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The polymerization of sickle hemoglobin in solutions and cells

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Abstract. The polymerization of sickle hemoglobin occurs by the same mechanisms in solutions and in cells, and involves the formation of 14 stranded fibers from hemoglobin molecules which have assumed a deoxy quaternary structure. The fibers form via two types of highly concentration-dependent nucleation processes: homogeneous nucleation in solutions with hemoglobin activity above a critical activity, and heterogeneous nucleation in similarly supersaturated solutions which also contain hemoglobin polymers. The latter pathway is dominant, and creates polymer arrays called domains. The individual polymers bend, but also cross-link, and the resulting mass behaves as a solid. The concentration of polymerized hemoglobin increases exponentially unless clamped by rate limiting effects such as oxygen delivery.
Key words. Polymerization; sickle hemoglobin; sickle cell disease; kinetics; thermodynamics; polymer domains; nucleation.

Introduction

Sickle cell disease holds a special position as the first disease for which an underlying molecular cause was identified. The consequences of the simple replacement of a charged amino acid (Glu $\beta 6$) with a hydrophobic, neutral Val are far ranging, and remain a vital area of current investigation. This review attempts to summarize what is understood about these consequences on the molecular level, in solutions and red cells. For compactness, it will not take a historical viewpoint, but will summarize our best understanding at this time. More detailed reviews on the physical chemistry[11] and pathophysiology[10,34] have recently appeared.

Structure and thermodynamics

Structure

The amino acid replacement leads to the formation of long, multi-stranded structures. The structures utilize a double stranded motif, which is also seen in crystals of HbS[40]. The double strand involves a variety of intermolecular contacts, but oddly enough only one of the replaced amino acids is involved in an up-down contact along the polymer. Unlike the crystal, the fibrous double strand found in solutions or cells is twisted, and the identification of the contacts between Hb molecules relies on understanding how the twist occurs and affects the crystal contacts.

The predominant structure seen in solutions or cells is composed of seven double strands: six wrapped around a central double strand[8,9] (fig. 1). The interplay of twist and contact energy has been proposed as the limiting feature on the 14-strand polymer[37]. In addition to the 14-strand structure, thicker structures are also seen, described as macrofibers and as fasicles[38].

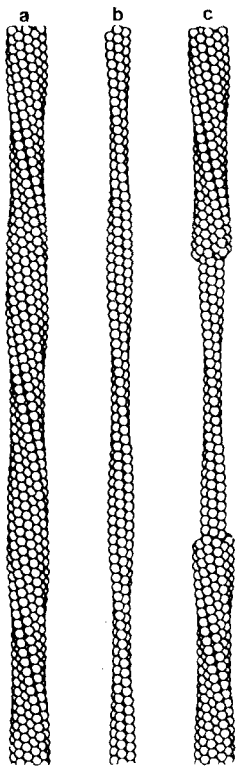

Figure 1. Model of the HbS polymer from image reconstruction of electron micrographs. The structure contains 14 strands in cross-section, with an inner core of 4 strands (shown in b) and an outer core of 10 (shown in a)[9].

Because only one of the two mutation sites is involved in the polymer, mixtures of HbA and HbS can copolymerize if they are able to reproportionate subunits[35]. In the absence of mixing subunits, or for other hemoglobins such as HbF even with subunits mixed with HbA, copolymerization does not occur.

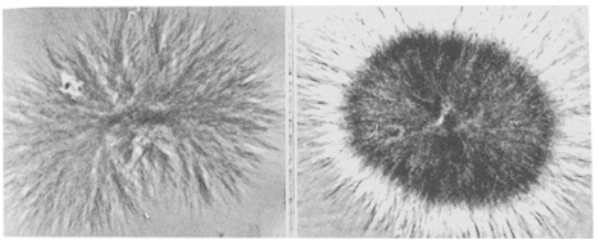

Figure 2. Polymer domains. Polymers are viewed with phase optics at ×1800 magnification. (Reprinted from White and Heagan[39] with permission.)

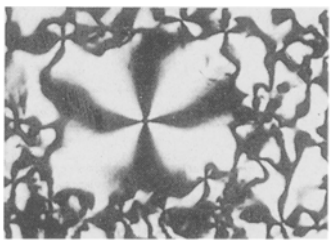

Figure 3. Polymer domain viewed between crossed polarizers. This domain has been made large (2 mm diameter) by heating a point in the center to induce nucleation there before the remainder of the sample polymerizes. Adjacent smaller domains have been formed spontaneously. (Reprinted from Sunshine et al.[36] with permission.)

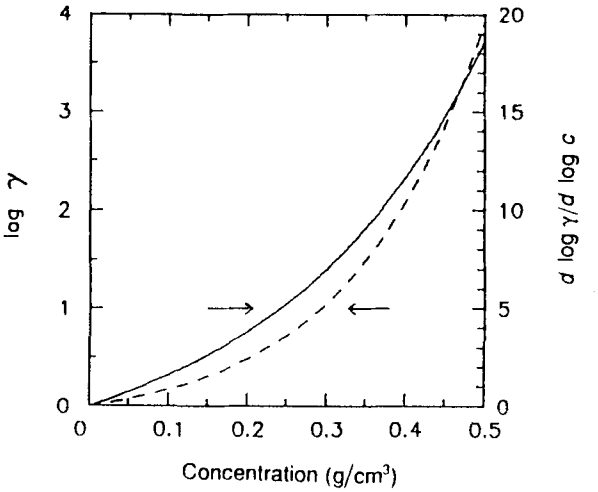

Figure 4. Activity coefficients calculated according to Minton[25]. Solutions of a given concentration behave thermodynamically as concentrations multiplied by the appropriate activity coefficient. The activity is entirely the result of volume exclusion by hemoglobin molecules, viewed as hard spheres.

The polymers form what is called a gel, which itself has structure. The polymers are cross-linked to one another, as well as linked in parallel[33]. There is enough flexibility to allow polymers to bend through angles at least as great as 23° in a few μm. Gels allowed to age a short time will show structures called polymer domains in which polymers radiate in all directions[39] (fig. 2). Such domains are readily viewed in thin solutions placed between crossed polarizers, which yield a characteristic Maltese Cross pattern (fig. 3)[2, 19, 24]. When fibers are placed on a grid for electron microscopy most of the interfiber contacts and even some of the lengthwise contacts are broken[5].

Thermodynamics of polymer formation

The polymerization can be well described by a solubility, in analogy with crystallization[20]. Polymer formation is favored by elevated temperature; at low temperatures hemoglobin is more soluble than at high temperatures. In understanding the solubility it is important to appreciate the molecular crowding in a Hb solution at concentrations typically found in an erythrocyte. At 34 g/dl, Hb molecules are 1.6 molecular diameters apart center to center, but only 0.6 diameters apart edge to edge. (Moreover, their natural motion assures that they will encounter each other every few nanoseconds.) The center to center distance is directly related to the number of particles per unit volume, which is a good measure of activity in dilute solutions. In concentrated solutions particles are much closer and activity must be measured by the product of concentration times an activity coefficient. Activity coefficients are extremely concentration-dependent, as seen in figure 4, but fortunately are approximated very well by calculations which only employ the hard sphere radii of the molecules[25]. (Longer range interaction, such as those dominated by electrostatics, involve temperature dependent activity coefficients.) Activity coefficients are not only important because polymerization occurs in a crowded milieu, but also because species incompetent to polymerize, such as HbO_2 will contribute to the crowding.

Relation to oxygenation

The thermodynamics and kinetics of oxygenation of dilute HbS is no different from HbA[16, 31]. However, in concentrated solutions which exceed the solubility, the interplay between function and assembly becomes apparent.

Polymer formation is inhibited by the presence of oxygen. This is understood in terms of the well established allosteric model for hemoglobin. Hb can assume two packings of its 16,400 MW subunits. Deoxygenated Hb packs in a tense or T conformation, whereas oxygenated Hb packs in relaxed or R conformation. The change in conformation is close to, but not exactly represented by the fraction of hemoglobin saturated with ligand. The change in structure is responsible for inter-subunit communication and hence cooperative oxygen binding[26]. The registry between polymer-stabilizing molecular contacts present in the T structure is absent when hemoglobin assumes the R structure. Thus it is the structural changes accompanying oxygenation which prevent polymer formation. Effectors, such as 2,3 diphosphoglycerate shift the equilibrium between R and T, and accordingly favor polymer formation at partial

saturations by biasing the equilibrium toward T (in addition to direct solution effects on the overall solubility)[32]. When hemoglobin polymerizes, it can still bind oxygen, but the binding is noncooperative (since the hemoglobin polymerized cannot change structure)[36]. The affinity is also about 3-fold lower than solution T state affinity[36], most likely as the result of imprisoning of the salt bridges (as is seen in crystals, in which hemoglobin binds oxygen with about 5-fold lower affinity than solution T state molecules[28].

Kinetics

The energetic benefit from assembling a monomer into a polymer is not very great. However, there is a significant entropic cost, for the monomer in solution has substantial motional freedom only partially compensated by the motion permitted within a polymer lattice. Each monomer contacts slightly more than four other monomers in a 14-stranded polymer structure, and a substantial fraction of these contacts typically must be made before the energetic gain exceeds entropic cost[12]. Since the entropic losses depend on the initial solution concentration, the energetic turning point will depend on initial concentration as well.

This interplay between entropic loss and bond gain means that small aggregates are less stable than the monomers. Many monomers must coalesce before adding one more monomer (growing the aggregate) is energetically more favorable than evaporating one monomer. Such a phenomenon is familar in the condensation of droplets in supersaturated vapor, and is referred to as nucleation.

The nucleation process just described differs from biological processes which involve nucleating centers. The nucleus described above represents a thermodynamic bottle-neck, which possesses intrinsically low stability, rather than a preferred structure which may be quite stable. The thermodynamic nature of the nucleus likewise implies that its size can vary as solution conditions are changed, since it represents no special arrangement or structure.

At a microscopic level, the formation of this unfavorable nucleus represents numerous attempts to form a large aggregate, one of which has randomly grown large enough to cross over to a region of increasing stability. This type of process is called homogeneous nucleation. In macroscopic samples, a large number of homogeneous nuclei form; in samples of cellular volume the number may be as low as one.

Once a nucleus forms, a polymer grows simply, i.e., without cooperativity or other complications. The polymer is not absolutely rigid, and its growing end dances about in Brownian motion, allowing the polymer to curve as it grows[33].

The process of forming a nucleus is somewhat simplified if other polymers are already present. An aggregate that begins growing on a polymer surface has a number of contact sites (and energies) already available, permitting a smaller size aggregate to become a nucleus. This means a second polymer can nucleate and grow alongside its 'parent'. The nucleus formed in this fashion is called heterogeneous, and accounts for the majority of polymers which are formed. The mechanism for polymer formation by homogeneous and heterogeneous nucleation pathways is called the double nucleation model, and is shown schematically in figure 5[12,14]. Not until concentrations near 40 g/dl are the equilibrium numbers of polymers formed by heterogeneous and homogeneous nucleation predicted to be equal. At lower concentrations heterogeneous nucleation is dominant[12].

When a large number of monomers must assemble a nucleus, the reaction will have a large concentration dependence. Activity coefficients further enhance the apparent reaction order. Consequently, due in almost equal part to activity coefficients and nuclear size, the

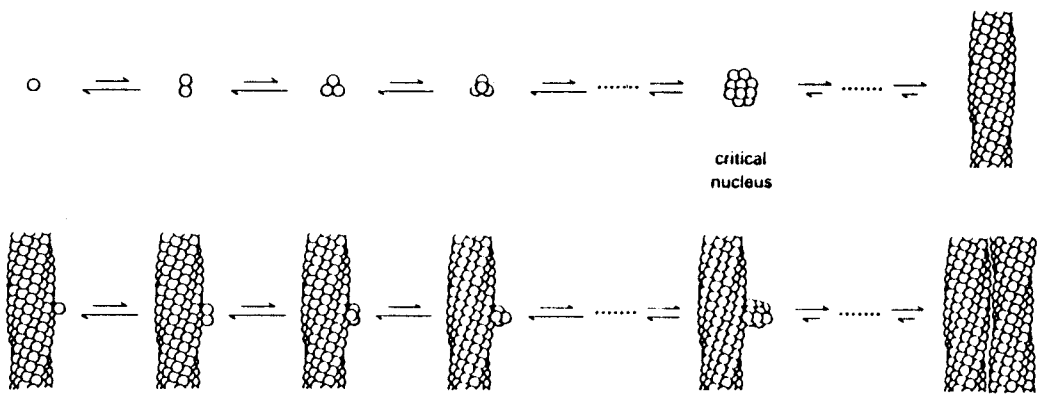

Figure 5. The double nucleation mechanism. Homogeneous nucleation occurs by a series of unfavorable steps in which monomers assemble a critical nucleus in solution. The nucleus is the species for which aggregation steps become increasingly favorable, and thus has only thermodynamic significance (upper diagram). Heterogeneous nucleation occurs by a series of unfavorable steps in which monomers form a nucleus attached to a polymer which has already been formed (lower diagram)[12].

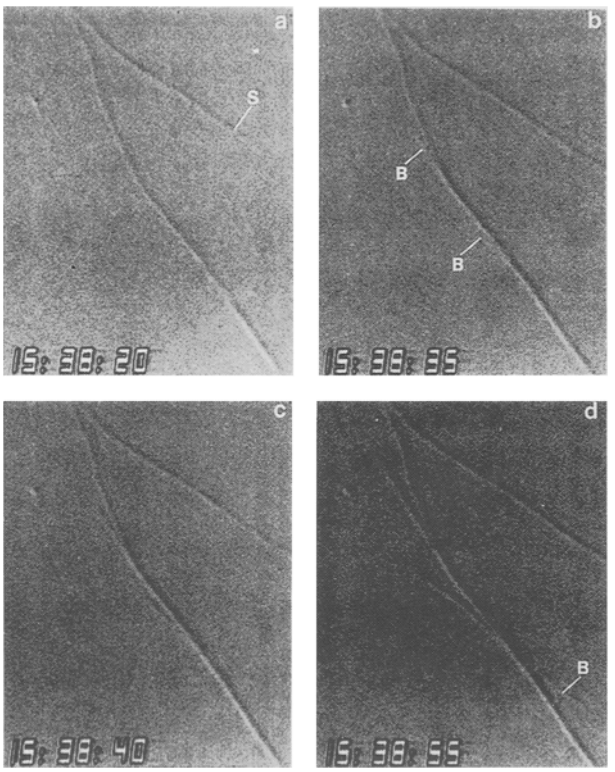

Figure 6. Direct observation of heterogeneous nucleation observed by Briehl and co-workers by differential interference contrast microscopy[33]. In this technique, the interference of the light passing around the fiber gives an image which can be used to visualize the growth of the fiber, though the image is much larger than the actual fiber size. Thickening of the fiber can be observed, and indicates heterogeneously nucleated polymers. Note also that the polymers will splay from the parent. Times are shown in the frames (h, min, s). Hb concentration is 22.2 g/dl, temperature is 21 °C. Frame size is 17 μm. (Reprinted from Samuel et al.[33] with permission.)

polymerization of sickle hemoglobin has a reaction order that varies from an average of 36th power in concentration at low concentrations (20 g/dl to 30 g/dl) to an average of 16th power at higher concentrations (30 to 40 g/dl)[13].

While the governing mechanism is not known, the polymers heterogeneously nucleated will commonly splay from the original polymer after a parallel run of some distance (fig. 6)[33]. This means that one homogeneous nucleus forms an initial polymer, which gives rise to additional polymers, that in turn form an expanding array. The array, or domain, initially resembles a bow tie, and if allowed to evolve long enough will form a spherulite[1].

From the foregoing description it is no surprise that the number of monomers taken up in polymers grows almost exponentially[3]. (It is exactly exponential if polymers are assumed to be infinitely thin; with a finite width and surface nucleation, the form is not quite exponential.) Observing the polymerized monomer content of a sample then shows an abrupt exponential growth. Since the initial polymers are difficult to detect, the exponential is seen in its growing phase at a time

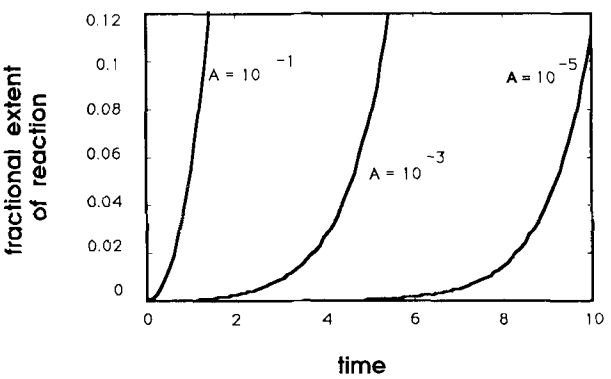

Figure 7. Exponential growth as predicted by the double nucleation mechanism. Despite an apparent delay, all three curves have begun at the same time zero. The curves differ in the magnitude of the paramter A in the expression A[exp(Bt) − 1]. When polymerization commences with an initial concentration of polymerized monomers c*, the A is replaced by $A + [c^*/2(c_o - c_s)]$, where c_o is the intial concentration and c_s is the solubility[3]. This has the effect of shortening or eliminating the apparent delay.

which appears to 'lag' the initiating event (fig. 7). This apparent delay is a useful way to characterize the kinetics of sickle hemoglobin polymerization, and is also physically instructive, for it implies that the sample characteristics are virtually unchanged during the delay period (due to the extremely small polymer mass). However, this is an instrumental effect, and a fine enough probe will detect polymers even at the start. If we take as a measure of this apparent delay a time to form 10% of the final polymerized monomer concentration, it is found that, at 35 °C, the tenth time varies from 10 ms to 10 ks as the concentration is varied from 20 g/dl to 40 g/dl (fig. 8)[13].

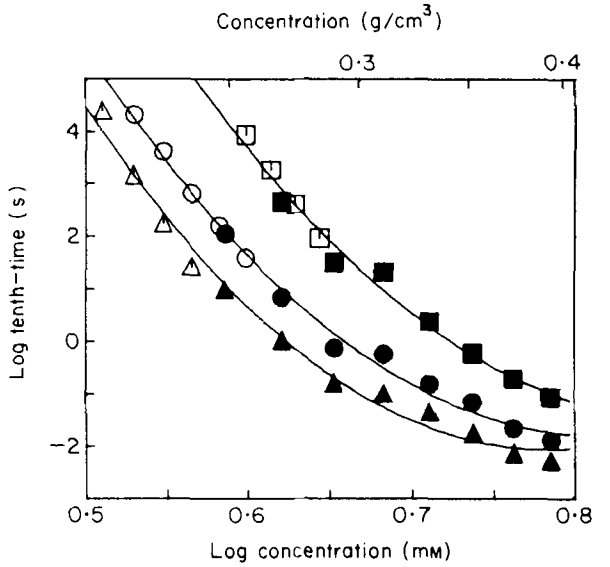

Figure 8. Tenth-times as a function of initial concentration. Open and filled symbols indicate difference in measuring technique. Triangles are measured at 35 °C, circles are measured at 25 °C, and squares are measured at 15 °C. Note the high concentration dependence, which slowly varies with concentration. Curves are drawn as an aid to visualization. (Reprinted from Ferrone et al.[13] with permission.)

A different type of delay arises in the wait for the first successful nucleation event. The unsuccessful attempts to form a nucleus leave no trace, and in that case, observation during the delay time can in principle give no clue as to the time elapsed (just as a radioactively decaying nucleus has no signature for its time of formation). This type of delay is intrinsically random[13, 14], and the distribution of these random delays allows the homogeneous nucleation process to be studied in a unique and sensitive way[18]. The nucleation event can be recorded because an entire polymer domain forms from each stochastic homogeneous nucleation event, and thus amplifies the original random process (fig. 9).

The double nucleation process also endows the reaction with a spatial dependence, since the rate of heterogeneous nucleation in a given volume depends on the number of polymers in that volume. In a given domain growth and heterogeneous nucleation may be occurring in the outer part while saturation has occurred in the inner part. At the same time, regions far from the domain may possess no polymers at all. Thus, even at early times in the reaction – during the apparent delay period – some saturation processes are likely to be operative as well as nucleation and growth.

Spatially dependent reactions allow for diffusion effects to play a role. The polymers are much less mobile than the monomers, which retain essentially their free diffusion coefficients[21]. As molecules are removed from the monomer pool to form polymers, new monomers will thereafter diffuse in to take their place. This makes the total hemoglobin concentration spontaneously rise at the center of polymer domains[6, 7].

Cellular considerations

Polymerization in the red cell occurs by the same processes which are active in solution. Studies performed on polymerization in cells show the same shape of the kinetic progress curves (in rapidly polymerizing solutions), a wide range of delay times consistent with the known distributions of intracellular concentrations, and the sharp increase in variability of the kinetics at long times, as seen in the stochastic variation of delay times. Furthermore, no additional processes appear to be operative. In particular, the erythrocyte membrane and its components exert no direct influence on the process (fig. 10)[17].

Although the mechanisms are the same, the conditions under which polymerization occurs in red cells are different in many respects from typical in vitro experiments. These are discussed below.

Presence of nonpolymerizing species

Physiologically the final oxygen delivery pressure is not zero. Typical values are 85 torr arterial, and 46 torr venous (versus 92 torr to 39 torr, for normal HbA)[22]. This means that polymer formation will always occur in the presence of nonpolymerizing species, as discussed above. This has the effect of increasing the crowding of the solution. However, as oxygen is delivered, the activity coefficient is constant since the total protein concentration remains fixed.

Non-equilibrium conditions

The transition through the circulatory system takes only a few seconds in the movement from arteries to veins, and 15 s to reach the lungs. From the range of delay times shown in figure 8 it is evident that not all cells will have even reached a tenth of the reaction in their passage through the smaller vessels, nor even in the overall transit between oxygenation. This argument can be made more quantitative. Morphological examination of arterial blood indicates about 10% of the circulating cells are sickled, and this is in reasonable agreement with equilibrium analysis that estimates about 5% sickle cells. On the other side of the capillaries, the mixed venous return shows about 20% sickled cells, in dramatic contrast to the expected equilibrium values of over 90% sickled cells at such pressures[27]. Polymer formation kinetics clearly have a pivotal role in determining which cells are eligible for potentially occlusive sickling. However, small though the percentage of cells which sickle in the circulation may be, a smaller fraction still must be responsible for occlusion, for otherwise the microcirculation would rapidly become permanently blocked[11].

Finite deoxygenation rates

Most kinetic laboratory experiments have been carried out on fully deoxygenated solutions. The few that have been done in the presence of ligands have predominantly been done in the presence of a fixed ligand concentration. Polymerization in vivo occurs in a dramatically different setting. Oxygen saturation is reduced from 95 to 50% in a few seconds, during which time the probability is greatest for becoming stuck inside a capillary or at its entrance.

There are several consequences to this fact. First of all, cells whose exponential growth is slow enough that they do not accumulate significant polymers until times longer than seconds have little direct risk of occlusion (though other damage, e.g., membrane transport, etc., may still occur). Secondly, highly concentrated cells which could sickle in times much less than a second in the laboratory encounter an obstacle to this rapid growth in the rate of oxygen removal. Such rapid polymerization will consume the available deoxyhemoglobin in forming polymers, and must then proceed at a rate limited by the rate of oxygen removal. This has the effect of softly clamping the rate of polymer formation to be near the rate of oxygen delivery as shown in figure 11[15].

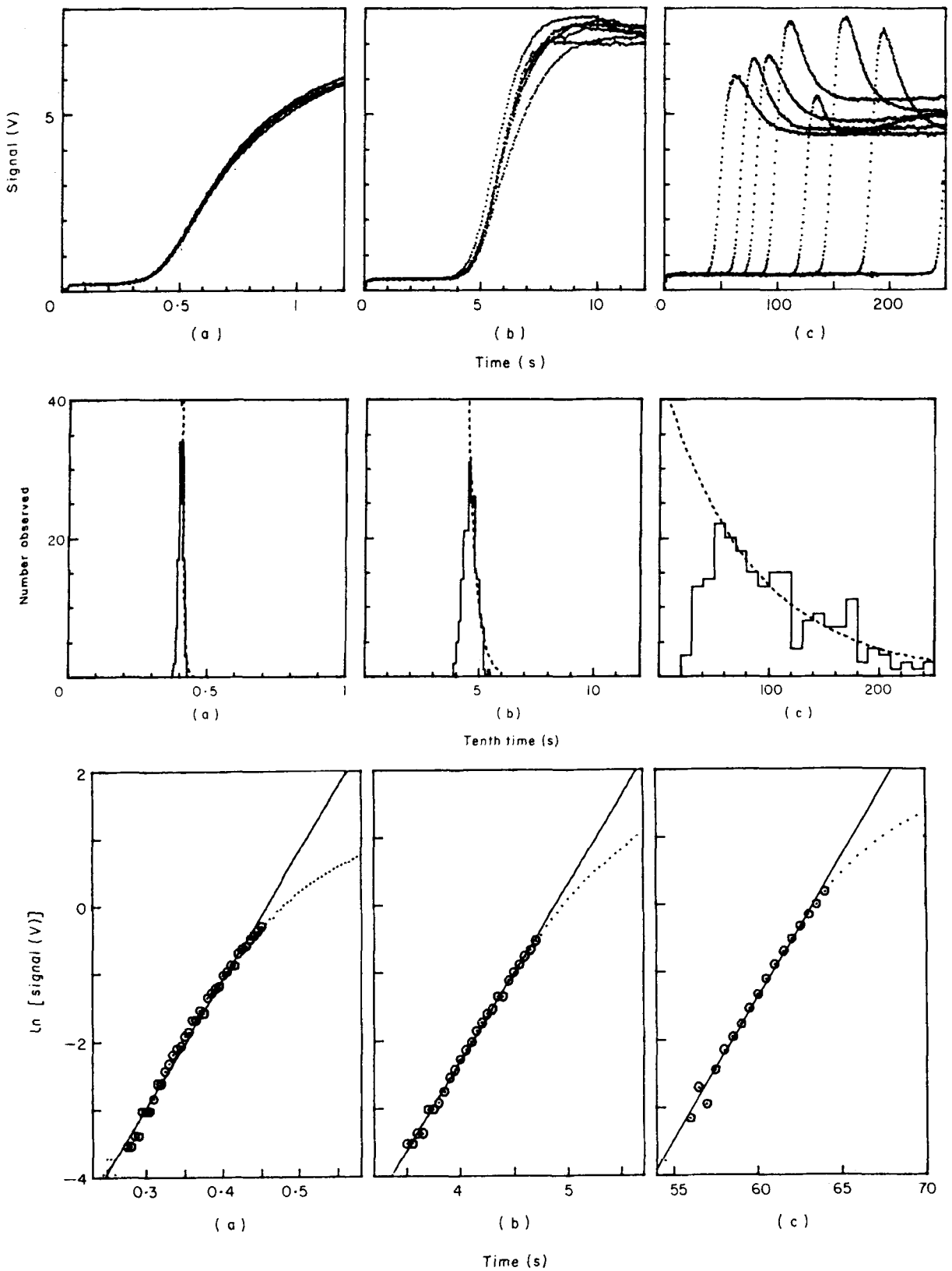

Figure 9. Polymerization progress curves for three different temperatures, showing onset of stochastic variation. Left panels are for 29.2 °C, center panels are for 18.9 °C, right panels are for 14.3 °C. 150–220 curves were collected at each temperature, and representative curves are shown at the top. As the temperature is lowered, the number of nuclei in the observed volume is smaller, and the variation in time of formation of the first homogeneous nucleation event becomes apparent. The second row shows the distribution of tenth times. The third row shows representative curves plotted semilogarithmically, showing the exponential character of the growth phase. Sample volume is about 10^{-10} cm^3. (Taken from Hofrichter[18] with permission.)

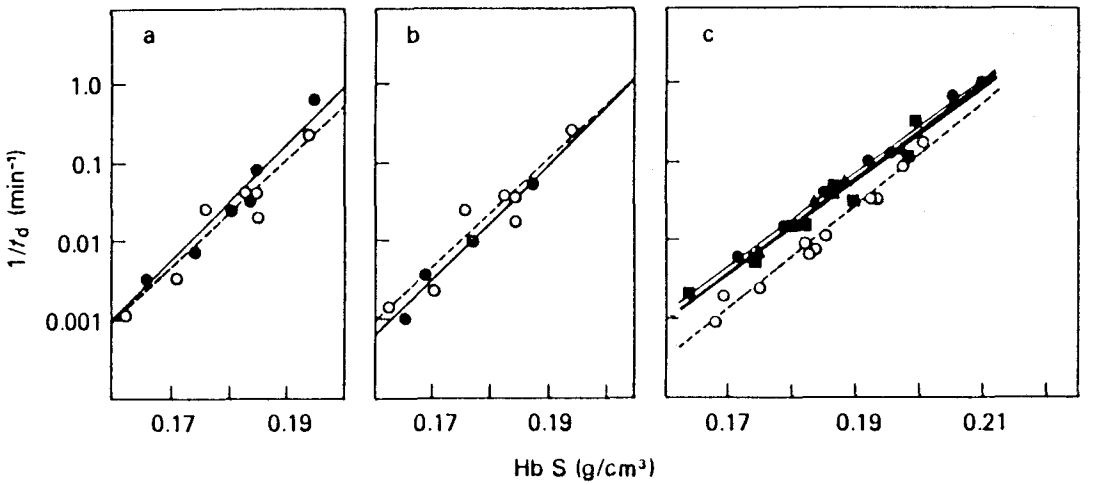

Figure 10. Effect of red cell membranes on the delay time (t_d) of HbS with correction for excluded-volume nonideality effects of added components. Control experiments without membrane components are shown as open circles. The delay time and tenth time are approximately equal (see text). *a* Effect of 0.51 mg/ml inside-out vesicles. *b* Effect of 0.60 mg/ml inside-out vesicles to which were added actin and spectrin. *c* Effect of added membrane ghosts: 0.67 mg/ml (filled squares), 3.7 mg/ml (filled triangles), 3.7 mg/ml (filled circles)[17].

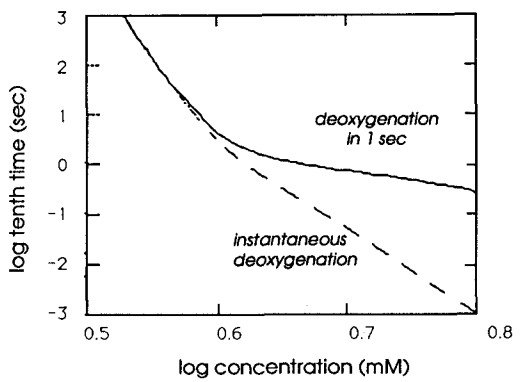

Figure 11. Predicted effect of finite rate of deoxygenation on tenth times[15]. Solid curve shows a calculation in which deoxygenation proceeds linearly, and takes 1 s to achieve its final value. The dashed curve is also a calculation, based on the double nucleation model. It is approximately equal to the observed data shown in fig. 8. Note that the effect of a finite deoxygenation rate is to eliminate the fastest tenth times[15].

The same effect militates against what is otherwise a dramatic consequence of unmelted polymer. Since reoxygenation may not be complete in the lungs, at high intracellular concentrations it is possible for some small amount of polymer to remain unmelted. When small amounts of polymer are present in solution experiments, it takes extremely little polymer to obliterate the apparent delay. However, in cells in which deoxygenation is not instantaneous, this leftover polymer will only recruit deoxygenated molecules which are provided by the slower deoxygenation process of the cell.

Finite cell volumes

Solution experiments and cellular polymerization also differ in that the volume of the red cell is small and limited. This is important because of the ability of monomers to diffuse much more rapidly than polymers locked into a domain. As a polymer domain consumes the monomers in the area in which it forms, the monomer concentration can reach the solubility in the interdomain spaces; in solution the interdomain concentration is replenished by diffusion from farther distances, allowing continued domain growth.

Rheological consequences

The formation of rigid, weakly cross-linked polymers creates a gel with solid-like properties, viz, the ability to hold stress indefinitely without flow, and the exhibition of a critical yield stress[4]. This is distinct from a system which is merely highly viscous. Because a gel is composed of interacting domains, the number of domains is an important variable. It appears that few domains are formed in each cell in vivo. Hence the rheology is dominated by a single domain, with perhaps a small number of domains interacting. Unfortunately, this could differ from bulk rheological measurements, which may be dominated by domain-domain interactions. Hence the most relevant experiments are likely to be single cell experiments, such as those using pipet aspiration techniques.

For cells showing morphological evidence of gelation indicating few domains, Nash et al. observed an exponential increase in the resistance to aspiration into pipets as oxygen partial pressure decreased and polymer concentration increased[29]. In fractioned cells Mackie and Hochmuth observed an increase in the rigidity which corresponded well to the concentration of polymerized hemoglobin[23]. These experiments indicate the clear potential for occlusion, but much remains to be

done to correlate fully the rheological effects with physiological variables (partial desaturation in seconds, etc.). On the other hand, small decreases in deformability are unlikely to have significant direct effects. For example, immature malarial parasites cause significant loss of deformability and can continue to circulate[30]. One immediate consequence of this observation is that small amounts of polymer are unlikely to pose significant problems.

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