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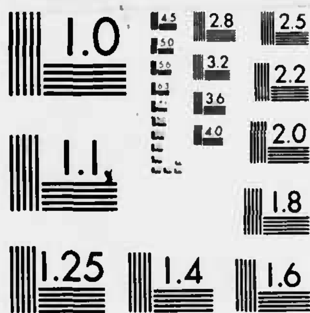

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EVALUATION OF THE STABILITY OF HEMOGLOBIN IN STORAGE BY ELECTRON SPIN RESONANCE

Annual and Final Report

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SEPTEMBER 30, 1983

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Summary

A series of hemoglobin (Hb) samples were received from LAIR. They were stored in two types of containers at 4°C and one set was frozen at dry ice temperatures. These samples were analyzed for metHb and hemichrome content as a function of storage time using ESR. The samples stored in sealed glass bottles at 4°C had a very uniform increase in metHb over the period of the study (17 months). This rate of increase appeared exponential in nature. The frozen samples, which were also stored in glass bottles, showed no increase in metHb over the period during which they were frozen. However, upon thawing, these samples gave an increase in metHb at a rate similar to those samples which had never been frozen. The samples, which had been stored at 4°C in a blood-pack, had a rate of increase in metHb identical to those stored in glass bottles.

The total hemichrome content of the storage samples is estimated at 30% to 45% of the metHb content for samples A and B, and 45% to 60% for samples C, D and E. However, all the hemichromes are in simple conformational equilibria with the metHb. Both the metHb and all the observed hemichromes can be converted *in vivo* back to Hb, and they are not expected to affect the viability of the solution as a resuscitation fluid.

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Introduction

Human hemoglobin (Hb A), the oxygen carrying pigment found in blood, holds the potential of being a viable resuscitation fluid when prepared in suitable solutions (1). Hb A can be stored over long periods of time, both in solution and in lypholyzed form, with retention of function as an oxygen carrier; in this aspect, it is superior to whole blood (2). Consequently, to insure the long term stability of Hb A, mechanisms of degradation as a function of preparation and storage conditions should be studied.

One mechanism of Hb A denaturation is the oxidation of Hb A by molecular oxygen to methemoglobin (metHb) and its subsequent conversion to hemichromes with a possible irreversible loss of function (3). The oxidation, itself, is of the iron, which is found in each of the four heme moieties, from the ferrous to the ferric form. When this process occurs in vivo, it may be reversed by enzymatic activity before the formation of hemichromes. However, in vitro, the oxidation is not reversed without the introduction of reducing agents.

ESR (Electron Spin Resonance) is a suitable tool for studying the progress of this degradative route (3,4,5). Though the ferrous form of the heme group is normally transparent to ESR, both metHb (high-spin ferric) and the various hemichrome derivatives (low-spin ferric) are observable and distinguishable at liquid helium temperatures (ca. 4.5°K) (6,7,8). Unlike standard analyses such as optical spectroscopy, the ESR transparency of the ferrous form of the heme group allows the detection and quantification of small amounts of metHb and hemichromes even in the presence of large amounts of Hb A.

In this study, we devised techniques to quantify the ESR data of stored Hb A and attempted to evaluate the conversion of Hb A to metHb and hemichromes as a function of storage time and storage conditions. From the g-values

observed for the hemichromes it is also possible to determine the nature of the distortion at the iron site. The samples for study and appropriate standards were supplied to us by LAIR.

Experimental Schedule

In February of 1982, LAIR prepared and delivered to us five groups of samples to be analyzed for metHb and hemichrome content over a period of storage time (17 months). Samples labeled A, B and D were each supplied in ten 50-ml glass bottles, fitted with serum caps, and were stored at 4°C in a walk-in refrigerator. Aliquots of sample C also arrived in glass bottles, but we stored them at dry ice temperatures in a special low-temperature commercial freezer. Sample A was entirely in a single 50 ml blood-pack and it was stored in the walk-in refrigerator at 4°C. All samples were supplied and stored under air at normal pressures. They were 0.1 M saline solutions at physiological pHs. All samples except B had a nominal Hb A concentration of 7%(g/dL). Sample B had been diluted.

For purposes of standardizing the ESR data, a 3.59% sample of cyanmetHb and a 7.68% sample of metHb were prepared by LAIR for this study.

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) to cool the sample. The temperatures 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 NMR gausmeter (FE-50). Dehumidified air constantly flowed through the Varian V4531 cavity to prevent the condensation of water, which would interfere with sensitivity. All quantitative measurements were made between 4.5 and 5.5° K and at low microwave power level to prevent saturation of the samples and non-linear response at signal intensity.

2. General Procedure

Samples were removed from their containers either by syringe in the case of samples stored in glass bottles or according to protocol if they were stored in blood-packs. They were then diluted 20% v/v with glycerine and placed in quartz ESR tubes, ca. 3mm I.D., and quickly frozen in liquid nitrogen. This was done to insure the formation of a "good glass" and to prevent further degradation if the samples were prepared on a day previous to ESR analysis. The added glycerine also helped to form good glasses, which would have a random orientation of molecules. In general, three ESR tubes were prepared for each sample, one of which contained an aliquot of the sample which had been centrifuged for 20 minutes at 30,000xg. The three seperate measurements allowed us to check the reliability of the measurement and to

determine if centrifugation altered the spectra. Concentrated NaOH was used in the cleanup procedure to minimize the possibility of hepatitis transmission from the samples.

The ESR spectra was then measured and the metHb intensities were compared to the soft glass standard's ESR intensity, which was measured several times in the course of an analysis, and the data reduced.

3. Standardization

A soft glass rod, 5mm in diameter, was the secondary standard for quantifying metHb concentrations from ESR observations. The high-spin ferric resonance ($g = 4.33$) due to iron impurities in the glass was found suitable for this purpose. The rod itself was calibrated against a metHb sample of a known concentration, which was supplied by Dr. Gerald Moore and Dr. Ross Tye of LAIR. The soft glass rod was chosen as a secondary standard for its stability, as compared to primary standards such as the metHb sample used to calibrate the rod. The same glass rod was used for the entire study.

ESR spectral data are presented as the first derivative of the absorption spectra. Peak to peak heights of the metHb ESR signals were the parameters for quantifying data. Because all the samples give the same metHb signals, the relative peak to peak heights are an excellent measure of their metHb concentrations for randomly oriented samples. The hemichromes, on the other hand, give a variety of spectra, and without an exact spectral analogue of known concentration the ESR spectrum of a sample of cyanmetHb, which was supplied by LAIR, was doubly integrated and compared as a standard with the doubly integrated hemichrome signals of the samples in the study. Also, a computer program was written to simulate the hemichrome resonances and estimate their relative amounts as a function of their downfield peaks.

4. Sources of Error

For all quantitative methods of analysis, sources of error must be recognized in order to minimize them. As we continue studying Hb A, we have come across several important sources of possible error in measurement.

A major source of error is found in tuning the ESR instrument itself. For each sample placed in the instrument, the phase and frequency of the microwaves irradiating the sample must be altered to maximize the sensitivity or to insure that sensitivity of one measurement is equal to the next. However, as this study progressed, standard settings were established and the problem has lessoned.

Variation in sample tube diameter proved a major concern. Signal intensity of ESR measurements is dependent on the amount of the sample in the microwave field. Ideally, one would use the same quartz tube for all measurements. However, tube breakage and the liquid helium and time-wasting method of preparing samples during a helium run make the use of one tube not efficient or possible. Commercially manufactured tubes seem to vary slightly enough to cause significant errors in measurements. Care is now taken in choosing tubes of a truly uniform shape.

Other origins of possible error include sample preparation and small variations in temperature.

By making repeated measurements on identical samples prepared in different tubes, we determined that a particular measurement may be in error by 30%, regardless of the magnitude of the sample concentration. In general, however, our estimate of error in signal intensity rarely exceeds 10%.

Results

a. Soft Glass Rod Calibration

In the method described above, the soft glass rod, which served as a secondary standard for quantifying the signal intensity of the metHb resonances, was calibrated against a primary metHb standard, supplied to us by LAIR. The standard was stated to be 100% of metHb at a concentration of 7.68%(g/dL). However, there was approximately 2.4% of H-metHb present in the standard. This fact may have affected the soft glass rod calibration.

Two tubes of this standard were prepared by the method above and analyzed by ESR and then diluted with a 100 mM solution of NaCl to three other concentrations (i.e., 1.506, .768 and .1536 % in metHb) and again analyzed for metHb. The results of these relative intensity (relative to the soft glass standard) measurements were averaged and plotted against their known concentrations of metHb. The slope, which was calculated by a least squares analysis, of this curve is the calibration constant correlating relative intensity measurements to actual metHb concentrations.

b. Growth of MetHb in Storage Samples

Five samples, which were prepared by LAIR were analyzed for metHb and for hemichrome content as a function of time over a period of 17 months. Fig. 1 contains a typical ESR spectrum of a sample. Evident in this spectrum are the resonances attributable to metHb and to hemichromes. Plots in Figs. 2 through 5 summarize the results of the metHb analyses. Table 1 contains the results of each analysis.

The metHb content of all samples except sample C, which was initially stored at dry ice temperatures, were observed to increase by at least fourfold

over the period of study. The metHb concentration of sample C remained constant until it was removed from the freezer and placed in the walk-in refrigerator at 4°C. Once thawed, the rate of metHb growth in this sample was commensurate with samples A, D and E.

The growth of the metHb in the samples appeared initially as a linear function of time, but later it was observed that an exponentially increasing function fit the data better. This became clear when the rate of metHb growth decreased at the larger times. The data for samples A, B and D were treated by least-squares analyses to estimate the rate constants for the exponential function. These results are found in Table 2. The curves drawn through the data points in Figs. 2 and 4 were calculated on the least squares results. The curve drawn in Fig. 5, which is of the data for sample E, was calculated on the least squares analysis of sample A. Sample E had too few data points for a reliable fit.

c. Estimation of Hemichrome Content

There were only two hemichrome species evident in all the samples under study. The first is called H-metHb, which is characterized by the principal g-values of 2.8, 2.27 and 1.69. The second is called O-metHb, and it is characterized by the g-values of 2.58, 2.19 and 1.85. O-metHb is commonly taken to be an adduct of the hydroxide ion and metHb. H-metHb is believed to be the resulting complex of the binding of the imidazole moiety of the distal histidine residue to the iron of the metHb heme (3,4,5,10). Both appear to be in a pH dependent equilibrium. The intensity of both hemichrome signals grew at the same rate as the increase in metHb content. Also, the ratio of intensities of the two hemichromes, as measured by the amplitudes of their most downfield resonances, remained constant for each sample over the period of study.

In order to evaluate the relative concentrations of these hemichromes in the storage samples, a computer program was written to simulate the lineshapes of these species. In this program, it is assumed that the relative transition intensities of the species are the standard function of their g-values, and the overall lineshape is taken to be a gaussian form (11,12). The output of one such simulation is in Fig. 6, where the relative concentration of O-metHb is set at 70% of H-metHb. From these simulations it is estimated that the two species are always present in equal amounts in samples A and B and that there is three times the amount of H-metHb as O-metHb in samples C, D and E.

The ESR spectrum of a 3.59% sample of cyanmetHb was doubly integrated and the result was compared to the hemichrome spectra of the storage samples to estimate the total amount of hemichromes present in the samples. At all times during our study the hemichrome contents of samples A and B are 30 to 45% of the metHb present, while in samples C, D and E they are 45 to 60% of their metHb.

Since the characteristics of samples A and B differ significantly with those of samples C, D and E, the pHs of all samples but E were measured in the sixty-second week of the study. Samples A and B had pHs of 7.4 and samples C and D, pHs of 6.8.

There was no residue remaining or spectral differences noted upon centrifugation of any of the samples (30,000xg, 20 min.).

TABLE 1

Data from ESR measurements on LAIR storage samples analyzed for metHb content (Feb. 17, 1982 through May 27, 1983)

Date	Number of Measurements	Average metHb conc. (g/dL)
<u>Sample A</u>		
2-17-82	2	0.50 ± .08
2-24-82	3	0.40 ± .08
3-8-82	3	0.68 ± .17
4-7-82	1	0.7
4-21-82	3	1.16 ± .14
5-20-82	2	0.95 ± .01
7-12-82	3	2.0 ± .16
9-27-82	3	2.4 ± .75
11-1-82	3	2.8 ± .3
3-8-83	3	3.2 ± .6
4-29-83	3	3.3 ± .3
<u>Sample B</u>		
2-17-82	2	0.14 ± .04
2-24-82	3	0.14 ± .04
3-8-82	3	0.18 ± .09
4-7-82	1	0.17
4-21-82	3	0.23 ± .01
5-20-82	2	0.31 ± .05
7-12-82	3	0.53 ± .03
11-1-82	3	0.6 ± .1
3-8-83	3	1.0 ± .15
4-29-83	3	1.0 ± .08
5-27-83	3	0.9 ± .1
<u>Sample C</u>		
2-17-82	2	0.39 ± .09
2-24-82	3	0.39 ± .07
3-8-82	3	0.29 ± .05

(table cont'd on next page)

Table 1, Cont'd

Date	Number of Measurements	Average metHb conc. (g/dL)
Sample C (cont'd)		
4-7-82	not measured	
4-21-82	3	0.34 ± .02
5-20-82	2	0.27 ± .04
7-12-82	3	0.53 ± .02
9-27-82	3	2.5 ± .4[a]
11-1-82	3	0.9 ± .05[b]
3-8-83	3	2.1 ± .3[b]
4-29-83	3	2.5 ± .6[b]
Sample D		
2-17-82	2	0.4 ± .1
2-24-82	3	0.4 ± .1
3-8-82	2	0.42 ± .01
4-7-82	not measured	
4-21-82	3	0.70 ± .08
5-20-82	2	1.0 ± .1
7-12-82	2	1.8 ± .1
9-27-82	not measured	
11-1-82	3	3.3 ± .12
3-8-83	3	3.2 ± .6
4-29-83	3	3.3 ± .07
5-27-83	3	3.5 ± .7
Sample E		
2-17-82	2	0.39 ± .04
2-24-82	3	0.39 ± .04
3-8-82	3	0.48 ± .01
4-7-82	not measured	
4-21-82	3	0.87 ± .06
5-20-82	2	0.94 ± .09
7-12-82	not measured	
9-27-83	3	2.3 ± .3

Notes: a. Sample never frozen.
b. Sample thawed on 9-23-82 and placed in 4°C refrigerator.

TABLE 2

Results from leasts squares analysis of metHb concentration data from LAIR storage study.
Feb. 10, 1982 is assumed to be the initial storage date.

Sample	C_0 g/dL	C_t g/dL	k $week^{-1}$	$\frac{0.69}{k}$ week
A	0.3	4.3	0.02	35
B	0.2	1.7	0.01	70
D	0.2	4.4	0.02	35

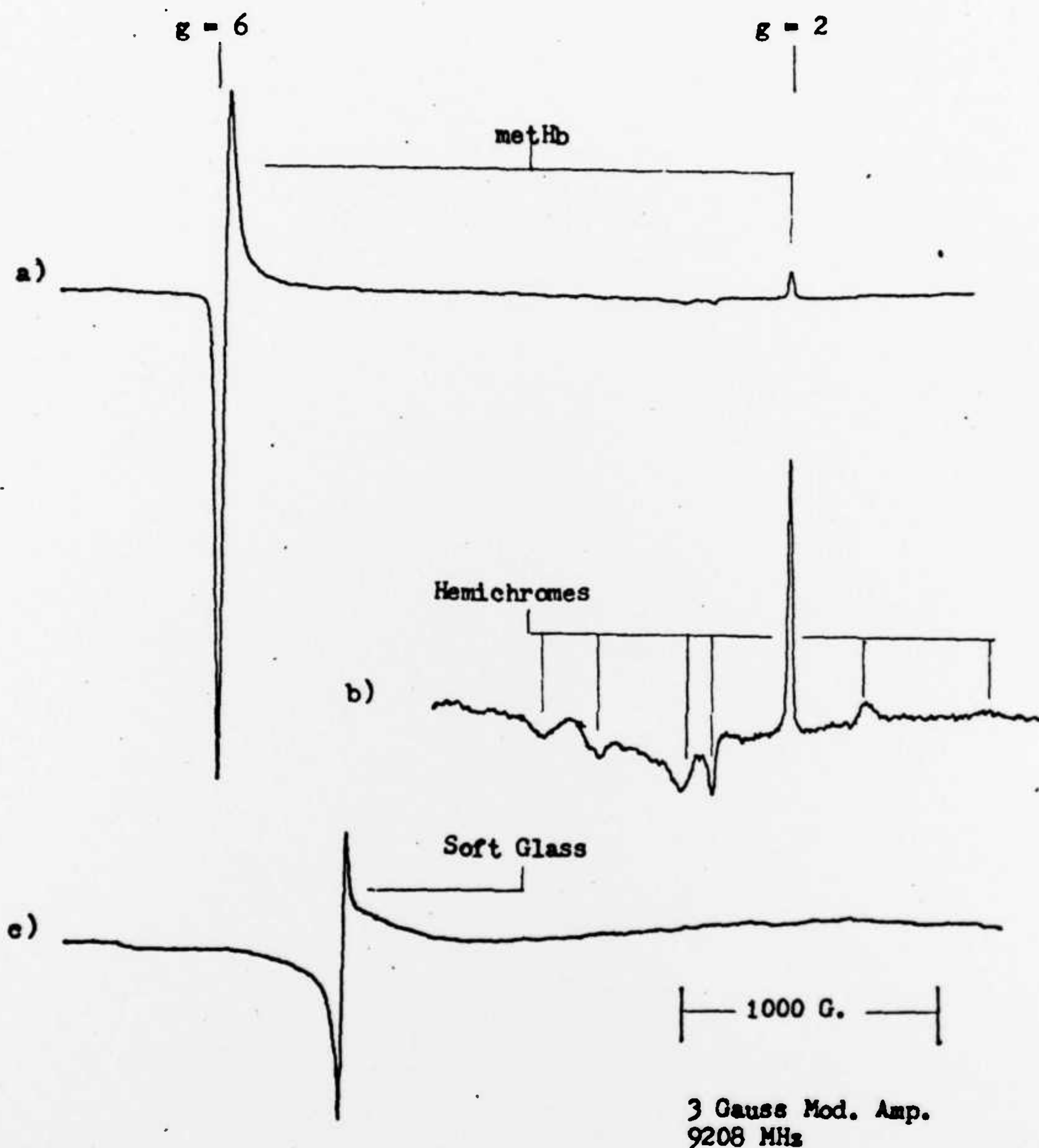


Fig. 1 a) ESR spectrum of sample A, measured on 3-8-83 at 4.5°K.
b) Same as a), but the gain has been increased by a factor of 10.
c) ESR spectrum of soft glass standard. Same conditions as a).

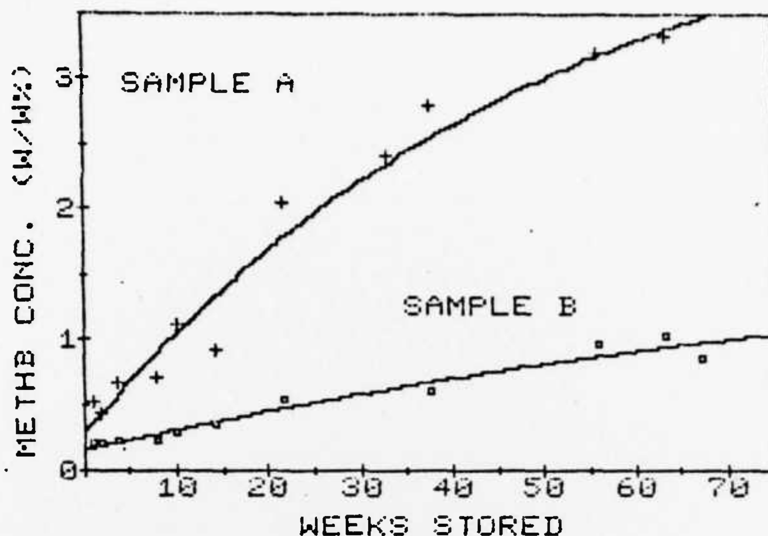


Fig. 2 Plots of metHb content in samples A and B as a function of time.

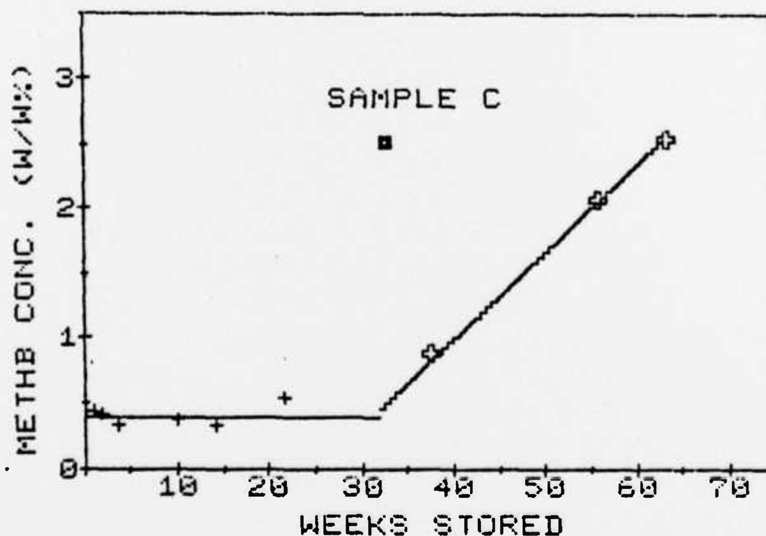


Fig. 3 Plots of metHb content in sample C as a function of time. Crosses indicate those aliquots which were frozen. Open crosses indicate those aliquots which were removed from the dry ice freezer in the thirty second week of storage. The solid square represents the aliquot which had never been frozen.

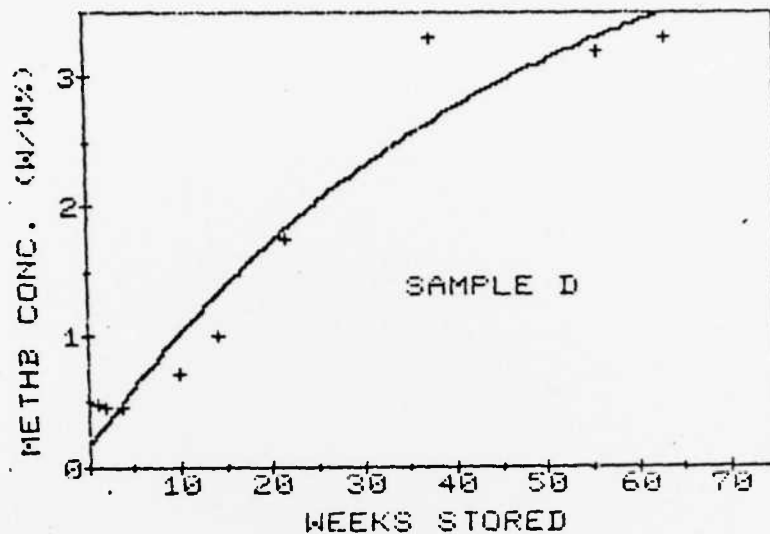


Fig. 4 Plot of the metHb content in sample D as a function of time.

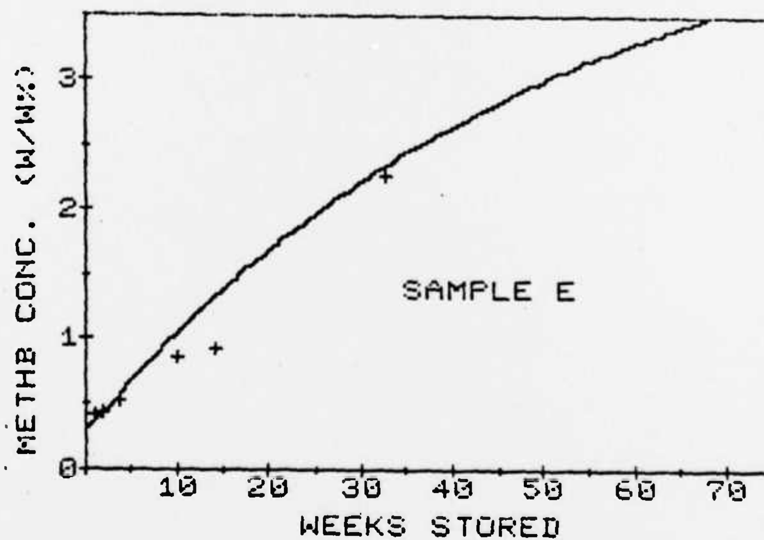


Fig. 5 Plot of the metHb content in sample E as a function of time.

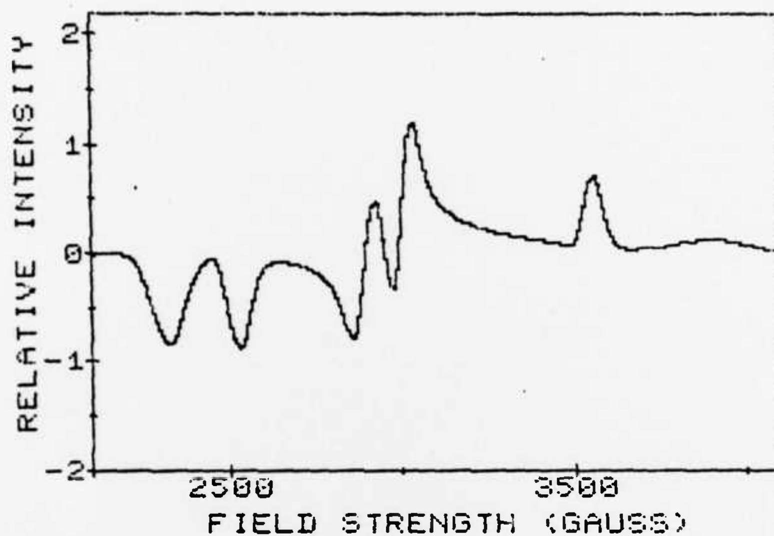


Fig. 6 Computer simulated ESR spectrum of hemichromes found in the storage samples.

Discussion

a. The Build-up of MetHb

We have now studied the build-up of the metHb in these samples over 17 months. During that time the initial growth slowed as shown in Figs. 2 and 4. They can be fit with a simple exponential growth curve of the type

$$C = C_0 + C_t(1 - e^{-kt})$$

where t is the time in weeks past Feb. 10 1982, and C_0 is the amount of metHb in the samples on that date. The values found for C_0, C_t and k for the A, B and D samples are given in Table 2. Also given are the half-lives which are equal to 0.69/k.

In sample C, which was kept frozen, there is no detectable formation of metHb until it is thawed. At this point the build-up follows the curve expected if t=0 is taken as the time for thawing. This is shown in Fig. 3.

In sample E, which was stored in a blood-pack, the build-up of metHb followed exactly the curve shown for sample A, which was sealed in glass. This is shown in Fig. 5, where the calculated curve was that for sample A and the points are those observed for sample E.

All of our observations about the build-up of the metHb in these samples can be summarized as follows:

1. When stored at 4°C under air, the half-life for the build-up of metHb ranged from 35 to 70 weeks.
2. When frozen at about -70°C, there is no build-up of metHb, and when thawed, the build-up then follows the normal pattern.

3. The build-up of metHb is the same in a blood-pack as it is in a sealed glass container.

The build-up of metHb in these samples is also accompanied by a build-up of certain hemichromes. The mechanism for the build-up of both these forms of oxidized hemoglobin is discussed in the next section.

b. The Build-up of Hemichromes

The various types of low-spin iron species which can be formed in both normal and modified Hb have been investigated over the last 15 years, most notably by J. Peisach and W. E. Blumberg. In 1981 a clear, concise review of this work was published (5).

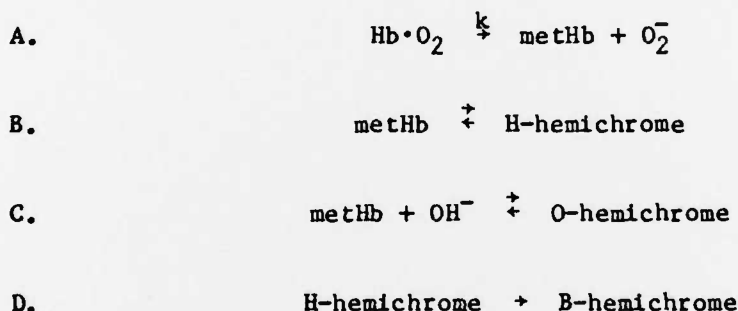
These authors have found that the EPR of the hemichromes can be used to divide them among distinct classes. These classes are distinguished by the distortions around the iron which force the metHb to go from its high-spin state with five unpaired electrons to the various hemichromes which have only one unpaired electron. These distortions can be judged by a complicated, and not completely unambiguous theoretical treatment based upon the g-values observed for the hemichromes.

If we used the classes developed by Peisach and Blumberg, we only have observed the formation of the hemichromes designated as the H- and O-hemichromes. The H- form is what we previously called the nitrogenous type hemichrome, where it is assumed that a histidine nitrogen has occupied an axial position around the iron. In the O- form it appears to be an OH^- in the axial position.

Both the H- and O- forms that we observe have only a small conformation change in the protein and they are in a pH dependent equilibrium with the metHb. This was clear in our previous report where we observed that they were

always in a fixed ratio for a given sample, but this ratio varied from sample to sample. From our pH measurements, we can conclude that the lower pH values observed for samples C and D can account for the fact that these samples contain more of the H-hemichrome.

Our observations about the simultaneous build-up of both metHb and the two hemichromes can be explained by the following mechanism, which is fully consistent with the studies of Peisach and Blumberg. In this mechanism steps B and C are equilibria, and step D has not been observed in our samples over the 17-month period.



A. $$\text{Hb}\cdot\text{O}_2 \xrightarrow{k} \text{metHb} + \text{O}_2^-$$

B. $$\text{metHb} \rightleftarrows \text{H-hemichrome}$$

C. $$\text{metHb} + \text{OH}^- \rightleftarrows \text{O-hemichrome}$$

D. $$\text{H-hemichrome} \rightarrow \text{B-hemichrome}$$

The O_2^- produced from the oxidation of the iron can be of concern for the stability of the Hb, but we have found no evidence that it plays a role in the chemistry around the iron site. The B-hemichromes have a further conformational change in the protein such that a denaturization occurs, and these B-hemichromes can not be returned either in vivo or in vitro to $Hb \cdot O_2$. However, up to step D these reactions are all considered to be reversible, and the build-up of metHb and the H- and O-hemichromes should not alter the viability of the samples for resuscitation purposes.

References

1. F. DeVenuto, H. I. Friedman, J. R. Neville and C. C. Peck, Surg. Gynecol. Obstet. 149, 417-36 (1979).
2. F. DeVenuto, Z. Zegna and K. Busse, ibid. 148, 69-75 (1979).
3. E. A. Rachmilewitz, et al., J. Biol. Chem. 246, 3356-66 (1971).
4. J. Peisach, et al., ibid., 246, 3342-55 (1971).
5. W. E. Blumberg, "Methods in Enzymology," (E. Antonini, L. Rossi-Bernardi and E. Chiaccone, Eds.), Vol. 76, 312-29, Academic Press (1981).
6. A. Ehrenberg, Arkiv. for Kemi 19, 119-28 (1962).
7. G. Palmer, "The Porphyrins," (D. Dolphin, Ed.), Vol. V, 313-53, Academic Press (1979).
8. J. F. Gibson, et al., Diss. Fara. Soc. 26, 72-80 (1958).
9. J. Peisach, et al., Proc. Natl. Acad. Sci. USA 63, 934-39 (1969).
10. J. Peisach, et al., Ann. NY Acad. Sci. 206, 310-27 (1973).
11. C. P. Scholes, J. Chem. Phys. 52, 4890-95 (1970).
12. R. Aasa and T. Vanngard, J. Magn. Res. 19, 300-15 (1975).

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