A TEMPERATURE-DEPENDENT LATENT-PERIOD IN THE AGGREGATION OF SICKLE-CELL DEOXYHEMOGLOBIN

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Received June 14,1974

SUMMARY

No viscosity differences have been found between the oxy and deoxy forms of hemoglobins A and S, or between like forms of the mutant and normal proteins prior to gelation of the HbS. However, measurements at concentrations up to 22 g/100 ml, at temperatures between 13.6° and 25.0°, show that no increase in viscosity occurs for periods between a few minutes at 25° to more than 14 hours at 13.6° after raising the temperature from 2°. When the viscosity finally rises, gelling occurs in 15 minutes or less. The length of the slow latent period depends strongly on temperature (indicating an energy of activation of over 60,000 calories), but the velocity of the faster process of viscosity-increase does not appear to depend on temperature. The dependence on concentration of the rates of the latent period reaction suggest a highly concerted process of aggregation without loss of compactness or radical change in molecular shape. An alternative mechanism, based on branched chain formation is also described.

Solutions of deoxygenated sickle-cell hemoglobin (HbS) at concentrations above the "minimum gelling concentration" (mgc) and temperatures above about 15°C are rapidly transformed to highly viscous gels (1-3). Normal human hemoglobin (HbA) does not show this behavior. The threshold concentration for this effect is sensitive to any impurities present, including other forms of hemoglobin (e.g. oxyhemoglobin or methemoglobin), sulfhydryl reagents, and protein denaturants. It is shown in this paper that there is also a time dependence which, under some conditions, may lead to erroneous reporting of the mgc.

We have made systematic measurements of the dependence of specific viscosity on concentration, temperature, and time, with emphasis on conditions of temperature and concentration which are close to or just below those at which gel formation takes place. The purpose of this investigation was to attempt to interpret the time course of viscosity change under various combinations of conditions, in terms of the mechanism of the aggregation process.

MATERIALS AND METHODS

Hemoglobin S was prepared from the pooled laked erythrocytes of homozygous donors whose blood was collected in Vacutainers over EDTA. The lysates were either centrifuged and dialyzed, or crystallized as oxyhemoglobin by a slight modification of the toluene method of Drabkin (4). Hemoglobin A was prepared

from citrated blood saturated with toluene and CO, by the method of Drabkin, crystallizing three times with 2.8 M phosphate buffer at pH 6.8. Stock solutions of either hemoglobin were concentrated if necessary with an Amicon high pressure ultrafiltration apparatus with a UM-2 membrane. All solutions used in the measurements contained pH 7.5 phosphate buffer at 0.05 ionic strength. Solutions were deoxygenated in a tonometer, attached to the viscometer tube, and to a pump and nitrogen supply.

Solutions were kept at 2° for a minimum of 15 minutes after deoxygenation, prior to being brought to higher temperatures.

Viscosities were measured in a modified low shear falling-ball viscometer (Gilmont #1) in a thermostat controlled to 0.01°. A steel falling-ball was used. The instrument constants of the two viscometers were 0.173 and 0.176. Most readings of η sp (the specific viscosity) were reproducible to within 5%.

RESULTS

Both hemoglobins at either 25° or 37°, in both oxygenated and deoxygenated forms, have indistinguishable specific viscosities over a concentration range of 2% to 22% (Fig. 1). The viscosities are proportional to concentration up to about 10-12 percent and then turn upward with a constantly increasing slope. The extrapolated reduced viscosities (intrinsic viscosities) are also identical within the experimental error, at about 0.04 ml/g. Although not shown in Fig. 1 oxy S has the same viscosities as deoxy S, below the mgc.

In the case of deoxy S, some of the more concentrated solutions, 17% to 22%, which gave measurable viscosities, identical with those of deoxy A, nevertheless eventually gelled and therefore had (instrumentally) infinite viscosity after longer times.

Although temperature has a marked effect on the mgc, which is lower at 37° than at 25°; there is little if any effect on the viscosity prior to gelling, although concentrations as high as 17% were used at 37° and 22% at 25°.

At concentrations well above the mgc, and at temperatures over 25°, aggregation to gels occurs very rapidly. With these, viscosity can not be measured prior to gelation. However by working at temperatures below 20° with a concentration of HbS (20.92 g/100 ml) which was close to the mgc at 20°, viscosities could be measured repeatedly over whatever period was required for a gel to form, up to 14 hours at the lower temperature. Most unexpected results were obtained, shown in Fig. 2.

At 20° the falling-ball became immobilized at about 0.5 hour after only two trials after bringing to temperature from 2°. At 15.8°, no clear rise in viscosity occurred for nearly 2 hours, after which a steady rise in η sp to about 2.5 occurred in about 15 minutes, and the solution gelled a few minutes

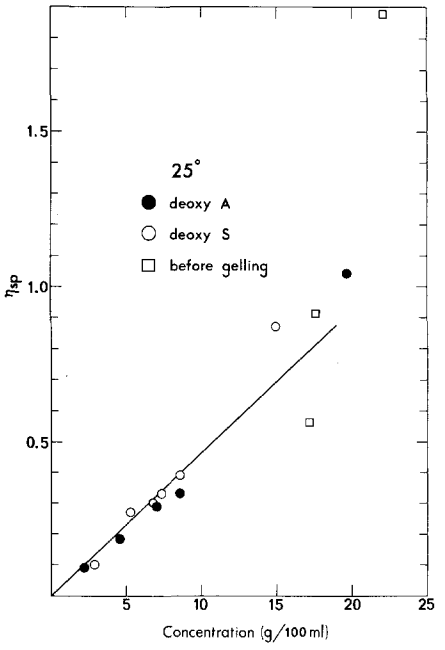


Figure 1: The specific viscosities at 25° of deoxy HbA and deoxy HbS as a function of concentration (g/100 ml). The points within squares remained constant over substantial time but gelled subsequent to measurement of viscosity.

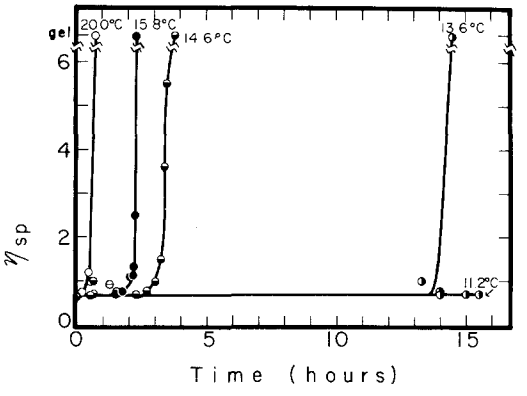


Figure 2: The specific viscosities as a function of time of a 20.9% solution of deoxy HbS at five temperatures: 20°, 15.8°, 14.6°, 13.6° and 11.2°.

later. At 14.6° the first rise in viscosity was detected after nearly 3 hours. η sp then rose rapidly in about 15 minutes, and a gel immobilized the ball a few minutes later. At 13.6°, no rise in η sp was observed for nearly 14 hours, although a gel formed at 14 hours. At 11.2°, no gel was observed in over 15 hours.

Thus, there appears to be a slow temperature-dependent reaction (which does not affect the viscosity) which must precede the gel-producing process. The latter appears to be fast, and its rate is essentially independent of temperature. Similar conclusions result from experiments at a number of other concentrations and temperatures selected to give ball-immobilizing gels in 2.2 hours or less. The results of these experiments are shown in Fig. 3.

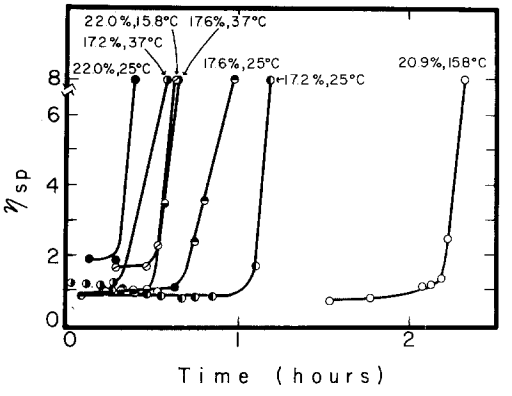


Figure 3: The specific viscosities as a function of time of a number of different concentrations of deoxy HbS at several temperatures:

	17.20%	17.60¢	20.92%	22.01%
15.8°C			○	⊘
25.0°C	◐	◍		●
37.0°C	◑	◍		

Both 22.01% at 15.8°C and 17.60% at 37.0°C had the same gel time.

The lowest concentration of HbS, 17.2%, was tested at high temperatures, 25.8° and 37.0°. At the lower temperature ηsp started to rise after about 0.9-1.0 hour. The viscosity was clearly higher at 1.1 hour, and a gel was produced a few minutes later. At the higher temperature, no increase in ηsp was visible at 0.3 hour, but a gel had formed in under 0.5 hour.

At a slightly higher concentration, 17.6%, 25.0° brought about an increase in η sp after 0.6 hour after which specific viscosities of 2.4 and 3.5 were obtained between 40 and 48 minutes, with gelling at about 54 minutes. At 37° the increase in η sp is detectable at 0.5 hour and a reading of 3.4 is obtained slightly later, just before gelling occurs.

With the highest concentration, 22.0%, a sharp jump to the gel viscosity occurs between about 18 and 21 minutes. For comparison the results with 20.9%, at 15.8°, already shown in the preceding figure, are repeated here. No increase

in η sp is visible until after 2 hours, although gelling occurs only 15 minutes later than the time at which the viscosity first rises. Another consistent, but less complete, set of results was obtained at 15.8°.

The results just described lead to the same conclusions as those in Fig. 2, but add an effect of concentration on the rates. Either low temperature or low concentration slow the initial step which precedes gelling, without affecting viscosity. Neither parameter appears to have an effect on the rate of viscosity increase, including gelling, once this process is initiated at the conclusion of the initial slow reaction.

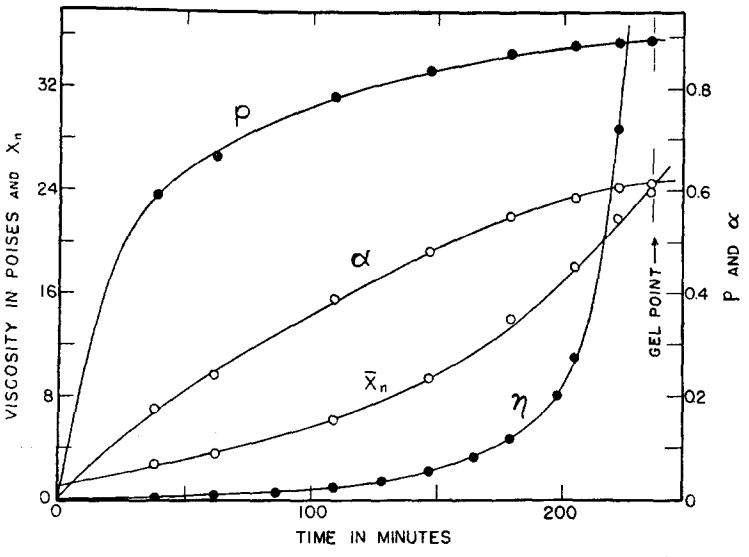
DISCUSSION

There are few published observations of a time-dependent increase in viscosity preceding the formation of a gel (5). Optical rotation effects, and changes in light-scattering, show that the protein in a gel continues to change for some hours after its formation is first detected. Such changes are not followed by the falling-ball method. Thus we are concerned only with the viscosity changes that precede gelling; as Figs. 2 and 3 show, a gradual increase in viscosity does occur prior to gelling, but it occurs rapidly and, at low temperatures or concentrations, follows a long period in which no viscosity change is detectable.

If the onset of a viscosity-rise represents a given constant effect, then the reciprocal of the time required to initiate an observable change can be interpreted as proportional to a rate, and the effect of temperature on this rate may be examined. An Arrhenius plot of the results in Fig. 2 show that the minimum value that can be assigned to the energy of activation of the first process is 61,000 calories. Protein denaturation reactions are often characterized by such high apparent energies of activation. However numerous observations of the optical properties (difference spectrum and ORD) of concentrated HbS deoxy solutions have failed to provide any evidence that unfolding occurs. There are only small differences in rates of unfolding of the two proteins at either acid or alkaline pH (6).

Our data on the second more rapid stage are sufficient only to show that the effect of temperature is small. Thus only physical rather than chemical processes may be involved.

The sharp dependence of the rate of the slow initial reaction on protein concentration (Fig. 3) suggests that the reaction may be highly concerted, proportional to a high power of the concentration (at least 4). An aggregation process may be highly concerted, but the absence of an increase in viscosity shows that the aggregated protein remains compact (no change in shape, partial specific volume or in hydration) during the latent period phase. The rapid



Firgure 4: The course of a typical three-dimensional polyesterification, taken from Flory (7). The reaction involves diethylene glycol, succinic acid, and tricarballylic acid.

η is the viscosity in poises.
p is the extent of the reaction determined tritrimetrically.
$\bar{X}_n$ is the calculated number average degree of polymerization.
α is the branching coefficient, defined as the probability that a given functional group of a branch unit leads via a chain of bifunctional units to another branch unit.

phase, which is less dependent on temperature and in which viscosity change occurs must then be interpreted as the result of expansion of the molecular domain ("swelling") or a change to a more asymmetrical shape.

A radically different alternative way of interpreting the rate data cannot be ruled out. A polymer composed of branching chains, grows at an ever increasing rate. There is very little effect on the viscosity until the aggregates have reached substantial degrees of polymerization, but thereafter the viscosity increases explosively, and gels are formed shortly after the viscosity increase becomes prominent (7). An example is shown in Fig. 4. With such a process there is only one reaction rather than two. Since such an analogy assumes that deoxy HbS (and not deoxy A etc.) is trifunctional (capable of aggregating in branched chains) it would help explain why the mgc rises as the fraction of the total hemoglobin which is deoxy HbS falls, and why deoxy HbS must always exceed at least one half of the total hemoglobins.*

*Sustained branching requires that the probability of adding a trifunctional (or higher functional) monomer is greater than the probability of adding a bifunctional one.

Neither of the two alternative kinetic mechanisms discussed above are to be confused with other purely conceptual two-stage hypotheses advanced recently, which bear on HbS viscosity data. In one of these (8) the initial process (identified with microtubule formation) is stated to be the faster of the two, contrary to the empirical results in Figs. 2 and 3.

ACKNOWLEDGEMENT:

This work was supported by the National Heart & Lung Institute. (grant HL12856)

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