THE INTRACELLULAR POLYMERIZATION OF SICKLE HEMOGLOBIN AND ITS RELEVANCE TO SICKLE CELL DISEASE

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In the last few years, the understanding of the molecular basis of sickle cell disease has progressed rapidly. It is now possible to describe the structure of the gel of polymerized deoxyhemoglobin S at molecular and atomic levels and to begin to understand the mechanism of formation of this gel from hemoglobin solutions. There are several current hypotheses for the pathophysiology of this disease based on molecular as well as cellular studies. Recently, it has become possible to measure the amounts of polymerized hemoglobin within erythrocyte populations and thereby demonstrate the existence of polymer in cells at very high oxygen saturation values. This unexpectedly low solubility of deoxyhemoglobin S in sickle erythrocytes can be largely accounted for by the theory of protein nonideality, with some contribution from cell heterogeneity. These results show that hemoglobin S in the erythrocyte behaves as it does in very concentrated hemoglobin solutions. There are strong indications, for both SS and AS cells, of the existence of cells with significant amounts of polymerized deoxyhemoglobin S but without morphological deformation (sickling). On the basis of these and other results, two models relating these biophysical findings to pathophysiology are discussed. First, it is likely that with deoxygenation there is a continuum of cellular changes. Initially, small amounts of polymer form; only with severe and prolonged deoxygenation do large domains of highly ordered, polymerized hemoglobin form in the erythrocyte and cause it to sickle. Second, we combine this model of cellular changes with information from rheologic studies to suggest an alternate model for the pathophysiology of the disease. We postulate that flexibility, governed by the amount and alignment of intracellular polymer, is the principal determinant of the flow of sickle erythrocytes. While cells that have large amounts of ordered polymer may be caught in the capillaries and venules, some cells at relatively high oxygen saturation, with polymer but no deformation, may have difficulty traversing the constriction of the precapillary arterioles. The implications of these recent molecular and cellular studies, as well as of the several models, for the therapy of sickle cell disease are discussed. In general, our analysis suggests that a therapeutically effective strategy is feasible and that certain approaches seem particularly propitious.

Most studies of sickle cell anemia during the first 40 yr after the description of the disease by Herrick concentrated on the properties of the sickle erythrocyte. Examination of the reversible morphological changes in these cells upon deoxygenation led to the classic hypothesis of Ham and Castle of a “vicious cycle of erythostasis” as the cause of the pathology of the disease. The discovery by Pauling and his associates of the abnormal hemoglobin molecule, hemoglobin S, in sickle cell anemia, later identified by Ingram as $^6$Glu$^{-}$Val$,^6$ initiated several decades of fertile investigation into the molecular basis of this disease. The observation that concentrated solutions of hemoglobin S gel upon deoxygenation suggested intracellular gelation as the basis for sickling of erythrocytes. The structure of this gel and the mechanism of its formation have since been studied extensively, especially using hemoglobin solutions.

In the first half of this review we will summarize insights into the pathophysiology of sickle cell disease that have resulted from these studies of hemoglobin solutions as well as critically evaluate other possible pathophysiologic factors in this disease. In the second half of this review we will present results of recent studies on the measurement of hemoglobin S gelation within erythrocytes and the explanation of these results. The relevance of these findings to understanding sickle cell disease will be presented in terms of several alternative models.
SICKLE HEMOGLOBIN POLYMERIZATION

The predominant structure of polymerized or aggregated deoxyhemoglobin S in the gel was originally thought to be a microtubular structure consisting of rings of six hemoglobin tetramers. However, recent structural analyses by electron microscopy have indicated that a more likely description of the polymerized hemoglobin fiber is a helical structure with 14 or possibly 16 hemoglobin tetramers in each layer (Fig. 1a). In addition, concurrent x-ray diffraction measurements of a true crystal of deoxyhemoglobin S indicated a molecular structure of paired strands of hemoglobin tetramers and defined the intermolecular contact regions within these strands (Fig. 1b). These paired strands seen in the crystal can be theoretically fit as an element of the helical fiber structure. These inferences about the structure of the polymer from electron microscopy and x-ray diffraction studies are compatible with suggestions about intermolecular contacts derived from studies of the effects of other mutations on the polymerization of hemoglobin.

Recent work has suggested that the polymer formed in hemoglobin solutions, and in cells as well, can transform into the crystal without major disruption of the molecular packing. These structural studies have been complemented by extensive analyses of the mechanisms of formation of the deoxyhemoglobin S gel. Minton has pointed out that the gelation process can be described as a phase equilibrium between a saturated solution of deoxyhemoglobin S tetramers and a solid polymer of these tetramers. Experimentally, the two phases can be separated by centrifuging a gelled deoxyhemoglobin S preparation at high speed. The polymer packs into a pellet that is in equilibrium with the concentrated deoxyhemoglobin S in the supernatant. In the uncentrifuged gel, and presumably in the cell, the two phases coexist without a physical separation. The essential validity of this two-phase model has been shown in extensive equilibrium studies.

Sickle cell disease may be viewed from this perspective as primarily due to the low solubility of deoxyhemoglobin S compared with that of deoxyhemoglobin A or oxyhemoglobins S or A. The solubility of pure deoxyhemoglobin S solutions close to physiologic conditions is about 16 g/dl, while the other forms have solubilities greater than 50 g/dl. When the solubility is exceeded upon deoxygenation of the sickle erythrocyte, polymer forms. Most of the information about the polymer discussed in this review originates from gelation studies at conditions thought to be close to physiologic. However, significant information has been obtained under other conditions, such as in high phosphate buffers. Analyses of the effects of additives on the solubility of deoxyhemoglobin S have become one of the standard methods for studying potential therapies for sickle cell anemia.

The equilibrium studies have been accompanied by detailed analyses of the kinetics of formation of the hemoglobin S polymer. Gelation may be initiated by deoxygenating hemoglobin S, increasing the concentration of deoxyhemoglobin S, or increasing the temperature of a concentrated deoxyhemoglobin S solution (deoxyhemoglobin S solubility decreases as
temperature increases up to 37°C). Most kinetic studies have been done using the temperature "jump" procedure. Characteristically, after the sudden increase in temperature of a concentrated deoxyhemoglobin S solution, there is a relatively long delay period during which no changes are observed followed by a rapid change in the solution properties, indicating the occurrence of gelation. This unusual kinetic behavior has been interpreted as evidence for a nucleation-controlled polymerization reaction. Such a mechanism has been observed for many phase transitions and accounts for previously perplexing observations on the biophysical behavior of deoxyhemoglobin S solutions. More recent work using laser photolysis has extended these observations to very short time ranges and has suggested that the actual mechanism may be somewhat more complex than that shown in Fig. 2.

The Kinetic Hypothesis

One important result of these kinetic studies has been the introduction of the concept of delay time as an important pathophysiologic parameter for describing the behavior of deoxyhemoglobin S. The delay time is inversely proportional to a very high power (30–40) of the total hemoglobin concentration and is very sensitive to other variables such as pH and temperature. In addition, the delay time can be theoretically related to the solubility of deoxyhemoglobin S. Experimental studies over a wide range of conditions have confirmed the validity of this relationship between delay time and solubility. These results led Eaton, Hofrichter, and Ross to postulate that the "probability of sickling inside capillaries is determined by the delay time of gelation and that a crisis occurs when the delay times are shortened enough or the capillary transit times lengthened enough to increase significantly this probability." Damage to tissues is believed to result when the cell becomes rigid while still in the capillary circulation and obstructs the microcirculation, presumably on the venous side. Since the delay time can be accurately determined from measurements of deoxyhemoglobin S concentration, this model has the potential of directly relating polymerization to microvascular transit rates.

OTHER POSSIBLE PATHOPHysiologic FACTORS

Membranes

In parallel to these studies of the reversible polymerization of sickle hemoglobin solutions, many investigators have tried to relate the pathophysiologic processes in patients with sickle cell disease to more complex phenomena involving the red cell membrane and the microcirculation. In addition to changes in the physical state of hemoglobin S upon deoxygenation, a variety of simultaneous metabolic alterations have been noted. These include efflux of potassium ions, influx of sodium ions, and changes in the ATPase-dependent pump mechanisms. The metabolic changes that have caused the greatest interest, however, are the marked increase in intracellular calcium ions and possible changes in ATP levels. During the last few years, a number of laboratories have studied the binding of hemoglobin to erythrocyte membranes. Binding was demonstrated under nonphysiologic conditions, but its significance under physiologic conditions is not clear. Binding seems to be somewhat greater for hemoglobin S than for hemoglobin A. These observations of abnormalities in sickle erythrocytes have been used in conjunction with the known decrease in filterability of sickle erythrocytes upon deoxygenation to suggest that the membrane properties of these cells is abnormal. This approach is supported by the findings of a variety of biochemical abnormalities in the composition of sickle erythrocyte membranes, and several therapeutic
strategies have been proposed based on this assumed decrease in membrane fluidity. However, understanding the effects of membranes specifically on pathophysiology requires rigorous biophysical measurement of the properties of the membranes in a manner independent of effects due to the physical state of the hemoglobin contained in the cell. In fact, recent data from a laser diffractometer (ektacytometer) suggest that changes previously ascribed to the membrane are really a reflection of the properties of the intracellular contents.

Irreversibly Sickled Cells

Much attention has also been focused on irreversibly sickled cells, cells that are morphologically abnormal even when fully oxygenated and the intracellular hemoglobin S is depolymerized. These cells, whose frequency varies greatly from patient to patient, have been found to have abnormal membranes. However, the relationship between these membrane protein abnormalities and the abnormal natural history of these sickle erythrocytes is not clear. Further, there is not a good correlation between the number of irreversibly sickled cells and the pathophysiology of this disease except for hemolysis. These cells tend to have elevated hemoglobin concentration, and the ultimate significance of irreversibly sickled cells in this disease may be a more general one concerning that subpopulation of erythrocytes with the high extreme in hemoglobin concentration.

Endothelial Adhesion

Recent sets of observations on the abnormally high adherence of sickle erythrocytes to endothelial cells in culture has raised the possibility of a pathophysiologic mechanism involving cell adhesion. This adherence is not enhanced in deoxygenated cells and is very dependent on cell density and the medium in which the red cells are suspended; indeed, fibrinogen appears to be a mediator of adhesion to the endothelial cells. Importantly, adherence correlates strongly with various parameters of sickle cell disease activity. It is not clear, however, that these studies are measuring more than an acute phase reactant, such as fibrinogen itself, and that the forces of the adherence phenomenon are comparable to the shear forces that occur in the microcirculation. Thus, further studies must be done before the significance of these observations can be evaluated.

**INTRACELLULAR SICKLE HEMOGLOBIN POLYMERIZATION**

Most of the above studies of possible pathophysiologic mechanisms are several steps removed from the biophysical description of the gelation of hemoglobin S solutions, discussed in the first part of this review. Understanding of the gelation of hemoglobin S within the erythrocyte and how gelation relates to cell abnormalities and abnormal rheology would provide an important connecting link between aggregation of hemoglobin and the pathophysiology of this disease. Unfortunately, until recently, very few methods were available to study this intracellular process. Birefringence, x-ray diffraction, and electron microscopic techniques were used to demonstrate, but do not quantitate, ordered polymer within the deoxygenated sickle erythrocyte. In addition, studies by water–proton nuclear magnetic resonance spectroscopy demonstrated changes in the cell upon deoxygenation, but the amount of intracellular gel could not be quantitated.

Four years ago we began work on a method, using natural abundance 13C nuclear magnetic resonance spectroscopy, that had a potential of quantitating intracellular polymerized hemoglobin S. This method uses various decoupling procedures to measure independently the amount of freely rotating and the amount of immobilized hemoglobin molecules. Because of the two phase nature of the polymerization process, the fraction of freely rotating hemoglobin molecules corresponds to the fraction of unpolymerized hemoglobin while the fraction of immobilized hemoglobin S corresponds to polymer fraction. The concentrations of the postulated critical nuclei and other intermediates are assumed to be very low relative to the concentrations of either or both of these two species. The validity of these assumptions, and the method in general, was established when we showed that the polymer fraction of gelled hemoglobin S solutions determined by nuclear magnetic resonance spectroscopy was the same as that determined by the more classical ultracentrifugation method.

When measurements were made of polymer fraction of sickle erythrocyte as a function of oxygen saturation, the result shown in Fig. 3 were obtained. Polymerized hemoglobin S was detected at >95% oxygen saturation and the amount of polymer increased with decreasing oxygen saturation to a maximum of 70% of the total hemoglobin at complete deoxygenation. These results, which were obtained using several different technical methods, as well as with blood from several different individuals with sickle cell disease, were at first quite surprising. For

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*Indeed, the spectroscopic method was more accurate, as it was found that the ultracentrifugation method required a 5%–10% correction due to the sedimentation of free hemoglobin tetramers as well as polymerized hemoglobin S.
fully deoxygenated hemoglobin S solutions, no polymer is detected until the deoxyhemoglobin S concentration exceeds the solubility (16 g/dl) at physiologic pH and temperature.25 In contrast, the data indicated that deoxyhemoglobin S had very limited solubility in the erythrocyte.

PROTEIN NONIDEALITY

The explanation of these experimental results resides in the description of the nonideal behavior of proteins at high concentration.24 Figure 4 is a scaled diagram comparing a hemoglobin solution at intracellular concentration (5 mM or 34 g/dl) with an isotonic sodium chloride solution (150 mM or 0.9 g/dl). It is apparent that compared to the ions of the salt the hemoglobin molecules are very large and very close together. Indeed, the ratio of solvent to solute on a weight basis is markedly decreased in the hemoglobin solution as compared to the salt solution. In order to correct for phenomena related to this molecular crowding, one uses activity rather than concentration in the relevant equations;89 the ratio of activity to concentration defines the activity coefficient. Minton, Ross, and Briehl90,91 have analyzed equilibrium sedimentation and osmotic pressure data for concentrated hemoglobin solutions and have determined the activity coefficients for hemoglobin solutions up to 40 g/dl. As hemoglobin concentration increases, the activity increases exponentially. For example, at 1 g/dl the activity coefficient is about 1, while at 34 g/dl the activity coefficient is 97.90

This high activity, which means that the hemoglobin in the red cell behaves chemically as if it were almost 100 times more concentrated than it actually is, has important implications for the behavior of intracellular hemoglobin S. For example, the kinetic studies discussed above can be used to estimate the size of the critical nucleus that controls the rate of polymerization (see Fig. 2). Whereas the size of the nucleus has been estimated at 30 or greater from the dependence of the delay time on concentration, considerations of activity results in a reduction in the calculated size of the critical nucleus to 10-15 hemoglobin molecules,92 a number more consistent with the knowledge of the polymer structure (Fig. 1a).

Another implication of the nonideal behavior of hemoglobin within the erythrocyte is that the presence of oxyhemoglobin S decreases the solubility of deoxyhemoglobin S. As noted above, a pure deoxyhemoglobin S solution has a solubility of 16 g/dl under physiologic conditions.25 However, as one first deoxygenates a sickle erythrocyte, the deoxyhemoglobin S formed is in the presence of almost 34 g/dl of oxyhemoglobin S. The latter markedly reduces the solubility
of the former.† The exact solubility of deoxyhemoglobin S as oxygen saturation is varied can be calculated from the thermodynamic analysis that has been developed to account for the gelation of hemoglobin mixtures.§ A schematic diagram of the results of this calculation is shown in Fig. 5. It can be seen that as deoxygenation occurs, the amount of polymer increases, as does the amount of deoxyhemoglobin S in solution. However, the presence of oxyhemoglobin S decreases the amount of deoxyhemoglobin S in solution compared with the amount in solution at complete deoxygenation.

These relationships are shown quantitatively by the solid line in Fig. 3, which is the theoretical curve predicted from protein nonideality for the polymer fraction within the sickle erythrocyte as a function of oxygen saturation. In general, the agreement between the experimental results and theory is excellent. However, we detect hemoglobin S polymer at even higher oxygen saturation (>96%) than theory would predict. This reflects cell heterogeneity in mean corpuscular hemoglobin concentration (MCHC).96,97 Our analysis is based on a homogenous population of cells with MCHC of 34 g/dl. Any significant subpopulation of cells with higher MCHC will skew the shape of the curve and shift the intercept toward 100% oxygen saturation.88 All individuals have cell populations with a distribution of MCHC values.93 Moreover, in individuals with sickle cell anemia, that distribution is greater and includes the “irreversibly sickled cells” that have a very high value of MCHC.55,98 Our theoretical analysis of polymer fraction for different MCHC values as a function of oxygen saturation for different MCHC values is shown in Fig. 6.

A very recent extension99 of the application of nonideality to hemoglobin polymerization, which includes a term for the nonideal behavior of water itself, suggests that these curves may slightly overestimate the amounts of intracellular polymer. In this case, the contribution of cell heterogeneity would be greater than that suggested by the small discrepancy between experimental results and the theoretical curve shown in Fig. 3. Further, variations in DPG levels, pH, concentrations of hemoglobins F and A2, levels of carboxyhemoglobin, and other factors also occur.97 All of these variables could have a significant effect on the amount of polymer in the erythrocytes of different individuals under similar conditions. Only experiments with fractionated cell populations will allow a defini-
We have also measured polymer fraction in erythrocytes from individuals with sickle cell trait. We find that polymer is detected only at oxygen saturation values below 60%, a result that we have confirmed with ultracentrifugation studies of sickle trait hemolyte. The curve for sickling of sickle trait erythrocytes as a function of oxygen saturation is shifted toward lower oxygen saturation and rises much more steeply, again indicating that morphological sickling and polymer formation are not always coincidental.

The existence of intracellular gelation without cell sickling has been suggested by Bradley and his colleagues. They found that flow through Nucleopore filters decreased at a time after partial deoxygenation prior to morphological deformation but coincident with intracellular polymer formation as determined by electron microscopy. Chien, in comparing microsiev and viscometry measurements, also concluded that stasis might occur before significant deoxygenation.

A MODEL FOR INTRACELLULAR POLYMERIZATION

We combine the information summarized above into a schematic representation of the formation of intracellular polymer in sickle erythrocytes upon deoxygenation (Fig. 7). We envision that the fully oxygenated cell has no polymerized hemoglobin S and that upon deoxygenation small amounts of polymer form inside the sickle erythrocyte. As deoxygenation progresses, the amount of polymer increases and larger domains are formed within the erythrocyte. As these domains grow, the cell becomes progressively more distorted and may assume the characteristic sickle shape or some other abnormal form. It should be noted that even on complete deoxygenation there are a significant number of free hemoglobin S molecules in equilibrium with polymer in each erythrocyte.

It is our belief that the physical state of the polymer (i.e., its amount and properties) are the major determinants of the abnormal flow properties of the sickle erythrocyte. Thus, it is likely that cells, such as those illustrated in Fig. 7b and c, which have isotropically distributed polymer, may have abnormal rheologic properties but appear morphologically normal. Although there are data in the literature to suggest this, further experiments to test this hypothesis are essential. It has been known for over 60 yr that slow deoxygenation of sickle erythrocytes leads to
Fig. 7. A diagram of the likely spectrum of intracellular polymerization of sickle erythrocytes at different oxygen saturation values: (a) 100%, (b) 75%, (c) 50%, (d) 25%, and (e) 0%. The cell in panel a has all free hemoglobin molecules; as deoxygenation progresses, polymer bundles appear, grow and align—eventually leading to distortion of the cell. The degree of alignment of the polymer and its properties is also expected to be dependent on the rate of deoxygenation and, possibly, on the past history of the cell (hysteresis). (A similar diagram, based on the kinetic studies in reference 104 has been published since we prepared this figure.)

formation of a larger number of cells with a characteristic sickle deformity, while rapid deoxygenation does not. We believe that as with a crystallization process, slow deoxygenation may lead to formation of highly aligned polymer due to the growth of only a few domains. Cells containing highly aligned polymers may have different rheologic properties as compared with cells with equal amounts, but less ordered, polymer. Studies of the rheologic or viscoelastic properties of gels of sickle hemoglobin and of sickle erythrocytes are needed.

AN ALTERNATE MODEL FOR THE PATHOPHYSIOLOGIC PROCESS

Mean arterial oxygen saturation values are significantly less than 100% in individuals with sickle cell disease. Thus, our measurements (Fig. 3) suggest that polymer of deoxyhemoglobin S is likely to always be present in the erythrocytes of these individuals (and might be detectable by electron microscopy). Given the presence of polymer in some, or possibly most, erythrocytes, the physiologic significance of the long delay period caused by the nucleation step found in the polymerization of hemoglobin S solutions is unclear. Indeed, our results suggest that rheologic problems may possibly occur at the high, but gradually diminishing, oxygen saturation values on the arterial side of the circulation, as well as on the venous side.

At the top of Fig. 8 is a schematic diagram of the present understanding of the microcirculation. The major constriction to flow occurs in the precapillary arteriole where erythrocytes must be deformed to pass through this small muscle-lined vessel. If deformability is impaired, as in a sickle erythrocyte with or without morphological deformation but with intracellular polymer, it is possible that passage will be hindered. It is likely in addition, however, that blockage of cells with polymer may also occur in the capillaries or venules at bifurcations or bends, where added resistance to flow can be envisioned.

We suggest this model (Fig. 8) as the pathophysiologic basis for sickle cell disease. Factors such as cell heterogeneity, the extent and rate of deoxygenation of the erythrocytes, and the resistance of the precapillary arterioles and other parts of the microcirculation (affected by humoral, neurologic, and other factors) would presumably determine the tendency towards stasis and tissue destruction. Such processes would likely explain the pain crises, but the stress imposed by deformation in the precapillary arterioles might also account for part of the chronic hemolytic anemia. This model also accords with microscopical observations of the conjunctival and retinal microcirculation, which showed obstruction to flow on the arteriole side of the microcirculation rather than the venule side as earlier theories had predicted.

CONCLUSIONS

We believe that our models for intracellular polymerization and for the pathophysiology of sickle cell
It will be noted that the therapeutic implications of our model are quite similar to those of the kinetic hypothesis.

**Fig. 8.** A model of the microcirculation basis of the pathophysiology of tissue damage in sickle cell anemia. The structural properties of the parts of the microcirculation are shown at the top (somewhat compressed in the long axis to save space). Normally erythrocytes must deform to squeeze through the precapillary arteriole; we hypothesize that such deformation may be impeded in sickle erythrocytes that have normal morphological appearance but significant amounts of intracellular polymer. This hypothesis would place a part of the lesion on the arterial side of the microcirculation, but obstruction to flow may also occur at bifurcations or bends in the capillary and venous microcirculation.

Disease are consistent with the current understanding of the biophysics of sickle hemoglobin solutions, the behavior of sickle erythrocytes, and the structure of the microvasculature. This analysis has definite implications for the therapeutic approaches to this disease.† It has been appreciated for some time that any agents that inhibit the aggregation of deoxyhemoglobin S molecules or lower the concentration of deoxyhemoglobin S within the cell should be beneficial. The latter goal might be approached through increasing oxygen affinity, but lowering total sickle hemoglobin concentration would be a better overall strategy because of the high activity coefficient when MCHC is 34 g/dl or greater. The recent work on the possible beneficial effects of therapeutically induced hypotension illustrates one approach to that goal. This analysis is also relevant to genetic approaches to sickle cell disease. It appears from our calculations (Noguchi and Schechter, unpublished) that adding a second hemoglobin (A, A₂, or F) to the sickle erythrocyte would be deleterious if MCHC were to be increased. A gene "insertion" or "switching" approach would work only if the hemoglobin S concentration was lowered by the technique. It would also seem that a systematic evaluation of agents with pharmacologic effects on the microvasculature would now be warranted.

The therapeutic objectives, as we envision it, are to decrease the amount of deoxyhemoglobin S polymer or its alignment in the erythrocytes of patients and/or to decrease resistance in the microcirculation. These are less stringent and probably more attainable goals than that of finding agents that unsickle sickled erythrocytes, for example, AS erythrocytes still sickle upon complete deoxygenation. Guidelines for some of these therapeutic objectives are available from comparisons of disease severity in the various sickle syndromes.  

Most importantly, these hypotheses concerning therapeutic approaches are now testable in sickle hemoglobin solutions and erythrocytes. Better experimental models of the microcirculation are needed, however.

The theory of protein nonideality, which explains so much about the behavior of hemoglobin S in the sickle erythrocyte, is very likely to be of relevance in the understanding of the behavior of other hemoglobin and nonhemoglobin molecules in the erythrocyte. The physical chemistry of all the interactions within the erythrocyte, for example, any interaction of hemoglobin molecules with the membrane, will be affected by the very high activity coefficients of the intracellular milieu. Indeed, nonideality will probably make significant contributions toward the physicochemical properties of any organelle or cell in which the concentration of soluble internal components is very high.

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