrals of high and low spin metHb EPR signals. Let H be the second integral of pure line shape of the high spin signal when $A_{g=6} = 1$, and let L be the second integral of pure line shape of the low spin signal when $A_{g=2.25} = 1$.

Then,

$$\left.\begin{aligned} I_{1.2\ mM} &= A_{g=6;\ 1.2\ mM} \times H + A_{g=2.25;\ 1.2\ mM} \times L \\ I_{0.5\ mM} &= A_{g=6;\ 0.5\ mM} \times H + A_{g=2.25;\ 0.5\ mM} \times L \end{aligned}\right\}$$

or,

$$\left.\begin{aligned} 1658 &= 2343\,H + 186\,L \\ 662 &= 909\,H + 110\,L \end{aligned}\right\}$$

From this system of equations H and L were found: $H = 0.6682$; $L = 0.4966$ (and thus $H/L = 1.35$). These values were used to derive a formula which could be used in determination of the partial concentration of the high spin form of metHb by measuring the parameter $A_{g=6}/A_{g=2.25}$, without double integration of pure line shapes of the high and low spin EPR signals separately:

$$\frac{[\text{High spin metHb}]}{[\text{Total metHb}]} = \frac{A_{g=6}\,H}{(A_{g=6}\,H + A_{g=2.25}\,L)} \times$$

$$\frac{(1/A_{g=2.25})}{(1/A_{g=2.25})} = \frac{(A_{g=6}/A_{g=2.25})\,H}{(A_{g=6}/A_{g=2.25})\,H + L} =$$

$$\frac{(A_{g=6}/A_{g=2.25})\,(H/L)}{(A_{g=6}/A_{g=2.25})\,(H/L) + 1} = \frac{1.35\,(A_{g=6}/A_{g=2.25})}{1.35\,(A_{g=6}/A_{g=2.25}) + 1}$$

where $A_{g=6}$ – the amplitude of the high spin g = 6 signal and $A_{g=2.25}$ – that of the signal of the low spin form g = 2.25 (Figure 1, C).

B. Derivation of algorithm for practical determination of the concentration of ROO$^{\bullet}$ and P$^{\bullet}$ radicals

Quantitation of free radicals was made by measuring the *a/b* parameter (see Figure 2). Such measurements were made at microwave power levels of 30 mW, which gave good signal-to-noise ratios. However, under these conditions the radical signals were saturated to differing degrees. There was no saturation for either radical upto 0.5 mW, but at this power the signal-to-noise ratio was poor, thus not allowing accurate measurement of the parameter *a/b*. By use of the saturation coefficients for the two different radicals (Figure 5), the second integrals of each component, determined at 30 mW, were converted to the expected corresponding values at 0.5 mW. This was then related to the second integral of the experimental spectrum measured at 0.5 mW. The procedure used is outlined below.

a) A spectrum of the reaction mixture was measured at 0.5 mW. No saturation occurs at this power, and hence the second integral gives the total amount of free radicals in the sample. This is the sum of the second integrals:

$$I_{T;\,0.5\,mW} = I_{ROO;\,0.5\,mW} + I_{P;\,0.5\,mW}$$

b) The spectrum of the same sample was taken at 30 mW (when the radical signals saturate to differing degrees), and the parameter *a/b* was determined. From this the percentage share of the second integral, attributable to ROO$^{\bullet}$ (α) and P$^{\bullet}$ (β), was calculated using the empirical formula from Figure 3 legend:

$$\alpha = f(a/b)$$
$$\beta = 100\% - f(a/b).$$

c) The second integrals of signals ROO$^{\bullet}$ and P$^{\bullet}$ at 30 mW can also be derived from $I_{ROO;\,0.5\,mW}$ and $I_{P;0.5mW}$ using the saturation coefficients S_{ROO} and S_P (Figure 3):

$$I_{ROO;\,30\,mW} = I_{ROO;\,0.5\,mW}\,(30/0.5)^{1/2}/S_{ROO} = 7.746\,I_{ROO;\,0.5\,mW}/1.825 = 4.244\,I_{ROO;\,0.5\,mW}$$
$$I_{P;\,30\,mW} = I_{P;\,0.5\,mW}\,(30/0.5)^{1/2}/S_P = 7.746\,I_{P;\,0.5\,mW}/2.221 = 3.488\,I_{P;\,0.5\,mW}$$

d) Two equations can now be formulated:

$$\frac{4.244 I_{ROO;\,0.5\,mW}}{4.244\,I_{ROO;\,0.5\,mW} + 3.488 I_{P;\,0.5\,mW}} = \frac{\alpha}{100\%}$$

$$\frac{3.448 I_{P;\,0.5\,mW}}{4.244\,I_{ROO;\,0.5\,mW} + 3.488 I_{P;\,0.5\,mW}} = \frac{\beta}{100\%}$$

These two equations are not independent ($\alpha + \beta \equiv 100\%$). So, either of them together with the equation $I_{T;0.5mW} = I_{ROO;0.5mW} + I_{P;0.5mW}$ ($I_{T;0.5mW}$ is a directly measured value) form a system of two equations with two unknowns, which can be solved.

Thus, $I_{ROO;0.5mW}$ and $I_{P;0.5mW}$ were calculated and converted into concentration terms by comparison with the second integral of metHb (100 μM) measured at 30 mW and interpolated to the microwave power of 0.5 mW according to a non-saturating dependence.