SICKLE CELL HEMOGLOBIN: MOLECULAR BASIS OF SICKLING PHENOMENON THEORY AND THERAPY

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Concern for man himself and his fate must always form the chief interest of all technical endeavors.
Albert Einstein

Part A. Theory

HISTORICAL INTRODUCTION TO THE MOLECULAR DISEASE CONCEPT OF SICKLE CELL ANEMIA BY LINUS PAULING

Thirty years ago, Linus Pauling suggested that sickle cell disease might be due to an abnormal hemoglobin molecule, and I think this was the beginning of molecular medicine. This is what he said about it in his Harvey Lecture,[69a] which he delivered in 1953:

"In the spring of 1945 I, together with eight men from medical schools of the country, was serving as a member of the Medical Advisory Committee which assisted in the preparation of the Bush Report. One evening Dr. William D. Castle, professor of medicine at Harvard University, mentioned to the other members of the committee the disease Sickle Cell Anemia, with which he has had some experience. He told about the discovery of the disease by Dr. J. B. Herrick[32] in 1910 and described the characteristic change in shape of the red blood corpuscles and the effect of oxygen in preventing the sickling and of carbon dioxide in accelerating it. I suggested that the action of carbon dioxide was to accelerate the dissociation of oxygen from oxyhemoglobin through the Bohr-Hasselbach effect (it had, in fact, been clearly stated by Hahn and Gillespie[28] in 1927 that sickling occurs only when the partial pressure of oxygen is small), and I pointed out that the relation of sickling to the presence of oxygen clearly indicated that the hemoglobin molecules in the red cell are involved in the phenomenon of sickling and that the difference between sickle cell anemia red corpuscles and normal red corpuscles could be explained by postulating that the former contained an abnormal kind of hemoglobin which, when deoxygenated, has the power of combining with itself into long rigid rods which then twisted the red cell out of shape."

Then in 1949 Pauling, Itano, Singer, and Wells,[66] proved by demonstrating the electrophoretic mobility the difference between sickle cell hemoglobin (Hb S) and the normal hemoglobin (Hb A) molecule. This was the discovery of the abnormal hemoglobin which Pauling had predicted to exist four years earlier. The important points are that Pauling, Itano, Singer, and Wells[66] discovered this abnormal hemoglobin and proved its existence in 1949; furthermore, it appears to the writer that this publication had triggered an information explosion in the general area of hemoglobin. The detailed mechanism of the

sickling process was suggested in the first paper on sickle cell anemia hemoglobin[66] as follows:

"We can picture the mechanism of the sickling process in the following way. It is likely that it is the globins rather than the heme of the two hemoglobins that are different. Let us suppose that there is a surface region on the globin of the sickle cell anemia hemoglobin molecule which is absent in the normal molecule and which has a configuration complementary to a different region of the surface of the hemoglobin molecule. This situation would be somewhat analogous to that which very probably exists in antigen-antibody reactions. The fact that sickling occurs only when the oxygen and the carbon monoxide are low suggests that one of the sites is very near to the iron atom of one or more of the hemes, and that when the iron atom is combined with either one of these gases, the complementariness of the two structures is considerably diminished. Under the appropriate conditions, then, the sickle cell anemia hemoglobin molecules might be capable of interacting with one another at these sites sufficiently to cause at least a partial alignment of the molecules within the cell, resulting in the erythrocytes becoming birefringent and the cell membrane's being distorted to accommodate the now relatively rigid structure within its confines. The addition of oxygen or carbon monoxide to the cell might reverse these effects by disrupting some of the weak bonds between the hemoglobin molecules in favor of the bonds formed between gas molecules and the iron atoms of the hemes."

The Harvey Lecture[69a] was delivered in April of 1954; this is an excellent review of the literature from Herrick up to this time with Pauling's analysis of the important works. Then in 1959, Dr. J. W. Harris[31] wrote an excellent and comprehensive review on the sickling phenomenon. The present review will cover mainly the theoretical aspect of the molecular basis for human red cell (with deoxy Hb S) to sickle. Therapy derived from this theory[62a] will be presented in Part B.

The postulated mechanism of interaction of deoxy Hb S (Deoxygenated Sickle Cell Hemoglobin) molecule was that of stacking by Pauling.[66,68] Because of the assumed complementariness in structure, the molecule of deoxy Hb S (without the oxygen molecule or other molecules attached) could interact to form long chains of molecules. These long chains of molecules could attract one another into parallel orientation, causing the formation of a crystal or liquid crystal. Evidence obtained by Harris[30] supports this picture through the observation that a solution of deoxy Hb S containing over 10% of the protein formed liquid crystals of the nematic type with the shape of double circular cones. Perutz and Mitchison[71] also made a quantitative study of the pleochroism of sickled cells and have shown that the pleochroism is compatible with this postulate. The orientation of the heme groups is such that the plane of the hemes is *parallel* to *the long* axis of the sickled cell.

Pauling made the following prediction:[67]

"In the course of time, through continued attack on the problem, the complete structure of the hemoglobin molecule will be discovered and the precise nature of the abnormalities that are present in the molecule of sickle cell anemia hemoglobin; .... we may feel confident that this knowledge will permit the deduction of improved therapeutic methods, and that in the future a similar attack on other diseases, through the determination of the structure of the molecules that are involved, can also be made."

It appears that this prediction has been fulfilled by Nalbandian,[62a] who deduced the therapeutic use of urea from the Murayama hypothesis on the molecular mechanism of the sickling process (see the section on therapy, Part B).

Pauling's Theory on Helical Aggregation of Globular Proteins[68]

Pauling's theory on helical aggregation of globular proteins is discussed below in light of the proposal of Bookchin and Nagel[13,14] that deoxy Hb S molecules aggregate into a packed helix with a low pitch having about six molecules per turn, which permits the existence of primary and secondary interacting sites. It should be noted that such round or circular hexamers of deoxy Hb S have not been observed by electron microscopy, although monomolecular filaments[25a,59,61] and microtubules have been reported.[10,23,25a,56,61,87]

Many globular proteins are known to polymerize, Pauling stated.[68] He pointed out that a monomer protein molecule containing a single polypeptide chain is asymmetric, and that a single pair of complementary combining regions on such a molecule would permit it to attach itself to equivalent molecules in such a way as to form a helical structure, giving rise to fibrils. If the pitch of the helix is zero or nearly zero, the helix degenerates into a circle, and a small polypeptide (dimer, trimer) is formed. A reasonable amount (about 5°) of freedom of angular motion in the bond between adjacent molecules would permit a

helical fibril of large diameter to change its length by as much as a factor of two, and would also permit tubular aggregates with walls one, two, or three molecules thick to be formed. Pauling suggested that some of the observed properties of globular proteins can be accounted for in this way.

Because the hemoglobin molecule consists of a tetramer and has a twofold axis of symmetry, it cannot aggregate to form a helix.

ENDOTHERMIC (THERMAL) AGGREGATION OF DEOXY HB S IN THE PROCESS OF SICKLING

Some aspects of the thermodynamics (or bioenergetics) of the sickling phenomenon to be presented will apply whichever way the molecule will stack . . . one on top of the other along the dyad (twofold axis of symmetry) or Y axis as proposed by this writer, or along the X axis of the molecule as proposed by Perutz and Lehman[70] and Finch, Perutz, Bertles, and Döbler.[25a] This second proposal for stacking of deoxy Hb S will be presented later.

The chemical and the three-dimensional structure of hemoglobin is given in detail in Chapter 1 of *Sickle Cell Hemoglobin: Molecule to Man,*[61] as well as in the first chapter of *Molecular Aspects of Sickle Cell Hemoglobin: Clinical Applications*[59] and therefore will not be presented here. A brief account will be presented here, however, in order to make this story on the molecular basis of the sickling process more complete.

It must be emphasized here that the original hypothesis of intramolecular cyclization from 1β Val to 6β Val via hydrophobic interaction has been abandoned, and intertetramic hydrophobic interaction has been proposed[56,57] because optical rotatory dispersion measurements with a modern instrument (Cary 60) indicate that there is no difference between Hb A ($\alpha_2 A \beta_2 A$) and Hb S ($\alpha_2 A \beta_2 6$ Glu $--> $ Val).[59,61] Again, it appears that there is consistent evidence suggesting that (predominantly) hydrophobic interactions are involved in the process of sickling, as will be presented here.

The hemoglobin molecule has a molecular weight of 65,458. It has a protein part called globin and a nonprotein part called heme; the heme group is technically known as the prosthetic group of the hemoglobin molecule. Normal adult human hemoglobin (Hb A) consists of a 4 polypeptide chains, 2 alpha chains (each containing 141 amino acids), and 2 beta chains (each containing 146 amino acids). It was discovered by Rhinesmith, Schroeder, and Pauling[79] that the normal adult hemoglobin (Hb A) molecule contains 4 polypeptide chains and that 2 of these chains are different from the other 2. Originally, these investigators decided to call these the A chain and the B chain, respectively; but the names were later changed to α and β by Schroeder, Rhinesmith, and Martin.[82]

The amino acid sequence studies of the human hemoglobin molecule were completed by Braunitzer[16] and Konigsberg[25c,43a] and their coworkers. Furthermore, Ingram[36] demonstrated an abnormal peptide in Hb S by a peptide mapping technique, and Hunt and Ingram[35] in 1959 provided evidence that in Hb S, glutamic acid is replaced by valine at the sixth position in each of the two β chains.

Scale Model Building

Shape of the Hemoglobin Molecule

The present reviewer constructed models of the human oxyhemoglobin molecule using Perutz'[73] x-ray diffraction data on the structure of horse oxyhemoglobin as previously described.[56,57,59,61] From the scale model it could be seen that the shape of the hemoglobin molecule is a truncated tetrahedron of a special sort. The lower half of the tetrahedron represents a pair of beta chains which are separated by about 7 Å. The "keys" are attached for close resemblance to the atomic model; very schematically, β6-Val is represented by "V" in Figure 1 and Figure 23. Three deoxy Hb S tetramers are stacked along the twofold axis of symmetry to represent a short segment of the monomolecular filament. Another proposal of aggregation along the X axis[25a,70] will be given later.

ELECTRON MICROSCOPY OF DEOXY HB S MONOFILAMENTS AND MICROTUBULES

Monomolecular Filament of Deoxy Hb S

It appears that the monomolecular filament of deoxy Hb S does form, and the 170 Å o.d. of the microtubule of deoxy Hb S[56,57] has been con-

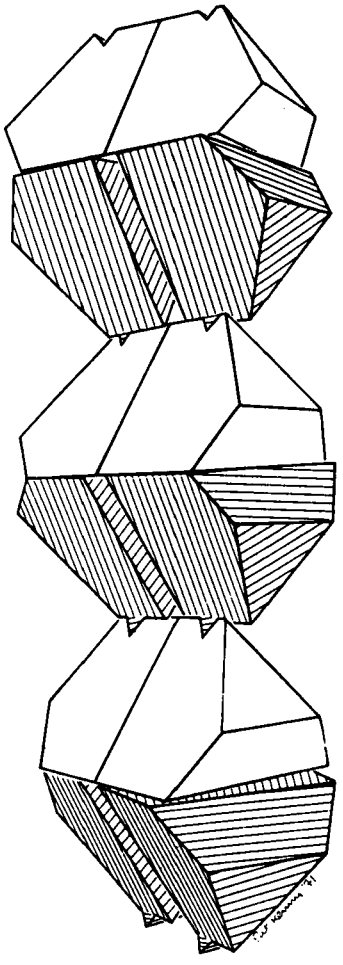

FIGURE 1. Schematic model of three deoxy Hb S tetramers illustrating molecular stacking along the Y axis (or the twofold axis of symmetry). (From Murayama, M., Molecular mechanism of human red cell (with Hb S) sickling, in *Molecular Aspects of Sickle Cell Hemoglobin: Clinical Applications,* Nalbandian, R. M., Ed., Charles C Thomas, Springfield, Ill., 1971, Chap. 1. With permission.)

firmed by Finch et al.[25a] and also by x-ray diffraction by Magdoff-Fairchild et al.[47]

In 1965, while Dr. B. Guy de Thé was visiting NIH, he demonstrated monomolecular filaments of deoxy Hb S using the Kleinschmidt technique, which consists of spreading a small amount of sickle cell hemoglobin on a Langmuir trough; then a small segment of the film is examined by the electron microscope. We did find a large number of fibers. These fibers have a diameter of only 50 Å and are extremely long.[59,61]

Microtubules of Deoxy Hb S

In view of the key position of molecular stacking in the proposed mechanism of human red cell sickling, an attempt has been made to demonstrate Hb S filament directly. Molecular threads of S hemoglobin are indeed found by electron microscopy in deoxygenated sickle cell hemolysates; however, their diameters are approximately 170 Å; whereas the monofilament has a diameter of 50 to 65 Å. A micrograph shows a central stripe of low opacity, suggesting that the Hb S fiber is hollow (see Figure 2). The outer diameter is 172 ± 11 Å; the inner diameter is 40 ± 6 Å. Steric and dimensional properties of the monofilament show that 6 strands of Hb S can be twisted together to form a hollow molecular cable of approximately the correct dimension.[56,57] This structure (Figure 3) is dimensionally similar to microtubules found in a wide variety of tissues. Furthermore, the postulated molecular cable provides a basis for the paracrystalline array of Hb S found by Stetson[87] and others.[10,23,25a,70,91,92]

Calibration of electron micrograph magnification by indanthrene olive TWP crystals with molecular planes facing of 24.9 Å was used (Figure 4) as a standard to measure O.D.'s and I.D.'s of deoxy Hb S microtubules. As stated above, O.D. = 172 ± 11 Å and I.D. = 40 ± 5.7 Å. Assuming that some of these are slightly out of focus, the dimensions might include the Fresnel fringes, thus giving rise to a higher value from the true one and, at the same time, decreasing the diameter of the hollow within. Dr. Labaw at NIH published a short note on the preparation of a 25 Å spacing crystal for magnification calibration above 18,000 x.[43c] In order to encourage everyone in this area to use a common reference, the procedure is appended at the end of this review.

S Hemoglobin Microtubules in Sickled Erythrocytes: Electron Microscopy of Ultrathin Section

Fibers whose O.D. of 170 Å have been found in ultrathin sections of sickled erythrocytes have been examined in conventional (transmission) electron microscopy.[10,23,37b,87,91]

Internal Structure of Sickled Erythrocytes as Revealed by the Freeze-Fracture Technique

Freeze-fractured sickled erythrocytes do show some microtubules as well as filaments (which are

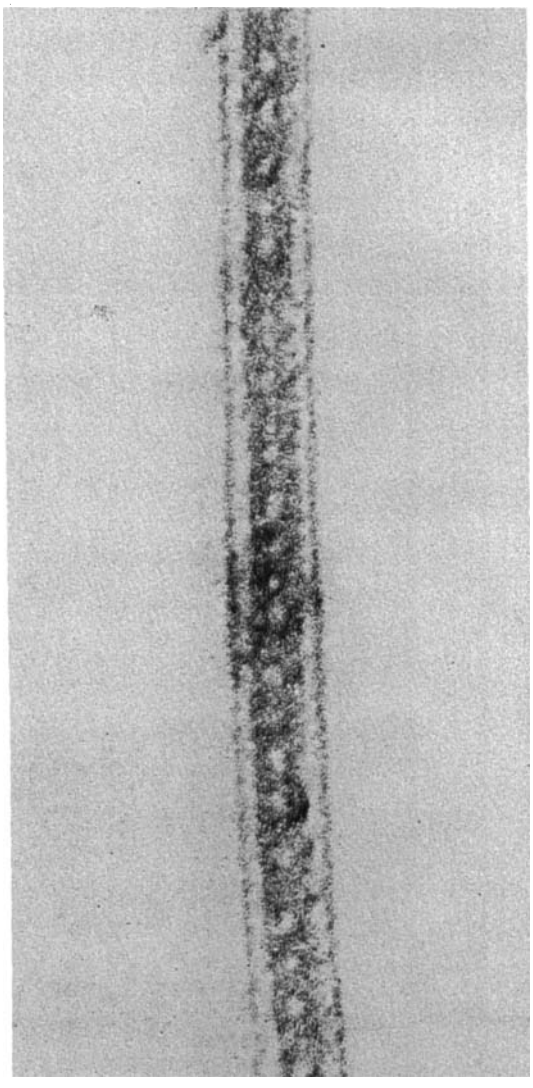

FIGURE 2. Electron micrographs of an Hb S microtubule (stained for positive contrast, interpreted as a hollow tubule). The microtubule has an O. D. of 170 A and I. D. of 40 A. Magnification is 12,500x (From Murayama, M., Molecular mechanism of red cell sickling, *Science*, 153, 145, 1966, Figure 5. Copyright 1966 by the American Association for the Advancement of Science.)

bundles of microtubules), which can be readily seen in Figure 5.

Scanning Electron Microscopy of the Cell Surface

Scanning electron microscopy has been used to study the cell surface of sickled erythrocytes by Farnsworth,[24b] Barnhart,[6] and others.[37b] Those of us who are interested in the internal structure of sickled erythrocytes must remove the membrane and look in to see what the structure of the fibers is like underneath the cell membrane. This has been done by "sandblasting" the membrane with ionized argon at 10^{-4} torr, accelerated at 4 keV as will be described next.

Scanning Electron Microscopy of the Ion Etched Sickled Erythrocyte

Lewis, Osborn, and Stuart[46] and Baker[5] used radio frequency pulses of inert gas ions to produce a sandblast effect on soft biological material prior to observation of surface structure in the scanning electron microscope. These investigators studied normal and sickled erythrocytes which had been fixed in glutaraldehyde. This problem was reinvestigated by the writer using controlled ion etching of human red cells (normal and sickled erythrocytes dried in nitrogen). Controlled ion etching was carried out with ionized argon at 10^{-4} torr accelerated at 4 keV. The sickled erythrocytes were magnetically oriented and then dried in a nitrogen atmosphere which gave additional control of the direction of etching. Results obtained on normal cells are essentially the same as those reported by others.[5,46] In sickled erythrocytes, after removing the cell membrane by etching, an array of parallel rods is seen. The tip of each rod has been partly etched away, but the diameter measured at the base is about 900 Å, suggesting that these are bundles of microtubules forming large fibers (see Figure 6). Possible arrangement of the microtubules to form this large fiber of about 900 Å is schematically shown in Figure 7.

Hydrophobic Interaction and Sickling

Tetramer-tetramer Interactions of Hb S

We turn now to the special peculiarities of Hb S, the beta-6-valyl residue, which is located at the bottom outside of the molecule to form the "key" on each of the beta chains. In this proposal, it is assumed that the key would then fit into a complementary site in the alpha chain of the adjacent Hb S tetramer. The complementary structure and stacking of the Hb S molecule make possible the head-to-tail stacking and aggregation of thousands of molecules into long chains.

The beta chains are separated by several angstrom units when deoxygenated; this is indicated in Figures 1 and 2. The tetramer-tetramer interaction (molecular stacking of 3 interacting tetramers) shown in Figures 1, 23, and 24 cannot occur upon oxygenation because the beta chains

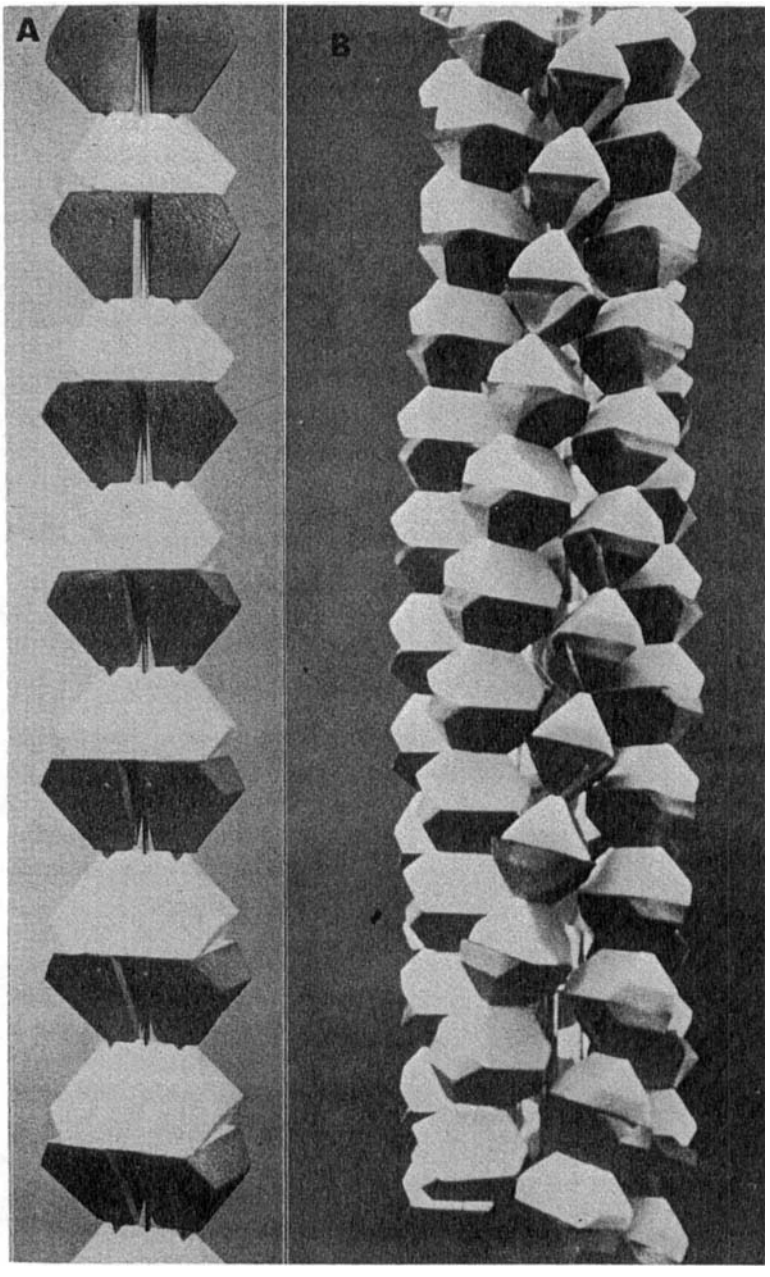

FIGURE 3. A. Monomolecular filament of deoxy Hb S formed by stacking "molecules" by the "lock-and-key" arrangement discussed in the text. Each "molecule" consists of a pair of α chains (light tone) and a pair of β chains (dark tone). Key is symbolized by a small "V" per beta chain. (From Murayama, M., Molecular mechanism of human red cell (with Hb S) sickling, in *Molecular Aspects of Sickle Cell Hemoglobin: Clinical Applications,* Nalbandian, R. M., Ed., Charles C Thomas, Springfield, Ill., 1971, Chap. 1. With permission.) B. Six strands of deoxy Hb S monofilaments twisted about a core to form a hollow cable. Each strand with 18 molecules stacked would be needed to make a complete turn according to this proposal. (From Murayama, M., Molecular mechanism of human red cell (with Hb S) sickling, in *Molecular Aspects of Sickle Cell Hemoglobin: Clinical Applications,* Nalbandian, R. M., Ed., Charles C Thomas, Springfield, Ill., 1971, Chap. 1. With Permission.)

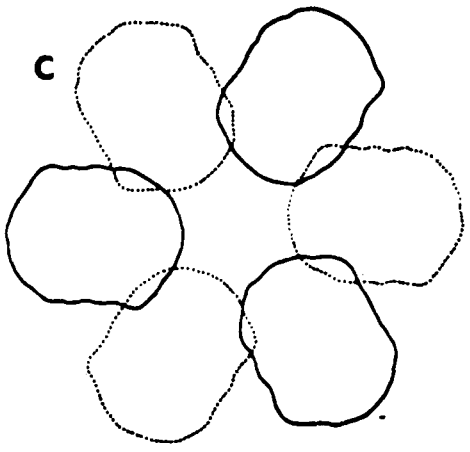

FIGURE 3c. Schematic drawing of cable cross section. (From Murayama, M., Molecular mechanism of human red cell (with Hb S) sickling, in *Molecular Aspects of Sickle Cell Hemoglobin: Clinical Applications*, Nalbandian, R. M., Ed., Charles C Thomas, Springfield, Ill., 1971, Chap. 1. With permission.)

move closer together by about 7 Å, the complementarity is lost, and the stacking crumbles. Oxygen appears to be the best unsickling agent.

Note that the cyclization from β-1 Val to β-6 Val by intramolecular hydrophobic interactions[52-54,56,57] has been abandoned[59,61] because the optical rotatory dispersion studies of purified fractions Hb A as well as Hb S give essentially identical results.[59] Thus, it appears that the intramolecular cyclization suggested earlier is not possible; so I have proposed intertetramic hydrophobic interactions for the aggregation of deoxy Hb S[59,61] as already stated.

Negative Temperature Coefficient of Gelation

Sherman[83] had reported that when susceptible cells were deprived of oxygen at low temperatures (4°C), sickling did not take place. The cells remained discoid, and no filaments were produced. In 1954, Allen and Wyman[1] reported their ob-

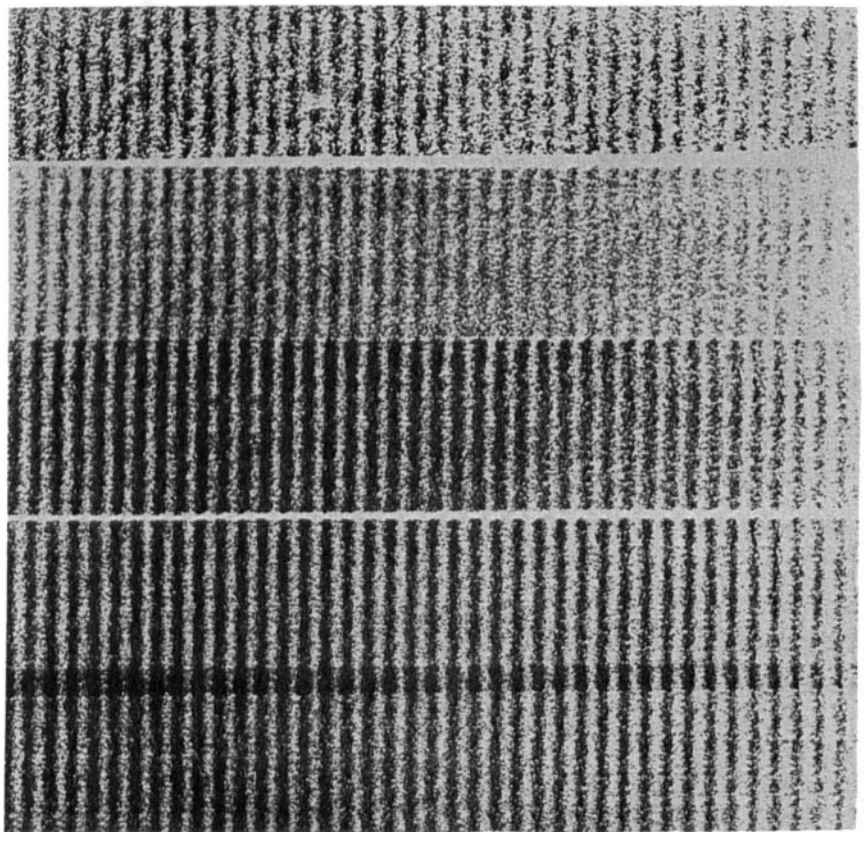

FIGURE 4. Transmission electron micrograph of 25A spacing of Indanthrene Olive TWP crystal for magnification calibration.

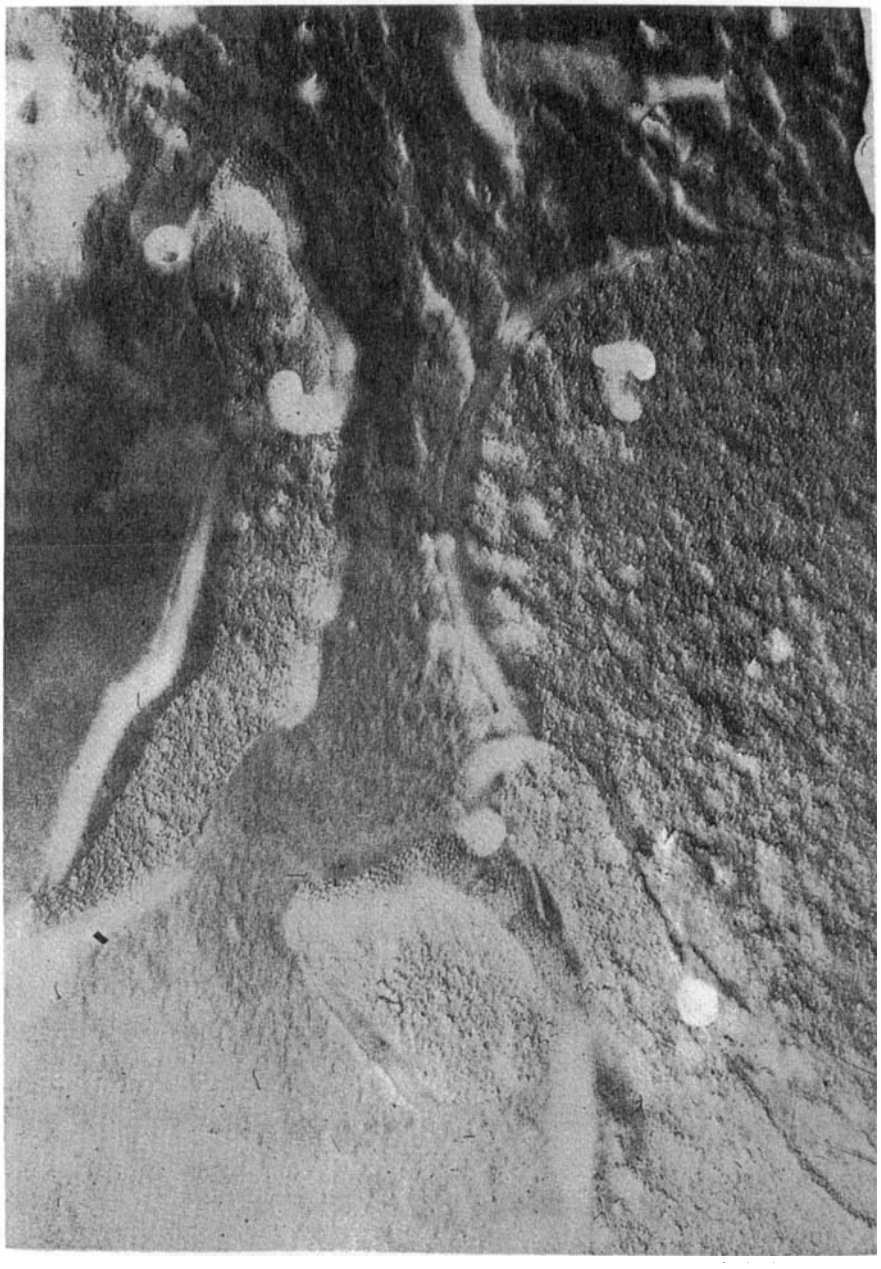

FIGURE 5. Transmission electron micrograph of inner structure of sickled erythrocyte by freeze-fracture (or freeze etching) technique showing bundles of filaments. Magnification 7,400x.

servation on the negative temperature coefficient of gelation (in French literature). Independently, Murayama[51] observed (while at Caltech as Linus Pauling's postdoctoral student) that deoxygenated S hemolysate has a negative temperature coefficient of gelation, i.e., the gel of deoxygenated Hb S liquefied reversibly when placed in ice water. Allison visited Caltech in 1954 at the very time when Murayama discovered the negative temperature coefficient of gelation of deoxy Hb S and extended this observation and reported[2] that approximately 30 g/100 ml concentration had a viscosity slightly but significantly greater than a corresponding solution of deoxy Hb A. Recently, Briehl[18] measured the negative temperature coefficient of gelation as the minimum gelling

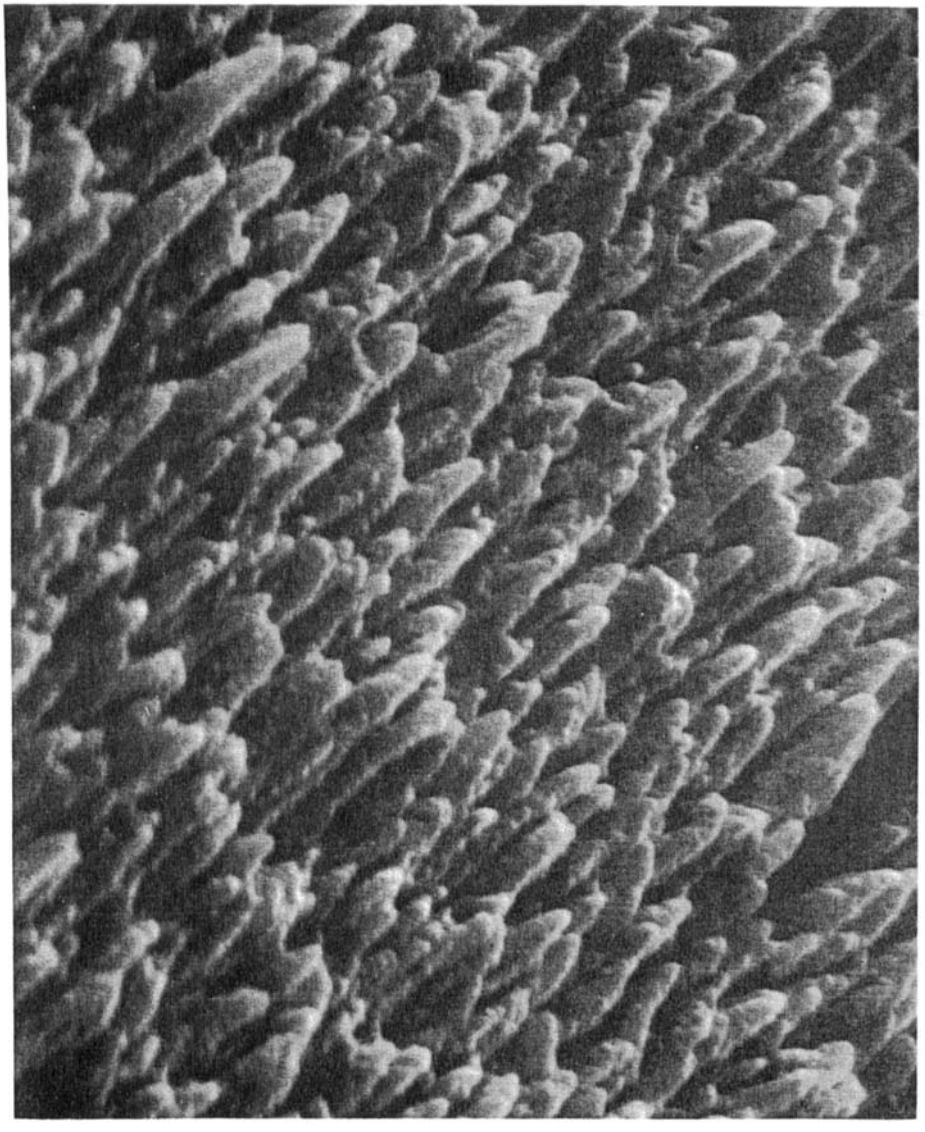

FIGURE 6. Scanning electron micrograph of the inner structure of sickled erythrocytes obtained by removing the outer membrane with controlled ion-etching (argon beam accelerated At 4 KEV for 4 min at 7 x 10^{-4} torr). The final magnification is 31,000x. (From Murayama, M., Molecular mechanism of human red cell (with Hb S) sickling, in *Molecular Aspects of Sickle Cell Hemoglobin: Clinical Applications,* Nalbandian, R. M., Ed., Charles C Thomas, Springfield, Ill., 1971, Chap. 1. With permission.)

concentration which falls about 2%/°C rise in temperature. This unique property of Hb S and Hb C-Harlem has been applied by Nalbandian[59,61] for the specific test for these hemoglobins (see Part B). In any case, deoxy Hb S (and Hb C-Harlem) will aggregate endothermically, which is the topic of the next section.

ENDOTHERMIC AGGREGATION OF DEOXY HB S

A quantitative study of the aggregation of deoxy Hb S was undertaken in relation to temperature. It was found that the endothermic aggrega-

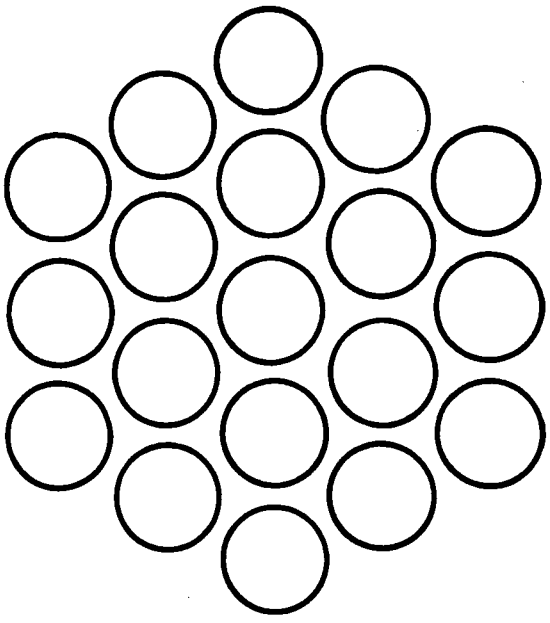

FIGURE 7. A theoretical close-packing arrangement of Hb S microtubules (each having O.D. of 170 A) that yields fibers whose O.D. is 850 A (5 x 170 A). This agrees well with 900 ± 50 A found for the fibers within sickled erythrocytes by scanning electron microscopy (after controlled ion etching. (See Figure 6.) (From Murayama, M., Bioenergetics of human red cell (with Hb S) sickling, in *Sickle Cell Hemoglobin: Molecule to Man,* Little, Brown and Co., Boston, Mass., 1973, Chap. 2. With permission.)

tion involves hydrophobic (or apolar) interactions between adjacent tetramers.

The enthalpy and the entropy changes during reversible thermal aggregation have been determined, as well as the minimum number of water molecules excluded from between the adjacent deoxy Hb S molecules in the process of polymerization.

The process of sickling is thought to take place due to tetramer-tetramer interactions via the hydrophobic residues of β-6 valyl with complementary binding pockets in the alpha-chains.[56, 59, 61] According to the theoretical work of Kliman,[42] nonpolar side chains of a macromolecule in aqueous solutions associate so as to reduce the surface of the nonpolar groups exposed to the water. This circumstance is produced by an entropy effect rather than by a favorable enthalpy. When a nonpolar molecule such as methane or ethane is transferred from a liquid nonpolar environment to water, there is a small release of heat and a large reduction in the entropy of the system. The entropy loss presumably arises from the ordering of water molecules around the nonpolar molecules in solution. One would predict that as the temperature is raised, the contribution of hydrophobic interactions to protein stability would increase up to the point where heat denaturation would overtake it.

Materials and Methods

Sickle cell hemoglobin was purified by column chromatography according to the method of Huisman and Dozy[34] with the following modifications: DEAE-Sephadex was replaced by QAE-Sephadex A-50 anion exchanger and tris-buffer by 0.05 *M* BIStris (bis-)2-Hydroxyethyl) imino-tris (hydroxymethyl)methane) buffer. The dilute solution of Hb S (the effluent) was concentrated in an ultrafiltration cell (AMICON) with a DIAFLO Type XM-100 membrane.

The object was to determine the rate of change of light scattering by the deoxygenated Hb S solution at different temperatures as indicated by a change in optical density (O.D.) or absorbance at the fixed wavelength of 305 nm. A standard silica cuvette sealed to a 50 ml round bottom silica flask (Figure 8) was used for spectrophotometry.[85] Two silica spacers were used, one (8 x 9 x 45 mm) providing a 1 mm light path length for the determination of degree of deoxygenation of Hb S by scanning the visible spectrum (700 to 400 nm), and the other (6 x 8 x 45 mm) providing a full 4 mm light path length for determining absorbance (O.D.) at 305 nm due to light scattering. Each spacer was attached to a 6 mm stainless steel rod 51 cm long by a short piece of rubber tubing for insertion into the cuvette. The rod passed through an ASCO® gland (AF Smith Co., Rochester, N.Y.), sealing the flask. The cuvette holder consisted of a copper block which could be thermostatted at any desired temperature. In the experimental procedure 0.1 ml of 16% Hb S solution and 3.9 ml of 0.2 *M* BIStris buffer at pH 6.7 were put into the cuvette, which was then cooled in an ice bath while the contents were deoxygenated by flowing water-saturated helium, or prepurified nitrogen, through the flask. The cuvette compartment of the recording spectrophotometer (Cary 14) was kept dry with a stream of dry nitrogen. When the solution was completely deoxygenated, as confirmed spectrophotometrically, the light path length was changed to 4.0 mm for the light scattering measurements at 305 nm. A typical record of absorbance change with time and

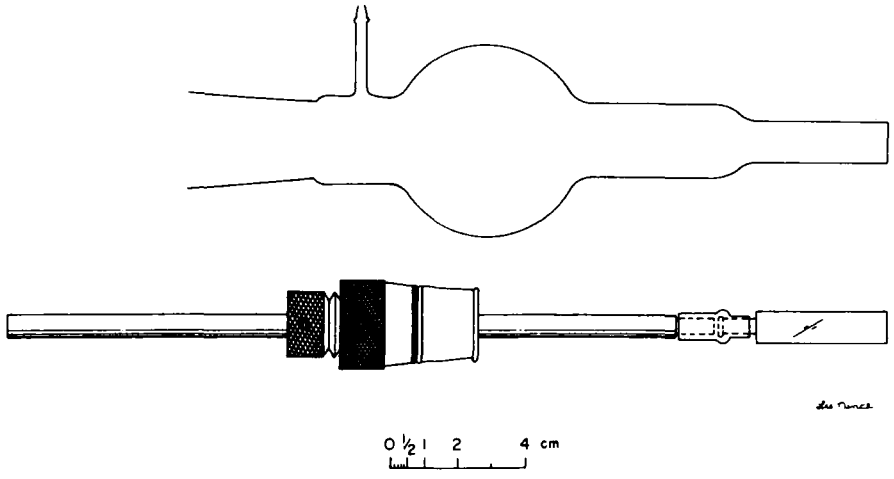

FIGURE 8. Equipment used in thermal aggregation studies of the deoxy Hb S molecules. (From Murayama, M. and Nalbandian, R. M., *Sickle Cell Hemoglobin: Molecule to Man*, Little, Brown and Co., Boston, Mass., 1973. With permission.)

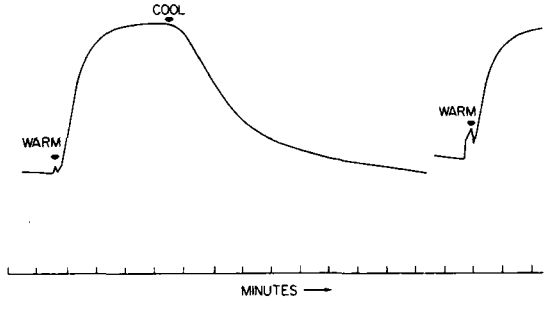

FIGURE 9. Warming and cooling curves for thermal aggregation of deoxy Hb S. "Warm" indicates the time when 38°C thermostat was turned on to affect a temperature change from 0° to 38°C. The wave length is 305 nm and the chart speed is 1/2 in/min. The line is drawn tangentially to the initial slope of the warm-up curve. The slope = Δ O.D./min = 0.39. (From Murayama, M. and Nalbandian, R., *Sickle Cell Hemoglobin: Molecule to Man*, Little, Brown and Co., Boston, Mass., 1973, 30. With permission.)

temperature is given in Figure 9, showing how k′, the rate constant (Δ O.D./min), was derived from the tangent to the initial slope.

Because turbidity (Δ O.D.) at first changes linearly with time (see Figure 9), it is assumed that the system is undergoing condensation polymerization according to the relationship $\tau = AN_0V_0^2(1 + k't)^{64}$ where τ is the turbidity, A is a scattering constant, N_0 the initial monomer concentration, V_0 the volume of the monomer, k′ the reaction rate constant, and *t* the time in minutes. This relationship appears to hold in the early phases of the aggregation where the particles formed are small compared to the 305 nm wave length used for the turbidity measurements.

For light scattering work at 305 nm, deoxygenate only with prepurified nitrogen, pure helium, argon, and the like, but not with dithionite ($Na_2S_2O_4$), ascorbic acid, or formamidine sulfinic acid. These reducing agents have a strong absorption band near 305 nm at 0°; as the temperature is increased, a red shift takes place and one obtains a change in absorbance at 305 nm "as if" aggregation is taking place. This artifact must be avoided.

Results

Optical densities (absorbances) at 13 equilibrium temperatures between 6 and 20°C are shown in Figure 10. The results show that the optical density is directly proportional to temperature, and that the thermal aggregation of deoxy Hb S is reversible.

The effect of increasing the concentration of KCl from 0.05 to 0.8 *M* on the rate of aggregation of deoxygenated Hb S at 38° is shown in Figure 11. This figure indicates that the rate of aggregation of deoxy Hb S increases with the ionic strength.

The enthalpy change = ΔH* = the energy of activation for the endothermal aggregation of deoxy Hb S was evaluated from the variation of the rate constant k′ with temperature. Plotting $-R \ln k'$ against 1/T, the slope of the straight line obtained is $\Delta H^* + RT$,[17] where R = the gas

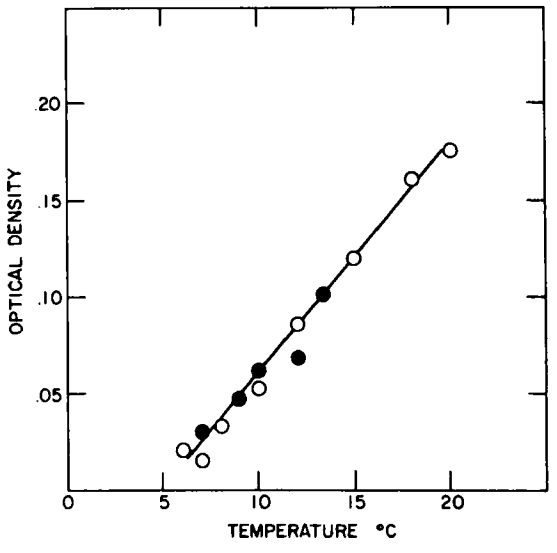

FIGURE 10. Reversible thermal aggregation of Hb S in 0.05 *M* BisTRIS buffer, pH 6.7. Open circles correspond to rising temperatures and closed circles to falling temperatures. (From Murayama, M. and Nalbandian, R. M., *Sickle Cell Hemoglobin: Molecule to Man*, Little, Brown and Co., Boston, Mass., 1973. With permission.)

constant, T = the absolute temperature, the $k' = \Delta$ O.D./min. A typical plot, leading to a value for ΔH^* of 17.3 ± 0.4 kcal/mole, is shown in Figure 12.

The change in entropy, ΔS^*, is defined as $\Delta H^*/T$, where ΔH^* for the thermal aggregation of deoxy Hb S = 17.3 kcal/mole and ΔS^* = 17,300 cal/mole/310° = 55 cal mole^{-1}/degree (entropy units). Assuming that the entropy change in the melting of ice (5.3 e.u.) would be essentially the same as for the freeing of water molecules from the hydration layer about the apolar (hydrophobic) group, the number of water molecules excluded from between the adjacent Hb S molecules would be 55/5.3 = 10.

Discussion

Although there are many macromolecules which undergo reversible thermal aggregation, only a few have been studied systematically. Lauffer[44,45] studied tobacco mosaic virus (TMV) A-protein which aggregates reversibly on warming. He found that the energy of activation (ΔH^*) is

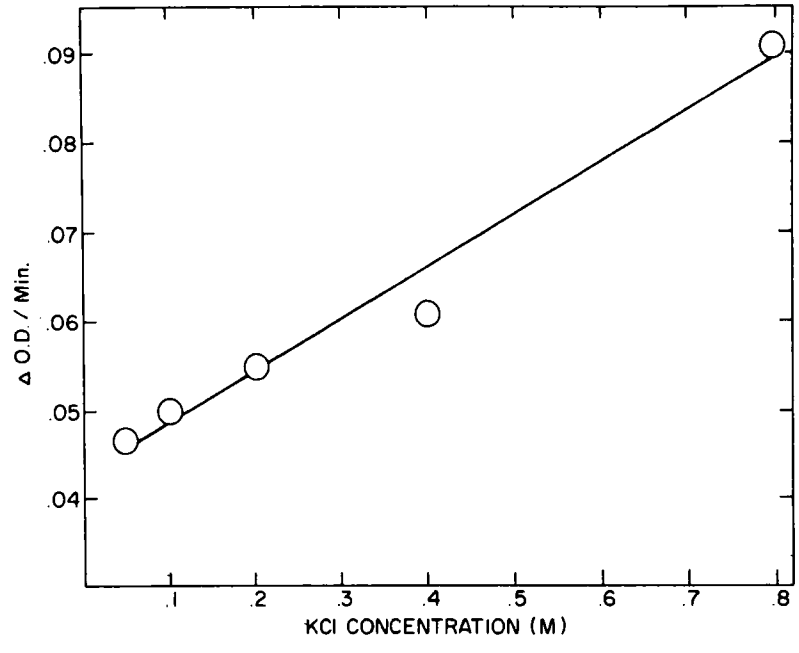

FIGURE 11. Effect of ionic strength on the rate of thermal aggregation of deoxy Hb S. As the concentration of KCl is increased from 0.05 *M* to 0.8 *M*, the rate of aggregation at 37°C increases. (From Murayama, M. and Nalbandian, R. M., *Sickle Cell Hemoglobin: Molecule to Man*, Little, Brown and Co., Boston, Mass., 1973. With permission.)

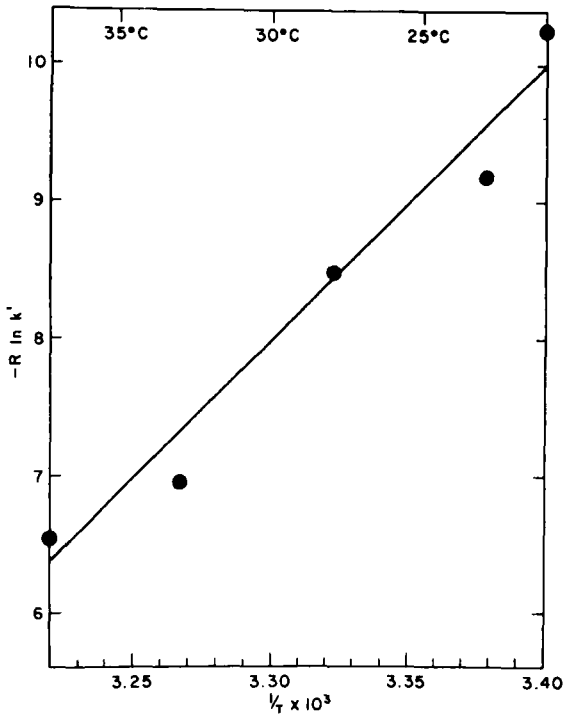

FIGURE 12. Enthalpy change for the thermal aggregation reaction of deoxy Hb S. $-R \ln k'$ is plotted against $1/T$; the slope of the straight line is $\Delta H^* + RT$. The slope of the curve was calculated by the method of least squares and is 17.4. Therefore, the enthalpy change is 16.8 kcal/mole. (From Murayama, M. and Nalbandian, R. M., *Sickle Cell Hemoglobin: Molecule to Man*, Little, Brown and Co., Boston, Mass., 1973. With permission.)

190 kcal/mole and the entropy change is 682 e.u. This increase in entropy suggests that 125 water molecules per monomer pair are excluded from between the adjacent TMV A-protein monomers during polymerization. The thermal aggregation reaction of TMV A-protein provides further evidence against association between polar residues (glutamyl and aspartyl) by hydrogen bonding. The fact that polymerization is favored by increased ionic strength, as shown by Smith and Lauffer,[44,45] can be interpreted as reflecting an important role played by electrostatic forces between reacting nomomers.

Poly-L-Valyl-Ribonuclease (PVRNase) undergoes thermal aggregation whereas the native RNase does not. The study of Kettman et al.[39] on the effect of salt concentration on the rate of change of absorbance (O.D.) of PVRNase suggests that thermal aggregation may be due primarily to attractive apolar (hydrophobic) interactions, since repulsive electrostatic forces would be diminished by higher ionic strengths. Kettman et al.[39] found that the rate of aggregation is diminished by applied hydrostatic pressure, which suggests that the transition state of the rate-limiting step exhibits a positive volume of activation (ΔV^*), as found also with Hb S.[58] The volume of activation for the thermal aggregation reaction of PVRNase can be determined by using the assumed relationship[29,89] between rate constants and pressure: $\frac{\partial \ln k'}{\partial P} = \frac{-\Delta V^*}{RT}$ where R = the molar gas constant, T = the absolute temperature, and k' = Δ O.D./min. The volume of activation for PVRNase is 259 cc/mole in 0.3 *M* NaCl but decreases to 203 cc/mole in 0.8 *M* NaCl. The large positive ΔV^* can be interpreted to mean that a large number of solvent molecules are released from the monomer's polyvalyl side chains in the rate determining transition state of aggregation. Our data on the high pressure chemistry of deoxygenated Hb S appear to be consistent with the findings of Kettman et al.:[39] ΔV^* for Hb S is 500 cc/mole in 0.2 *M* NaCl; when the ionic strength is increased to 0.8 *M* NaCl, ΔV^* is decreased to 300 cc/mole.[58] The number of water molecules apparently eliminated during tetramer-tetramer interactions is actually a minimum of about 10 molecules. Much more water must be freed because the ordering of the large hemoglobin molecules results in a large entropy loss.

Kauzmann[38] has pointed out that transfer of hydrocarbons from water solution into nonpolar solvents is accompanied by a volume increase, e.g., + 18.1 cc/mole for ethane into hexane. This rather large volume change has been interpreted to be due to the release of water molecules "bound" about the alkane molecules. The reaction occurs spontaneously at about room temperature in the direction of forming hydrophobic interactions, i.e., noncovalent alkyl-alkyl interactions with the exclusion of solvating water molecules.

Galactothermin is a protein of human milk which shows reversible heat-precipitation at neutral pH.[81] My (unpublished) study of this thermal (endothermic) polymerization reaction indicates that the heat of activation is $\Delta H^* = 70$ kcal/mole. The entropy change, therefore, is 220 e.u., indicating that the number of water molecules excluded from between the adjacent monomers is about 42. The high pressure chemistry of galactothermin indicates that the volume of activation, ΔV^*, is 70 cc/mole.[60]

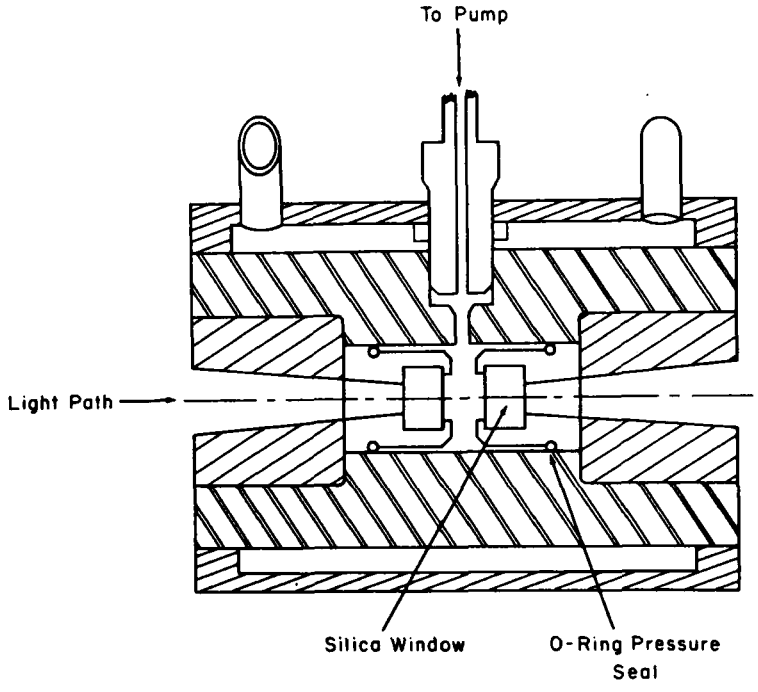

FIGURE 13. Schematic cross-section drawing of the high pressure cell used in the study of the high pressure chemistry of deoxy Hb S. (From Murayama, M. and Nalbandian, R. M., *Sickle Cell Hemoglobin: Molecule to Man*, Little, Brown and Co., Boston, Mass., 1973. With permission.)

In summary, the effect of ionic strength on the rate of thermal aggregation of deoxygenated Hb S is thought to be due to reduction in electrostatic repulsive forces between the Hb S monomers. Our findings concerning the thermal aggregation of deoxygenated Hb S and of galactothermin appear to be consistent with the study on the A-protein of TMV by Lauffer et al.[44,45] and also with the work of Kettman et al.[39] on poly-Valyl-RNase. Thermal aggregation of such proteins appears to take place only when water is set free from the subunits in the process of polymerization. Findings of this type emphasize the importance of the water of the molecular environment in biochemical reactions. Accordingly, it is suggested that the water structure, clathrate structures, and related problems ought to be vigorously investigated by neutron diffraction[89a] and related probes.

Heat produced by the washed erythrocytes was measured with the LKB flow microcalorimeter, and it was found that metabolic heat production in sickle cell anemia and in SC and CC diseases was in the descending order; this finding appears to be consistent with the notion of endothermic aggregation of Hb S molecules (Boyo and Ikomi-Kumm).[15]

Unsickling Under High Hydrostatic Pressure

Because Marsland[48] had shown that protoplasmic-gel structures are weakened under high hydrostatic pressure, it was suspected that sickled erythrocytes would unsickle under high hydrostatic pressure. As suspected, sickled erythrocytes do unsickle reversibly under pressure of about 50 atm; they become spherical under high pressure and then sickle again when decompressed slowly. This observation is also consistent with the slowing of the rate of aggregation of Hb S in solution under hydrostatic pressure.

The influence of high hydrostatic pressure on deoxy Hb S solutions was investigated in a jacketed high-pressure optical cell with silica windows (Figure 13). It was observed that "unsickling" is promoted by a rise in pressure up to 50 atm, but that still higher pressure again facilitated sickling; that is, there is an optimum pressure of unsickling. Sickled erythrocytes unsickle under hydrostatic pressure of 50 atm, suggesting that the

aggregated form of the Hb S molecule is more voluminous than the monomer. From measurements of turbidity of Hb S solutions in UV light, the rate of aggregation can be determined at different applied pressures. Calculations of the data indicate that the molecule appears more voluminous (400 ml/mole) up to 50 atm. It appears that at such a pressure, a phase change takes place (reversal of pressure effect), and the molecule is less voluminous (~500 cc/mole) for pressures higher than 50 to 60 atm. This finding is consistent with the slowing of the aggregation reaction of poly-L-valyl-ribonuclease by a factor of 10 as the hydrostatic pressure is increased to 300 atm. Neither the normal hemoglobin nor the native ribonuclease will aggregate at all, either with pressure changes or by thermal aggregation.

Haptene Blocking by Alkane Gases

If the proposed intermolecular hydrophobic interactions between deoxy Hb S tetramers are essentially correct, then saturating blood containing sickle cell hemoglobin with propane, methane, ethane, or isobutane might cause unsickling because the gas would undergo hydrophobic interactions with isopropyl groups of the valyl side chain residues, and thereby block valyl intermolecular interactions. These gases can be looked upon as the specific binding group, the haptene. This, then, is essentially a haptene-blocking experiment. The complementary binding site or the complementary binding pocket in each of the alpha chains would in principle be plugged up by molecules of these gases, one might say, preventing the beta-6-valyl from interacting. Accordingly, stacking would no longer be possible. Propane (and the other named gases) does in fact unsickle and block sickling of erythrocytes containing deoxy Hb S.[53]

Freedman et al.[25b] reported that the tris(hydroxymethyl) aminomethane and also sugars inhibit gelation of deoxy Hb S; this finding is consistent with the notion that hydrophobic as well as other "lilliputian" interactions are involved. "Lilliputian" interactions mean those weak forces which have profound manifestation because of the large number involved. Accordingly, the process of sickling involves predominantly hydrophobic interactions and to a lesser extent, electrostatic forces and hydrogen bonding. The latter tends to stabilize the hydrophobic interactions by a "scaffolding" effect. But above all, deoxy Hb S aggregation is not due to a favorable ΔH^*, but it appears to be entropy driven.

The minimum gelling concentration (MGC) of deoxy Hb S would decrease inversely with the ionic strength in some instances. Due to the specific ion effect, sodium iodide, for instance, has an opposite effect; in fact, NaI behaves "as if" it might be an excellent desickling agent: this is one of my unpublished data using the light scattering method described herein. A systematic study of alkali metal halides on the process of aggregation of deoxy Hb S is needed.

MOLECULAR ORIENTATION IN SICKLED ERYTHROCYTES

It now appears that most investigators in this area do agree that deoxy Hb S does form monofilaments and also forms microtubules from 6 strands of monomolecular filaments whose O.D. is 170 Å and I.D. is 50 Å. But there are differences of opinion as to how the molecules are stacked: along the dyad Y axis,[56,59,61] or along the X axis as proposed by Perutz and Lehmann,[70] Finch, Perutz, Bertles, and Döbler,[25a] and Hofrichter, Hendricker, and Eaton.[33] In the final analysis, the proposed model of the deoxy Hb S microtubule must account for its optical property and account for the fact that sickled erythrocytes are magnetically orientable perpendicular to the magnetic field and that the direction of strong light absorption is perpendicular to the needle axis. Concerted effort on the part of everyone concerned would be needed to solve the problem. It appears that in the area of paramagnetism of the heme, no one is able to determine the direction of the "M" vector with respect to the heme plate.

If the heme plate is perpendicular to the axis of the deoxy Hb S fiber, then the stacking must be along the X axis (or between the Z axis and the X axis). This proposal is due to Perutz and Mitchison[71] in 1950, who determined the dichroism of the sickled erythrocytes and reported that between crossed nicol prisms without compensation, the erythrocytes lit up in a deep blue color and became extinguished completely at every 90° rotation of the stage. Normal red cells show no birefringence at all. The direction of strong absorption is normal to the needle axis. Note that everyone agrees that the optical axis is perpendicular to the long axis of the sickled erythrocytes. Then in 1951, Perutz, Liquori, and

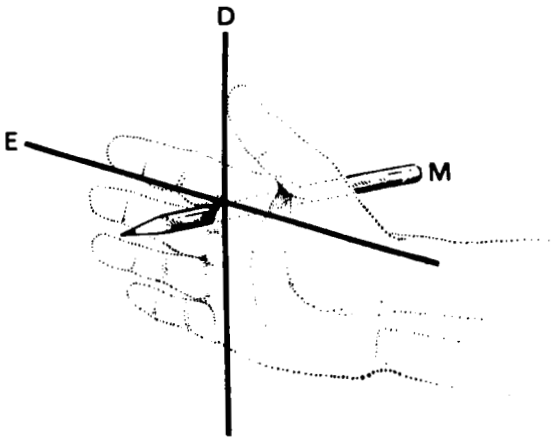

FIGURE 14. Schematically indicating the "E" vector perpendicular to the dyad axis of symmetry "D." Then the magnetic vector "M" would be perpendicular to both "E" and "D." If "D" is in fact parallel to the long axis of the sickled erythrocyte, it would be magnetically orientable, perpendicular to the magnetic field. (See Figure 16.)

Eirich[72] reported that the dichroism of the sickled cell was caused by the Hb S molecules lined in such a way that the plane of the heme group is normal to the long axis of the sickled erythrocyte. Pauling[69a] commented that there is conflict between these conclusions.[66,68]

Murayama, Olson, and Jennings[54] reinvestigated this problem and observed essentially the same phenomenon as reported by others,[4,71,72] but it was concluded that the heme plates are oriented parallel with respect to the long axis of the sickled erythrocytes. It was assumed that the E-vector is a linear oscillator; then we assumed that deoxy Hb S molecules stack along the twofold axis of symmetry (the dyad, indicated "D" in Figure 14). The electrical vector "E" is perpendicular to the dyad "D"; therefore, it was assumed that the "M," the magnetic vector, would be perpendicular to the heme plate (and also to both "D" and "E"). Accordingly, it was predicted and experimentally found that sickled erythrocytes are magnetically orientable, perpendicular to the magnetic field.[55] This prediction may have been fortuitous because the problem of paramagnetism of the heme group appears much too complex today. It appears that the problem of paramagnetism of the heme group remains essentially unsolved.

Since heme is a planar oscillator, not linear, then the heme plates must be oriented perpendicular to the long axis of the sickled erythrocyte. The orientation of the deoxy Hb S molecules in microtubules will be presented in detail later.

Orientation of Sickled Erythrocytes in a Magnetic Field

The dichroic ratio measurement data above suggested that the heme plates are parallel to the long axis of sickled erythrocyte. In addition to these observations, scale model building experiments clearly showed that the shape of the hemoglobin molecule is, in fact, a truncated tetrahedron of a special sort. It can easily be seen from the model that the heme plates are essentially parallel to the long axis of the sickled erythrocyte. This idea was also deduced from the dichroic ratio measurements. If the hypothesis on a molecular mechanism of sickled erythrocyte formation is correct, then it follows that the magnetic vector of each of the heme plates would be essentially perpendicular to the long axis of the sickled erythrocyte.

Since it was found by Pauling and Coryell[65] in 1936 that ferro-hemoglobin is paramagnetic, whereas oxyhemoglobin and carbon-monoxy-hemoglobin are diamagnetic, it occurred to me that sickled erythrocytes (which can only "sickle" when deoxygenated) should orient themselves perpendicular to the magnetic lines of force.

Conventional methods were used to observe microscopically the phenomenon of sickling. The stage of a compound microscope was removed in order that the wet preparations could be observed in the magnetic field (Figure 15). The magnet* used in the experiment has a field-strength of 3,500 G with 30 mm gap between the pole pieces. The diameter of the pole pieces is 40 mm. A sheet of brass was bent with an appropriate hole for the light to pass and was used to hold the wet preparation in between the pole pieces.

It has been observed experimentally that the sickled erythrocytes do in fact orient themselves, each with its long axis, perpendicular to the magnetic lines of force (Figure 16).[55] Oriented sickled erythrocytes can be readily randomized in a solenoid actuated by 60 Hz 110 VAC. It has also

*A large permanent magnet with adjustable pole pieces was made by General Electric Co., Schenectady, New York, Catalog No. 7766115, Serial No. 100086. It was calibrated by the manufacturer, who gave the flux density as 2205G at a gap of 38 mm.

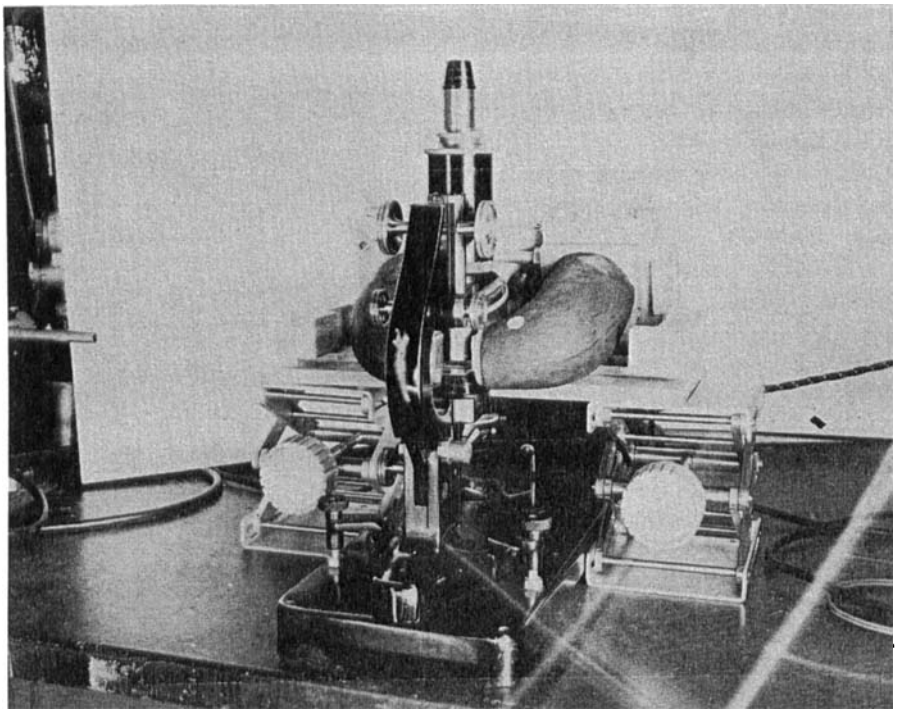

FIGURE 15. The Alnico magnet with a wet preparation in the "stage" so that the observation is possible while the sickled erythrocytes are in the magnetic field.

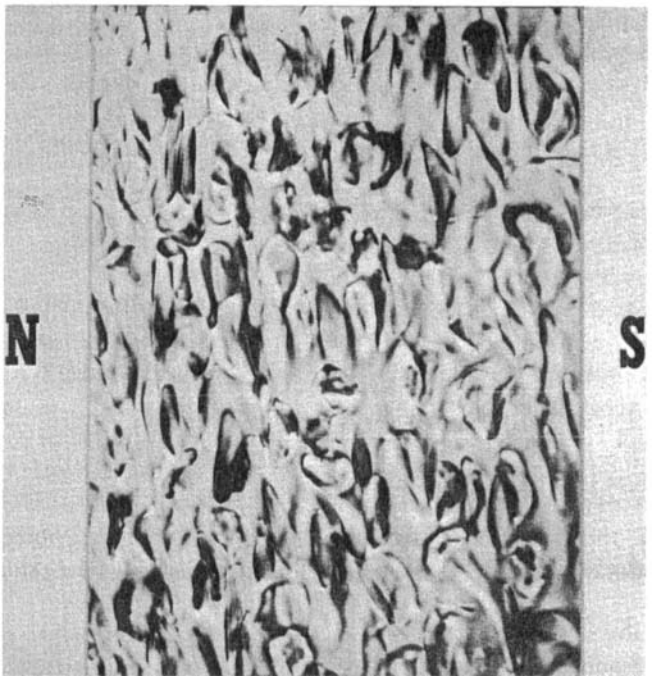

FIGURE 16. Intact sickled erythrocytes containing Hb S are orientable in a magnetic field. Note that the orientation of the cells is east to west, i.e., the long axis of the distorted cells is perpendicular to the north to south magnetic lines of force.

been observed that deoxy Hb S molecules are magnetically steerable (see Figure 17).

Deoxygenated erythrocytes containing the normal human hemoglobin do not show any apparent orientation in the magnetic field.[55]

In any event, the sickling process appears to involve linear stacking of the hemoglobin molecules with the plane of the heme plates parallel to the twofold axis of symmetry of the molecule and also parallel to the long axis of the sickled erythrocytes. The magnetic vectors could only be perpendicular to the heme plate, thus perpendicular to the long axis of the sickled erythrocytes, because sickled erythrocytes orient themselves perpendicular to the magnetic lines of force.*

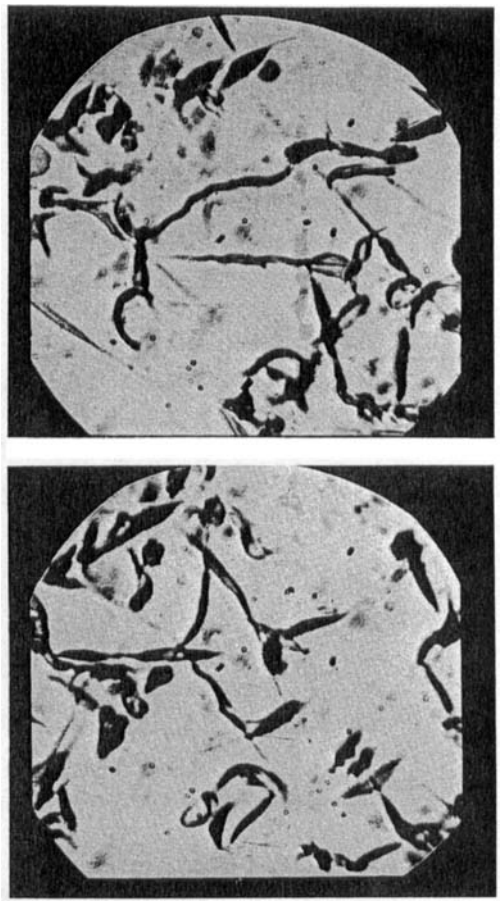

FIGURE 17. Deoxy Hb S molecules appear to be magnetically steerable; a test-tube containing blood from a patient known to be homozygous for Hb S was placed overnight in 51.7 k gauss superconducting magnet (Varian HR2-20 NMR apparatus). Oxygen was metabolized by the cells and the resulting sickled erythrocytes are almost spirochete-like in morphology.

Cofactor for Deoxy Hb S Polymerization

With Hasegawa, we found that a highly purified solution of deoxy Hb S does not aggregate at all,[59] but when the residue from the diffusate is put back, Hb S aggregates as expected. We suspected that a nonprotein, low molecular weight, dializable substance was responsible for the aggregation because sickle cell hemoglobin, purified by gel filtration, also does not aggregate. Work is still in progress to isolate, characterize, and determine the biochemical origin and other properties of this dialyzable cofactor. Johnson et al.[37c] have a notion that prostaglandin-E_2 might be the cofactor.

Desickling by Urea

Oligodynamic Action of Urea in Desickling Process

The molecular mechanism of red cell sickling appears to involve hydrophobic "bonds." Tetramer aggregation is essentially due to hydrophobic interactions between beta-6-valyl groups and corresponding complementary binding sites on adjacent tetramers. Accordingly, the theory implies that reversal of sickling is possible by breaking this specific hydrophobic interaction, an insight which Nalbandian[63] recognized. He successfully deduced the use of urea for this purpose from molecular information[38] (see Part 2 on therapy).

It is assumed in this discussion that an ideal hydrophobic group would be surrounded by a clathrate of water. A clathrate[27] is a molecular cage structure (Figure 18) which surrounds the "guest" molecule (xenon, methane, etc.). In order to disrupt hydrophobic interactions, it is quite clear that urea[38] is one of the best reagents to alter the clathrate of the precisely arranged water structure (see also section on therapy) and, hence, to disrupt the specified hydrophobic "bonds." Since this hydrophobic interaction results from only a single substitution at the beta-6-(valyl for glu), very little urea ought to be required to break the bonds (2 residues per 574 in each tetramer). Of course normal hemoglobin, Hb A, does not aggregate at all. Contrary to the general belief that

*This could be a fortuitous deduction because nematic crystals do orient in a magnetic field due to diamagnetism. Diamagnetism of nematic liquid crystal of deoxy Hb S is not known.

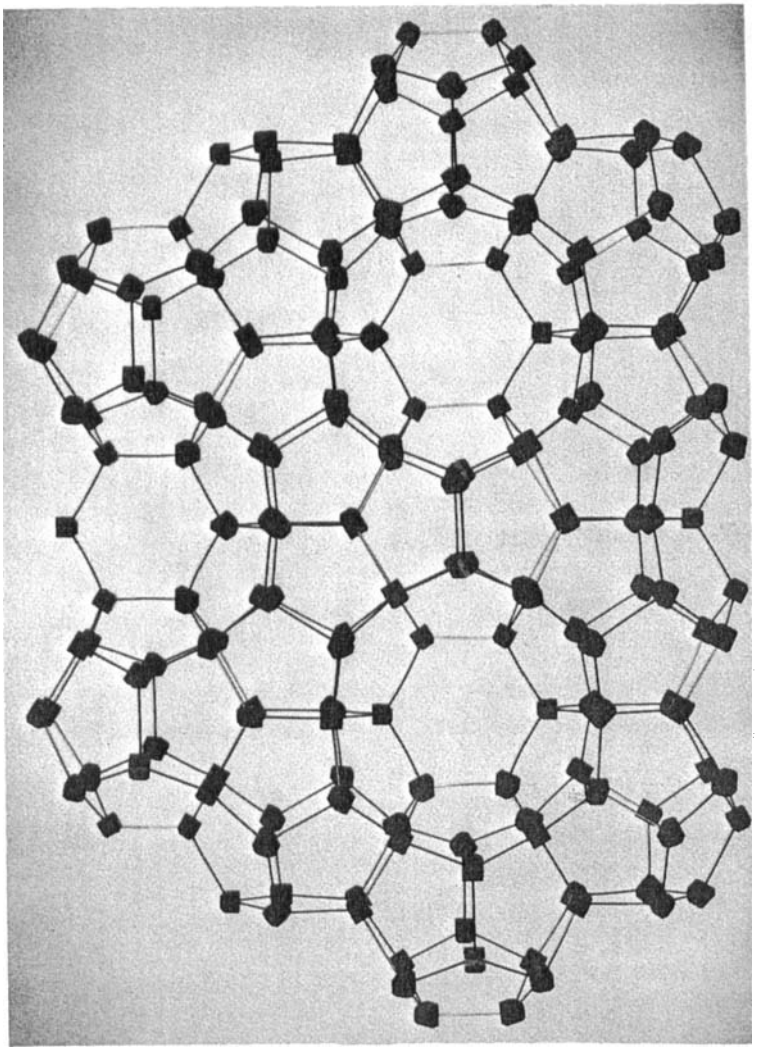

FIGURE 18. The structure of type I hydrate. The tetrakaidecahedral cavities (12 pentagonal and 2 hexagonal faces) are formed when layers of dodecahedra arranged as shown here are stacked on top of each other in such a manner that the hexagonal openings line up perpendicular to the plane of the page. Certain amino acid residues locate in these hexagonal cavities to form hydrophobic interactions or "bonds." (From Murayama, M. and Nalbandian, R. M., *Sickle Cell Hemoglobin: Molecule to Man*, Little, Brown and Co., Boston, Mass., 1973. With permission.)

6 to 8 molar urea would be needed to denature proteins, only about 10 m*M* of urea is required[59] to desickle sickled erythrocytes (Figures 19 and 20).

It appears foolish of urea "critics" to discount the action of 10 m*M* urea in desickling, on the grounds that 6 to 8 *M* urea is needed to *denature* protein. Denaturation and desickling are orders of magnitude apart; therefore, the urea concentration should also be orders of magnitude apart. Denaturation involves breaking perhaps 50 hydrophobic and several hundred H-bonds, whereas desickling involves only 2 hydrophobic bonds per molecule of deoxy Hb S.

In 1967, Refoyo[78] was the first investigator to show that urea in low (millimolar) concentrations could disperse macromolecular polymer systems in aqueous solutions. Three years later, Nalbandian[61] showed that urea had a similar effect at comparable low concentrations on another macromolecular, polymerized system, sickled hemoglobins S. In 1972, Ratner and Miller[77] made

FIGURE 19. Human red cells containing Hb S sickled by N_2 in 2.5% glucose/water. Magnification 1,400x. (From Murayama, M. and Nalbandian, R. M., *Sickle Cell Hemoglobin: Molecule to Man,* Little, Brown and Co., Boston, Mass., 1973. With permission.)

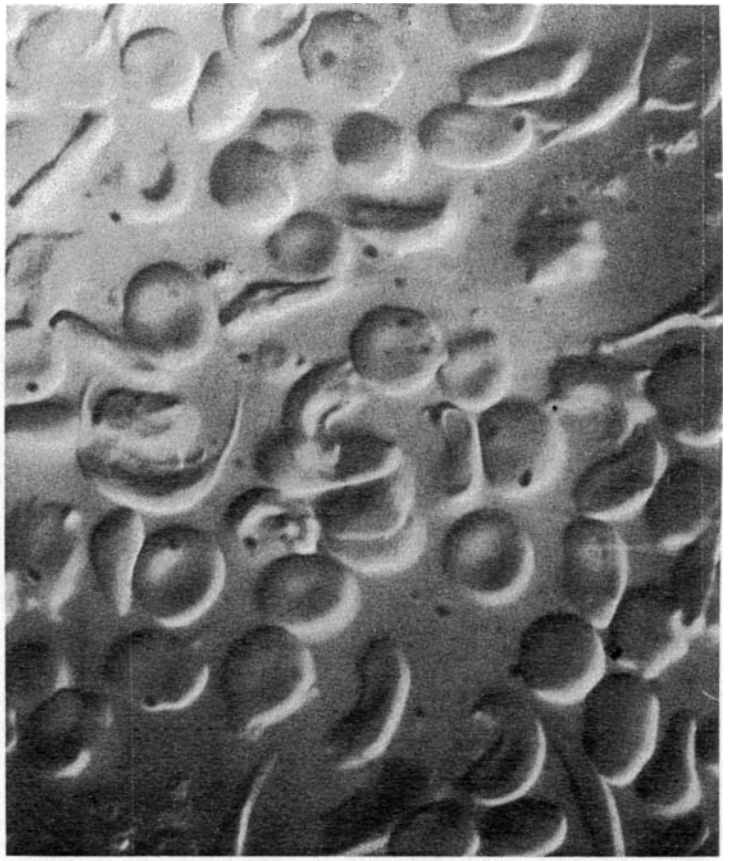

FIGURE 20. Same system as shown in Figure 19 to which has been added urea (equivalent to a blood urea nitrogen level of 150 mg per 100 ml of blood). Note the restoration to essentially normal erythrocyte morphology. Magnification 1,400x. (From Murayama, M. and Nalbandian, R. M., *Sickle Cell Hemoglobin: Molecule to Man,* Little, Brown and Co., Boston, Mass., 1973. With permission.)

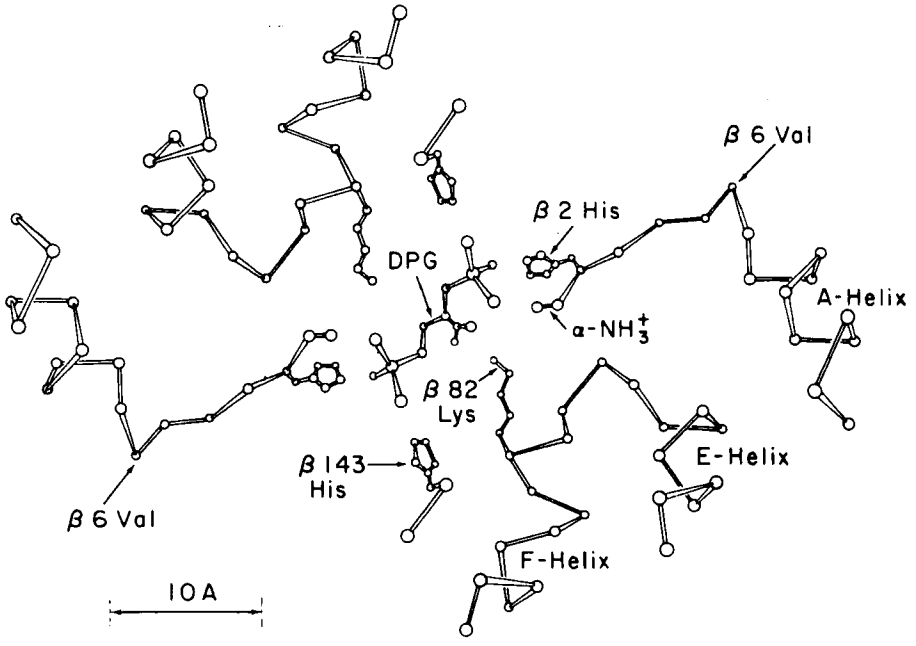

FIGURE 21. The binding of 2,3-Diphosphoglycerate to human deoxy Hb S is shown in the sketch. The stereochemistry of DPG complements the basic residues of the central cavity to form salt bridges with Valines 1 and Histidines 2 and 143 of both β-chains, and with Lysine 82 of one β-chain. This binding pulls the A-helix and the hemoglobin S site of mutation (6-β Val) towards the E-helix and the EF corner (β-79 Asp). Redrawn from Arnone, A., X-ray Diffraction Study of Binding of 2,3-Diphosphoglycerate to Human Deoxyhaemoglobin, *Nature*, 237, 146, 1972.

observations similar to those of Refoyo but differed on the molecular mechanism of urea. I have chosen to refer to this heretofore unappreciated effect of the low concentrations of urea as an "oligodynamic action." Much sophisticated, fundamental work must be done especially on the molecular structure of water before the molecular mechanism of urea in this regard is well understood.

This specificity of action of urea on sickled Hb S has been examined by the removal of urea of its specific enzyme, urease.[61,62b] Urease completely removes the oligodynamic action of urea and urea-treated cells will sickle again (See Figure 21). A possible mode of action of urea, therefore, is by the formation of additional products (adducts).

BINDING OF 2,3-DIPHOSPHOGLYCERATE BY DEOXY Hb S

It is generally known now that hemoglobin and 2,3-diphosphoglycerate (DPG) are the principal organic constituents inside the human erythrocyte. Both molecules have an in vivo concentration of 5 m*M*, but the function of DPG was unknown until the discovery[7,21] that it lowers the oxygen affinity of hemoglobin and thus facilitates the unloading of oxygen. Subsequent investigation has shown that DPG regulates oxygen transport by binding preferentially to deoxyhemoglobin.

Thus, it appears that DPG is stereochemically complementary to a constellation of 6 positively charged groups in the β chains which face the central cavity of the hemoglobin molecule. DPG has 3 negatively charged groups with an average pK_a of 2.8 and 2 with an average pK_a of 7.1, whereas the 3 pairs of positively charged protein groups facing them have pK_a of 10.5, 6.8, and 7.6, respectively.[8] In this way, the charges on the DPG could be compensated by those of the protein. This could also explain why DPG does not alter the Bohr effect. On dissociation, the number of proteins bound by DPG would roughly equal the number lost by the 2 α-amino groups and 2 histidines. (For fully stretched chains, the distances between the terminal phosphates are 6.5 Å in 2,3-DPG, 5.5 Å in ATP, and 2.8 Å in ADP.)

Upon oxygenation, the distance between the α-amino groups increases from 16 Å to 20 Å, so that they can no longer make contact with the phosphates of the DPG, and the helices close up so that DPG is expelled from the central cavity. In oxyhemoglobin, this particular site, therefore, is closed; but at this stage we cannot be sure that oxyhemoglobin does not contain an alternative site, with a weaker binding constant, to which DPG can become attached.

It is beyond the scope of this article to review all the fascinating aspects connected with the discovery and implications of 2,3-DPG in the physiology of hemoglobin; however, the pertinent role it plays in the process of sickling will be presented below.

Arnone's x-ray study[3] shows that one molecule of DPG binds to one molecule of deoxyhemoglobin tetramer, and that it takes up a stereochemically complementary position on the two-fold axis of symmetry (the dyad axis of symmetry), plugging the entrance to the central cavity. Its anionic groups form salt bridges with seven cationic groups of the 2-chains which include the valines 1, histidines 2 and 143, and one of the lysine 82 (Figure 21). This complementary stereochemistry is specific for the quaternary deoxy structure and is lost on transition to the oxygenated form. Thus, DPG stabilizes the former at the expense of the latter, thereby shifting the allosteric equilibrium towards the form with the lower oxygen affinity. This finding has implications for the mechanism of human red cell sickling, lowering the solubility, etc.

Kilmartin and Rossi-Bernardi[40] showed that the reaction with cyanate blocks the interaction between DPG and the α-amino groups of valine 1β by forming $-NHCONH_2$ groups in their place. Hence, the inhibitory effect which cyanate has on the sickling process[20] is likely to be due to a reduction of the affinity of deoxyhemoglobin S for DPG, and a consequent shifting of the allosteric equilibrium towards the oxy-form (as suggested by Perutz).[75]

Beutler, Paniker, and West[12] reported that the interaction between 2,3-DPG and deoxy Hb S does not produce configuration changes which influence the aggregation leading to sickling. This work needs to be repeated.

Briehl[18] studied aggregation of deoxy Hb S by equilibrium ultracentrifugation and found that the 6 m*M* 2,3-DPG lowers the minimum gelling concentration as compared to DPG-free solution of deoxy Hb S. In all experiments where aggregation of deoxy Hb S is to be investigated, it appears that equimolar amounts of 2,3-DPG ought to be added so that all results would be comparable.

In our laboratory it was found that 2,3-DPG is not the cofactor we are looking for by the method of light scattering.

SOLUBILITY OF DEOXY Hb S

Solubility and gelation of deoxy Hb S appear to be interrelated. In order for deoxy Hb S solution to gel, it must have some of the conditions similar to those needed for low solubility. Solubility of deoxy Hb S has been studied always in a phosphate buffer system. Essentially the same study but in other nonphosphate buffer systems should yield additional useful information, such as the effect of different anions and cations.

Perutz and Mitchison[71] in 1950 reported that there is a marked solubility difference between the deoxy Hb A and Hb S. The solubilities were measured at 20° in phosphate buffer at pH 6.8. They found that the solubility of deoxy Hb A is about half that of the oxy and met Hb A. But the solubility of deoxy Hb S is less than 1/100 of that of oxy Hb S. From their findings[71] they concluded that the deoxy Hb S in sickled erythrocytes is in a crystalline state. Extremely low solubility of deoxy Hb S suggested to them a simple mechanism for the reversible changes in shape undergone by the cells. It appeared to them as though on deoxygenation, Hb S crystallizes inside the red cell, and that on oxygenation, it goes back into solution. The change in shape would be forced upon the cells partly by the habit of the crystals, and partly by the loss of water which would be expected to accompany crystallization (while red cells contain 34% hemoglobin, crystals contain between 50 and 60% of the pigment).[71]

Itano[37a] in 1953 provided a simple way of determining roughly the amount of Hb S present in the mixture of hemoglobin. Note that he used 2.34 *M* phosphate buffer. White and Beaven[90] in 1954 reported solubilities of deoxyhemoglobins in pH 6.7 phosphate buffer at high ionic strength. Solubility curves of deoxy Hb A/A, Hb S/A, and Hb S/S are reproduced with permission (Figure 22). It can be seen that the solubility in general decreases with the increase of ionic strength. Again note that his study is also in phosphate buffer.

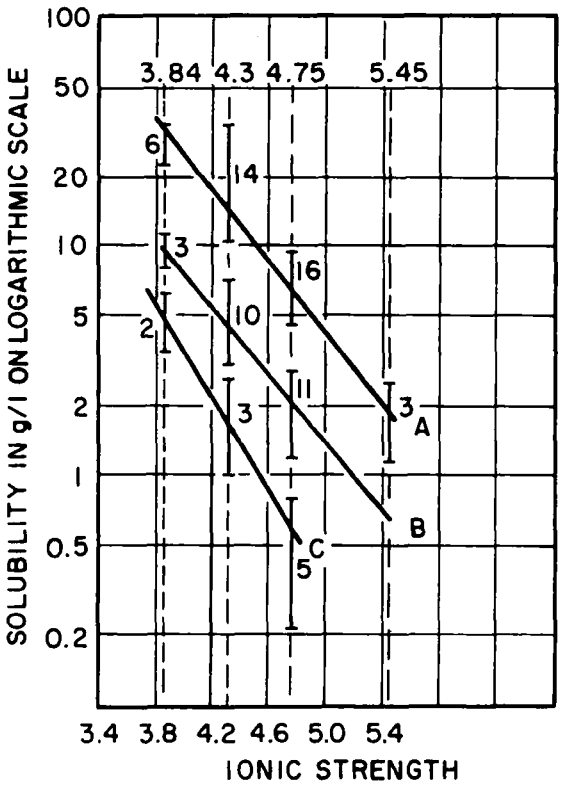

FIGURE 22. Solubility of deoxy Hb S in pH 6.7 phosphate buffer. The regression lines are fitted to experimental values for hemoglobin derived from normal adult blood, Hb A/A (A), from Hb S/A, heterozygous for S (B) and Hb S/S, homozygous for S (C). The range of values for each group at different ionic strengths is shown by the vertical bars. The figures indicate the number of observations in each group. (From White, J. C. and Beaven, G. H., A review of the varieties of human haemoglobin in health and disease, *J. Clin. Path.*, 7, 175, 1954. With permission.)

Solubility of deoxy Hb S without phosphate would be informative; furthermore, the influence of 2,3-DPG on solubility of Hb S could be investigated.

In a phosphate buffer of sufficiently high ionic strength, oxygenated S hemoglobin as well as Hb A will aggregate; White[92] found that they, in fact, form fibers or microtubules essentially similar to those seen in deoxygenated Hb S solution. The conditions used by White[92] could be hardly physiological, but it is extremely interesting to note that many globular proteins have great propensity to form microtubules.

Gelling of Deoxy Hb S

Singer and Singer[84] studied the gelling phenomenon of Hb S and found that the presence of Hb A decreases the minimal amount of Hb S required for gelation; Hb C even reduced this further. Hb F seems to exert no significant influence on the MGC (see also Bertles et al).[9b] Murayama[51] found that the deoxy Hb S has a negative temperature coefficient of gelation; this was found independently of Allen and Wyman[1] who published their observation in a French journal which I did not read until my discovery.

The fact that Hb A participates in the gelling process has been confirmed by a direct analysis of the solid phase obtained upon gelation of hemoglobin mixtures (Bertles, Rabinowitz, Döbler).[9b] The gelling properties of binary mixtures of hemoglobins have been studied by Bookchin et al.[13] and Bookchin and Nagel,[14] who measured the minimum total concentration of hemoglobin at the gelling point (MGC) as a function of the relative proportion of the two hemoglobin species.

Minton[49] analyzed the data of Bookchin et al.[13] and those of Bookchin and Nagel[14] and therefrom he proposed the following: the process of "gelation" in concentrated solutions of Hb S and Hb C_H (β-6 Glu → Val, β-73 Asp → Asn) and their mixtures with Hb A is considered as a 2-step process: step 1 is the linear association of monomeric protomer to form a rod-like oligomer, and step 2 is the side-by-side association of oligomer to form nematic phase of "gel." As a first-order approximation, it is assumed that both steps may be treated as reversible equilibria. On this basis, a qualitative description of the composition dependent behavior of a generalized binary system in the presence of each of the postulated equilibria is developed.

Minton[49] proposes that an amino acid substitution at one of the positions, β-6 or β-73, primarily affects one of the two association steps. This proposal leads to two limiting models: in model I the β-6 position influences step 1 association and β-73 position influences step 2 association, whereas in model II the roles of the two positions are reversed. Minton states that additional experimental information will be required to determine which (if either) of the limiting models provides the more appropriate description.

Studies of Deoxy Hb S Aggregation By Ultracentrifugation

Williams[93] as well as Briehl[18] investigated the

aggregation process of deoxy Hb S by equilibrium ultracentrifugation. Williams[93] used a purified Hb S solution for his study, while Briehl[18] used sickle cell hemolysate.

Briehl reported that by high speed analytical ultracentrifugation of fully deoxygenated Hb S solution at high concentrations (initial concentration about 7 m*M* in heme) at equilibrium, three zones can be observed: 1) beginning at the meniscus, where concentration was near zero, a concentration increase with radial position consistent with the known molecular weight of hemoglobin in lower concentration regions and indicative of non-ideality effects of higher concentration portions, 2) a narrow transition zone in which the concentration gradient increases very sharply indicating a large degree of aggregation, and 3) an opaque region consisting of gelled deoxy Hb S. Carbonmonoxy Hb S, and deoxy Hb A and carbonmonoxy Hb A showed only one zone.

In zone 1, deoxy Hb S showed higher weight average apparent molecular weight than deoxy Hb A, indicative of some aggregation at concentrations under that required for gelation. The concentration of Hb S at the phase change (the bottom of zone 2) was taken as the minimal gelling concentration. MGC falls about 2%/°C rise in temperature (the negative temperature coefficient of gelation). Lowering the pH toward 6.5 lowers MGC; 6 m*M* 2,3-DPG also lowers MGC as compared to DPG-free solutions. Thus DPG and increasing acidity in the physiological pH range favor gelation of deoxy Hb S as judged by equilibrium ultracentrifugation.[18]

Williams' study[93] is essentially the same as Briehl's[18] but he used a purified solution of Hb S. He measured weight-average molecular weights of Hb S at high concentrations by equilibrium ultracentrifugation. Carbonmonoxy Hb S appears to exist as a solution of unassociated molecules, as does carbonmonoxy and deoxy Hb A. Deoxy Hb S, however, was seen to exist in a gel-like state at concentrations above 14 g/dl, but no aggregates smaller than the gel were observed in solutions which are in equilibrium with the gel. Carbamylation of Hb S produced a solution of unaggregated molecules, as did cooling of uncarbamylated Hb S to 5°C. It is concluded that the gel of Hb S is formed in a stoichiometrically concerted manner, and that the size of the smallest stable aggregate is greater than 20 hemoglobin molecules.[93]

Since high hydrostatic pressure does have an effect on the process of aggregation of deoxy Hb S,[58] it would be important to find out if indeed the high hydrostatic pressure tends to slow down the rate of aggregation. By a static method, hydrostatic pressure tends to decrease the rate of aggregation of deoxy Hb S to about 50 atm. At around 50 atm, a phase change appears to take place; pressure above 50 atm increases the rate of aggregation. There is an optimum pressure for desickling; beyond this point, ΔV^* changes its algebraic sign and then would facilitate the process of aggregation.[58]

It should be mentioned that a purified solution of Hb S is essential for nearly all studies; accordingly, a very rapid method of purification of the pigment would be most helpful. Then finally, ways and means of stopping the process of "aging" (oxidizing of the Fe^{2+} to Fe^{3+}) would be most helpful.

Summary

Precision scale models of sickle cell hemoglobin molecules indicate that the genetic substitution of valine for glutamic acid at the sixth position in the beta chains allows an intermolecular hydrophobic "bond" to form. The negative temperature coefficient of aggregation and the unsickling both by propane and by high hydrostatic pressures are consistent with the presence of such a bond. Hydrophobic "bonding" permits tetramer-tetramer interactions in such a way as to allow head-to-tail molecular stacking by a "lock-and-key" arrangement. Molecular orientation along the long axis of sickled erythrocytes is deduced from dichroic ratio measurements in polarized light, which also show that the plane of the heme groups is parallel to the long axis of the cell. Consequently, it was predicted and experimentally confirmed that sickled erythrocytes are orientable in a magnetic field perpendicular to the magnetic lines of force. It follows that the plane of the heme plates is parallel to the twofold axis of molecular symmetry. Filaments interpreted as hollow cables of 6 Hb S monofilaments have been demonstrated by electron microscopy. Also, a single monofilament has been demonstrated on a Langmuir trough. Recently, a dialyzable cofactor for deoxy Hb S aggregation has been found. Highly purified Hb S by dialysis does not aggregate at all; even its optical rotatory dispersion is essentially the same as Hb A at 0°C and 38°C.

When a small amount of the residue from the diffusate is added to purified Hb S, aggregation takes place dramatically. The technique and findings of the high pressure chemistry of Hb S aggregation and the determination of the volume of activation (ΔV^*), are discussed in detail. Thermodynamic treatment of studies on the endothermic aggregation of deoxygenated Hb S (ΔH^*) indicate that the system is entropy driven and that a minimum of 10 molecules of H_2O are set free when a tetramer-tetramer interaction occurs. Desickling can be achieved by use of alkane gases (methane, ethane, propane, etc.) under slight atmospheric pressure. The oligodynamic action of urea to desickle sickled erythrocytes (with deoxy Hb S) is reasonably related to its disruptive effect on water clathrates essential for hydrophobic interactions.

MOLECULAR ORIENTATION OF DEOXY Hb S MOLECULES IN MICROTUBULES ACCORDING TO PERUTZ ET AL.

Another proposal for deoxy Hb S to stack along the X axis has been suggested by Perutz and Lehman[70] and Finch, Perutz, Bertles, and Döbler.[25a] Finch et al.[25a] confirmed my finding that six strands of monofilaments of deoxy Hb S twist around a hollow and my observation that the O.D. is 170 Å has been confirmed also by x-ray diffraction by Magdoff-Fairchild.[47] The difference in the interpretation of the optical data by us is the source of the difference of opinion between Perutz et al.[70,25a] and by this reviewer as well as Pauling.[67,69a] As already stated, the polarized light study of the sickled erythrocyte by Perutz and Mitchison[71] states that the heme groups are perpendicular to the long axis of the microtubule; accordingly, the molecules must stack side to side, so to speak, along the X axis (or between the X and Z axes). Finch et al.[25a] suggested that the monomolecular filament of deoxy Hb S is formed by stacking along the X axis, which is perpendicular to the twofold axis of symmetry (the Y axis) and runs along parallel to the longer dimension of the spheroid. The physical shape of the hemoglobin is that of an oblate or elongated spheroid of the dimensions 65 x 50 x 55 Å, along the X, Y, and Z axes, respectively. The lack of significant shape anisotropy causes the molecule to appear essentially spherical in electron micrographs and permits much freedom in matching the observed x-ray diffraction patterns with those calculated from molecular models. Neither the x-ray diffraction of the sickled erythrocyte nor electron microscopy has yet provided concrete information on the orientation of the hemoglobin molecules within the microtubule. Finch et al.[25a] proposed that the stacking of deoxy Hb S is along the X axis, as shown in Figure 23. It has been suggested[25a] that the cross section of the tube would be such that the dyad axis would be oriented radially (see Figure 24), and it appears that the magnetic vector would be parallel to the

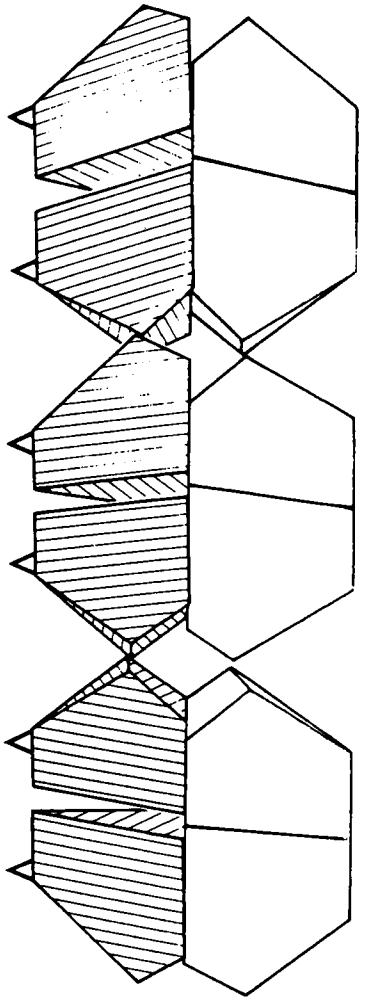

FIGURE 23. Schematic model of three deoxy Hb S tetramers illustrating molecular stacking along the X-axis. Compare with Figure 1 which illustrates stacking along the Y axis (or the twofold axis of symmetry).

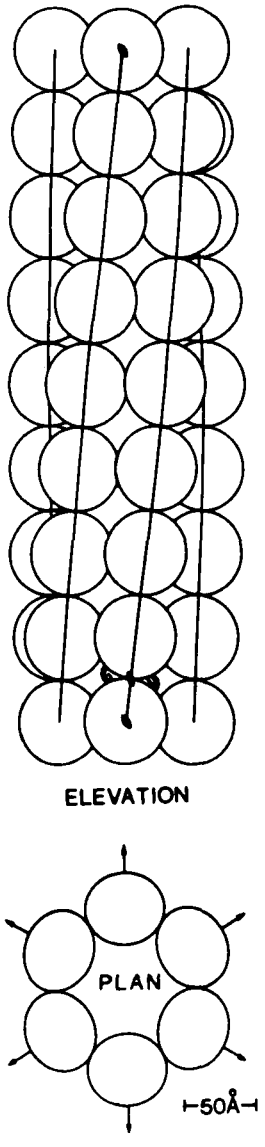

FIGURE 24. Structure of superhelical microtubule of deoxy Hb S according to Finch, Perutz, Bertles, and Döbler. The arrows and signs indicate the probable positions of the molecular dyads perpendicular to the fiber axis. A length of eight rings is shown corresponding to the approximate repeat of the structure. The superhelix was found to be right-handed. (From Finch, Perutz, Bertles, and Döbler, *Proc. Natl. Acad. Sci. USA*, *70*:718, 1973. With permission.)

heme plate (or essentially congruent with the twofold axis of symmetry). This may not be because β-6 valine residues are either pointing inside of the tubule or pointing out. Hydrophobic interactions cannot occur between the tetramers in a stacking of this sort.

Nearly all evidence suggests that sickling is an allosteric one, that it is entropy driven, and that predominantly hydrophobic interactions appear to be involved. It is an allosteric effect in the sense that the aggregation of the deoxy Hb S molecule depends upon the change of quaternary structure which the hemoglobin molecule undergoes on deoxygenation. Factors that bias the allosteric equilibrium towards the quaternary deoxy structure, such as binding of 2,3-DPG and low pH, favor the gelling; and factors that bias it toward the quaternary oxy structure, such as inhibition of the terminal salt bridges by reaction of the α-amino group with cyanate, oppose the gelling.[8,18,20,93]

Hofrichter, Hendricker, and Eaton,[33] using a microspectrophotometer, measured the polarized absorption spectra through the Soret band on approximately one square micron area of sickled erythrocytes. From their dichroic ratio measurements, they conclude that the heme plates are oriented perpendicular to the long axis of the sickled erythrocyte (because they assume the early conclusion of Perutz and Mitchison[71]). The dichroic ratio is defined as the ratio of the absorbance measured with light polarized perpendicular to the long axis of the cell to that measured with light polarized parallel to this axis. The dichroic ratio is greater than unity in all cells measured along the long axis. Assuming that the arrangement of the four hemes of the hemoglobin molecule is unchanged in the microtubule, the maximum possible value of the dichroic ratio of a single microtubule is 3.9. The highest dichroic ratio is 3.0 ± 0.1, indicating that the X axis of the molecule is less than 20° from the long axis of the microtubule. However, lack of perfect alignment of microtubules in the cell, as well as possible experimental errors, acts to decrease the observed dichroic ratio from the true value for a single microtubule. The value of 20° is thus an upper limit. This result eliminates 95% of the allowed orientations[56,57,59,61] for the X axis and, therefore, would rule out the only detailed proposal for intermolecular bonding in the microtubules.

Finch et al.[25a] state that optically, therefore, the fiber axis of gels or deoxy Hb S corresponds to the X axis of the molecule in the gel which must be parallel to, or enclose a small angle with, the fiber axis. As already stated, the molecule is a spheroid with diameters of about 65 x 50 x 55 Å along X, Y, and Z directions, respectively. The observed spacing of 64 Å along the fiber axis

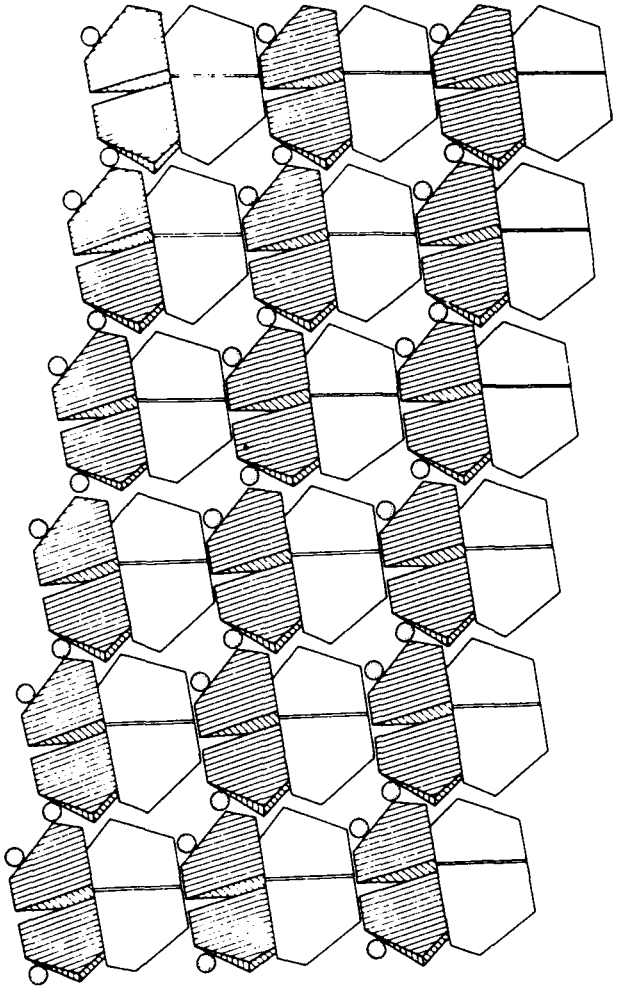

FIGURE 25. This is another proposal for the structure of the deoxy Hb S microtubule. A schematic drawing of the microtubule is based on available experimental data; the heme groups are essentially perpendicular to the fiber axis. The tetramers are arranged so that the specific binding sites (β-6 Valyl) could participate in the fiber formation. The specific binding site of β_1 polypeptide chain could participate in the stacking of the tetramers; stacking takes place essentially along the X-axis (between X and Z axis). The specific binding site of β_2 polypeptide chain could participate in the cross-linking between the monomolecular filaments of deoxy Hb S. Then deoxy Hb S would have a greater propensity to stack because contacts of large surface areas between the tetramers could be involved in addition to the hydrophobic interactions due to the specific binding site (β-6 Valyl). This schematic drawing shows the microtubule "cut" into half along the longitudinal axis and then flattened. The structure is not proven.

shows that Y or Z cannot be parallel to that axis. It could be X or some direction between X and Z. When hemoglobin molecules aggregate to form straight filaments, we should expect the bonds between them to be strongest if the molecular dyads (Y) were normal to the fiber axis; such an arrangement would generate, at each interface, a second set of dyads with two equivalent contacts at either side.[25a]

The structures of the helical tubule (microtubule) of deoxy Hb S, as shown in Figure 24 from Finch et al.[25a] are essentially the same as the model built by Jensen et al;[37b] the pitch of the super helix must be altered to comply with parameters given by Finch et al.[25a] (Incidentally, monomolecular filaments do not twist into a loose alpha helix, as stated by Jensen and Lessin.[37b]) As indicated by Finch et al., the molecular dyad axes are to be radially perpendicular to the core of the microtubule such that *none* of the β-6 Val will participate in hydrophobic interactions (particularly so if the β-6 Val are to be "wrapped up inside" of the microtubule).

Another possible arrangement of deoxy Hb S molecules in the microtubule is suggested here (see Figure 25). It is so arranged that β-6 Val may participate in the stacking (acting synergistically with the large surface area contact between the adjacent tetramers), and β-6 Val can participate in the cross-linking with the adjacent monomolecular filament of deoxy Hb S. This structure is only a probable one and has not been proven; however, the arrangement of the deoxy Hb S molecules is consistent with the notion that the aggregation involves predominantly hydrophobic interactions. It is also consistent with Pauling's theory[68] of aggregation of globular protein molecules in that they are expected to polymerize if a force of interaction could operate between 2 molecules with energy corresponding to a bond of about 20 kcal/mole. The interaction of the 2 molecules is the sum of a number of small interactions (Lilliputian) bonds – van der Waal's forces, hydrogen bonds, and the attraction of positively charged and negatively charged groups and *hydrophobic interactions.* In the instance of deoxy Hb S molecules, polymerization is essentially entropy driven and the interaction is *predominantly* hydrophobic.

Comment on Deoxy Hb S Microtubule

It is interesting that the deoxy Hb S microtubule is an exceedingly rigid structure. Thus it is possible to study the microtubule in an electron microscope without the supporting membrane (which causes the loss in the resolving power of the instrument). This has been achieved by placing the supporting membrane which is strong enough to support the deoxy Hb S solution but so weak that it can be broken by the electron beam at the cross-over. In this way it would be possible to have the microscopists' dream come true. . . the object of study just "floats" in midair with no support. And a more "true" structure could be realized by drying them by the critical point method. This would eliminate the high pressure effect which results from drying by the conventional method.

Appendix

PREPARATION OF A 25 Å SPACING CRYSTAL FOR MAGNIFICATION CALIBRATION ABOVE 18,000 X

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If one part (by volume) of the dyestuff Indanthrene Olive TWP Ex. Con. Pdr. available from General Aniline and Film Corp. is boiled with stirring in two parts of Quinoline (b.p. 235-237) for 15 to 30 min, tiny, thin, lath-like crystals are formed having a molecular plane spacing of 24.9Å (determined by x-ray diffraction) in the direction of their width. Most of these are sticking out like bristles from clumps of dye but some are free. These latter can be concentrated by alternate low- and high-speed centrifugation. After concentration, the small crystals are washed twice

*Labaw, L. W., *J. Appl. Physics,* 35, 3076, 1964.

TABLE 1

Physical and Chemical Agents Which Affect the Sickling Process*

Name of agent	Site of action	Induction (I) block (B) reverse (R)	Ref.
Urea	Hydrophobic β-6 Val	B + R	62a
Pot cyanate	α-NH_2	B	20
Carbamyl phosph.	α-NH_2	B	43b
Nitrogen mustard	α-NH_2	in vitro blocking	80b
Alkane gases Methane Ethane Propane	Hydrophobic β-6 Val	R + B	53
Cooling	Hydrophobic	R + B	1, 2, 56
High pressure	β-6 Val	R + B	58
Sodium nitrite	Oxidation of Fe^{2+} to Fe^{3+}	B	11, 69b
Hg^{2+}	β-93 cys	B	25d
PCMB(p-chloromercuri-benzoate)	β-93 cys	B	80a
K+	?	I	26
Botanical (Fagara)	?	B + R	86
Hypertonic soln	?	I	9a
Ascorbic acid	-0_2	I	22
Glutathione	-0_2	I	88
Prostaglandin E_2	?	I	37c

*(From Nalbandian et al., *Clin Chem.*, 18, 961, 1972. With permission.)

with quinoline to remove a red contaminant soluble in quinoline. A glass rod is dipped into a rather viscous concentration of washed crystals in quinoline and then touched to a distilled water surface. A thin layer of crystals over the water surface results as can be seen by reflected light. The crystals are then picked up on carbon-coated specimen grids in the usual way and are ready to be used for calibration.

Comment on Table 1: Physical and Chemical Agents Which Induce Sickling, Block Sickling, and Reverse the Sickling Process.

There are ways and means to block sickling but not reverse it, as in the instance of carbamylation. In the instance of urea, the sickling process may be blocked and/or reversed. It is exceedingly important to keep this in mind. The term "inhibition" of sickling used by Cerami and Manning[20] has caused much confusion, and this type of semantic trick ought to be avoided at all cost because communication is difficult even with the best of intentions. In Table 1 is a summary of some of the known physical and chemical means to block and/or reverse the sickling process, as well as some of those which are known to induce the process of sickling. Because we know that deoxy Hb S aggregates endothermically, heat will induce sickling; on the other hand, cooling will reverse as well as block the sickling process. The table is not expected to be complete.

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INTRODUCTION

The hypothesis which I have proposed for the molecular mechanism of sickling[33,37] has apparently had some clinical value.[45] Both therapeutic and diagnostic gains in sickle cell disease by the use of this concept have been made by Nalbandian and his co-workers.[33,37,45] In Nalbandian's view of my hypothesis, he saw at least four implications of therapeutic significance for sickle cell disease:[33]

Principle of steric hindrance – Since an appropriate configuration was causally related to the process of sickling, another appropriate chemically induced conformational change of the Hb S tetramer which did not interfere with oxygen transport should block or inhibit the sickling process.

Principle of chemical interference with formation and stability of pathologic hydrophobic interactions – Because the entire molecular population of an erythrocyte (with Hb S) is physically transformed to a hydrophobic-interaction-dependent nematic liquid crystal, any chemical attack on the intertetramic hydrophobic "bonds" which eliminates or reduces the stability of such interactions should disperse the system and reverse and/or block the sickling phenomenon.

Principle of the manipulation of the sickling cofactor – once the sickling cofactor is fully characterized and identified, its action and role in the sickling process may be nullified by chemical or even immunological methods [or inhibit the enzyme(s) which will convert its precursor(s)].

Principles of alteration of the quaternary structure of the hemoglobin molecule by manipulation of genetic mechanisms – This principle is discussed in detail elsewhere,[33] and since it involves activation and inhibition of genetic mechanisms, a detailed discussion is not germane here.

DEDUCTION OF UREA AS A DESICKLING AGENT

Any candidate molecule for chemotherapeutic action in sickle cell disease had to have certain properties according to Nalbandian:[33,35-37,40]

1. Nontoxic
2. Water soluble
3. Easily diffusible through body fluids and cell membrane
4. Readily excreted
5. No uncorrectable, serious side effects
6. Chemical capability of breaking hydrophobic interactions.

The last feature indicated that the candidate molecule had to have a cluster or a linear array of hydrogen atoms at the surface as an essential feature of the molecular structure. An additional molecular feature became obvious to me in subsequent personal discussions with Nalbandian, namely, that the molecule had to have an electric dipole moment greater than that of water. To the above list an additional criteria may now be added:

7. Electric dipole moment of the molecule greater than that of water.

Between 1961 and 1972, several groups of physical chemists, Brunning and Holtzer,[3] Whitney and Tanford,[56] Mukerjee and Ray,[34] Refojo,[51] and Ratner and Miller[50] showed by various studies that urea did break weakly hydrophobic interactions. Coupling this observation with the first six criteria listed above, Nalbandian perceived[33,35-37,40] that urea, a compound made in human liver, fulfilled the specifications of a theoretically defined chemotherapeutic agent. Furthermore, the electric dipole moment of urea is 4.6 compared to that of water which is 1.8. However, while aqueous solutions of urea proved most effective as a desickling agent, Nalbandian found it caused lysis of erythrocytes after an exposure of about 30 min at 1.0 *M*. Although unknown to Nalbandian at this time, Ponder and Ponder in 1954,[49] in a brief comment had shown the same thing, i.e., that aqueous solutions of urea first reversed sickled cells and then lysed them. This was an observation made incidentally by these workers in their investigations of the effect of urea on the red cell membranes of birds, animals, and man. In 1956 Javid[21] had reported that urea in 10% invert sugar could be given intravenously for the treatment of cerebral edema.[20] Using such a preparation of urea Nalbandian avoided the therapeutic paradox of urea: an effective desickling agent which may be subsequently lethal due to hemolysis. Details of

such studies have been published elsewhere.[33, 35-37,40]

Nalbandian and co-workers also showed that it requires less urea to block sickling than it does to reverse sickling.[33,36,39,41,44] The clinical implications of these simple experiments are of considerable clinical importance. Small elevations of the BUN through daily medication with modest doses of urea should be of prophylactic benefit by *blocking* the sickling event, while larger intravenous doses of urea in sugar solutions should *reverse* sickling in the management or termination of the acute sickle cell crisis. Urea, both orally and intravenously, has been used successfully in the therapy of sickle cell disease by others.[19,28,29, 32,52,55] A few have reported failures,[27,47] but perusals of such studies show that the required BUN levels specified by the original protocol were never obtained in these instances. One group has reported both successes and failures with urea therapy.[31] Such failures are quite understandable, for the therapeutic effectiveness of urea is a concentration-dependent action. The application of the lessons of the laboratory bench to clinical problems of sickle cell disease has been astonishingly simple and direct.

Traditionally, physical chemists and others have used urea in 6 and 8 molar concentrations for a great variety of chemical and biochemical reactions. The *oligodynamic action* of urea at the relatively low concentrations (6 to 8 m*M*) required for therapy is only now being appreciated.[50,51] It is a fortuitous circumstance that urea breaks hydrophobic interactions in the sickling process only to a "modest" degree. For this, it is precisely the low level of chemical activity required if urea is to have therapeutic validity. Obviously, any vigorous rupture of all or most hydrophobic interactions will denature the hemoglobin molecule and forfeit the critical function of oxygen transport. Orten and Nalbandian[37] have shown that one mole of urea, a concentration almost 15 times greater than a maximum therapeutic blood level, has no detectable effect on the electrophoretic mobility of hemoglobin S and therefore, presumably, no adverse effect on the tertiary or quaternary structure of the hemoglobin molecule.

Molecular Mechanism of Urea

The molecular mechanism by which urea *blocks and reverses sickling* is not known for certain, and additional work will be required to elucidate this information. One may conjecture[33,37] that urea will form hydrophobic-interaction-mediated combinations with hemoglobin S tetramers at one or more of the four critical combining sites per Hb S tetramer. Not more than one molecule of urea theoretically need react with a given Hb S tetramer to hinder sterically the sickling event.

An alternate and perhaps more likely mechanism of action arises from the consideration of the relative electric dipole moments of the molecules of urea (4.6) and water (1.8).[33] The hydrophobic interaction is a function of the interaction between nonpolar amino acid residues such as valine, and the precisely ordered structure of water molecules arranged in a tetrakaidecahedral-like conformation. The greater electric dipole moment of urea may randomize this critical molecular array of water molecules, thereby dispersing the "stacked" or nematic liquid crystal phase of interacting molecules of deoxy Hb S.

Perhaps both mechanisms may be at work. Additional studies on this point are indicated.

CARBAMYLATION

In 1971, Cerami and Manning suggested[6] that urea acted as a desickling agent, not for the reasons originally advanced by Nalbandian, but because urea hydrolyzed to cyanate ion in an aqueous media. Drawing on the work of Stark, Stein, and Moore in 1960,[54] Cerami and Manning believed that the cyanate ion formed by hydrolysis from urea reacted with amino and sulfhydryl groups of proteins including the N terminal valine residues of both alpha and beta globin chains. Their data and that of others[15,30,55] show that indeed carbamylation does occur with Hb S as they proposed. A careful reading of their original paper, however, will provide evidence from their own data that urea and cyanate operate by distinctly different molecular mechanisms.

Molecular Action of Cyanate

The molecular mechanism of the action of the cyanate ion on the sickling is of clinical importance. A series of studies initiated in my laboratory in the summer of 1971[33] and later confirmed and extended by Nalbandian and co-workers was helpful.[33,42,43] Aliquots of SS erythrocytes were deoxygenated by each of three physiologic methods (nitrogen, helium, and carbon dioxide). In an oxygen-free atmosphere, three sickled

aliquots (Figure 19 of Part A) were desickled with 25 m*M* of urea in sugar solutions (BUN equivalent of 70 mg/100 ml), as shown in Figure 20 of Part A. Upon the addition of urease to urea treated cells, sickling reappeared equivalent in severity and extent to that originally induced by the sickling agents (dithionite or sodium metabisulfite) used. Furthermore, SS erythrocytes carbamylated by the method of Cerami and Manning were not detectably affected or altered morphologically by urease. Because of his clinical interests, Nalbandian and associates repeated the method devised and used by me, changing only the BUN concentration, elevating it to 50 m*M* (BUN equivalent of 140 mg/100 ml). The only difference between the results of my studies and those by Nalbandian is that the degree of reversal of sickling was greater in his studies[33,34] reflecting the concentration-dependent aspect of the chemotherapeutic efficacy of urea.

The comparison of the effects of urea and cyanate on sickling has been summarized in Table 1 and leads unavoidably to the conclusion that

Comparison of Effects of Urea and Potassium Cyanate on Sickling*

Urea	Potassium cyanate
Reverses and blocks sickling	Blocks but does not reverse sickling
Effects are reversible	Effects are irreversible
Sickled cells, reversed with urea, are again sickled after action of urease	Urease has no effect on cyanate-treated cells
Effects are immediate	Effects require time
Effects are apparent in the deoxygenated state	Initially hemoglobin S must be oxygenated before carbamylation will occur

*From Nalbandian et al., *Clin. Chem.*, 18, 961, 1972. With permission.

both of these agents act by separate and distinct molecular mechanisms. Even in their original report,[6] Cerami and Manning expressed uncertainty in their discussion that urea was mediating its therapeutic activity through cyanate, but this demurral appears to have been overlooked by some voluble writers, and for a short time in the literature, the desickling action of urea was "explained" by carbamylation by cyanate.

Studies by Kilmartin and Rossi-Bernardi undertaken in 1969[22,23] antedate the work of Cerami and Manning, but are in essential agreement. These workers found that carbamylation of horse hemoglobin (a) increased oxygen affinity, (b) interfered with CO_2 binding to the hemoglobin, and (c) involved the alpha amino terminal residues.

While the steric configuration of the hemoglobin S molecule is altered by carbamylation since all four terminal valine amino acid residues of both the alpha and beta globin chains (among others) have reacted with the cyanate ion, it appears that the "inhibition of sickling" is related in some way to the left shift of the oxygen dissociation curve. This, of course, is reflected in an increased oxygen affinity as several workers have confirmed.[15,30,53] Increasing the oxygen affinity of hemoglobin is a change theoretically adverse to the delivery of oxygen peripherally. However, this does not seem to be a significant flaw because Gillette and his co-workers have shown increased survival of sickle cells treated by cyanate in patients.[16-18] May and co-workers,[30] however, have reported deleterious effects on intraerythrocytic ribonuclease.

In these studies which ultimately will have clinical application, the persistent use of the term "inhibits sickling" in reference to the action of the cyanate ion has obscured an important distinction.[17,30] Cyanate *blocks* but does not *reverse* sickling, whereas urea does both. Carbamylated hemoglobin S will then be useful only in a prophylactic role. It cannot be effective in terminating an acute sickle cell crisis.

Effect on Partitioning

Carbamylation of Hb S gives rise to two distinct bands upon electrophoresis[10] and on DEAE-Sephadex column in contradiction to urea-treated hemoglobin which maintains its original hemoglobin electrophoretic mobility *in toto* (see

above). Furthermore, it has been noted that carbamylated Hb S will sickle.[10] The observation has been made that this fact reduces the clinical value of potassium cyanate and that "a drug that really does inhibit gelation would seem more desirable."[10] It is of pertinent interest that as early as 1970 it was reported and apparently overlooked that urea did precisely that under the conditions of the Murayama Test as shown by Nalbandian and co-workers.[36,37]

Effect on Blood Viscosity

In 1957, Allison investigated[1] the effect of urea among other factors on the specific viscosity of sickle cell hemoglobin in a deoxygenated hemolysate system and found that urea did reduce the specific viscosity. However, using essentially the same concentration of urea, Segel et al.[53] obtained negative results and, hence, are in disagreement with the earlier findings of Allison. Cooper and Ransom[11] in 1972 noted that urea at concentrations above 400 mg/100 ml did significantly reduce the viscosity of sickle cell blood, but they found that cyanate was substantially more effective in this property.

Safety of Cyanate Therapy

The toxicity of cyanate has been studied,[12] and the target organ appears to be the central nervous system, producing a depressed sensorium and ultimately convulsions. In studies conducted here at NIH by Dr. Kinoshita,[24] 5 m*M* cyanate produces opacity in the lens protein by a tissue culture technique (rabbit lens). On the other hand, the studies of Gillette[18] and associates seem to reveal that in the dosage used clinically, cyanate is safely tolerated. The work of May and associates has shown a deleterious effect on erythrocytic enzymes and erythrocytes.[30]

Urea is virtually nontoxic but does have a major side effect, diuresis, which can be offset by appropriate intravenous fluid therapy. It is a physiological curiosity that mole for mole, cyanate is 30 times more potent as a diuretic than is urea.[43]

Carbamyl Phosphate

At the suggestion of J. L. Wood and on the basis of the reports of the therapeutic action of urea and cyanate, Kraus and Kraus used carbamyl phosphate in a series of investigations as a therapeutic agent.[25,26] They found that this compound "inhibited sickling" in a manner analogous to that by cyanate discussed above. In their original paper, they described the ionization of carbamyl phosphate to cyanic acid and thence to cyanate ion which penetrated the red blood cell membrane and acted thenceforth as the cyanate ion does as described above. This action of carbamyl phosphate has been confirmed.[5,8,9] Chabas and Grisolia[8] have shown that carbamyl phosphate significantly inactivates glutamate dehydrogenase, but the clinical effect of this action is not yet known. Kraus and Kraus[25,26] have indicated that carbamyl phosphate carbamylates hemoglobin at lower concentrations and proceeds more readily than does cyanate. However, Carreras-Barnes et al.[5] have reported opposite findings in their studies.

It is evident that carbamyl phosphate has the same limitations but potentially very important clinical applications of prophylaxis as sodium or potassium cyanate. The statements made in Table 1 above and elsewhere[33,43] hold true also for carbamyl phosphate. Like cyanate, it can be safely predicted that carbamyl phosphate will be of no value in aborting acute sickle cell crisis. However, the very important clinical role of prophylactic medication in sickle cell disease may be filled by one or the other, or perhaps both, of these compounds.

COMMENT

Innovative ideas in science invite attack, and properly so. Meritorious concepts will survive; meretricious ones will not thrive. When Nalbandian and his group advanced urea[35-37,40,46] as a candidate chemotherapeutic agent for sickle cell disease, challenges of unusual severity developed which have passed through three stages: (1) that urea was not a desickling agent but actually caused sickling; (2) that urea was a desickling agent but had this action because of hydrolysis to cyanate and subsequent carbamylation of hemoglobin S (and A); and (3) that urea acts as a desickling agent after all but at BUN levels higher than those put forward originally.

In my judgment, the intensity of the criticism launched against the proponents of urea[4,9,14] has been a boon for the proposed therapeutic role of urea since it appears to have survived an extraordinarily rigorous challenge.

It is an oft-noted event in the history of science

that with the emergence of a new scientific paradigm, intense and vigorous opposition is generated. In the consequent fervor of debate, a scientific milestone is passed almost unnoted. In my opinion, in 1970, with the advent of urea therapy, there was a recurrence of this ritual. For the first time in the history of medicine, insofar as I can determine, a combination of specific molecular information and my hypothesis for the pathogenesis of an abnormal molecular event expressed in submolecular detail resulted in specifications for an ideal chemotherapeutic agent which, by deduction, led directly to the specific selection of urea by Nalbandian. Cerami and Manning[6] advanced potassium cyanate therapy, again resolving the rationale in molecular terms, no matter that their original reasoning was based on an incorrect premise. The proposal by Kraus and Kraus[25] of carbamyl phosphate is analogous to potassium cyanate, but again is conceived in terms of molecular information. Medicine now has demonstrated the capability of deducing rational therapy from specific molecular information just as Pauling predicted 20 years ago.[48] A major forward step of this order of magnitude arms the sickle cell research worker with a powerful principle, and, at the same time, transcends the relatively narrow borders of sickle cell disease with potential application along the broader front of medical disease.

The chemotherapeutic use of urea in sickle cell disease in my opinion is a valid first step in the evolution of an ideal chemotherapeutic agent for this affliction. Within one year of its introduction in 1970, it has led to two additional candidate therapeutic agents. All three of these agents have a rational basis in the molecular pathology and physiology of sickle cell disease. The pace of progress in this dread medical entity should be rapid henceforth.

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