PROGRESS IN INTERPRETING THE PHASE TRANSITIONS OF SICKLE-CELL HEMOGLOBIN

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Continuous viscosity measurements of high accuracy at very low shear rates have been used at a number of concentrations and temperatures to study the process of gelation of sickle-cell hemoglobin (HbS) both in the presence and absence of inositol hexaphosphate. The latter lowers the critical gelling concentration and also the length of the latent period by factors of about 2 and 30 respectively. The inhibition of gelling by Tris is also partially counteracted by IHP. It is shown that all of the viscosity phenomena may be explained by the requirement that an energetically unfavorable formation of nuclei consisting of 33 ± 6 tetramers precedes the rapid aggregation phase. Measurements have also been made of the solubility, enthalpies, entropies, and free energies of crystallization of crystals of deoxyHbS, with results that are in partial agreement with similar measurements for gels. Sub-solubility concentrations of deoxyHbS can be made to crystallize by adding suitable amounts of deoxyHbA. Such additions increase the yield of crystals, i.e., diminish their solubility, at all concentrations of deoxyHbS. The interactions between sets of deoxyHbS tetramers are much stronger than between sets of deoxyHbA, but are not as strong as between deoxy A and S in sets of certain fixed ratios which may be related to the lattice geometry.

ISBN 0-12-164350-6

INTRODUCTION

For the last four or five years we have been concerned with the difference in aggregating properties of sickle-cell hemoglobin (HbS) and its normal human counterpart, HbA. This work was initially the result of our partially successful effort to correlate the pH stability functions of various forms of hemoglobins with the ligands attached to them, later extended to an attempt to determine the effect of individual amino-acid substitutions. In this work we took advantage of the close resemblance of the hemoglobin of humans to that of Rhesus monkeys (12 amino-acid substitutions) and the smaller resemblance to that of the horse (over 60 amino-acid substitutions).[1] It became evident that definitive results would require working systematically with species that differed in far fewer amino-acid locations. The gorilla is such a species (n = 1). Far more diversified and accessible are the large number of human mutant hemoglobins, including the most plentiful one of all, HbS.

Having quickly learned that there were only small differences in stability between the various liganded forms of HbA and HbS, even although the apoproteins do show a substantial difference, we turned to attempting to understand why the deoxy form of S had the unique potentiality of forming gels. Our general plan was to study the effects of various environmental parameters on such cogent properties as viscosity, light-scattering, solubility, and electrical birefringence of the two hemoglobins *in pure form*, with the intention of later studying these same properties in mixtures. It was hoped the application to such data of models taken from the physical chemistry of high polymers would illuminate the nature of the gelling process, and explain why a single amino-acid substitution in each of the two β subunits could produce this effect.

The physical methods we emphasized are:

1. Changes of viscosity with time (inferences on size and shape).
2. Changes in light-scattering with time and at equilibrium (for inferences primarily on particle size).
3. Changes in the relaxation of electric birefringence (Kerr constants and retardations for rotational diffusion constants).
4. The solubility studies referred to above.

[1] *There are wide differences, both in kinetic and in equilibrium relations to pH, among the hemoglobins of these species.*

All of the above measurements were carried out over a wide range of temperature and at total concentrations of hemoglobin up to 25 weight percent.

In this presentation we wish to describe our most recent accurate redetermination of the time-dependent viscosity changes, and also the results of our solubility measurements, especially those in which mixtures of deoxyHbS and nongelling hemoglobins are co-crystallized. We merely mention in passing our light-scattering work which lead to the demonstration of deoxyHbS crystallization and its rate dependence, because all our solubility work has been carried out on crystals. Figures 1 and 2 show the crystals. Those interested will find the experimental details and a theoretical treatment of the crystallization kinetics elsewhere (1).

Three years ago we (2) described kinetic aspects of the aggregation process in which deoxyHbS molecules are converted into gels. Solutions of deoxyHbS which were only slightly below the usually measured "minimum gelling concentration (MGC)" were observed to require "latent periods" of between minutes to 14 hours, depending on the concentration and temperature. The dependence of the duration of the latent period on temperature yielded an apparent activation energy of over 60 kcal/mole and a dependence on the 4th (or greater) power of the concentration. During the latent period, no change in viscosity could be detected prior to the rapid increase in viscosity when gelation finally occurred.

The existence of the latent period has subsequently been verified by a number of investigators, using various techniques. Moffat and Gibson (3) used turbidity measurements to observe a latent period which was found to depend on the 15th power of the deoxyHbS concentration when IHP was present. Hofrichter *et al.* (4) utilized changes in optical birefringence and calorimetry to detect a variable latent period proportional to the 33rd power of the deoxyHbS concentration and which depended on temperature in accordance with an activation energy of 90 kcal/mole. The calorimetric work yielded a heat of polymerization of about 4 kcal/mole (5). In addition, Eaton *et al.* (6) concluded that essentially parallel results were obtained when gelation was studied by changes in a number of other properties.

A recent viscometric study of the latent period preceding gelation was reported by Harris and Bensusan (7). However, since their latent periods were found to be shear-rate dependent in the range of their instrumental capabilities, only qualitative results were discussed.

In all of our subsequent work both HbS and HbA have usually been prepared from the blood of single heterozygous donors by a slight modification of the chromatographic method of Williams

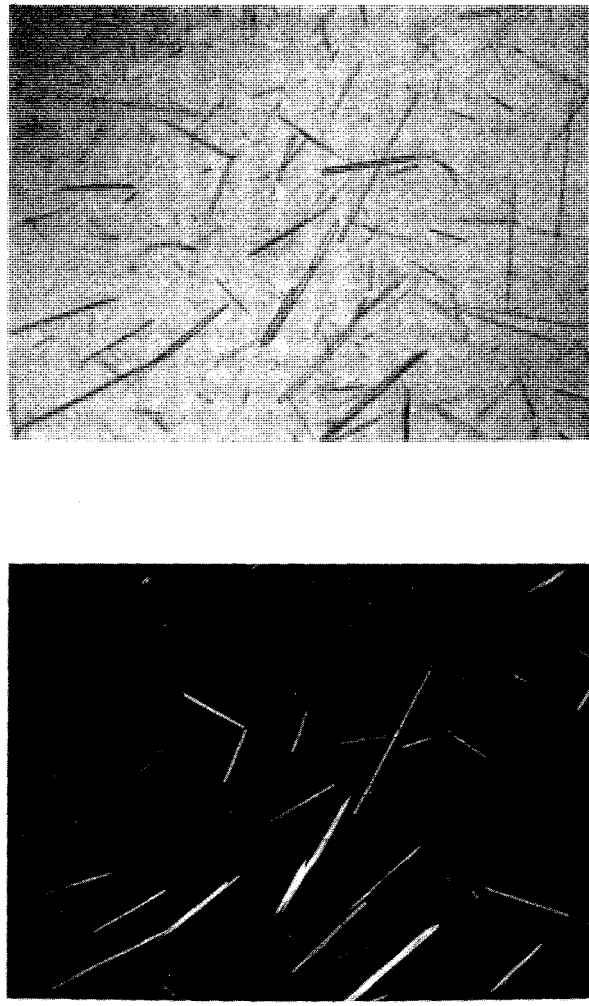

FIGURE 1. Crystals from a stirred solution of deoxyHbS at a total concentration of 17 g/dl formed and kept at 23°C (upper photograph). Solvent: 0.1 phosphate buffer (pH 7.0) with 5 mM IHP and 5 mM EDTA. Lower photograph: same field viewed between crossed polarizers.

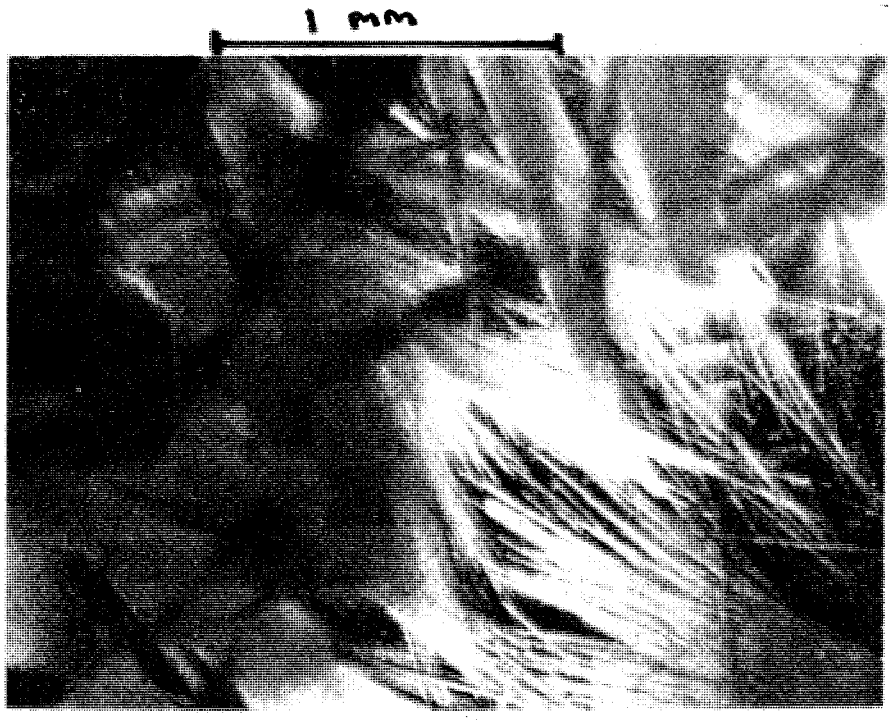

FIGURE 2. Crystals of deoxyHbS, three weeks after formation (by stirring for 2 hours) at 23°C. Solvent was distilled water.

et al. (8), highly effective in removing other impurities such as HbF, and in "stripping" the hemoglobin of phosphates and metabolites.

We (9) have extended and refined the experiments of Malfa and Steinhardt (2) with a very low shear viscometer so that interpretation of the data avoids an important source of ambiguity. The higher sensitivity of the new viscometer-densimeter employed has been exploited to detect the presence of intermediate stages of aggregation during the latent period.

TIME-DEPENDENT VISCOSITY CHANGES

Measurement of Solution Viscosity

Viscosities were measured in a specially constructed modification of the magnetic suspension, coaxial-cylinder rotational viscometer described by Hodgins and Beams (10). 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} dynes/cm^2 provided by another magnetic field. It is described in detail by Kowalczykowski (11). The *relative viscosity* was determined by dividing the time per revolution in the protein solution by the time per revolution in the solvent.

The instrument 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 at rigidly controlled temperatures.

Solutions were deoxygenated by passage of humidified helium, followed by addition of sodium dithionite as an oxygen scavenger.[2] The hemoglobin solutions were filtered into the viscometer through 0.2 μ Nucleopore filters. All manipulations were performed at approximately 0°C to prevent premature aggregation of the HbS.

Rate Measurements

Gelation was initiated by rapidly raising the temperature from 3.5°C to the desired higher temperature. Two to three minutes were required to bring the viscometer to temperature equilibrium at each change. 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.

The degree of deoxygenation was determined spectrophotometrically after each experiment. The total hemoglobin concentration was determined by converting to cyanomethemoglobin with Drabkins' reagent (13) and applying an extinction coefficient of 11.0 $mM^{-1}cm^{-1}$ at 540 nm and a molecular weight of 16,110 daltons per heme group (14).

a. Viscosity vs. Time Profiles. Early attempts to use a Zimm-Crothers floating rotor viscometer (Beckman Instrument Co.) were abandoned when it was found that an apparent

[2] *The use of ascorbate in place of dithionite has been described (12).*

irreversible gradual increase in viscosity occurred in solutions of both oxy- and deoxy-hemoglobin (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 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 viscosity of solutions of either oxy- or deoxyHbA in concentrations up to 20 gm % is continuously recorded, no detectable change occurs over periods of up to six hours. Similarly, with solutions of oxyHbS (up to 20 gm %) and with solutions of deoxyHbS below the concentration representing the solubility of deoxyHbS crystals or gels as determined by Pumphrey and Steinhardt (1) and by Magdoff-Fairchild *et al.* (15), no change in the solution viscosity occurs. However, when the solubility limits of deoxyHbS are exceeded, results typical of those shown in Figure 3 are obtained. Solutions of deoxyHbS of the indicated concentrations have been rapidly brought from 3.5°C to 27.20°C; the times indicated are measured from the temperature jump. In all cases, the kinetic curves (viscosity/time) consist of two separate stages: first, a latent period of variable duration (ranging from minutes to at least 15 hours) which is highly dependent on both the deoxyHbS concentration and the temperature. During this latent period, there is a significant gradual increase in viscosity which is apparent almost immediately after the temperature is raised. The latent period is followed by a stage in 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. A more gradual second stage is observed 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 (Figure 4) is obtained. Rotation of the rotor resumes after partial melting of the gel. The viscosity drops rapidly at first and then continues to decrease slightly for a period of 4-6 hours before leveling off. The length of time required is longer for gelled solutions which have been kept for longer times at higher temperatures.

b. Concentration and Temperature Dependence in the Presence of IHP. If the reciprocal length of the latent period is assumed to represent the rate of aggregation or other underlying process, as proposed by Malfa and Steinhardt (2), then the rate is readily measured since the length of the latent period may be unambiguously determined as the time

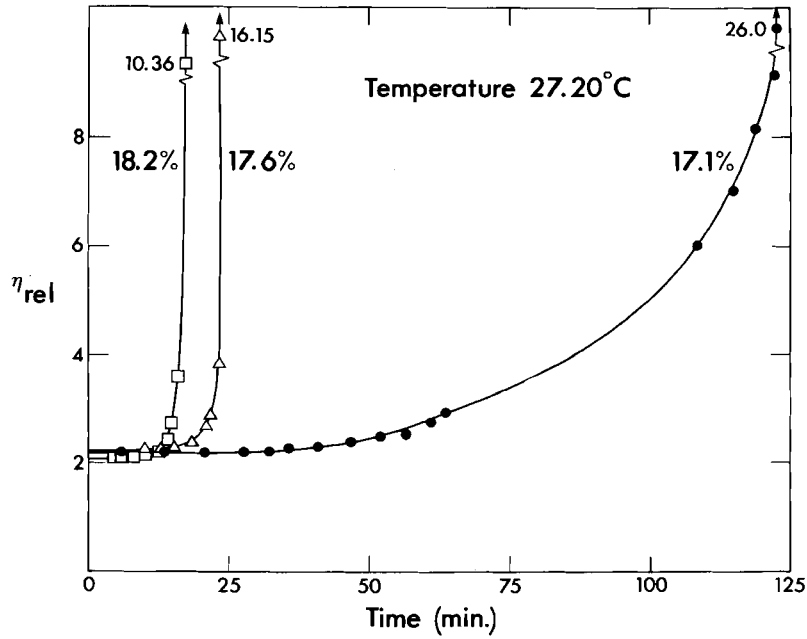

FIGURE 3. The relative viscosity vs. time profiles for the gelation of three deoxyhemoglobin S solutions of the indicated concentrations. The time indicated is the time after the temperature has been rapidly changed from 3.5°C to 27.20°C. At the end point rotation of the rotor was prevented by the formation of gel. The buffer employed was sodium phosphate, pH 7.0, ionic strength 0.1, containing 5 mM inositol hexaphosphate (IHP), 5 mM EDTA, and approximately 0.05 M sodium dithionite.

required for immobilization of the rotor: the rotor will always be immobilized at a given viscosity which represents a fixed amount of aggregate.

The dependence of the duration of the latent period on the HbS concentration is demonstrated in Figure 5. The slope shown specifies the formal order of the reaction; 33.2 ± 6 by means of a linear least squares fit of the data. When crystals are formed, instead of gels, a 15th power dependence of rate on concentration prevails (1). However, two different measures of rate are involved in this comparison.

The dependence of the latent period on temperature at a constant hemoglobin concentration is illustrated in Figure 6. This Arrhenius plot shows an extraordinarily high dependence on temperature, yielding an activation energy of 96 ± 10 kcal/mole. Pumphrey and Steinhardt (1) found that a similarly high

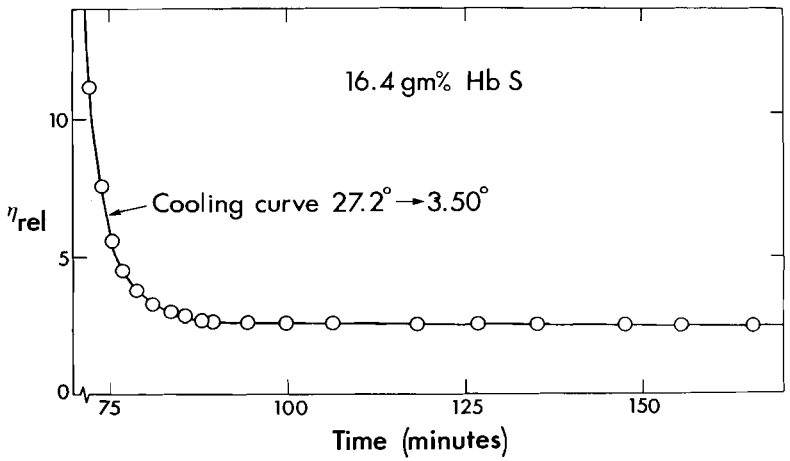

FIGURE 4. Cooling curve obtained by dropping temperature of a solution which has gelled at 27.20°C to 3.5°C. The hemoglobin S concentration was 16.4% and the solvent was the same as indicated in Figure 3.

activation energy, about 80 kcal/mole, appeared at low temperature when they formed HbS crystals rather than gels. At higher temperatures much smaller effects were found.

c. Concentration and Temperature Dependence in the Absence of IHP. When inositol hexaphosphate (IHP) is omitted from the buffer solution, qualitatively similar kinetics of gelation are observed. However, the latent period is approximately 30 times longer in the absence of IHP than in its presence, when all other conditions are the same.

A plot of log reciprocal latent period against log [HbS] (Figure 7) has a slope of 26 ± 6 at 27.20°. An Arrhenius plot of the temperature dependence at 20.7 gm/percent in the absence of IHP is shown in Figure 8. The plot is non-linear; the slope at the high temperature end of the curve yields an activation energy of about 200 kcal/mole, while at the low temperature end of the curve, an activation energy of 100 kcal/mole is obtained. When crystals are formed, in the *presence* of IHP another non-linear Arrhenius plot is obtained (1) but the curvature is of opposite sign; it is possible that stirring results in changing the rate-limiting step in the aggregation process.

d. Results with Tris Buffer. When a buffer system containing 0.1 ionic strength Tris buffer was substituted for phosphate efforts to induce gelation in solutions of up to

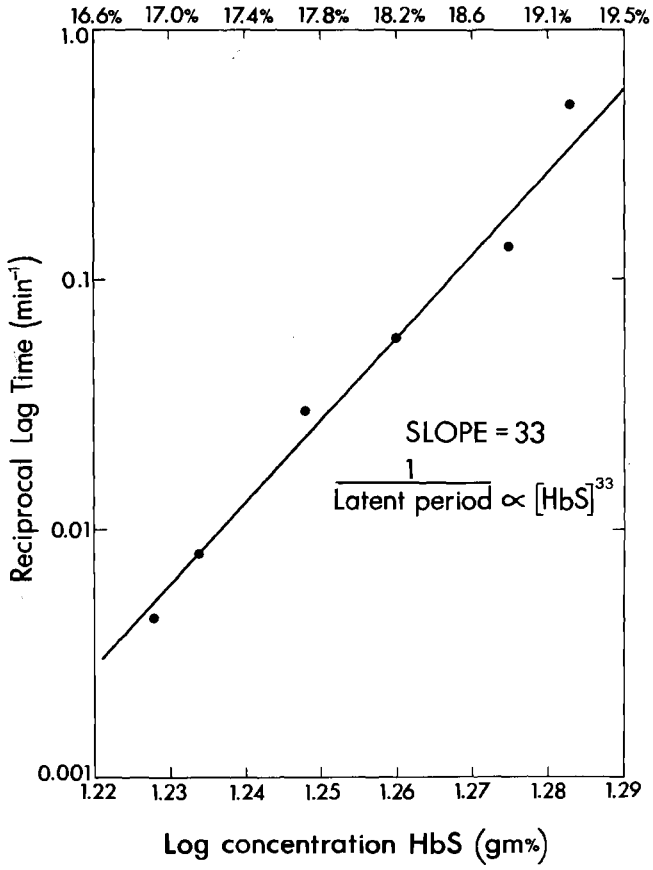

FIGURE 5. Log-log plot showing the concentration dependence of the latent period at 27.20°C. The buffer used was 0.1 ionic strength phosphate buffer, pH 7.0 with 5 mM IHP, 5 mM EDTA, and about 0.05 M sodium dithionite. A least-squares fit of the data yielded a straight line, y = 33.2X, with a correlation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

20 gm % deoxyHbS failed. Stirring also failed to induce gelation or crystallization. The inhibition of gelation by Tris buffer has been observed in several laboratories, including our own (e.g., see Freedman *et al.* [16]). However, when 5 mM IHP is added to this Tris buffer system, gelation of the deoxyHbS is again observed, and the kinetics of gelation are similar to those in the phosphate buffer system. The length of the latent period, however, is now about *40 times longer* than when phosphate buffer plus IHP is employed. The

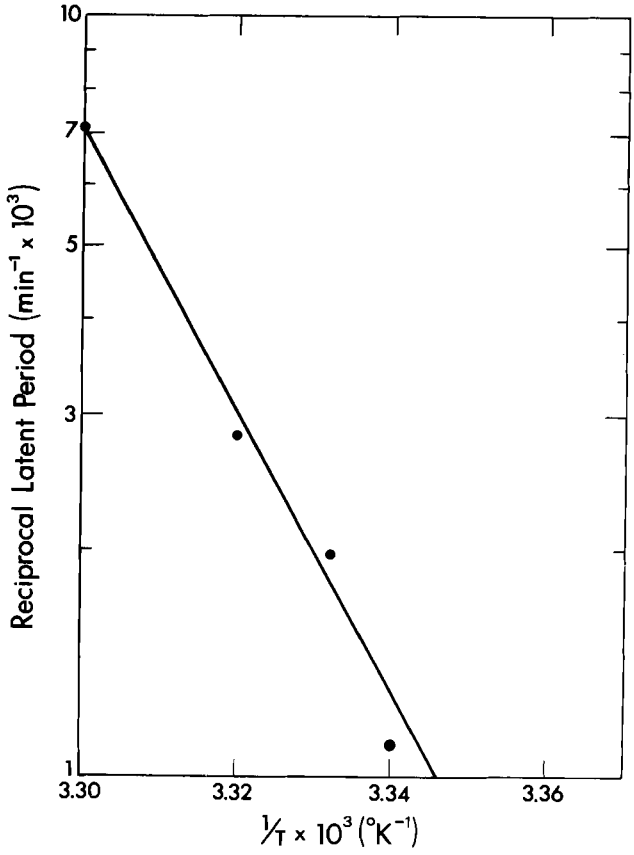

FIGURE 6. Arrhenius plot of the temperature dependence of the latent period at a hemoglobin S concentration of 15.7 gm %. The buffer system described in Figure 5 was employed. A least-squares fit of the data yields the straight line, y = 1.93 x 10^4x + 61.5, with a correlation coefficient of 0.989.

concentration dependence of the latent period is again large, inversely proportional to about the 25th power of HbS concentration.

e. Density Measurements. Within experimental error, there is no detectable change in density on gelation at any temperature, hemoglobin concentration or solvent composition. Any volume change upon aggregation cannot exceed 1 part in 10,000.

The high sensitivity of the rotational viscometer 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

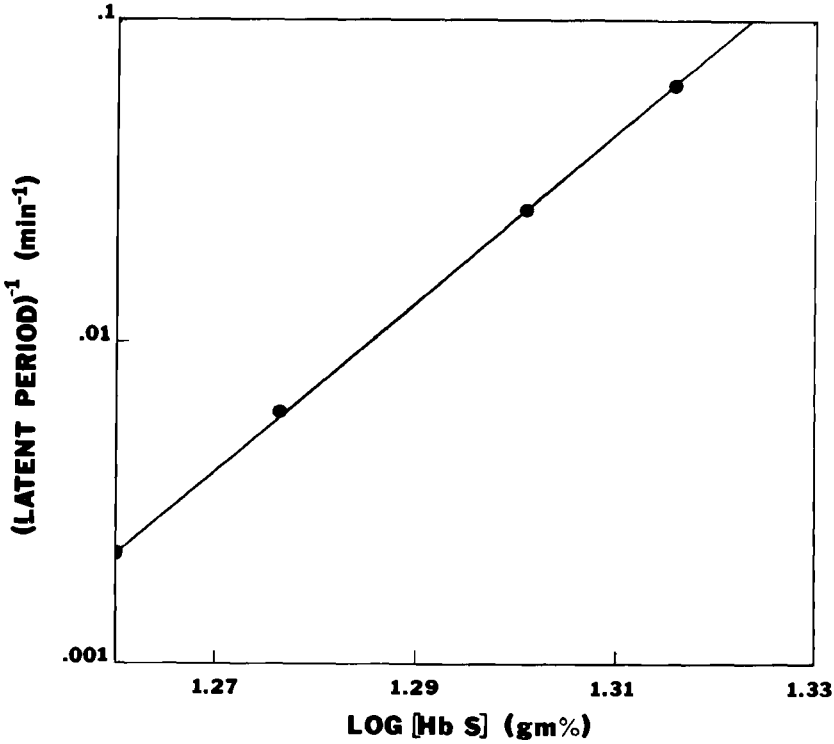

FIGURE 7. Log-log plot showing the concentration dependence of the latent period at 27.20°C in the absence of IHP. The same buffer system employed in Figure 5 was used except that the IHP was omitted. A least-squares fit of the data resulted in the straight line, y = 26.3x - 25.73, with a correlation coefficient of 0.999.

viscosity during the latent period shows that intermediates are formed throughout this period; thus, aggregates must be forming which possess hydrodynamic asymmetry; either the asymmetry or 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 NMR) has detected any significant changes during the latent period, we conclude that there is a gradual increase in the concentration of a relatively few highly asymmetric particles, rather than an increase in a much larger number of less asymmetric aggregates.

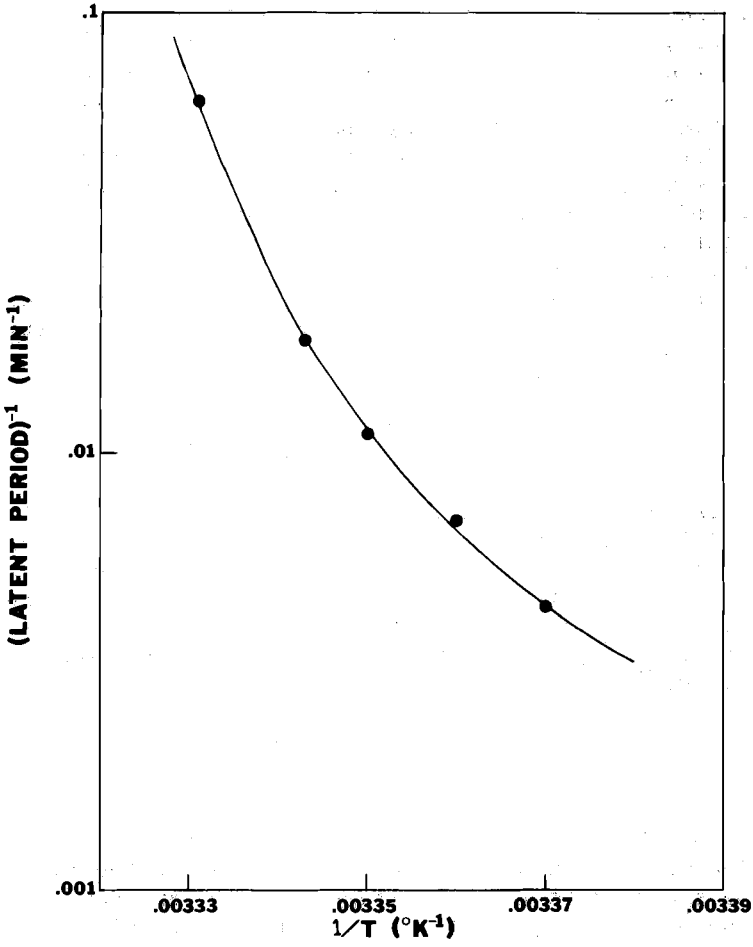

FIGURE 8. Arrhenius plot of the temperature dependence of the latent period in the absence of IHP at a hemoglobin S concentration of 20.7 gm%. The buffer system is the same as described in Figure 5 except that the IHP was omitted. A forced least-squares fit of the data results in the straight line, $y = 3.31 \times 10^4 x + 109$, *with a correlation coefficient of 0.982.*

Interpretation

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 solutions. Hofrichter *et al.* (4) 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 (17) 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:

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

where σ is the interfacial surface tension, V is the molecular volume, 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.

$$J = A_{exp}\left[-\frac{16\pi\sigma^3 V^2}{3h^3T^3(\ln S)^2}\right] \qquad (2)$$

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 (18). The resulting complex expression for the nucleation rate contains an additional term, τ, referred to by Toschev (18) as an induction time or 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:

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

which predicts that a log (τ) vs $(\log S)^{-2}$ plot should yield a straight line. This prediction is tested by Figure 9, in which the log of the latent period is plotted against $(\log S)^{-2}$. The value of c_e used for the equilibrium HbS solubility was obtained from Pumphrey and Steinhardt (1) and was confirmed by later work.

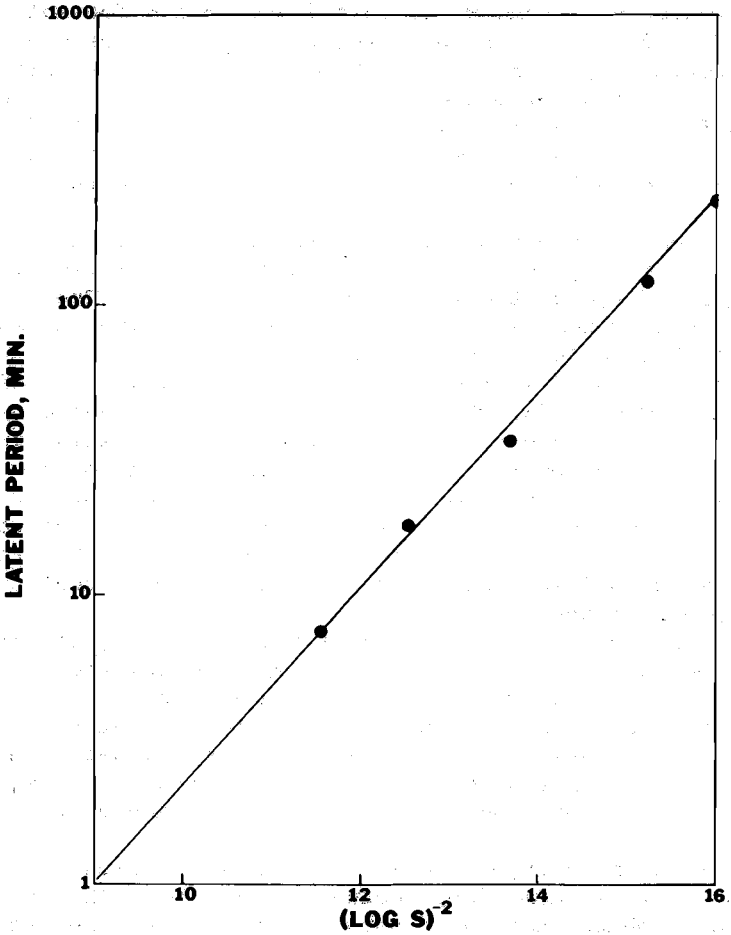

FIGURE 9. Log of the reciprocal latent period versus (log S)$^{-2}$. A value of 9.5 gm% was used for $c_e = [HbS]_e$. Temperature is 27.20°C and the buffer system is that described in Figure 5. A linear least-squares fit of the data yield the line, y = .33x - 2.95, with a correlation coefficient of 0.999.

The approximate size of the critical nucleus (n) can be obtained from equation 4:

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

where n is the size of the critical nucleus which depends on the observed linearity of the log J plotted against log c (19). Thus, the slope of the log reciprocal latent period vs log [HbS] plot yields the size of the critical nucleus, which, from Figures 6, 7, and 9, is 33 ± 6 and 26 ± 6 for solutions with IHP and without, respectively. So, a nucleus consisting of approximately 30 hemoglobin S molecules, arising from the stepwise addition of monomers to the growing cluster, must form before the rapid growth phase can commence. In drawing this conclusion, no allowance has been made for possible effects attributable to the non-ideality of concentrated protein solutions.

The temperature dependence of the latent period yields an activation energy which represents the energy barrier to the formation of the critical nucleus (equation 1). The values of $\Delta\varepsilon$ are 96 ± 10 kcal/mole for solutions containing IHP and 125 ± 10 kcal/mole for solutions without IHP.

The apparent discrepency with the turbidity study of Moffat and Gibson (3) who obtained a much lower value, 15, for n, appears to be due to the fact that these investigators defined the latent period as the time for one-half of the total optical density change to occur. Oosawa and Asakura (20) 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.

A comparison with the study of Pumphrey and Steinhardt (1) cannot be made since, in their studies, the solutions were stirred, which resulted in crystallization and 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.

Nucleation theory explains the observed decrease in latent period, at fixed hemoglobin concentration, when IHP is present. Equation 2 shows that, at constant temperature, the rate of nucleation depends on the supersaturation ratio and on 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. Both alternatives appear to play a part in the effect.

The molecular basis of the inhibition of gelation by Tris buffer is unknown. Since gelation with Tris present in the absence of IHP is 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.

Finally, since no changes in solution density were detected during gelation, only an upper limit on the volume change can be determined. Based on the density sensitivity of our apparatus and a deoxyHbS solubility of 9.5 gm percent at 27.20°C, it is calculated that a 60 cc/mole volume change would be detected in a 5 ml sample of 19.5 gm % HbS, if 50% of the available hemoglobin had aggregated. We note, for comparison, that the volume change for the polymerization of flagellin is 150 to 300 cc/mole (22) and about 160 cc/mole for the polymerization of tobacco mosaic virus (23).

Kauzman (24) and others have shown that the volume change of protein reactions arises largely 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 cc/mole. If one makes the simple assumption that all of the ΔV of aggregation is due entirely to hydrophobic bonding, 2-3 "bonds" would form per tetramer. This simple assumption does not take into account possible offsetting effects such as changes in the electrostriction of the solvent.

In conclusion, the kinetic results obtained with 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 the slow approach to steady-state conditions. The latent period represents the time during which the critical nucleus is built-up; only a small number of asymmetric 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 a HbS microtubule (25). 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 by 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.

SOLUBILITY EXPERIMENTS

Our preoccupation with solubility determinations derives, in part, from a conviction that in equilibrium systems, the "MGC" of gels and their solubilities are identical, and that the well-defined solubilities of our crystals in water or dilute salt-solutions give a reliable and understandable basis for characterizing aggregating behavior.

Solubilities of crystals have been determined as a function of total HbS. Initially HbS solutions (in 0.1 μ phosphate buffer, 5 mM IHP, 5 mM EDTA, pH 7.0 at 23°C), ranging in concentration from 5 - 20 g/dl, were deoxygenated, stirred until turbidity marked the onset of crystallization, gently rotated for 1 week at 23°C and then centrifuged to pellet the crystalline mass. Supernatants were then assayed as cyanmetHb. When the concentration of HbS in the supernatants was plotted against total HbS concentration in the samples, a classic phase rule solubility was obtained (Figure 10). The solubility so determined was = 11.0 g/dl.

Because these crystals of deoxyHbS obey the Gibbs phase rule and because they are highly asymmetric, a simple turbidometric method readily yields the same solubility measurements. At any given temperature crystals are induced by gently

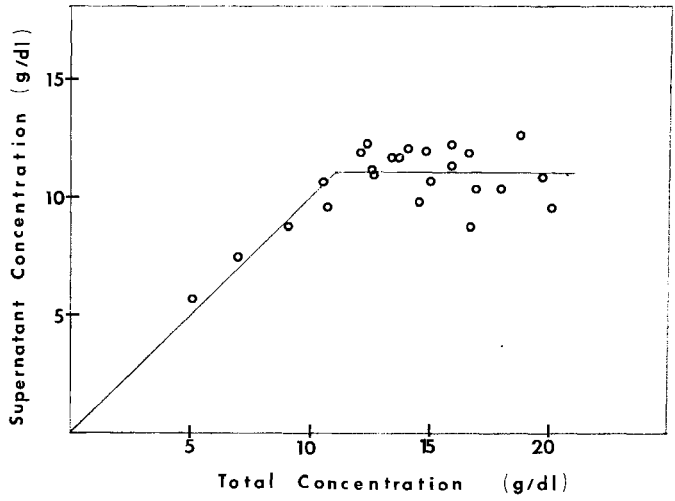

FIGURE 10. Solubility plot for deoxyHbS crystallized from stirred solutions at 23.0°C. Solvent: 0.1 μ phosphate buffer (pH 7.0) with 5 mM IHP and 5 mM EDTA. 10 mM sodium dithionite was added following deoxygenation under nitrogen.

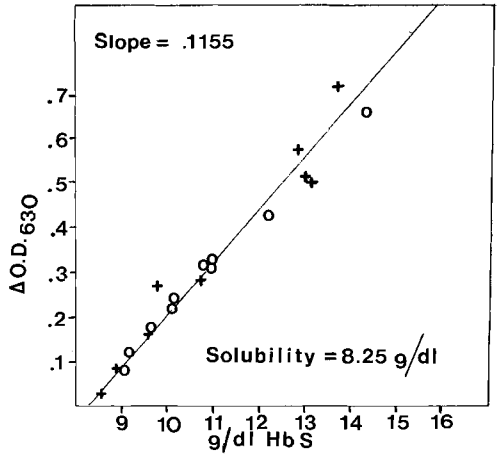

FIGURE 11. Solubility of deoxyHbS in the presence of IHP at 22.9°C as determined by turbidometric technique. ΔOD_{630} represents the excess turbidity observed between the suspension of crystals of deoxyHbS formed by gentle agitation and the same sample chilled to dissolve the crystals.

stirring initially cooler, therefore supsaturated, solutions of deoxyHbS. The suspension of crystals is anaerobically transferred to a spectrophotometric cell of 0.1 mm path length. The turbidity is measured at 630 nm. The HbS crystals are dissolved by immersing the cell in an ice bath. The extinction at 630 nm is again measured (see plot of typical raw data in Figure 11), and the difference between the two measurements ($\Delta\varepsilon_{630}$-excess turbidity) is found to be directly proportional to the mass of suspended crystals (total initial mass minus the mass of dissolved Hb) in the sample. That is, the amount of scattered light is not only theoretically (26,27) but empirically proportional to the amount of crystals present and is due directly to the size and shape of the crystals. Extrapolation of the excess turbidity to zero on a plot of $\Delta\varepsilon_{630}$ vs total HbS concentration yields the solubility of crystalline HbS at that temperature.

The validity of this method of solubility determination for crystalline HbS was demonstrated at a single temperature (23°C, where a solubility of 10.8 ± 0.2 g/dl was found in the presence of IHP). The method was then assumed to be valid at other temperatures in order to measure ΔH of crystallization (1). The new work confirms the validity of that assumption for other temperatures and also for solubility determinations in the absence of IHP. The agreement between the slopes at

TABLE I. Comparison of Crystalline HbS Solubility (g/dl) in the Presence and Absence of Inositol Hexaphosphate

T °C	*+IHP*	*-IHP*
30.1°	*7.30*	*12.80*
27.3°	*7.82*	*13.60*
22.9°	*8.25*	*14.10*
19.1°	*8.75*	*14.78*
15.7°	*10.80*	*16.50*

all temperatures is evidence that the distribution of sizes and shapes of the crystals formed in the presence of IHP is the same over that range of temperatures.

The same proportionality has been found at five temperatures in the absence of IHP. The solubility of crystalline deoxyHbS in the presence of IHP is slightly more than half of the solubility in its absence. A direct comparison of these solubilities at each temperature appears in Table I.

Comparison of the solubilities of crystalline HbS (without IHP) and those determined for HbS gels in the absence of IHP by Magdoff-Fairchild *et al.* (15) shows the solubility of HbS crystals is lower than that of gels at all temperatures studied. The difference in solubility between the two states (21.8 g/dl for gel; 14.10 g/dl for crystal at room temperature) in the absence of IHP is somewhat larger, however, than the difference in solubility between states observed in this laboratory in the presence of IHP (11.3 g/dl for gel; 8.25 g/dl for crystals). Nevertheless, while such data indicate that the crystalline form of HbS is thermodynamically more stable than the gel, the increase in stability due to the contribution of long-range order in the crystals is small in both cases.

The relationship between solubility of HbS (in the presence and absence of IHP) and temperature is depicted in Figure 12. The slopes of those lines correspond to a ΔH of crystallization of 2.8 kcal/mole and to 2.2 kcal/mole, respectively. There is a break in slope near 19.1°C; as in the data of Magdoff-Fairchild *et al.* (15) for HbS gels in the absence of IHP at pH 7.20. The solubility of gels was reported to be temperature-independent above 22°C (15). However, the ΔH of crystallization which is calculated from the slope of the line in Figure 12 is 2.2 kcal/mole, a value which is consistent with the 2 to 4 kcal/mole reported by others (15,5) for the enthalpy of gelation of HbS, without IHP. Using this value of ΔH = 2.2 kcal/mole, it is possible to calculate values of the free energy change and entropy change associated with the

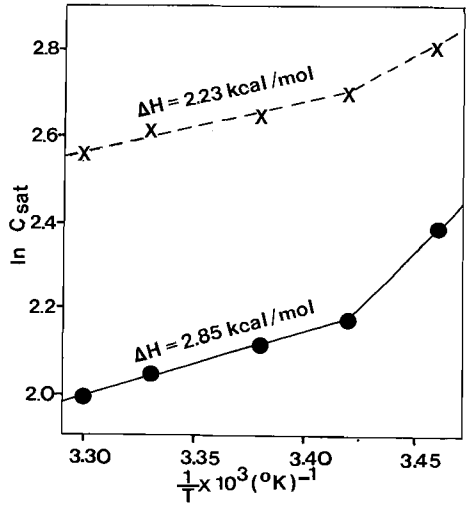

FIGURE 12. The relationship between the solubility of deoxyHbS (in g/dl) and temperature (over the range 15.7°C - 30.1°C) in the presence (——•——) and absence (— — X — —) of IHP.

crystallization of HbS from saturated solutions. Those values are given in Table II, together with values for HbS crystallization in the presence of IHP.

ΔG is more negative the higher the temperature. The entropy change is independent of temperature. In arriving at these values the term (RT ln C_{solid}) has been combined with the free energy change.[3] No allowance for non-ideality has been made.

It should be possible, as earlier investigators have realized (28-31), to learn more about the structure of these crystals (or gels) from solubility measurements, if the crystals are induced to form from mixtures of deoxyHbS with other non-aggregating unliganded or liganded forms of hemoglobin (i.e., mixtures of solutions of deoxyHbS and deoxyHbA, or mixtures of deoxyHbS with metHbS or cyanmetHbS). It has long been known that the "minimum gelling concentration" is reduced when small progressively higher amounts of non-gelling forms (except HbF) are added to fixed amounts of deoxyHbS. This is clearly equivalent, in terms of our solubility concept, to a decreasing solubility of deoxyHbS in the presence of deoxyHbA or liganded HbS. (All the previous work with mixtures was done

[3]*Using this value of ΔG in calculating ΔS is equivalent to assigning the value S=1 to the entropy of the solid.*

TABLE II. Changes in Free Energy and Entropy upon Crystallization of HbS from Saturated Solutions with and without IHP Present

	ΔH (kcal/mol)	ΔG (kcal/mol)	ΔS (e.u.)
No IHP	*2.23*	*-3.7 to -3.5*	*20.1*
With IHP	*2.85*	*-4.1 to -3.7*	*22.9*

with gels rather than crystals.) Observations of this phenomenon have extended to determinations of the variable composition of the gels as the ratios of the components of the mixtures have been altered (31,32). A presumption has been raised that amino-acid positions 73 and 121 in the β chains are involved in gelling (33-35); and that outer portions of the α chains may also have an effect (36,37). Bunn (38) and Park (39) demonstrated that rapid hybridization of oxy dimers of A and S occurs when they are mixed before deoxygenation, and Benesch *et al.* (40) and Thomas and Edelstein (41) explained why hybridization does not occur when deoxygenated hemoglobins are mixed. Several investigators have reported that the presence or absence of hybrids does not affect the MGC, but Moffat, who developed a method assigning "weights" to the aggregating tendencies of homogeneous and heterogeneous pairs of dimers from his mixture data and that of others, claimed that significant differences did exist. Goldberg *et al.* (32) have suggested that Moffat's data did not represent the attainment of true equilibria. They have presented data of their own, obtained by analysis of supernatants after ultracentrifugation of gels, which tends to contradict the conclusions of Moffat.

Although our work with mixtures is still in its early stages, some of our experiments seem to definitely exclude one possibility, that the inclusion of either deoxyHbA or liganded forms of HbS or HbA can be due to trapping of, e.g., deoxyHbA in deoxyHbS lattices. Thus, experiments have been performed in which the quantity of deoxyHbS was only about 0.9 as great as required for the induction of crystals under the conditions chosen (for example see Figure 13). Crystals were nevertheless induced to form by gentle stirring when the total concentration of deoxyHb was raised by about one-third by adding deoxyHbA. Smaller amounts of deoxyHbA were without effect.

When the initial concentration of deoxyHbS is above the solubility at the temperature studied, small additions of deoxyHbA always diminish the solubility, i.e., increase the amount of crystals formed, consistent with the effect

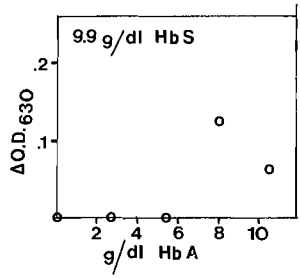

FIGURE 13. The effect of increasing amounts of deoxyHbA on the excess turbidity observed at 630 nm between crystalline suspensions and solutions of deoxyHbS. The initial concentration of deoxyHbS was insufficient to produce crystals.

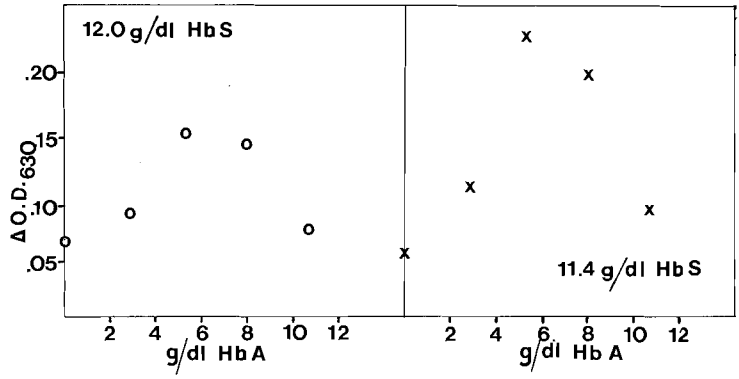

FIGURE 14. The effect of deoxyHbA on the excess turbidity observed at 630 nm between crystalline suspensions and HbS solutions plotted as a function of the ratio of the concentrations HbS to HbA for two independent sets of data. HbS was fixed at 12.0 g/dl for pts 0; 11.4 g/dl for pts X.

described above with subsolubility concentrations of deoxyHbS (Figure 14). However, larger additions *diminish* the crop of crystals.

There is an optimum concentration or concentration-ratio (Figure 15) for effectiveness.[4] These ratios must be related to the structure of the crystals, probably the short-range structure, in view of the small differences in free energy

[4]*This is a logical requirement if small additions reduce the solubility, since pure non-gelling forms have, by definition, very high solubilities.*

between crystals and gels. The analysis is complicated by the fact that the composition, and possibly the crystallographic integrity, vary with the composition of the mother-liquor. Such a system is not easily formulated by the familiar methods of classical solution-theory in which an invariant solid-phase of constant thermodynamic activity greatly simplifies analysis.

Our current program envisages systematic application of three approaches, the first of which (varying deoxyHbA at several fixed values of deoxyHbS) has been partially described above. A second series deals with the substitution of various liganded forms of both HbS and HbA for deoxyHbA. A third series will study mixtures of deoxyHbS and deoxyHbF since HbF appears to be largely excluded from sickle-cell aggregations unless it is present in the cyanmet form. The question of the effects of hybridization on our results will not arise with cyanmet and NO forms, but may be very troublesome with some other mixtures.

From the work already described, which is very much what the MGC data would lead one to expect, it is clear that there exist much stronger attractive interactions between molecules of deoxy S with most other forms than exist between any pairs of the other molecules whether like or unlike. In the language of solubility deoxyHbS is much less soluble than other forms of hemoglobin but solid solutions *which include predominantly deoxyHbS* approach it in insolubility. Determination of the tolerable limits of non-HbS inclusions in such solid solutions should lead to analyses which will contribute to learning the arrangements of HbS tetramers in gels and crystals.

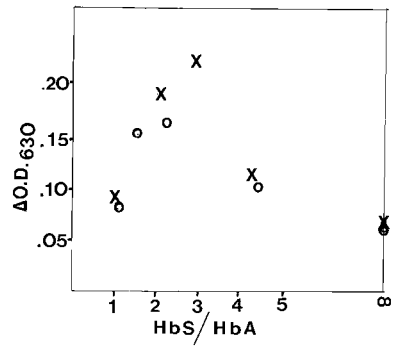

FIGURE 15. The effect of increasing amounts of deoxyHbA on the excess turbidity observed at 630 nm between crystalline suspensions and homogeneous solutions of deoxyHbS at two fixed concentrations of HbS. Deoxygenating either before or after mixing produces the same result.

ACKNOWLEDGMENTS

Valuable technical assistance from Mrs. Kathleen Bayne and Mrs. Joan Scott is gratefully acknowledged.

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DISCUSSION

HOFRICHTER: If I recall correctly, you showed that the activation energy for the crystallization reaction *decreased* at low temperatures while the temperature dependence of the solubility *increased* at low temperatures. This result is not compatible with the standard supersaturation dependence of the rate which is expected for crystallization reactions. Could you comment on this?

STEINHARDT: The figure you refer to is based on the effect of T on viscosity kinetics. Nevertheless, there is either a real anomaly here, or we have something more to learn.

HOFRICHTER: I have another question. You indicated that there was no shear dependence at the low shear values which you generate in the Beams viscometer. Is that demonstrated experimentally?

STEINHARDT: We have done one experiment using a larger shear force.

HOFRICHTER: And there was no effect?

STEINHARDT: There was no effect we could see. Our experimental accuracy on the viscosity is a little better than 1%. And, there is always this question of what happens while you are heating it up, because that requires about two minutes. You are not only heating up the solution, but you are also heating up the container, which has a fairly massive piece of metal as well as the glass.

ARNONE: Your work indicates that IHP enhances gelation. Have you looked at DPG?

STEINHARDT: We did some experiments with it but when we found that IHP gave so much larger effects, we stopped using the DPG.

ARNONE: But did DPG give small effects in the same direction?

STEINHARDT: Yes, in the same direction.

ARNONE: How do your findings compare with those of Bookchin, indicating that it is the pH and not the cofactor that effects gelation?

STEINHARDT: I cannot answer that question because I do not recall the pH's, but our pH's were held within 1/300 of a pH unit.

WATERMAN: When you compare "extender" concentrations of deoxyHbA and deoxyHbS, do you observe the same enhancement of crystallization for a given starting concentration of deoxyHbS? In other words, you mentioned a maximum effect was observed when 25% of the lattice points were occupied with deoxyHbA. However, this enhancement of crystallization could be a concentration effect and I wonder whether added deoxyHbS shows the same enhancement as added deoxyHbA, or whether the effect is one of a greater enhancement?

STEINHARDT: Adding HbS gives more crystals than adding HbA.