FORMATION OF NEEDLE-LIKE AGGREGATES IN STIRRED SOLUTIONS OF HEMOGLOBIN S[1]

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SUMMARY. When concentrated solutions of hemoglobin S are gently stirred under conditions such that a gel would be formed in the absence of stirring, gellation does not occur. Instead, as light scattering measurements have shown, after an initial latent period, which can range from minutes to several hours -- depending upon the experimental conditions -- a highly turbid but fluid suspension of particles of aggregated hemoglobin is rapidly produced. The turbidity is fully reversed upon cooling to 0° C, and subsequent gellation can be induced by refraining from stirring upon raising the temperature. The turbid solutions are stable for weeks under the conditions of their formation, even when in contact with gels of hemoglobin S. The particles giving rise to the turbidity have been viewed and photographed by light and electron microscopy, and are seen as highly asymmetrical rigid filaments with lengths ranging to about 15 microns.

Much progress has recently been made toward an understanding of the phenomenon of gellation in Hb S.[2] Electron microscopy has revealed the probable molecular architecture of the gel (1), which consists of long microtubules formed from six-membered rings of Hb molecules stacked in a helical fashion. Malfa and Steinhardt first showed by viscometry that an initial lag period, sensitive to temperature and concentration, precedes the formation of gels. (2,3) Similar observations have been subsequently made by a variety of other techniques. (4,5,6)

While studying the kinetics of Hb S gellation by light scattering, we observed that gentle stirring prevents gellation. When warmed, instead of undergoing a transition from an isotropic liquid phase to a gel, we discovered that stirred solutions of deoxygenated Hb S become turbid while remaining fluid, owing to the presence of microscopic Hb aggregates suspended in solution. A lag period -- which is highly sensitive to temperature and hemoglobin concentration -- precedes the rapid aggregation process; but the length of the lag period is much

[1] The production of fluid suspensions of aggregates of sickle cell hemoglobin by stirring was reported at the 170th National Meeting of the American Chemical Society, Chicago, Illinois, August 24-29, 1975.

[2] Abbreviations used: Hb = deoxyhemoglobin; IHP = inositol hexaphosphoric acid; EDTA = ethylenediaminotetraacetic acid.

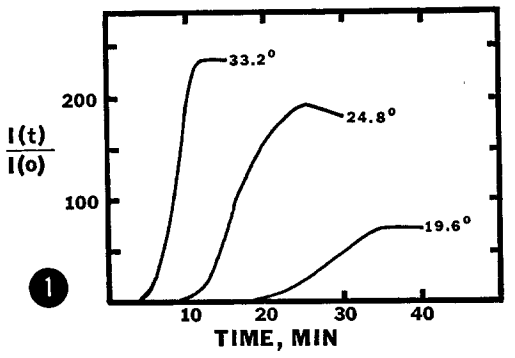
shorter in stirred solutions than in unstirred solutions under the same conditions. This paper discribes the kinetics and characterizes the particles formed.

MATERIALS AND METHODS. Hb S was purified from laked erythrocytes of patients with sickle cell disease by chromatography on DEAE-Sephadex A-50 (7). The solutions contained phosphate buffer of ionic strength 0.1, pH 7.0 (at 23° C), with 5 mM EDTA (to chelate Cu^{2+}, [8]), and 5 mM IHP.

Light scattering measurements were made at 90° to the incident beam in thermostated 10 mm square fluorescence cuvettes mounted so that the incident and scattered light beams gave a light path of only 2 mm close to one of the corners of the cell. A Brice-Phoenix light scattering photometer was modified by the substitution of a Spectra-Physics 5 mW helium-neon laser light source with associated optics and a selected, red sensitive photomultiplier (EMI 9781A). The scattering volume was about 10 μl, and the short light path permitted solutions of hemoglobin to concentrations of up to 20 g/dl. The solutions were stirred by small Teflon coated magnetbars in the cuvettes.

For viewing in the light microscope, specimens of the turbid, stirred solutions of Hb S were prepared in a nitrogen-filled glove bag, and cover slips were sealed to the microscope slides with epoxy cement. Electron micrographs were made of anaerobically dessicated films of the stirred solutions without staining.

RESULTS AND DISCUSSION. Figure 1 shows the light scattering behavior of Hb S solutions at three different temperatures under continuous, gentle stirring. The solutions were rapidly raised to the indicated temperatures from 2° C at zero time. There is in each case a lag period -- which is found to depend critically upon hemoglobin concentration and temperature -- followed by a rapid increase in light scattering to an equilibrium value. The increase in turbidity is completely reversed within a few minutes at 0° C or by oxygenating the sample. Both the slope of the rapidly increasing protions of the curves and the levelling-off value of the scattered intensity are also concentration and temperature dependent. There is also a simultaneous sharp increase in the inten-



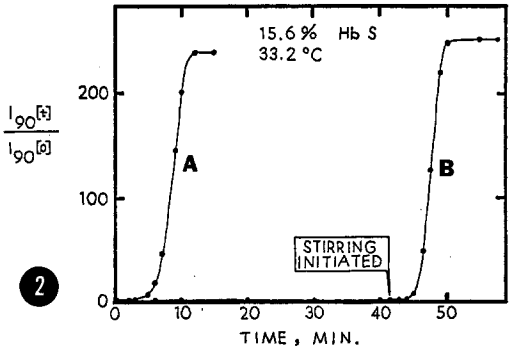


FIGURE 1. Light scattering kinetics of aggregation in stirred solutions of 15.6 g/dl Hb S at three temperatures. The scattered intensity at 90° relative to the initial scattered intensity is plotted as a function of time.

FIGURE 2. The effect of stirring on the light scattering kinetics of aggregation of 15.6 g/dl Hb S at 33.2°C. The data of curve A were obtained with continuous stirring throughout the experiment. In the experiment shown in curve B, stirring was begun only after 42 minutes.

sity of the horizontally polarized component of the scattered light from an almost undetectable level (appropriate for symmetrical, globular proteins), to a large value -- indicating either highly asymmetrical particles or particles which are large compared to the wavelength of the scattered light, or both. Although these solutions become highly turbid, they do not gel. They remain fluid (although the viscosity rises), and are stable for long periods of time under the conditions of their formation, even when placed in contact with gelled Hb S under the same conditions.

Figure 2 shows that the lag time is greatly shortened by stirring. Without stirring, a 15.6 g/dl Hb S solution at 33.2° C will gel (with a resultant sharp increase in turbidity) in several hours; the onset of the process leading to rapid production of turbidity in stirred solutions begins within five minutes of either raising the temperature from 2° C to 33.2° C while stirring, or initiating stirring in a static solution at 33.2° C prior to the onset of gellation.

If the reciprocal of the lag time is assumed to be proportional to the rate

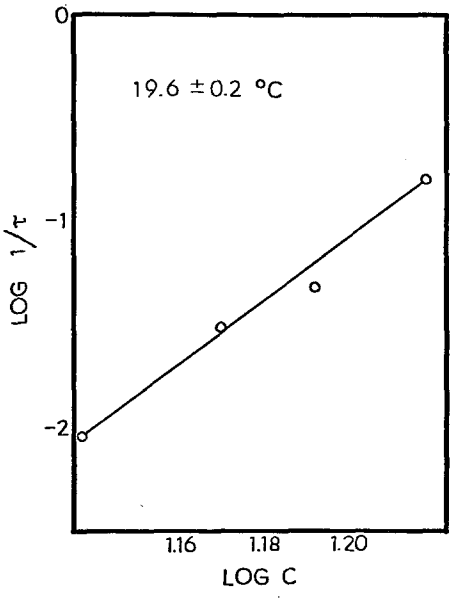


FIGURE 3. The dependence of the lag times in the light scattering aggregation kinetics data upon the concentration of Hb S at 19.6°C. The logarithm of the reciprocal of the lag time, τ, is plotted as a function of the hemoglobin concentration.

of a rate-limiting process leading eventually to the rapid growth of turbidity in the stirred Hb S solutions (2,3), the concentration dependence of this rate at constant temperature can be assessed as in Figure 3. The slope of this plot yields an inverse 15h power dependence of the observed lag times upon the hemoglobin concentration. High power dependences of the lag times upon Hb S concentration in unstirred solutions have been observed in other studies (2,3,4,5). The similarity between this process and precipitation from supersaturated solutions -- which involves rare nucleation events and subsequent rapid growth of nuclear aggregates -- has been noted (4). If this analogy is applicable, it is plausible to attribute the shortened lag period in stirred solutions to the proliferation of available nuclei by disruption of growing post-nuclear aggregates by shearing forces set up by stirring. The resulting larger number of smaller aggregates thus produced (relative to unstirred Hb S solutions) might limit or forestall the formation of larger network gels.

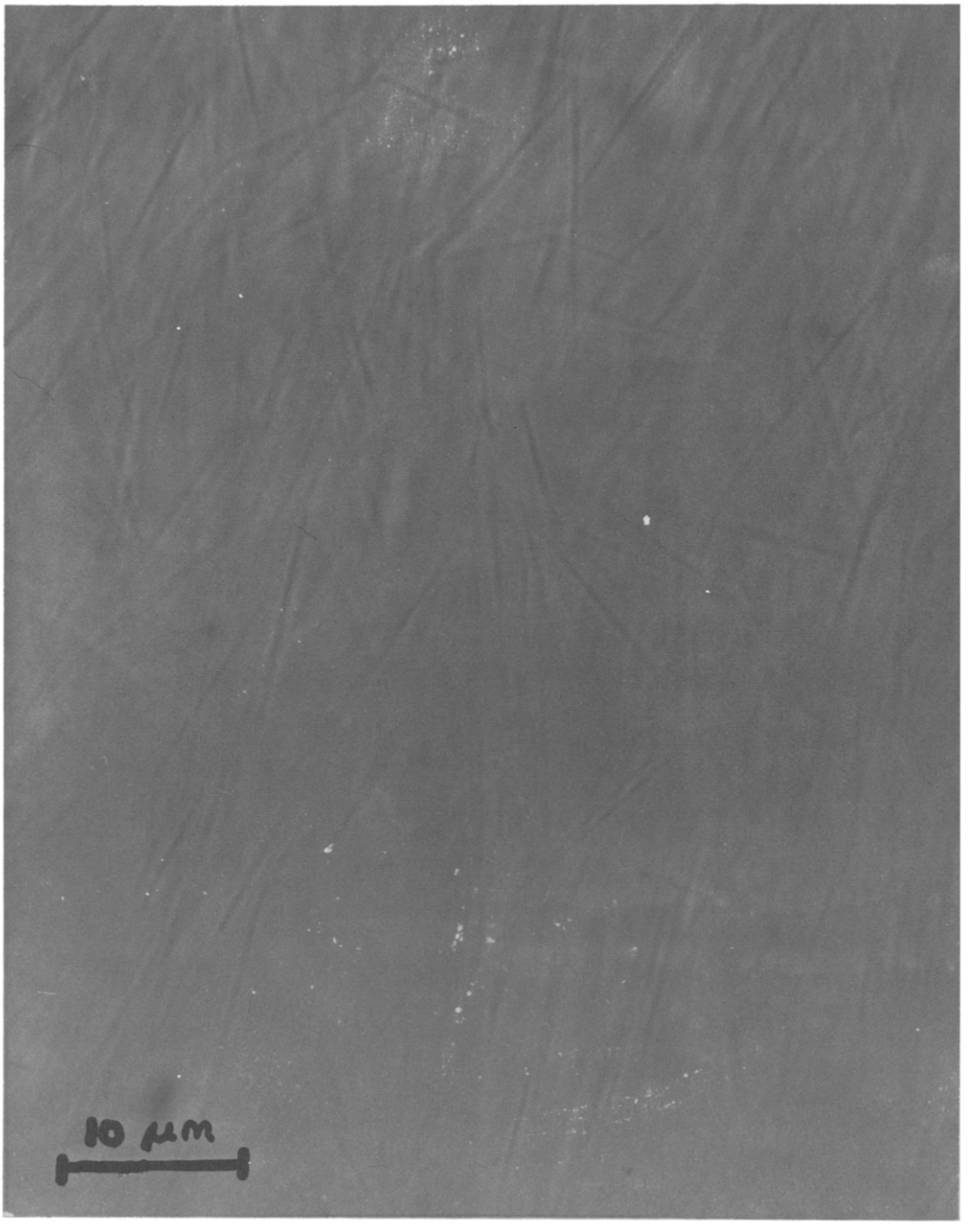


FIGURE 4. Light micrograph of a thin film from the suspension of Hb S aggregates produced by stirring a 16.4 g/dl hemoglobin solution at 23°C. (2500X)

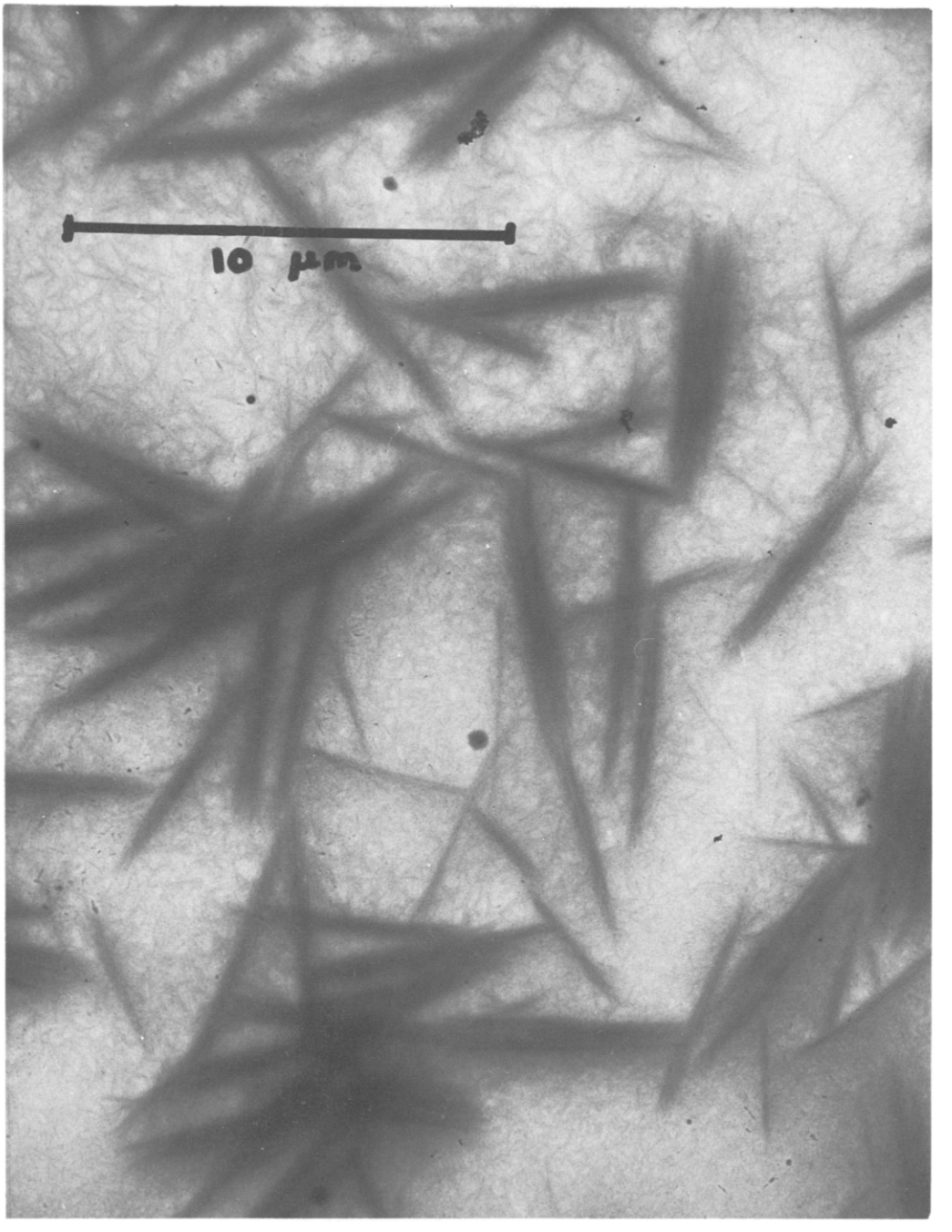


FIGURE 5. Electron micrograph of a dried film from the suspension of Hb S aggregates produced by stirring a 16.4 g/dl hemoglobin solution at 23°C. (6500X)

Figure 4 is a light micrograph of a thin aqueous film of a turbid Hb S solution resulting from stirring at 23° C. Needle-shaped, highly asymmetric particles averaging about 15 μm in length can be seen. Their suspensions are not detectably birefringent when observed between crossed polarizers; thus tactoid formation does not occur. The particles can be dissolved while under observation by cooling to near 0° C or by breaking the seal of the cover slip and allowing oxygen to diffuse in.

The electron micrograph of Figure 5 -- made from a dried film of the turbid Hb S solution -- shows particles which appear to be comprised of bundles of smaller rigid elements. The average length of the measureable bundles is about one-half the length of the needles observed under the light microscope. This discrepancy may be an artefact due to dehydration of the sample during preparation. The possibility that the filaments making up the bundles may themselves be aggregates of still smaller filamentous particles which can just be resolved in Figure 5 is being investigated.

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