

Kinetics of Hemoglobin S Gelation Followed by Continuously Sensitive Low-shear Viscosity

Changes in Viscosity and Volume on Aggregation

STEVE KOWALCZYKOWSKI† AND JACINTO STEINHARDT

*Department of Chemistry, Georgetown University
Washington, D.C. 20057, U.S.A.*

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The kinetics of gelation of deoxyhemoglobin S were investigated as a function of temperature, concentration of hemoglobin, and solvent composition. Measurements were made by continuously monitoring the changes in viscosity with time, after polymerization had been induced by rapidly raising the temperature. A specially constructed low-shear viscometer was used. The solution density was also measured continuously to determine whether a volume change accompanied aggregation.

The results confirm earlier work in showing that the time-dependence of the viscosity is composed of a variable latent period (several minutes to tens of hours) during which there is only a slight and very gradual increase in viscosity, followed by a stage in which the viscosity rises very sharply within a very short time. The length of the initial latent period is highly dependent upon the HbS‡ concentration ($33^{\text{rd}} \pm 6$ power) and temperature. If the duration is interpreted as the inverse of a reaction rate, the activation energy is 96 ± 10 kcal/mol for solutions containing inositol hexaphosphate. Unlike measurements reported by others, no dependence of the latent period on shear rate was observed at the low shear rate employed. When IHP is omitted from the hemoglobin solutions, qualitatively similar results are obtained; however, the latent period depends on the $26^{\text{th}} \pm 6$ power of the deoxyhemoglobin S concentration and yields an average activation energy of 125 ± 10 kcal/mol. The length of the latent period is increased 40-fold. Tris is known to prevent gelation but the inhibition can be partly reversed by adding IHP. When this is done, highly concentration-dependent latent periods are again observed. The results may be interpreted in terms of nucleation kinetic theories: a critical nucleus composed of approximately 30 hemoglobin molecules is required for gelation; and the energy barrier (which is larger in the absence of IHP) to the formation of this critical aggregate is approximately 100 kcal/mol.

Gelation is not accompanied by a detectable volume change (limits 5×10^{-5} g/ml). This indicates that the volume change of the reaction must be less than ± 60 cm³/mol when the aggregates represent one half of the HbS available for polymerization.

1. Introduction

Malfa & Steinhardt (1974) described kinetic aspects of the aggregation process in which deoxy HbS‡ molecules are converted into gels. Solutions of deoxy HbS which were only slightly below the usually measured "~~maximum~~
MINIMUM gelling concentration"

† Present address: Institute of Molecular Biology, University of Oregon, Eugene, Oregon.

‡ Abbreviations used: Hb, hemoglobin; IHP, inositol hexaphosphate.

were observed to require "latent periods" ranging from minutes to 14 hours, depending on the concentration and temperature. The dependence of the reciprocal of the duration of the latent period on temperature yielded an activation energy of over 60 kcal/mol and a dependence of the latent period on the fourth (or greater) power of the concentration. During the latent period, no change in viscosity was detected prior to the rapid increase in viscosity when gelation finally occurred. These results suggested that a highly concerted aggregation process was in progress with little or no change in molecular shape during the latent period.

The existence of the latent period has subsequently been verified by a number of investigators, using various techniques. Moffat & Gibson (1974) used turbidity measurements to observe a latent period which was found to depend on the 15th power of the deoxy HbS concentration when IHP was present. Hofrichter *et al.* (1974) utilized changes in optical birefringence and calorimetry upon gelation to detect a variable latent period which was proportional to the 33rd power of the deoxy HbS concentration and which varied with temperature in accordance with an activation energy of 90 kcal/mol. The calorimetric work yielded a heat of polymerization of about 4 kcal/mol (Ross *et al.*, 1975). In addition, Eaton *et al.* (1976) compared the results of latent-period measurements by changes in optical birefringence, turbidity, and water proton nuclear magnetic resonance linewidths and concluded that essentially parallel results were obtained.

A recent viscometric study of the latent period preceding gelation was reported by Harris & Bensusan (1975). However, since their latent periods were found to be shear-rate dependent in the range of their instrumental capabilities, only qualitative results were discussed. Extensive observations were made on the effects of the admixture of HbF 2,3 diphosphoglycerate, and sodium chloride, and the highly viscous gelled solutions were observed to be markedly non-Newtonian in behavior.

The present paper extends and refines the experiments of Malfa & Steinhardt with a very low-shear viscometer so that interpretation of the data avoids an important source of ambiguity. The length of the latent period has been determined as a function of several environmental parameters over a wide range of temperature, concentration and organic phosphate. For the first time, efforts to measure changes in density have also been investigated. Most important, the higher sensitivity of the new viscometer-densimeter employed has been exploited to attempt to detect the presence of intermediate stages of aggregation during the latent period.

2. Materials and Methods

(a) Purification of HbS

Erythrocytes from whole blood, obtained from donors with either sickle-cell disease or sickle-cell trait, were washed by the method of Drabkin (1946) and lysed with 1 vol. each of toluene and distilled water, equal to the packed cell volume after centrifuging. The HbS was purified chromatographically on Sephadex A50 by the method of Williams & Tsay (1973) using a 7.5 cm \times 60 cm column and eluting with 0.05 M-tris(hydroxymethyl) aminomethane buffer at pH 7.80 (23°C) instead of pH 7.60; the column could be loaded with up to 6 g of protein at one time and still effect good separation of A₂S, A and F components. Before layering on the A50 the lysate was equilibrated with 0.05 M-Tris buffer (pH 8.0) by passage down a 2.5 cm \times 45 cm column of Sephadex G25. The purified HbS, stripped free of organic phosphates, was concentrated after elution with an Amicon ultrafiltration unit which employed a PM-30 membrane. Equilibration with

the final buffer used in the experiments was achieved using a Sephadex G25 column, followed by reconcentration. All chemicals used were reagent grade.

(b) *Measurement of solution viscosity*

Viscosities were measured in a specially constructed modification of the magnetic suspension, coaxial-cylinder rotational viscometer described by Hodgins & Beams (1971). This apparatus is capable of measuring the viscosity of protein solutions to a precision of 0.1% at shear stresses as low as 3×10^{-4} dyn/cm². It is described in detail by Kowalczykowski (1976). The time for each rotation of the inner cylinder (rotor) was obtained by either stop-watch timed determination of the rotation time, or by detecting the rotation rate of the rotor by an optical device and recording the rotation on chart paper. The *relative viscosity* (η_{rel}) was then determined by dividing the time per revolution in the protein solution by the time per revolution in the buffer solution.

(c) *Measurement of solution density*

The magnetic-suspension viscometer also measures solution densities to a precision of 1 part in 10^5 by calibrating the voltage across the support coil with solutions of known density. Such standard KCl solutions are tabulated by Beattie *et al.* (1928); the KCl used was obtained from the National Bureau of Standards as a primary standard, and used after vacuum drying at 70°C for several days. The temperature was controlled to ± 0.02 deg. C, by circulating water. As described in Results, the precision of the density measurements is degraded when solutions of high viscosities are encountered.

(d) *Deoxygenation*

Solutions of HbS were deoxygenated by passage of humidified helium, followed by addition of sodium dithionite as an oxygen scavenger. The hemoglobin solutions were filtered into the viscometer through 0.2 μ m Nucleopore filters. All manipulations were performed at approximately 0°C to prevent premature aggregation of the HbS; the apparatus was prechilled to 3.5°C before hemoglobin was added.

(e) *Rate measurements*

The process of gelation was initiated by rapidly raising the temperature from 3.5°C to the desired higher temperature by switching the temperature control from the cold bath to another. Viscosity and solution density were simultaneously recorded as a function of time until just after the gel point (infinite apparent viscosity). Melting (cooling) curves were obtained by rapidly dropping the temperature back to 3.5°C. 2 to 3 min were required to bring the viscometer to temperature equilibrium at each change.

The degree of deoxygenation was determined after each experiment by anaerobically transferring the solution to a 0.1 mm path-length cylindrical cuvette and scanning the visible absorption spectrum in a Cary 14 spectrophotometer. The total Hb concentration was determined by converting to cyanomethemoglobin with Drabkins' (1946) reagent and applying an extinction coefficient of $11.0 \text{ mmol}^{-1} \text{ cm}^{-1}$ at 540 nm. A molecular weight of 16,110 per heme group (Van Assendelft, 1970) was used to express concentration in g%.

3. Results

(a) *Viscosity versus time profiles*

Early attempts to use a Zimm-Crothers floating rotor viscometer (Beckman Instrument Co.) were abandoned when it was found that an apparent irreversible gradual increase in viscosity occurred in solutions of both oxy and deoxyhemoglobin (both A and S types). The increase could be temporarily eliminated by simply stirring the solution. These observations and others led to the conclusion that rigid films of denatured protein were gradually formed at the surface. By completely

submerging the rotor in the hemoglobin solution by means of a self-regulating magnetic field the effect of surface films was completely eliminated.

When the magnetic suspension viscometer-densimeter is used to study solutions of either oxy or deoxy HbA in concentrations up to 20 g%, no detectable change in viscosity is observed when monitored for periods of up to six hours. Similarly, with solutions of oxy HbS (up to 20 g%), and for solutions of deoxy HbS below the concentration representing the solubility as determined by Pumphrey & Steinhardt (1977) and by Magdorff-Fairchild *et al.* (1976), there is also no change in the solution viscosity with time. However, when the solubility limits of deoxy HbS are exceeded, results typical of those shown in Figure 1 are obtained. Solutions of deoxy HbS of the

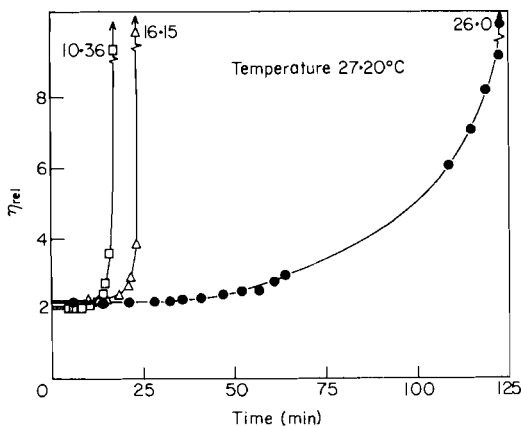


FIG. 1. The relative viscosity (η_{rel}) versus time profiles for the gelation of 3 deoxy HbS solutions of the following concentrations: (\square) 18.2 g%; (\triangle) 17.6 g%; (\bullet) 17.1 g%. The time indicated is the time (min) after the temperature has been rapidly changed from 3.5°C to 27.20°C. At the end-point, rotation of the rotor was prevented by the formation of gel. The buffer employed was sodium phosphate (pH 7.0, ionic strength 0.1) containing 5 mM-IHP, 5 mM-EDTA, and approx. 0.05 M-sodium dithionite.

indicated concentrations had been rapidly brought from 3.5°C to 27.20°C; the times indicated are measured from the time of the temperature-jump. In all cases, the kinetic curves (viscosity/time) obtained consist of two separate stages: first, a latent period of variable duration (ranging from minutes to at least 15 h) which is highly dependent upon both the deoxy HbS concentration and the temperature. During this latent period, there is a significant gradual increase in viscosity which is apparent almost immediately after the temperature has been raised. The latent period is followed by a stage during which the viscosity increases very abruptly within a relatively short time (minutes), resulting in the formation of a gel which prevents the rotor from turning. The second stage is more gradual in solutions with longer latent periods.

If the temperature of a solution which has gelled is subsequently dropped to 3.5°C, a cooling curve (Fig. 2) is obtained. Rotation of the rotor resumes after partial melting of the gel. The length of time required is longer for gelled solutions which have been kept for longer times at higher temperatures. The viscosity drops rapidly at first and then continues to decrease slightly for a period of four to six hours before levelling off.

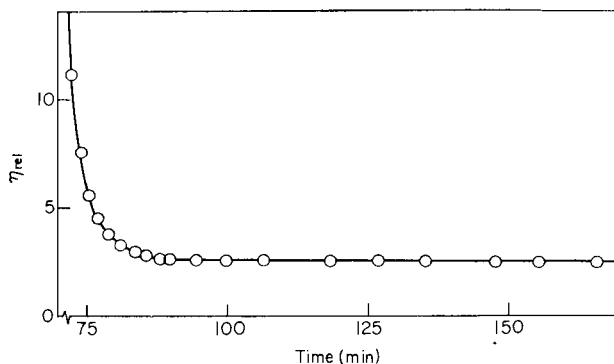


FIG. 2. Cooling curve obtained by dropping the temperature of a solution, which has gelled at 27-20°C, to 3.5°C. The HbS concentration was 16.4 g% and the solvent was the same as indicated in Fig. 1.

(b) *Concentration and temperature-dependence in the presence of inositol hexaphosphate*

If the length of the latent period is assumed to represent a constant extent of transformation (aggregation), then the reciprocal of the latent period should be proportional to the rate of aggregation or other underlying process, as proposed by Malfa & Steinhardt (1974). The length of the latent period may be unambiguously determined as the time required for immobilization of the rotor, since the rotor will be immobilized at a given viscosity which represents a fixed amount of aggregate. Essentially the same values for the length of the latent period are also obtained from the point of intersection of the linear extrapolation of the initial part of the latent period and the latter part of the rapidly rising viscosity phase; this is due to the nearly vertical increase in viscosity of the second phase.

The dependence of the latent period on the HbS concentration is demonstrated in Figure 3, in which the reciprocal of the latent period is plotted logarithmically against $\log[\text{HbS}]$. The slope of this line specifies the formal order of the reaction; it is found to be 33.2 (± 6) by means of a linear least-squares fit of the data, indicating that the latent period is proportional to the inverse of the 33rd power of the HbS concentration. When crystals are formed, instead of gels, a 15th-power dependence of rate on concentration prevails (Pumphrey & Steinhardt, 1977).

The dependence of the latent period at a constant hemoglobin concentration as a function of temperature is illustrated in Figure 4. This Arrhenius plot shows that the latent period is highly dependent upon temperature, yielding an activation energy of 96 ± 10 kcal/mol. It was found by Pumphrey & Steinhardt (1977) that a similarly high activation energy, about 80 kcal/mol, was found at low temperature when HbS crystals rather than gels were formed, but the effect of temperature was much smaller at higher temperatures.

(c) *Concentration and temperature-dependence in the absence of inositol hexaphosphate*

When inositol hexaphosphate is omitted from the buffer solution, qualitatively similar kinetics of gelation are observed: there is a variable latent period followed by a phase of rapidly increasing viscosity. However, the latent period is approximately

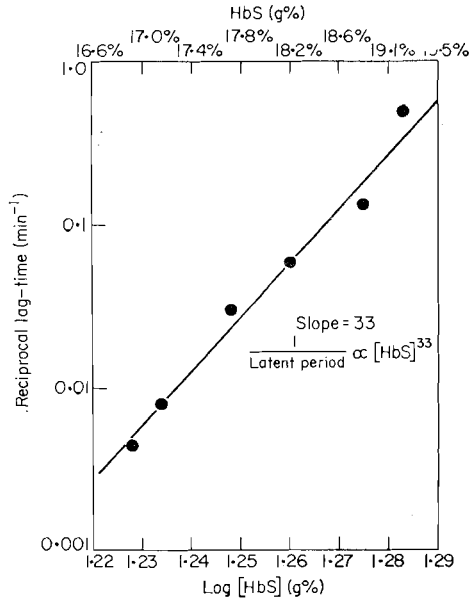


FIG. 3. Log-log plot showing the concentration-dependence of the latent period at 27.20°C. The buffer used was 0.1 ionic strength phosphate buffer (pH 7.0) with 5 mM-IHP, 5 mM-EDTA, and about 0.05 M-sodium dithionite. A least-squares fit of the data yielded a straight line, $y = 33.2x - 43.1$, with a correlation coefficient of 0.995.

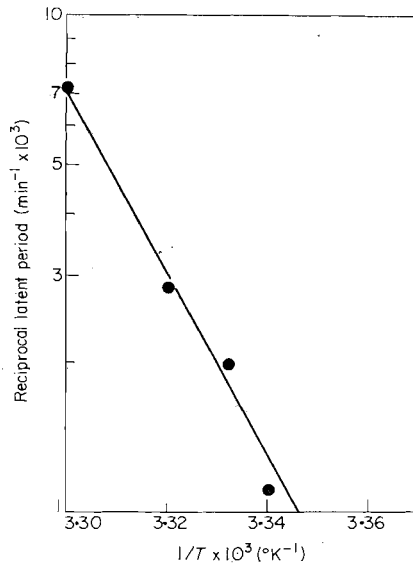


FIG. 4. Arrhenius plot of the temperature-dependence of the latent period at an HbS concn of 15.7 g%. The buffer system described in Fig. 3 was employed.

30 times longer in the absence of IHP than in its presence, all other conditions being the same.

A plot of log reciprocal latent period against log[HbS] (Fig. 5) has a slope of 26 ± 6 , indicating that the latent period in the absence of IHP is also inversely proportional to approximately the 30th power of the HbS concentration.

An Arrhenius plot of the temperature-dependence of the latent period in the absence of IHP is shown in Figure 6. The plot is definitely non-linear (as it is when crystals are formed in the presence of IHP); the slope at the high-temperature end of the

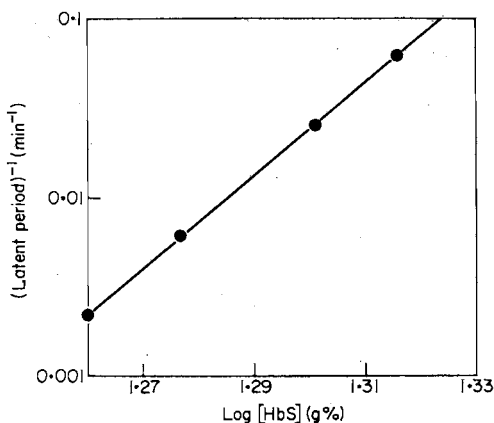


FIG. 5. Log-log plot showing the concentration-dependence of the latent period at 27.20°C in the absence of IHP. The same buffer system as employed in Fig. 3 was used except that the IHP was omitted. A least-squares fit of the data yielded the straight line, $y = 26.3x - 25.73$, with a correlation coefficient of 0.999.

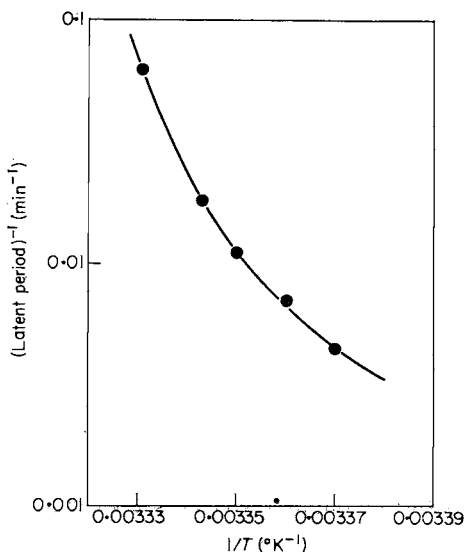


FIG. 6. Arrhenius plot of the temperature-dependence of the latent period in the absence of IHP at an HbS concn of 20.7 g%. The buffer system is the same as described in Fig. 3 except that the IHP was omitted.

curve yields an activation energy of about 200 kcal/mol, while at the low-temperature end of the curve an activation energy of 100 kcal/mol is obtained. A forced single linear least-squares fit of the data yields an activation energy of 126 kcal/mol.

(d) *Results with Tris buffer*

In order to see if the inorganic phosphate ion used as a buffer in the studies already described had any effect on the kinetics, a buffer system containing 0.1 ionic strength Tris buffer (pH 7.0) and 5 mM-EDTA was employed. Efforts to induce gelation in solutions of up to 20 g% deoxy HbS failed even when they were kept at 37°C for 20 hours; agitation also failed to induce gelation or crystallization. The inhibition of gelation by Tris buffer has been observed in several laboratories, including our own (see e.g. Freedman *et al.*, 1973).

However, when 5 mM-IHP is added to this Tris buffer system, gelation of the HbS is again observed, and the kinetics of gelation are similar to those previously obtained in the phosphate buffer system; a variable latent period, followed by a phase of rapidly increasing viscosity. The length of the latent period, however, is now about 40 times longer than with the system employing phosphate buffer plus IHP. The concentration-dependence of the latent period is again large, inversely proportional to about the 25th power of HbS concentration.

(e) *Density measurements*

Typical continuous density measurements of HbS solutions which eventually gel are shown in Figure 7. The density of this solution is shown against time; the arrow indicates the time of gelation. Within experimental error, there is no detectable change in the density; other experiments showed that no change is observable at any temperature, hemoglobin concentration, or solvent composition. Thus, volume change upon aggregation cannot exceed one part in 10,000 (see Materials and Methods).

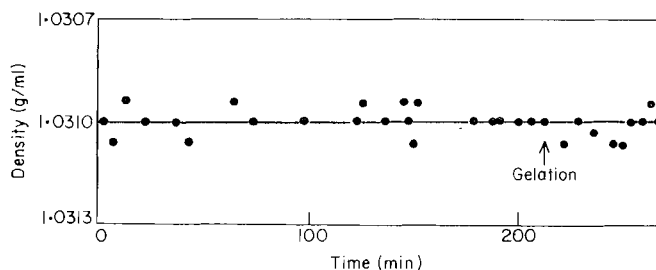


FIG. 7. Density *versus* time plot for a 12.9 g% deoxy HbS solution at 20.60°C. The buffer is the same as described in Fig. 3. Rotational immobilization (gelation) occurs at the arrow; density measurements beyond that point are subject to great uncertainty.

4. Discussion

The results obtained with the magnetic suspension viscometer demonstrate that this apparatus is free of the surface denaturation effects which plague the use of other rotational viscometers for measurements with protein solutions. Thus the observed changes in viscosity can be confidently interpreted as arising from the bulk solution rather than only from the surface. The Brookfield model LVT1/2 used by Harris &

Bensusan (1975) is subject to the effects of surface denaturation†; our own work has shown that the Zimm-Crother design has this limitation also.

The higher sensitivity of the rotational viscometer over that of the falling-ball viscometer used by Malfa & Steinhardt (1974) in their initial studies permits the detection of a gradual increase in viscosity during the latent period which is never observed in solutions which do not ultimately gel. The gradual, reproducible increase in viscosity during the latent period indicates that intermediates are being formed throughout this phase; thus, aggregates must be forming which possess hydrodynamic asymmetry; either the asymmetry or the volume fraction, or both, of these particles changes with time. Since the viscosity is particularly affected by aggregates of high axial ratios, and since no other technique (optical birefringence, calorimetry, turbidity or nuclear magnetic resonance) has detected any significant changes during the latent period, we may conclude that during this period there is a gradual increase in the concentration of the relatively few highly asymmetric particles, rather than an increase in the much larger number of less asymmetric aggregates (which would have a much smaller effect on the viscosity).

The effects of temperature, concentration, and solvent composition can be understood if they are considered in the context of concepts employed to interpret the kinetics of homogeneous nucleation of solid crystals from supersaturated solution. Hofrichter *et al.* (1974) used an analogous theory, that of the condensation of water droplets from saturated water vapor, to interpret their results. The homogeneous nucleation theory proposes that there are two separate kinetic events: a slow, thermodynamically unfavorable nucleation step during which a nucleus grows by the stepwise addition of monomers to form a critical nucleus of size n . Once the critical nucleus composed of n monomers is formed, subsequent rapid addition of monomer occurs stepwise through a series of larger and larger aggregates, the formation of which is thermodynamically favored (Mullin, 1972). Mullin has demonstrated that the free energy difference between a solid aggregate and the solute in solution has a maximum value at n . The size of the critical nucleus is given by:

$$\Delta G_{\text{crit}} = \frac{16\pi\sigma^3 V^2}{3(kT \ln S)^2} \quad (1)$$

where σ is the interfacial surface tension, V is the molecular volume, k is the Boltzmann constant, T is the absolute temperature, and S is the supersaturation ratio; the latter is defined as $S = c/c_e$, where c is the total solute concentration and c_e is the equilibrium solution concentration (solubility). Thus, there exists an initial free energy barrier to nucleation, and to the subsequent growth phase.

The steady-state rate of nucleation, J , can be expressed in the form of an Arrhenius rate expression by using the above expression for the free energy, giving:

$$J = A \exp\left\{-\frac{16\pi\sigma^3 V^2}{3k^3 T^3 (\ln S)^2}\right\} \quad (2)$$

This expression does not predict the existence of a latent or induction period. However, when one introduces the condition that the approach to a steady-state is slow, an induction period becomes apparent (Toschev, 1973). The resulting complex expression for the nucleation rate contains an additional term, τ , which is referred to by Toschev

† Charache *et al.* (1971) noted that artifacts due to surface denaturation were ~~not~~ encountered unless a "guard ring" was incorporated.

as an induction time or a non-stationary time-lag. This time-lag represents the time required by the system to achieve a steady-state distribution of clusters by size; during this time-period the probability of formation of a critical-sized nucleus is very low. Consequently, the overall rate of aggregation is low during this time-period and is limited by the time required to achieve the steady-state.

Thus, the resulting latent period, τ , is essentially the time required for nucleation (i.e. appearance of stable nuclei of size n) and, therefore, is proportional to the reciprocal of the rate of nucleation, J^{-1} . The substitution of τ^{-1} for J in expression (2) results in the following proportionality:

$$\log(\tau) \propto \frac{\sigma^3}{T^3(\log S)^2} \quad (3)$$

which predicts that a $\log(\tau)$ versus $(\log S)^{-2}$ plot should yield a straight line. This prediction is shown by Figure 8 in which the log of the latent period is plotted against $(\log S)^{-2}$. The value of c_0 used for the equilibrium HbS solubility was obtained from Pumphrey & Steinhardt (1977).

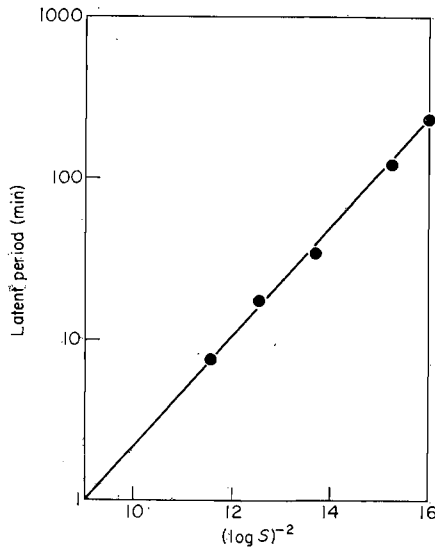


FIG. 8. Log of the reciprocal latent period versus $(\log S)^{-2}$. A value of 9.5 g% was used for $c_0 = [\text{HbS}]_e$. Temperature was 27.20°C and the buffer system was that described in the legend to Fig. 3. A linear least-squares fit of the data yielded the line, $y = 0.33x - 2.95$, with a correlation coefficient of 0.999.

The approximate size of the critical nucleus can be obtained by making use of a simplifying empirical observation made by Nielsen (1964), that $\log J$ varies linearly with $\log S$ over several decades of J . Using this approximation, it can be shown that

$$\frac{d \log J}{d \log c} = n, \quad (4)$$

where n is the size of the critical nucleus. Thus, the slope of the log of the reciprocal latent period versus $\log[\text{HbS}]$ plot yields the size of the critical nucleus, which, from

Figures 4 and 5, is 33 ± 6 and 26 ± 6 for solutions with IHP and without, respectively. So, a nucleus consisting of approximately 30 hemoglobin S molecules, which arises from the stepwise addition of monomer to the growing cluster, must form before the rapid growth phase can commence.

The temperature-dependence of the latent period yields an activation energy which represents the energy barrier to the formation of the critical nucleus. The values of ΔE are 96 ± 10 kcal/mol for solutions containing IHP and 125 ± 10 kcal/mol for solutions without.

The results obtained by other investigators, who used other methods, are in qualitative agreement with those described here. In the first kinetic study by Malfa & Steinhardt (1974), it was concluded that n must be larger than 4 and ΔE must be over 60 kcal/mol. Hofrichter *et al.* (1974) obtained much better values of n (33 ± 5) and ΔE (90 kcal/mol).

There is an apparent discrepancy with the turbidity study of Moffat & Gibson (1974) who obtained a much lower value, 15, for n . However, these investigators defined the latent period as the time for one-half of the total optical density change to occur. Oosawa & Asakura (1975) have shown that the initial rate of polymerization is proportional to the n th power of the total concentration, but that the half-time of polymerization is inversely proportional to the $(n/2)$ power of the concentration. The value of 15 should be doubled in order to make a comparison with our results; the resultant value of 30 agrees very well with those obtained in other studies.

A comparison with the study of Pumphrey & Steinhardt (1977) cannot be made since, in their studies, the solutions were stirred, which resulted in much shorter latent periods. These authors have proposed that the rate of aggregation in stirred solutions is not determined solely by a nucleation rate but also by the rate of formation of secondary nuclei that result from the shearing of the aggregates already in solution.

Harris & Bensusan (1975) in their viscometric study of gelation show that there is a pronounced effect of shear rate on the length of the latent period, which decreases with increasing shear rate. A fourfold increase in the latent period was obtained by changing the shear rate from 77 s^{-1} to 19 s^{-1} . This phenomenon was also observed by Pumphrey & Steinhardt (1977) who noted that stirring greatly reduced the length of the latent period. In the range of the much lower shear stresses attainable with the apparatus used in this study, there was *no* effect on the latent period when the shear stress was varied from $8 \times 10^{-4} \text{ dyn/cm}^2$ to $2 \times 10^{-3} \text{ dyn/cm}^2$ (approx. 0.08 s^{-1} to 0.2 s^{-1}). This insensitivity suggests that agitation must exceed a threshold value in order to affect the latent period significantly. Similar behavior was observed for the G \rightarrow F transformation of actin, another nucleation-controlled process: shear rates above 100 s^{-1} , but not below, accelerated the rate of polymerization of G actin to F actin (Kasai *et al.*, 1960). In general, it has been observed that stirring reduces the length of induction periods in a nucleation-limited rate process and induces nucleation at much lower values of supersaturation (Mullin, 1972).

Nucleation theory explains the observed decrease in the latent period, at a fixed hemoglobin concentration, when IHP is present. Equation (2) shows that, at constant temperature, the rate of nucleation depends upon the supersaturation ratio and upon the interfacial surface tension. Thus, in the presence of IHP, either the supersaturation ratio is higher and/or the surface tension is lower than without IHP. That the first alternative plays a part is shown by solubility studies previously referred to (Pumphrey & Steinhardt, 1977) which indicate that the solubility of HbS

in the presence of IHP is much lower (about $\frac{1}{2}$) than in its absence. However, the second alternative must also participate: the $\log \tau$ versus $(\log S)^{-2}$ curves with and without IHP are not superimposable, indicating that the molecular changes upon IHP binding, observed by Arnone & Perutz (1974), are sufficient to cause a change in the surface properties of the molecule and thereby result in different values of σ , the interfacial surface tension.

The molecular basis of the inhibition of gelation by Tris buffer is unknown. Since gelation with Tris present, in the absence of IHP, was not possible, Tris obviously affects the solubility of HbS. Even in the presence of saturating levels of IHP the latent period is longer in Tris than in phosphate buffer; thus IHP is not capable of completely reversing the inhibitory effects of Tris. Competition for the IHP binding site is unlikely because of the positive charge of the Tris ion; one must look for another effect of the Tris ion.

Finally, since no changes in solution density were detected during gelation, only a lower limit on the volume change can be determined. Based on a sensitivity of 5×10^{-5} g/ml and an HbS solubility of 9.5 g% at 27.20°C, it can be calculated that a 30 cm³/mol volume change would be detected in a 5 ml sample of 19.5 g% HbS, if 100% of the available hemoglobin had aggregated. Since 50% conversion is a more acceptable estimate of the amount aggregated before a rigid gel forms, this would raise the maximum possible volume change to less than +60 cm³/mol (a positive sign is indicated by the observation of Murayama (1966) that sickled erythrocytes unsickled at pressures of 200 to 300 atmospheres). For comparative purposes, the volume change for the polymerization of flagellin is 150 to 300 cm³/mol depending upon the temperature (Gerber & Noguchi, 1967), and about 160 cm³/mol for the polymerization of tobacco mosaic virus (Lauffer, 1971).

Kauzman (1959) and others have shown that the volume change of protein reactions arises almost entirely from changes in the structure of the solvent, since the protein is less compressible. Studies on model compounds have shown that the volume change associated with the formation of a hydrophobic bond is about +20 cm³/mol. Since HbS polymerization is entropy-driven, hydrophobic interactions have always been suspected of playing a part in the aggregation. Thus if one makes the simple assumption that all of the ΔV of aggregation is due entirely to hydrophobic bonding, two to three "bonds" would form per molecule. This simple assumption does not take into account possible offsetting effects such as electrostriction of solvent induced by the appearance of new charged groups which might arise during the aggregation process.

In conclusion, the results obtained for the kinetics of HbS gelation, as measured by changes in viscosity, are consistent with a mechanism of homogeneous nucleation from supersaturated solutions. The apparent biphasic nature of the viscosity-time profiles results from a slow approach to steady-state conditions. The latent period represents the time during which the critical nucleus is being built-up; only a small number of critical nuclei capable of rapid growth into large aggregates are present. For example, the 10% change in viscosity during the latent period can be accounted for by only a 0.1% volume fraction of aggregates which have the dimensions of an HbS microtubule (Josephs *et al.*, 1976). The change to a very rapid increase in viscosity represents the point at which steady-state conditions have been achieved and the rate of production of nuclei is at a maximum. The viscosity rises sharply due to an increase in volume fraction of aggregate as well as an increasing contribution from

higher-order terms in concentration in the viscosity-concentration equations. Consistent quantitative application of this model of gelation serves to explain many of the phenomena related to gelling.

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