QUASI-ELASTIC LASER LIGHT SCATTERING FROM SOLUTIONS AND GELS OF HEMOGLOBIN S

ZVI KAM AND JAMES HOFRICHTER
Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT Quasi-elastic light scattering has been used to examine solutions and gels of deoxyhemoglobin S. The autocorrelation function is found to decay with a characteristic exponential relaxation which can be ascribed to the diffusion of monomer (64,000 molecular weight) hemoglobin S molecules. In the absence of polymers, the relaxation time is in good agreement with previous measurements of the diffusion coefficient for solutions of normal human hemoglobin. In the presence of the polymer phase, a large (>200-fold) increase in the scattered intensity is observed but no contribution to the decay of the autocorrelation function from the motion of the aligned polymer phase can be detected. Heterodyning between the time-independent scattering amplitude from the polymers and the time-dependent scattering of the diffusing monomers results in a twofold increase in the relaxation time arising from monomer diffusion.

INTRODUCTION

Gels of deoxyhemoglobin S (Hb S) are mixtures of a monomeric solution phase and an aligned polymer phase (1–3). The macroscopic structure of these gels is a space-filling collection of spherulitic domains in which the polymer long axes project radially from the center of the spherulite (3, 4). The high resolution structure of the gels is assumed, based on the strong similarities in the thermodynamics and kinetics of polymerization (2, 5–8), to be identical to that observed for polymer crystals obtained from stirred solutions. The polymers in these crystals contain seven pairs of double strands (9, 10) each of which is similar to those seen by Wishner et al. (11) in single crystals of Hb S. Studies of the gelation of a large number of mutant hemoglobins (12) confirm this assumption. Nonetheless, the details of the low resolution structure of the gel remain largely undescribed. Very little is known, for example, about either the packing of the polymers or the detailed interactions (i.e., cross-links) between polymers in the gel.

The unusual kinetics of Hb S polymerization, characterized by a delay with a very high concentration dependence, have been interpreted in terms of a double-nucleation mechanism (13). The mechanism postulates that homogeneous nucleation is necessary to initiate polymerization in polymer-free solutions but that, once even a small amount of polymer is present, new polymers are formed much more rapidly by heterogeneous nucleation on the surface of existing polymers. As a result, the polymerization progress curves are autocatalytic, and there is an apparent delay before the appearance of detectable amounts of polymer. The observed broadening of the polymerization progress curves and the increased number of polymer domains formed when polymerization is rapid have been rationalized as resulting from changes in the relative rates of homogeneous nucleation and heterogeneous nucleation (4, 14). It has also been suggested that the macroscopic ordering of polymers and the size of the spherulitic domains is controlled by the kinetics of nucleation and growth during the formation of the gel (4, 14). These arguments could be extended to suggest that the mechanical properties of the gel, and hence the mechanical properties of red cells when they are sickled, may also be dependent on the kinetics of gel formation.

Quasi-elastic laser light scattering (QELS) is a sensitive probe of fluctuations resulting from diffusion in solutions or from the elastic deformation of semi-rigid structures (e.g., polyacrylamide gels) (15, 16). A recent report of the effect of cell sickling (caused by the intracellular polymerization of Hb S) on the autocorrelation function suggested that the fluctuations in the scattered light disappeared when the cells sickled (17). This result was interpreted as suggesting that "Brownian motion of the hemoglobin was essentially stopped by extensive aggregation in the cell" (17). From thermodynamic studies of the monomer-polymer equilibrium in vitro, it is expected that a significant fraction (20–60%) of the Hb S molecules in red cells

Dr. Kam's permanent address is Polymer Department, Weizmann Institute of Science, Rehovot 76100, Israel.

from patients with homozygous sickle cell (SS) disease remains unpolymerized at 37°C (2), and these molecules should still be capable of diffusing. For these reasons we decided that it would be interesting to examine the QELS of well characterized gels of Hb S.

We find that the only observable decay of the autocorrelation function measured in QELS experiments on Hb S gels arises from the motion of hemoglobin S molecules which remain in the solution phase. The motion of these molecules is not detectably altered by the presence of polymerized Hb S. We are not able to detect any contribution to the decay of the autocorrelation function arising from viscoelastic fluctuations of the polymerized Hb S.

METHODS

Oxyhemoglobin S (oxyHb S) was prepared in 0.15 M potassium phosphate, pH 7.4 buffer as previously described (18). Because of the high extinction coefficient of hemoglobin at 632.8 nm (ϵ = 1,050 M^{-1} cm^{-1}) and the high concentrations of Hb S required for gels to form, the samples were prepared inside thin glass capillaries. Square microcells (Vitro Dynamics, Inc., Rockaway, NJ) with an internal path length of 0.4 mm were used. After deoxygenation by addition of an aliquot of 1 M sodium dithionite in the same buffer to a final concentration of 0.05 M, the sample capillaries were filled by aspirating the cold Hb S solution under a nitrogen atmosphere and sealing them with varnish (Red GLPT insulating varnish; G.C. Electronics, Rockford, IL). At the final Hb S concentration of 0.255 g/cm^3, the optical density of the sample at the 632.8 nm He-Ne laser line is ~0.6, and a gel forms at room temperature (20–22°C) after a delay of a few minutes. When examined under the polarizing microscope, the gel was observed to have domains with an approximate size of 0.1 mm.

Uniform and well-aligned gels were prepared by pulling capillaries from a temperature of 5°C to room temperature through a temperature gradient of ~1°C/cm at a speed of 0.5 cm/h. The gels formed in this way started growing at the warmer end of the capillary and the polymer long axes were aligned parallel to the capillary axis. Using these samples, the dependence of the scattering properties on the orientation of the polymers can be studied by changing the angle of the polarization and the scattering vectors with respect to the capillary axis and hence to the long axes of the Hb S polymers in the gel. The degree of alignment was estimated from the order parameter, $<P_2(\cos\theta)>$, obtained from the polarization ratio of the absorbance of light polarized perpendicular and parallel to the capillary at 632.8 nm (19, 20). The measured polarization ratio was 1.3. The order parameter calculated using a polarization ratio of 3.0 for the polymers (20) and the predicted polymer fraction of 0.34 at 20°C (2) is ~0.7.

For light scattering measurements the sample capillary was held by a micromanipulating xyz stage (three model 450-A micrometric stages; NRC Corp, Fountaindale, CA) inside an nuclear magnetic resonance (NMR) tube (0.5 cm o.d.) which was filled with microscope immersion oil. The oil acts as both an index matching medium to reduce scattering from the capillary surfaces and as a conductive medium between the capillary and the NMR tube. The tube was mounted in a copper sleeve, drilled to provide paths for the incident and scattered light. The sleeve was thermostated using a Peltier heat pump and bipolar controller (Cambion Inc., Cambridge, MA). ~2 mW of polarized incident laser light from a Helium-Neon laser (model 80-2H; Coherent Radiation, Palo Alto, CA) were passed through a polaroid film polarizer and focused onto the sample using a microscope objective lens (Leitz 4×/0.12). The scattering angle was determined by the alignment of the system to be either 90 or 45°. A long working distance microscope objective (Leitz UM 10/0.22) with a variable numerical aperture (minimum ≈0.02) was used to image the scattering cell onto a 0.2-mm diameter pinhole, which defined the scattering volume. Alignment of the system was facilitated by an insertable prism, which diverted the image from its direct path to the photomultiplier into an eyepiece. By setting the objective numerical aperture to its minimum value the angular aperture of the detection system could be limited to a small number of coherence areas.

Absorption of laser light by the sample results in a significant increase in the sample temperature. To estimate the temperature rise in the sample, ΔT, we approximate the illuminated region of the capillary as a small sphere, the surface of which is maintained at a constant temperature, $T_o + \Delta T$, and equate the heat flux from this sphere to a larger sphere, the surface of which is maintained at a constant temperature, T_o (21) to the laser power absorbed by the sample. The result is:

$$\Delta T \simeq \frac{F}{4\pi\kappa}\left(\frac{1}{a} - \frac{1}{b}\right).$$

In our experiment, the laser power absorbed in the sample, F, is 1.5 mW; the heat conductivity for immersion oil, κ, is ~0.8 mW/cm °C. If we take into account the higher conductivity of glass, we estimate b, the radius of the heated volume, as 0.25 mm; and a, the radius at which the temperature is regulated, as 2 mm. The calculated temperature rise is thus ~6°C. This result is consistent with the observed temperature at which the polymer phase dissolves in the deoxyHb S samples (see below), which suggests a temperature rise of at most 2–3°C.

Photon pulses from the photomultiplier (model 9659QC; Thorn EMI GENCOM Ltd., Plainview, NY) in a model 62/2A44 housing/amplifier; Pacific Photometric Instruments, Div. Pacific Instruments, Inc., Concord, CA) were fed into a multibit correlator (model K7025; Malvern Instruments Ltd., Framingham, MA). The time-averaged autocorrelation function was first normalized using the total number of photons counted. To obtain the autocorrelation relaxation time, the logarithm of the correlation function, calculated after subtraction of a constant baseline amplitude, was fitted to a straight line using a calculator (9815A; Hewlett-Packard Co., Palo Alto, CA). The baseline value was initially estimated from the final amplitude of the measured autocorrelation. The fit yielded a final amplitude that was lower than the initial estimate and this value was input as a new estimate of the final amplitude. This procedure was iterated until the estimated value was unchanged in the final fit. The difference between the theoretical baseline given by the square of the average scattered intensity and the fitted baseline, which reflects the nonfluctuating immobile component, was used to estimate the heterodyne correction (see DISCUSSION).

RESULTS

Scattering experiments were carried out on 0.255 g/cm^3 samples of oxy and deoxyHb S at temperatures that varied from 3° to 33°C. The correlation functions measured at 6.6°C and 33°C are presented in Fig. 1. The decay of the autocorrelation is clearly observed to take place in all four experiments. Note, however, that while the relaxation rates for the oxy and deoxy samples are identical at 6°C, the results for the two samples differ significantly at 33°C. At the higher temperature, the amplitude of the normalized autocorrelation is much smaller and the relaxation time is significantly shorter for the deoxy sample than for the oxy sample. The temperature dependence of the apparent diffusion coefficients is plotted in Fig. 2. The temperature dependence for the oxyHb S sample (Fig. 2 *a*) is very similar to that expected from the temperature dependence of the water viscosity. The diffusion coefficient measured for the same solution of oxyHb S at 20°C was 4.9×10^{-7} cm^2/s, somewhat smaller than the value (5.6×10^{-7} cm^2/s) obtained by extrapolation from previously reported measurements for lower concentrations of Hb A (22).

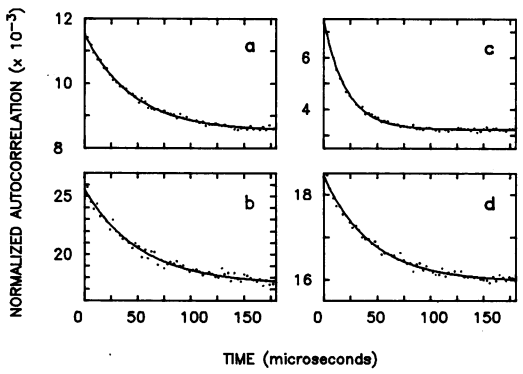

FIGURE 1 Normalized intensity autocorrelation functions for laser light scattering from 0.255 g/cm³ samples of oxy and deoxyHb S. The measured data for (*a*) and oxyHb S and (*b*) a deoxyHb S solution at 6.6°C, and (*c*) an oxyHb S solution and (*d*) a deoxyHb S gel at 33°C are shown as the points. The fitted single exponential relaxation is shown as the solid line in each panel. The reproducibility of the fitted relaxation time was ±4–5% in repeated experiments. The decay time for oxyHb S decreases by a factor of ~2 at the higher temperature as a result of the temperature dependence of the viscosity of water. Collection numerical apertures were 0.22 for the three solutions, and 0.02 for the gel.

The temperature dependence of the apparent diffusion coefficients measured for the deoxyHb S sample is shown in Fig. 2 *b*. The concentration of this sample (0.255 g/cm³) is equal to the solubility of Hb S at a temperature of ~8°C (2), and all polymer is expected to disappear when the sample is cooled below this temperature. At temperatures above 8°C, polymer is present in the sample at equilibrium.

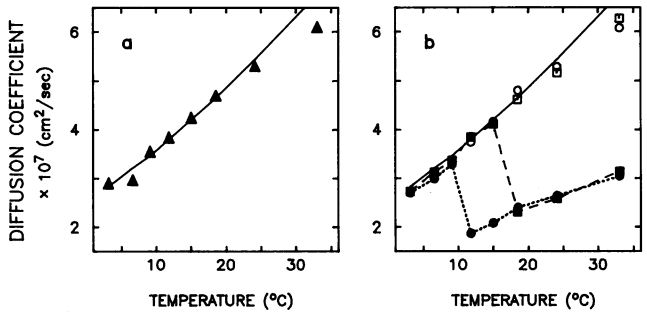

FIGURE 2 The temperature dependence of the apparent diffusion coefficient for 0.255 g/cm³ solutions of oxyHb S and solutions and gels of deoxyHb S. (*a*) Results for oxyHb S. The dependence of the diffusion coefficients on temperature expected from the temperature dependence of the viscosity of water is plotted as the solid line, where the theoretical values have been calculated relative to the measured value of 4.8×10^{-7} cm²/s for the oxyHb S solution at 20°C. (*b*) Results for deoxyHb S obtained by raising (open square) and lowering (open circle) the temperature. When a cold deoxyHb S solution is heated in a series of steps which include (*i*) raising the temperature, (*ii*) waiting for 10 min for temperature equilibration, and (*iii*) measuring the autocorrelation function, supersaturated solutions were obtained at temperatures of 11 and 15°C. The diffusion coefficients at these temperatures in the presence of polymer were measured by cooling already formed gels. Diffusion coefficients obtained by multiplying the values measured in the presence of polymer by a factor of two (heterodyne correction) are plotted as the open symbols. The calculated curve from (*a*) is included as a reference.

When the temperature is raised from a temperature below 8°C, however, the nucleation-controlled kinetics of polymerization result in a delay time which is of the order of days at 11°C, shortens to hours at 15°C, and is only a few minutes at room temperature (13). Consequently, in an experiment in which the sample temperature is raised in a series of steps and the autocorrelation function is measured at each temperature, polymer is not expected to appear until the temperature is well above 8°C. The filled squares are the results of experiments in which the temperature was raised from 2°C. The apparent diffusion coefficients are identical to those measured for the oxyHb S solutions at temperatures of 15°C and below. At temperatures above 15°C, the apparent diffusion coefficients decrease by a factor of two from those measured for the corresponding oxy sample. At these temperatures it was clear that gelation had occurred, as evidenced by a very large (<200-fold) increase in the scattered intensity. As shown in Fig 1, the decrease in the apparent diffusion coefficient is also associated with a large decrease in the normalized autocorrelation amplitude. When the temperature of the deoxyHb S sample is decreased, high scattering amplitudes and reduced apparent diffusion coefficients are observed for all temperatures above 10°C. At temperatures below 10°C, the scattering amplitude decreases dramatically and the apparent diffusion coefficient again becomes identical to the values measured for the oxyHb S sample. In all experiments we found no correlation decay other than a single relaxation described by the apparent diffusion coefficient reported in Fig 2 in the time range between 1 μs and 20 ms.

In an effort to determine whether a decay resulting from motion of the polymerized Hb S was being masked by the high amplitude of the decay resulting from monomer diffusion, the intensity and autocorrelation of the depolarized scattering was measured. Strong depolarized scattering has been predicted to result from the shape anisotropy of Hb S fibers, with a depolarization ratio close to 1 for scattering of light polarized at 45° to the fiber axis (23). Since depolarized scattering from hemoglobin monomers is very weak, the autocorrelation of the depolarized scattering might, therefore, be used to enhance fluctuations arising from viscoelastic modes of the polymer phase. We observed, however, that the depolarized scattering from well oriented gels was much weaker than the polarized scattering, independent of the orientation of the polarization and scattering vectors relative to the fiber axis. The decay rates for the polarized light correlation function were also found to be independent of the orientation of both the polarization and scattering vectors.

DISCUSSION

The decay in the autocorrelations for oxyHb S can be ascribed solely to the diffusion of 64,000-mol-wt monomers. For deoxyHb S under conditions where no polymer is present, the autocorrelation function decays with an identi-

cal relaxation time and this decay can also be assigned to monomer diffusion. When polymer is present, however, the relaxation times for the deoxyHb S sample decrease by a factor of two and there is a large decrease in the normalized autocorrelation amplitude. We shall see that this result can be straightforwardly explained as arising from heterodyning between the fields scattered from the diffusing monomeric hemoglobin and the immobile polymer phase.

When light scattered by mobile particles is the only contribution to the measured intensity (called "homodyne detection"), the scattered intensity is the square of the interfering scattered field strengths. If the spectrum of the scattered field is broadened by Doppler shifts resulting only from the diffusive motion of the particles, then the spectrum is a Lorentzian centered about the incident light frequency. In this case the fluctuations in the scattered intensity which arise from the beating frequencies of the scattered field have a frequency spectrum which is twice as broad as the spectrum of the scattered field (15). If a strong light field containing only the frequency of the incident light is coherently added to the scattered field, then the scattered intensity is, to first order, the square of the strong field and the fluctuations in the scattered intensity result from interference between the weak, broadened field and the intense, monochromatic field. In this case (called "heterodyne detection") the frequency spectrum of the scattered intensity is identical to that of the weak, broadened field, and thus has a width which is only half that observed if the same broadened field is measured using homodyne detection (15). In conventional heterodyne experiments the strong field arises from an independent scatterer, such as a teflon wedge, which is inserted into the scattering volume. If the frequency spectrum of the scattered intensity is characterized by measuring an autocorrelation function, then the decay time for the homodyne autocorrelation function is equal to $(2q^2D)^{-1}$ where D is the diffusion coefficient of the Hb S monomers and q is the scattering vector, $(2\pi/\lambda)\sin(\theta/2)$. This decay time is exactly half that for the heterodyne autocorrelation function, which is $(q^2D)^{-1}$ (15). The normalized amplitude of the heterodyne autocorrelation is also inversely proportional to the total scattered intensity (which arises from the monochromatic field).

This phenomenology provides a straightforward explanation of our results. In the absence of polymer, the scattered intensity from both the oxyHb S and deoxyHb S samples arises from Hb S monomers and the decay time for the autocorrelation function is related to the diffusion coefficient by the homodyne expression. In the presence of polymer, the intense scattering from the polymers provides an internal source of an unbroadened, intense scattered field which interferes with the broadened field produced by scattering from the monomers. The decay time is now related to the diffusion coefficient by the heterodyne expression and is exactly twice that observed in the absence of polymer. When the observed decay is corrected for strong heterodyning by multiplying the apparent diffusion coefficient by a factor of two (Fig 2 *b*), the diffusion coefficients for the monomers in the gelled samples are identical, to within experimental error, to those measured for oxyHb S at the same temperature. The factor of two decrease in the apparent diffusion coefficient for the polymerized samples thus does not result from a change in the diffusion coefficient of the Hb S monomers in the polymerized sample, but from the presence of a strong, unbroadened contribution to the total scattered intensity.

The are two limiting models for the distribution of polymers in the Hb S gel. In the first, polymers are densely packed and occupy only a small fraction of the total volume, leaving the monomers to diffuse in relatively large volumes of polymer-free solution. This model predicts that the formation of polymers will increase the monomer diffusion coefficient by ~6% relative to the oxyHb S control because the concentration of monomer in the solution phase of the gel is ~0.07 g/cc lower than that of the control (22). In the second, polymers are interspersed uniformly throughout the sample volume and the monomers diffuse through the partially occupied volume. The theory for diffusion of small particles in partially occupied media was originally treated (with reference to heat conductivity of inhomogeneous media) by Maxwell (24), and has been reexamined for several specific cases (25). The effect of introducing an array of uniformly distributed, fixed barriers into the sample volume is to slow down diffusion by a factor which, for dilute barriers, depends only on the volume fraction of the barriers, v. The apparent diffusion coefficient, D', is related to the diffusion coefficient for free solution, D, by

$$D' = \frac{(1 - v)}{(1 + v/2)} D.$$

The volume fraction of polymer is ~0.17 at 33°C, where 47% of the Hb S molecules are assembled into polymers, so diffusion in the gel is predicted to be ~23% slower than in the polymer-free solution. When combined with the decreased concentration of the solution phase, the prediction is that the measured diffusion coefficient should decrease, relative to oxyHb S at the same concentration, by ~15%. If the Hb S polymers are uniformly distributed over dimensions of q^{-1}, then the apparent diffusion coefficient should be reduced by this amount. In our data, after correction for the effect of heterodyning, there is no systematic difference between the diffusion coefficients for the oxy and gelled deoxy samples. Since the predicted 15% decrease in D is considerably larger than the uncertainty of the measurements, this result argues against the presence of randomly distributed polymer in the gel and favors the first model in which polymers are locally packed with high density, leaving dimensions comparable to q^{-1} in which free diffusion occurs. More precise information concerning

the dependence of the monomer diffusion coefficient on the volume fraction of polymer could clearly be obtained from experiments in which the volume fraction of the polymers is varied by changing the total Hb S concentration at a fixed temperature so that the concentration of monomers in the solution phase is kept constant.

Measurements of the correlation functions from oxygenated and deoxygenated sickle red blood cells have previously been reported by Nishio et al. (17). The autocorrelations were presented with the amplitude at zero correlation time normalized to unity and the reported correlation from deoxygenated sickle cells appears not to decay at all. Our results show that this normalization procedure will decrease the amplitude of the scaled decay by a factor which is equal to the ratio of the scattered intensity from the polymerized and unpolymerized samples, which is a factor of 200–1,000 for scattering at 90°. As a result, any small but real decay is surpressed by normalizing the signal for the large, constant amplitude. This result is clearly misleading, since our results show that the autocorrelation functions from Hb S gels can be readily measured and its amplitude and decay rate can be accurately interpreted as arising from scattering from the freely diffusing hemoglobin S monomers.

As mentioned above, the hysteresis in the values for the deoxy sample is expected from the known kinetics of Hb S polymerization. At temperatures below 15°C the delay time is sufficiently long that the scattering data can be collected before any significant amount of polymer has formed. The diffusion coefficients measured under these conditions, where the solution is supersaturated, permit upper limits to be placed on the amount of aggregated material which is in rapid equilibrium with the monomeric Hb S. Our results indicate that there can only be extremely limited aggregation of Hb S before gel formation. Since the apparent diffusion coefficient, for particles of similar size, is the z-average of the distribution of coefficients in the sample, an upper limit on the concentration of aggregates under these supersaturated conditions can be calculated. For example, if the diffusion coefficient is within 1% of that of monomers, <0.5% of the molecules can be present in form of dimers (assuming that the diffusion coefficient of dimers is ~0.75 that of monomers), or <0.001% of the molecules can be present in the form of 10-mers.

The remaining problem is to explain the absence of any decay in the autocorrelation function which can be ascribed to the polymer phase. The decay time of the autocorrelation of light scattered from gels (16) is predicted to have a diffusive, q^2 dependence. The decay time for polarized (depolarized) scattering is proportional to the ratio between the longitudinal compression modulus (transverse shear modulus), and the frictional force between the gel fibers and the solvent. Our measurements show no relaxation which can be assigned to viscoelastic fluctuations of the polymers in the gel. In preliminary experiments we observed decays with amplitudes of ~0.1% of the correlation baseline and relaxation times of ~0.07 ms in the autocorrelation function measured at 90° for 4.6% polyacrylamide (PA) gels (cf. 16). Relatively simple considerations[1] led us to expect that the decay times for the Hb S and PA gels would not differ by more than an order of magnitude. A major difference between HbS and polyacrylamide gels is that the scattered intensity at 90° from Hb S gels is larger by at least a factor of 1,000. If, as suggested by the measurements of the monomer diffusion coefficients, the increased scattering efficiency of the Hb S gels arises from a course-grained polymer network in which the inhomogeneities occur over dimensions which are comparable to or larger than the wavelength of light (such as reflections from crystal faces), the motion of the polymers might be largely restricted by the side-to-side aggregation into microcrystallites. On the other hand, even if the absolute amplitude of the gel viscoelastic fluctuations were comparable to that of PA gels, the high scattered intensity resulting from a course-grained polymer network may cause the relaxation amplitude from polymer motion to be unresolvable, since it is masked by the decay in the autocorrelation function which results from monomer diffusion, which has an amplitude of ~0.1% for the Hb S gel.

Dr. Kam would like to acknowledge the members of the Laboratory of Chemical Physics for their warm hospitality and the stimulating atmosphere experienced while visiting the National Institutes of Health. We thank, in particular, Dr. William Eaton, Dr. Attila Szabo, and Dr. Andrea Mozzarelli for their interest, involvement, and many stimulating discussions and suggestions. We also thank Dr. Boon Chock for permitting us to use his light scattering apparatus for these studies.

Received for publication 10 April 1986 and in final form 17 June 1986.

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[1]Crude estimates suggest that the decay times for gel viscoelastic fluctuations should be similar for HbS and PA. The frictional drag on an Hb S polymer is expected to be somewhat larger than PA because of its increased thickness. The restoring forces to motion perpendicular to the fiber axis depend on both the interaction between parallel fibers, and the stiffness of the fibers themselves. The only basis on which to estimate these forces is from the stiffness of the two gels. For unoriented Hb S gels rheological measurements of the storage modulus (26) have given values of $\sim 10^4$ dynes/cm^2, which is comparable to the reported value for 5% PA gels (16).

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