Hemoglobin S Polymerization and Gelation Under Shear II. The Joint Concentration and Shear Dependence of Kinetics

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The kinetics of hemoglobin S gelation are critical in sickle disease because microvascular obstruction can be avoided if red blood cells pass these vessels during the delay time, before polymerization and gelation occur in sufficient degree to rigidify the cells. Kinetics, including the delay time and the closely related exponential progress rate, are highly sensitive to hemoglobin concentration and degree of deoxygenation. Kinetics are also greatly accelerated by shear, an effect that may contribute to pathogenesis, since red blood cells deform and can undergo shear in vivo. Here we examine the joint dependence of kinetics on shear and hemoglobin concentration. As shear rate increases, the concentration dependence of the exponential progress rate decreases. The large decrease in concentration dependence supports the conclusion that acceleration of gelation by shear is due to breakage and not to enhancement of heterogeneous nucleation. Under shear, new fibers are created by breakage of existing ones, as well as by heterogeneous nucleation. At high shear, the rate of new fiber creation by breakage is very great and dominates that by heterogeneous nucleation. Therefore, if breakage depended only on shear rate and were independent of the concentration of hemoglobin in solution, the concentration dependence of kinetics should vanish. Although it decreases, it does not disappear. The concentration dependence that remains at high shear arises from (1) the direct contribution of fiber growth rate to the exponential progress rate, (2) the dependence of breakage rate on fiber growth rate, and (3) the dependence of solution viscosity on hemoglobin concentration. © 1993 by The American Society of Hematology.

Polymerization and gelation of hemoglobin S are the initial events in the sequence that produces sickle cell crises and its many pathological sequelae in sickle cell disease. The occurrence and rapidity of gelation depend strongly on the extent of deoxygenation and on hemoglobin concentration. Since the development of crises depends on whether red blood cells pass the microvasculature before gelation occurs, the rapidity of gelation is of paramount importance in clinical events and in attempts to prevent crises and find a means of treatment for the disease. We have shown that shear greatly accelerates polymerization and gelation, because it causes fiber breakage with the result that there are more fiber ends at which polymerization occurs. Since the intraerythrocytic milieu is subject to shear as red blood cells pass through the vasculature, shear may also be a critical factor in clinical events.

Here we examine the joint effects of shear and hemoglobin concentration on gelation kinetics. Since one or the other factor may be dominant depending on conditions, characterization of their combined action may have clinical relevance. Examination of the joint effects of shear and concentration also offers information on the mechanism by which shear acts on hemoglobin S gels.

Materials and Methods

Sickle hemoglobin, obtained from therapeutic exchange transfusions, was purified chromatographically, deoxygenated (near 0°C) under nitrogen in a tonometer with an absorption cell and introduced into a Wells-Brookfield LVT cone-plate viscometer (cone angle 1.565 degrees) (Brookfield Eng Labs, Stoughton, MA) in an anaerobic glove box as previously described. Full deoxygenation was confirmed spectrophotometrically. Gelation was commenced with a temperature jump and progress curves were obtained under steady-state shear. Excess viscosity (that above the baseline viscosity before polymerization commenced) was measured. After each run, the sample was fully melted by lowering the temperature to near 0°C, also as previously described. An average of seven runs (range, three to 22 except for one single run) was performed under each set of conditions (hemoglobin concentration and shear rate).

Viscometry in the presence of an air-solution interface presents the risks of an undesired contribution of surface viscosity to the total measured viscosity and of protein denaturation at the surface. Although we did not use a guard ring, these artifacts were not significant in the results, because the results under fixed conditions were reproducible on repeated runs and hence any possible denaturation that might have occurred did not affect the viscosity progress curves significantly. Further, the viscosity of interest was excess viscosity due to polymer formation so that any changes in baseline viscosity (including surface viscosity) did not enter into the progress curve. Also, baseline viscosity was 3 to 4 centipoise (cps), consistent with reported results for hemoglobin in the concentration range used (12.1 to 14.5 mmol [heme]/L). Finally, the unprotected gap between the cone and cup in the Wells-Brookfield viscometer was 2.5 mm. For this geometry, the torque due to a bulk viscosity of 1 cps requires 3 surface cps to produce an equal torque (and to affect results such surface viscosity would have to be an excess viscosity that developed only during polymerization and was reversible, which is unlikely).

Gelation progress was analyzed as before, with the exception that data were collected from the viscometer directly into a personal computer. Progress curves showed a delay time followed by a rapid increase in viscosity and were exponential in shape. Results reported are the rate of exponential increase as measured by nonlinear least-squares fit to a single exponential up to a viscosity of 10 cps, except as noted in the legend to Fig 1. All runs were in 0.1 mol/L potassium phosphate, pH 7.0, at 20°C.
RESULTS

Figure 1 shows exponential progress rates as functions of total hemoglobin concentration at different shear rates. Progress rate increases with concentration, but does so less at high shear rates than at low ones. The power dependence of exponential progress rate on concentration ranges from 16 at 5.8 s⁻¹ to 5 at 115 s⁻¹, values that are significantly lower than the concentration dependence in the absence of shear (see Discussion).

Figure 2 plots the slopes in Fig 1 against shear rate to show the shear rate dependence of concentration dependence. The concentration dependence decreases markedly as shear rate is increased. Figure 3 presents the same information in a reciprocal fashion, as the concentration dependence of shear rate dependence. The shear rate dependence is of the order of 1 (ie, a first power dependence of exponential progress rate on shear rate), but decreases from approximately 1.5 at the lowest concentration used to approximately 0.7 at the highest.

To represent the joint dependence of exponential rate on concentration and shear rate, two kinds of regression are used in Fig 2 (with similar analyses in Fig 3). (1) In “regression of regressions” the slopes of the linear regressions of Fig 1 (d log B/d log C) are themselves linearly regressed against log G (where B is exponential progress rate, C is hemoglobin concentration, and G is shear rate). The resultant regression slope (equal to −8.1) is therefore the second derivative, d² log B/d log C d log G. The regression in Fig 3 is the same second derivative and, hence, its slope (−9.0) should be the same as that in Fig 2, ie, the shear dependence of concentration dependence and the concentration dependence of shear dependence must be and are the same. This procedure assumes that the data can be satisfactorily fitted by linear regression. The plots of Fig 1 support the assumption over the narrow range of concentrations observed.

(2) A multivariate fit of the data (as opposed to sequential regression of regressions) can also be made under the same assumption of linearity based on the following equation (see Fig 2 legend):

\[ \ln B = K \ln C \ln G + k_1 \ln C + k_2 \ln G + k, \]

where \( K, k_1, k_2 \) and \( k \) are constants. In Figs 2 and 3, the results are shown as additional regression lines. The second derivative for this fit in both figures is \( d^2 \ln B/d \ln C d \ln G = 2.303 \times d^2 \ln B/d \ln C d \ln G = -5.3 \). That the multivariate second derivative, \( K \), is negative and similar to the regression of regression results in Figs 2 and 3 supports the conclusion that the concentration dependence of kinetics decreases as shear rate increases (and, reciprocally, that the shear rate dependence decreases as concentration increases). Because the assumption of linearity is empirical and approximate, equation 1 is only a useful representation of the results and has no theoretical basis or mechanistic implication.

DISCUSSION

In the absence of shear, exponential progress rates for gelation show a high power dependence on hemoglobin concentration under conditions similar to those used here (20°C, 14 mmol [heme]/L, pH 7.0, 0.1 mmol/L phosphate). Under the same conditions, Briehl and Christoph⁶ found a 45th power dependence. Ferrone et al⁷ found approximately a 50th power dependence at 25°C, pH 7.35, and 0.15 mol/L phosphate. In the present results this, dependence is markedly reduced by shear, until at 115 s⁻¹ it is only fifth power.

Increases in exponential progress rate and inverse delay time, a closely related measure of gelation kinetics,⁹ due to shear have previously been observed by Fieschko et al⁴ and Wenger and Balcerzak.⁵ However, those studies compared only one shear condition (in which shear rate was variable with both position in the sample and time) with unheated
hence, a In B/a In C is, to a first approximation, a function of In G where \( u(C) \), \( U(C) \), \( v(G) \), and of exponential rate. The slopes of the Fig 1 regressions are plotted against log shear rate. The slope of the solid regression line is \(-8.1\) ± \(1.4\) with correlation coefficient, \( r^2 = .92 \). The effect of shear rate on concentration dependence can also be obtained directly from the individual data points (rather than from their regressions) by a multivariate regression (text equation 1) under the following reasoning: In Fig 1, the data fit approximately to straight lines; hence, \( \alpha \) In B/a In C is, to a first approximation, a function of In G only (and not of In C). Similarly, from a plot of In B against In G at different concentrations, \( \beta \) In B/\( \beta \) In G is a function of In C, but not of In G. Therefore, In B = \( v(G) \ln C + v(G) \ln G \) and In B = \( u(C) \ln G + U(C) \) where \( u(C) \), \( U(C) \), \( v(G) \), and \( V(G) \) are undetermined functions. Equating these two expressions requires that In B = \( K \ln C + k_1 \ln C + k_2 \ln G + k \), where \( K \), \( k_1 \), \( k_2 \), and \( k \) are constants. Applying this equation to the data by a multivariate linear regression gives \( K = -2.3 \pm 1.4 \), \( k_1 = 20.2 \pm 4.8 \), \( k_2 = 6.9 \pm 3.7 \) and \( k = -59 \) with \( r^2 = .89 \). Converted to log units, \( K = \alpha^2 \log B/\alpha \log C \), \( \log G = -5.3 \) and \( k = -25 \). The dotted line shows \( \beta \log B/\beta \log C = k_1 + k \log G \) obtained by this regression. In summary, the solid line is a regression against a single variable (log G) of the regression results against the other independent variable (log C), whereas the dotted line is a multivariate regression simultaneously against log C and log G.

From Fig 2, of the effect of shear in reducing concentration dependence.

Mechanisms for the effect of shear on gelation kinetics. Any proposed mechanism for the action of shear must explain (1) acceleration of gelation kinetics by shear, with (2) maintenance of the exponential shape of the progress curve, and (3) the marked decrease of the concentration dependence of kinetics under shear, with (4) a small residual concentration dependence even at high shear rates.

Models for the acceleration of gelation by shear attribute the effect to fiber breakage.\(^{10,12}\) Fiber breakage results in more ends and, hence, more sites for polymerization. If the rate of production of new fibers by breakage is proportional to polymer mass, the progress curve will maintain its exponential shape. Shear-induced breakage is thus a third way of producing new fibers, added to the homogeneous and heterogeneous nucleations that occur in the double nucleation model\(^{13}\) for gelation.

In our previous rheological study of hemoglobin S,\(^{2}\) we concluded that of the three principal reactions in polymerization (homogeneous nucleation, heterogeneous nucleation, and monomer addition), shear acts either on heterogeneous nucleation or on a process (such as fiber breakage) that would be subsumed within the heterogeneous nucleation rate parameter in the equations of the double nucleation model. We then argued that there is no plausible mechanism by which shear could affect heterogeneous nucleation itself and, hence, the effect must be on fiber breakage.

The present results strengthen this conclusion in respect to the exclusion of enhancement of heterogeneous nucleation by shear as the cause of shear induced acceleration of gelation: if the shear dependent acceleration of gelation were due to shear augmented heterogeneous nucleation, gelation kinetics would be expected to retain the high concentration dependence seen in the absence of shear (see also Appendix). Since this is not the case, the results show that shear does not act on heterogeneous nucleation (even though it is the major determinant of exponential rate in the absence of shear). This experimental evidence replaces (or supplements) the argument that there is no plausible mechanism by which shear could accelerate heterogeneous nucleation.\(^{2}\) Taken with the previous evidence,\(^{2}\) the conclusion that shear acts by breaking fibers is reinforced.

In the present results, the exponential progress rate under high shear is much greater than that at low shear. Hence, the rate at high shear is at least that much greater than the rate in the absence of shear. If this increase results from breakage, it follows that the rate of new fiber end creation by breakage under high shear is much greater than the rate of fiber creation by heterogeneous nucleation.

Shear might also have other effects.\(^{2}\) Enhancement of the rate of cross-linking\(^{14}\) might increase viscosity and thus contribute to the shear dependence of the exponential progress of viscosity. But an increase in number of cross-links (as opposed to an increase in polymer mass in this autocatalytic samples and, hence, did not provide a quantitative measure, as in Fig 2, of the effect of shear in reducing concentration dependence.

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reaction) does not seem a likely explanation for the effect of shear in maintaining an exponential viscosity progress curve and vastly increasing its rate. In addition, the opposite action, breakage of cross-links, would also probably occur as the gel is sheared.

Fiber alignment and tactoid formation, observed in hemoglobin S fibers and enhanced by shear, would of themselves decrease rather than increase viscosity as a result of phase separation and the production of mobile domains of aligned fibers within a less dense phase. A contrary effect might also arise from phase separation: the monomer-polymer equilibrium necessarily shifts so as to favor polymerization in the aligned phase. If the equilibrium shift were associated with a faster aggregation reaction (rather than slower depolymerization), this effect might be hypothesized to explain some acceleration of gelation by shear. In this situation, shear would act to accelerate the rate-limiting step, alignment of fibers in the isotropic phase to form an anisotropic phase. But it is not likely that such a mechanism would explain an order of magnitude change in rate and that the shape of the progress curve would remain exponential, unaltered but for rate from its shape in the absence of shear.

Spherulitic domains are known to occur in hemoglobin S fibers and their packing, if its rate were enhanced by shear, could affect the progress of viscosity. But it is also unlikely that any such effect would produce the large changes in rate and the maintenance of shape that we observe over a range of shear rates.

**Concentration dependence of kinetics under shear.**

The basic equation for the exponential progress of polymerization in the absence of shear, $B_0$, is [developed in the Appendix]:

$$B_0 = \sqrt{r(C)g(C)}.$$  
(2)

where $r(C)$ is the net ("on" minus "off") rate of fiber growth, $C$ is the concentration of monomeric hemoglobin in solution, and $g(C)$ is the heterogeneous nucleation rate. Heterogeneous nucleation is highly cooperative and its rate is therefore highly concentration-dependent. Fiber growth is not cooperative and, hence, the concentration dependence of exponential progress in the absence of shear depends primarily on heterogeneous nucleation and to lesser extent on the rate of fiber elongation.

When fiber breakage occurs, the rate of new fiber creation is (apart from homogeneous nucleation) the sum of heterogeneous nucleation and fiber breakage rates. Since polymerization occurs at fiber ends, the rate of polymer formation depends on the total fiber creation rate. Modification of equation 2 to include fiber breakage followed by differentiation results in the basic equation for the concentration dependence of exponential progress rate, $B$, expressed as a power dependence of $B$ on $C$ and $\ln(C)$ [developed in the Appendix, equation A(4)]:

$$\frac{\partial \ln B}{\partial \ln C} = \frac{1}{2} \left[ \left(1 + \frac{d \ln \gamma}{d \ln C} \right) \frac{S}{S - 1} + \frac{g}{g + b} \frac{d \ln g}{d \ln C} + \frac{b}{g + b} \frac{\partial \ln b}{\partial \ln C} \right],$$  
(3)

where $S$ is the supersaturation ratio (the ratio of the hemoglobin activity to activity at equilibrium), $\gamma$ is the activity coefficient of monomeric hemoglobin in solution, and $b(C, G)$ is the concentration and shear rate ($G$)-dependent rate of fiber breakage expressed as breakage frequency per unit polymer concentration.

Equation 3 states that the power dependence of exponential progress rate on the concentration of monomeric hemoglobin in solution depends on three terms: $(\gamma/\gamma)(1 + d \ln \gamma/d \ln C) S/(S - 1)$ represents the concentration dependence of the net growth rate. When $S$ is near 1 there is a high power dependence, but this does not represent cooperativity; when $S$ is high this term contributes (apart from activity effects) only a first power dependence. $(g/(g + b)) d \ln g/d \ln C$ is the true power dependence of heterogeneous nucleation on concentration and is the major component of $\partial \ln B/\partial \ln C$, except when shear rate is high (b $\gg$ g implies g/(g + b) is small). $(b/(g + b)) d \ln b/\partial \ln C$ represents the effect of concentration on the breakage parameter, $b$, and is, of course, zero in the absence of shear.

**Kinetics at high shear rates.**

When the shear rate is sufficiently high, new fiber-end creation by breakage greatly exceeds that by heterogeneous nucleation, $b \gg g$, and equation 3 becomes

$$\frac{\partial \ln B}{\partial \ln C} \approx \frac{1}{2} \left[ \left(1 + \frac{d \ln \gamma}{d \ln C} \right) \frac{S}{S - 1} + \frac{b}{g + b} \frac{\partial \ln b}{\partial \ln C} \right].$$  
(4)

That this condition is approached in the present experiments is evidenced by the order of magnitude increase of exponential rate at high shear over that at low shear, which must itself be equal to or greater than the purely nucleation dependent rate in the absence of shear (see Appendix).

If the concentration dependence of kinetics at high shear ($b \gg g$) remained large, it could be ascribed to enhanced heterogeneous nucleation and not to fiber breakage. Conversely, if it vanished, it could be ascribed to concentration-independent breakage, but not to heterogeneous nucleation. In fact, it decreases greatly, but does not vanish.

The residual concentration dependence might be attributed to any of the three terms in equation 3. (a) Fiber growth rates, either through activity or the ratio $S/(S - 1)$ when $S$ is near 1, give rise to a concentration dependence which, however, is not large enough to account for the residual dependence observed (demonstrated quantitatively below and in Fig 4). (b) If the rate of fiber creation, although dominated by breakage, is in small but significant part due to heterogeneous nucleation, this will contribute to concentration dependence. This term also is not large enough to account for the observed residual concentration dependence. (3) Finally, fiber breakage rates are dependent on the concentration of monomeric hemoglobin in solution for two reasons. First, at high concentration, fibers grow and reach the critical breaking length more rapidly and hence break more frequently. Second, at high concentration, viscosity is increased and the force on fibers in a shear field is thereby increased and breakage rate is increased. Since the two terms (a) and (b) are not sufficient to explain the observations, breakage (and the parameter $b$) must show a significant concentration dependence.
The homogeneous nucleation rate makes no contribution to the exponential progress rate and, hence, is not directly implicated in the rates we observe under shear\textsuperscript{2} (except very early in the reaction, before any measurable increase in viscosity).

However, the possibility of an indirect effect of homogeneous nucleation must be addressed. As concentration increases, gel structure changes because the highly cooperative homogenous nucleation rate increases much more rapidly than does fiber growth rate. Hence, there are more domains\textsuperscript{23} and relatively shorter fibers. If this altered structure produces a gel that breaks more readily under shear (due, for example, to different patterns and extents of cross-linking), breakage and, hence, exponential progress could become concentration-dependent. However, under shear, new fibers are created almost wholly by breakage and only negligibly by homogeneous nucleation; any gel structure that might depend on homogeneous nucleation rate would probably be minimal and would also be eliminated by shear breakage. Hence, such an indirect effect is not a likely explanation of the persistence of concentration dependence of kinetics under shear.

An estimate of the contributions of the three terms to concentration dependence at high shear can be obtained under the conditions of these experiments (see Appendix for details). (1) Based on the virial coefficients of Ferrone et al\textsuperscript{13} and the data of Goldberg et al\textsuperscript{23} and Briehl\textsuperscript{26} for values of $S$ under the conditions of the present study, the first term, $(\gamma d \ln \gamma/d \ln C)/S(S-1)$, contributes approximately 3.8 to $\partial \ln B/\partial \ln C$. (2) Assuming 2% of fiber creation rate is due to heterogeneous nucleation at high shear and the concentration dependence of heterogeneous nucleation, $d \ln g/d \ln C$, is approximately 130 (see Fig 4 legend), the second term is approximately 1.3. (3) In the Appendix, the growth rate–dependent portion of $\partial \ln B/\partial \ln C$ is shown to be $(\gamma d \ln \gamma/d \ln C)/S(S-1)$, equal to the first term. Finally, using the Mooney equation\textsuperscript{23} to approximate the concentration dependence of viscosity, the viscosity-dependent portion of the third term is approximately 1.5.

Thus, the total power dependence at high shear, when breakage rate greatly exceeds heterogeneous nucleation rate, is approximately $\partial \ln B/\partial \ln C = 10$. This is similar to the observed values at high shear rates (Fig 2).

Consequently, most of the observed persistence of a concentration dependence of kinetics at high shear can be attributed to the properties of fiber growth rate itself and to its effect on breakage rate.

**Predicted and observed concentration dependence of kinetics under shear.** Quantitative comparison of observed and predicted results can be made in two ways. For the concentration dependence of B, values of $\partial \ln B/\partial \ln C$ can be taken from either the regressions of $\ln B$ against $\ln C$ at each shear rate or from the multivariate regression fit. In both cases, the predicted results are based on equation 3. The results of comparison by these methods are described in Fig 4. The average value of the ratio of observed to predicted values of $\partial \ln B/\partial \ln C$ is close to 1, supporting the conclusion that the substantial residual concentration dependence of kinetics at high shear results primarily from the noncooperative process of fiber elongation.

**Shear rate dependence of kinetics.** We have previously shown that the power dependence of the exponential progress on shear rate is about 0.8 at approximately 14 mmol (heme)/L.\textsuperscript{2} The present results confirm this (Fig 3) and show, in addition, a decrease in this parameter from approximately 1.5 to 0.7 as concentration increases. As previously discussed,\textsuperscript{2} a power dependence of 0.5 is expected if fibers in solution are broken under shear, while a dependence of 1.0 is anticipated if the fibers are cross-linked in a solid-like network. The present results, as the previous ones, are thus most consistent with solid-like structure.

When $f$, the fraction of fiber creation due to heterogeneous nucleation is high (ie, at high concentrations), shear and its changes have lesser effect on the exponential progress rate [equation A(7), Appendix], consistent with the de-
crease in $\partial \ln B/\partial \ln G$ with increasing concentration in Fig.

Relative importance of heterogeneous nucleation and breakage in vivo. Whether shear-induced breakage, as compared with heterogeneous nucleation, is a major contributing factor to gelation rates in vivo and hence pathogenesis depends on the differences between in vivo conditions and those used here.

Principal differences include: (1) Hemoglobin concentrations are approximately 1.5 times higher in vivo than in the present studies. (2) Unsaturation is of the order of 25% in vivo (although varying from tissue to tissue) as compared with 100% here. (3) The range of intraerythrocytic shear rates in vivo is difficult to estimate and may or may not be higher than the shear used here. However, shear is a significant factor in kinetics in these results at levels as low as $10^{-5}$ s$^{-1}$, which are probably reached or exceeded in vivo in the microvasculature. (4) Due to the higher hemoglobin concentration in vivo, intraerythrocytic viscosity is higher than viscosity in the present experiments. (5) The presence of the cell membrane may have a mechanical effect that influences breakage.

The first condition favors heterogeneous nucleation as the dominant factor in vivo. The second and fourth and probably the third favor breakage. However, extrapolation from the present data cannot be made with sufficient accuracy to determine whether breakage rate is a significant factor in vivo.

The need for extrapolation of nucleation rates can be avoided by comparing delay times in the absence of shear under the present solution conditions with times in vivo. For obstruction to occur in vivo, delay time must be less than the transit time for red blood cells in the microvasculature, about 1 second. In the present data, delay times at the lowest shear rates are approximately 1,000 seconds. Hence, in the absence of shear, they would be this long or longer. Since delay time and heterogeneous nucleation rate are approximately inversely proportional, heterogeneous nucleation rates in vivo are of the order of 1,000 times faster than under the present conditions in the absence of shear. Whether breakage is a major factor in fiber-end creation in vivo could then be estimated if intraerythrocytic shear rates were known and breakage rates could be estimated from them. But this is also not presently possible, because shear rates in red blood cells are uncertain and because there are no observations that show fiber breakage or reflect intraerythrocytic kinetics under shear. Hence, the extent of shear induced breakage in vivo and its role in clinical events cannot yet be quantitatively defined.

APPENDIX

Exponential progress rate under shear. The basic equation (text, equation 2) for the rate of polymerization in the absence of shear, $B_0$, (neglecting the correction for homogeneous nucleation that is needed only very early in the reaction) is$^{13}$:

$$B_0 = \sqrt{r(C)g(C)}, \quad \text{A(1)}$$

where $r(C) = k_r(\gamma C - \gamma_0 C)$ is the net rate of fiber growth $(k_r$ is a rate constant, $C$ and $\gamma$ are the concentration and the activity coefficient for monomeric hemoglobin in solution respectively and $C_0 = C_{\text{eq}}$, and $\gamma_0$ are the monomer concentration and activity coefficient at equilibrium), and $g(C)$ is the heterogeneous nucleation rate.

If shear acted to accelerate the reaction by increasing the heterogeneous nucleation rate itself, $g(C)$ should be replaced by $h(G)g(C)$, where $h(G)$ is the shear-rate-dependent factor by which shear facilitates heterogeneous nucleation. In this event, the concentration dependence of rate should remain unchanged, contrary to the present observations.

If, on the other hand, shear produces fiber breakage, $g(C)$ in equation A(1) must be replaced by the sum of heterogeneous nucleation and breakage rates, $g(C) + b(C, G)$, where $b(C, G)$ is the fiber breakage rate (as breakage frequency per unit polymer concentration), which depends on monomer concentration, $C$, as well as on shear rate, $G$. Then:

$$B = \sqrt{r(C)(g(C) + b(C, G))} \quad \text{A(2)}$$

or

$$\ln B = \frac{1}{2} \left[ \ln r(C) + \ln(g(C) + b(C, G)) \right] \quad \text{A(3)}$$

The concentration dependence (at constant shear rate), expressed as the power dependence of $B$ on $C$, is $\partial \ln B/\partial \ln C$, obtained by differentiating equation A(3), to obtain the basic equation for analyzing the present results,

$$\partial \ln B/\partial \ln C = \frac{1}{2} \left[ 1 + \frac{\partial \ln \gamma}{\partial \ln C} \right] \frac{S}{S - 1}$$

$$+ \frac{g}{g + b} \frac{\partial \ln g}{\partial \ln C} + \frac{b}{g + b} \frac{\partial \ln b}{\partial \ln C}, \quad \text{A(4)}$$

where $S$ is the supersaturation ratio expressed in terms of activity, $S = \gamma C/\gamma_0 C_0$. In the absence of shear, $b = 0$ and equation A(4) reduces to

$$\frac{d \ln B_0}{d \ln C} = \frac{1}{2} \left[ 1 + \frac{\partial \ln \gamma}{\partial \ln C} \right] \frac{S}{S - 1} + \frac{d \ln g}{d \ln C}, \quad \text{A(5)}$$

in which the first term on the right, $(\partial \ln \gamma)$, reflects the concentration dependence of net growth rate and the second (and much larger) term, $(\partial \ln g/d \ln C)$, the concentration dependence of heterogeneous nucleation. At very high shear rates, $b \gg g$ and equation A(4) becomes

$$\frac{\partial \ln B}{\partial \ln C} = \frac{1}{2} \left[ 1 + \frac{\partial \ln \gamma}{\partial \ln C} \right] \frac{S}{S - 1} + \frac{\partial \ln b}{\partial \ln C}. \quad \text{A(6)}$$

This equation predicts only a small dependence of $B$ on concentration, deriving from the first (growth rate) and last (breakage) terms.

Finally, the power dependence of exponential rate on shear rate is

$$\frac{\partial \ln B}{\partial \ln G} = \frac{1}{2} \left[ \frac{b}{g + b} \frac{\partial \ln b}{\partial \ln G} \right]. \quad \text{A(7)}$$

Kinetics at high shear rates. At high shear rates, the dominance of fiber-end creation by breakage over that by heterogeneous nucleation can be estimated from the data. Dividing equation A(1) by equation A(2) to obtain $f$, the fraction of new fiber creation that is due to heterogeneous nucleation:

$$f B_0 = \frac{g}{g + b} = \frac{g + b}{g + b} = f. \quad \text{A(8)}$$

Since measurement of $B_0$ requires the absence of shear, it and therefore $f$ cannot be obtained by viscometry. However, the data do provide $f_{\text{max}}$, an upper limit for $f$, if $B_0$, the value of $B$ at the lowest shear rate used at each concentration, is substituted for $B_0$. 

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\[ \gamma < \left( \frac{B_1}{B} \right)^2 = \gamma_{\text{max}}, \quad \text{(A9)} \]

Reference to Fig 1 shows that \( \gamma \) is a small fraction (<0.1) for many of the data points.

**Concentration dependence of fiber breakage.** To evaluate \( \delta \ln b/\delta \ln C \) from equation A(4) (text equation 3), the concentration dependence of the fiber breakage rate, \( \delta \ln b/\delta \ln C \), must be obtained where \( b = b(C, G) \) is a function of both monomer concentration and shear rate. Fibers are assumed to break when the force on them reaches a critical level, which, under constant shear and solution conditions, occurs when they reach a critical length. Thus, the breakage rate is proportional to the fiber growth rate, \( \tau(C) \).

\[ b(C, G) = \tau(C)b(C, G), \quad \text{(A10)} \]

where \( b(C, G) \) represents breakage rate per unit polymer per unit length.

The dependence of \( b' \) on \( C \) arises from the viscosity dependence of breakage rate. The force on a fiber in a shear field (at any fixed orientation) is maximal in the middle of the fiber and proportional to fiber length (L) in two separate ways: (1) because longer fibers extend to regions of higher flow velocity, and (2) if the fiber is in solution rather than in a solid like layer of a gel, because the fibers have greater lengths exposed to shear. The critical breaking force \( (F_c) \) is also proportional to viscosity \( (\eta) \) and shear rate. Therefore,

\[ F_c \propto L = \eta \eta' \quad \text{(A11)} \]

where the exponent \( m = 2 \) for a solution of fibers (because there are two ways length affects force) and 1 for a solid-like gel. Therefore, breakage rate, \( L \), varies inversely as the square root of \( \eta \) in the former case, and as \( \eta' \) in the latter,

\[ L \propto \frac{1}{(\eta'G)^{1/2}} \quad \text{(A12)} \]

Breakage rate increases in inverse proportion to length because the time to elongate to the breaking length is shorter. But breakage rate is additionally inversely proportional to length, because shorter average length implies more (but shorter) fibers. Hence, breakage rate is inversely proportional to the square of length,

\[ b' \propto 1/L^2 \quad \text{(A13)} \]

and therefore depends on \( \eta'G \) according to

\[ b' = b_0(\eta'G)^m \quad \text{(A14)} \]

Therefore,

\[ b = b_0(\eta(C)(\eta'G)^m, \quad \text{(A15)} \]

where \( b_0 \) is a constant.

The viscosity of hemoglobin solutions at high concentrations fits the Mooney equation:25,25

\[ \eta = \eta_0 \exp \left( \frac{[\eta]k}{1 - k + \frac{(\eta'G)}{c}} \right) \quad \text{(A16)} \]

(\( \eta \) is viscosity, \( \eta_0 \) is solvent viscosity, \([\eta]\) is the intrinsic viscosity of hemoglobin, c is hemoglobin concentration in g/mL, k is a "crowding factor," and \( \eta' \) is Simha’s factor28). The power dependence of viscosity on concentration is then:

\[ \frac{d \ln \eta}{d \ln c} = m + \frac{\eta'G}{1 - \frac{k}{c}(\eta'G)} \quad \text{(A17)} \]

Differentiating equation A(15) and substituting equation A(17) in it:

\[ \frac{\delta \ln b}{\delta \ln C} = \left( 1 + \frac{d \ln \gamma}{d \ln C} \right) \frac{S}{(S - 1)} + \frac{m[\eta]c}{1 - \frac{k}{c}[\eta]c} \quad \text{(A18)} \]

The predicted value of the concentration dependence of exponential progress rate, \( \delta \ln b/\delta \ln C \), can now be obtained by substituting equation A(18) in A(4) and using \( \gamma_{\text{max}} \) to approximate \( f \) (equations A(8) and A(9)). For hemoglobin, \([\eta] = 3.6 \text{ ml/g}, \) and \( k/v = 0.4.23 \)

Figure 4 shows comparison of predicted and observed values of the residual concentration dependence at high shear under both assumptions \( m = 1 \) and \( m = 2 \).

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**References**

Hemoglobin S polymerization and gelation under shear II. The joint concentration and shear dependence of kinetics

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