Journal of Magnetic Resonance **142,** 266–275 (2000)
doi:10.1006/jmre.1999.1935, available online at http://www.idealibrary.com on IDEAL®

A New Method for Quantitation of Spin Concentration by EPR Spectroscopy: Application to Methemoglobin and Metmyoglobin

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Received April 21, 1999; revised August 30, 1999

A new method of EPR spectral analysis is developed to quantitate overlapping signals. The method requires double integration of a number of spectra containing the signals in different proportions and the subsequent solution of a system of linear equations. The result gives the double integral values of the individual lines, which can then be further used to find the concentrations of all the paramagnetic species present. There is no requirement to deconvolute the whole spectrum into its individual components. The method is employed to quantify different heme species in methemoglobin and metmyoglobin preparations. A significantly greater intensity of the high-spin signal in metmyoglobin, compared to methemoglobin at the same heme concentration, is shown to be due to larger amounts of low-spin forms in methemoglobin. Three low-spin types in methemoglobin and two in metmyoglobin are present in these samples. When their calculated concentrations are added to those of the high-spin forms, the results correspond to the total heme concentrations obtained by optical spectroscopy. © 2000 Academic Press

Key Words: **EPR; hemoglobin; myoglobin; methemoglobin; metmyoglobin; metHb; metMb; low spin heme; high spin heme; hemichrome; alkaline metHb; alkaline metMb; quantitation.**

INTRODUCTION

Heme proteins, when oxidized to the ferric (Fe^{III}) state, can exist in either high- ($S = \frac{5}{2}$) or low- ($S = \frac{1}{2}$) spin forms. While both forms are paramagnetic, they exhibit very different EPR signals. The high-spin (HS) heme EPR signal is characterized by three effective g factors, two close to the value of 6 and the third close to 2. The first two, one higher and one lower than $g = 6$, can be so close as to be indistinguishable, especially when X-band spectroscopy (~9 GHz) is used. This is the case with methemoglobin (metHb) and metmyoglobin (metMb) (*1, 2*). The low-spin (LS) forms have two effective g factors higher and one g factor lower than $g = 2$ (*3*). The LS heme state is formed when the 6th coordination site in the heme is occupied by a ligand which is either a small molecule or an amino acid residue of the protein itself. In the latter situation, the tertiary structure must be altered (*3, 4*). The variability of the LS forms, which can be seen in soluble proteins such as the hemoglobins and myoglobins (*2, 3, 5*) and in membrane-bound proteins such as cytochrome oxidase (*6*), gives rise to a multiplicity of EPR signals. Analysis of such spectra, especially quantitation of different species with overlapping EPR signals, has often proven difficult (*7, 8*). The present work stems from attempts to understand the different intensities of the HS $g = 6$ signals observed in low-temperature EPR spectra of metHb and metMb samples prepared at equal concentrations. We decided to investigate methodically how the intensities of the EPR signals obtained under specified conditions were related to the concentrations of the paramagnetic species responsible.

This apparently simple objective is however difficult to achieve, since all the signals overlap. One of the strategies which might be used to solve this problem is to obtain the pure line shapes of all the signals, integrate these line shapes, and then measure how much of each there is in every spectrum. The latter operation can be performed using subtraction with a variable coefficient. For example, if a bulk spectrum R contains a pure line shape signal R_1 in an unknown amount x, then R can be expressed as

$$R = xR_1 + \Sigma(\text{other signals}). \qquad [1]$$

To subtract with a variable coefficient, a spectrum kR_1 is subtracted from spectrum R using successive values of the coefficient k until no components of signal R_1 can be seen in the difference spectrum. The value of k at this point equals the unknown amount x of signal R_1. We use this procedure in the present paper. However, the first part of the strategy, obtaining the pure line shapes of all the signals, is very difficult. We therefore propose a novel technique to calculate the integrals of the overlapping signals which does not require prior deconvolution of the bulk spectrum. This technique merely requires accurate measurement of the second integral of a number of spectra containing the overlapping signals in differing proportions.

RESULTS AND DISCUSSION

1. Spectra of Metmyoglobin and Methemoglobin

Figure 1 shows the typical spectra for metHb and metMb prepared at 110 μM and pH 7, frozen in liquid nitrogen and measured at 25 K. The HS heme signal with $g_{x,y} = 6$ and $g_z =$

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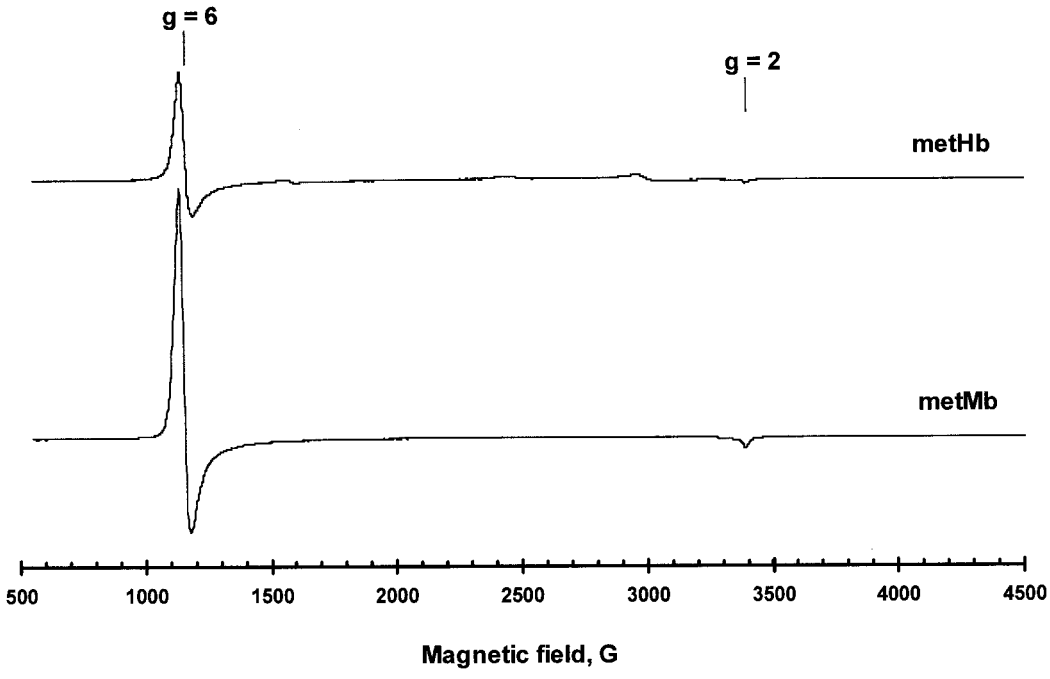

FIG. 1. The EPR spectra of metHb and metMb (110 μM heme, pH 7.0) measured at 25 K and microwave frequency 9.4865 GHz.

2 has a greater peak-minus-trough intensity in the metMb sample than in the metHb sample. This result was confirmed at three different pH values (Fig. 2). In all three cases, pH 6, 7, and 8, the intensity of the EPR signal of the HS form of heme is significantly lower in hemoglobin than in myoglobin. Although the peak-to-trough distance was larger in the $g = 6$ region of metHb (52 G, compared to 46 G for metMb), this small difference was not sufficient to account for the observed difference in intensity.

The simplest explanation for the difference is that the hemoglobin preparations contain low spin (LS) forms in higher concentration than the myoglobin preparations (cf. also Fig. 2). Three distinct LS forms were present in metHb and two in metMb. The effective g-factor values of the two HS and the four LS signals in these metHb and metMb spectra are presented in Table 1. However, it is far from obvious from the spectra that the differences in LS signal intensities can account for the big difference in the HS intensities seen in Fig. 1. To determine this we need to accurately determine the concentrations of the overlapping species in the spectra in Figs. 1 and 2. Temperature and power saturation effects on the separate species need to be considered first.

2. Temperature Dependences of the HS and LS Forms

To quantify the concentrations of the paramagnetic centers responsible for the different heme protein forms, it is first necessary to ensure that the signals are not saturated. This requires the choice of a proper combination of temperature and microwave power for each measurement. Further complications arise for high-spin species that do not obey the Curie Law. Ideally, the two-dimensional dependence of the second integral of each signal should be plotted on the Z axis of the X–Y plane (temperature, microwave power), and such dependences should be found individually for each pH value. Then a temperature/power combination can be chosen where none of the signals are saturated. Appropriate corrections can also be made for the differences in temperature dependence for high- and low-spin species. However, as we do not have the pure line shapes of these signals necessary to accomplish such integrations, we are forced to use approximations as described below.

2.1. HS heme forms. The HS signal temperature dependences were measured by integration of the EPR spectra in the range 1–2200 G, up to the field position where the LS signals start to contribute to the value of the integral (Fig. 1). This field range does not cover the whole HS signal (which actually extends to ~3500 G), but corresponds to about 75% of the total integral. This percentage is constant in the absence of power saturation, i.e., it does not change with change of power below some critical value, when the temperature is fixed, and does not change with temperature above some critical value, when the power is fixed. The HS heme EPR signal only begins to saturate at both a very high power and a very low temperature (see below) and these power saturation characteristics are independent of pH. The line shapes of the $g = 6$ signals in metHb and metMb were similar to each other and did not change as the pH was varied from 6 to 8. Therefore, as the overall line shapes and power saturation characteristics of the metHb and metMb $g = 6$ signals were essentially identical in all the experimental conditions studied, the second

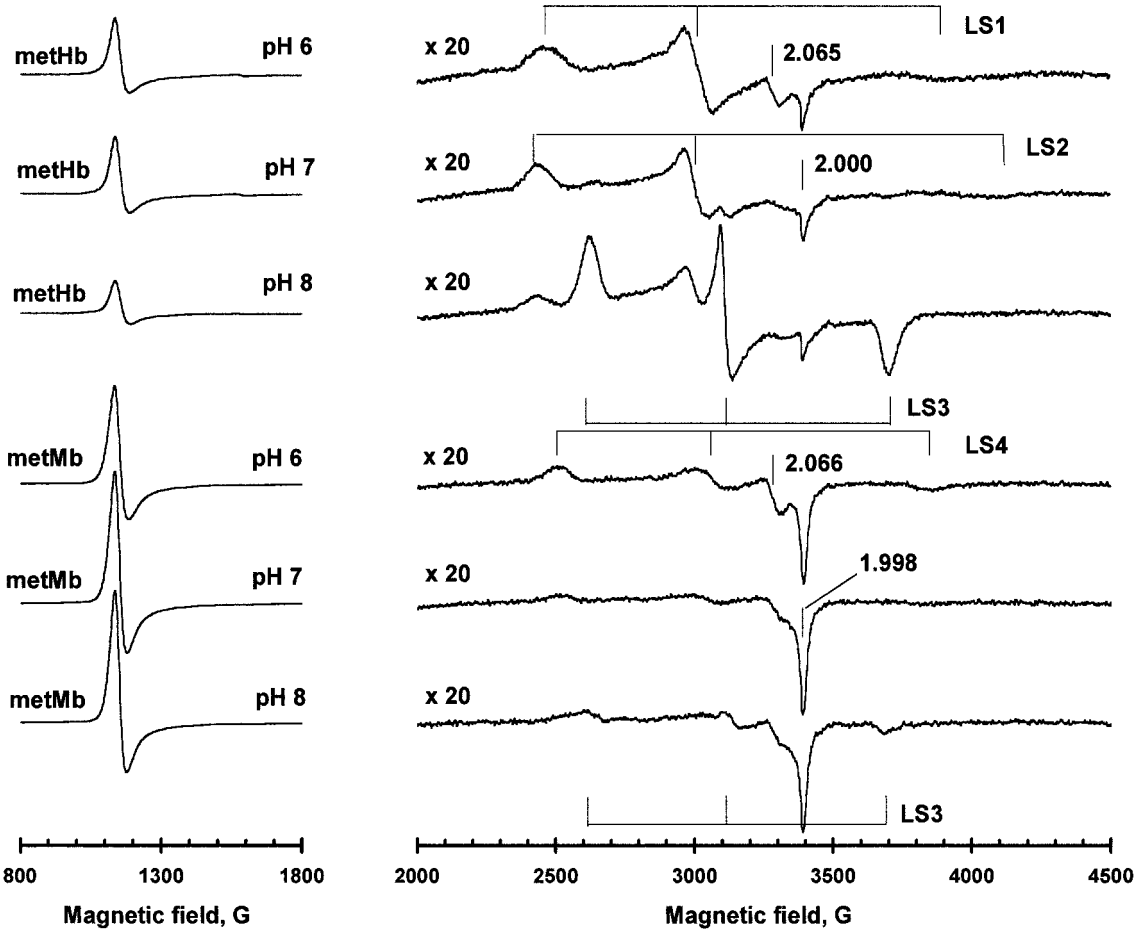

FIG. 2. The EPR spectra of metHb and metMb prepared at three different pH values and measured at 25 K. The concentration of each preparation was 110 μM. The LS region of the EPR spectra is shown at 20-fold gain. The effective g factors of the signals are given in Table 1. The microwave frequency was 9.4865 GHz.

integral values measured in this 1–2200 G range correspond accurately to a constant fraction (~75%) of the total second integrals and therefore to the relative concentrations of the HS forms. The coincidence of this fraction (75%) for the two proteins is fortuitous; it is important to note that for the further analysis described here to be valid it is only necessary that this fraction is

TABLE 1

Effective g Factors of the HS and LS Forms of Methemoglobin and Metmyoglobin Measured at 9.4865 GHz and 25 K[a]

	HS (metHb)	HS (metMb)	LS1[b]	LS2[b]	LS3[c]	LS4[d]
g_x	5.82 (zero line crossing) 5.96 (peak maximum)	5.85 (zero line crossing) 5.97 (peak maximum)	2.7236	2.7853	2.5860	2.6981
g_y	indistinguishable from g_x	indistinguishable from g_x	2.2442	2.2593	2.1795	2.2113
g_z	2.000	1.998	1.73–1.74	1.65–1.66	1.8319	1.758
5th ligand	Proximal histidine	Proximal histidine	Proximal histidine	Proximal histidine	Proximal histidine	Proximal histidine
6th ligand	Water	Water	Distal histidine	Distal histidine	OH^-	Distal histidine

[a] The $g_{x,y}$ and g_z for the HS forms seem to be pH-independent between pH 6 and 8. Since the real value of $g_{x,y}$ for the HS forms is between the peak maximum and zero line-crossing, the values measured in both ways are given. The indicated 5th and 6th ligands of heme iron (see below) were attributed on the basis of previously published data (*1–3*).

[b] LS1 and LS2 signals were attributed to the histidine hemichromes of metHb.

[c] LS3 was attributed to the alkaline heme form (i.e., the species with an hydroxyl anion at the 6th coordination position), indistinguishable in metHb and metMb.

[d] LS4 was attributed to the histidine hemichrome of metMb.

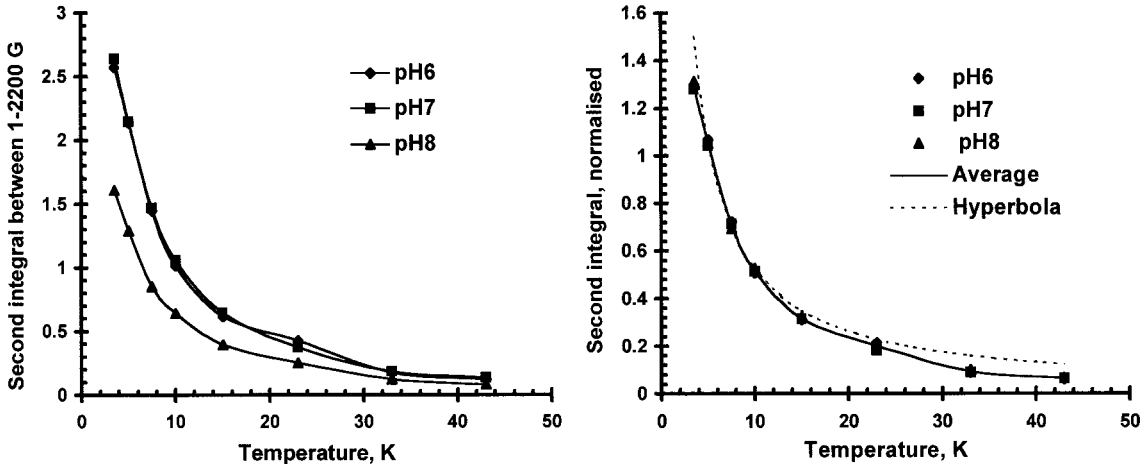

FIG. 3. (left) Temperature dependences in arbitrary units of the second integrals of the metHb spectra measured in the range 1–2200 G. This corresponds to approximately 75% of the total second integral of the HS form spectra. (right) Normalized temperature dependences. The dependences on the left were multiplied by factors to make all three dependences superimposable. These factors were used to determine the ratio of the concentrations of the HS metHb in the three samples: [HS metHb (pH 6)]:[HS metHb (pH 7)]:[HS metHb (pH 8)] = 1.63:1.67:1.00. The hyperbola (dashed) represents the expected Curie law dependence for a nonsaturated EPR signal. The microwave power for measurement was 0.0503 mW.

constant for each protein under the different experimental conditions, not that it has the same numerical value for the different proteins.

Figures 3 and 4 show the temperature dependences of the HS heme EPR signals measured as above. The HS heme signals diminish with increasing temperature more steeply than predicted by the Curie law (Intensity $\propto 1/T$) for a nonsaturated EPR signal (right-hand plots, Figs. 3 and 4), because the middle and upper Kramers doublets are becoming appreciably populated at temperatures above 10 K (*4*). This must be taken into account in any quantitative analysis, since the apparent EPR signal at $g = 6$ and $g = 2$ represents only part of the total HS heme population when the spectrum has been measured at a temperature above 10 K. The left-hand plots show the second integrals of the HS signals at the three pH values examined. The right-hand plots represent the normalized dependences; the factors required to plot the curves in this superimposed form reflect the actual proportions of the HS heme forms at the different pH values. These factors do not however indicate how the HS concentration in metHb (Fig. 3) relates to the HS concentration in metMb (Fig. 4).

2.2. LS heme forms. The LS signal temperature dependences are shown in Fig. 5. The approximation used is that the

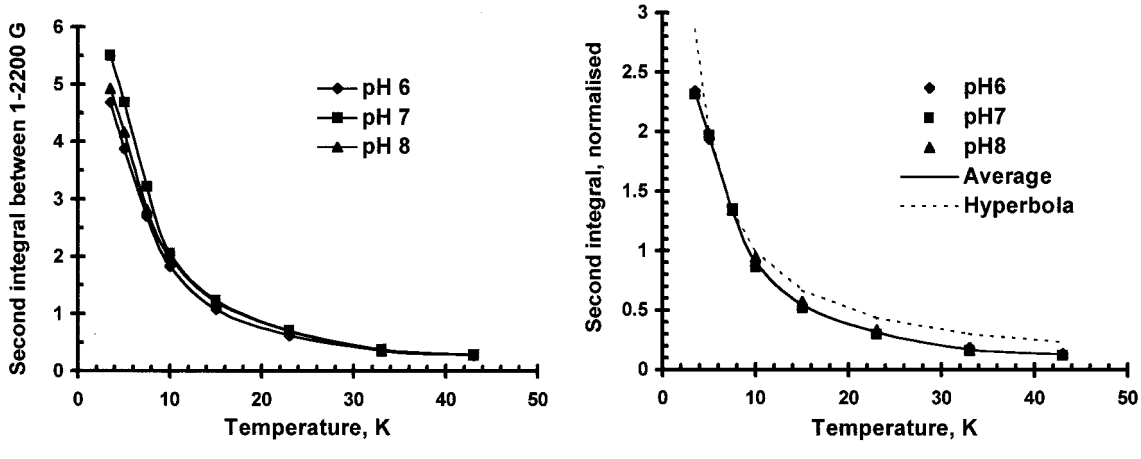

FIG. 4. (left) Temperature dependences in arbitrary units of the second integrals of the metMb spectra measured in the range 1–2200 G. This corresponds to approximately 75% of the total second integral of the HS form spectra. (right) Normalized temperature dependences. The dependences on the left were multiplied by factors to make all three dependences superimposable. These factors were used to determine the ratio of the concentrations of the HS metHb in the three samples: [HS metMb (pH 6)]:[HS metMb (pH 7)]:[HS metMb (pH 8)] = 0.94:1.05:1.00. The hyperbola (dashed) represents the expected Curie law dependence for a nonsaturated EPR signal. The microwave power for measurement was 0.0503 mW.

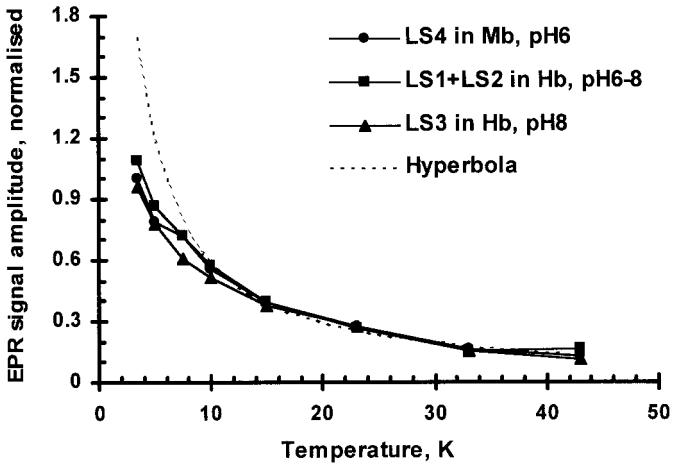

FIG. 5. Temperature dependences of the LS forms of metHb/metMb. The signal peak intensities were measured (see Fig. 2). The whole curves were then normalized using multiplication by appropriate factors to make the curves superimposable in the nonsaturated hyperbolic range, >15 K. The microwave power for measurement was 0.0503 mW.

line shapes do not change with temperature or pH under nonsaturating conditions. With this assumption, the peak intensities of the signals were measured using the method of subtraction with variable coefficient (see Introduction). At pH 7 and 8 the LS1 form is at such low concentrations that it does not interfere with measurements of the LS2 species. At pH 6 the two forms are present at similar concentrations and one cannot be sure of measuring the temperature dependence of each signal separately. However, the shape of the temperature dependence of the two forms, LS1 and LS2, measured at pH 6 was very close to the shapes of the temperature dependence of the LS2 form. It was concluded, therefore, that these two LS forms, LS1 and LS2, in metHb have identical temperature dependences at the different pH values. They were therefore averaged. As expected, the Curie Law is obeyed for the LS species; there is a nonsaturating region which fits a hyperbola at $T > 15$ K for all LS forms in both metHb and metMb (Fig. 5). This gives us confidence that we have not introduced an artefact by using peak intensities, rather than double integrals, in determining the proportion of the LS species at the different pH values.

We can now choose a temperature and a nonsaturating microwave power at which the EPR spectra can be integrated. Values of 0.05 mW and 25 K were selected. A temperature of 25 K belongs to the nonsaturating hyperbolic part of the temperature regimes of all the LS forms when measured at 0.05 mW. These are also nonsaturating conditions for the HS forms, although, as described above, only a fraction of the total HS population is detected at this temperature and power. When the temperature dependences for the HS forms have been determined, it is possible to find the necessary correction factors.

3. Mathematical Treatment

We now propose a new method to quantify paramagnetic species with overlapping EPR signals. Instead of obtaining the pure line shapes for each center, the method involves a double integration of a number of EPR spectra containing the species in different proportions and the subsequent solution of a linear system of equations to give the integrals of the individual signals. Both the spectra of metHb and those of metMb may be treated in this way, as described below.

3.1. Methemoglobin. The total second integral I of a metHb spectrum can be represented as a linear combination of the unknown second integrals (I_{HS}, I_{LS1}, I_{LS2}, and I_{LH3}) of the individual EPR signals

$$I = a_1 I_{HS} + a_2 I_{LS1} + a_3 I_{LS2} + a_4 I_{LH3}. \quad [2]$$

For the three pH values, we have

$$\text{pH } 6: 1.038 = 1.63 I_{HS} + 8.21 I_{LS1} + 1.30 I_{LS2} + 0.00 I_{LS3} \quad [3]$$

$$\text{pH } 7: 1.066 = 1.67 I_{HS} + 1.50 I_{LS1} + 1.89 I_{LS2} + 0.08 I_{LS3} \quad [4]$$

$$\text{pH } 8: 1.000 = 1.00 I_{HS} + 1.00 I_{LS1} + 1.00 I_{LS2} + 1.00 I_{LS3} \quad [5]$$

The left-hand sides of Eqs. [3]–[5] (integrals I) were obtained by double integration of the overall spectra (Fig. 2) in the range of 500−4500 G. This was seen to encompass the complete spectrum as increasing the integration range to 1–5500 G did not result in a significant increase of the integral values but instead resulted in a poorer reproducibility (results not shown). The values in the left-hand sides of Eqs. [3]–[5] were determined with a standard error <6% (see Methods). The double integrals were normalized such that the value at pH 8 was 1.000.

The a_1 coefficients for the integrals of the HS forms were found as in the caption to Fig. 3. The coefficients a_2, a_3, and a_4 for the LS forms were obtained using the procedure of spectral subtraction with a variable coefficient. The spectrum of the pH 8 sample was multiplied by such a coefficient and subtracted from the pH 6 and pH 7 spectra. The coefficient was then varied until LS1, LS2, or LS3 signals were not seen in the difference spectrum. This way of determining coefficients a_2, a_3, and a_4 forces each coefficient to the value of unity in the pH 8 spectrum.

The system of three Eqs. [3]–[5] contains four unknowns. Therefore, an additional condition is needed to relate two of the unknowns. In principle, a measurement at another pH could be made. However, it is also possible to make use of the fact that the hydroxide-ligated metHb species (LS3) has zero intensity

TABLE 2
Partial Contributions of Individual EPR Signals to the Second Integral of the EPR Spectra of Methemoglobin Frozen at Three Different pH Values

metHb	Total	HS	LS1	LS2	LS3
pH 6	1.0379	0.8052	0.1010	0.1317	0.0000
pH 7	1.0663	0.8250	0.0184	0.1915	0.0314
pH 8	1.0000	0.4940	0.0123	0.1013	0.3924

Note. See legend to Fig. 2 for conditions.

TABLE 3
Second Integrals and Their Sums for Individual Methemoglobin EPR Signals[a]

metHb	Total	HS	LS1	LS2	LS3
pH 6	1.2526	1.0199	0.1010	0.1317	0.0000
pH 7	1.2863	1.0450	0.0184	0.1915	0.0314
pH 8	1.1317	0.6257	0.0123	0.1013	0.3924

[a] Calculated using the (oversimplified) assumption that 100% of the HS form populates exclusively the lowest Kramers doublet at 25 K.

at pH 6. Thus, an additional relationship between I_{LS1} and I_{LS2} can be determined as follows. The pH 8 spectrum was subtracted from the pH 7 spectrum with a coefficient which was varied to eliminate I_{LS3} in the difference spectrum. Neither this spectrum, nor the pH 6 spectrum, contains the I_{LS3} signal. These two spectra were therefore integrated over the range 2200–4400 G, and the parts of the integrals caused by the tail of the HS spectrum were subtracted. Two equations are thus obtained with two unknowns, I_{LS1} and I_{LS2}. Solving this system gives the ratio of the two reference integrals $I_{LS2}/I_{LS1} = 8.236$, which represents the necessary fourth condition to solve the system of Eqs. [3]–[5].

The solutions of the system of Eqs. [3]–[5] were therefore found to be

$$I_{HS} = 0.4940$$
$$I_{LS1} = 0.0123$$
$$I_{LS2} = 0.1013$$
$$I_{LS3} = 0.3924. \qquad [6]$$

After substitution of these values into the equations, the second integral is partitioned between the different signals in the three samples as shown in Table 2.

The values presented in Table 2 are not yet exactly proportional to the concentrations of the corresponding paramagnetic species. This is because (i) the HS signal is generated by a fraction of the total HS heme states, and (ii) species with very different effective g values (such as the HS and LS heme states) give EPR signals with different integral intensity per unit spin. This latter proviso means that although the second integral is proportional to its concentration for any particular species, the coefficient of proportionality differs for signals with different g values. To estimate the concentrations correctly and completely quantitatively, these two facts must be taken into consideration (*9*).

A temperature of 25 K is within the nonsaturating part of the temperature-dependence for all the LS forms (Fig. 5). However, for HS forms at 25 K, a factor must be introduced to take into account the fact that the $g = 6$ signal corresponds only to a fraction of the HS states, since two other Kramers doublets play a role at this temperature. For the metHb case this factor (at 25 K) equals the ratio of the integrated values of the expected (hyperbolic) dependence to the experimental temperature dependence, as shown in Fig. 3 (right-hand plot). Its numerical value of 1.2667 means that the apparent intensity of the HS signal is that much lower than if there were no other transitions. Including this factor into the system of Eqs. [3]–[5] permits a calculation of the corrected integrals of the HS forms and their effect upon the total integrals. Table 2 then becomes Table 3, which illustrates what the HS and total signal integrals would be were the middle and the upper Kramers doublets not populated.

The values in Table 3 are still not quite proportional to the concentrations of the paramagnetic species. For field-swept EPR spectra, the second integral of the signals should be divided by a factor which is a function of the principal g values to make it proportional to the concentration (*9*). This factor, called g_P, is determined as in Eq. [7]

$$g_P \approx \frac{2}{3}\sqrt{\frac{g_x^2 + g_y^2 + g_z^2}{3}} + \frac{1}{3} \times \frac{g_x + g_y + g_z}{3}. \qquad [7]$$

For three equal g factors (an isotropic line) the g_P value is thus equal to the measured g value. The g_P values for both metMb and metHb are presented in Table 4.

The calculated values for all the signals detected (Table 3) are then divided by the corresponding g_P values from Table 4

TABLE 4
The g_P Values Calculated for the EPR Signals in Methemoglobin and Metmyoglobin

	g_x	g_y	g_z	g_P
HS (Hb)	5.871	5.871	2.000	4.814
HS (Mb)	5.884	5.884	1.998	4.823
LS1	2.724	2.244	1.735	2.258
LS2	2.785	2.259	1.655	2.265
LS3	2.586	2.180	1.832	2.213
LS4	2.698	2.211	1.758	2.244

Note. See Eq. [7] and associated text.

TABLE 5

Total Concentration and Concentrations of Individual Paramagnetic Centers in Methemoglobin Expressed in Relative Units[a]

metHb	Total	HS	LS1	LS2	LS3
pH 6	0.315	0.212	0.044	0.058	0.000
pH 7	0.324	0.217	0.008	0.085	0.014
pH 8	0.357	0.130	0.005	0.045	0.177

[a] Calculated from the second integrals of the individual signals after corrections for different Kramers doublet populations and using the correction for magnetic field swept EPR spectra (see text).

TABLE 6

Total Concentration and Concentrations of Individual Paramagnetic Centers in Metmyoglobin Expressed in Relative Units[a]

metMb	Total	HS	LS3	LS4
pH 6	0.292	0.254	0.000	0.038
pH 7	0.296	0.283	0.000	0.013
pH 8	0.292	0.270	0.013	0.009

[a] Calculated from the second integrals of the individual signals after corrections for different Kramers doublet populations and using the correction for magnetic field swept EPR spectra (see text).

to give the further corrected values shown in Table 5. Table 5 now represents, in relative units, the concentrations of the individual paramagnetic centers in the metHb preparation at pH values 6, 7, and 8.

3.2. Metmyoglobin. In a similar way, the total second integral $\mathcal{J}$ of a metMb spectrum can be represented as a linear combination of second integrals of individual EPR signals as in

$$\mathcal{J} = b_1\,\mathcal{J}_{HS} + b_2\,\mathcal{J}_{LS4} + b_3\,\mathcal{J}_{LH3}. \quad [8]$$

For the three pH values, Eqs. [9]–[11] are the myoglobin equivalent of Eqs. [3]–[5]

$$\text{pH 6: } 0.980 = 0.94\,\mathcal{J}_{HS} + 4.29\,\mathcal{J}_{LS4} + 0.00\,\mathcal{J}_{LS3} \quad [9]$$

$$\text{pH 7: } 1.027 = 1.05\,\mathcal{J}_{HS} + 1.43\,\mathcal{J}_{LS4} + 0.00\,\mathcal{J}_{LS3} \quad [10]$$

$$\text{pH 8: } 1.000 = 1.00\,\mathcal{J}_{HS} + 1.00\,\mathcal{J}_{LS4} + 1.00\,\mathcal{J}_{LS3}. \quad [11]$$

Both integrals on the left-hand sides and the coefficients on the right-hand sides were determined with a standard error of less than 2% (see Methods).

In this case we have three unknowns and three equations. The solutions for this system [9]–[11] are:

$$\mathcal{J}_{HS} = 0.951$$

$$\mathcal{J}_{LS4} = 0.020$$

$$\mathcal{J}_{LS3} = 0.029. \quad [12]$$

These values were substituted into the equations. The correction factor for the fractional population of the middle and upper Kramers doublets at 25 K was 1.368 (Fig. 4, right-hand plot) and the g_P value was 4.823 (Table 4). The table of concentrations for metMb in common units is thus obtained (Table 6).

We have chosen the units for I (hemoglobin) and $\mathcal{J}$ (myoglobin) arbitrarily. To compare the values in Table 5 with those in Table 6 we need to know the value of $I/\mathcal{J}$. This can be done by comparing the second integrals of the complete spectra of metHb and metMb from 1 to 4500 G, which gives a value of $I/\mathcal{J}$ of 0.886. Correcting for this number we can determine the relative concentrations of all species in all six samples. In order to convert this to absolute concentrations we need to have an independent measure of the heme concentration (or use an external standard of known concentration). All samples were ostensibly made up at 110 μM (using optical extinction coefficients). Therefore, the total integrals were averaged and this mean value set to 110 μM. This finally allows us to present the data as absolute concentrations for each molecular species (Table 7).

The fact that the total heme concentration in all the samples was within 10% of the mean value (110 μM) demonstrates that, despite the apparent large differences in the intensities of the high-spin signals (Fig. 2), the total concentration of heme is essentially the same in all cases (Table 7). The pH dependences of the concentrations of HS form and all LH forms in metHb and metMb are shown in Fig. 6. LS forms constitute a greater proportion of total protein in metHb than in metMb at all three pH values. At pH 8 the total concentration of all the

TABLE 7

Total Concentration and Concentrations of Individual Heme Species in Methemoglobin and Metmyoglobin Measured by Low-Temperature EPR Spectroscopy

		Total	HS	LS1 (Hb)	LS2 (Hb)	LS3	LS4 (Mb)
metHb							
pH 6	in μM	104	70	15	19	0	0
	in %	100	67	14	19	0	0
pH 7	in μM	107	72	3	28	5	0
	in %	100	67	3	26	4	0
pH 8	in μM	119	43	2	15	59	0
	in %	100	36	1.5	12.5	50	0
metMb							
pH 6	in μM	109	95	0	0	0	14
	in %	100	87	0	0	0	13
pH 7	in μM	111	106	0	0	0	5
	in %	100	96	0	0	0	4
pH 8	in μM	109	101	0	0	5	3
	in %	100	92	0	0	5	3

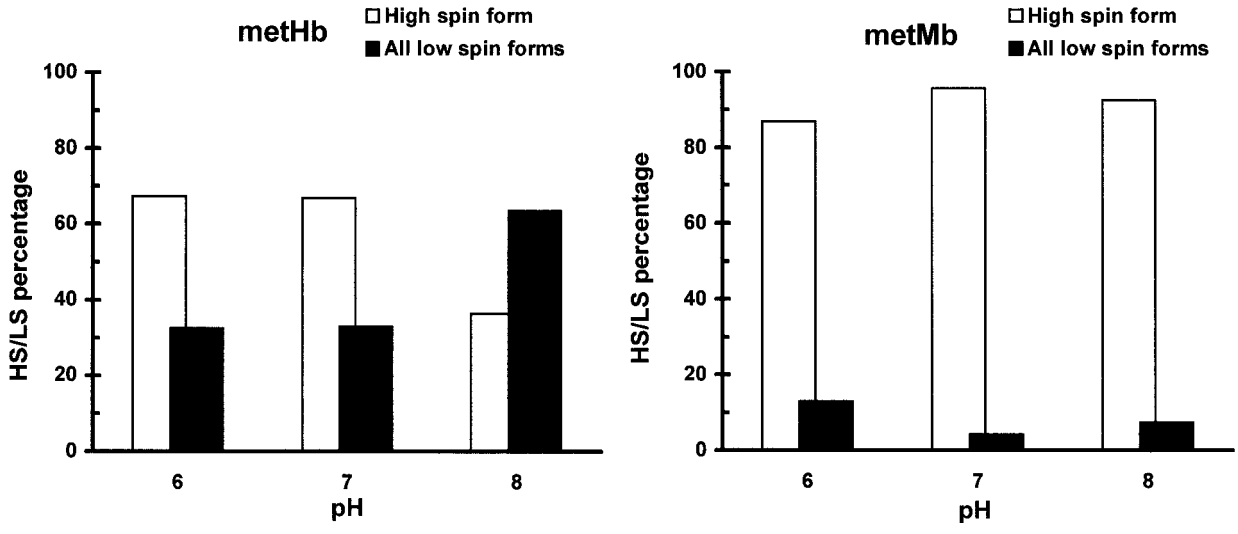

FIG. 6. Relative concentrations of the HS form and all LS forms as a function of pH, expressed as percentages of the total concentrations in metHb and metMb (calculated from data in Table 7).

LS forms of metHb is even higher than the concentration of HS metHb. This is mainly due to increased formation of the LS3 metHb (hydroxide complex) at pH 8 as the pK for this transition is known to be significantly lower in hemoglobin than myoglobin.

The heterogenity observed in the EPR spectra is not noticeable in room temperature optical spectra where at pH 7 the lineshapes of the hemoglobin and myoglobin optical spectra are very similar. The LS1 and LS2 and LS4 forms described here are therefore likely to be formed at low temperature only (*10*).

4. Using the Method to Test Unknown Solutions

The metHb and metMb samples employed in this work are now used in this laboratory as standards to determine the concentration of any preparation of metHb or metMb. In each case the procedure of subtraction with variable coefficient is used to measure the intensity of an EPR signal in the sample under study; this is directly compared to that in the standard sample. Since the concentration of each ferric heme in the standards is known, the measured intensity of any other signal can be expressed as an absolute concentration.

Several other hemoglobin and myoglobin preparations have been tested to check whether the protein concentration determined by optical spectroscopy is always consistent with the results obtained using the present method. Table 8 shows that the EPR procedure of concentration measurement is in good correspondence with the data based on the extinction coefficients for optical spectroscopy. MetHb is always characterized by a higher proportion of LS forms than are present in metMb when measured at the same pH. As a result, the HS heme EPR signal ($g = 6$) is always lower in metHb than in metMb at the same pH. This difference is probably a direct indication to the fact that the pK value for the "acid-alkaline" transition in metMb is almost 1 unit higher than in metHb (pK $\approx$ 9 and 8, respectively) (*11*).

Another fundamental problem which arises when estimating concentration of species with very different EPR line shapes is the fact that integration of the experimental spectra may lead to a systematic error which could have a strong dependence on the line shape. Ideally, every unknown signal under study should be compared to a reliable concentration standard with a similar line shape to minimize the systematic error. Unfortunately, in most experiments it is not possible to always use a standard of identical line shape to the unknown solution. As a further test of our method we therefore compared the concentrations determined by our new quantitation method with those obtained using a very different EPR standard. A 100-μM $CuSO_4$ standard was measured against the pH 8 metHb sample (Table 7), using 0.05 mW power at 43 K under nonsaturating conditions. A factor of 2 was used for the HS form, to take into account the middle and upper Kramers doublets at 43 K (cf. Fig. 3, right-hand plot). The copper g_P coefficient was 2.147 (effective g factors at $g = 2.306$, 2.064, and 2.064). The estimate gave an apparent concentration for the copper sample of 109 μM in reasonably good correspondence with the value of 100 μM—the concentration at which the copper sample was prepared. We conclude that the method described is accurate to within 10%, even when standards of very different line shapes are used.

CONCLUSIONS

A method has been developed to quantify paramagnetic species with overlapping EPR signals. This method has general application. In the specific case of hemoglobin and myoglobin

TABLE 8
Total and Component Concentrations[a] in Four Methemoglobin and Metmyoglobin Preparations Measured by Optical and EPR Spectroscopy

Sample	Optical spectroscopy[b]	EPR spectroscopy[c]					
	Total protein	HS Fe^{III}–OH_2 (Hb/Mb)	LS1 Fe^{III}–His (Hb)	LS2 Fe^{III}–His (Hb)	LS3 Fe^{III}–OH^- (Hb/Mb)	LS4 Fe^{III}–His (Mb)	Total protein
Human metHb, pH 7 (Sigma)	47	29	0	13	2	0	44
Human metHb, pH 7.4 (purified in this laboratory)	50	38	2	10	2	0	52
Horse skeletal muscle metMb, pH 7 (Sigma)	50	44	0	0	0	2	46
Horse heart metMb, pH 6.5 (Sigma)	50	48	0	0	0	1	49

[a] Concentrations in μM heme.
[b] 298 K.
[c] 10 K.

we have demonstrated that differences in the intensities of the HS EPR signal in metHb and metMb, when prepared at equal concentrations, are found to be entirely consistent with a variation in the amount of the low-spin forms present.

EXPERIMENTAL

1. Protein Samples

Human hemoglobin (Sigma H-7379) and horse heart myoglobin (Sigma M-1882) were prepared in 100 mM potassium phosphate buffer (pH 6.0) at an approximate heme concentration of 1.5 mM. Essentially identical results were achieved, if instead of using Sigma, human hemoglobin was purified fresh from a healthy donor's blood according to (*11*). The samples were fully oxidized to the Fe^{III} (met) form by 2 mM ammonium persulphate. Each sample was then passed down a Sephadex G-25 column and the resulting concentration was measured using a Hewlett Packard 8453 diode array spectrophotometer. Aliquots were reduced with 10 mM dithionite and then bubbled with CO for 30 s. The following extinction coefficients for the CO-ferroheme forms were used to calculate the protein concentrations: $\epsilon_{540} = 15.4\ mM^{-1}\ cm^{-1}$ (horse CO-ferromyoglobin) and $\epsilon_{540} = 13.4\ mM^{-1}\ cm^{-1}$ (human CO-ferrohemoglobin) (*11*). The oxidized stock solutions were diluted to 110 μM in 100 mM potassium phosphate buffer containing 20 μM diethylenetriamine pentaacetic acid (DTPA) for chelating adventitious metal ions at the pH values 6.0, 7.0, and 8.0.

2. Copper Standard Sample

Copper sulfate was used as a concentration standard (*12*). Four 1-liter solutions of 100 μM $CuSO_4$ were prepared: (1) in 0.1 M imidazole, pH 8; (2) in 0.1 M imidazole, pH 9.5; (3) in 0.01 M ethylenediamine-tetraacetic acid (EDTA), pH 3.5; and (4) in 0.01 M EDTA, pH 8. Four EPR samples were made of the aliquots taken from each solution. It was shown that the second integrals of the copper EPR signal in every EPR sample made of the same solution were identical within a 2–4% error. Although the line shape of the copper EPR signal was somewhat different for the four different solutions, the second integral value was essentially the same (standard error/mean = 0.093). Sample 3 was used for the comparative quantitation described in Section 4 of Results.

3. EPR Sampling and Measurement

Wilmad SQ EPR tubes were used for the EPR samples. Only selected tubes with o.d. = 4.053 ± 0.064 mm (1.58%) and i.d. = 3.116 ± 0.044 mm (1.41%) were used. The tubes containing aerated protein solutions (or water for blanks) were quickly frozen in methanol pre-cooled on dry ice. Once frozen, the samples were transferred to liquid nitrogen where they were stored prior to the EPR measurements. The spectra were measured using a Bruker EMX spectrometer equipped with an Oxford Instruments liquid helium system and the high-quality spherical Bruker resonator SP9703. The instrumental conditions (cf. Figs. 1 and 2) were: microwave power, $P = 0.8$ mW; microwave frequency, $\nu = 9.4865$ GHz; modulation frequency, 100 kHz; modulation amplitude, $A_m = 5$ G; sweep rate, $SR = 23.8$ G/s; time constant, $\tau = 0.082$ s; number of scans, NS = 2 (per spectrum). Accurate g values were obtained using the built-in microwave frequency counter and a DPPH (2,2-diphenyl-1-picrylhydrazyl) powder standard, $g = 2.0037 \pm 0.0002$ (*12*). Three spectra per protein sample were

measured at each pH (each spectrum being recorded after reloading the sample into the resonator). Two separate blanks (water samples) were used for baseline recording, and again three separate spectra of each blank were recorded. We integrated different combinations of "protein minus blank" spectra. The 18 possible combinations (3 samples and 6 blanks) gave a standard error for the value of the double integral of 6% for metHb and 2% for metMb.

APPENDIX: NOMENCLATURE

Hb	hemoglobin
Mb	myoglobin
LS	low spin
HS	high spin
EPR	electron paramagnetic resonance
ν	microwave frequency, GHz
P	microwave power, mW
A_m	modulation amplitude, G
SR	EPR spectra sweep rate, G/s
τ	time constant, ms
NS	number of scans in EPR spectra acquisition
T	absolute temperature, K
His	histidine

ACKNOWLEDGMENTS

This work was supported by a Wellcome Trust Research grant (MTW, CEC) and a Wellcome Trust University Award (CEC). We thank Jacqueline Dunn for providing a purified human metHb sample and Dr. Brandon Reeder for providing a horse skeletal muscle metMb sample. The technical assistance of Neil Barnard is also acknowledged.

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