

# SICKLE HEMOGLOBIN GELATION

## REACTION ORDER AND CRITICAL NUCLEUS SIZE

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**ABSTRACT** Sick cell hemoglobin (Hb S) gelation displays kinetics consistent with a rate-limiting nucleation step. The approximate size of the critical nucleus can be inferred from the order of the reaction with respect to Hb S activity, but determination of the reaction order is complicated by the fact that Hb S activity is substantially different from Hb S concentration at the high protein concentrations required for gelation. Equilibrium and kinetic experiments on Hb S gelation were designed to evaluate the relative activity coefficient of Hb S as a function of concentration. These experiments used non-Hb S proteins to mimic, and thus evaluate, the effect on activity coefficients of increasing Hb S concentration. At Hb S concentrations near 20% the change in Hb S activity coefficient generates two-thirds of the apparent dependence of nucleation rate on Hb S concentration. When this effect is explicitly accounted for, the nucleation reaction is seen to be approximately 10th-order with respect to effective number concentration of Hb S. The closeness of the reaction order to the number of strands in models of Hb S fibers suggests a nucleus close to the size of one turn of the Hb S fiber.

These experiments introduce a new approach to the study of Hb S gelation, the equal activity isotherm, used here also to show that Hb S·Hb A (normal adult hemoglobin) hybrids do incorporate into growing nuclei and stable microtubules but that A·S hybridization is neutral with respect to promotion of Hb S nucleation and the sol-gel equilibrium.

### INTRODUCTION

After deoxygenation, sick cell hemoglobin (Hb S)<sup>1</sup> in concentrated solutions aggregates to form extended microtubules (1,2). The kinetics of the gelation display a latent period (delay time), during which no change in solution properties is evident, followed by large, rapid changes in viscosity (3), heat absorption (4), birefringence (4), etc. Hofrichter et al. (5) interpreted this in terms of a rate-limiting nucleation step followed by rapid microtubule growth (see Fig. 1).

Several investigators have found the delay time ( $t_d$ ) to be proportional to a very

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<sup>1</sup>*Abbreviations used in this paper:* BSA, bovine serum albumin;  $\gamma$ , protein activity coefficient;  $\gamma[\text{Hb S}]$ , Hb S activity;  $\gamma_r$ , relative protein activity coefficient; Hb A, normal adult hemoglobin; Hb S, sickle hemoglobin;  $[\text{Hb S}]_\gamma$ , the concentration of Hb S at a constant activity coefficient;  $n$ , order of the nucleation reaction; T- and R-state Hb A, the allosteric quaternary forms characteristic of normal deoxy and liganded Hb A;  $t_d$ , delay time.

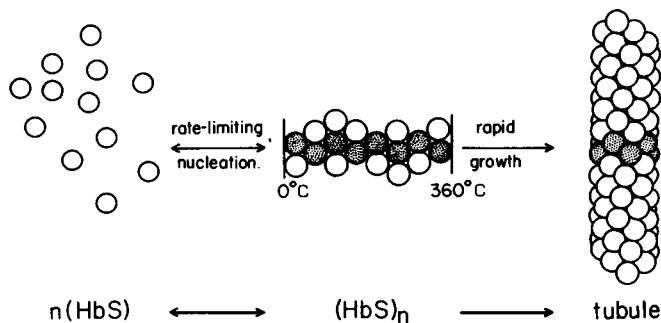


FIGURE 1 Deoxy Hb S monomers associate through a series of unfavorable reactions to reach a stable nucleus that can support fiber growth. Measured reaction order,  $n$ , for the nucleation reaction suggests that 10–18 unfavorable steps are required to achieve a stable nucleus, apparently by closing the growing ring. The microtubule model is from Josephs et al. (22).

large power of the Hb S concentration (5,6). If solution ideality is assumed, the apparent order of the reaction with respect to Hb S concentration can be obtained from the slope of a plot of  $\log (1/t_d)$  vs.  $\log [\text{Hb S}]$ . Hofrichter et al. (5), working in the 19–23% Hb S concentration range, found a slope of 30–40 and interpreted this as indicating that a critical nucleus 30–40 Hb S molecules in size must be formed before microtubule growth can begin. Several researchers (7–9), and most completely Ross and Minton (10), have shown that at high protein concentrations Hb solutions are nonideal due to excluded volume effects, and that protein activity coefficients increase rapidly with rising protein concentration. Thus, to find the true reaction order one must construct the plot of  $\log (1/t_d)$  against  $\log (\text{Hb S activity})$ .

Ross and Minton (10) have previously computed Hb activities from published data on sedimentation (8,9) and osmotic pressure (7) at high Hb concentrations. We have studied the activity-concentration relationship by measurements on the Hb S gelation system itself in both kinetic and equilibrium modes. Our results are generally consistent with Ross and Minton's (10), and lead to a value for the reaction order of about 10.

## MATERIALS AND METHODS

Blood was collected in heparinized tubes from co-workers and from three individuals known to be homozygous for Hb S and to have negligible Hb F levels. Hemolysates were prepared by lysis of the saline-washed erythrocytes in distilled water and centrifugation to remove erythrocyte membranes. The hemolysates, used without further purification, gave delay time values identical to those reported by Hofrichter et al. (5) for purified Hb S. Bovine serum albumin (BSA) was purchased as a 10% solution from Sigma Chemical Co. (St. Louis, Mo.), and BSA concentrations were calibrated by absorbance at 278 nm. Iodoacetamide-reacted des-His-Tyr HbA, which remains in the oxy (R-state) conformation (11), even when unliganded, was prepared by treating native Hb A with carboxypeptidase A in 0.1 M Tris-HCl buffer at pH 8 (12). The reaction was followed by observing the decrease with time in the deoxy/oxy ratio of the Soret band (13). The protease was removed by passage of the reaction solution through a col-

umn of DEAE-Sephadex equilibrated with 20 mM phosphate, pH 6.9. All foreign (non-Hb S) proteins were labeled by incubation with [ $^{14}\text{C}$ ]iodoacetamide for 24 h in 0.25 M phosphate, pH 7.15. Excess reagent was rigorously removed. All proteins were dialyzed against 0.25 M phosphate at pH 7.15 and concentrated in a Schleicher Schuell collodion bag apparatus (Schleicher & Schuell, Inc., Keene, N.H.) to 35% for Hb and to 30% for BSA.

Kinetic experiments were performed to determine protein concentrations that yield a constant delay time when Hb S and foreign protein are mixed in varying proportions. For the experiments with BSA and R-state Hb A, accurately measured amounts of concentrated stock solutions of Hb S and foreign protein were pipetted into a small test tube, and 10  $\mu\text{l}$  of a freshly prepared solution containing 0.75 M sodium dithionite and 0.25 M phosphate at pH 6.9 was added to deoxygenate the solution at 0°C. The final pH was 6.9. For native Hb A, the species most likely to form stable hybrids with Hb S (14,15), similar experiments were performed in which Hb A and Hb S were either mixed in the oxy-state and allowed to hybridize or were deoxygenated with dithionite before mixing to inhibit hybridization. Under the conditions of the experiment, the half time for hybridization of native deoxy Hb is several hours (14,15), compared with the 10 min or so necessary to complete the kinetic experiment. The experimental solutions, under argon, were repeatedly gelled at 37°C, reliquified in an ice bath, diluted by judicious addition of buffer, and gelled again at 37°C, until the delay time was  $150 \pm 20$  s. Protein concentrations at that point were recorded and plotted (Fig. 3). Because the dependence of delay time on protein concentration is so large, the uncertainty of 15% in delay time corresponds to an uncertainty of <1% in Hb S concentration and thus does not compromise experimental accuracy, set at about the 3% level by summed pipetting errors. The total volume of the solutions ranged from 125 to 175  $\mu\text{l}$ . Temperature equilibration from 0° to 37°C required 30 s. The delay time end point was determined simply by observing failure of the solution to flow when the test tube was tilted. In this method, solutions are subjected to no significant shearing force.

In equilibrium experiments performed to determine the equilibrium Hb S solubility in Hb S-foreign protein mixtures, concentrated Hb S and labeled foreign protein were mixed in varying proportions and deoxygenated with dithionite while at 0°C. Again separate experiments were done in which Hb A and Hb S were either mixed in the oxy form to allow hybridization or were deoxygenated before mixing to inhibit hybridization. Solutions were placed in a 4-cm length of thin quartz EPR tubing (16) (40  $\mu\text{l}$  in volume; Amersil Inc., Sayreville, N.J.) and transferred to a 37°C water bath for 10 min to obtain equilibrium gelation ( $t_d \sim 20$  s). They were then equilibrated for 20 min at 20°C and spun in glycerol-filled centrifuge tubes, topped by a rubber ring to center the quartz tubing, in an SW 60 Ti Beckman rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 40,000 rpm (150,000 g) for 1 h (2) with the temperature carefully maintained at 20°C. The quartz tube was then broken 2–3 mm above the pelleted gel and the supernate removed. Supernatant Hb concentration was determined by absorbance at 540 nm. Foreign protein was determined by liquid scintillation counting of the  $^{14}\text{C}$ -label after pepsin digestion of the acidified sample. A color quench correction was applied. The centrifugation conditions used lead to an artifactual decrease in supernatant protein concentration by 5%, owing to protein striking and sliding down the wall of the nonsector-shaped tubes. In the text Fig. 2 shows the directly measured protein concentrations, but the calculations leading to Figs. 4, 5, and 6B incorporate this small correction.

The dependence of delay time on Hb S concentration was determined by successive small dilutions of concentrated Hb S with buffer, noting the delay time and concentration of Hb S at each step. To determine the dependence of delay time on Hb S concentration at constant total protein concentration, Hb S was diluted by successive additions of a solution of BSA equal in concentration to the initial Hb S solution, and the delay time and concentration of Hb S in the successively diluted mixtures were noted.

## RESULTS

### *Use of Hb S Surrogate Proteins*

Solutions of deoxy Hb S containing deoxy Hb A (17), liganded hemoglobins (18) or ovalbumin (19) gel at lower Hb S concentration than solutions of pure deoxy Hb S. We have observed that myoglobin and even nonproteinaceous macromolecules (polyethylene glycol and a high molecular weight polyacrylamide-polyacrylic acid copolymer) can also promote the gelation of dilute Hb S solutions very effectively. Apparently these dissimilar compounds exert their effect not by specific involvement in the process of gelation but rather by increasing the activity coefficient of the Hb S in solution. An analogous activity coefficient effect must occur when the concentration of Hb S itself is increased. The following experiments were designed to evaluate this effect.

These experiments used non-Hb S proteins, which we hoped would not alter Hb S-Hb S interactions directly but would influence the Hb S activity coefficient in just the same way as added Hb S does. BSA, R-state Hb A, and native Hb A were used to promote Hb S gelation kinetics and the Hb S gelation equilibrium. These molecules obviously have very nearly the same size and shape as Hb S, and Ross and Minton (10) have argued convincingly that the activity coefficient effect for Hb depends solely on these parameters. The results obtained support the Ross-Minton conclusions and demonstrate the validity of using surrogate proteins to study such effects.

When the effect of concentration on the Hb S activity coefficient is extracted, one can evaluate the remaining dependence of gelation kinetics on Hb S number concentration per se, i.e., the true reaction order of the Hb S gelation process. This leads to an estimate of the size of the Hb S nucleus necessary for gelation. The results also relate to current questions on the involvement in the gelation process of Hb S hybrids and other non-Hb S proteins.

### *Equilibrium Experiments*

When deoxy Hb S gels, equilibrium is established between sol phase and gel phase Hb S. The equilibrium concentration of sol phase protein can then be measured after centrifuging down the gelled material (2). Because the two Hb S phases are in equilibrium, their chemical activities are equal. The activity of sol phase Hb S is equal to  $\gamma C$ , where  $C$  is the concentration of Hb S and  $\gamma$  is its activity coefficient. If the sol phase Hb S activity coefficient is changed, for example by adding foreign protein, the concentration of Hb S in the sol phase must change correspondingly to maintain equilibrium with the gel phase.

In equilibrium experiments, mixtures of Hb S and foreign protein were gelled, and the gelled material was pelleted by centrifugation. The concentration of sol phase Hb S in equilibrium with the gel is plotted in Fig. 2 as a function of measured foreign protein concentration in the sol. The curves for the three foreign proteins used are identical within experimental error. Also, data for solutions of Hb S and Hb A mixed in the

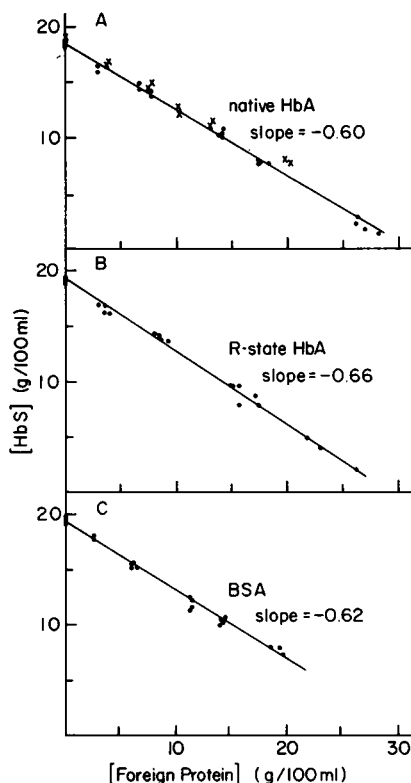


FIGURE 2 Equal activity isotherms for Hb S in the sol phase of equilibrium gelation mixtures plotted against foreign protein in the sol phase. The slopes indicate the amount of T-state Hb A, R-state Hb A, and BSA needed to keep Hb S activity constant as Hb S concentration decreases. In A, ● represents data points for Hb A and Hb S mixed while liganded; × represents data points for Hb A and Hb S mixed after deoxygenation.

oxy state and hybridized fall on the same line as the points for solutions of the two hemoglobins mixed in the deoxy state and therefore unable to hybridize.

It is seen that the presence of increasing amounts of foreign protein in the sol causes a depression of the concentration of Hb S in equilibrium with the gel. However, if the chemical activity of the gel remains constant (see Discussion), the activity of the sol phase Hb S remains constant because at all points on the curves sol phase Hb S remains in equilibrium with the gel. Therefore, the curves in Fig. 2 define an equal activity isotherm for Hb S. At any two points on the isotherm,  $\gamma_1 C_1 = \gamma_2 C_2$  or  $\gamma_1/\gamma_2 = C_2/C_1$ . The ratio of the activity coefficients at any two points on the curve can be calculated from the concentrations of sol phase Hb S at the two points.

#### *Kinetic Experiments*

The above experiments deal with solutions at equilibrium. Similar results come from kinetic experiments. Kinetic experiments were designed to determine protein solu-

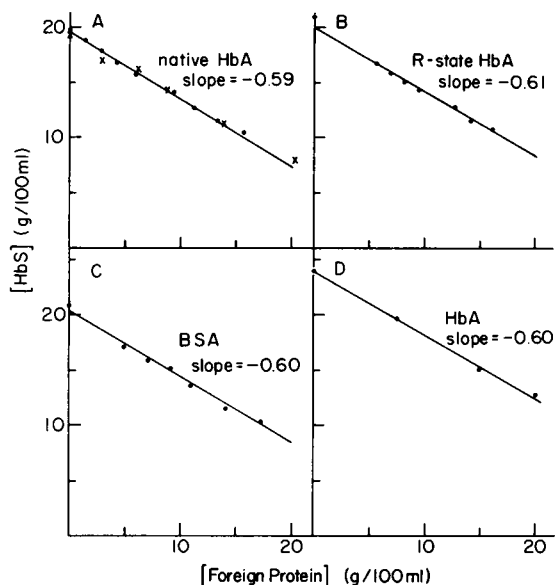


FIGURE 3 Equal activity isotherms for Hb S in terms of the composition of mixed protein solutions which gel in  $150 \pm 20$  s. The lower right-hand panel shows the isotherm calculated from data of Bookchin and Nagel (18), which were obtained as a kinetically determined approximation to the equilibrium Hb S solubility for solutions of Hb S mixed with Hb A. In A,  $\bullet$  represents data points for Hb A and Hb S mixed while liganded;  $\times$  represents data points for Hb A and Hb S mixed after deoxygenation.

tions that gel after a delay time of 150 s at 37°C. Mixtures having this property are shown in Fig. 3. As in the equilibrium experiments, the presence of increasing concentrations of foreign protein decreases the concentration of Hb S necessary to maintain constant Hb S activity, defined kinetically by equal delay times. As in the equilibrium experiments, there is no difference in the results for solutions of Hb S with Hb A mixed in either the oxy or deoxy forms.

The curves obtained for BSA, R-state Hb A, and native Hb A (Fig. 3) are identical within experimental error. Also, there is no statistical difference between the equal activity isotherms defined by the kinetic data and the equilibrium data. (It appears, however, that the curve obtained from the equilibrium experiments is displaced on the concentration axes by  $-5\%$  owing to the artifactual loss of supernatant protein during the centrifugation run.) It should be noted that the curves of Figs. 2 and 3 happen to fall essentially at the same level on the Hb S axis only because of the differing conditions of the experiments (20°C for the equilibrium experiments, 37°C and 150 s for the kinetic experiments).

#### *Calculation of $\gamma$ , vs. Hb S*

Both the kinetic and equilibrium experiments show that added foreign protein increases the Hb S activity coefficient. Let us assume that the presence of a molecule of

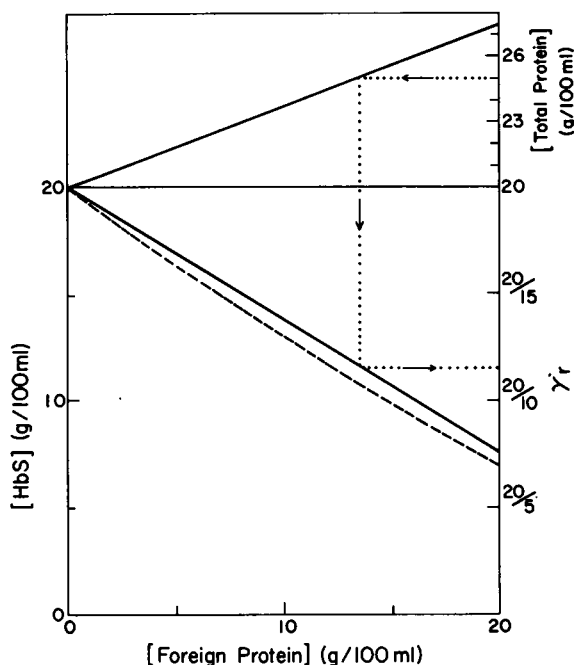


FIGURE 4 Summary curves for calculation of relative activity coefficients as a function of total concentration of Hb S-like protein. The rising curve shows total protein concentration along the activity isotherm. —, Summary equal activity isotherm from Figs. 2 and 3; ----, isotherm predicted from Ross and Minton's results (10). The arrows indicate the pathway taken in the sample calculation (see text) for relative activity coefficient,  $\gamma_r$ , in 25% solution.

foreign protein in an Hb S solution has the same effect on the Hb S activity coefficient (not total activity) as the presence of a molecule of Hb S itself (see Discussion). That is, the activity coefficient of Hb S in a mixture with foreign protein at a defined total protein concentration is equal to the Hb S activity coefficient in a solution of pure Hb S at the same protein concentration. Then, using the equal activity isotherm, one can calculate the relative activity coefficient of Hb S in pure solution as a function of Hb S concentration.

The summary isotherm shown in Fig. 4 is an average of the lines in Figs. 2 and 3 after correction for the centrifugation artifact. It has a slope of  $-0.61$  and an intercept at 20% Hb S. The rising curve shows the total protein concentration in the mixtures. As stated previously, the ratio of the activity coefficients,  $\gamma_2/\gamma_1$ , at any two points on the isotherm, is equal to the inverse of the ratio of the Hb S concentrations at the two points,  $C_1/C_2$ . To calculate the relative activity coefficient,  $\gamma_r$ , of Hb S along the isotherm,  $C_1$  is defined as the concentration of Hb S at the intercept; i.e.,  $\gamma_1 = 1.0$  when  $C_1 = 20\%$ . The arrows in Fig. 4 show the pathway of a sample calculation. In the presence of a total protein concentration of 25%, the activity of 11.5% Hb S equals the activity of pure Hb S at 20%. The activity coefficient of Hb S at 25% relative to the activity coefficient of Hb S at 20% is then  $20/11.5$ , or 1.7. The relative activity of Hb S,

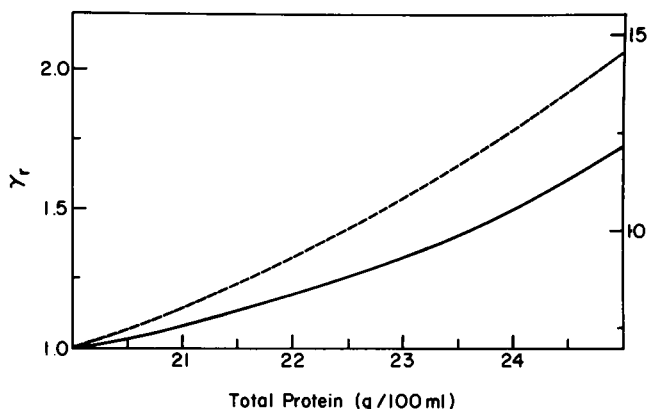


FIGURE 5 Relative activity coefficient,  $\gamma_r$ , plotted against total concentration of Hb S-like protein. Activity coefficient of pure Hb S at 20 g/100 ml is normalized to unity. —, From our experiments; ----, calculated from Ross and Minton's results (10). The right-hand ordinate shows absolute activity coefficients from Ross and Minton's results (10).

$\gamma_2 C_2 / \gamma_1$ , in a 25% solution of pure Hb S is then  $1.7 \times 25$  or over twice that at 20% Hb S, defined as  $1.0 \times 20$ .

The solid line in Fig. 5 shows a plot of  $\gamma_r$  vs. total protein concentration calculated in this manner. The dashed line gives analogous  $\gamma_r$  values for Hb A calculated from the results of Ross and Minton (10), who used published osmotic pressure (7) and equilibrium centrifugation (8, 9) data to calculate absolute values of Hb activity coefficients as a function of concentration. The discrepancy between the two curves may be largely due to an unfortunate sensitivity of our calculation to the exact slope and intercept of the isotherm. If the preceding calculation is reversed, Ross and Minton's data can be used to compute an equal activity isotherm for Hb comparable to our measured curve. The dashed line in Fig. 4 was generated in this way and compares closely with our measured data.

### Reaction Order

If ideality is assumed, the apparent reaction order for the rate-limiting nucleation step of Hb S gelation is given by the slope of a plot of  $\log (1/t_d)$  vs.  $\log [\text{Hb S}]$ . Fig. 6 A shows delay time-concentration data plotted in this manner. A slope of 32 was obtained in agreement with Hofrichter et al. (5). As pointed out earlier, however, Hb S activity must be considered rather than Hb S concentration. Because of the properties of the log function, absolute values of Hb S activities are not needed to obtain the correct slope. If all  $\gamma_r$  values are normalized to the same point, as in Fig. 5, the reaction order is given by the slope of  $\log (1/t_d)$  vs.  $\log [\gamma_r \cdot \text{Hb S}]$ . Fig. 6 B gives such a plot. An analogous plot using  $\gamma_r$  values from Ross and Minton's work (10) is also shown. The slope indicates that the nucleation reaction is about 10th order with respect to Hb S activity.

Alternatively, the reaction order can be obtained using Hb S concentration if the



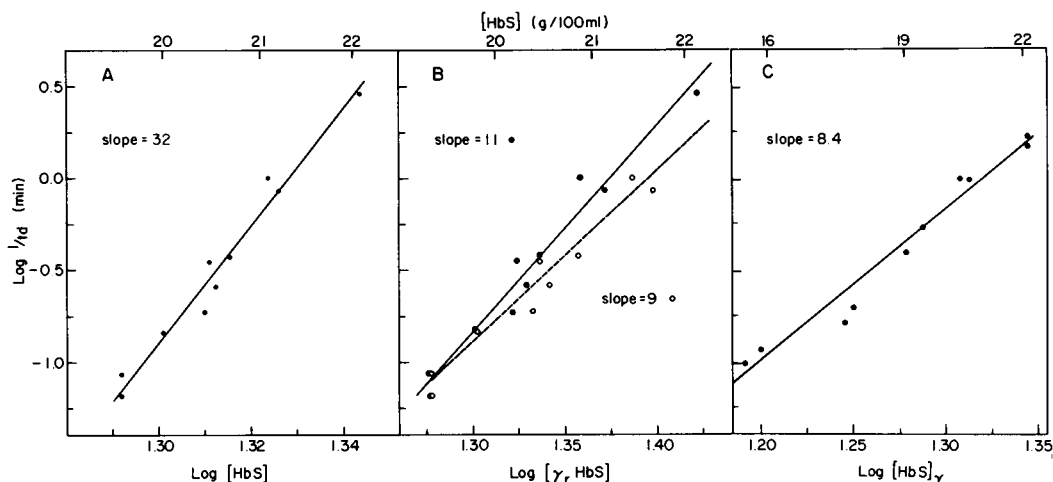


FIGURE 6 Delay time ( $t_d$ ) log-log plot to find the order of the nucleation reaction. Log(inverse delay time) in pure Hb S solutions is plotted against Hb S concentration (A) to give apparent reaction order, and against Hb S activity (B) to give true reaction order with respect to the number concentration of Hb S. —, From the present results; ----, using activity coefficients calculated by Ross and Minton (10). Panel C shows a delay time-Hb S concentration plot for solutions of Hb S in which the total protein concentration was kept constant by the addition of appropriate amounts of concentrated BSA.

activity coefficient of Hb S is kept constant as the concentration of Hb S is varied. The previous experiments show that when foreign protein is present, the activity coefficient of Hb S is determined by the total protein concentration. Therefore, if total protein concentration is kept constant, the Hb S activity coefficient will not change. Additionally, if this approach is adopted, the activity coefficients of any prenuclear Hb S aggregation intermediates will also be kept constant. Fig. 6C shows a plot of  $\log(1/t_d)$  vs.  $\log[\text{Hb S}]$  in which Hb S concentration was varied, but total protein concentration was kept constant by the addition of appropriate amounts of BSA to the gelation mixture. A slope of 8.4 was obtained in good agreement with Fig. 6B. Hofrichter (personal communication) has obtained similar results using  $\text{CO} \cdot \text{Hb S}$  to maintain constant protein concentration.

## DISCUSSION

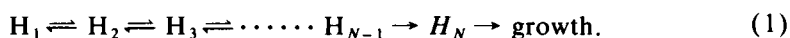
### *Reaction Order and Critical Nucleus Size*

Hb S gelation displays kinetics characteristic of a nucleation-dependent condensation reaction (5). When conditions favoring gelation are imposed upon a concentrated Hb S solution (e.g. deoxygenation, or warming above the critical temperature), there follows a lengthy delay period ( $t_d$ ) and then a shorter period (about  $t_d/2$ ) of rapid growth (5). The length of the delay period is highly determinate (e.g. see Figs. 2 and 6 A) and terminates very reproducibly. It has proved difficult to detect any Hb S association intermediates during the delay period (19), but the onset of rapid tubule

growth that marks the end of this period is apparent even to the unaided eye and has been studied by many physical and optical techniques.

The great difficulty of detecting prenuclear association products requires that any such species be kinetically unstable so that they do not reach experimentally measurable levels during the long delay period. Nevertheless, the reproducibility of the delay time requires that a statistically large number of sequential, multimolecular reactions must proceed in a regular way during the delay period and eventually generate a number of stable structures, the critical nuclei, which in short order bring the delay period to a close by supporting rapid fiber growth.

The simplest kinetic scheme for producing sizable nuclei after a defined delay period involves the sequential addition of Hb S molecules to a growing sequence of prenucleation aggregates, as in Eq. 1.



In this scheme prenuclear aggregates are kinetically unstable owing to their susceptibility to a fast dissociation reaction, but at the level of the nucleus ( $H_N$ ) the back reaction is essentially abolished and the nucleus forms a stable structure capable of supporting rapid growth. The regularity of Hb S nucleation kinetics appears to require some such process, though the details may be more complex than suggested in Eq. 1.

Hofrichter et al. (5) considered the special case of Eq. 1 in which the development of the critical nucleus is slow compared to growth, so that the observed delay time corresponds to the period necessary to begin to produce a number of nuclei through the sequence of unfavorable, rate-limiting steps. The formal solution for this simple kinetic situation has not been rigorously worked out, and a searching examination also must consider possible side and branching reactions. However, it seems intuitively likely that the apparent order of the overall reaction for generating nuclei, and thus ending the delay period, will correspond roughly to the number of rate-limiting addition reactions required to form the critical nucleus, and therefore approximately to the number of Hb S molecules in the critical nucleus. Hofrichter et al. (5) have suggested that for this case the nucleus size will be equal to the reaction order plus one.

More recently, Eaton (W. A. Eaton, personal communication), following Oosawa's treatment of actin polymerization (20), has considered the case in which the kinetics for generating the nuclear and prenuclear aggregates is fast, so that they are effectively at equilibrium with  $H_1$  from zero time. In this scheme the apparent end of the delay period is taken to occur at some preset point in the growth phase. Here the nucleus size turns out to be twice the reaction order (though Eaton points out that in special cases the nucleus might be a larger multiple of reaction order).

Our results indicate that the reaction order is close to 9. Given the kinetic approaches just described, it appears that a reasonable estimate of Hb S nucleus size is 10–18. Until the difficult problems in this area are solved, the possibility that this tentative conclusion is misleading must be kept open, but present knowledge suggests that the final answer will not be far outside this range.

In light of present knowledge of the structure of the Hb S fiber, these numbers are

quite suggestive. The basic macrostructure forming the Hb S gel is known to consist of a long fibrillar aggregate (1,2), like that diagrammed in Fig. 1, and developing knowledge in this area has changed the estimate of the cross-sectional extent of the most abundant fiber form observed in Hb S gels from 6 Hb S molecules (21) to 8 (22) and most recently to 14 (S. J. Edelstein, personal communication). It also appears that tubules differing in size from the abundant form can occur with lesser frequency (22).

It is hard to ignore the correspondence between the size of a ring (21,22) or plate (Edelstein, personal communication) forming one turn of the Hb S fiber and the apparent size of the first stable nucleus necessary to support growth of the fiber. That the two may be synonymous is also suggested by consideration of the fundamental principles of a nucleation process. The prenuclear aggregates ( $H_2$  through  $H_{N-1}$  in Eq. 1) are all, by definition, unstable. They dissociate rapidly to smaller forms. When the structure  $H_N$  is achieved, something new occurs; the addition of one extra Hb S monomer to the unstable form,  $H_{N-1}$ , suddenly and discontinuously abolishes the fast back-reaction and thereby imposes kinetic stability against dissociation upon the newly formed nucleus. If the stable nucleus represents just one turn of the microtubule, then an obvious structural explanation for this striking behavior is apparent. Prenuclear aggregates can degrade rapidly because the incomplete ring or plate is readily dissociated, whereas the completed structure is bound to be much less susceptible to degradation. No comparable event occurs at any other structural stage. In the several prenuclear structures one can draw, instability exists because monomers at the growing ends of prenuclei make few contacts and therefore dissociate relatively easily, and because a single separation reaction between two internal monomers can separate the whole structure. When ring closure is achieved, this situation changes discontinuously. The closing of the ring removes the previously vulnerable ends, and also requires at least two internal separation reactions to occur simultaneously to degrade the structure. Furthermore, this picture provides a ready explanation for minor forms (22) consisting of tubules of smaller or larger dimension than the abundant form. Closure may occasionally occur with an aberrant number of monomers. Though such a structure may be strained relative to the abundant form, it can still be stable against rapid dissociation, for the reasons just outlined, and provide a template upon which a fiber of the same dimension can grow.

To summarize, the apparent molecularity of the Hb S nucleation process measured in this work is close to the size of one turn of the Hb S fiber. This and related considerations suggest that the critical nucleus consists of one completed turn of the fiber.

### *The Use of Molecular Mimicry*

In this section we discuss the principles that make possible the use of surrogate proteins for mimicking and thus evaluating Hb S activity coefficient effects.

Ross and Minton (10) have shown that, under the conditions we used, the activity coefficient of Hb in concentrated solution can be wholly accounted for by excluded volume effects. For illustration, let us consider a single molecule of Hb S in a solution of Hb at a given concentration. The volume excluded to that molecule of Hb S (23),

defined by its distance of closest approach to other molecules, is the same whether the rest of the proteins in solution are Hb A, Hb S, or a mixture of both. An identical situation prevails in a mixture of Hb S with BSA, because the two proteins are very similar in size. Owing to the excluded volume, the Hb S acts as though it is more concentrated than its straightforwardly measured concentration. For example, Hb S molecules will collide more frequently than if the extra protein were replaced by free solvent. Mechanistically this is so because, with the other protein molecules present, an Hb S molecule needs to search (diffuse) through a lesser volume before achieving collision with another Hb S molecule; the accessible volume is only a fraction of the total apparent volume. Conventionally, the chemist compensates for this apparently increased activity by assigning an activity coefficient multiplier to the measured Hb S concentration. The appropriate coefficient can be computed a priori if size and shape are known, as Ross and Minton have done (10), or the coefficient can be extracted from measurements of some activity-dependent parameter as a function of concentration, for example, from equilibrium sedimentation or osmotic pressure data taken at high protein concentration (10).

In concentrated solutions of Hb S, a major role in the gelation process is played by the volume excluded by the Hb S molecules themselves. The influence on both gelation kinetics and gelation equilibrium can be conveniently and quantitatively expressed as a concentration-dependent activity coefficient effect. We have calibrated the Hb S activity coefficient effect by systematically substituting for Hb S molecules the surrogate proteins BSA, R-state Hb A and native deoxy Hb A, all of which have closely the same size and shape as Hb S. Our approach was to add known amounts of surrogate protein and to see how much this was worth in terms of the reduction in Hb S concentration necessary to keep Hb S activity constant at the original level (20% in Fig. 4). The decreased Hb S concentration could then be used to calculate the increase in its activity coefficient as in Fig. 4. The assumption that the effect of added surrogate protein is equal to the effect due to the same concentration of Hb S enters in constructing and using the curve in Fig. 4 for total protein, wherein Hb S and foreign protein are considered interchangeable.

The discussion just given outlines the rationale for the use of non-Hb S proteins in a study of activity coefficients in concentrated Hb S solutions as a result of excluded volume effects. For this approach to succeed, it is necessary that the added proteins: (a) closely mimic (all) activity coefficient effects due to Hb S, and (b) impose no other effects, either positive or negative, upon Hb S gelation. Evidence that these criteria are met can be found in Figs. 2 and 3, where all three proteins used (T-state Hb A, R-state Hb A, and BSA) are shown to exert quantitatively the same effect. This result seems to require that just as T-state Hb A by necessity accurately mimics the size and shape (and any other activity coefficient effectors) of deoxy Hb S, so must the other surrogate proteins used. Also, if any of the surrogate proteins exercise some further specific interaction effect (outside of the excluded volume influence) on Hb S nucleation and growth, then all three proteins must exert quantitatively the same effect. Because one of the three proteins is BSA, the apparent conclusion is that the influence

of specific interactions, exerted in common by all three proteins, is essentially zero. Further evidence that the three proteins influence Hb S gelation solely through an excluded volume effect is found in the agreement between the measured isotherm (Fig. 4) and the isotherm predicted from the results of Ross and Minton (10) because the Ross-Minton calculation is based only on the Hb-excluded volume effect.

It may be noted in passing that the other major possible source for activity coefficient effects, charge-charge interaction, seems quite unlikely on the basis of prior work by Scatchard and Pigliacampi (24). These investigators have shown that at low ionic strength, BSA and CO·Hb A have differing second virial coefficients and interact electrostatically, but that this effect is rapidly attenuated by increasing salt concentration. The ionic strength of solutions in our experiments was purposely kept high (0.25 M  $\text{PO}_4$ ) to preclude such interactions.

In summary, the approach used here to separately evaluate concentration-dependent solution nonideality and the intrinsic concentration dependence of the Hb S gelation process is well grounded in theory and experiment.

The conclusion that Hb A exerts no specific effects on the Hb S gelation process does not necessarily require that Hb A fails to become incorporated into growing nuclei and the gel phase, but merely that if it does, then the effect of the incorporation must be neutral with respect to gelation kinetics and equilibrium. This issue is discussed in the next section.

### *Hybridization and Incorporation*

The results discussed in this paper have implications for some current questions concerning the possible incorporation of non-Hb S hemoglobins into Hb S gels. It is difficult to distinguish non-Hb S incorporation into the Hb S gel by direct experimental measurement of the gel phase (2,25). The present experiments provide an alternative approach to this issue.

The hemoglobin molecule is a tetramer consisting of two identical  $\alpha\beta$  dimers, and liganded hemoglobin is in rapid association-dissociation equilibrium with its dimers. When Hb A and Hb S are mixed in the oxy state, their  $\alpha\beta$  dimers will reassociate in seconds (14) to form a fraction of A·S hybrids in which the  $\beta$  subunit in one  $\alpha\beta$  dimer carries the sickle mutation and the other is normal. The proportion of hybrids is simply statistically determined, e.g. when equal amounts of liganded Hb A and Hb S are mixed, the resultant solution contains Hb A, A·S hybrids, and Hb S in a 1:2:1 ratio (26,27). Unlike the liganded form, deoxy Hb dissociates only very slowly, so that Hb A and Hb S when mixed in the deoxy form will reassociate with a half-time of 1 h or so at 37°C and even more slowly at lower temperature (14,15).

In some of our experiments Hb A and Hb S were initially mixed in the oxy state. In these solutions, all three hemoglobin species (A·A, S·S, and A·S hybrids) were present before the solutions were deoxygenated and processed as described. In other experiments, the Hb A and Hb S were deoxygenated before mixing so that no hybrids were present when the experiments began. Given our protocols of time and temperature, essentially no rehybridization occurred in any of these solutions before the kinetic

experiments were terminated and only a small amount ( $\sim 5\%$ ) before the sol-gel separation in the equilibrium experiments. The same results were found whether hybrids were present or not, and this is true of both the kinetic and equilibrium experiments. All the results match those for BSA which, of course, cannot hybridize with hemoglobin. Bookchin et al. (28) and Goldberg et al. (25) have also noted that the minimum gelling concentration for mixtures of Hb S with Hb A is independent of the presence or absence of A·S hybrids. Moffat (29) reported slightly lower minimum gelling concentration values for hybridized Hb S-Hb A mixtures than for the unhybridized situation.

Do A·S hybrids incorporate into the gel? If A·S hybrids are excluded from specific participation in the gelation process, then the total hybrid population would behave as foreign protein in our experiments. Hybridization would act to decrease the effective Hb S concentration, and for a given initial mixture the delay time would be lengthened. To recover the standard 150-s delay time, a compensatingly higher proportion of Hb S would have to be initially added, so that the kinetically determined isotherm (Fig. 3) would appear shifted upward compared to the nonhybridized case (i.e. intercept still at 20% Hb S, and slope displaced toward the horizontal). The same effect would occur for the equilibrium isotherm (Fig. 2) because more Hb S must be left behind in the sol phase to compensate for that removed into hybrids. Calculation shows that the effect would be easily measureable. That no such effect occurs demonstrates that A·S hybrids cannot have been excluded. They must participate actively in gelation. Several researchers have reached this conclusion (25,28–33), and it has been experimentally demonstrated by Bookchin et al. (31).

However, a hybrid molecule cannot participate in the kinetic and equilibrium processes as effectively as pure Hb S. For every S·S molecule that hybridizes, two A·S hybrids capable of forming polymers are produced. If the specific polymerizability of each hybrid equaled that of Hb S, hybridization would increase the effective Hb S concentration, and the isotherms would all be shifted downward compared to the nonhybrid case (intercept at 20% Hb S, slope displaced toward the vertical). The fact that hybridization is quantitatively neutral with respect to gelation (Figs. 2A and 3A) requires that each of the two hybrid molecules, produced by hybridization from one Hb S molecule, must retain exactly half of the specific interaction effectiveness of pure Hb S.

The halved effectiveness of A·S hybrids finds an obvious explanation in terms of known hemoglobin structure. Hb A and Hb S have two-fold symmetry about their  $\alpha_1\beta_1 \cdot \alpha_2\beta_2$  cleavage plane. Clearly, Hb S can be incorporated into gels in either of its two equivalent orientations. The A·S hybrid has halves that are not quite identical. They differ at one point; the position of the  $\beta 6\text{Glu-Val}$  mutation. Our results can be explained by picturing that, owing to the various contacts the Hb S molecule makes in the gel, the  $\beta 6\text{Val}$  on one face of the molecule is necessary for incorporation, whereas the  $\beta 6\text{Glu}$  or Val residue on the other face is irrelevant. If we refer to the contact regions in this model as face 1 and face 2, then at face 1 the Hb A dimer ( $\beta 6\text{Glu}$ ) is excluded, but the stereochemistry about face 2 does not distinguish  $\beta 6\text{Glu}$  from Val.

With respect to the kinetics of gel formation, the fraction of collisions that will successfully join an incoming A·S tetramer to the growing structure will then be reduced by half. The very same postulate also explains the halved effectiveness of the A·S hybrid with respect to equilibrium incorporation. An A·S hybrid can occupy any position in the fiber in one orientation but not in the other, whereas a pure Hb S molecule can of course bind in its two identical orientations, and thus enjoys a twofold advantage in the competition for site binding. An evident corollary of this view is that pure Hb A could not be incorporated into Hb S gels to any significant extent.

In summary, the present results support the conclusion (25,28–33) that A·S hybrids enter Hb S gels and incipient nuclei. Our results support the qualitative prediction of Bookchin et al. (31) that the polymerizing tendency of A·S hybrids will be smaller than Hb S tetramers, and quantify this relationship. However, the implication that Hb A tetramers are not included in the gel contradicts several researchers' findings (2,25,29). It may be noted that among these reports, one suffers from neglect of solution nonideality (29), and data in the others are subject to large corrections (2,25). Further results relating to these issues will be discussed elsewhere.

### *Implications for Therapy*

The discovery of the true reaction order for Hb S gelation has implications for the clinical treatment of the disease. Hofrichter et al. (5), when they initially found the apparent 30th-power dependence of delay time on Hb S concentration, put forward the fertile suggestion that the nucleation reaction might provide a sensitive point at which to attack sickling disease. The present results help to refine that suggestion.

Eaton et al. (34) pointed out that small changes in erythrocyte volume will greatly affect delay time because Hb S concentration is changed proportionately. The apparent 30th-power dependence of gelation time was measured by Hofrichter et al. (5) in a relatively low concentration range, where Hb S concentration never exceeded 24%. Even in this range the change in activity coefficient of Hb S with concentration accounts for most of the concentration dependence of the delay time. In erythrocytes an effect much greater than 30th-power in concentration will prevail because Ross and Minton's results (10) display a rapidly accelerating Hb S activity curve in the concentration region of about 30–35% commonly found in the erythrocyte. Thus dehydration would greatly promote sickling speed, whereas even a slight erythrocyte expansion, if it could be managed, would be highly beneficial. On the other hand, chemical approaches, which seek to block critical Hb S contact residues, will profit by only a smaller though still substantial 10th-power dependence on "inactivated" Hb S because total protein concentration would remain constant.

Our results also help roughly to define the relationship between increase in delay time and clinical benefit. Fig. 6B and C show that delay time is proportional to the concentration of Hb S to the 10th power, at constant total protein concentration. The delay time for a solution of Hb A and Hb S mixed in a 60:40 ratio would therefore be  $2.5^{10}$  times longer than that in a pure Hb S solution of the same total protein concentration. This is the situation for Hb S heterozygotes. The delay time in their erythro-

cytes is extended by a factor of nearly  $10^4$ , and they rarely experience sickling symptoms. To achieve this delay time extension in Hb S homozygotes, 60% of their cellular Hb S would have to be chemically inactivated because only the concentration of polymerizable Hb S, not total protein, would be affected, so a 10th-power dependence would hold. The same effect could be obtained by increasing erythrocyte volume by just 10%, as both the concentration and the activity coefficient of the intracellular Hb S would be lowered, and the delay time would respond with an 80th-power dependence.

We are grateful to Doctors W. A. Eaton, S. J. Edelstein, and J. Hofrichter for providing us with manuscripts in advance of publication. This work was supported by research grant AM 11295 from the National Institutes of Health.

*Received for publication 14 February 1978.*

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