Molecular Mechanism of Red Cell "Sickling"

The crumpling of sickled erythrocytes is due to aggregation of the hemoglobin molecules into tubules.

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In deoxygenated blood from human patients with the hereditary disease, sickle-cell anemia, the erythrocytes shrink or crumple into crescentic or holly-leaf shapes. In oxygenated blood the cells assume the normal form of biconcave discs. Over 20 years ago Pauling (*1*) suggested that the abnormal "sickling" behavior might be due to the presence of a chemically different type of hemoglobin molecule which would, upon deoxygenation, aggregate into rods and thereby twist the cell out of shape. In 1950 Perutz and Mitchison (*2*) found that deoxygenated sickle-cell hemoglobin (Hb S) has a much lower solubility than deoxygenated normal hemoglobin (Hb A) or than either type of oxyhemoglobin. Accordingly they suggested that the sickling phenomenon involved actual nonspecific crystallization of the hemoglobin in the susceptible cell. Simultaneously, Harris (*3*) observed spindle-shaped liquid crystals, 1 to 15 microns long, in deoxygenated solution of Hb S.

In 1952 Pauling (*4*) amplified his hypothesis by suggesting, still in a nonspecific way, that Hb S molecules might be able to aggregate or stack together in long chains because of having a complementary surface conformation. Meanwhile, a series of brilliant investigations had shown that each of the four heme groups in the normal Hb A molecule is associated with a polypeptide chain.

The polypeptide chains are of two kinds—one, designated the alpha chain, containing 141 amino acid residues and the other, designated the beta chain, containing 146 residues (*5*). Further, Ingram (*6*) and Hunt and Ingram (*7*) provided evidence that, in Hb S, glutamic acid is replaced by valine at the 6th position in each of the two beta chains. Nevertheless, even as late as 1959 Harris was impelled to write (*8*) that this essentially complete knowledge of the structure of the Hb molecule "does not at present help in the explanation of the abnormal behavior of S hemoglobin or the mechanism of the sickling phenomenon other than to make more probable the hypothesis that hemoglobin polymers are formed through the opposition of a complementary surface." It is the purpose of the present article to present several pieces of experimental evidence leading to a consistent hypothesis explaining how the seemingly minute chemical difference between sickle-cell hemoglobin and normal hemoglobin—namely, two amino acid residues out of 574—can alter the three-dimensional structure of the hemoglobin molecule and thereby cause sickling.

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Scale Model Building

Tentative atomic models of human oxyhemoglobin were built from the x-ray diffraction data of Perutz on horse oxyhemoglobin, summarized by Perutz in 1965 (*9*). Fractional coordinates of amino- and carboxyl- terminal ends of helical and nonhelical segments in horse oxyhemoglobin were combined with the atomic coordinates of sperm whale myoglobin. The nonhelical regions were built by copying the conformation of the corresponding region of a model of sperm whale myoglobin, built to the same scale. As Perutz (*9*) remarked, it is impossible to solve all steric problems by means of such a three-dimensional jig-saw puzzle. However, the hemoglobin model has an accuracy between 1 and 2 angstroms, and it does enable one to draw some tentative conclusions about the general conformation of the hemoglobin unit.

It has been suspected for a long time that the four polypeptide chains are tetrahedrally arranged. It is difficult, however, to see in the model anything resembling a regular tetrahedron. In view of the fact that the *X* and *Z* axes are at an angle of 110 degrees (*10*), it could be postulated that the basic molecular plan is derived from a tetrahedron which has been bisected by a plane parallel to two edges and then rotated upon itself (Fig. 1, *A* and *B*). According to this plan the beta chains, which form a fairly well defined edge in the molecular model, would occupy the lower half. The top half of the model, occupied by the two alpha chains, would be rotated 70 degrees clockwise with respect to the beta chain half. If now the four apexes of the original tetrahedron are truncated (Fig. 1*C*), a surface conformation is obtained which agrees reasonably well with the shape of the molecular model if the surface contours are crudely smoothed by "sanding off" projecting amino acid residues.

To turn now to the special peculiarities of Hb S, three trial amino ends for the beta chain were built, one for Hb A, one for Hb S, and one for Hb C Georgetown, another sickling type of hemoglobin. From steric considerations it appears that in Hb S a hydrophobic intramolecular bond could form between the terminal residue (valyl) of the beta chain and the genetically substituted 6th residue (valyl) in the same chain (Fig. 2). In Hb C Georgetown, essentially the same conformation as that shown in Fig. 2 could occur, but the intramolecular bond would be ionic. In the normal molecule, however, no corresponding bond would form. When the valyl-valyl bond forms in the beta chains, the molecule changes in surface conformation so as to present a projecting "key," represented by the triangles filled with broken lines in Fig. 1*C*. The intramolecular bonds would also allow cyclization from the 1st $-\overset{|}{C}=O$ to the 4th $-\overset{|}{N}-H$ by hydrogen bonding. As the model indicates, the "key" would then fit into a complementary site in the alpha chains of an adjacent Hb S molecule.

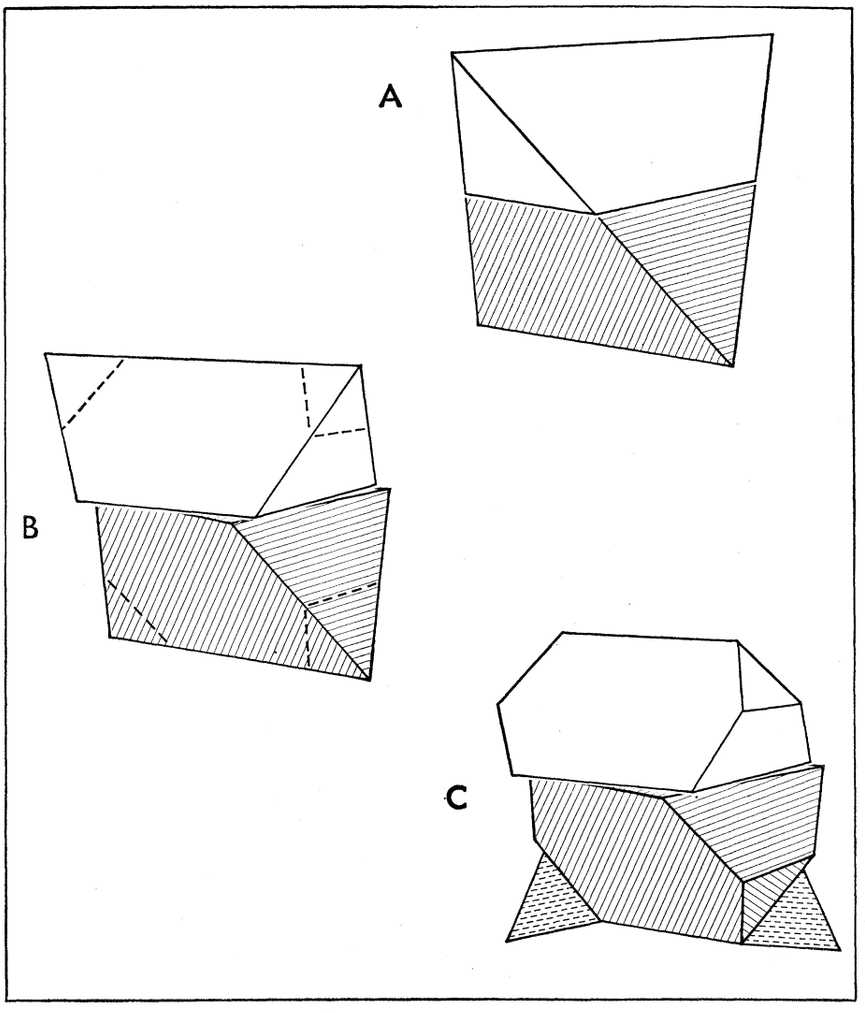

Fig. 1. Derivation of a truncated tetrahedron that resembles the S hemoglobin scale model. (*A*) A regular tetrahedron is bisected by a plane parallel to the edges formed by the hypothetical pair of "alpha chains" (the upper half) and the pair of "beta chains" (the lower half). (*B*) The edge of the upper half is rotated 70 degrees clockwise with respect to the lower edge, as shown here. The apexes are truncated to obtain *C*. (*C*) The triangular tabs are attached for close resemblance to the atomic model of S hemoglobin.

Negative Temperature Coefficient of Gelation

Before the genetic substitution of amino acids in the Hb S molecule was known, it was observed that Hb S solution has a negative temperature coefficient of gelation—that is, the deoxygenated Hb S solution gels at 38°C but melts reversibly when placed in an ice bath (*11*). This observation is consistent with the hypothesis that a hydrophobic bond may be involved in sickling.

Because Marsland (*12*) had shown that protoplasmic gel structures are weakened when high hydrostatic pressures are applied, it was suspected that sickled erythrocyte would "unsickle" under a high hydrostatic pressure. As suspected, sickled erythrocytes do "unsickle" reversibly under pressure of about 200 to 300 atmospheres: they become spherical under a high pressure and then sickle again when decompressed slowly. This observation is also consistent with the slowing of aggregation reaction of poly-L-valyl-ribonuclease by a factor of nearly 10 as the hydrostatic pressure is increased to 300 atmospheres (*13*), as observed by Becker and his co-workers.

Optical Rotatory Dispersion Studies

Sickle-cell hemoglobin solution was studied by optical rotatory dispersion at 0° and 38°C because this method is generally recognized to be one of the best for detecting conformational change

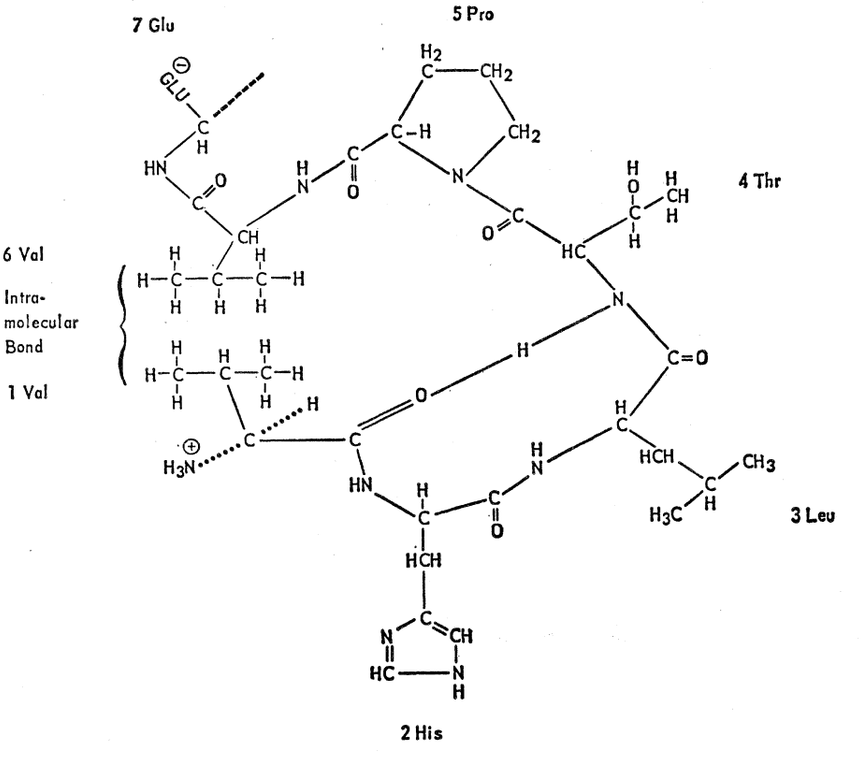

Fig. 2. Schematic representation of the binding site near the amino terminus on the beta chain of sickle-cell hemoglobin. Depicted is a hydrophobic bonding between valyl residues and the hydrogen bonding from the 1st —Ć=O to the 4th —Ń—H. [From *Biochim. Biophys. Acta* **94**, 198 (1965), reproduced with permission]

in optically active molecules. At 0°C the magnitude of the positive Cotton effects at 560 millimicrons and at the Soret band are essentially the same as in Hb A solution. But when deoxygenated Hb S solution is warmed to 38°, the magnitude of rotation is reversibly increased by a factor of about 3. The oxygenated Hb S solution also shows an increase in the amplitude of the positive Cotton effect. The amplitude of the positive Cotton effect in Hb A solution is the same at 38° as at 0°, and the magnitude of rotation at either temperature is about the same as it is for Hb S solution at 0° (*14*). These observations are in accord with the well-known Kauzmann and Eyring (*15*) rule that optical activity is increased by bond restriction and consistent with the postulated valyl-valyl hydrophobic bonding in the beta chains. Since a hydrophobic bond is weaker at lower temperature (*16*), the hypothesis is consistent with cyclization of Hb S molecules at 38° but not at 0°. Since the magnitude of optical rotation of Hb A did not change significantly with temperature, it was concluded that no significant intramolecular change takes place in the normal molecules between 0° and 38°C (*14*).

"Unsickling" by Propane

If the proposed intramolecular hydrophobic bonding between the 1st valyl and the 6th valyl in Hb S (Fig. 2) is essentially correct, then saturating blood containing sickle-cell hemoglobin with propane might cause "unsickling" because the propane would form hydrophobic bonds with the isopropyl groups of the valine side chain residues and thereby block the intramolecular valyl-valyl interactions. Propane does in fact both unsickle and block sickling of erythrocytes containing Hb S molecules. Further, it fails to affect erythrocytes containing Hb C Georgetown, in which an ionic bond has been postulated.

Molecular Orientation in Sickled Erythrocytes

Dichroic-ratio measurements of sickled erythrocytes containing either Hb S or Hb C Georgetown indicate that the plane of the heme groups is oriented essentially parallel to the long axis of the sickled erythrocyte. My associates and I concluded that the resultant extinction coefficient of the tetrahedral array of heme groups is perpendicular to the long axis of sickled erythrocytes (*17*). This deduction is consistent with the molecular model of Hb S.

Magnetic Orientation of Sickled Erythrocytes

Dichroic-ratio measurements seemed interpretable only on the hypothesis that the Hb S molecules in the sickled erythrocytes were preferentially aligned. Pauling and Coryell (*18*) had found that ferrohemoglobin is paramagnetic, whereas oxyhemoglobin and carbonmonoxyhemoglobin are diamagnetic. Hence, if the Hb S molecules are stacked along the long axis of deoxygenated sickled erythrocytes with the heme groups essentially parallel to the long axis of the erythrocytes, the cells ought to orient themselves in a magnetic field with their long axes perpendicular to the lines of force. It was observed that sickled erythrocytes in a magnetic field of 3.5 kilogauss do in fact orient themselves with their long axes perpendicular to the magnetic lines of force, as shown in Fig. 3 (*19*). Normal, human red blood cells do not show any orientation in the magnetic field.

A Stacking Hypothesis of Sickling

The behavior of sickled erythrocytes in polarized light and in a magnetic field strongly suggests that the Hb S molecules are linearly arranged. The structure for the Hb S molecule deduced from model building, and sup-

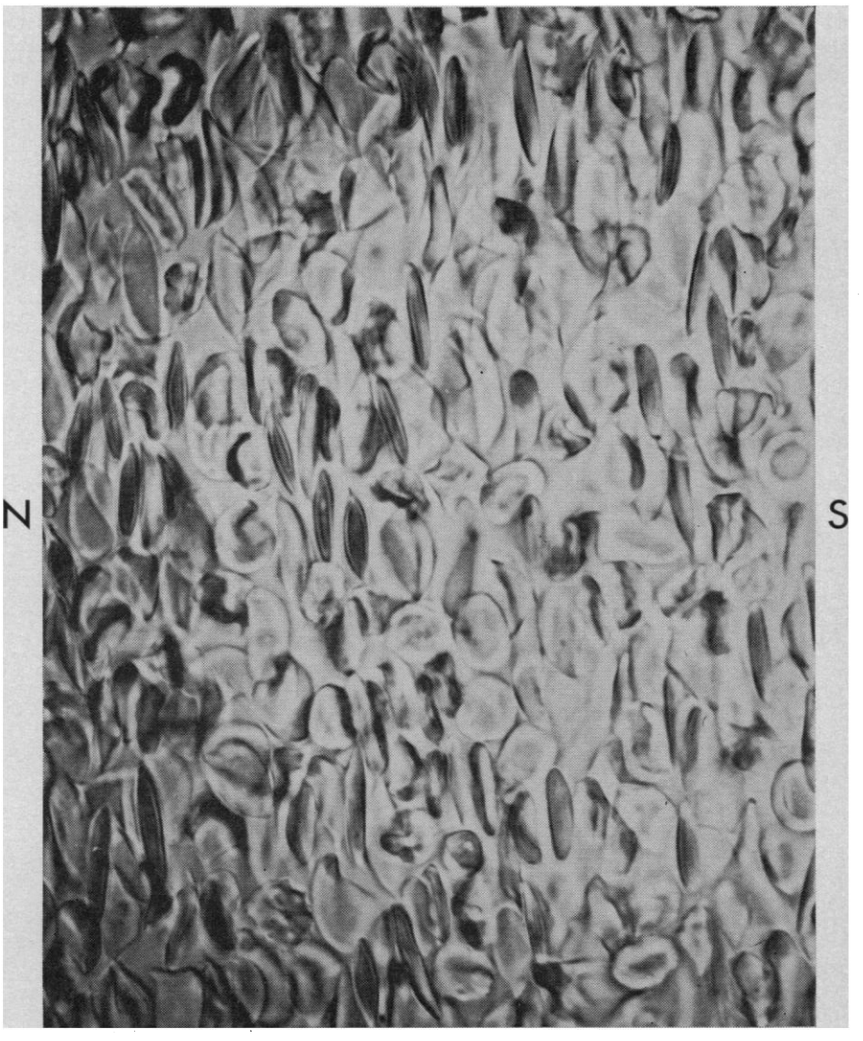

Fig. 3. Magnetically oriented sickled erythrocytes. The magnetic lines of force run from north (*N*) to south (*S*). Sickled erythrocytes are oriented with the long axes perpendicular to the magnetic lines of force.

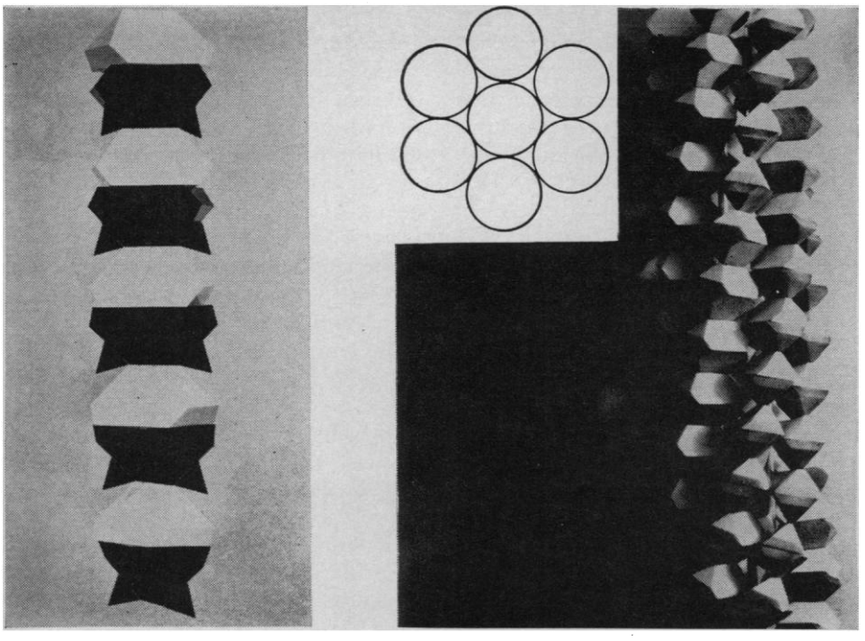

Fig. 4. (Left) Five molecular models of Hb S stacked to represent a segment of a monofilament. Each molecule consists of a pair of alpha chains (light tone) and a pair of beta chains (dark tone). The "key" is symbolized by a small triangle, one per beta chain. Note the offset between the edges of the alpha and beta portions of a molecule. (Right) The six strands of S hemoglobin monofilaments twisted about a "core" to form a cable. Each strand with 18 molecules stacked would be needed to make a complete turn.

ported by the temperature studies, optical rotatory dispersion studies, and propane experiments, provides a possible mechanism for molecular filament formation. The proposed cyclization in Hb S (Fig. 2) forms the "key" in a "lock-and-key" conformation permitting molecules to mate in complementary fashion. Head-to-tail stacking along the dyad axis of symmetry could thus result in an Hb S monofilament (Fig. 4, left). The postulated stacking would also be consistent with the observed behavior of sickle-cell erythrocytes in relation to oxygen. Sickling takes place upon deoxygenation, suggesting that the complementarity between the beta chains and the adjacent alpha chains is lost upon oxygenation. Such a conformation change, in which the beta chains of normal hemoglobin move closer together by as much as 7 angstroms upon oxygenation, was found by Muirhead and Perutz (*20*).

In view of the key position of molecular stacking in the mechanism of sickling proposed, an attempt was made to demonstrate Hb S filaments directly. Hemoglobin S molecular threads were found with the electron microscope (Fig. 5). However, they have diameters of approximately 170 angstroms, whereas the hypothetical monofilament (Fig. 4, left) should have a diameter of the order of 65 angstroms. The micrographs show a central stripe of reduced opacity (Fig. 5, right), suggesting that the Hb S filament is hollow. Steric and dimensional properties of the monofilament show that six strands of S hemoglobin could be twisted together to form a hollow molecular cable of approximately the correct dimensions (outer diameter, 172 ± 11 angstroms; inner diameter, 40 ± 6 angstroms) (Fig. 5, right). This structure is dimensionally similar to the microtubules found in a wide variety of tissues (*21*). Furthermore, the postulated molecular cable provides a basis for the paracrystalline honeycomb array of Hb S found by Chandler Stetson, Jr. (*22*) in ultrathin cross section of sickled erythrocytes. Each cell in this honeycomb was about 150 angstroms across and had a central hole 50 angstroms in diameter.

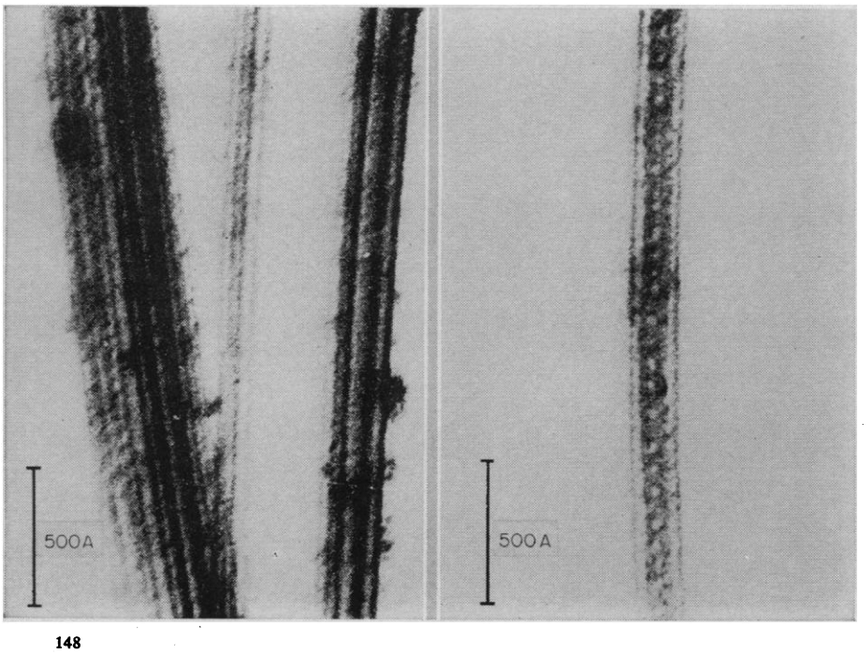

Fig. 5. (Left) Electron micrograph of S hemoglobin "microtubules." (Right) Electron micrograph of S hemoglobin "microtubule" interpreted as a hollow small tubule. The outer diameter of the "microtubule" is 170 angstroms and the inner diameter is 40 angstroms.

Summary

Precision scale models of sickle-cell hemoglobin molecules indicate that the genetic substitution of valine for glutamic acid at the 6th position in the two beta chains allows an intramolecular hydrophobic bond to form. This changes the conformation in such a way as to allow molecular stacking. Optical rotatory dispersion studies and the results of subjection of Hb S solution to temperature change and to propane are consistent with the presence of such a bond. Examination of sickled erythrocytes in a magnetic field and in polarized light indicates that the Hb S molecules are aligned *in situ*. Filaments interpreted as hollow cables of six Hb S monofilaments have been demonstrated by electron microscopy.

Experimental Procedure

Hemoglobin S solution was purified as described elsewhere (*11*). A 2-percent solution was deoxygenated in a 50-milliliter round-bottomed flask with a cuvette attached at the bottom, similar to the flask described by Smith (*23*). The solution was alternately deoxygenated under reduced pressure (by an aspirator) and refilled with carbon dioxide (gas from Dry Ice placed in a stoppered suction flask) to pressure of 1 atmosphere. A recording spectrophotometer (Cary model 14) was used to determine the degree of deoxygenation. When completely deoxygenated, the contents of the flask exhibited a flow birefringence. The preparation of electron-microscopic grids was carried out inside a dry box with blocks of Dry Ice placed in it to maintain an anaerobic and anhydrous atmosphere. A small drop of Hb S solution (10^{-2} percent) was placed on each carbon-coated grid and then frozen, on a block of brass pre-cooled on Dry Ice; then the brass block, with grids, was placed in a desiccator and dried from the frozen state under reduced pressure.

Some of the grids were stained with potassium phosphotungstate (*p*H 7.2) for negative contrast or stained positively with uranyl acetate (2 percent), and some were shadowed by platinum vapor by means of a conventional technique. The grids were examined under an electron microscope (RCA EMU-3G).

In ten purified specimens of Hb S, each from a different donor, very small tubules were found in all instances. Micrographs yielding many details of the "microtubules" were obtained in certain uranyl acetate preparations where the supporting film had ruptured. In such preparations the electron beam was focused on an area adjacent to the small tubule under study, then the tubule was quickly moved into the field for photography.

For calibration, Indanthrene Olive TWP crystals (*24*) with molecular spacing of 24.9 angstroms, as determined by x-ray diffraction, were used.

References and Notes

1. L. Pauling, *Harvey Lectures Ser.* **12(1953-54)**, 216 (1955).
2. M. F. Perutz and J. M. Mitchison, *Nature* **166**, 677 (1950).
3. J. W. Harris, *Proc. Soc. Exp. Biol. Med.* **75**, 197 (1950).
4. L. Pauling, *Amer. Phil. Soc.* **96**, 556 (1952).
5. G. Braunitzer, R. Gehring-Muller, N. Hilschmann, K. Hilse, G. Hobson, V. Rudloff, B. Wittmann-Liebold, *Z. Physiol. Chem.* **325**, 283 (1961); W. Konigsberg, G. Guidotti, R. J. Hill, *J. Biol. Chem.* **236**, PC 55 (1961); J. Goldstein, W. Konigsberg, R. J. Hill, *ibid.* **238**, 2016 (1963).
6. V. M. Ingram, *Nature* **180**, 326 (1957).
7. J. A. Hunt and V. M. Ingram, *ibid.* **184**, 640 (1959).
8. J. W. Harris, in *Progress in Hematology*, L. M. Tocantins, Ed. (Grune & Stratton, New York, 1959), vol. 2, p. 47.
9. M. F. Perutz, *J. Mol. Biol.* **13**, 646 (1965).
10. ———, *Nature* **149**, 491 (1947).
11. M. Murayama, *J. Biol. Chem.* **228**, 231 (1957).
12. D. Marsland, *Intern. Rev. Cytol.* **5**, 199 (1956).
13. M. S. Kettman, A. H. Nishikawa, R. T. Morita, R. R. Becker, *Biochem. Biophys. Res. Commun.* **22**, 262 (1966).
14. M. Murayama, *Nature* **194**, 933 (1962).
15. W. Kauzmann and H. Eyring, *J. Chem. Phys.* **9**, 41 (1941).
16. H. A. Scheraga, in *The Proteins*, H. Neurath, Ed. (Academic Press, New York, 1965), vol. 1, p. 522.
17. M. Murayama, R. A. Olson, W. H. Jennings, *Biochim. Biophys. Acta* **94**, 194 (1965).
18. L. Pauling and C. D. Coryell, *Proc. Nat. Acad. Sci. U.S.* **22**, 159 (1936).
19. M. Murayama, *Nature* **206**, 420 (1965).
20. H. Muirhead and M. F. Perutz, *ibid.* **199**, 633 (1963).
21. D. B. Slautterback, *J. Cell Biol.* **18**, 367 (1963); M. C. Ledbetter and K. R. Porter, *Science* **144**, 872 (1964); D. W. Fawcette and F. Witebsky, *Z. Zellforsch. Mikroskop. Anat. Abt. Histochem.* **62**, 785 (1964); S. I. Wolve, *J. Cell Biol.* **25**, 408 (1966).
22. C. A. Stetson, Jr., *J. Expt. Med.* **123**, 341 (1966).
23. M. H. Smith, *Nature* **192**, 722 (1961).
24. L. W. Labaw, private communication.

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NEWS AND COMMENT

NIH: Demand Increases For Applications of Research

At the White House on 15 June, President Johnson discussed Medicare with influential medical and hospital leaders in a meeting that combined elements of a progress report and a pep rally. The President has taken a personal, almost paternal, interest in Medicare, and he and other federal officials were concerned over the possibility that a sizable number of hospitals in the South might be barred from participating in Medicare by failure to comply with civil rights legislation which applies to the program.

The meeting and the President's appeal for unstinting support were not surprising. What was unexpected was the President's announcement—obviously not an offhand remark—that he was calling a meeting of top officials in the health and medical research hierarchy of the administration to reason together on ways in which the results of federally supported biomedical research can be applied more widely, rapidly, and effectively.

These remarks received little notice in the press at the time, but they generated shock waves in the upper reaches of the federal health and medical research establishment.

Mentioned specifically by the President was the National Institutes of Health. NIH in Bethesda has a billion-plus annual budget, administers the largest complex of laboratories and clinics involved in health research in the world, and through its extramural programs now finances about 40 percent of national expenditure on biomedical research.

In his statement at the meeting the President made it clear that he expects NIH to do more applied or developmental research. What the President was driving at is clear in the following excerpt from the text of his remarks, released by the White House.

I am calling, very shortly, a meeting (I want to serve notice on Secretary Gardner publicly because I don't want to give him a chance to object privately) of the Director of the National Institutes of Health and the directors of the nine individual institutes as well as the Surgeon General of the Public Health Service. I