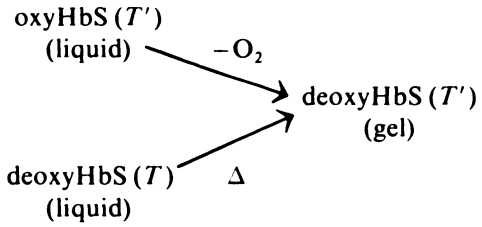
EDITORIAL HYPOTHESIS

Delay Time of Gelation: A Possible Determinant of Clinical Severity in Sickle Cell Disease

By William A. Eaton, James Hofrichter, and Philip D. Ross

THE FACTORS which determine the frequency of crises and overall severity in sickle cell disease are still not clear. Although Allison,[1] Charache and Conley,[2] and others[3,4] have considered that the kinetics of sickling may be important clinically, accurate measurements of the rates of hemoglobin S gelation and cell sickling have only been made recently.[5-15] Measurements on cells under physiologic conditions,[8] and extrapolation of gelation data to near physiologic conditions,[6,11] indicate that sickling in vivo may take place in times comparable to capillary and venous transit times. Furthermore, the rate of both gelation and sickling are found to be sensitive to very small changes in physiologic parameters.[6-14] These results suggest the hypothesis that sickling kinetics play an important role in determining the clinical course of sickle cell disease. In this editorial we briefly describe the molecular basis for the observed kinetics and comment on possible clinical and therapeutic implications of these new findings.

Gelation consists of a highly concerted polymerization of hemoglobin S molecules into fibers and the alignment of these fibers to form some kind of crystalline phase.[16-18] The aligned fibers rigidify the red cell and produce the distortion referred to as morphological sickling. Gelation may be induced either by deoxygenating a concentrated oxyhemoglobin S solution, or by heating an already deoxygenated solution from a temperature (T), where deoxyhemoglobin S is completely soluble, to some elevated temperature (T') at which it will eventually gel. If the deoxygenation and temperature "jump"



are both accomplished in a time which is short compared to the time required for gelation, then the two experiments should give identical kinetic results. The temperature jump method is technically easier for solution experiments. For measurements on red cells, the temperature jump method cannot be used since the total intracellular hemoglobin S concentration in most cells exceeds the

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solubility of deoxyhemoglobin S even at 0°C, and the deoxygenation method must be employed.

The time course of the gelation reaction is very unusual. When gelation of a hemoglobin S solution is thermally induced, the reaction does not start as soon as the temperature is raised. Prior to the onset of the reaction there is a delay period, during which there is no measurable fiber formation. This delay period has been observed with a variety of techniques, including viscosity,[9,10,14] birefringence,[6,11,19] turbidity,[12,20] heat absorption,[6,11,19] and nuclear magnetic resonance water linewidth measurements.[20] Once polymerization does start, it proceeds rapidly and is over 90% complete in a time equal to, or less than, the delay time. By varying the temperature or total deoxyhemoglobin S concentration, the delay time can be made to vary from less than a second to many hours, or even days.[6,11] If the temperature is changed from 40°C to 30°C the delay time increases by a factor of about 10, and changing the temperature from 30°C to 20°C further increases the delay time by a factor of about 100 (Hofrichter, Ross, and Eaton, manuscript in preparation). The sensitivity to concentration is even more striking. The delay time is inversely proportional to about the 30th power of the total deoxyhemoglobin S concentration.[11] This finding means that a 20% decrease in the concentration will increase the delay time by about a factor of 250! To our knowledge this is the largest concentration dependence ever measured for a molecular process.

The unusual time course and extraordinary concentration dependence suggest that the gelation reaction can be explained by a mechanism very similar to a condensation or crystallization process. It is common experience to prepare a supersaturated solution and observe that at first nothing happens—there is a delay period then crystals suddenly appear. Each crystal grows from a small molecular aggregate, called a nucleus. The formation of these nuclei is the rate-limiting process in most crystallizations. In the case of hemoglobin S gelation we have recently proposed that nucleation is also rate-limiting, and that each fiber, which may be considered a microcrystal, grows from a separate nucleus (Fig. 1).[6,11] In this mechanism the nuclei are formed in a series of bimolecular steps that are thermodynamically unfavorable. The 30th power concentration dependence then implies that the nucleus consists of about 30 hemoglobin S molecules. Once the nucleus is formed, polymerization proceeds by the stepwise addition of (64,000 MW) monomers, which is now thermodynamically favorable. As the fibers grow they spontaneously align to form a crystalline phase. This mechanism adequately accounts for the existing experimental data. Several important aspects of the gelation reaction remain to be explored, particularly the direct observation of prenuclear aggregates, the measurement of the gelation rate as a function of oxygen saturation, and the oxygenation-induced degelation kinetics.

The postulated mechanism suggests that there should be other analogies to crystallization processes in addition to nucleation. In general, the rate of crystallization is intimately related to the solubility—the more supersaturated the solution, the faster is the rate. In the case of a gel, an accurate estimate of the relevant solubility can be determined by measuring the supernatant concentration after sedimenting the fibers in an ultracentrifuge.[19,21,22] The degree

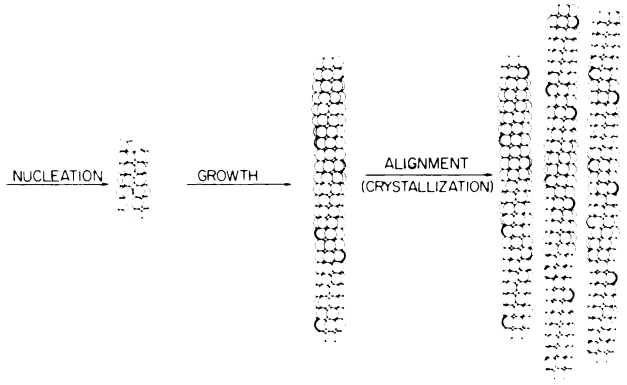

Fig. 1. Proposed mechanism of hemoglobin S gelation (from Hofrichter et al.: Proc Natl Acad Sci USA 71:4864, 1974). The structure of the individual fibers is that proposed by Finch et al.[18]

of supersaturation may then be expressed by the ratio of the total hemoglobin S concentration (c_t) prior to gelation to the solubility (c_s), measured after the gelation reaction is complete. This ratio, called the supersaturation ratio (S), can be related to the delay time by a very simple expression:[11]

$$1/t_d = \gamma S^n, \qquad S \equiv \frac{c_t}{c_s} \tag{1}$$

where t_d is the delay time, and γ ($\sim 10^{-7}$ sec^{-1}) and n (~ 30) are experimentally determined constants. This equation accounts for both the observed concentration dependence, and, because the solubility decreases with increasing temperature, most of the observed temperature dependence of the delay time. The important property of the equation is that both the total concentration and the solubility are raised to a very high power. Because n is so large, very small changes in either the total concentration or solubility can produce very large changes in the delay time. The equation is useful for predicting how the delay time will depend on variables that affect the solubility.

The kinetics of sickling in vitro is very similar to that observed for isolated hemoglobin S. A delay time of 30 msec has been measured by Rampling and Sirs,[5] who studied the deformation of cells following instantaneous deoxygenation. Zarkowsky and Hochmuth have also studied the rate of sickling at zero oxygen tension.[7,13] Although their method has less time resolution, they also find that cells exhibit a delay period, and they have further observed a marked sensitivity of the delay time to cell density, osmolarity, pH, and temperature. As found in gelation studies, the temperature dependence of the sickling rate becomes less with increasing temperature. The kinetics of sickling after partial deoxygenation has been observed by Messer and co-workers.[8] Using both morphological and rheologic techniques they find delay periods in the time range of seconds that are very sensitive to the cell density. The results on cells can be understood from the results of the gelation studies. The cell density is proportional to the intracellular hemoglobin concentration[23] and, because of the 30th power concentration dependence, the sickling rates are predicted to be

very sensitive to cell density. Osmolarity and pH, on the other hand, influence both the intracellular concentration[24] and the hemoglobin solubility.[25,26] The net effect of changes in pH and osmolarity on the rate of sickling can be predicted from equation (1). Thus, decreasing the pH decreases the solubility more than it increases cell volume, predicting that the delay time of sickling will decrease, as is observed. Similarly, increasing the osmolarity by increasing the salt concentration decreases the cell volume more than it increases the solubility, accounting for the observed decrease in the delay time.

The cell experiments of Hochmuth, Messer, and co-workers are important in at least two respects: (1) they provide kinetic data under conditions which are close to physiologic; (2) they establish that the kinetic behavior of cells and isolated hemoglobin S solutions are essentially the same. This means that the same molecular mechanism controls the rate of both gelation and sickling.

To relate the kinetic findings in vitro to sickling in vivo, it is necessary to consider both the deoxygenation and oxygenation steps of the circulatory cycle as initiators of gelation and degelation, respectively. The capillary transit time is of the order of 1 sec,[27] and saturation of the cellular hemoglobin on leaving the capillary is less than 75%.[28] The cell then remains in the venous return at relatively constant saturation for varying lengths of time, averaging about 15 sec,[29] before reaching the lungs where it is rapidly reoxygenated. In vitro studies on hemoglobin S solutions have shown that the rate of temperature-induced degelation is quite rapid even at 0°C,[11] and studies on cells at 37°C have shown that melting of the gel by oxygen is extremely rapid, taking place in less than 0.5 sec.[4,8] We assume that, with the possible exception of the most concentrated cells, oxygenation in the lungs produces complete intracellular degelation, and that, because of the high arterial pO_2, the hemoglobin remains ungelled until the cell reaches the capillary.[30,31] From the time the cell enters the capillary the oxygen saturation decreases continuously. As a result, the solubility of the intracellular hemoglobin continuously decreases, the supersaturation ratio increases, and the delay time for sickling decreases. If the delay time becomes shorter than the time required for the cell to escape the capillary, sickling will occur inside the capillary, with the possibility of vasoocclusion. On the other hand, the delay time may not become so short, and the cell will reach the venous system before sickling has begun. Here again there are two possibilities. Cells having low supersaturation ratios (i.e., low hemoglobin concentrations or high oxygen saturation) and/or short venous return times may reach the lungs before sickling occurs at all. Cells having higher supersaturation and/or longer return times may sickle within the venous system. These cells, while not occluding the circulation, may suffer membrane damage as a result of sickling, and there is the possibility of an increase in intracellular hemoglobin concentration.[3]

Although our description of in vivo sickling is probably oversimplified, it does suggest the importance of the rate processes to the pathophysiology of vaso-occlusive crises. It is generally accepted that such crises occur as a result of sickling within capillaries and consequent blockage of the microcirculation by the rigidified cells.[32] We have proposed that the probability of sickling inside capillaries is determined by the delay time of gelation. It follows, then,

that a crisis occurs when *the delay times are shortened enough or the capillary transit times lengthened enough to increase significantly this probability.*[11] When a patient is not in crisis, there is a much lower probability of cells sickling within the microcirculation. There is some support for this kinetic hypothesis in the clinical literature. Acidosis, dehydration, and fever, as well as oxygen deprivation, all of which are predicted to reduce markedly the delay time, have been implicated as factors that can precipitate a crisis.[33-38] (These parameters also affect the solubility and, therefore, the extent of sickling; consequently, the clinical observations do not unambiguously distinguish between the importance of thermodynamic and kinetic factors.) The kinetic hypothesis would also predict that tissue damage is more likely in organs where the delay times are short, or where the capillary transit times are long.[39,40] Thus, for example, in the kidney, where the delay time is predicted to be substantially shortened by the hypertonicity in the renal medulla, functional impairment is common.[39,41] In contrast, myocardial damage from infarction is infrequent, even though the capillary oxygen tension is low, presumably because of the rapid blood flow in the coronary circulation.[39,42]

The kinetic hypothesis suggests strategies for finding an effective therapy for sickle cell disease. First, it becomes important to screen potential therapeutic agents for their influence on both the gelation delay time and on solubility. A preliminary screening on this basis might point to important structural features of the ideal drug. An entirely new approach, suggested by the enormous concentration dependence of the delay time, is to explore ways of permanently decreasing the intracellular hemoglobin concentration. The gelation data predict that a 2% increase in the cell volume will nearly double the delay time. Drugs which produce only a small increase in red cell volume could thus have a large effect on the delay time. Another way of decreasing intracellular concentration might be to introduce an iron deficiency. Although a recent case report is not encouraging,[43] an iron-deficiency anemia might "switch" the clinical status of the patient to that of a much milder anemia. This idea has been previously proposed,[44,45] but it was rejected on the grounds that iron deficiency could only be effective with a dangerously large decrease in intracellular concentration.[45] The sensitivity of the delay time to concentration suggests that such a large decrease may not be necessary.

Before clinical studies employing such pharmacologic approaches are undertaken, it is necessary to demonstrate directly the importance of kinetics as a determinant of the state of cells in vivo. The possibility remains that sickling in vivo is not adequately modeled by the in vitro experiments discussed here. An important feature of the kinetic hypothesis is that it suggests clinical investigations to test its validity. The great sensitivity of the gelation delay time to hemoglobin concentration predicts that the lifetime of cells whose intracellular hemoglobin concentration has been only slightly reduced[46,47] should be considerably longer than that of undiluted cells. One might also expect to find a correlation between clinical severity and the distribution of sickling delay times. A knowledge of the distribution is important because of the wide range of observed intracellular concentrations[23] and delay times.[7,13] This distribution could be measured directly by the techniques of Rampling and Sirs,[5] Zarkowsky

and Hochmuth,[13] or Messer et al.,[8] or indirectly, by measuring the intracellular concentrations and solubilities, i.e., supersaturation ratios, on fractionated cells. Such studies could also lead to an estimate of the increase in the delay time required to produce a clinical effect.

If the delay time of gelation does turn out to be a major determinant of clinical severity, then there is cause for optimism, for the great sensitivity of the gelation kinetics to small perturbations suggests that a variety of approaches could lead to effective therapy.

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