SATURATION TRANSFER ELECTRON PARAMAGNETIC RESONANCE DETECTION OF SICKLE HEMOGLOBIN AGGREGATION DURING DEOXYGENATION

P. THIYAGARAJAN AND MICHAEL E. JOHNSON
Department of Medicinal Chemistry and Pharmacognosy, Health Sciences Center, University of Illinois at Chicago, Chicago, Illinois 60680

ABSTRACT Spin-label saturation transfer EPR (ST-EPR) methods have been used to study the sickle hemoglobin (HbS) aggregation behaviors induced by slow deoxygenation at a constant temperature of 30°C, and by a rapid temperature increase from 1° to 30°C for fully deoxygenated HbS. For slow deoxygenation at 30°C, we find that the effective HbS correlation time exhibits a continuous increase, without any abrupt transitions, suggesting that polymer formation in concentrated HbS at high temperature occurs even at high oxygenation levels. Upon a rapid temperature increase, fully deoxygenated HbS exhibits a short delay time, then an abrupt increase in effective correlation time. These results also indicate that ST-EPR provides a useful method for probing the molecular dynamics of HbS aggregation.

INTRODUCTION

The aggregation of deoxygenated sickle hemoglobin (HbS)[1] is the major cause for the clinical symptoms of sickle cell anemia. The kinetic and equilibrium characteristics of HbS aggregation have been studied by a wide variety of biophysical techniques, including viscometry (1, 2), optical turbidity (3), laser light scattering(4), water NMR relaxation time and line width measurements (5–8), microcalorimetry and linear birefringence measurements (9), and the combined use of optical and sedimentation methods (10, 11). Most of these studies have been carried out either on samples at constant levels of partial oxygenation, or on completely deoxygenated samples, using temperature change to induce aggregation. Thus, they give information on the aggregation behavior of HbS at a constant level of deoxygenation after temperature change, but not on the behavior during the transition from an oxygenated to a deoxygenated state at constant temperature. An understanding of this aspect of HbS aggregation will be helpful in understanding the molecular events that occur in vivo when the HbS molecules become deoxygenated.

The problem of characterizing HbS aggregation during deoxygenation has recently been approached through the use of $^{13}C/^{1}H$ double-resonance NMR spectroscopy to measure the amount of HbS polymer within sickle and sickle-trait erythrocytes as a function of oxygen saturation (12, 13). This work has shown that polymer formation in sickle erythrocytes can be detected even at deoxygenation levels of <10%, and that the fractional HbS polymer content increases monotonically with increasing deoxygenation. However, there is still little information available at the molecular level on HbS motional characteristics during deoxygenation-induced aggregation. Such information should be valuable in understanding HbS aggregation under physiological conditions.

In this work we report the use of slow-motion spin-label saturation transfer EPR (ST-EPR) (14–16) to compare the motional characteristics of concentrated normal adult hemoglobin (HbA) and HbS during slow deoxygenation at constant temperature. We find that within minutes after deoxygenation is initiated the average correlation time of HbS increases significantly beyond that of HbA under equivalent conditions. On a logarithmic scale, the increase in average HbS correlationtime is continuous, and approximately hyperbolic in shape. No abrupt transition in ST-EPR spectral behavior is observed during the time course of deoxygenation. For fully deoxygenated HbS, we find that upon rapidly increasing the temperature of a fully deoxygenated HbS solution from 1° to 30°C, the ST-EPR spectra indicate an abrupt decrease in HbS motional freedom, consistent with the gelation observed by other

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A preliminary account of this work was reported at the 25th Annual Biophysical Society Meeting, Denver, CO, February, 1981.

Dr. Thiyagarajan's current address is the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, IL 60439.

Please address communications to Dr. Johnson.

[1] *Abbreviations used in this paper:* EPR, electron paramagnetic resonance; ST-EPR, saturation transfer EPR; NMR, nuclear magnetic resonance; Mal-6, 4-maleimido-2,2,6,6-tetramethylpiperidin-1-yloxy; HbA, normal adult hemoglobin; HbS, sickle hemoglobin; HbF, fetal hemoglobin; IHP, inositol hexaphosphate; metHb, methemoglobin; COHb, carbonmonoxy Hb; TPX, methylpentene polymer; EDTA, Ethylenediamine tetraacetic acid.

physical methods (1–10). The rapid and continuous increase in average HbS correlation time observed during deoxygenation, even at very high levels of oxygenation, suggests that polymer formation in concentrated HbS solutions begins even at very high levels of oxygenation. These results also indicate that ST-EPR methods provide a sensitive technique for probing HbS aggregation mechanisms at the molecular level.

MATERIALS AND METHODS

HbS was prepared from sickle blood samples obtained from homozygous donors with less than ~5% HbA and HbF. Membrane-free carbonmonoxy HbA and HbS were prepared and spin labeled with Mal-6 following published procedures (17, 18). Inositol hexaphosphate (IHP) was added to give a constant IHP/Hb molar ratio of 4:1 in 0.05 M sodium phosphate buffer at pH 6.7.

Before use in deoxygenation studies, the carbonmonoxy hemoglobin solutions were fully oxygenated by flushing slowly with oxygen in a round-bottom flask on a rotary evaporator under a flood lamp. After checking the completeness of Hb oxygenation through the optical spectra, samples were concentrated to the required levels by ultrafiltration; concentrations are noted below in the discussion of experimental results. We have used gas-permeable methylpentene polymer (TPX) capillaries (19, 20) for deoxygenating the hemoglobin samples within the EPR cavity. After filling the capillaries with Mal-6- (4-maleimido-2,2,6,6-tetramethylpiperidin-1-yloxy) labeled oxy Hb, one end was sealed, and the other end snugly fitted into a small Teflon holder. The samples were then placed in the EPR cavity at 30°C for the ST-EPR measurements; deoxygenation was achieved by flushing the EPR cavity with nitrogen, and allowing it to equilibrate with the sample through the gas-permeable capillary.

For temperature-increase experiments with fully deoxygenated samples, the CO ligand was first exchanged for O_2 as described above and solutions then deoxygenated under humidified nitrogen. After deoxygenation, samples were loaded into 20-μl glass capillary pipettes, and sealed. Samples were kept in an ice-water bath for at least 2 h before beginning EPR measurements.

Second harmonic out-of-phase absorption (V_2') ST-EPR spectra were measured using an X-band Varian E-4 spectrometer (Varian Associates, Inc., Palo Alto, CA) at a modulation frequency of 100 kHz; a PAR model 126A lock-in amplifier (EG & G Princeton Applied Research, Princeton, NJ) was used for phase-sensitive detection at 200 kHz. During the phase null–setting procedure (15, 16), oxygen was used (instead of nitrogen) with the gas-permeable capillaries for EPR temperature control, to keep the sample fully oxygenated until EPR measurements were begun. A modulation amplitude of 5 G (gauss) was used for the measurement of all spectra. Other procedures and spectrometer settings were equivalent to those of Thomas et al. (15). Temperature was measured using a digital thermometer and a copper-constantin thermocouple (Fluke, Inc., Mountlake Terrace, WA).

Measurement of ST-EPR spectra and nitrogen flow through the EPR cavity were started simultaneously on the fully oxygenated Hb samples at 30°C. EPR spectra were accumulated on a time averager (Nicolet Instrument Corp., Madison, WI, model 1174) and recorded on magnetic tape every 2 min. About 90–100 spectra were measured for each sample; after that, the spectral behavior reached a plateau. Upon completion of the deoxygenation experiments, the HbS samples in the TPX capillary were found to be solidly gelled, while the HbA samples were fully fluid. Dual samples were subjected to similar conditions and transferred to small optical cells with a short path length for the measurement of optical spectra at intervals of ~30 min after initiation of deoxygenation. The results indicated that deoxygenation proceeded monotonically with time, and was essentially complete after 1–1 1/2 h. Experiments using TPX capillaries filled with buffered nitroxide solutions (no Hb) indicated that oxygen diffusion out of the capillary was ~90% complete within ~6–8 min. Thus, the time course of deoxygenation is probably controlled primarily by the rates of O_2 "unloading" from HbO_2, and rebinding in partially deoxygenated solutions, rather than by a diffusion barrier in the TPX capillary. At the end of the runs (~3–4 h), the samples generally contained ~70–75% deoxy Hb, ~20% methemoglobin (metHb), and <5% HbO_2. (MetHb formation may be due to an oxidation-reduction reaction involving the spin label, oxygen, and the hemes. Addition of EDTA to the solution does not effect metHb formation in this system.)

For the temperature-increase experiments, the samples were placed in the EPR cavity at 1°C, and the phase null adjusted as described above. After recording several ST-EPR spectra at 1°C, the temperature was increased to 30°C; temperature stabilization occurred within ~1 min. The HbS samples gelled under this condition.

For obtaining a quantitative picture of the molecular motions of HbA and HbS under various conditions, we have converted the ST-EPR parameter, L''/L (15), into apparent correlation times (for definition, see Fig. 1). This was done by measuring the ST-EPR spectra and viscosities for hemoglobin solutions in different glycerol-water mixtures, representing motions with a wide range of correlation times. From the viscosity data (18) the rotational correlation times were calculated using Stoke's relation. The best polynomial regression relation was obtained for each of the spectral parameters in terms of the correlation time, and the relation then utilized to calculate the average apparent correlation times for all of the measured spectra. It should also be noted that the estimated apparent correlation times are the mean of the contributions from aggregated and nonaggregated forms of hemoglobin solution.

RESULTS

To compare results obtained by ST-EPR methods with those obtained by other physical measurement techniques, we have performed "conventional" temperature increase experiments on deoxy HbS solutions at various concentrations. The ST-EPR spectra for deoxy HbS at a concentration of 21 g/dl are shown in the left column of Fig. 1 for

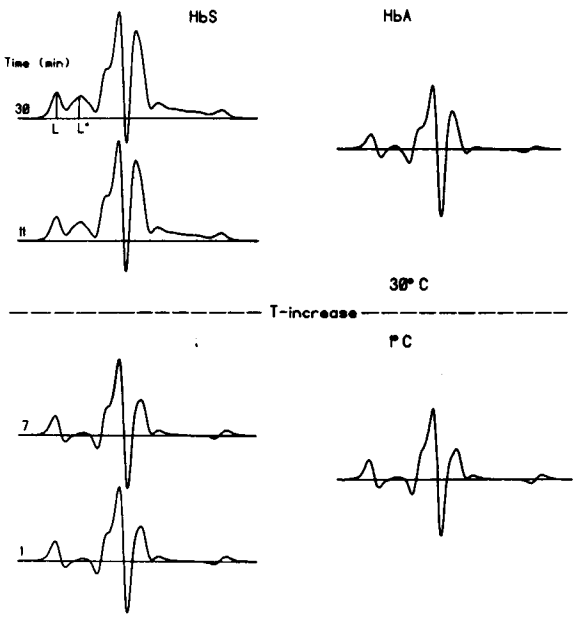

FIGURE 1 Representative V_2' spectra measured during temperature jump experiments with HbA and HbS. The times corresponding to the HbS spectra are marked beside each spectrum. The HbA spectra showed no significant time dependence. The positions where the spectral heights were measured to determine the L''/L ratio are also shown.

various times before and after the temperature increase. A comparison of these spectra with those reported by Thomas et al. (15) for various correlation times indicates that increasing the temperature from 1° to 30°C produces a rapid and substantial decrease in the apparent HbS motional rate. In contrast, the spectra of deoxy HbA, shown in the right side of Fig. 1, exhibit a slight trend toward faster motion upon an increase in temperature from 1° to 30°C.

The apparent correlation time, τ_L, has been used to obtain a more detailed description of the HbS motional behavior before and after the temperature increase. Results for two deoxy HbS samples at concentrations of 18 and 21 g/dl are shown in Fig. 2. The behavior of the HbS apparent correlation time, τ_L, is quite similar to that of various gelation parameters observed in other studies (9, 10): τ_L exhibits a concentration-dependent delay time during which it remains relatively constant; after that, it rapidly increases by ~2 orders of magnitude, and again goes to a relatively constant value. The high-temperature limiting value is also concentration dependent, as would be expected, since more concentrated HbS solutions exhibit a higher fractional degree of polymerization (21, 22). In contrast, the apparent correlation time for deoxy HbA subjected to a similar increase in temperature shows only a slight shift to faster motion. Thus ST-EPR methods are clearly also quite sensitive to the solution-to-gel phase transition observed by other techniques (9–11, 21).

Fig. 3 shows the spectra of HbS (37 g/dl) and HbA (38 g/dl) after increasing time periods of deoxygenation in gas-permeable capillaries within the EPR cavity at 30°C. It is apparent from this figure that the HbS line shapes change very substantially during deoxygenation, going toward a very slow-motion spectral shape. In contrast, the spectral changes for HbA under similar conditions are much smaller. This indicates that there is a continuous and

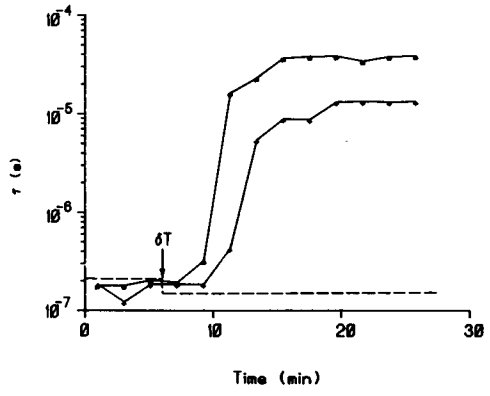

FIGURE 2 Apparent correlation time, τ_L, as a function of time for fully deoxygenated HbS at concentrations of 21 g/dl (*) and 18 g/dl (+). The temperature was kept at 1°C during the phase-nulling procedure, and the first 8 min of each run. At the vertical mark the temperature was rapidly increased to 30°C. The dashed line corresponds to the correlation time of HbA at ~20 g/dl. Note the slight decrease in τ upon raising the temperature. Compare this behavior with that of Figs. 3 and 4.

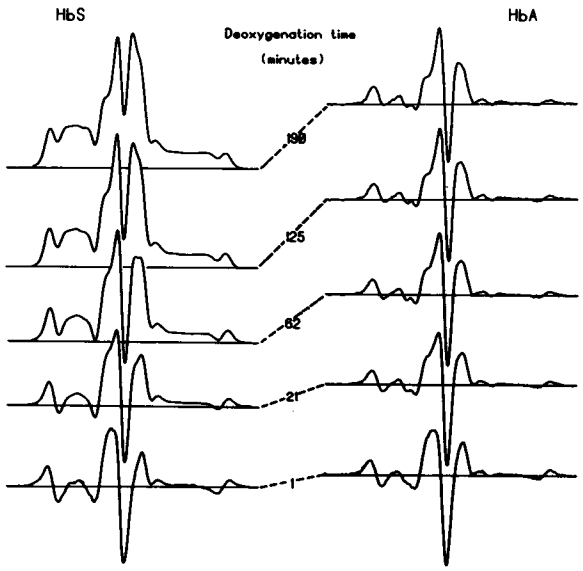

FIGURE 3 Representative V_2' spectra measured during deoxygenation of HbS and HbA. The deoxygenation times corresponding to the individual spectra are shown.

substantial decrease in average HbS motional freedom occurring during deoxygenation. However, the change in motional rate appears much more continuous than that observed for the temperature-increase experiment. Because the deoxygenation of HbS and HbA is occurring under equivalent conditions, the very large relative motional restriction exhibited by HbS must result from a continuous HbS aggregation process.

Fig. 4 is a plot of the apparent correlation time, τ_L, as a function of deoxygenation time for HbS at concentrations of 37 and 23 g/dl, and for HbA at 38 g/dl. From this plot, it appears that HbS undergoes a smooth and continuous decrease in apparent mobility, with the decrease in average motional freedom starting upon the initiation of deoxygenation. For HbA undergoing deoxygenation, the change in

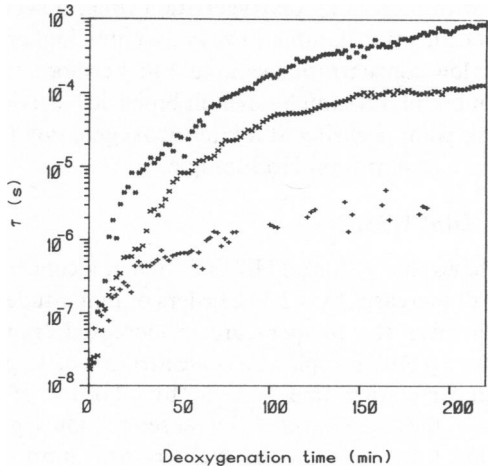

FIGURE 4 Apparent correlation times, τ_L, as a function of deoxygenation time for HbS at concentrations of 37 (*) and 23 (×) g/dl, and for HbA at a concentration of 38 g/dl (+).

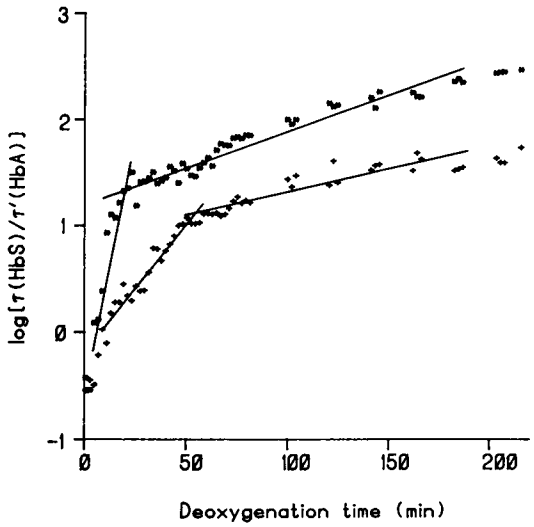

FIGURE 5 The variation of log $[\tau_L(\text{HbS})/\tau'_L(\text{HbA})]$ as a function of deoxygenation time for HbS at concentrations of 37 (*) and 23 g/dl (+). We speculate that the break points in the curves may correspond to the macroscopic phase change of gelation.

apparent τ_L is substantially restricted from that exhibited by the two HbS systems. HbS at concentrations below the minimum gelation concentration also exhibits behavior equivalent to that of HbA. Under the nitrogen purge conditions of this experiment, carbonmonoxy Hb (COHbA) exhibits a nearly constant apparent correlation time (data not shown).

Fig. 5 gives a semilog plot of $\tau_L(\text{HbS})/\tau'_L(\text{HbA})$ as a function of deoxygenation time. $\tau'_L(\text{HbA})$ is a least-squares fit of the data for HbA undergoing deoxygenation (Fig. 4) to an exponential function of the deoxygenation time. This plot allows us to "normalize" the ST-EPR spectral behavior for the effects of spin exchange produced by the paramagnetic oxygen, and enables us to estimate the extent to which HbS aggregates during deoxygenation. As expected, the ratio for both HbS samples increases rapidly with increasing deoxygenation time; however, the high concentration sample always assumes higher values than the low concentration sample. Furthermore, the ratio curves of both HbS samples exhibit biphasic behavior, with the break point occuring at a later deoxygenation time for the lower concentration HbS sample.

DISCUSSION

Fig. 2 shows that τ_L for an HbS solution at a concentration of 21 g/dl increases by ~2 1/2 orders of magnitude within 6–8 min after the temperature is increased from 1° to 30°C. For an HbS sample at a concentration of 18 g/dl the apparent correlation times increase by ~2 orders of magnitude, when the temperature is increased to induce gelation. The delay time between the temperature jump and the steep increase in apparent correlation time is 2–3 min for the lower concentration sample, and <2 min (the scan repeat time) for the higher concentration sample. Thus, these results are qualitatively quite similar to those of other studies in which the gelation transition is monitored by other physical methods (9, 10). Hence, the spin-label ST-EPR method provides a reliable probe of HbS motional behavior during aggregation.

Many of the studies reported to date on HbS gelation have been carried out either on samples at a constant level of partial oxygenation, or on completely deoxygenated samples using temperature or concentration change to induce aggregation. Thus, they yield information on the aggregation behavior of HbS at a constant level of deoxygenation. The work reported in Figs. 3–5 of this study differs from most previous work in that the level of Hb oxygenation is continuously varied at a constant temperature and concentration, and HbS aggregation is observed by ST-EPR.

The results from Fig. 4 clearly show that both HbA and HbS at similar concentrations initially exhibit nearly the same apparent correlation time, $\tau_L = 2.5 \times 10^{-8}$ s, and as deoxygenation progresses, they show similar increases in correlation time during the first few minutes. At the end of the initial 6–8 min of deoxygenation they attain apparent τ_L values ~4–5 times that of the initial value.The similarity of the apparent motional behaviors of both HbA and HbS during the initial phase suggests that the apparent increase in the correlation time during the initial phase is probably due to the decrease in the content of unbound oxygen present in the hemoglobin solutions. This is supported by the observation that the ST-EPR spectra of nitroxide spin probes in lipid bilayers show faster motion (shorter correlation times) in the presence of oxygen than in its absence (20). Plachy and Windrem (19) have further observed that a time of ~8–10 min is required to deoxygenate a buffer solution in a gas-permeable capillary system having a geometry similar to that utilized here. Thus, it appears that the oxygen concentration in the buffer drops from saturation level to a level controlled by its rate of release from Hb and its rate of diffusion out of the capillary during the first 8–10 min of nitrogen purge. While the change in HbA spectral behavior during deoxygenation is probably primarily due to the reduction of Heisenberg exchange between the nitroxide and the paramagnetic oxygen molecules, Hb conformational change may also play a role. The limiting L''/L ratios differ slightly between deoxy Hb and COHb in nitrogen-saturated buffer (not shown).

Significant deoxygenation of the hemoglobins appears to begin after the initial 5–10 min of nitrogen purge. The correlation time profiles of HbA and HbS hemoglobins start diverging after the first 5–10 min of nitrogen purge. The apparent correlation time for HbS increases rapidly compared with that for HbA. The increases in apparent correlation time for both hemoglobins observed in this phase may include contributions from a small amount of dehydration occurring through the gas-permeable capil-

lary, and some residual oxygen–spin label Heisenberg exchange as the ligand oxygen diffuses out of the system. However, the contribution due to dehydration seems to be rather small, as we find that a sample of COHbA exhibits only a small increase in apparent correlation time (not shown).

As deoxygenation progresses past the initial few minutes, the apparent correlation time for HbS continues to increase rapidly, while that for HbA levels off. This rapid increase in τ for HbS as compared with HbA must be due to the formation of deoxy HbS aggregates.

Thus, the constant temperature HbS deoxygenation results summarized in Fig. 5 indicate that HbS aggregate formation begins even at very high levels of oxygen saturation and moderate HbS concentrations. The curves of Fig. 5 show biphasic behavior; after an initially steep, but continuous, rise in the apparent correlation time ratio, the curves level off to more gradual increases with time. If deoxygenation follows an approximately exponential time course, as would be expected for these sample conditions and geometry, then we might also expect the correlation time to follow a similar time course (if motional restriction and deoxygenation are linearly related). In a logarithmic plot, this should produce a simple linear dependence, clearly in disagreement with the results shown in Fig. 5. Thus, we suggest that these breaks in slope may correspond to the macroscopic phase change of gelation. This behavior also leads to the speculation that HbS aggregate formation at the molecular level, and the macroscopic phase change commonly known as gelation, may be two distinct events that can be separated in time. This appears to be consistent with $^{13}C/^{1}H$ nuclear magnetic double-resonance studies that have found that sickling and HbS polymer formation within homozygous SS red cells exhibit differing dependencies on HbS oxygenation (12).

In summary, the present study demonstrates the usefulness of ST-EPR in resolving the large differences in the motional behaviors of HbA and HbS during deoxygenation at constant temperature and concentration. This is of particular relevance in examining the question of what happens to HbS molecular motion during deoxygenation, and has a direct bearing on understanding the phenomena occurring in vivo where the hemoglobin is deoxygenated at constant temperature and concentration. Our results indicate that HbS exhibits aggregation even when the solution is highly oxygenated, and that aggregation of HbS at high concentration appears to proceed continuously with deoxygenation. These conclusions are in basic agreement with $^{13}C/^{1}H$ magnetic double-resonance studies on intact sickle erythrocytes (12).

We thank Dr. L. Fung, Loyola University of Chicago, for the gift of the TPX capillaries used in these studies. This work was supported in part by grants from the National Institutes of Health (HL-23697) and the Chicago Heart Association.

Dr. Johnson is an Established Investigator of the American Heart Association.

Received for publication 29 September 1982 and in final form 2 February 1983.

REFERENCES

1. Harris, J. W., and H. B. Bensusan. 1975. The kinetics of the sol-gel transformation of deoxyhemoglobin S by continuous monitoring of viscosity. *J. Lab. Clin. Med.* 86:564–575.
2. Kowalczykowski, S., and J. Steinhardt. 1977. Kinetics of hemoglobin S gelation followed by continuously sensitive low-shear viscosity: changes in viscosity and volume on aggregation. *J. Mol. Biol.* 115:201–213.
3. Moffat, K., and Q. H. Gibson. 1974. The rates of polymerization and depolymerization of sickle cell hemoglobin. *Biochem. Biophys. Res. Commun.* 61:237–243.
4. Wilson, W. W., M. R. Luzzana, J. T. Penniston, and C. S. Johnson, Jr. 1974. Pregelation aggregation of sickle cell hemoglobin. *Proc. Natl. Acad. Sci. USA.* 71:1260–1263.
5. Waterman, M. R., and G. L. Cottam. 1976. Kinetics of the polymerization of hemoglobin S studies below normal erythrocyte hemoglobin concentration. *Biochem. Biophys. Res. Commun.* 73:639–645.
6. Cottam, G. L., M. R. Waterman, and B. C. Thompson. 1977. Kinetics of polymerization of deoxyhemoglobin S and mixtures of hemoglobin A and hemoglobin S at high hemoglobin concentrations. *Arch. Biochem. Biophys.* 181:61–65.
7. Shibata, K., M. R. Waterman, and G. L. Cottam. 1977. Alteration of the rate of deoxyhemoglobin S polymerization: effect of pH and percentage of oxygenation. *J. Biol. Chem.* 252:7468–7474.
8. Eaton, W. A., J. Hofrichter, and P. D. Ross. 1976. Delay time of gelation: a possible determinant of clinical severity in sickle cell disease. *Blood.* 47:621–627.
9. Hofrichter, J., P. D. Ross, and W. A. Eaton. 1974. Kinetics and mechanism of deoxyhemoglobin S gelation: a new approach to understanding sickle cell disease. *Proc. Natl. Acad. Sci. USA.* 71:4864–4868.
10. Hofrichter, J., P. D. Ross, and W. A. Eaton. 1976. Supersaturation in sickle cell hemoglobin solutions. *Proc. Natl. Acad. Sci. USA.* 73:3035–3039.
11. Sunshine, H. R., J. Hofrichter, F. A. Ferrone, and W. A. Eaton. 1982. Oxygen binding by sickle cell hemoglobin polymers. *J. Mol. Biol.* 158:251–273.
12. Noguchi, C. T., D. A. Torchia, and A. N. Schechter. 1980. Determination of deoxyhemoglobin S polymer in sickle erythrocytes upon deoxygenation. *Proc. Natl. Acad. Sci. USA.* 77:5487–5491.
13. Noguchi, C. T., D. A. Torchia, and A. N. Schechter. 1981. Polymerization of hemoglobin in sickle trait erythrocytes and lysates. *J. Biol. Chem.* 256:4168–4171.
14. Hyde, J. S., and D. D. Thomas. 1973. New EPR methods for the study of very slow motion: application to spin-labeled hemoglobin. *Ann. NY Acad. Sci.* 222:680–692.
15. Thomas, D. D., L. R. Dalton, and J. S.Hyde. 1976. Rotational diffusion studied by passage saturation transfer electron paramagnetic resonance. *J. Chem. Phys.* 65:3006–3024.
16. Hyde, J. S., and L. R. Dalton. 1979. Saturation-transfer spectroscopy. *In* Spin Labeling: Theory and Application. L. J. Berliner, editor. Academic Press, Inc., New York. 2:1–70.
17. Abraham, E. C., D. Walker, M. Gravely, and T. H. J. Huisman. 1975. Minor hemoglobins in sickle cell anemia, β-thalassemia, and related conditions: a study of red cell fractions isolated by density gradient centrifugation. *Biochem. Med.* 13:56–77.

18. Johnson, M. E. 1978. Librational motion of an "immobilized" spin label: hemoglobin spin labeled by a maleimide derivative. *Biochemistry.* 17:1223–1228.

19. Plachy, W. Z., and D. A. Windrem. 1977. A gas-permeable ESR sample tube. *J. Magn. Reson.* 27:237–239.

20. Popp, C. A., and J. S. Hyde. 1981. Effects of oxygen on EPR spectra of nitroxide spin-label probes of model membranes. *J. Magn. Reson.* 43:249–258.

21. Ross, P. D., J. Hofrichter, and W. A. Eaton. 1977. Thermodynamics of gelation of sickle cell deoxyhemoglobin. *J. Mol. Biol.* 115:111–134.

22. Noguchi, C. T., D. A. Torchia, and A. N. Schechter. 1979. ^{13}C NMR quantitation of polymer in deoxyhemoglobin S gels. *Biochemistry.* 76:4936–4940.