Abnormality of Hemoglobin Molecules in Hereditary Hemolytic Anemias

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ABNORMALITY OF HEMOGLOBIN MOLECULES IN
HEREDITARY HEMOLYTIC ANEMIAS*

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Twenty years ago, after having worked for a decade on the
determination of the structure of relatively simple inorganic
and organic molecules, I became interested in hemoglobin. This
interest arose from the consideration of the structural origin of the
sigmoid oxygen equilibrium curve. It was soon extended to include
the denaturation of hemoglobin and other proteins and the mag-
etic properties of hemoglobin and its derivatives. The study of
magnetic properties has been especially fruitful in providing in-
formation about the nature of the bonds formed by the iron atoms
in hemoglobin with the neighboring atoms of the porphyrin ring
system, the globin, and attached molecules such as the oxygen
molecule.

The discovery of the abnormal hemoglobins was the result of
the consideration of hypothetical molecular mechanisms of the
disease. 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 B. Castle, Professor
of Medicine in Harvard University, mentioned to the other mem-
bers of the Committee the disease sickle-cell anemia, with which
he had had some experience. He told about the discovery of the
disease by Dr. J. B. Herrick, in 1910,17 and described the character-
istic change in shape of the red 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-
Hasselmach effect (it had in fact been clearly stated by Hahn and
Gillespie in 1927 that sickling occurs only when the partial pres-

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sure 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 contain an abnormal kind of hemoglobin, which when deoxygenated has the power of combining with itself into long rigid rods, which then twist the red cell out of shape. The opportunity to test this idea arose when Dr. Harvey A. Itano came to the California Institute of Technology, in the fall of 1946. He had been a student of Professor Edward A. Doisy, of St. Louis University School of Medicine, where Dr. Itano had received his M.D. degree in 1945. Dr. Doisy suggested that he work with me, and the opportunity for doing so arose in the course of his year as an intern, when he was awarded an American Chemical Society Predoctoral Fellowship in Chemistry, for the three years 1946 to 1949. In a letter to Dr. Itano I suggested that he investigate the hemoglobin from the red cells of sickle-cell-anemia patients, in order to see whether it was different from normal adult human hemoglobin. On his arrival in Pasadena in September, 1946, he began this investigation. He verified the published report \textsuperscript{15} that carbonmonoxyhemoglobin, like oxyhemoglobin, prevents sickling of the red cells, and found that some other hemoglobin derivatives, including alkyl isocyanide-ferrohemoglobin, ferrichemoglobin, ferrihemoglobin azide, and ferrihemoglobin cyanide similarly prevent sickling. He developed a rapid diagnostic test for sickle-cell anemia and sickle-cell trait, based on the use of a chemical reducing agent.\textsuperscript{16} Most of the properties of the hemoglobin from the blood of sickle cell-anemia patients were found to be the same, to within the error of determination, as those of hemoglobin from normal individuals, but it was finally clearly shown, by careful measurement of electrophoretic mobility, that the blood of the patients contains nearly 100 per cent of an abnormal hemoglobin, differing from normal adult human hemoglobin, and that the blood of the parents of patients contains an approximately half-and-half mixture of the abnormal hemoglobin and normal adult human hemoglobin.\textsuperscript{17} This electrophoretic work was carried out with the collaboration of Dr. S. J. Singer and Dr. Ibert C. Wells.
THE INHERITANCE OF SICKLE-CELL ANEMIA

The electrophoretic patterns reported in the first publication\textsuperscript{30} are shown in Fig. 1. They were made by electrophoresis for 20 hours at a potential gradient of 4.73 volts per centimeter of solutions of carbonmonoxyhemoglobins in phosphate buffer of 0.1 ionic strength and pH 6.90. The peaks \( \alpha \) and \( \beta \), representing normal hemoglobin and hemoglobin from the red cells of patients with sickle-cell anemia, are single peaks, corresponding in each case to an electrophoretically homogeneous material. The electrophoretic mobilities are different for the two hemoglobins; in fact, at this pH the molecules of normal hemoglobin move toward the anode, showing that they have a negative electric charge, and those of sickle-cell-anemia hemoglobin move toward the cathode, showing that they have a positive charge. The isoelectric points in phosphate buffer of ionic strength 0.1 were found to be 6.87 (in pH units) for normal adult human carbonmonoxyhemoglobin and 7.09 for sickle-cell-anemia hemoglobin. The difference between these values is nearly the same as that between the observed values 6.68 for normal ferrohemoglobin and 6.91 for sickle-cell-anemia ferrohemoglobin.

![Fig. 1. Longsworth scanning diagrams of carbonmonoxyhemoglobin in phosphate buffer of 0.1 ionic strength and pH 6.90, taken after 20 hours electrophoresis at a potential gradient of 4.73 volts per centimeter.](image)
The electrophoretic diagram of a solution containing a mixture of normal carbonmonoxyhemoglobin and sickle-cell-anemia carbonmonoxyhemoglobin, in equal amounts, is shown as d in Fig. 1, and that of hemoglobin from the red cells of a parent of a patient is shown as c. The blood from which this hemoglobin was obtained showed the characteristic properties of sickle-cell trait (sicklemia); the cells could be made to sickle, on removal of oxygen, but less readily than the cells of a sickle-cell-anemia patient. It is seen that the sicklemic hemoglobin is a mixture of two hemoglobins, presumed to be normal adult human hemoglobin and sickle-cell-anemia hemoglobin, with the normal hemoglobin present in an amount somewhat greater than 50 per cent.

The indication of a genetic basis for the sickling of erythrocytes had been recognized by Emmel in 1917; and Taliaferro and Huck, at a time when the distinction between sicklemia and sickle-cell anemia was not clearly understood, suggested that a single dominant gene was involved. The inheritance of sickle-cell disease was then clarified by Neel, who in 1947 had suggested "that there is present in the colored population a certain factor which, when heterozygous, may have no discernible effect, but usually results in sickling, and, when homozygous, tends to result in sickle cell anemia." In 1949 he reported that every one of 42 tested parents of children with sickle-cell anemia was found to be sicklemic, their blood containing red cells which could be made to sickle, though less readily than that of the sickle-cell-anemia patients. He concluded that sickle-cell anemia is the result of the homozygous condition of the sickle-cell gene, and sicklemia the result of the heterozygous condition. Beet arrived at the same conclusion independently and almost simultaneously. The electrophoretic patterns shown in Fig. 1 had permitted this inference to be drawn before Neel's paper and Beet's paper were published. Moreover, the gene responsible for the sickling process could be identified with an alternative pair of alleles of which neither one is recessive or dominant, one allele being responsible for a part of the process of manufacture of normal adult human hemoglobin, and the other for the manufacture of sickle-cell-anemia hemoglobin. The fact that all the red cells of a sicklemic individual can be made to sickle by removal of oxygen shows that the cells are not of two
classes, one containing normal hemoglobin and the other abnormal hemoglobin, but that each cell contains a mixture of the two kinds of hemoglobin. The presence of a larger amount of normal than of abnormal hemoglobin in the blood of sicklemic individuals indicates that the process of manufacture of the abnormal hemoglobin is somewhat less efficient than that of normal hemoglobin. It was suggested in the first paper on sickle-cell anemia hemoglobin that the two genes in the heterozygous individual might compete for a common substrate in the synthesis of two different enzymes essential to the production of the two different hemoglobins, or that competition for a common substrate might occur at a later stage in the series of reactions leading to the synthesis of the two hemoglobins themselves.

An investigation of the amount of abnormal hemoglobin in the blood of sicklemic individuals was carried out by Wells and Itano, who found, using the electrophoretic method, that the amount of sickle-cell anemia hemoglobin varied from 24 per cent to 45 per cent in 42 individuals with sickleemia. Neel, Wells, and Itano reported a study of 32 sicklemic individuals who were members of 7 Negro families, comprising 74 individuals altogether. The amounts of abnormal hemoglobin, ranging from 22.3 per cent to 45.2 per cent, showed significant differences between family means. A postulate to explain the apparent inheritance of a factor determining the amount of abnormal hemoglobin in sicklemic blood was made by Itano. He suggested that the differences can be attributed to differences in the rate of synthesis of normal hemoglobin, and that the evidence requires that there be at least three rate-determining modifications of the mechanism of synthesis of normal hemoglobin.

Additional contributions to the problem of the genetics of normal and abnormal hemoglobins have been made by Neel and other workers.

The Properties of Sickle-Cell-Anemia Hemoglobin

Sickle-cell anemia hemoglobin is closely similar to normal adult human hemoglobin in most of its properties. The two proteins have approximately the same sedimentation and diffusion constants,
and hence nearly the same molecular weights. The acid-base titration curves of both hemoglobins in the neighborhood of neutrality are linear, a change of 1 pH unit of the solution being associated with the change in charge of the hemoglobin of about 13 electronic charges per molecule. The normal molecule has about three more negative charges than the abnormal molecule in this region. In the search for the structural basis for this difference samples of porphyrin dimethyl esters were prepared from the two hemoglobin, and the samples were shown by their x-ray powder photographs and by identity of their melting points and mixed melting points to be identical. The difference in structure was hence attributed to a difference in the globins.

An investigation by Schroeder, Kay, and Wells of the amino acid composition of normal adult human hemoglobin (from normal Negro individuals) and sickle-cell-anemia hemoglobin gave results indicating that the hemoglobins do not differ with respect to their content of basic and acidic amino acids; the investigators concluded that sickle-cell-anemia hemoglobin probably contains slightly less leucine and more serine than normal hemoglobin, and possibly less valine and more threonine. These amino acids do not contribute directly to the net charge of the proteins, but they might affect the folding or coiling of the polypeptide chains in such a way as to change the acid or basic constants of other groups. Havinga investigated the phosphorus content, optical rotation, ease of separation of hemes and globin, and number of terminal amino acid residues of normal adult human hemoglobin and sickle-cell-anemia hemoglobin, and found no significant differences between the two proteins. Globins carefully prepared from the two hemoglobins were investigated electrophoretically by Havinga and Itano and found to have the same difference in electrophoretic mobility as the hemoglobins themselves. On denaturation by treatment with 4 N guanidinium chloride for 1 hour at 4°C and removal of the guanidinium chloride by dialysis, the globins were found to have increased markedly in heterogeneity, and to have essentially the same electrophoretic properties. These results indicate that the normal and abnormal hemoglobin molecules might be composed of the same polypeptide chains, folded, however, in different ways, and that on denaturation with guanidinium ion the
resulting denatured proteins have the same complex of configurations. The interesting possibility exists that the gene responsible for the sickle-cell abnormality is one that determines the nature of the folding of polypeptide chains, rather than their composition.

It was pointed out by Sherman in 1940 that sickled red cells are observed under the polarizing microscope to be birefringent, whereas normal cells are optically isotropic. Ponder suggested, on the basis of this observation, that in sickled cells the hemoglobin molecules assume an orderly or paracrystalline arrangement, which is responsible for the sickling. A detailed mechanism of the sickling process was suggested in the first paper on sickle-cell-anemia hemoglobin, 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 hemes of the two hemoglobins that are different. Let us propose 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 partial pressures of oxygen and carbon monoxide are low suggests that one of these 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 erythrocyte's 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 iron atoms of the hemes."

A more detailed discussion of the effect of oxygen was made possible by the results of an investigation of the combination of hemoglobin with alkyl isocyanides. It was found that ethyl iso-
cyanide, isopropyl isocyanide, and tertiary butyl isocyanide differ greatly in their combining powers with hemoglobin, although they have essentially the same combining power with heme; and this fact was interpreted as showing that the four hemes in the hemoglobin molecule are buried within the globin. "Our postulate provides an obvious explanation of the action of oxygen in preventing the sickling of sickle-cell-anemia erythrocytes. We have visualized the sickling process as one in which complementary sites on adjacent hemoglobin molecules combine. It was suggested that erythrocytes containing oxyhemoglobin or carbonmonoxyhemoglobin do not sickle because of steric hindrance of the attached oxygen or carbon monoxide molecule. This steric-hindrance effect might be distortion of the complementary sites through forcing apart of layers of protein, as is suggested by the isocyanide experiments."

Substantiation of this picture was soon obtained through microscopic investigations. Rebuck, Sturrock, and Monaghan, substantiating the work of Sherman, observed that in the early stages of sickling the intracellular hemoglobin forms anisotropic aggregates, suggestive of incipient crystallization. Perutz and Mitchison, at the suggestion of Dr. C. A. Stetson of the Rockefeller Institute, compared the dichroism of sickled cells and hemoglobin crystals, and additional studies of the same sort were reported by Perutz, Liquori, and Eirich. These investigators found that the dichroism of the sickled cells corresponds to an orientation of the hemoglobin molecules such that the normal to the plane of the heme groups is perpendicular to the long axis of the crystal needles and of the sickled cells. (This statement is based on the paper of Perutz, Liquori, and Eirich; there is some conflict with the earlier paper.) They also pointed out that the solubility of sickle-cell-anemia hemoglobin is much smaller than that of normal hemoglobin or of either normal oxyhemoglobin or sickle-cell-anemia oxyhemoglobin. A detailed study of the solubilities of mixtures of sickle-cell-anemia hemoglobin and other hemoglobins has been made by Itano, who has shown that a solubility measurement provides a simple way of determining roughly the amount of sickle-cell-anemia hemoglobin present in a mixture of hemoglobins. A most significant investigation was then reported by
Harris.\textsuperscript{46} He showed that a stroma-free solution of sickle-cell-anemia hemoglobin with concentration 15.2 or 23.5 g. per 100 ml. on deoxygenation forms birefringent spindle-shaped bodies varying in length from 1 to 15 μ. He identified these bodies as tactoids (liquid crystals) of the nematic type. His photomicrograph is shown as Fig. 2, which may be compared with Fig. 3, a similar photomicrograph (375 X magnification) of sickled erythrocytes.

It hence seems probable that sickle-cell anemia can be described as a molecular disease, resulting from the difference in molecular structure of sickle-cell-anemia hemoglobin and normal adult human hemoglobin. The properties of the abnormal hemoglobin are such that when deoxygenated the molecules combine with one another to form long molecular strings, which, through intermolecular attraction, aggregate into tactoids. These tactoids have enough mechanical strength to distort the red cell, changing the viscosity of the blood, and causing the clinical and pathological manifestations of the disease.

The close approximation in structure of sickle-cell-anemia hemoglobin to normal adult human hemoglobin is strikingly shown by the antigenic properties, investigated by Goodman and Campbell.\textsuperscript{40} They found that, whereas large differences in serological specificity are shown by human fetal hemoglobin and human adult hemoglobin, suggesting that only a few antigenic groups are shared in common by these two hemoglobins, only a small difference in antigenic specificity could be demonstrated between sickle-cell-anemia hemoglobin and normal adult human hemoglobin. Antisera obtained by injection of rabbits with these two forms of adult human hemoglobin showed no differences in antigenic specificity. The injection of chickens with these two forms of adult hemoglobin produced antiseraums with a significant, though small, difference in properties, indicating that the two hemoglobins have a predominance of antigenic groups in common, but that a small number are different.

The results of an investigation by Ingbar and Kass\textsuperscript{47} of the number of titratable sulfhydryl groups (two groups per molecule in normal hemoglobin, and three in sickle-cell-anemia hemoglobin) may provide an additional clue as to the difference in structure of the molecules.
OTHER ABNORMAL HEMOGLOBINS, FETAL HEMOGLOBIN,
SYNERGY WITH THALASSEMIA

During the five years since the discovery of sickle-cell-anemia hemoglobin three other abnormal varieties of adult human hemoglobin (named hemoglobin C, D, and E, respectively; A represents normal adult human hemoglobin, and S sickle-cell-anemia hemoglobin) have been discovered. These abnormal hemoglobins, either alone or in synergistic interaction with sickle-cell-anemia hemoglobin or with thalassemia, have been observed in association with five diseases, which, together with a sixth disease resulting from the simultaneous existence in the individual of sicklemia and thalassemia minor, had not previously been recognized as clinical entities. Another interesting complication in the constitution of the blood has also been recognized, the continued manufacture of fetal hemoglobin by anemic individuals.

Hemoglobin C was discovered by Itano and Neel and has been further investigated by Neel, Spaet et al., Ranney et al., and Smith and Conley. The difference in electrophoretic mobility between hemoglobin C and normal adult human hemoglobin is nearly twice as great as the difference between sickle-cell-anemia hemoglobin and normal hemoglobin: the electric charge of hemoglobin C differs by about 5 electronic charges from that of A. Hemoglobin C was first found to be present together with S in the blood of some anemia patients. These patients may be described as carrying one allele for C and one for S: they are heterozygous in hemoglobin C and also in hemoglobin S, having inherited one of the two abnormalities from each parent. On investigation, the red cells of one parent were found to contain a mixture of A and C, and of the other parent to contain a mixture of A and S. Dilution of S with C does not inhibit the sickling tendency so much as dilution of S with A, and in consequence the individuals of type SC are anemic, their disease being called sickle-cell:hemoglobin-C disease. The heterozygous condition in C does not lead to a pathologic state. Sickle-cell:hemoglobin-C disease can be readily differentiated from sickle-cell anemia on clinical grounds. The characteristic differences in electrophoretic behavior of A, S, and C are shown in the paper electrophoresis patterns of Fig. 4, repro-
duced from the work of Smith and Conley. Under the conditions of this investigation, which was carried out in veronal buffer at pH 8.6 and ionic strength 0.06, all three hemoglobins are negatively charged, with A having the largest negative charge and C the smallest charge.

![Paper electrophoresis patterns of hemoglobins.](image)

**FIG. 4.** Paper electrophoresis patterns of hemoglobins. Veronal buffer, 0.06 ionic strength, pH 8.6; Whatman paper No. 3, 6 X 6 inches, 15 mils, 380 volts. The migration begins at the dotted line; hemoglobin A is the fastest moving, hemoglobin S has intermediate mobility, and hemoglobin C migrates most slowly. From Ernest W. Smith and T. Lockard Conley, *Bull. Johns Hopkins Hosp.* 93, 94 (1953).

Using paper electrophoresis, Smith and Conley made an investigation of the hemoglobins of 500 white persons and 500 Negroes. In the 500 white persons they found no evidence of the presence of any hemoglobin differing electrophoretically from normal adult human hemoglobin. Hemoglobin S was found to occur in 8.4 per cent of the 500 Negroes surveyed, and hemoglobin C in 2 per cent. Seven patients with sickle-cell hemoglobin-C disease were found among the 500 Negroes.

If the incidence of the heterozygous state AC is 2 per cent, the
Occurrence of the C allele is 0.01, and it can be predicted that homozygous hemoglobin-C disease should occur among Negroes with frequency 0.01², or one in 10,000. It was reported by Spaet and co-workers⁴⁵ and Ranney and co-workers⁵³ that patients with a mild hemolytic anemia have been found with only hemoglobin C in their red cells, and it is probable that they are homozygous in hemoglobin C, and that their disease is homozygous hemoglobin-C disease.

An interesting complication in the hemoglobin pattern has been resolved through the discovery that anemic individuals may continue to manufacture fetal hemoglobin long after they have passed the fetal stage of development. Wells and Itano, in the course of their investigation of the ratio of S to A in sicklemic individuals,⁴⁶ discovered that there is present in the blood of patients with an abnormally mild form of sickle-cell anemia a small amount, 5 per cent to 20 per cent of the total, of a protein other than S. They identified this protein as A, on the basis of its electrophoretic pattern, but they pointed out that the patients could be assumed, from the facts that both parents were sicklemic and that hemoglobin S predominated, to be homozygous in S, and that the presence of A was anomalous. Itano and Neeld⁵⁸ reported that one patient with sickle-cell:hemoglobin-C disease gave a hemoglobin electrophoretic pattern showing S and C in large amounts (39 per cent and 48 per cent, respectively) and an additional hemoglobin, 13 per cent, with the electrophoretic mobility, at the pH used (pH 6.50), of A. The electrophoretic pattern of this patient is given in Fig. 5. It was pointed out by Singer, Chernoff, and Singer⁵⁸ that there is present in the blood of many patients with sickle-cell
anemia and other hematologic disorders a hemoglobin fraction that is resistant to alkali denaturation, and that the properties of this fraction permitted it to be identified with fetal hemoglobin, F, in particular, these authors suggested that the hemoglobin reported by Wells and Itano to be present in small amounts in the blood of some sickle-cell anemia patients is hemoglobin F, and not A. At about the same time Liquori reported that he had found that as much as 50 per cent of the hemoglobin in the blood of patients with thalassemia is fetal hemoglobin, and Rich found that two patients with thalassemia major had in their red cells no hemoglobin other than F. It is now recognized that anemic patients may continue to manufacture significant amounts of F throughout their lives, and that the presence of F ameliorates their disease. Hemoglobin F is identified, in relation to A, only with difficulty by electrophoretic methods, but it is easily identified by its resistance to alkali denaturation and by its characteristic ultraviolet absorption spectrum. Goodman and Campbell verified the identification of the minor hemoglobin in patients with sickle-cell anemia by studying its antigenic properties, which were found to be either identical with or closely similar to those of fetal hemoglobin.

Thalassemia (Cooley’s anemia, Mediterranean anemia) is a hereditary anemia that results from a gene-controlled interference with the process of synthesis of adult human hemoglobin. It was first clearly recognized by Cooley and Lee. The disease is largely confined to persons derived from the northern shores of the Mediterranean. The thalassemia allele in the homozygous form produces a very serious anemia, thalassemia major, which usually terminates fatally in childhood. In heterozygous form the allele produces a mild anemia, called thalassemia minor. In some parts of Italy, notably the region around Ferrara, the incidence of thalassemia minor is about 10 per cent, corresponding to a probability of the allele of 5 per cent.

In 1953 Powell, Rodarte, and Neel reported one case of a disease due to the combination of thalassemia minor and sicklemia. The resulting sickle-cell:thalassemia disease is a more serious disease than thalassemia minor. Other patients with the disease have been described by Sturgeon, Itano, and Valentine and by Neel, Itano, and Lawrence. A case of thalassemia:hemoglobin-C disease
Abnormality of Hemoglobin Molecules in Anemia

The third abnormal hemoglobin to be discovered, hemoglobin D, was found by Itano in five members of a single family. Two of the family were patients with a disease resembling sickle-cell anemia but somewhat milder than the normal form of this disease. The electrophoretic patterns of the blood of these patients were closely similar to those of hemoglobin S. The hemoglobin from the red cells of the three other individuals gave an electrophoretic pattern like that obtained from sicklemics. It was found, however, by measurement of the solubility of the hemoglobins that an abnormal hemoglobin is present, with electrophoretic properties like that of S, and solubility like that of A. This hemoglobin, hemoglobin D, seems not to interfere with the process of sickling so much as does A, so that the state of double heterozygosity SD leads to a moderately serious anemia. The blood of each of the two patients with sickle-cell-hemoglobin-D disease was found to contain a small amount (6 per cent to 12 per cent) of F.

Two individuals containing the fourth abnormal hemoglobin, E, have been discovered by Itano, Bergren, and Sturgeon. One of them is an anemic patient with thalassemia-hemoglobin-E disease. The mother of the patient has thalassemia minor, and no hemoglobin other than A in her red cells. The red cells of the patient contain 41 per cent F and 59 per cent E. The father of the patient is of type AE, with 72 per cent A and 28 per cent E (no F); he is a carrier of E (personal communication from Itano, Bergren, and Sturgeon). The patient has inherited thalassemia minor from the mother and the allele for E from the father. The thalassemia allele seems to have completely suppressed the manufacture of A.

Hemoglobin E shows a striking change in electrophoretic mobility with change in pH. At pH 6.5, in cacodylate buffer of ionic strength 0.1, its mobility is greater than that of A and slightly less than that of S, and at pH 8.8, in 0.01 F disodium hydrogen phosphate, its mobility is nearly identical with that of C. The absorption spectrum, solubility, and lability to alkali denaturation of E are similar to those of A.

A summary of the hereditary hemolytic anemias related to abnormal hemoglobins is presented in Fig. 6. There are five forms of
adult human hemoglobin: A, S, C, D, and E. Assuming that these five correspond to five alleles occupying the same locus in a chromosome, there are fifteen possible combinations, the five homozygous states AA, SS, CC, DD, and EE, and the ten heterozygous states AS, AC, AD, AE, SC, SD, SE, CD, CE, and DE. Of these fifteen states nine have been found to occur. In addition to sickle-cell anemia, six other types of hereditary anemia involving an abnormality of hemoglobin have been recognized.\textsuperscript{11}

\begin{align*}
&\text{A}\quad \text{AA}  \quad \text{Th} > \text{AS} > \text{SD} > \text{AC} > \text{Th} > \text{SE} > \text{CC} \\
&\text{S}\quad \text{AS}  \quad \text{SS} \\
&\text{C}\quad \text{AC}  \quad \text{SC} \\
&\text{D}\quad \text{AD}  \quad \text{SD} \\
&\text{E}\quad \text{AE}  \quad \text{SE} \\
&\text{\textsc{Talassemia major}} \quad \text{AA} > \text{AC} > \text{AD} = \text{AE} > \text{Th} \\
\end{align*}

Fig. 6. A chart representing possible combinations of the alleles A, S, C, D, and E. The horizontal row at the bottom represents simultaneous occurrence with thalassemia minor. The amount of fetal hemoglobin usually present is also indicated: $F$ means a few per cent, $F'$ means 10 per cent or more. Observed conditions are shown within heavy borders. At the top the seriousness of different kinds of anemia is indicated.

The seriousness of these diseases (not including AC + Th and E + Th, for which the number of patients is too small to permit an estimate) is indicated at the top of Fig. 6. There is some variability in the seriousness of the diseases, in part as the result of the extent to which compensation of abnormal hemoglobins is achieved through the manufacture of fetal hemoglobin. The mildest anemias are SC and CC. Not all SC and CC individuals are anemic. Although they probably have a greater than normal rate of hemolysis, they may be able to compensate completely with a greater than normal rate of production of red blood cells.\textsuperscript{32-72}

The conditions CD, DD, SE, CE, DE, EE, and DA + Th have not yet been observed; it may be found that they are associated with anemia.
Except for the fetal-hemoglobin fraction, the hemoglobin of patients with thalassemia major has properties reported to be the same as those of normal adult human hemoglobin, and it is usually considered that thalassemia is not associated with the production of an abnormal hemoglobin. There are some facts, however, that indicate that thalassemia hemoglobin is a fifth abnormal kind of adult human hemoglobin, with properties so closely similar to those of normal adult human hemoglobin that the differences have escaped detection. It has been observed that thalassemia minor involves a greater interference with the synthesis of the normal hemoglobin than of abnormal hemoglobin. For example, whereas in sicklemic individuals the ratio $A:S$ is always greater than 1, this ratio is much less than 1 in thalassemia:sickle-cell disease. Two patients reported by Neel, Itano, and Lawrence were found to have the ratios $A:S:F$ equal to 20:61:19 and 11:84:5, respectively. The hemoglobin of the patient with thalassemia:hemoglobin-E disease contains no detectable amount of normal adult hemoglobin, but only E and F. The thalassemia allele interferes with the manufacture of normal adult hemoglobin, and seems not to interfere seriously with the manufacture of the abnormal hemoglobins. The simplest explanation of this fact is that the thalassemia allele occupies the same locus in the chromosome as the alleles for the other abnormal hemoglobins, and is itself responsible for an abnormal globin in which the abnormality is of such a nature as to interfere with the step of inclusion of the hemes in the molecule. If this postulate is correct it should be possible to show a difference between thalassemia hemoglobin and normal adult human hemoglobin.

Under this circumstance there would be six alleles occupying the same locus, $A$, $S$, $C$, $D$, $E$, and $Th$ (more than six if there is more than one thalassemia allele). The six states at the bottom of the triangle in Fig. 6 should then be written $ATh$, $STh$, $CTh$, $DTh$, $ETH$, and $ThTh$. Of the possible twenty-one combinations of the six alleles, fourteen would be ascribed to known individuals.

The possibility that the thalassemia gene is allelomorphic with the sickle-cell gene has been discussed by Neel. He pointed out that both of the children of a patient with thalassemia:sickle-cell disease and married to a normal woman exhibited thalassemia minor,
and mentioned that if either of these children had been normal (without either thalassemia minor or sickleemia) the hypothesis of allelomorphism would have to be abandoned. Silvestroni and Bianco, who a number of years ago described the disease resulting from simultaneous inheritance of thalassemia minor and sickleemia, have also discussed the genetic aspects of thalassemia and sickling, and have concluded that the two are not allelomorphic.

**Sickle-Cell Anemia Hemoglobin and Malaria**

A number of interesting questions about the origin and heredity of the abnormal hemoglobins remain to be answered. It has, for example, been pointed out by Lehmann that sickle-cell anemia seems to be a far less serious disease in Africa than in America. A possible explanation may be that the patients in Africa for some reason manufacture a larger amount of fetal hemoglobin than those in America; the question can presumably be answered by a thorough investigation of the hemoglobins of Africans, which has not yet been carried out.

The question of the continued high incidence of the sickle-cell allele, despite its continued loss because of the lethal character of the homozygous condition, has been raised by Neel, who has suggested three alternative explanations: (1) continued production of the allele through mutation; (2) the existence of an abnormal genetic mechanism that favors the heterozygous condition, AS, over the normal condition, AA; (3) a positive selection of the heterozygote, perhaps through increased fertility. The first explanation has to be rejected because the rate of mutation that would be required is far greater than any that has ever been observed for any organism. There now exists evidence indicating that the third alternative provides the correct explanation, and that malaria is involved. It was first suggested by Brain that the presence of S in the red cells might give protection against malaria parasites, and thus confer an advantage to the sicklemic individual that would balance the disadvantage of the lethal homozygosity. Lehmann wrote that "The lethal tendency of a gene potentially causing sickle cell anemia may thus be counteracted by its conferring a resistance to malaria similar to that found in early infancy." A test of the hypothesis was carried out recently by Allison, who in-
ected 15 healthy adult Africans with the sickle-cell trait and 15 similar healthy adult Africans without the sickle-cell trait with \textit{P. falciparum}, by subinoculation with 15 ml. of blood containing a large number of trophozoites or by being bitten by heavily infected anopheles mosquitoes, in which the presence of sporozoites was confirmed by dissection of the mosquito. The infection was established in 14 out of the 15 Africans without the sickle-cell trait, and in only 2 of the 15 with this trait. It was concluded by Allison that the abnormal erythrocytes of individuals with the sickle-cell trait are less easily parasitized by \textit{P. falciparum} than are normal erythrocytes, and that accordingly those who are heterozygous for the \textit{S} allele have a selective advantage over normal individuals in regions where malaria is hyperendemic. It is, of course, not unreasonable that the abnormal hemoglobin may be less effective than normal hemoglobin in nourishing the parasites.

The question of the origin of sickle-cell anemia has been investigated especially by Lehmann, who has studied the incidence of the ability of red cells to sickle in several parts of the world. In Africa the incidence of the sickle-cell allele is highest in the north and east, diminishing somewhat toward the west and south, and being virtually zero in South Africa itself. In India the major groups of the population, classed as Dravidians, show no sickling, and among the pre-Dravidians living in the hills of Southern India the Veddoids alone have a high incidence of sickling. A high incidence of sickling was found also in a Veddoid community of Southern Arabia, although sickling is absent among the Semitic Arabs. Some sicklemic individuals are found in Italy, and in Greece there are a few communities, each of a few hundred individuals, with a high incidence of sickling. Lehmann concluded that it is not unlikely that the sickle-cell gene originated, presumably through a mutation, in a Veddoid community in Southern Arabia, and that it spread from this point to India, the Mediterranean region, and especially to Africa.

Molecular Diseases

Sickle-cell anemia has been described as a molecular disease. It may be that all diseases can be described as molecular diseases, inasmuch as the human body and the vectors of disease are all com-
posed of molecules. For example, carbon monoxide poisoning is the result of combination of molecules of carbon monoxide with molecules of hemoglobin. Erythroblastosis fetalis involves the interaction of the molecules or haptenic groups of an Rh antigen on red cells with molecules of the homologous antibody. An inborn error of metabolism such as alcaptonuria results from the failure of the body to manufacture molecules of the enzyme that, if present, would catalyze the oxidation of homogentisic acid in the body. Any hereditary disease may be said to be a molecular disease, if the genes are described as molecules, in that it involves an abnormality of a gene.

There is, however, one sense of the expression molecular disease that permits it to be applied to sickle-cell anemia and the other diseases associated with the abnormal hemoglobins. These diseases have been shown to result from the manufacture by the patient of abnormal molecules, in the place of normal molecules which are manufactured by normal individuals, and the abnormal molecules have been characterized. I think that it is not unlikely that many diseases will in the course of time be found to be molecular diseases in this sense. The discovery of the abnormal molecules responsible for other molecular diseases may be far more difficult than the discovery of the abnormal hemoglobins. Hemoglobin is unique among the proteins of the human body because of its presence in very large amount, about 1 per cent by weight of the body, and because of the ease with which it can be obtained from the individual and characterized. Other substances in general are present in far smaller amounts. I have estimated that there are of the order of 100,000 different kinds of proteins in the human body. If the number of substances making up the human body is about 100,000, the average amount of each substance present (aside from water) is about 100 mg., and some important substances may be present in far smaller amounts. The isolation and identification of abnormal forms of molecules of these substances might be extremely difficult. Because of the insight into the nature of disease that would be provided by the discovery of additional molecular diseases, however, it seems important to prosecute investigations along this line with much vigor.
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