A New Understanding of Sickle Cell Emerges

X-ray, kinetic studies paint a comprehensive picture of sickle cell disease to the level of atomic interactions

The last decade, and particularly the last 5 years, have been a very fruitful period for research on sickle cell disease. For the first time, it is now possible to paint a comprehensive picture of the molecular events that lead to the chronic anemia, severe, painful crises caused by blockage of blood flow, and, often, death that characterize this affliction. More is known about the genesis of sickle cell disease than any other human illness, says Alan N. Schechter of the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD). Some 30 years ago, Nobel laureate Linus Pauling coined the term "molecular disease" to describe sickle cell and similar afflictions because their causes were understood on a molecular level. Today, says William A. Eaton of NIAMDD, sickle cell can be considered "the first atomic disease" because it is becoming understood at the level of atomic interactions.

Sickle cell disease takes its name from the characteristic sickled shape assumed by some affected red blood cells (erythrocytes) when they are deoxygenated. Most such cells, however, simply assume distorted shapes and become fairly rigid. Since erythrocytes must be flexible enough to pass through capillary blood vessels smaller than they are, those that become rigid tend to wedge in the capillaries and block blood flow. Surprisingly, relatively little is known about why different organs are affected by this blockage, about the normal age of onset of the disease, about sex differences, and so forth. Most of the available data, says Wendell F. Rosse of the Duke University Medical Center, "are anecdotal, retrospective, and lack statistical validity." Last year, therefore, the National Heart, Lung, and Blood Institute launched a 5-year, prospective study of 3500 sickle cell patients at 23 hospitals around the country. The study, coordinated by Rosse, is designed to determine the natural history of the disease by clinical evaluation of victims.

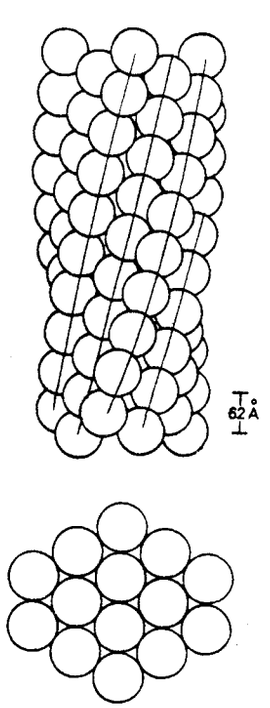
The sickle cell defect occurs in hemoglobin, the oxygen-carrying constituent of erythrocytes. All adult hemoglobins are composed of four polypeptide chains, which form the hemoglobin tetramer: two α chains, each with 141 amino acids, and two non-α chains, each with 146 amino acids. Each chain surrounds an iron porphyrin molecule to which oxygen can be reversibly bound. In the healthy adult, 96 to 97 percent of the non-α chains are β chains, which combine with α chains to form hemoglobin A (HbA). The remainder are δ chains, which differ from the β at ten amino acid residues, and γ, which differ at 39 residues. Hemoglobin containing γ chains (HbF) is found primarily in fetuses; hemoglobin containing either δ or γ chains is essentially normal.

Sickle cell hemoglobin (HbS) differs from normal hemoglobin in only one amino acid on each β chain. Vernon M. Ingram of the Massachusetts Institute of Technology demonstrated in 1956 that the defect involves substitution of a valine residue for the normal glutamic acid residue at the sixth position of the β chain, yielding a loss of two negative charges per hemoglobin tetramer. In 1977, Sherman Weissman, Bernard Forget, and their colleagues at Yale University sequenced messenger RNA that codes for normal β chain and found that the glutamic acid at the 6 position is coded for by the ribonucleotide triplet GAG (G, guanine; A, adenine). Conversion of the single base adenine to uracil would produce the messenger codon for valine, but the presence of such an abnormal codon has not yet been demonstrated in either messenger RNA or cellular DNA.

The mutant gene coding for HbS occurs on one chromosome in about 9.5 percent of American blacks and in as many as 20 percent of black Africans; it is also present in many individuals from Mediterranean countries. Individuals with the mutant gene from only one parent are said to have sickle cell trait. About 40 percent of the hemoglobin in such individuals is HbS. There are no ill effects arising from the substitution of this amount of HbS except under the most unusual of circumstances. In fact, the mutation appears to provide some protection against malaria (see box). Sickle cell disease occurs when the individual inherits the mutant gene from each parent, so that virtually all the hemoglobin is HbS. There are about 50,000 such individuals in the United States and a correspondingly higher number in Africa.

In 1951, Max F. Perutz of Cambridge University demonstrated by low-resolution x-ray crystallography that the structures of oxygenated HbS and HbA are "indistinguishable"; he and others also found that the solubilities and oxygen affinities in dilute solution of the two hemoglobins are virtually identical. Investigators were puzzled by these findings and, in the words of one of the investigators, the field "languished" for nearly 15 years.

In the mid-1960's, several investigators, including Makio Murayama of NIAMDD, Jack F. Bertles of St. Luke's Hospital in New York City, the late Chandler A. Stetson of New York University, and James G. White of the University of Minnesota, began to observe by electron microscopy ordered arrange-



The HbS polymer

Most investigators now believe that 14 strands of HbS combine to form a long fiber.

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ments of hemoglobin molecules in sickled erythrocytes or solutions of HbS that had polymerized. In 1973 Bertles and Beatrice Magdoff-Fairchild, also of St. Luke's, obtained the first x-ray diffraction patterns of polymerized HbS. Several different structures have since been proposed for the polymers.

Three years ago, using new, more sophisticated techniques for image reconstruction in electron microscopy, Stuart H. Edelstein and his colleagues at Cornell University suggested a 14-stranded model, a densely packed structure in which ten strands of HbS polymer surround a core of four strands. More recently, Robert Josephs of the University of Chicago has proposed a 16-stranded model, but most investigators still think the 14-stranded structure is correct.

Meanwhile, in 1972, Barry C. Wishner, working in the laboratory of Warner E. Love of Johns Hopkins University, grew high-quality crystals of deoxygenated HbS for x-ray studies. In a series of papers since then, Wishner, Love, and their colleagues have shown that the basic unit of the polymer is a double-stranded fiber in which the β^6 valine of one HbS molecule is hydrophobically bonded to a specific region of an adjacent tetramer. Only one of the two β^6 valines in each tetramer participates in this intermolecular hydrophobic bonding; all other bonding between tetramers involves amino acids that are present in both HbS and HbA. The double-stranded polymer then becomes part of the 14- (or 16-) stranded fiber. The fact that deoxygenated HbA does not polymerize even at very high concentrations indicates that the bonds involving the β^6 valine contribute a substantial fraction of the energy of polymer formation.

The relevance of the crystal structure to conditions within the erythrocyte was suspected for some time. Recently, however, Magdoff-Fairchild has shown by x-ray diffraction studies of gels within erythrocytes that the same spatial relationships exist. Furthermore, she has found that the polymers are slowly converted to crystals with only a very small change in the x-ray diffraction pattern, indicating that the two different structures are very similar.

Another line of evidence is provided by Ronald Nagel and Robert Bookchin of the Albert Einstein College of Medicine. They studied the polymerization of 30 β-chain mutants of HbS whose amino acid sequences are known. Twenty of the 30 had no effect on polymerization; the mutant amino acid residues in each of these are not in any of the contact regions. Ten of the mutant hemoglobins did interfere with polymerization; in nine of these, the mutant amino acid is in a contact region observed in Wishner's crystals, and the tenth is on the edge of a contact region. Similarly, Reinhold Benesch and Ruth Benesch of Columbia University have studied α chain mutants of HbS. They have found three such mutants that interfere with polymerization, and in each case the mutant amino acid is in a contact region.

These results together, Love says, provide "as tightly nailed a coffin as you can get" proving that the contact regions identified in the crystals are those that occur in sickled erythrocytes. The relation between polymer formation and cell

Why Does Sickle Trait Persist?

Because of the high mortality and disability rates among people who receive the sickle cell gene from both parents, the frequency of appearance of the mutant gene would be expected to decline in any gene pool. The fact that the incidence has remained stable at about 20 percent in African populations suggests that the sickle cell gene provides some benefit. Several possible explanations have been offered, but the most likely, first advanced in 1949 by several investigatons, is that sickle cell trait provides some protection against *Plasmodium falciparum*, the parasite responsible for the most severe form of malaria. Much clinical evidence to support this hypothesis was developed by Anthony C. Allison of the Medical Research Council in London and Lucio Luzzatto of the International Institute for Biophysics and Genetics in Naples, but the mechanism remained a mystery until recently.

The key breakthrough occurred in 1976 when William Trager and James Jensen of Rockefeller University and J. David Haynes and his colleagues at the Walter Reed Army Institute of Research independently developed techniques for growing *P. falciparum* in culture (*Science*, 22 April 1977, p. 413). Milton J. Friedman of the University of California at San Francisco, who was then at Rockefeller, quickly found that the parasite would grow as well in cells containing oxygenated sickle cell hemoglobin as in cells containing normal hemoglobin. He also found, however, that when the sickle cell cultures were deoxygenated, parasites within cells that sickled were killed, probably by disruption of their metabolism.

Previously, Luzzatto had obtained erythrocytes from individuals with both sickle cell trait and malaria, and observed that the presence of the parasite caused the cells to sickle faster. Following up on this observation, Friedman found that the parasite lowers the *p*H of an infected cell by about 0.4 *p*H units; this decrease, under the proper conditions, increases sickling about 20-fold. About one-third to halfway through the 48-hour reproductive cycle of *P. falciparum*, infected cells attach themselves to blood vessel walls in a low oxygen environment. Under such conditions, about 2 to 4 percent of sickle trait cells will normally sickle. Because of the lowered *p*H produced by parasite infection, however, about 40 percent of the infected cells sickle, killing in the process the parasites they contain. The effect of the mutant gene is thus not completely protective, but it does lessen the severity of the disease and may be sufficient to prevent death.

Interestingly, using recombinant DNA techniques, Yuet Wai Kan and Andree M. Dozee of the University of California at San Francisco (who have used similar techniques to develop a safe, effective prenatal test for sickle cell disease) have shown that there are two distinct sickle genes that apparently arose from independent mutations in malaria-prone areas. One gene, contained in a DNA fragment of about 13,000 base pairs, arose in West Africa and spread through North Africa into Mediterranean populations. The second, contained in a fragment about 7,600 base pairs in length, originated in East Africa and spread to the Middle East and Asia. Most American blacks have the gene in the 13,000 base pair fragment. Investigators agree that since the gene provides no protective effect for American blacks, its incidence in the gene pool will decline, albeit very slowly.

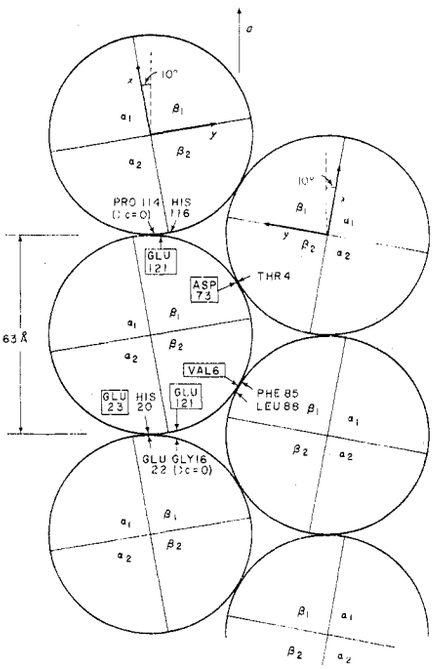
—T.H.M.

sickling is less clear, but the simplest explanation is that the growing polymer merely distorts the shape of the cell, causing tenting of the membrane. There are about 270 million HbS molecules in each erythrocyte, Schechter says, and the free energy released during polymerization should be more than enough to deform the cell. Some investigators, however, think that there are also secondary effects on erythrocyte membranes that contribute to sickling. In any case, the new understanding of the interactions in the contact regions provides a new approach to therapy. It should be possible, says Schechter, to design oligopeptides or other agents that will bind specifically in the contact region, disrupting contact between HbS tetramers. This approach will be discussed in a second article on new therapeutic approaches.

Another area that has undergone major developments in recent years is the study of the kinetics of HbS polymer formation. This has been achieved primarily by Eaton, James Hofrichter, and Philip D. Ross of NIAMDD, but also by Jacinto Steinhardt of Georgetown University and others. They have found that the behavior of HbS in solution can explain many aspects of its behavior in erythrocytes.

If, for example, a concentrated solution of deoxygenated HbS (about 25 grams per 100 milliliters) is cooled to 0°C, the individual hemoglobin tetramers remain separated. When the solution is warmed to about 20°C, however, there is a variable delay period followed by a rapid, autocatalytic polymerization of HbS into the long fibers characteristic of sickled cells. The delay period can vary from milliseconds to days, depending upon such physiological parameters as HbS concentration, fractional saturation with oxygen, *p*H, and the fraction of HbA or HbF, but it is highly reproducible for any particular set of conditions. Recent experiments by Frank A. Ferrone, Eaton, and Hofrichter suggest that the delay time is the time required to form nuclei large enough to permit thermodynamically favorable growth of polymers.

These observations led Eaton and Hofrichter to formulate what is known as the kinetic hypothesis of sickle cell disease. In the arterial system, erythrocytes contain an oxygenated solution of HbS. As the cell squeezes through a narrow capillary to reach the venous system, it releases its oxygen to the tissues. Eaton and Hofrichter have observed that the delay time for HbS under normal physiological conditions is of the same order of magnitude as the time required for the erythrocyte to pass through the capillaries. Under normal conditions, then, most cells will sickle after they leave the capillaries, and will regain normal flexibility upon reoxygenation in the lung.



Intermolecular interactions in HbS

The HbS molecule is represented as a sphere divided into four quadrants, one for each chain.

Under conditions of stress, however, oxygen is released from the erythrocytes sooner and polymerization may occur while the cell is still in the capillary. No longer flexible, the cell blocks the capillary and oxygen deprivation and tissue damage ensue. Simply stated, then, the kinetic hypothesis argues that the delay time of intracellular polymerization, relative to the capillary transit time, is the critical variable determining clinical severity in sickle cell disease.

There is, says Eaton, a strong correlation between the delay time of HbS solutions in vitro and clinical severity of the disease. Factors that shorten the delay time in vitro, such as higher acidity (acidosis), increased concentration of HbS (dehydration), and increased temperatures (fever), may precipitate sickle crises. Increased concentrations of HbA or HbF, in contrast, can produce large increases in delay time in vitro, and the presence of these hemoglobins is associated with decreased clinical severity.

In an effort to quantify the effects of delay time, Eaton, Hofrichter, and Helen Sunshine examined the delay times of hemoglobins from three naturally occurring conditions: sickle cell trait, sickle cell disease with hereditary persistance of fetal hemoglobin (S/HPFH), and sickle cell combined with β^+ thalassemia (S/β^+ thalassemia). In this thalassemia, HbS accounts for about 70 to 85 percent of total hemoglobin, the clinical course of the disease is less severe than that of normal sickle cell, and the in vitro delay time is increased by a factor of 10 to 100. In S/HPFH, HbS accounts for 70 to 80 percent of total hemoglobin, the clinical course of the disease is much less severe, and the delay time is increased by a factor of 10^3 to 10^4. And in sickle cell trait, HbS accounts for only 40 percent of total hemoglobin, there is no disease, and the delay time is increased by a factor of 10^6. Thus, the threshold for a therapeutic effect in sickle cell disease would result from a method that increases in vitro delay time by a factor of 10 to 100. An increase of 10^3 to 10^4, says Eaton, should produce a major therapeutic effect, and an increase of 10^6 would produce a "cure."

These findings help to explain some previously confusing therapeutic results. Many investigators, for example, had once thought that urea might be an effective therapeutic agent because it readily disrupts hydrophobic bonding. A major clinical trial, however, finally proved that this is not the case. Eaton, Hofrichter, and Sunshine have demonstrated that the concentration of urea used in therapy increases the delay time by only a factor of 2, insufficient for a therapeutic effect. Phenylalanine is a much more effective inhibitor of polymer formation than urea, but the concentration required to produce a 10- to 100-fold increase in the delay time is known to be toxic to very young children. Cyanate is particularly interesting, says Eaton; at the concentrations used orally in patients, it increases delay time by a factor of 7 to 30. Such an effect, he says, should correlate with some clinical improvement, but not with either a major clinical effect or no effect at all. That is precisely what was observed in clinical trials before they were stopped because of toxic side effects.

As indicated by these results, studies of polymerization provide an important new way to study the efficacy of potential new therapeutic agents without exposing patients to risk. In the past year or two, in fact, most laboratories studying therapeutic agents have adopted some variant of the kinetic studies to measure efficacy. This approach should make it easier to screen large numbers of compounds for their potential effects and to discard those which show little or no promise.—THOMAS H. MAUGH II