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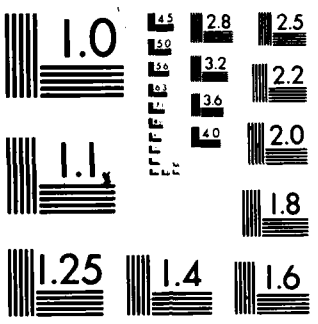

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REPORT NUMBER 1

EVALUATION OF THE STABILITY OF HEMOGLOBIN IN PREPARATION AND STORAGE BY ELECTRON SPIN RESONANCE

ANNUAL SUMMARY REPORT ([redacted])

ROLLIE J. MYERS

AUGUST 31, 1981

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Summary

Techniques have been developed to quantitatively measure the methemoglobin content of hemoglobin samples using electron spin resonance spectroscopy. It is also possible to follow the buildup of the hemichromes as the methemoglobin decays. This technique was applied to hemoglobin supplied by Fischer and also to samples prepared for us by LAIR. The Fischer samples came sealed in glass ampules. They contained relatively large amounts of hemichromes but their hemichrome content seemed independent of storage times. Their hemichrome signals also fell into two distinct types. The LAIR samples, supplied in blood-packs, had a well defined halflife for methemoglobin decay of 39.1 ± 3.2 days. Their hemichromes' signals started out small but increased as the methemoglobin decayed. They did not also fall into the simple two type pattern of the Fischer samples. The differences in stability and hemichrome content between the Fischer and LAIR hemoglobin samples must be due to differences in preparation and storage conditions.

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Introduction

Hemoblobin (Hb), the oxygen carrying pigment found in human blood, holds the potential of being a viable resuscitation fluid, when prepared in suitable solutions.[1] Hb has an advantage over whole blood in that it may be stored for long periods of time both in solution and in lyophilized form with retention of function.[2] Consequently, to insure the long term functional stability of Hb, mechanisms of degradation as a function of preparation and storage conditions should be studied.

One mechanism of Hb denaturation is the oxidation of Hb by molecular oxygen to methemoglobin (metHb), which then slowly converts to hemichromes with irreversible loss of function as an oxygen carrier. The oxidation, itself, is of the iron, in each of the four heme groups, from the ferrous form to the ferric. This process occurs in vivo and is reversed by enzymatic activities. However, in vitro the oxidation is not reversed without the use of reducing agents.

ESR (Electron Spin Resonance) is a suitable tool for evaluating the progress of the degradative route.[3] Though the ferrous form of the heme group is transparent to ESR, both metHb (high-spin ferric) and hemichromes[5,6,7] (low-spin ferric) are easily observable and distinguishable at liquid helium temperatures (approximately 4.5°K).

In this study, we devised techniques using external standards to quantify the ESR data of stored Hb and used these techniques to evaluate the conversion of metHb to low-spin forms as a function of storage time of samples stored at 4°C. The samples, which were studied, were either commercially available or prepared by LAIR for the study.

Experimental Schedule

The original schedule called for us to examine the useability of the Heli-tran cooling system for the ESR measurements of metHb and hemichromes in variable hemoglobin samples. For this purpose LAIR first supplied us with a test hemoglobin sample, Dane XII. With this sample we developed our sample handling techniques and discovered that our ESR spectrometer and the Heli-tran cooling system gave us excellent sensitivity. This sample was very clean and little or no hemichromes could be detected. Since we did not handle this sample by sterile techniques no storage studies were attempted. We could conclude, however, that is was not necessary to go to 1.4°K with a double Dewar set-up and our more convenient Heli-tran system could be used for all of our studies.

LAIR next supplied us with Dane XIV, which was stated to be 6.3 gm/dL in Hb (total) and 2.6% in metHb. This sample was used for the standardization of our apparatus. We were also supplied with several commercial Fischer hemoglobin samples. We were pleased to find hemichrome signals in these samples and so we were now doubly sure that our ESR techniques were satisfactory for a study of selective metHb-hemichromes storage effects.

In the middle of our contract period, LAIR supplied us with samples to be used to study the effects of storage. Instead of LAIR supplying us these samples in EPR tubes they gave us four blood packs which we stored, sampled and analyzed over a period of three months. After this time, the trends in metHb and hemichromes were well established. These trends were quite different between the LAIR and Fischer samples, but the LAIR samples were all consistent with one another.

During the last two months of the contract we did not obtain any further samples and we were not able to test the effects of reducing agents or other chemicals on the stability of hemoglobin samples. We were also not supplied with lyophilized samples. This last period of our contract was spent on data reduction and analysis.

Experimental Methods

1. Instrumentation

The spectra of all samples were measured with a Varian V4502 ESR spectrometer equipped with an Airproducts and Chemicals' Liquid Transfer Heli-Tran System (LTD-3-110). The temperature of the samples were determined by a calibrated carbon resistor and a digital ohmmeter. A Hewlett Packard frequency counter (5245L) with an X-Band plug-in (5255A) measured the microwave frequencies. Magnetic field strength was calibrated with the use of a Harvey-Wells precision gaussmeter (FE-50). Dehumidified air constantly flowed through the Varian V4531 cavity to reduce the condensation of water, which would interfere with sensitivity. All quantitative measurements were made between 4.5° and 5°K.

2. Sample Preparation

a. General Procedure

Samples were stored in their original air tight containers at 4°C in a walk-in refrigerator until ESR measurements were to be made. At that time, a sample was removed and it was diluted 20% v/v with glycerine, placed in a quartz EPR tube, 3 mm I.D., and quickly frozen in liquid nitrogen. The glycerine was added to insure the formation of a good "glass."

b. Specific Procedures

1. Samples prepared by Fischer Chemicals

Fourteen 2 ml glass ampules of Hemoglobin prepared by Fischer were obtained from LAIR. These samples had been prepared and stored under various conditions and on various dates. At the time of measurement, these ampules were broken open and made ready for ESR analysis in the manner described above. For these one-time samples, two ESR tubes were prepared and two spectra obtained to secure reproducibility.

2. Samples prepared by LAIR

For long term storage studies, large volume (ca. 500 ml) samples of Hemoglobin were prepared for us by LAIR. These were stored in Blood-Packs fitted with sample site couplers. Aliquots for study were extracted by sterile syringe to prevent contamination by bacteria and molecular oxygen, both of which could interfere with time dependent studies of metHemoglobin (metHb) and hemichrome concentrations. Final preparations were as mentioned above. Generally, only one ESR tube was prepared from each sample on each occasion of measurement. However, if a certain

measurement was in doubt, another tube was prepared. Concentrated NaOH was used in the cleanup procedure to minimize the possibility of Hepatitis transmission from the samples.

3. Standardization

A soft glass rod, 5 mm in diameter, was the standard for quantifying metHb concentrations from ESR observations. The high spin ferric resonance (g_e = 4.33) due to iron impurities in the glass was found to be suitable for this purpose. The rod itself was initially standardized against a hemoglobin sample, whose concentration of metHb was known, which was supplied by LAIR. At the time of this writing, a suitable ESR standard for hemichrome resonances has not been found, though several possibilities, including a polycrystalline sample of $K_3Co(CN)_6$ doped with Fe^{3+}, were tried, but without success.

Peak to peak heights of various ESR resonances were the parameters, gleaned from the recorded spectra, used in quantifying spectral measurements. Because all the metHb give the same ESR signals, the relative peak heights are an excellent measure of their concentrations. The hemichromes, on the other hand, give a variety of spectra and only the growth of the hemichrome signals could be followed.

Results and Discussion

1. Results

a. Study of Fischer Hemoglobin Samples

In the method described above, several samples of hemoglobin, supplied by LAIR and from Fischer Chemicals, were examined by ESR at 5°K for metHb and denatured hemoglobin (hemichromes) content. The spectra of all the samples examined showed both strong metHb and clearly visible hemichrome resonances, regardless of their previous storage times and manner of preparation. The concentration of metHb ranged from 0.7 to 1.4 g/dL in these samples. For example, on December 17, 1980, we analyzed two Fischer samples. Sample #5, prepared by Fischer July, 1979 and stored at 4°C, from our analysis, had metHb = 1.4 gm/dL, while Sample #11, prepared by Fischer November 18, 1980, also stored at 4°C, analyzed to have metHb = 1.3 gm/dL. The hemichrome signal, however, was twice as strong in Sample #11 as it was in Sample #5. A spectrum of Sample #11 is shown in Fig. 1.

b. Storage Study on Samples prepared by LAIR

Four hemoglobin samples were obtained from and prepared by LAIR for the purpose of long term storage studies. A typical LAIR ESR spectrum is shown in Fig. 2. These samples were examined over a 3 month period by ESR for their metHb and hemichrome content. The metHb concentrations in these samples were observed to decrease greatly over this period. This decrease is modeled reasonably well by a single exponential decay, the half-life of which is approximately 39 days for all four samples, independent of the initial concentrations of metHb. The exponential character of the decay of the metHb content and the half-lives were determined graphically, as shown in Figs. 3 and 4 along with numerical least squares treatment. See Table I for the summary of the results.

As the metHb content of the samples decreased with time, the signals from the hemichromes were seen to increase. The hemichrome signals went from almost nothing initially to a large signal at the end of the three month period. Quantitation was difficult, since the hemichrome resonances, unlike those of the Fischer samples, were not clearly resolved. However, the envelope containing the resonances was consistent with those of the Fischer samples, in that it extended from g = 2.8 to g = 1.8. The reasons for the lack of resolution of the hemichrome resonances are unclear. Most likely a variety of slightly differing hemichromes were produced.

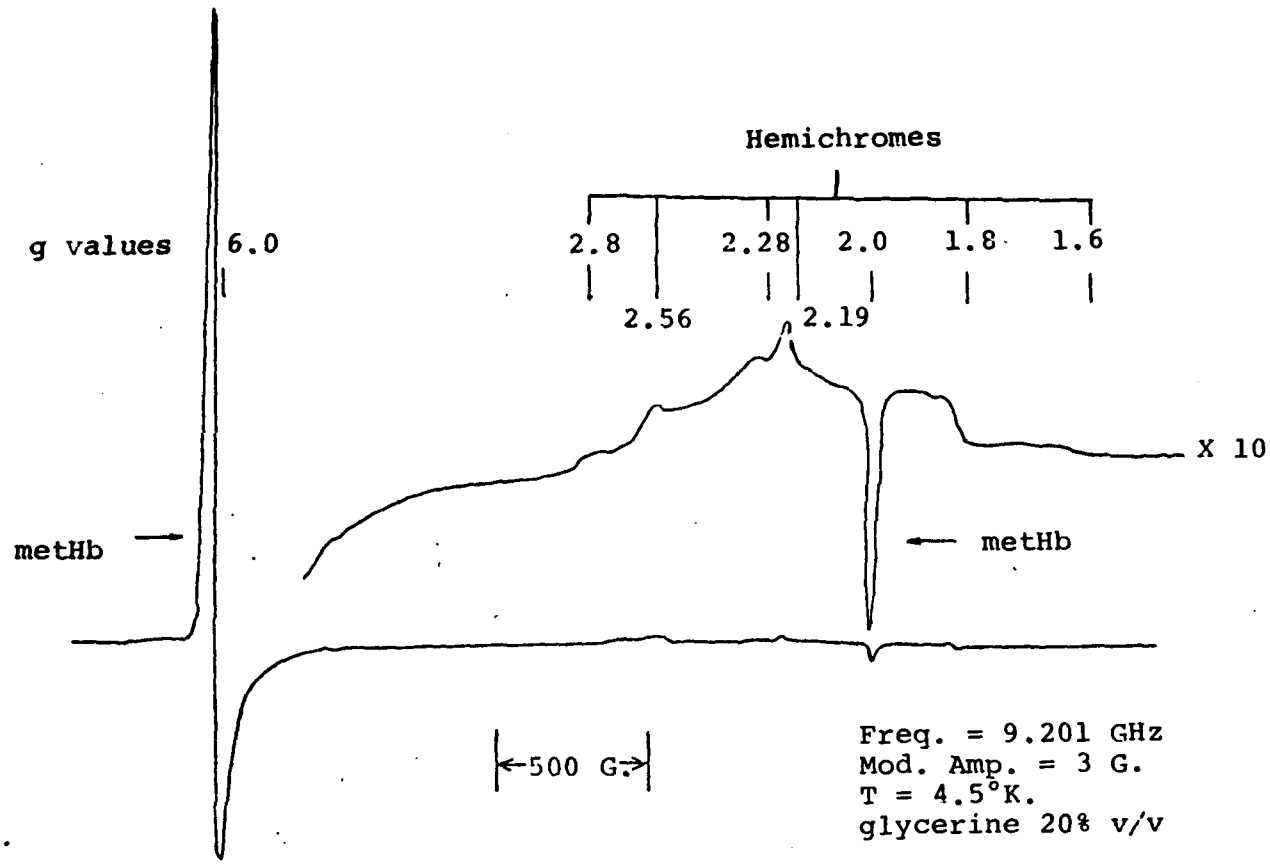

Figure 1. ESR spectrum of Fischer sample #11 in a frozen solution. Spectrum measured 12-17-80.

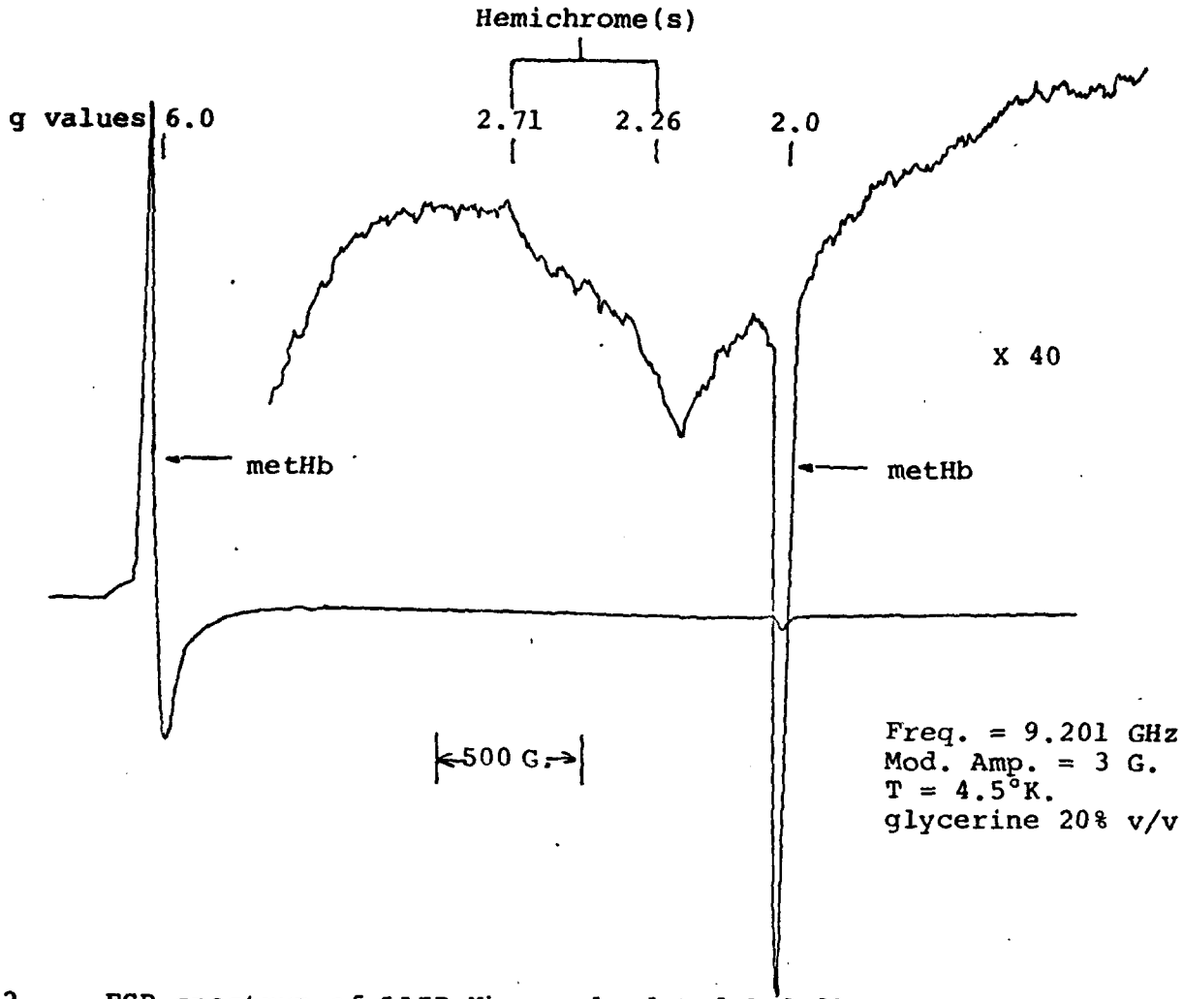

Figure 2. ESR spectrum of LAIR Hb sample dated 1-8-81. Spectrum is of a frozen solution measu- 21-81.

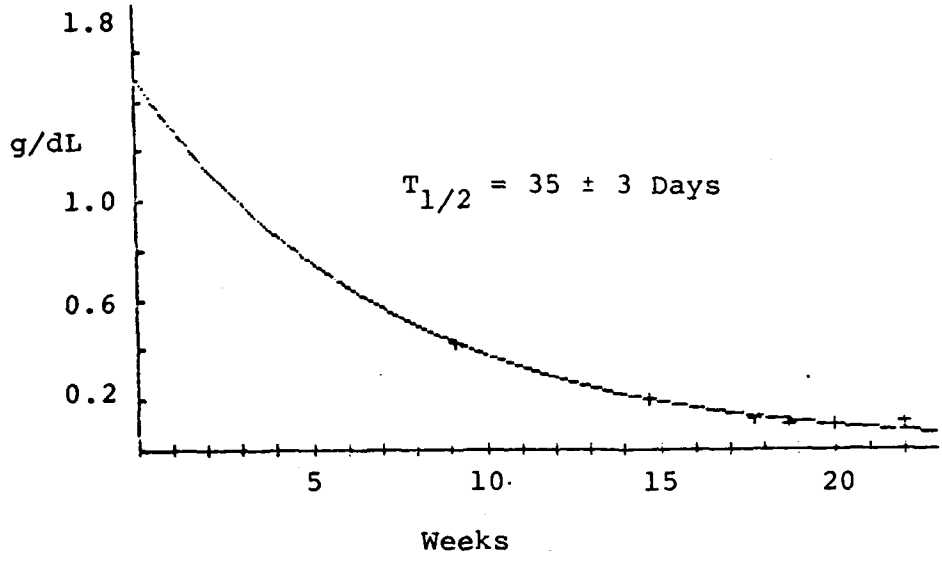

Figure 3. MetHb content of LAIR Hb sample dated 1-3-81 as a function of storage time at 4°C. Of the four LAIR samples, these data fit a single exponential the best.

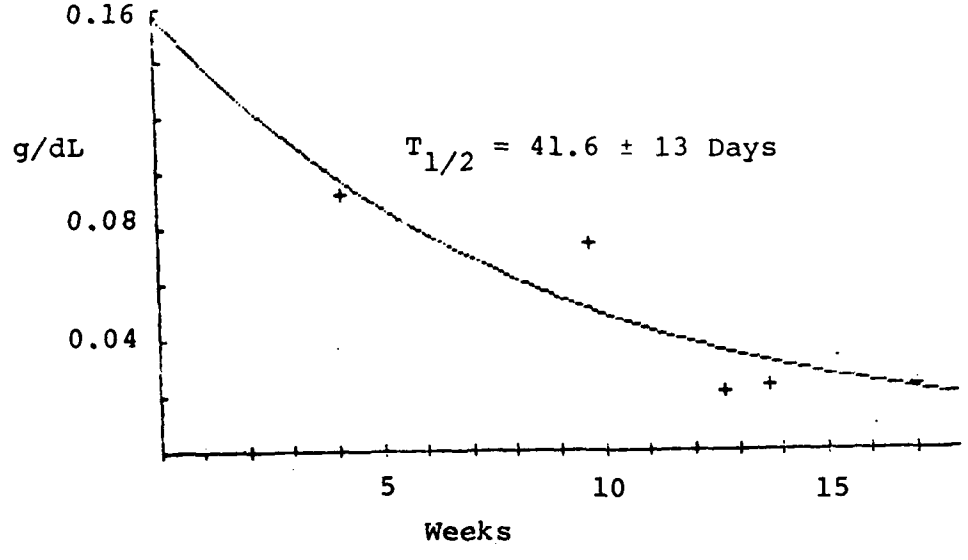

Figure 4. MetHb content of LAIR Hb sample dated 2-12-81 as a function of storage time at 4°C. These data fit a single exponential the worst.

Table I. Results of a 3 month study of metHb concentrations as a function of storage time in air-tight Blood-Packs as obtained from and prepared by LAIR.

Sample	Points Taken	metHb Conc. (g/dL) on 3-13-81	Half-life (days)
1-8-81	6	0.42	35.5 ± 3
1-29-81	5	0.15	37 ± 12
2-5-81	6	0.70	42.2 ± 9
2-15-81	5	0.092	41.6 ± 13
Average	-	-	39.1 ± 3.2

2. Discussion

While this project up to now has been largely explorative into the methods and techniques of quantifying EPR data of hemoglobin and it derivatives, several of our results are quite interesting.

The approximately equal concentrations of two species of hemichrome found in all the Fischer samples studied may be a consequence of the tetrameric nature of hemoglobin, which consists of two pairs of subunits, alpha and beta. The stucturally different subunits might have different hemichromes associated with each: one of which probably has a histidyl moiety bound to the distal position of the heme, the other an oxygeneous ligand. Peisach and Rachmilewitz[3,8] have shown that the isolated subunits do have characteristically different EPR spectra in the high spin form as well as in the low spin. However, the fact that we observed two hemichromes in equal amounts may be the result of other factors.

The metHb content of the samples prepared by LAIR decreased exponentially with a half-life of 39 days, while the Fischer samples are more stable. The single exponential function which characterized the metHb decay affirms earlier results of Peisach and Rachmilewitz[3] that the mechanism of the decay is first order or pseudo-first order.

$$P(\text{heme } d^5_{5/2}) \cdot OH_2 \dashrightarrow P(\text{heme } d^5_{1/2}) \cdot X$$

The mechanism they proposed consists of the irreversible formation of hemichromes from metHb. Our data, that the content of metHb decreased exponentially and that hemichrome content increased as metHb decreased, support this scheme. However, the possibility of another mechansism besides hemichrome formation cannot be disregarded, until quantitative studies on the increase of hemichromes during long term storage can be made and correlated to the disappearance of metHb. Peisach et al.[3] also found that over a period of 7 months only one-half of the metHb in a Hb sample incubated at 4°C was converted to hemichromes. Their result differs from ours, in which the conversion of half the metHb took place in 39 days. This difference in half-life is probably the result of different preparative techniques and storage conditions. The relatively large quantities of metHb in the Fischer samples after their storage for over a year indicates that either storage conditions or preparation has a strong effect upon the stability of the metHb.

The short half-life of the LAIR samples could be possibly due to contamination in handling. Since all four samples had very similar half-lives for their metHb it would seem unlikely that this contamination came about after the samples were placed in their respective blood-packs. This can be confirmed by measuring the ratio of the metHb and hemichrome signals in the spectra

that we took. This ratio should be proportional to their relative concentrations. When this ratio for each sample is extrapolated back to the dates of origin shown in Table I, it indicates, in every case, that only a small amount of hemichrome should have been present on the dates of origin. This means that the decay of the metHb in the LAIR samples was more-or-less continuous during the entire period after they were prepared. This decay could not have been affected by our sampling methods.

Another interesting fact about the hemichrome signals in the LAIR samples is that they seem to reach a maximum value close to 120 days after the dates shown in Table I. This is a tentative observation but it may be the result of some change in the protein after the hemichromes have built up through the decay of the metHb.

It would have been better if each sample had been divided into smaller units when they were prepared by LAIR. It would be also a good idea to place some of these original samples in glass containers since the plastic material in the blood-packs might be a key element in the difference in stability between the Fischer and LAIR samples.

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Appendix - The Presentation of ESR Spectra

Figures 1 and 2 show typical ESR spectra. What is shown in these figures is a plot of the first derivative of the ESR absorption versus the magnetic field. Since absolute absorption is not shown, the indicated signals are only proportional to the changes in the absorption with respect to the magnetic field. The samples that we use are frozen glasses with the heme groups held at all possible angles with respect to the magnetic field. In such a glass spectrum, the largest signals in the ESR spectrum occur when the heme groups are either parallel or perpendicular to the magnetic field. The molecules at these pareticular angles contribute the largest changes in the absorption since they represent the edges or limits of the actual ESR absorption. The molecules with their heme groups between about 5° and 85° with respect to the magnetic field may contribute very little to the ESR signals since their ESR absorption changes little with magnetic field.

The ESR absorptions from a metal ion complex with an odd number of electrons, like ferric ion, follow the formula

$$h\nu = g(\theta)\mu_B H$$

where h is Planck's constant, ν is the microwave frequency, μ_B is the Bohr magneton, another constant, and H is the magnetic field. The g-value, called $g(\theta)$ here, is a proportionality constant which is a characteristic of the ferric ion in the complex. We show it as $g(\theta)$ to indicate that it depends upon the angle between the heme group and the magnetic field. If the odd electrons in the ferric ion have no net contributions from their orbital motions, then $g(\theta)$ would be a constant equal to 2.00. The deviations of $g(\theta)$ from this value is an indication of the interaction between the ferric ion and the heme group and any other ligands which surround it.

In metHb the ferric ion has five odd unpaired electrons. This could lead to at least three separate ESR spectra. By going to almost 4°K we depopulate all of the possible states for these five electrons except for the lowest pair. This allows only one ESR spectrum. However, this spectrum shows very large orbital contributions to the g-value. When the magnetic field is parallel to the plane of the heme group, and not too large in value, $g(\theta) = 6$. On the other hand, when the magnetic field is perpendicular to the heme group, $g(\theta) = 2$. While all possible $g(\theta)$ values are possible between 6 and 2, the derivative nature of the ESR signals only give strong signals near $g = 6$ (the low field left side end) and near $g = 2$ (the right middle portion). The $g = 6$ signal for metHb is stronger than the $g = 2$ signal because there are more heme groups which are orientated close to having the magnetic field in their planes rather than being perpendicular to their planes. This is simple geometry since there is more

relative volume perpendicular to an axis rather than parallel to it.

The hemichromes have only one unpaired electron. This is a result of a strongly interacting ligand becoming bound to the ferric iron above the plane of the heme group. This greatly decreases the orbital contributions to the $g(\theta)$ values. As a result they all are fairly close to 2.0. This ligand also destroys the nice four-fold symmetry around the plane of the heme group and three distinct $g(\theta)$ values, called g_x, g_y and g_z, are possible. Their values are characteristic of the particular hemichrome, although they are all just slightly larger than 2.0 in value. Unfortunately, the strengths of the ESR signals from the hemichromes have almost no simple relation to those from the metHb. A smaller number of unpaired electrons gives a weaker signal and having three distinct g-values instead of two distinct g-values also gives a weaker signal. We would like to develop a good standaard for the hemichromes like we have for the metHb.