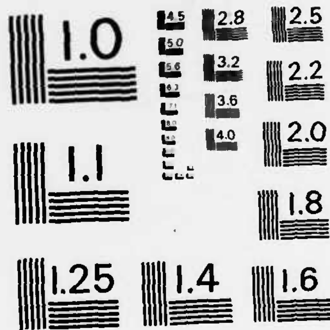
AD-A139 165 EVALUATION OF THE STABILITY OF HEMOGLOBIN IN PREPARATION AND STORAGE BY ELECTRON SPIN RESONANCE(U) CALIFORNIA UNIV BERKELEY R J MYERS 31 AUG 82 1/1

UNCLASSIFIED DAMD17-80-C-0142 F/G 6/5 NL


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

AD A139165

(1)

AD

REPORT NUMBER 2

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

ANNUAL SUMMARY REPORT ([illegible])

ROLLIE J. MYERS

AUGUST 31, 1982

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-80-C-0142

University of California
Berkeley, CA 94720

DTIC
ELECTE
MAR 2 1 1984
S D
B

Approved for Public Release
Distribution Unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

DTIC FILE COPY

84 03 15 084

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

Field	Entry
1. REPORT NUMBER	
2. GOVT ACCESSION NO.	AD-A139165
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle)	Evaluation of the Stability of Hemoglobin in Preparation and Storage by Electron Spin Resonance
5. TYPE OF REPORT & PERIOD COVERED	Annual Report September 1981 - Sept. 1982
6. PERFORMING ORG. REPORT NUMBER	
7. AUTHOR(s)	Rollie J. Myers
8. CONTRACT OR GRANT NUMBER(s)	DAMD17-80-C-0142
9. PERFORMING ORGANIZATION NAME AND ADDRESS	University of California, Berkeley M11 Wheeler Hall Berkeley, California 94720
10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS	62772A.3S162772A874.AC.145
11. CONTROLLING OFFICE NAME AND ADDRESS	Commander US Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701
12. REPORT DATE	August 1982
13. NUMBER OF PAGES	20 pages
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	
15. SECURITY CLASS. (of this report)	Unclassified
15a. DECLASSIFICATION/DOWNGRADING SCHEDULE	

16. DISTRIBUTION STATEMENT (of this Report)

Approved for Public Release; Distribution Unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

Summary

A series of hemoglobin 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 using ESR for metHb and hemichrome content as a function of storage time. The 4°C samples stored in glass bottles had a very uniform increase in metHb which was linear in time over the five months they were studied. The frozen samples had no changes in their metHb with time once they were frozen. The 4°C sample stored in a blood-pack had a noticably slower increase in metHb with time.

The hemichrome content of only a special sample was analyzed by double integration of its ESR spectrum and we found its metHb to be 4.5 0.4% (g/dL) and its hemichrome to be 5 1% (g/dL). It is proposed that the two different types of hemichromes are both in chemical equilibrium with the metHb in the smaples. The two equilibrium constants appear to vary with the nature of the samples.

Accession For
NTIS GRA&I ☑
DTIC TAB ☐
Unannounced ☐
Justification PER CALL JC
By
Distribution/
Availability Codes
Dist A-1 | Avail and/or Special

TABLE OF CONTENTS

page

Introduction....................4

Experimental Schedule....................5

Experimental Methods....................6

Results....................8

Discussion....................17

References....................19

Tables and Figures

Table 1: "Results of 5 month study of LAIR Samples"....................10

Table 2: "Least Squares Analysis of Results"....................11

Figure 1: "Soft Glass Calibration"....................12

Figure 2: "ESR Spectrum of Special Sample....................13

Figure 3: "ESR Spectrum of Sample A"....................14

Figure 4: "MetHb Contents of Samples A and B"....................15

Figure 5: "MetHb Contents of Samples C and D"....................15

Figure 6: "MetHb Content of Sample E"....................16

Introduction

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

One mechanism of HbA denaturation is the oxidation of HbA 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 (3,4). Though the ferrous form of the heme group is normally transparent to ESR, both metHb (high-spin ferric) and hemichromes (low-spin ferric) are easily observable and distinguishable at liquid helium temperatures (approximately 4.5° K) (5,6,7). Unlike standard analyses such as vis/uv spectroscopy, the transparency of the ferrous form of the heme groups allows the detection and quantification of small amounts of ferric forms even in the presence of large amounts of the ferrous forms.

In this study, we devised techniques using external standards to quantify the ESR data of stored HbA and used these techniques to evaluate the conversion of HbA to metHb and from MetHb to hemichromes as a function of storage times and storage conditions. The samples, which were studied, and the standards were prepared by LAIR for the study.

Experimental Schedule

In the first part of the current contract period, LAIR prepared and delivered to us a concentrated sample of cyanmethemoglobin as a possible standard for quantifying ESR data on hemichromes in storage samples. The stated concentration of this sample was 3.59% in cyanmetHb. We measured the ESR spectrum of this sample and used it as a double integration standard for the special sample which we received later from Dr. Neville.

In February of this year, LAIR next supplied us with five groups of samples to be analyzed for metHb and hemichrome content over the following eight months. Samples labeled A, B, and D were each supplied in ten 50 ml glass bottles, which were fitted with serum caps, and were stored at 4°C in a walk-in refrigerator. Aliquots of sample C also arrived in glass bottles and were stored at dry ice temperatures in a special commercial freezer. Sample E was entirely in a 50 ml blood-pack and stored in the walk-in refrigerator at 4°C. All samples were stored under air at normal pressure.

A special 50 ml sample stored at 4°C was received from Dr. Ryan Neville and was analyzed for metHb and hemichrome content in April of the contract period. The cyanmetHb received earlier served as the hemichrome standard for the hemichromes in this sample.

In July a metHb standard was received from Dr. Gerald Moore and used to calibrate the soft glass standard, which was used as the relative standard for metHb ESR intensities of the stored samples.

The analyses of the stored samples will be continued until all the samples are depleted.

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 30,000 x g. The three separate 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 in late July. 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.

ESR spectral data are presented as the first derivative of the absorption

spectra. Peak to peak heights of the metHb ESR resonances were the parameters for quantifying data. Because all the samples give the same metHb signals, the relative 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 double integration of hemichrome spectra of a sample of cyanmetHb, which was supplied by LAIR, was doubly integrated numerically and compared as a standard to the double integration of the hemichrome resonances of the Neville sample. Otherwise, only the relative growth of hemichrome content was followed.

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 HbA, 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 lessened.

Variation in tube diameter proved itself a major concern. Signal intensity of ESR measurements is dependent on the amount of placement of the sample in the irradiating 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, the estimate of error rarely exceeds 10%.

Results

1. 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). There appeared in the ESR spectra of this standard some discernable traces of hemichromes.

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. Fig. 1 contains this plot. 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. The Special Sample

We received from Dr. Ryan Neville of LAIR a sample to be analyzed on 20 April. The analysis was run on 22 April. The ESR of this sample is displayed in Fig. 2. The results of this analysis indicated that the metHb concentration was 9.4% 0.4. Using the cyanmetHb sample we had received earlier from LAIR the total hemichrome concentration was estimated to be 5% ±1. There were two hemichromes that were present in the sample in approximately equal concentrations and can be seen in Fig. 2 near g = 2. Upon centrifugation, this sample left a small residue, but centrifugation did not appear to affect the ESR spectra.

c. Storage Studies on Samples Prepared by LAIR

Five samples prepared by LAIR were analyzed for metHb and hemichrome content as a function of storage time over a period of 22 weeks. Fig. 3 is a typical ESR spectra of a sample evincing the metHb and hemichrome resonances. Plots in Fig.s 4 through 6 summarize the results of the metHb analyses. Table 1 contains the results of all the analyses.

The metHb content of all samples except sample C, which was stored at dry ice temperatures, was observed to increase by at least 100% over the period of study. The metHb concentration of sample C appeared to remain constant to within the precision of measurement. The growth of the metHb intensities appeared linear with respect to storage time. The data was subjected to a least squares numerical treatment and the results are summarized in Table 2. The lines plotted through data points in Fig.s 4 - 6 were calculated from the least squares results.

The formation and growth of two sets of hemichrome resonances were observed over the period of study in all samples except C. Sample C's hemichrome content appeared to remain stable. The inner set of hemichrome resonances (g_z = 1.8, g_y = 2.19 and g_x = 2.8) most certainly belong to the dihistidyl form of the heme group and irreversibly converted from metHb (8). These two forms are found in approximately equal concentrations in samples A and B and their relative growth rates are equal. Samples C, D and E contain primarily the dihistidyl form over the hydroxyl in a ratio of approximately 2.5/1. However, the relative growth rates of the two forms in these samples are equal, so that their concentration ratio remains constant. The difference in hemichrome ratios between samples A and B, and samples C, D and E is probably a reflection of a pH difference, where A and B are more basic than C, D and E.

None of the samples in the storage study left the residue upon centrifugation.

Table 1:

Data from ESR measurements on LAIR storage samples analyzed for metHb content (Feb. 17 through July 12 1982).

Date (1982)	No. of Measurements	Average metHb conc. (g/dL)
Sample A		
2-17	2	0.50 ± .08
2-24	3	0.40 ± .08
3-8	3	0.68 ± .17
4-7	1	0.7
4-21	3	1.16 ± .14
5-20	2	0.95 ± .01
7-12	3	2.0 ± .16
Sample B		
2-17	2	0.14 ± .04
2-24	3	0.14 ± .04
3-8	3	0.18 ± .09
4-7	1	0.17
4-21	3	0.23 ± .01
5-20	2	0.31 ± .05
7-12	3	0.53 ± .03
Sample C		
2-17	2	0.39 ± .09
2-24	3	0.39 ± .07
3-8	3	0.29 ± .05
4-7	not measured	
4-21	3	0.34 ± .02
5-20	2	0.27 ± .04
7-12	3	0.53 ± .02
Sample D		
2-17	2	0.4 ± .1
2-24	3	0.4 ± .1
3-8	2	0.42 ± .01
4-7	not measured	
4-21	3	0.70 ± .08
5-20	2	1.0 ± .1
7-12	2	1.8 ± .1

(table cont'd next page)

Table 1, Cont'd

Date (1982)	No. of Measurements	Average metHb conc. (g/dL)
Sample E		
2-17	2	0.39 ± .04
2-24	3	0.39 ± .04
3-8	3	0.48 ± .01
4-7	not measured	
4-21	3	0.87 ± .06
5-20	2	0.94 ± .09
7-12	not measured	

Table 2:

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

Sample	Extrapolated Initial Conc. (g/dL)	Rate of Conc. Increase (g/dL/week)	R
A	0.3 ± .1	0.07 ±.01	0.23
B	0.09 ± .03	0.018±.003	0.2
C	0.33 ± .06	0.005±.005	0.02
D	0.24 ± .09	0.063±.008	0.26
E	0.33 ± .04	0.047±.005	0.14

N.B. R is the ratio of the rate of increase over the initial conc. and can be used as a measure for a sample's relative tendency to produce metHb. For example, the R value of 0.23 for sample A means that the metHb conc. of sample A increases by 23% of the initial conc. per week. An R of 0.02 for sample C suggests that its metHb conc. is relatively stable.

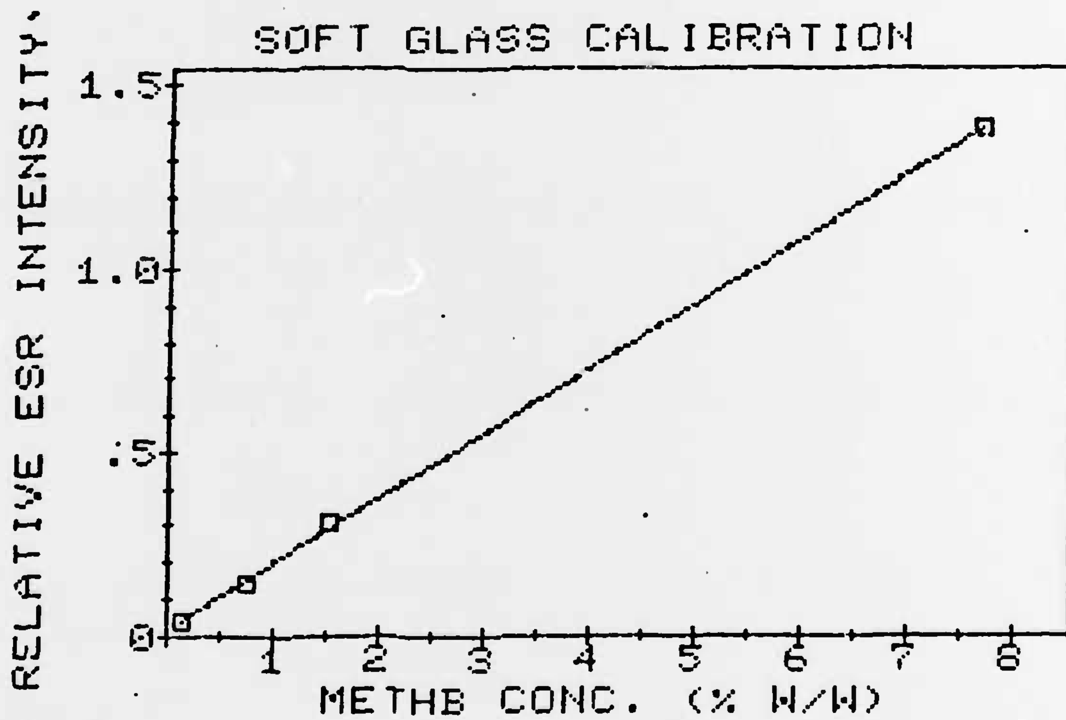


Fig. 1. MetHb ESR signal intensity, relative to the soft glass standard, as a function of metHb concentration. This curve was used to calibrate the soft glass rod standard.

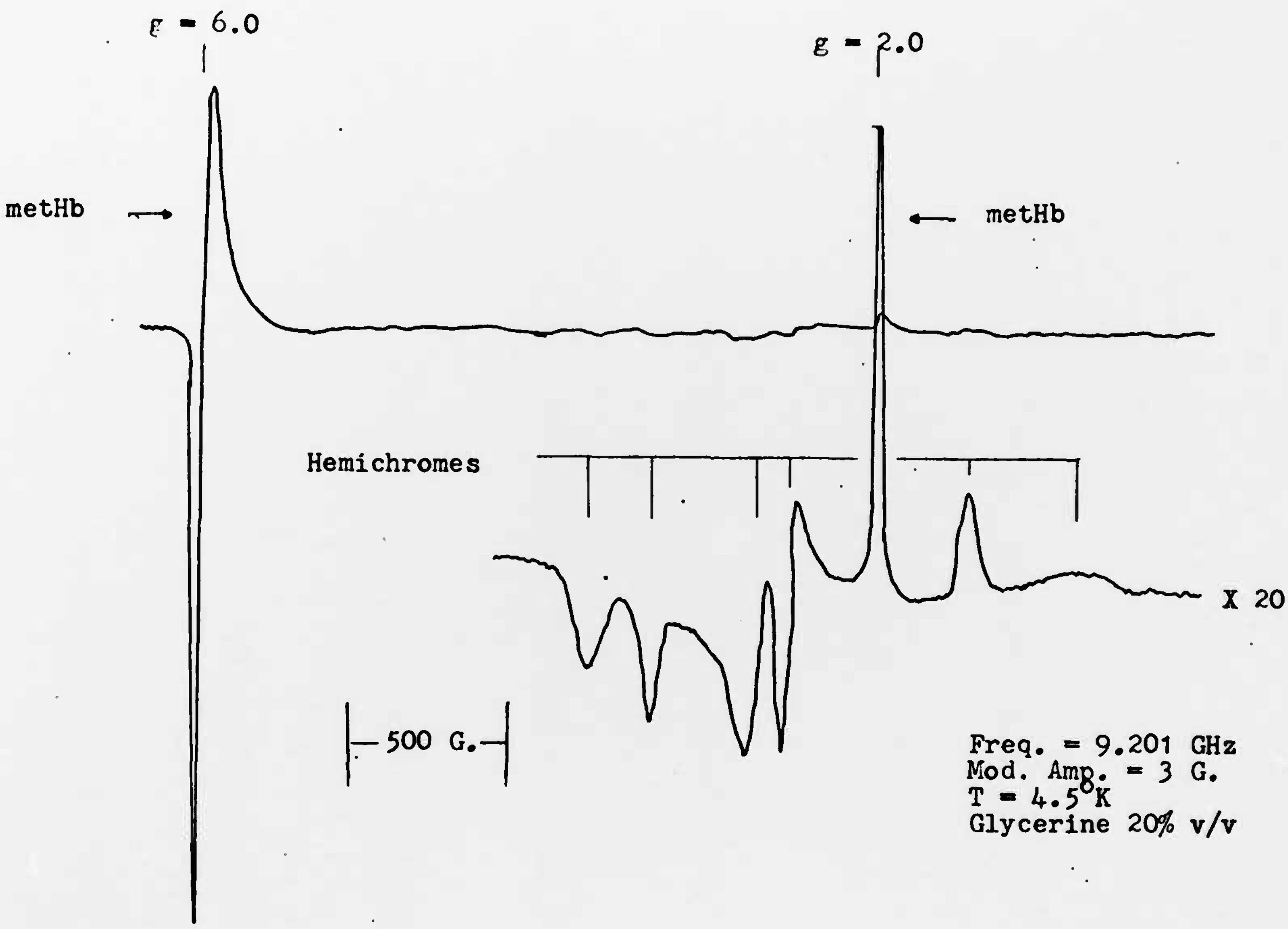


Fig. 2. ESR spectrum of Neville's sample. Spectrum was measured 4-22-82.

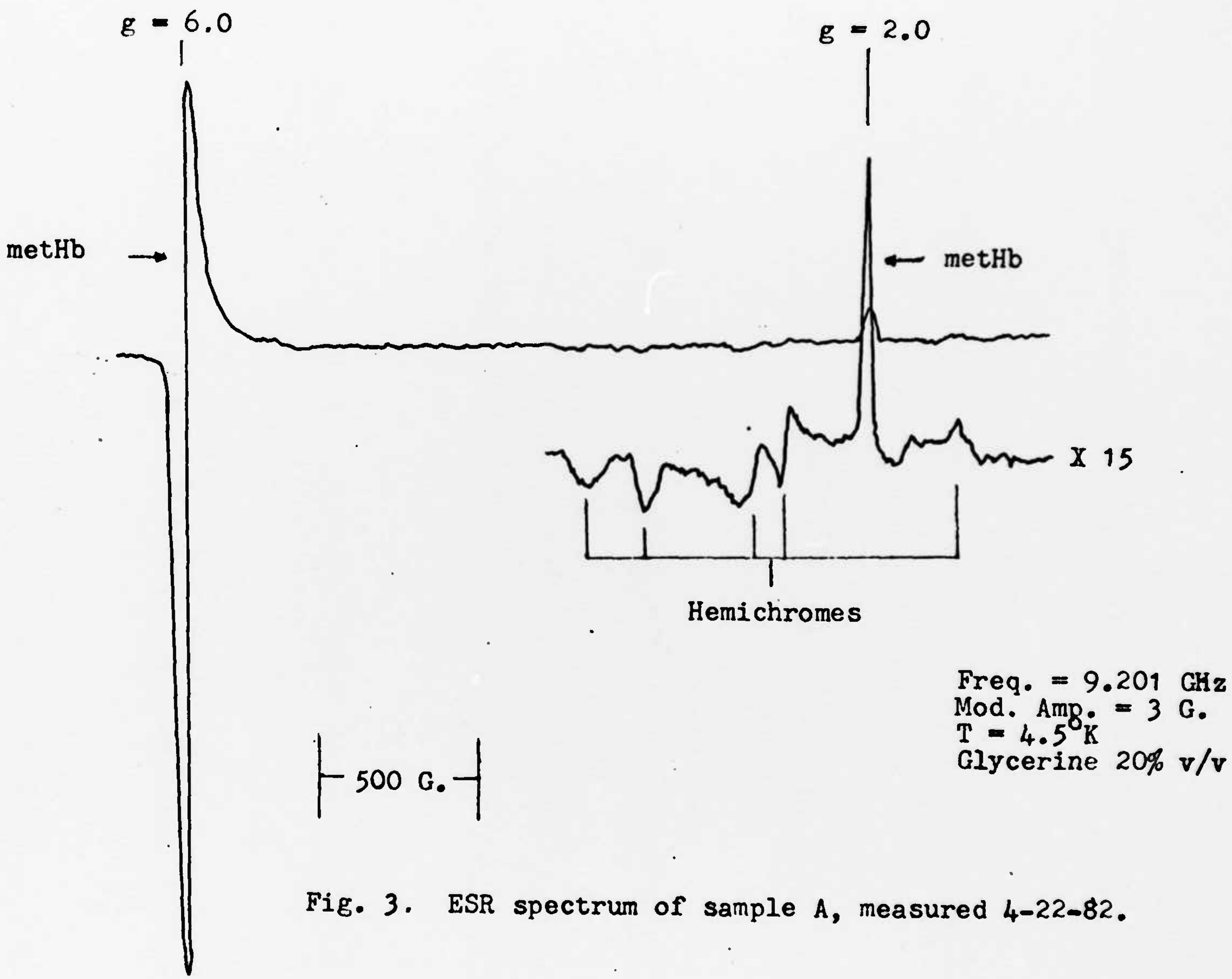


Fig. 3. ESR spectrum of sample A, measured 4-22-82.

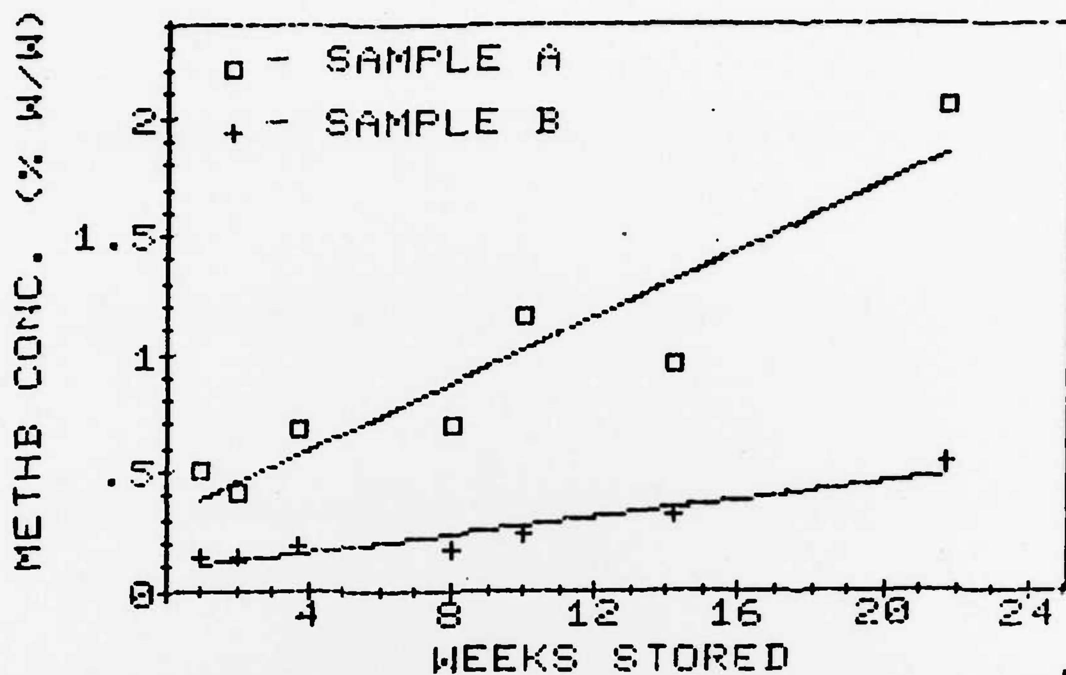


Fig. 4. Plots of the MetHb content of samples A and B as a function of storage time. Day 0 is assumed to be 2-10-82

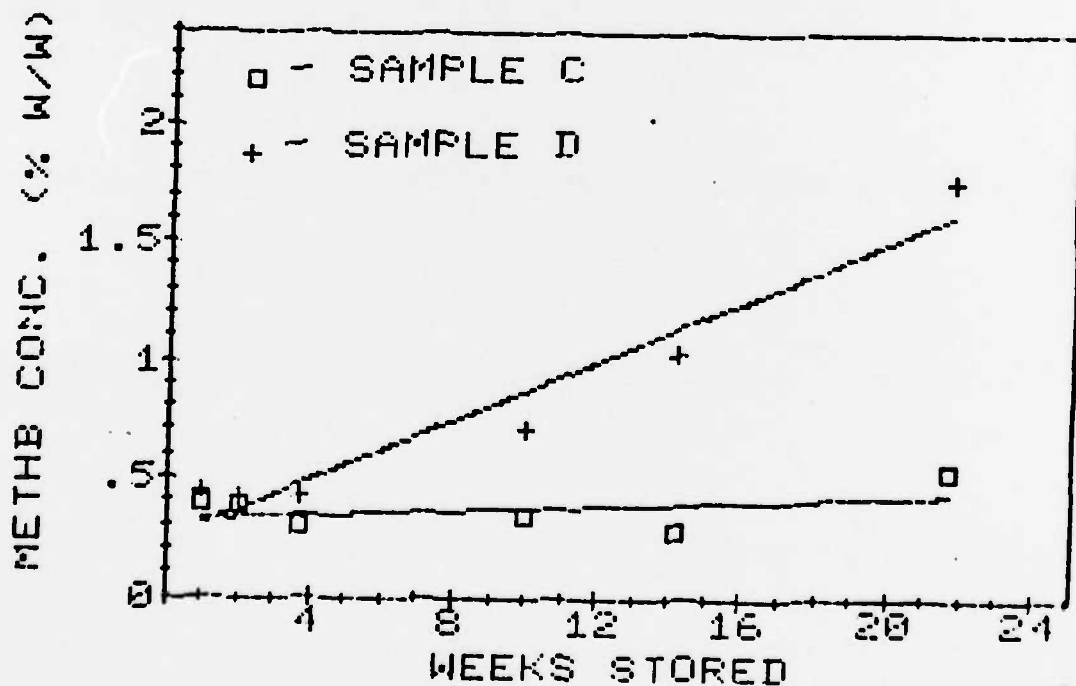


Fig. 5. Plots of the metHb content of samples C and D as a function of storage time. Day 0 is assumed to be 2-10-82.

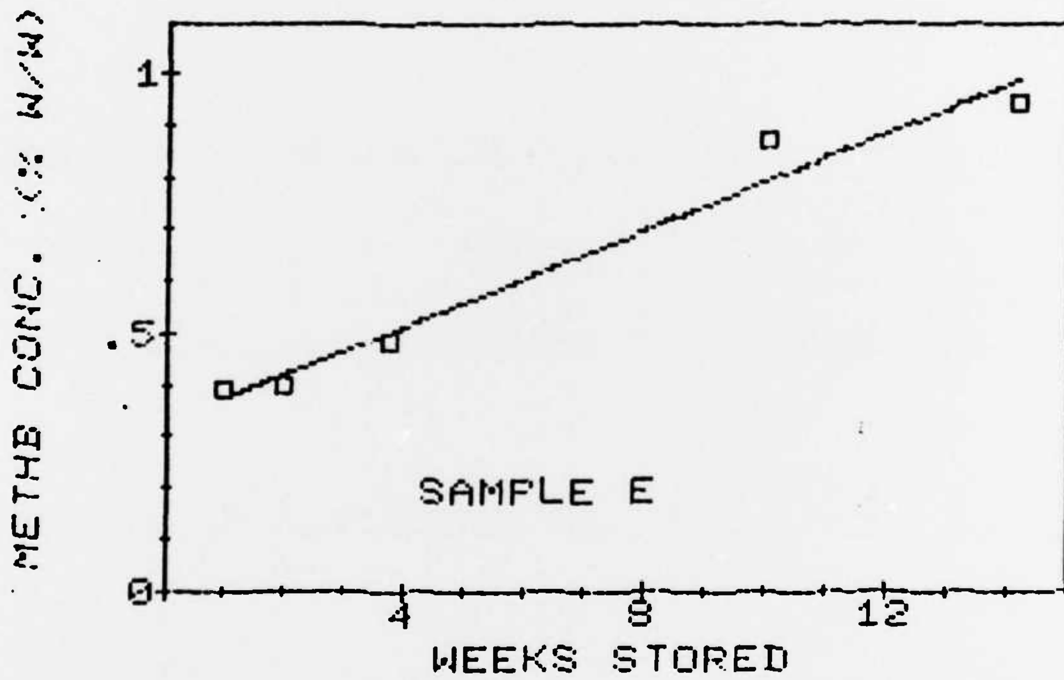


Fig. 6. Plot of the metHb content of sample E as a function of storage time. Day 0 is assumed to be 2-10-82.

2. Discussion

a. The build-up of metHb

All the samples stored at 4° C had increases in their metHb concentrations which are linear with time. In chemical kinetic terms, a linear change is called zero-order kinetics. This is in contrast to the more usual first-order kinetics which is only linear if a log plot is utilized. In addition, in samples A, B and D the relative rate R is the same for all these samples. This relative rate R is defined as the actual rate divided by the apparent initial metHb concentration on Feb. 10, 1982. If we can assume that the initial metHb was derived from the HbA in the sample by some mechanism which has always had the same zero-order rate, then the metHb concentration in samples A, B and D at any time is always proportional to its value at any other time. Furthermore, if we extrapolated our linear build-up of metHb back in time past the date we received them, we can conclude that samples A, B and D were all "born" 4 weeks before Feb. 10, 1982 and the metHb has been building up since that time.

Extrapolation of the data for sample E, stored in a blood-pack, back in time gives an earlier date: 7 weeks before Feb. 10, 1982. It seems most likely to us that the build-up of metHb in sample E has lowered since it was placed in its blood-pack on Feb. 10, 1982. It is possible that the blood-pack inhibits the build-up of metHb relative to that in glass.

Since sample C was given to us unfrozen, it is very logical that the data shown in Fig. 5 for samples C and D cross at about the time that we received the samples and froze sample C. It is very clear from Fig. 5 that low temperature freezing completely inhibits the build-up of metHb.

Since we do not know the total HbA concentrations in any of our samples, we can not determine if the rate of formation of metHb is always proportional to the total HbA in each sample. Since the HbA is presumably much larger than the metHb in these samples, zero-order kinetics would be expected since the HbA would change very little during the period of study. It is possible, however, that the rates are not proportional to the HbA in each sample, and that they depend upon the amount of a catalyst in the sample. This could be a pH dependence or some other factor which is the same for samples A and C but different for sample B. It is our understanding that samples A and C have quite different origins, but we do not know if they have the same pH or the same HbA concentrations. Samples C, D and E were apparently all the same material although sample D in the blood-pack has a lower rate for the build-up of metHb.

In conclusion, the metHb has increased linearly with time in all the samples stored at 4°C, but not changed in the sample frozen at dry ice temperatures. This linear build-up could be expected to continue for perhaps one year where either the depletion of HbA or the production of hemichromes could be expected to affect its rate. The sample stored in a blood-pack, while identical to others stored in glass, had a slightly lower rate for the build-up of metHb. This indicates a small effect of this storage method, compared to glass, on the chemistry of the stored HbA.

b. The build-up of hemichromes

The hemichromes are also oxidized HbA, but the ferric iron assumes a low-spin state. This low spin is due to a change in the groups coordinating the iron with a water, in the case of metHb, being replaced by an oxygeneous ligand or a nitrogenous ligand. The ESR changes a great deal wien the iron goes to low spin and the resulting spectrum can be resolved into oxygeneous and nitrogenous forms. Both forms are usually seen, but for reasons which we do not understand the relative amounts in each form seems to be characteristic of each HbA sample.

A quantitative measure of the amount of hemichromes has depended so far on a sample of cyanmetHb and a double integration of the ESR spectra. this has only been seriously attempted for the special sample which was very concentrated. Approximate analysis leads us to believe that the regular HbA samples had hemichrome contents which were 10-20% of the amount of metHb present. In the future we hope to bypass the double integration requirement.

If we use the ESR signals as a quantity which is proportional to the amounts of the two hemichromes we can follow their build-up relative to the metHb. Our numbers are not tabulated, but they indicate tha the hemichromes also increase linearly with time. This was somewhat surprising since we expected the product of the metHb which itself increased linearly with time, to perhaps increase quadratically with time. In fact, our data indicate that the hemichromes are essentially a fixed percentage of the metHb. As a result, when the metHb increases, the hemichromes increase proportionately.

It has been reported (8) that the oxygeneous ligand is an OH^- and that this is in a chemical equilibrium, depending upon pH, with the metHb. Since we see <u>fixed</u> ratios in each sample of the oxygeneous and nitrogenous forms which do not change with time, we are forced to conclude that <u>both</u> forms are in a chemical equilibrium with the metHb. We don't know what factors determine the relative amounts of each, but it again may be pH.

From the signal heights we estimate the following:

For A and B (Minnesota)

$$\frac{\text{Nitrogenous}}{\text{Oxygeneous}} \sim 1$$

For C, D and E (LAIR)

$$\frac{\text{Nitrogenous}}{\text{Oxygeneous}} \sim 2.5$$

These ratios do not vary as the total hemichromes and metHb increase.

In conclusion, the hemichromes appear to be a fixed fraction of the metHb, and they have two forms, an oxygeneous and a nitrogenous form. The relative amounts of these two forms varies with the sample but is fixed in time. All these features are consistent with the hemichromes being formed as part of a chemical equilibrium from the metHb with the equilibrium constants dependent upon factors such as pH.

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., J. Biol. Chem. 246, 3342-55 (1971).

5. A. Ehrenberg, Arkiv. för Kemi 19, 119-28 (1962).

6. G. Palmer, "The Polyphyrins," (D. Dolphin, Ed.) Vol. V, 313-53, Academic Press (1979).

7. J. F. Gibson et al., Diss. Fara. Soc. 26, 72-80 (1958).

8. J. Peisach et all, Proc. Natl. Acad. Sci. USA 63, 934-39 (1969).

END

FILMED

4-84

DTIC