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Structure of Sickled Erythrocytes and of Sickle-Cell Hemoglobin Fibers

(electron microscopy/x-ray diffraction/molecular arrangement/helices)

J. T. FINCH*†, M. F. PERUTZ*, J. F. BERTLES‡, AND JOHANNA DÖBLER‡

* Medical Research Council Laboratory of Molecular Biology, Cambridge, England; ‡ Medical Service, St Luke's Hospital Center, and Department of Medicine, Columbia University College of Physicians and Surgeons, New York, N.Y. 10025

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ABSTRACT **Deoxyhemoglobin from patients homozygous for sickle-cell anemia (deoxyhb S) aggregates into long straight fibers. These may extend through most of the length of the sickled cell, forming either square or hexagonally packed bundles with lattice constants of 170–180 Å. Each fiber is a tube made up of six thin filaments, which are wound around the tubular surface with a helical pitch of about 3000 Å. Each filament is a string of single hemoglobin molecules linked end to end at intervals of 62 Å in dry and 64 Å in wet fibers. Molecules in neighboring filaments are in longitudinal register so that they form flat hexagonal rings; these rings are stacked so that successive ones are rotated about the fiber axis by 7.3°. The whole structure repeats after about eight rings. In this structure each molecule makes contact with four neighbors. The likely orientation of the molecules and points of contact between them are discussed. Similar filaments are also observed in normal deoxygenated erythrocytes, but in much lower concentration and aggregated into fibers of irregular diameter. No filaments appear in oxygenated sickle, or normal, adult cells, nor in oxygenated or deoxygenated fetal cells.**

Sickle-cell anemia is due to a mutation in the globin gene that causes the replacement of one pair of amino-acid residues in the β chains [Glu A3(6)$\beta \rightarrow$ Val]. This replacement leaves the solubility of the abnormal hemoglobin in its oxygenated form (oxyhb S) unchanged, but drastically reduces the solubility of deoxyhb S so that it precipitates in the erythrocytes, causing them to become elongated and rigid. The deformation and stiffness of the erythrocytes is the primary cause of the disease and is the feature that distinguishes sickle-cell anemia from the anemias caused by other abnormal hemoglobins. If a method could be found of preventing the precipitation of deoxyhb S, it might lead to a possible therapy. We are approaching this problem by a structural study of the deoxyhb S precipitate.

In sickled cells and in cell-free solutions deoxyhb S has been reported to aggregate into fibers with diameters of between 140 and 170 Å (1–5, 20). Oriented preparations give x-ray fiber diagrams with a marked periodicity of 64 Å along the fiber axis (6). We wish to report electron microscope studies of thin sections of sickled cells and of deoxyhb S that show the general arrangement of the fibers. These are followed by studies of negatively stained preparations in which the arrangement of the individual hemoglobin molecules is resolved, and by further x-ray diffraction work. Several features of the x-ray fiber diagrams can be explained from our findings, but the stereochemical mechanism of aggregation remains to be discovered.

Abbreviations: DPG, 2,3-diphosphoglycerate; deoxyhb S, deoxyhemoglobin S.

† Reprint requests to: Dr. J. T. Finch.

METHODS

For electron microscopy of thin sections, deoxygenated sickled cells and ultracentrifuge pellets of deoxyhb S were prepared as described in refs. 3 and 5.

Negatively stained specimens of deoxyhb S were obtained by deoxygenating washed erythrocytes from a sickle-cell homozygote suspended in 0.1 M NaCl–1 mM 2,3-diphosphoglycerate (DPG) in a nitrogen-filled glove box for 3 hr. This suspension was applied to a carbon-coated grid, lysed either by washing with 0.03 M NaCl or with a solution of phospholipase C in 0.1 M NaCl, stained with a 1% solution of sodium phosphotungstate (pH 7.0), and dried while still in a nitrogen atmosphere. Alternatively, a concentrated suspension of erythrocytes from a sickle-cell homozygote in 1% NaCl was lysed by addition of an equal volume of water and left for 15 min. NaCl was then added to bring the total concentration of NaCl to 2%. The cell walls were removed by spinning for 0.5 hr at 15,000 rpm (27,000 $\times$ g), decanting the supernatant, and spinning it once more as before. The hemoglobin solution was then dialyzed against either 0.01 M NaCl or 0.1 M sodium phosphate (pH 6.5), giving solutions with a Hb concentration of about 10%. To these, DPG was added to a concentration of 1 mM. They were then deoxygenated in the nitrogen-filled glove box for 3 hr, diluted to various concentrations down to 1%, applied to the grids, washed with water, stained, and dried as described above. Electron micrographs of negatively stained specimens were taken with a Philips EM 300. Low angle x-ray diffraction pictures were taken of concentrated gels of Hb S prepared as described in ref. 6. Optical diffraction pictures of electron micrographs were taken as described in ref. 7.

RESULTS

Thin sections through a sickled cell show fibers of deoxyhb S that extend along most of its length and wrap themselves around the inner surface of the cell wall. In cross-section, the fibers are packed in either square or hexagonal arrays at center-to-center distances of about 180 Å (3). By high-speed centrifugation of cell-free solutions, deoxyhb S fibers can be packed into pellets. Thin sections of these show well-ordered bundles of straight fibers, often packed in square lattices with center-to-center distances of about 230 Å (Fig. 1*a* and *b*).

Negatively stained micrographs obtained from dilute solutions of deoxyhb S show thin filaments resolved into

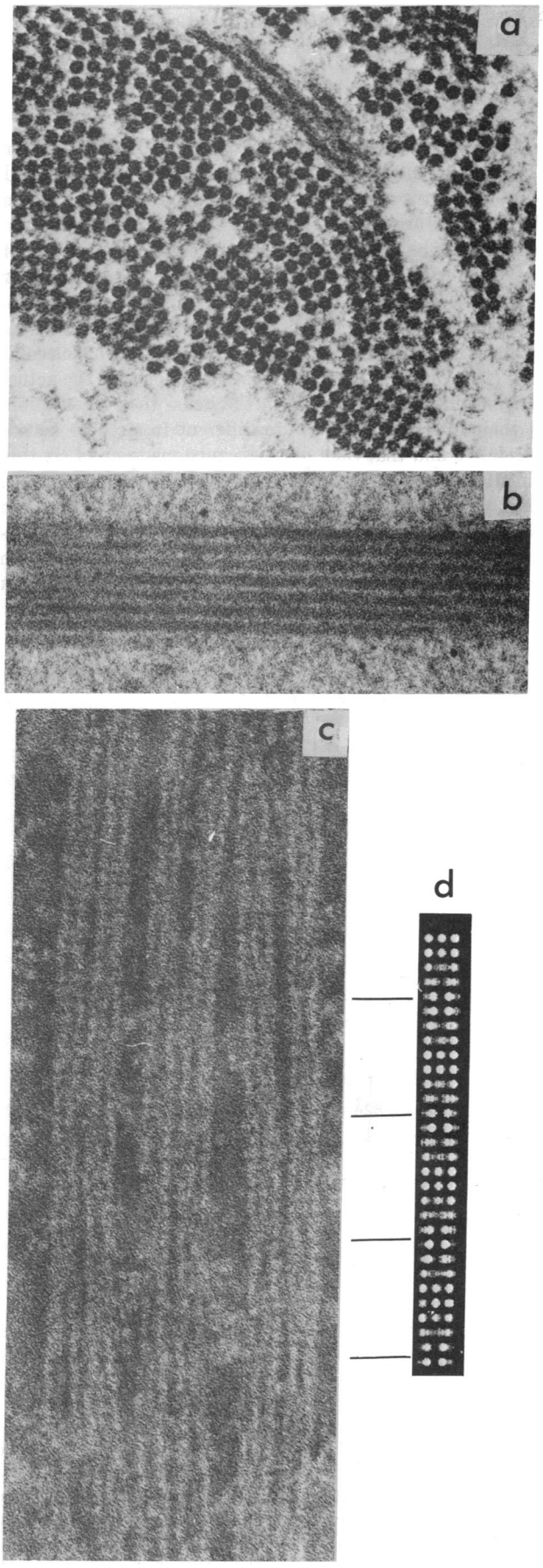

FIG. 1. (*a*) Transverse section through fibers in centrifuged pellet of deoxyhb S (×100,000). (*b*) Longitudinal section through same (×105,000). (*c*) Negatively stained fibers of deoxyhb S obtained directly from sickled cell lysed on the grid (×355,000). (*d*) Projected image simulated by computer of six filaments forming a helical fiber, as seen in *c*.

strings of beads of about 60 Å diameter, corresponding to the size of single hemoglobin molecules (Fig. 2). Some of the filaments are single, but most of them aggregate into fibers with a wide variety of diameters. All these fibers have transverse striations at intervals of about 62 Å, showing that hemoglobin molecules in neighboring filaments are in exact longitudinal register.

Fibers from erythrocytes lysed directly on the electron microscope grids had a more ordered structure (Fig. 1*c*). Their thickness was uniformly 170 Å, in agreement with the fibers seen in thin sections, and the arrangement of the hemoglobin molecules was resolved. The fibers show transverse striations at intervals of 62 Å, like the ones deposited from salt-free solutions, and they appear hollow as though the molecules formed rings that were stacked on top of each other to build a long tube. Along the length of the fibers a helical pattern that repeats at about every eighth ring is just discernible by the eye; its reality is confirmed by optical diffraction pictures taken of the electron micrographs (Fig. 3). As would be expected for such a pattern, these diffraction pictures show strong reflections on a layer line of 510 (8.2×62) $Å^{-1}$ spacing. The electron microscope images have a mirror symmetry about their axial lines that shows that the number of molecules in each ring is even. The peak on the first layer line (510 $Å^{-1}$) occurs at a distance from the center that corresponds to a spacing of 63 Å. If this is the first peak of a Bessel function of even order, than the mean radii of electron-transparent material would be 53, 75, and 97 Å for a well-preserved helical structure made up of four, six, or eight filaments, respectively. The observed maximum diameter of 180 Å clearly rules out the four- and eight-stranded structures. In a helical pattern that repeats every 8.2 rings, the difference in azimuth between successive rings is $60/8.2 = 7.3°$. In summary, the fibers are tubular aggregates made up of six filaments of about 60 Å diameter, wound around each other in a helix with a repeat of about $8 \times 62 = 496$ Å (Fig. 4). A projected image of such a fiber, simulated by computer, closely resembles the electron microscope images (Fig. 1*d*).

The low-angle x-ray diffraction diagram ($2\theta < 3°$), which we have obtained from oriented gels of deoxyhb S, are consistent with this structure. On the equator of our x-ray diagrams are three sharp lines that correspond to the 10, 11, and 20 reflections from a square lattice with $a = 225$ Å, in agreement with the spacing of 230 Å observed in the electron micrographs of sections of fixed gels. These arise from the regular side-by-side packing of the fibers. The distances between the fibers in oriented gels are greater than in the erythrocytes, because the hemoglobin concentration is lower. It may seem surprising at first that such loosely packed fibers should pack in a regular lattice, but this is a common feature of hydrated gels that are made up of long rigid particles, such as for instance tobacco mosaic virus (9). However, in all such cases the packing is hexagonal, and the reason for the square lattices formed by deoxyhb S is not clear. The structure of the fibers proposed here implies that meridional reflections should not appear on any layer line except those with spacings that

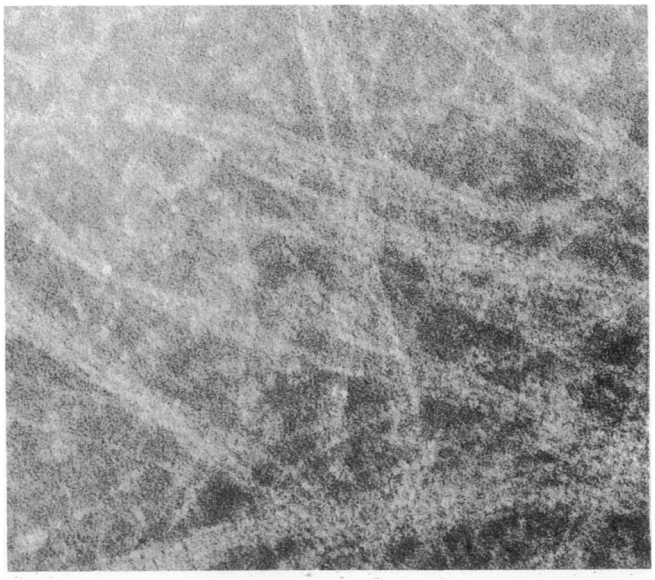

FIG. 2. Negatively stained filaments, either single or aggregated into fibers of varying thickness, deposited from a solution of deoxyhb S (×230,000).

are orders of 64 Å, but Magdoff–Fairchild *et al.* (6) reported a near-meridional reflexion at 9.8 Å and a diffuse meridional one at 18 Å, being the 7th and 13th orders of a basic spacing of 128 Å. We have re-examined these reflections on our x-ray diagrams and find that they are both off-meridional, so that intensity on the meridian occurs only for orders of 64 Å, as would be expected for stacks of rings of that thickness.

When examined under the polarizing microscope, oriented gels of deoxyhb S are dichroic, showing strong absorption when the electric vector of the incident light is normal to the fiber axis, and weaker absorption when the vector is parallel to the fiber axis.

Electron micrographs of lysed deoxygenated normal cells also showed filaments of Hb molecules similar to those seen in lysed sickled cells, but with certain important differences. Their concentration was much lower, so that many grids had to be searched before any filaments could be found. They did aggregate side by side in longitudinal register, but the number of filaments aggregating was irregular (Fig. 5). No filaments were found in oxygenated sickle, or normal, adult

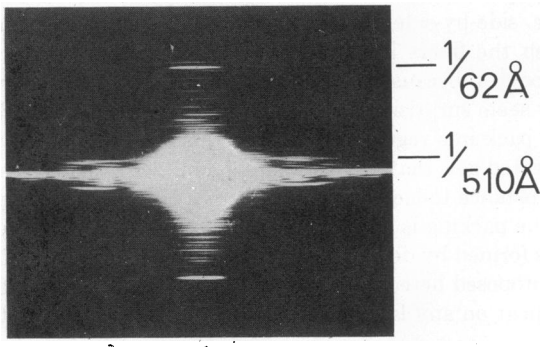

FIG. 3. Optical diffraction pattern obtained from a single fiber of deoxyhb S as shown in Fig. 1c. The meridional reflection at 62 $Å^{-1}$ corresponds to the spacing between successive rings along the fiber. The reflection on the layer line of 510 $Å^{-1}$ corresponds to the axial repeat of the helical pattern along the fiber, i.e., to the vertical distance between the two dyads marked in Fig. 4.

cells, nor in oxygenated or deoxygenated cells from the cord bloods of two Caucasian fetuses that contained less than 10% Hb A.

DISCUSSION

Deoxyhb S forms long tubular fibers of about 170 Å diameter that may extend through the entire erythrocyte or be wrapped around its inner surface. Through much of the volume of the cell these fibers are packed closely together in parallel bundles, forming either hexagonal or square lattices (3). The growth of these semi-crystalline structures is clearly responsible for the characteristic deformation or "sickling" of erythrocytes. To account for the observed diameter of the fibers, Murayama proposed that deoxyhb S forms 6-stranded hollow molecular cables (20). Our results confirm his suggestion, but the actual arrangement of the molecules in the helix, their orientation, and their internal structure are different from those he advanced. We find that each fiber is a tube made up of six thin filaments that are wound around the tubular surface with a helical pitch of about 3000 Å. Each filament, in turn, is a string of single hemoglobin molecules (Fig. 4). The six filaments are in logitudinal register, so that the six hemoglobin molecules at any one level form a planar hexagon 62 Å thick

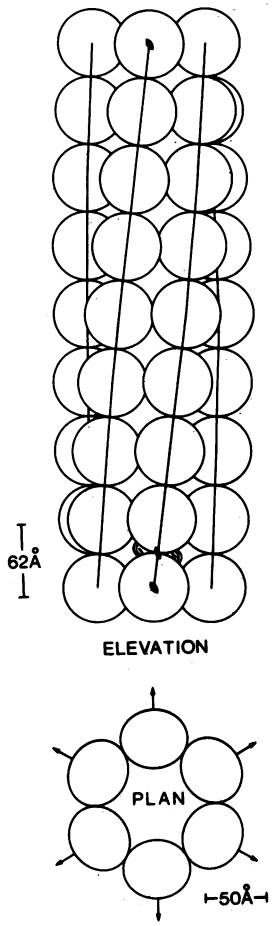

FIG. 4. Structure of helical tube of deoxyhb S. The arrows and signs indicate the probable positions of the molecular dyads normal to the fiber axis. A length of eight rings is shown corresponding to the approximate repeat of the structure. The hand of the helix was investigated by tilting the specimen about the fiber axis and observing the shift of the superposition pattern. It was found to be right-handed.

in dried, and 64 Å thick in wet, specimens. In precipitates from cell-free solutions the fibers are sometimes frayed into single filaments (Fig. 2), but we have never seen single hexagons, which suggests that aggregation into filaments comes first.

When six filaments aggregate into a fiber, each molecule is linked to four neighbors. We do not yet know what the points of contact between neighboring moleculars are, but certain restrictions can already be placed upon them. The first question to be considered is the structure of the Hb S molecule itself. Glutamate 6, which is replaced by valine in Hb S, lies on the surface of the molecule and is, therefore, unlikely to alter its internal structure. This is supported by x-ray studies of oxyhb S that show its crystal structure to be indistinguishable from that of oxyhb A (8). All the evidence suggests that sickling is an allosteric effects, in the sense that the aggregation of Hb S molecules depends upon the change of quaternary structure that the hemoglobin molecule undergoes on deoxygenation. Factors that bias the allosteric equilibrium towards the quaternary deoxy structure, such as DPG and low pH, favor the gelling, and factors that bias it towards the quaternary oxy structure, such as inhibition of the terminal salt bridges by reaction of the α-amino groups with cyanate, oppose the gelling (10–12).

The next question concerns the possible orientation of the deoxyhb S molecules in the fibers. This is restricted by their optical properties and by the external dimensions of the molecules. Both sickled cells and oriented gels of deoxyhb S exhibit negative birefringence and polarization dichroism, which means that the refractive index and absorption coefficient are low when the electric vector of the incident light is parallel to the length of the fibers and high when it is perpendicular to the fibers (8, 13). Suppose we refer a hemoglobin molecule to an orthogonal axial system as indicated on the right of Fig. 6, then the normals to the heme planes in deoxyhb enclose angles of about 25° with the X-axis. The hemes themselves are strongly polarizable when the electric vector of the incident light is approximately parallel to their plane, i.e., when it lies in the Y Z plane, and weakly polarizable when it is normal to their plane, or nearly so, i.e., when the vector is parallel to X. In crystals of human deoxyhb A, the X-axes of the molecules enclose a small angle with the crystallographic a axis, whence refraction and absorption are smallest when the electric vector of the incident light is parallel to a. Optically, therefore, the fiber axis of gels of deoxyhb S corresponds to the a axes of crystals of deoxyhb A, which means that the X-axis of the molecules in the gels must be parallel to, or enclose a small angle with, the fiber axis. A look at the shape of the molecule leads to the same conclusion. The molecule resembles a spheroid with diameters of about 65, 50, and 55 Å along the X, Y, and Z directions, respectively (14). The observed spacing of 64 Å along the fiber axis 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, as indicated by the hooks between the two molecules at the bottom of the helix in Fig. 4.

The position of valine A3(6)β is shown in Fig. 6. The replacement of the glutamate A3 by either lysine or alanine fails to produce sickling on deoxygenation (11, 15), which suggests that it is caused by the presence of valine, rather than the absence of the negatively charged glutamate. Various substitutions in other positions on the surface of the molecule affect sickling, and would therefore be expected to form part of contacts between neighboring molecules. Re-

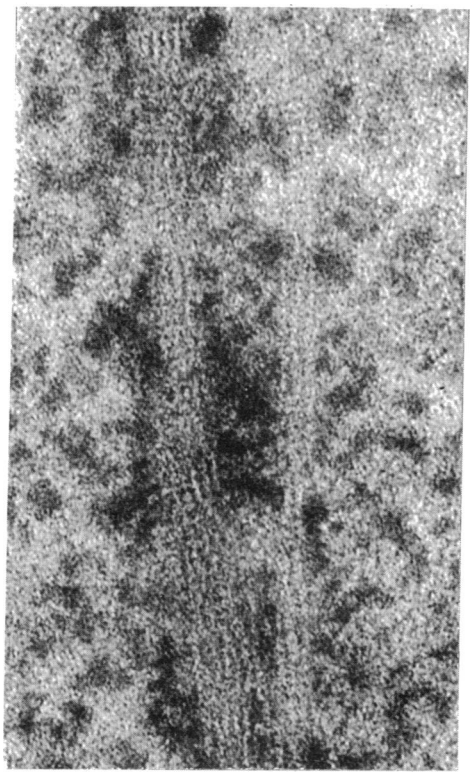

FIG. 5. Negatively stained fibers of deoxyhb A obtained direct from normal human erythrocytes lysed on the grid (×250,000).

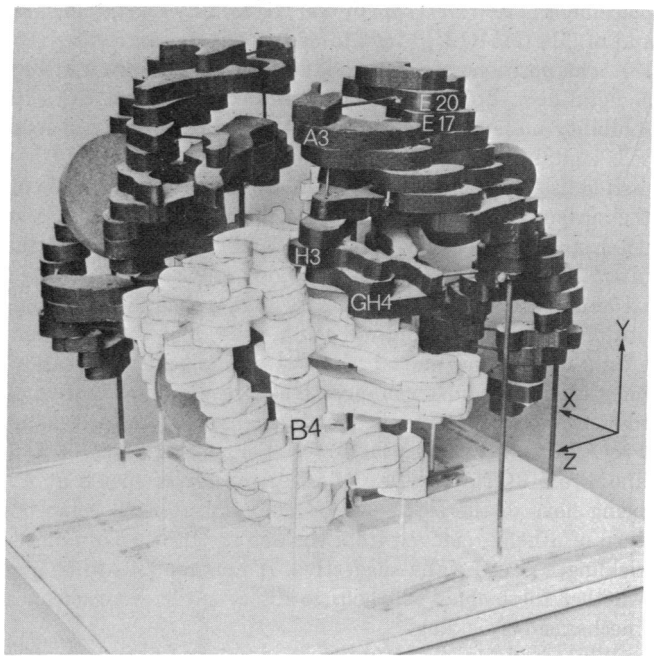

FIG. 6. Model of human deoxyhb A, showing position of various residues. The *white segments* indicate the α, the *black* ones the β chains. The *grey disks* represent the hemes.

placement of Asp E17(73)β or Glu B4(23)α by Gln diminishes, and of Glu GH4(121)β by Gln or Lys enhances, sickling (16, 17). The positions of these residues are also indicated in Fig. 6. Fetal deoxyhb, when mixed with deoxyhb S, increases its solubility more than deoxyhb A (18) and deoxygenated fetal cells contain no Hb filaments. Most of the substitutions that distinguish the γ from the β chain are conservative and lie on the surface of the molecule, while the internal residues that determine the tertiary structure, and the residues at the interfaces with the α chains that determine the quaternary structure, are almost unaltered. It follows that the structures of deoxyhb A and F must be the same, and their different effects on sickling must be due to amino-acid substitutions on the surface. Nonconservative replacements that may influence the contacts between neighboring molecules are Pro A2(5) → Glu; Asp D3(52) → Ser; Ala E20(76) → Lys; and Pro H3(125) → Glu. Of these the replacements at A2, being next to the sickle-cell residue, and at E20, being one turn of α-helix removed from the one at E17 that diminishes sickling, are especially suggestive. It now remains to be seen whether these clues will help to discover the stereochemical mechanism of sickling.

White and Heagan (19) observed tubular fibers in gels of deoxyhb A and of oxy and deoxyhb S precipitated with concentrated phosphate buffer. However, the thickness of their fibers was 240 Å, rather than 180 Å as found by ourselves and others in deoxygenated sickle cells. It appears that the 180-Å fibers are the ones present *in vivo* in sickled cells. Our results confirm the occurrence of filaments similar to those of deoxyhb S in deoxygenated normal cells, but in much lower concentration and more irregularly aggregated. This finding suggests that the substitution of glutamate by valine in position 6β does not change the mode of aggregation of deoxyhb into filaments, but rather stabilizes a normal contact between molecules in the deoxy structure, perhaps by promoting the crystallization of the filaments into regular helical fibers of 180 Å diameter. It may be this crystallization that is responsible for the drastic reduction in solubility.

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