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Kinetics and Mechanism of Deoxyhemoglobin S Gelation: A New Approach to Understanding Sickle Cell Disease*

(calorimetry/pathophysiology/nucleation theory/sickle cell therapy/macromolecular assembly)

JAMES HOFRICHTER, PHILIP D. ROSS, AND WILLIAM A. EATON

Laboratories of Chemical Physics and Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

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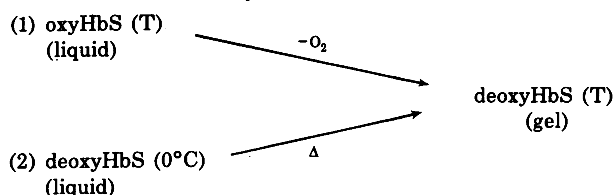
ABSTRACT We report the results of a kinetic investigation on the gelation of purified deoxyhemoglobin S. Gelation was induced by raising the temperature and was monitored by measuring both the heat absorbed, with a microcalorimeter, and the appearance of linear birefringence, with a microspectrophotometer. The kinetics are unusual. Prior to the onset of gelation there is a delay period, followed by a sigmoidal progress curve. The delay time is formally dependent on approximately the 30th power of the deoxyhemoglobin S concentration; a decrease in concentration from 23 to 22 g/dl increases the delay time by a factor of four. It is also extremely temperature dependent; a 1°C temperature rise in the range 20–30°C almost halves the delay time. From these results we conclude that the initial rate is controlled by the nucleation of individual fibers. We present a kinetic model that accounts for the concentration, temperature, and time dependence of the initial phase of the gelation reaction. Extrapolation of our data to physiological conditions predicts that changes in intracellular hemoglobin concentration and oxygen saturation, realizable *in vivo*, produce enormous changes in the delay time. The range of delay times spans both the mean capillary transit and total circulation times. This result points to the delay time as an extremely important variable in determining the course of sickle cell disease, and suggests a new approach to therapy.

Hemoglobin S in concentrated solutions aggregates to form a highly viscous material, referred to as a gel. It is the formation of this gel that rigidifies and distorts deoxygenated erythrocytes of patients with sickle cell anemia. Considerable new insight into the structure and equilibrium behavior of this system has been gained through recent electron microscope (1), x-ray diffraction (1, 2), optical (3), solubility (4), sedimentation (5–7), and theoretical (8, 9) studies. The gel may be tentatively described as consisting of two phases in reversible equilibrium: a liquid phase containing mainly monomeric (molecular weight of 64,000) hemoglobin and a solid phase containing polymeric hemoglobin in the form of bundles of long straight fibers (compare ref. 8). The solid phase, which exhibits optical birefringence and other properties of a liquid crystal, is favored by low oxygen concentrations and high temperatures.

Much less is known about gelation kinetics. The rate of gelation of deoxyhemoglobin S at a fixed temperature T may be measured in two ways:



In the first experiment gelation is induced by removing oxygen from an oxyhemoglobin S solution. In the second, gelation is induced by raising the temperature of an already deoxygenated hemoglobin S solution from 0°C, where it is a nonbirefringent liquid, to the higher temperature T. Although the first experiment may be considered more relevant to the *in vivo* sickling process, the two experiments should give identical results if the deoxygenation and temperature change are both fast when compared to the rate of gelation. Neither experiment has previously been carried out on hemoglobin S solutions, but the deoxygenation experiment has been attempted several times with cells (10–13). In none of these experiments has the intrinsic rate of sickling been resolved. In this paper we present a kinetic study of the thermally induced gelation reaction in solutions of deoxyhemoglobin S.

MATERIALS AND METHODS

Erythrocyte lysates were prepared from freshly drawn blood of patients with homozygous S disease according to the method of Perutz (14), except that lysis of the packed cells was accomplished by freeze-thawing with liquid nitrogen followed by hypotonic shock with deionized-distilled water. Separation of oxyhemoglobin S was carried out by DEAE-cellulose chromatography with minor variations of the method described by Huisman and Dozy (15). The fraction containing hemoglobin S was dialyzed against 0.25 M potassium phosphate (pH 6.9), and concentrated by ultrafiltration. Scanning starch gel electrophoresis of the final material revealed a single band of hemoglobin S, indicating >98% purity. There was less than 4% methemoglobin S. Deoxygenation was carried out under nitrogen by the addition of 0.5 M sodium dithionite (a kind gift from Dr. Graham Palmer) in potassium phosphate buffer. For optical measurements 10- to 15-μl drops of cold solution were sealed between glass coverslips with dental wax. For calorimetric measurements 1.6 ml of cold solution were sealed in a stainless steel calorimeter cell. All sample handling was carried out in a nitrogen atmosphere.

Hemoglobin concentrations were determined spectrophotometrically after conversion with potassium ferricyanide and potassium cyanide to cyanomethemoglobin. Concentrations are expressed in g % (g/dl) and are based on $\epsilon = 11{,}000$ M^{-1} cm^{-1} per heme at 540 nm and, for convenience, a

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molecular weight of 16,000 g/mole of heme. The final composition of the solvent for all measurements was defined by the 0.25 M potassium phosphate, pH 6.9 dialysate, plus 0.05 M sodium dithionite. The number of moles of deoxyhemoglobin S in the polymerized phase of the gel was determined by measuring both the concentration of monomeric hemoglobin in the supernatant after centrifugation (140,000 × *g* for 90 min) and the volume fraction of polymerized material. At the completion of an optical, calorimetric, or sedimentation experiment, the hemoglobin was shown to be completely in the deoxy form by measuring the visible absorption spectrum of a small aliquot sealed between coverslips, using a double-beam recording microspectrophotometer similar to the instrument previously described (16).

Calorimetric experiments were carried out in the instrument previously described (17). The areas of the calorimetric emf-time curves were evaluated by planimetry, and correction (18) for the normal decay time of the instrument was made in order to obtain true kinetic data. Optical birefringence was detected by measuring the light transmitted through a sample placed between crossed linear polarizers. The measurements were made with a Leitz Ortholux-Pol microscope equipped with a photometric attachment, employing a RCA 1P28 photomultiplier tube and Keithley 414S picoammeter. The temperature was controlled by placing a sample, sealed between coverslips, in a bilayer copper housing, mounted on a thermostatted stage. The half-time for temperature equilibration was measured by monitoring the transmittance at 589 nm of a sample of cresol red in Tris buffer after introduction into the copper housing, and was found to be 9 ± 2 sec for jumps from 20°C to 2°C.

The observed light intensity transmitted through the sample placed between crossed linear polarizers may be related to the amount of hemoglobin that has been incorporated into the ordered, solid phase, and it can be shown that (Ross, Hofrichter, and Eaton, to be published):

$$[I(t)/I(\infty)]^{1/2} = N(t)/N(\infty) \equiv f \qquad [1]$$

is a good first approximation. In this equation $I(t)$ is the light transmitted through the analyzer at time t, $I(\infty)$ is the light transmitted at infinite time, $N(t)$ is the number of moles of aligned hemoglobin molecules in the volume under observation at time t, $N(\infty)$ is defined analogously, and f is the fractional extent of the reaction.

RESULTS

A 23.3 g % solution of deoxyhemoglobin S is a liquid at 0°C. When the temperature is increased, the solution becomes extremely viscous, ceases to flow, and exhibits linear birefringence. These are the well-known changes that accompany deoxyhemoglobin S gelation (19, 20). In order to examine the kinetics of gelation we performed "temperature-jump" experiments. In these experiments the temperature of either a liquid solution or a gel is rapidly changed, and the time evolution of the system at a new fixed temperature is monitored either optically or calorimetrically. If the temperature of a gel, initially at 20°C, is rapidly changed to 2°C, the birefringence disappears with a half-time of 26 sec (Fig. 1, Curve 1). This half-time is somewhat longer than the 9-sec half-time required for thermal equilibration; thus, the true half-time for the birefringence decay must be slightly smaller than 26 sec. When, however, the temperature of a sample, initially at 0°C, is rapidly raised to 20°C, a totally different kinetic behavior in observed (Fig. 1, Curves 2 and 3). No heat or

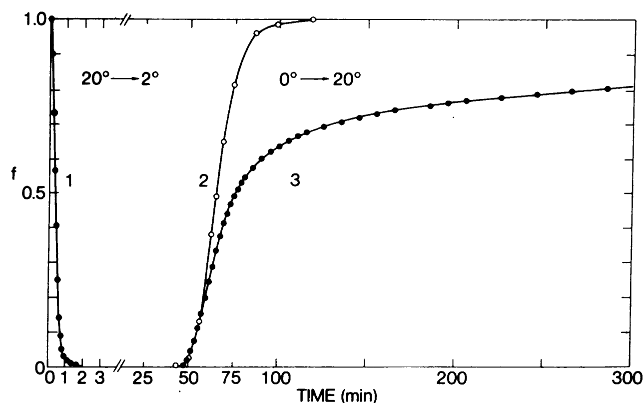


FIG. 1. Fractional extent of gelation versus time. Curve 1 results from rapidly changing the temperature of a 23.3 g % deoxyhemoglobin S gel from 20°C to 2°C in an optical experiment. Curves 2 and 3 were obtained by rapidly changing the temperature of the same sample from 0°C, where it is a nonbirefringent liquid, to 20°C in calorimetric and optical experiments, respectively. The total birefringence is taken as the birefringence at infinite time, estimated by extrapolation of the last part of the curve which approaches a limiting value exponentially.

optical signal is measurable for about the first 45 min. After this delay period the two monitoring techniques begin to show a signal almost simultaneously†. The calorimeter measures a rapid absorption of heat, which is 90% complete after only 30 min. The birefringence also rises rapidly, but after about 50% completion, increases slowly for many hours.

The data in Fig. 1 show that the birefringence and the heat are following the identical process initially, but the continued increase in the birefringence indicates that the two techniques are seeing the details of the process somewhat differently. This interpretation is further supported by the results shown in Fig. 2, where we present normalized tracings from a series of experiments in which the final temperature was varied. A striking feature of these data is that the heat curves are virtually superimposable when translated along the log time axis. The birefringence curves are also similar over the first 60% of the reactions, even though the delay time changes by a factor of 200.

Another striking feature of the data in Fig. 2 is the temperature dependence of the delay time. Changing the final temperature from 30°C to 16°C increases the delay time from 0.5 min to 540 min, a factor of 1000. In Fig. 3a we have plotted the reciprocal delay time, $1/t_d$, on a logarithmic scale versus the reciprocal of the absolute temperature. Data derived from both the birefringence and heat measurements fall on the same straight line, from which an activation energy of about 90 kcal/mole is calculated.

The rate of gelation also shows an enormous concentration dependence. In Fig. 3b we have plotted the reciprocal of the delay time on a logarithmic scale versus the logarithm of the concentration at four different temperatures. The concentration dependence is given by the slopes, which vary between 38 and 28. If, for the present, we simply average the four

† In optical experiments, where only 10–15 μl of solution was generally used, some scatter in the observed delay times was observed in repeated experiments on the same or separately prepared samples. We interpret this scatter as arising from concentration inhomogeneity in the sample, which could not be removed by mixing this small volume.

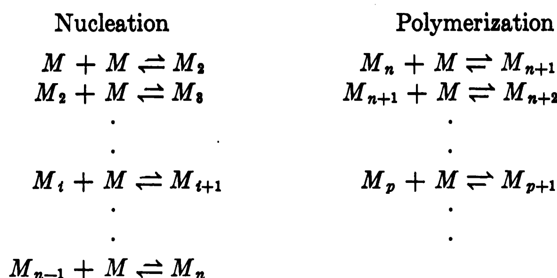
slopes, the result is 33 ± 5, including estimates of the experimental uncertainties. We see, then, that the delay time depends on about the 30th power of the deoxyhemoglobin S concentration!

Preliminary experiments, carried out on erythrocyte lysates in the same buffer, led to the systematic study on purified deoxyhemoglobin S described above. We have as yet found no qualitative difference between the behavior of lysates and purified deoxyhemoglobin S. Also, we should point out that a highly temperature-dependent delay time prior to a rapid increase in viscosity has been recently observed by Malfa and Steinhardt in hemoglobin S solutions deoxygenated with nitrogen (21).

DISCUSSION

The major result of our kinetic experiments is that there is an enormously concentration- and temperature-dependent delay time, prior to the onset of rapidly increasing heat absorption or birefringence. Because the heat absorption and birefringence begin almost simultaneously, we conclude that both techniques are observing the same kinetic process in the first part of the gelation reaction. The invariance of the kinetic form in Fig. 2 is good evidence that the kinetics of the system are controlled by one basic mechanism, which is responsible for the concentration, temperature, and time dependence. The time course of the reaction further suggests that this mechanism must involve either a nucleation or an auto-catalytic process.

We suggest that these dramatic effects can be explained by using a theory similar to that developed for the condensation of water from supersaturated air (22). The kinetic scheme is:



Aggregation begins via a series of thermodynamically unfavorable bimolecular steps, which eventually form a nucleus of critical size, n. Each addition reaction is described by a forward rate constant k_f and a backward rate constant k_b. The initial step is the dimerization of monomers. The aggregate grows by stepwise addition of monomers until the critical nucleus M_n is formed; polymerization then proceeds by a series of thermodynamically favorable monomer-addition steps. The prenuclear steps are characterized by the constant $K = k_f[M]/k_b$, where $K < 1$, while the analogous constant for polymerization steps is σK, where $\sigma K > 1$. Solving the steady-state rate equations by the methods previously used (23, 24) the rate of formation of the critical nucleus is

$$\frac{[M]^{n-1}k_f^{n-2}\,(\sigma K - 1)(1 - K)}{k_b^{n-3}\,(\sigma - 1)} \qquad [2]$$

Since the monomer concentration is raised to the $(n - 1)$ power, this expression immediately accounts for the concentration dependence of the reaction. The observed 30th power dependence indicates that the nucleus consists of about 30 hemoglobin molecules. The reciprocal temperature derivative of the above rate expression yields an apparent activation energy that is composed of a sum of energy terms, the largest contribution arising from the term $(n - 2)\Delta H$, where ΔH is the equilibrium enthalpy change for the addition of monomer to the prenuclear aggregates. Crudely estimating ΔH as the measured heat of polymerization of about 3 kcal/mole, and using the observed n of 30, we calculate an activation energy of about 90 kcal/mole, providing an adequate explanation of the observed temperature dependence.

In order to explain the time dependence of the reaction, characterized by a delay period which is followed by the rapid development of birefringence and heat absorption, we consider the approach of the system described by this kinetic scheme to the steady state. The large number of coupled differential equations must, in general, be solved by numerical techniques. For short times, however, the concentration of the aggregate of i monomers, when treated by the above scheme, can be shown by iterative integration to be given by

$$[M_i] = [M]^i\,(k_f t)^{i-1}/(i - 1)! \qquad [3]$$

At longer times, additional terms become important, and the rate of appearance decreases, the concentration of i-mer eventually becoming invariant when the system approaches the steady state. This time dependence is very similar to that described in detail by Abraham (25) for the condensation of water from supersaturated air. If we assume that the rate of polymerization is rapid compared to the relatively slow rate of formation of nuclei of size n, we predict that the amount of polymer formed should be proportional to t^k, where k is large and approaches an upper limit of $(n - 1)$ as $t \rightarrow 0$. In fact, the calorimetric progress curves are of this form for the first 10–20% of the reaction, with $k = 15$. This large value of k supports the basic mechanism. The observation of a delay period and a value of k less than $n - 1$ (about 30) arises from the finite sensitivity of the measurements.

Our proposed mechanism of gelation is summarized schematically in Fig. 4. Monomers aggregate to form a nucleus. This constitutes the rate-limiting step in the mechanism. Monomers then add rapidly to the nucleus to yield long fibers. As the fibers grow they align parallel to each other, forming a liquid crystalline phase. The large number of monomers in the nucleus, approximately 30 under our experimental conditions, argues against a number of possible nucleation schemes, e.g., the formation of a six-membered ring nucleus, and suggests that the nucleation may involve growth of a two-dimensional aggregate which eventually closes to form a microtubular structure similar to that shown in Fig. 5. We recognize that our model is oversimplified, since a more realistic description of the system will require more than three constants. Nonetheless, we believe that the model presented here accounts for the major features of the gelation kinetics.

In vivo sickling

In order to relate our results to near physiological conditions, we employ an empirical equation which accounts for the observed concentration- and most of the observed temperature-dependence of the delay time, and which, we predict, should also account qualitatively for the effect of other variables, such as pH, ionic strength, and oxygen concentration. The relation is:

$$1/t_d = \gamma S^n, \text{ where } S \equiv \frac{[\mathrm{Hb}]_{\mathrm{total}}}{[\mathrm{Hb}]_{\mathrm{solubility}}} \qquad [4]$$

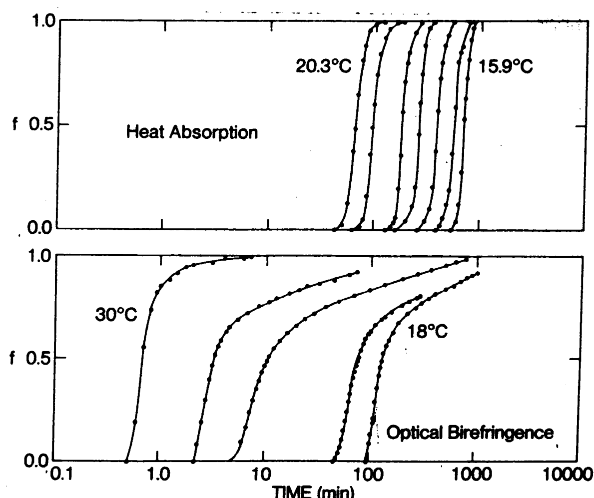


FIG. 2. Fractional extent of gelation versus time at different temperatures. The temperature of a 23.3 g % deoxyhemoglobin S solution was rapidly changed from 0°C to some final temperature. Time is on a logarithmic scale. The ordinate, f, is the fraction of the total heat absorbed, or the square root of the fraction of the total birefringence. In the calorimetric experiments the final temperatures were 20.3, 19.6, 18.7, 17.8, 17.0, 16.3, and 15.9°C, and the total observed heats were between −7 and −8 mcal. In the birefringence experiments, the final temperatures were 30, 25, 22.5, 20, and 18°C.

The quantity S may be called the supersaturation ratio, and is defined as the total hemoglobin S concentration prior to gelation divided by the equilibrium solubility, which may be approximated by the minimum gelling concentration. It is worth noting that for small changes in S within the range of our experiments, expressions similar to Eq. **4** may be derived either from Eq. **2** or from a condensation model for the nucleation process. If, as a first approximation, we treat γ and n as parameters which are independent of experimental conditions, then the entire temperature-, ionic strength-, pH-, oxygen concentration-, etc.-dependence is incorporated into the solubility term, $[Hb]_{solubility}$. This enables us to predict delay

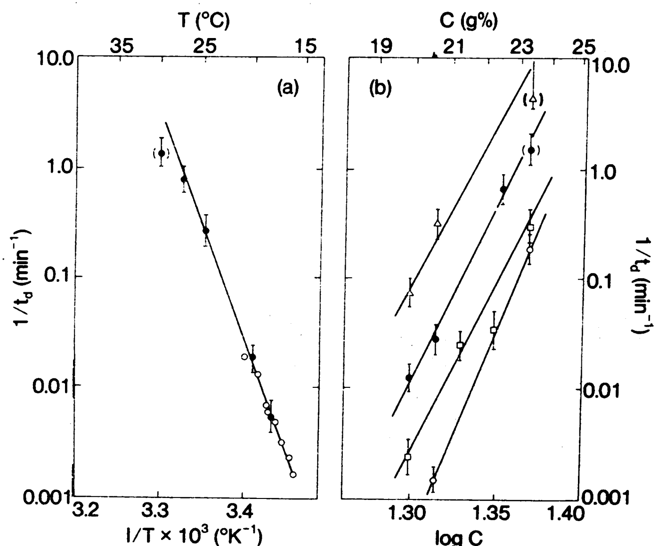


FIG. 3. (a) Temperature and (b) concentration dependence of the delay time. Fig. 3a is an Arrhenius plot of the reciprocal delay time, showing both calorimetrically (open circles) and optically (closed circles) determined delay times. The error bars indicate the range in which 80% of the birefringence delay times were observed in repeated experiments. Fig. 3b shows the observed concentration dependence at a series of temperatures: 35°C, △, slope = 29; 30°C, ●, slope = 32; 25°C, □, slope = 31; 22.5°C, ○, slope = 38. Points in parentheses are upper limits for the true delay time because the measured thermal response time of the instrument prohibits a true measurement.

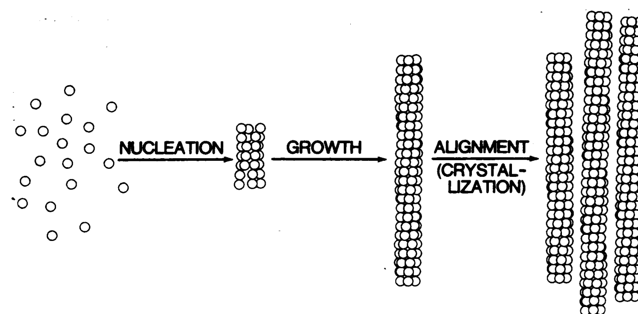


FIG. 4. Proposed mechanism of deoxyhemoglobin S gelation. Monomeric hemoglobin in solution, depicted on the left side of the figure, aggregates in supersaturated solutions to form the compact nucleus for a single fiber. The aggregation takes place by a sequential addition of monomer or small aggregates and is the rate-limiting step in the formation of the fiber. The fiber then grows by the relatively rapid addition of both monomer and small aggregated species. The fibers, even when present in quite low concentrations, align and crystallize into the parallel, ordered arrangements shown on the right.

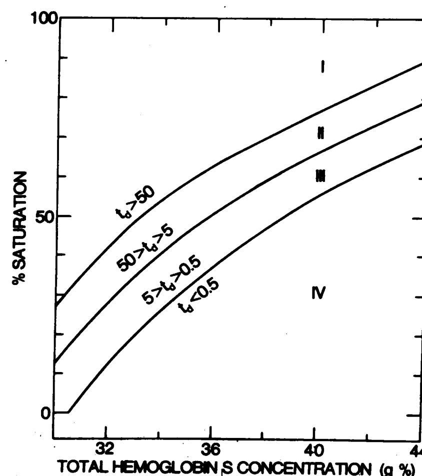


FIG. 5. Map of delay times calculated as a function of oxygen saturation and total hemoglobin S concentration. Using the solubility data of Bookchin and Nagel (25°C, pH 7.35, 0.15 M potassium phosphate) (4, 27) and Eq. 4, the delay time is calculated. The 37°C value is then obtained by extrapolation using our experimental activation energy. Contours are drawn through points of equal delay time. The map is divided into four regions. Region I contains delay times that are greater than the normal circulation time in the body; it also spans conditions under which cells never sickle. The delay times in Region II are less than the time interval between deoxygenation of a cell in the tissues and its reoxygenation in the lungs, where unsickling is essentially instantaneous (13). Region III contains delay times which are comparable to the mean transit time of a red cell through a capillary. In Region IV, cells sickle instantaneously, and the sickling rate is almost completely governed by the deoxygenation rate. Cells in this region have the highest probability of sickling inside the capillary, and thereby occluding the microcirculation.

times by using our experimentally determined γ (about 10^{-6} sec^{-1}) and n (about 30), and the available data on the ligand-, ionic strength-, and pH-dependence of the solubility (4, 7, 26).

The delay times, estimated as a function of total hemoglobin S concentration (28) and the percent saturation with oxygen at 37°C are shown in Fig. 5 in the form of a map. The solubilities of deoxyhemoglobin S-oxyhemoglobin S mixtures are not yet known, but they should be close to the values for deoxyhemoglobin S-cyanmethemoglobin (A or S) mixtures (4, 27). The map shows that at intracellular hemoglobin concentrations and oxygen saturations found *in vivo*, the predicted delay times span both the mean capillary transit (0.5–2 sec) and total circulation times (10–40 sec) (29). While we strongly suspect that the boundaries on the map will move considerably once true experimental values are determined, we do not expect the basic feature of the map, that is, the strong dependence of the delay time on the deoxyhemoglobin S concentration, to change. Since gelation is accompanied by a viscosity increase, the map also predicts the delay time for rigidification of cells that precedes morphologic sickling. The sparse data on cells (13) are consistent with our estimates.

It has not yet been established what role kinetic factors play in sickle cell disease. The rate of sickling has been previously suggested to be an important determinant of the clinical severity (12). Our finding of the enormous sensitivity of the gelation delay time to small changes in physiological parameters points to the delay time as an extremely important variable in the disease. A shift of the erythrocyte population from Region I toward Region IV increases the *probability* of sickling within the microcirculation or venous return. This shift may be brought about by several factors: the delay time is predicted to be halved by increasing the intracellular hemoglobin concentration from 34 to 35 g %, by increasing the temperature from 37 to 38.5°C, or by decreasing the intracellular pH by 0.03 unit. Although these factors have long been known to influence the *thermodynamics* (extent) of sickling, our results show that their effect on the thermodynamics is minor compared to their dramatic effect on the *rate* of sickling. It is tempting to speculate that small changes in the body temperature, intracellular hemoglobin concentration, or intracellular pH—in addition to decreased oxygen saturation—may shorten the gelation delay time sufficiently to precipitate the painful vaso-occlusive episode known as a sickle cell crisis. This hypothesis may explain the association of fever, dehydration, and acidosis with the onset of crises (30). It may also explain the generally milder course of the disease observed in patients whose erythrocyte lysates have a higher solubility, such as in sickle-thalassemia and the various other double-heterozygous conditions (4, 12).

Our hypothesis on the role of the gelation delay time in influencing the clinical course of sickle cell anemia suggests a new approach to therapy. First, it is necessary to establish that the delay time is an important variable in the patient. A correlation of clinical severity with the measured supersaturation ratios on fractionated erythrocytes would be suggestive. If this, or more convincing clinical investigations, show that the delay time is important, then a screening of additives for their effect on the delay time would be worthwhile, keeping in mind that they may have a very small or no effect on the solubility‡. For this purpose the optical technique used in this investigation could be extremely useful, since it requires only a few microliters of lysate, and the instrumentation is simple and inexpensive.

We are particularly grateful to Terrell L. Hill for valuable discussions. Discussions with Elliot Charney, Floyd Kregenow, Arthur W. Nienhuis, Alan N. Schechter, and Steven B. Zimmerman were also extremely helpful. We thank Pongrac Jilly and Arthur W. Nienhuis for providing us with sickle cell blood, and Robert L. Scruggs for assistance in the early stages of this work.

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‡ If the gelation delay time turns out to be an important variable, then our work already suggests that certain measures, advocated in the past, might be expected to provide some therapeutic benefit. For example, increase of daily water intake (to prevent an increase in intracellular hemoglobin concentration), maintenance on alkalinizing agents (to prevent a pH-induced decrease in hemoglobin solubility), and administration of antipyretics immediately at the onset of fever might be of some value.