Structure-function relations of human hemoglobins

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In 1949 Pauling and his associates showed that sickle cell hemoglobin (HbS) belonged to an abnormal molecular species. In 1958 Ingram, who used a two-dimensional system of electrophoresis and chromatography to break down the hemoglobin molecule into a mixture of smaller peptides, defined the molecular defect in HbS by showing that it differed from normal adult hemoglobin by only a single peptide. Since then, more than 200 variant and abnormal hemoglobins have been described. Furthermore, the construction of an atomic model of the hemoglobin molecule based on a high-resolution x-ray analysis by Dr. Max Perutz at Cambridge has permitted the study of the stereochemical part played by the amino acid residues, which were replaced, deleted, or added to in each of the hemoglobin variants. Some of the variants have been associated with clinical conditions. The demonstration of a molecular basis for a disease was a significant turning point in medicine. A new engineered hemoglobin derived from crocodile blood, with markedly reduced oxygen affinity and increased oxygen delivery to the tissues, points the way for future advances in medicine.

Hemoglobin has played a spectacular role in the history of biology, chemistry, and medicine. This paper, written primarily for the clinician, is a brief outline of the complex problems associated with abnormal hemoglobins. The thalassemias have been intentionally omitted and will be presented in a separate publication.

Hemoglobin is a two-way respiratory carrier, transporting oxygen from the lungs to the tissues and facilitating the return transport of carbon dioxide. In the arterial circulation, hemoglobin has a high affinity for oxygen and a low affinity for carbon dioxide, organic phosphates, and hydrogen and chloride ions. In the venous circulation, these relative affinities are reversed. To stress these remarkable properties, Jacques Monod conferred on hemoglobin the title of "honorary enzyme." If we call heme its active site, oxygen its substrate, and hydrogen ions its inhibitors, then hemoglobin mimics the properties of an enzyme. Therefore, it became evident that unraveling the properties of hemoglobin was necessary to understanding the mechanism of hemoglobin function as it pertains to respiratory physiology.

In 1937, Dr. G. S. Adair gave Dr. Max Perutz crystals of horse hemoglobin (personal communication, Max Perutz, 1966). This started Dr. Perutz on the path that led to the elucidation of the structure of hemoglobin (1). For this endeavor he was awarded the Nobel Prize in chemistry in 1962.

In 1957 Ingram demonstrated that sickle cell anemia was caused by the replacement of one of the 287 amino acid residues in the half molecule of hemoglobin (2). This finding facilitated understanding of disease at the molecular level, since for the first time a point mutation in a structural gene was shown to cause the substitution of one amino acid in the protein controlled by that gene. Furthermore, the accumulation of the sickle cell gene in malarial regions of the world became a convincing illustration of evolution by natural selection (3). Persons with the sickle cell trait (HbA/S) have a selective advantage over normal individuals when they contract falciparum malaria because the parasite count remains low and lethal cerebral malaria is avoided.

To date, well over 200 hemoglobin variants have been described. The term "variant" rather than "abnormal" is preferred because most hemoglobins are not associated with disease. The late Professor Herman Lehmann at Cambridge University in England and his "musketeers" in different parts of the world have been responsible for discovering many of these variants. Furthermore, as knowledge accumulated, it became evident that the structure-function relations of various hemoglobins in stereochemical terms could be related to clinical symptomatology (4, 5).

STRUCTURE OF HEMOGLOBIN

Hemoglobin comprises four subunits, each having one polypeptide chain and one heme group *(Figure 1)*. All hemoglobins carry the same prosthetic heme group iron protoporphyrin IX associated with a polypeptide chain of 141 (alpha) and 146 (beta) amino acid residues. The ferrous ion of the heme is linked to the N of a histidine. The porphyrin ring is wedged into its pocket by a phenylalanine of its polypeptide chain. The polypeptide chains of adult hemoglobin themselves are of two kinds, known as alpha and beta chains, similar in length but differing in amino acid sequence. The alpha chain of all human hemoglobins, embryonic and adult, is the same. The non-

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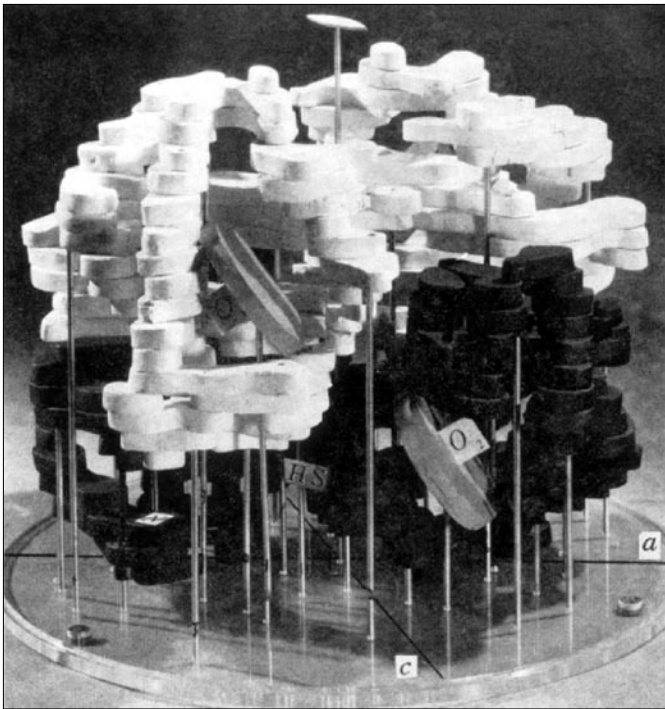
Figure 1. Model of the hemoglobin molecule. Two identical white (alpha) polypeptide chains and two identical black (beta) polypeptide chains form a complete molecule. The hemes are shown as discs. O_2 marks the oxygen binding site. Reprinted courtesy of Dr. Max Perutz.

Figure 2. Diagrammatic representation of oxygen equilibrium curves of the lug worm, man, and hemoglobin Scuba. The effect of hydrogen ions, 2,3-bisphosphoglycerate, and carbon dioxide (H^+ + BPG + CO_2) is to promote a right shift. If man had the hemoglobin of the lug worm (left shift), he would die of anoxia.

alpha chains include the beta chain of normal adult hemoglobin ($\alpha_2\beta_2$), the gamma chain of fetal hemoglobin ($\alpha_2\gamma_2$), and the delta chain of HbA_2. In some variants, the gamma genes are duplicated, giving rise to two kinds of gamma chains.

Oxygen binds reversibly to the ferrous iron atom in each heme group. The heme group that has become oxygen bound varies with the partial pressure of oxygen. The sigmoid shape of the oxygen equilibrium curve shows that there is cooperative interaction between oxygen binding sites. Hence, as oxygenation proceeds, combination with further molecules of oxygen is made easier. The oxygen equilibrium (or dissociation) curve is not linear but S-shaped and varies according to environments and species *(Figure 2)*. At a partial pressure of oxygen of 100 mm Hg, the hemoglobin in the red cell is fully saturated with oxygen. The dissociation curve is plotted as percentage of oxygen saturation against partial pressure.

The structure of hemoglobin has been extensively studied by x-ray analysis (6). The arrangement of the subunits—which is known as the quaternary structure—differs in the oxy- and deoxyhemoglobin.

In human hemoglobin, the fit between the polypeptide chain is critical because the gap between two of the polypeptide chains in the hemoglobin molecule becomes narrower when oxygen molecules become attached to the ferrous atoms. This has been likened by Max Perutz to a molecular form of paradoxical breathing: unlike the lungs, the hemoglobin molecule contracts when oxygen enters and expands when oxygen leaves.

Compounds other than oxygen, such as nitric oxide and carbon monoxide, also are able to combine with the ferrous atom of hemoglobin. Carbon monoxide attaches itself more firmly to
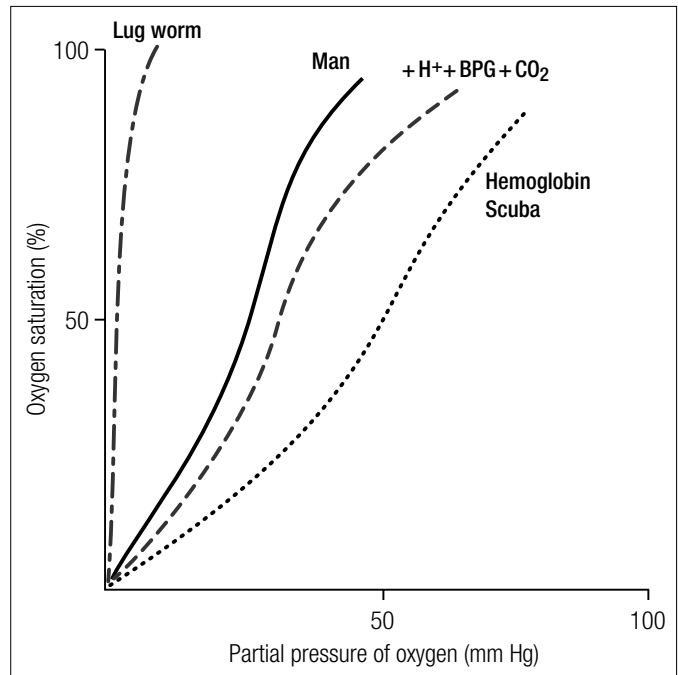
the ferrous atom than oxygen does. Once carboxyhemoglobin is formed, oxygen cannot displace carbon monoxide to any extent. This forms the molecular basis of coal gas poisoning.

In the body, the adequacy of the oxygen transport system depends on the adequacy of oxygenation of blood in the lungs, the rate and distribution of blood flow, the oxygen-carrying capacity of the blood (hemoglobin concentration), and the affinity of hemoglobin for oxygen so as to allow unloading of oxygen in peripheral capillaries. Hence, the availability of oxygen to the body may be altered by abnormalities at any point in this physiological pathway. In this paper, only the role of hemoglobin affinity for oxygen will be considered as variant forms of hemoglobin are discussed.

SICKLE CELL HEMOGLOBIN

Sickle cell hemoglobin (HbS) has existed in humans for thousands of years. Dr. Konotey-Ahulu, a Ghanaian physician, reports that among West African tribes, specific names were assigned to clinical syndromes identifiable as sickle cell anemia (7). However, sickle cells were first described in the peripheral blood of an anemic patient from the West Indies by the Chicago physician Robert Herrick in 1910 (8). While homozygous sickle cell anemia is the most common and severe form of sickle cell disease (SCD), other sickling disorders combining HbS with beta or alpha thalassemia, hemoglobin C, hemoglobin D, and other hemoglobins share a similar pathophysiology with common as well as distinguishing clinical features.

HbS results from a single base-pair mutation in the gene for the beta-globin chain of adult hemoglobin. An adenine-to-thymine substitution in the sixth codon replaces glutamic acid with valine in the sixth amino acid position of the beta-globin

chain (9, 10). This substitution yields the electrophoretically distinct hemoglobin described by Linus Pauling in 1949 (11). In the deoxygenated form of HbS, the beta-6 valine becomes buried in a hydrophobic pocket on an adjacent beta-globin chain, joining the molecules together to form insoluble polymers (9). In sufficient concentration, these insoluble polymers give rise to the classical sickle morphology. This process causes severe damage to the red cell membrane. Sickled red cells may then aggregate and go on to cause microvascular obstruction. Also, these abnormal red cells adhere to endothelial cells (12) and can interact with various cytokines (13).

The process of microthrombosis and microembolization is the foundation of SCD pathology. Occlusion of the microvasculature by sickled erythrocytes causes painful crises, priapism, pulmonary emboli, and osteonecrosis, and ultimately damages every organ system including the retinae, spleen, liver, and kidneys. Many patients with SCD have hematocrits of 20% to 35% and chronic reticulocytosis. Clinical symptoms can be precipitated by fever, infection, excessive exercise, temperature changes, hypoxia, and hypertonic solutions. The clinical severity of the symptoms experienced is related to the concentration of HbS in the red blood cell and expression of other hemoglobins, endothelial factors, nitric oxide and other factors. Also, patients with SCD have a higher proportion of dense, dehydrated erythrocytes (14).

In about 11% of SCD patients under 20 years of age, stroke occurs because of stenotic cranial artery lesions, demonstrable by transcranial Doppler ultrasonography. A regular program of transfusion aimed at reducing the sickle cell population to <50% prevents about 90% of stroke cases. Unfortunately, the high risk of stroke returns after transfusion is discontinued (15).

The surface of HbS consists mainly of hydrophilic amino acid side chains together with some smaller hydrophobic side chains. Since adult hemoglobin is present at a very high concentration within the red cell and yet appears to remain free from aggregation at all levels of saturation with oxygen, the amino acids on the surface of the molecule must be arranged so as to avoid attraction between adjacent molecules. Of the majority of hemoglobin variants with surface amino acid substitutions, only a minority are associated with any significant clinical abnormalities. Except for HbS, none of those more common hemoglobins found in the homozygous state, such as hemoglobins C, D, and E, are associated with any greater abnormality than mild anemia. The surface of hemoglobin A is therefore able to accommodate a variety of different amino acid changes without its structure or function being affected (16).

The valine-for-glutamic acid substitution has very little effect on the oxygenated form of HbS (17). However, when the concentration of deoxygenated HbS becomes sufficiently great, its properties differ markedly from those of deoxygenated hemoglobin A, causing the formation of insoluble fibers and bundles, which distort the red blood cell into the sickle shape.

Since the discovery of HbS, the clinical symptomatology and associated pathophysiology of SCD have gradually been elucidated (18). SCD is characterized by anemia and four types of crises: painful (vasoocclusive), sequestrative, hemolytic, and aplastic. Damage to the red blood cell membrane gives rise to reduced cell survival and chronic hemolytic anemia. If severe enough, this damage increases the risk of bilirubin gallstone formation, stroke, and heart failure. Also, the anemia is aggravated by the mechanical impedance to blood flow caused by sickled red blood cells, resulting in widespread vasoocclusive complications. Interestingly, the anemia to some degree can be protective against vasoocclusive complications, as it moderates the increase in viscosity associated with sickling in the microcirculation. Hence, judicious exchange transfusion therapy and blood transfusion is indicated for the prevention of pain crises, stroke, pulmonary hypertension, and other related conditions (19).

Blood transfusion not only increases the oxygen-carrying capacity of blood but also decreases the percentage of cells capable of sickling. It is recommended that transfusion should be carried out with phenotypically matched, leuko-reduced, sickle-cell–negative blood in order to attain a posttransfusion hematocrit of about 36%. (20). The complications of transfusion are well known and include allo- and autoimmunization, iron overload, and the transmission of infectious diseases such as hepatitis and HIV. Also, a considerable number of patients with sickle cell anemia worldwide have undergone successful bone marrow transplantation (21). Only selected patients are eligible for the procedure. Even then, bone marrow transplantation was associated with a 5% to 10% mortality, mostly from graft-versus-host disease.

Another approach to reducing the effect of HbS polymer formation has been to augment the production of fetal hemoglobin (HbF). Through population and clinical observation, it has long been recognized that higher blood HbF levels correlate with fewer clinical manifestations of SCD. Pharmacologic manipulation of HbF in the therapy of sickling disorders has been proposed since the mid-1950s. To date, several agents have been tried, but the safest and most effective has proven to be hydroxyurea (22). The mechanism of increased HbF production by hydroxyurea is not fully understood. Also, recent studies have found that hydroxyurea contributes to the production of nitric acid, a potent endothelial relaxing factor (23).

Numerous inflammatory markers associated with endothelial surfaces and white blood cells are elevated in SCD, including C-reactive protein. Baseline granulocyte counts are often increased. Leukocytosis itself is a risk factor for increased mortality (24). Finally, laminin, a constituent of the endothelial matrix that binds to the Lutheran antigen on red cells, is expressed on sickled red blood cells in greater quantities than on normal red blood cells (25).

Almost every aspect of hemostasis tending to hypercoagulability has been described in SCD (26). However, it is not known whether the hypercoagulability is the cause or the result of vasoocclusion. Thrombocytosis is due to hyposplenia, and platelet aggregation is increased (27). Antiphospholipid antibodies may be elevated, and protein C and S levels are decreased (28). Also, high levels of von Willebrand factor and factor VIII can be found (29). Therapeutic trials of heparins, coumadin, and antiplatelet agents have been limited, yielding inconclusive information, but they are ongoing.

Table 1. Examples of hemoglobins with increased oxygen affinity

Hemoglobin	Substitution	Site in molecule affected	p50	Reference
Hb Chesapeake	α92 (Arg→Leu)	$\alpha_1\beta_2$ contact	19.0	Charache et al, 1966 (32)
Hb J Capetown	α92 (Arg→Gln)	$\alpha_1\beta_2$ contact	↓	Botha et al, 1966 (33)
Hb Yakima	β99 (Asp→His)	$\alpha_1\beta_2$ contact	12.0	Jones et al, 1967 (34)
Hb Brigham	β100 (Pro→Leu)	$\alpha_1\beta_2$ contact	19.6	Lokich et al, 1973 (35)
Hb Rainer	β145 (Tyr→Cys)	C-terminal	12.9	Adamson et al, 1969 (36)
Hb Bethesda	β145 (Tyr→His)	C-terminal	12.8	Bunn et al, 1972 (37)
Hb Syracuse	β143 (His→Pro)	BPG β contact	11.0	Jensen et al, 1975 (38)

p50 indicates 50% saturation of hemoglobin.

Table 2. Examples of hemoglobins with reduced oxygen affinity

Hemoglobin	Substitution	p50	Reference
Hb Kansas	β102 (Asn→Thr)	70	Reissmann et al, 1961 (39)
Hb Yoshizuka	β108 (Asn→Asp)	↑	Imamura et al, 1969 (40)
Hb Agenogi	β90 (Gln→Lys)	↑	Imai et al, 1970 (41)

p50 indicates 50% saturation of hemoglobin.

HEMOGLOBINS WITH ALTERED OXYGEN AFFINITY

The hemoglobin loading and unloading of oxygen can be expressed by an oxygen dissociation curve. The physiologic consequences of the abnormal hemoglobins depend on the oxygen affinity, which defines the point of 50% saturation (p50). The oxygen dissociation curve of normal hemoglobin represents the reaction of hemoglobin with oxygen as modified by hydrogen ions (Bohr effect) and 2,3-bisphosphoglycerate (BPG) (30, 31). Hemoglobin oxygen affinity increases with falling temperature and decreases with rising pH and 2,3-BPG. Hence, red blood cells containing such an abnormal hemoglobin may have an abnormal oxygen dissociation curve because of 1) an intrinsic abnormality of hemoglobin-oxygen dissociation, 2) an altered interaction of hemoglobin with BPG, 3) an altered Bohr effect, or 4) a combination of any or all of the above. It is common to speak of the oxygen-dissociation curve as being shifted to the left (increased oxygen affinity) or to the right (decreased oxygen affinity).

Increased oxygen affinity

Some hemoglobins have been described in which the associated clinical manifestations can be ascribed to an increased oxygen affinity *(Table 1)*. High-affinity hemoglobins bind oxygen more readily and deliver less oxygen to tissues.

Several hemoglobins with increased oxygen affinity have substitutions affecting the $\alpha_1\beta_2$ contact of the tetramer. Others have substitutions involving the C-terminal residues of the beta chain or of the BPG binding sites. All these substitutions favor the oxygenated conformation and cause a left shift of the oxygen dissociation curve, which reflects an increased blood affinity for oxygen. Therefore, it follows that the red cells of such

individuals give up less oxygen to the tissues. The relative anoxia increases erythropoietin production and causes polycythemia.

Most of the abnormal hemoglobins with increased oxygen affinity manifest themselves by causing polycythemia in the carrier. The increased oxygen affinity reduces tissue oxygen delivery, causing an increase in erythropoietin secretion and in red cell mass. The possibility of an abnormal hemoglobin with high oxygen affinity should be considered in those atypical patients with polycythemia in which the white blood cell and platelet counts are not elevated and splenomegaly is absent. The importance of establishing the correct diagnosis is mainly to protect the patient from the chemotherapeutic treatment of polycythemia. Family members should be advised that their children may be affected. The life expectancy of affected individuals is essentially normal, and most patients are symptom free. However, if such patients become symptomatic and their hematocrit rises towards 60%, then phlebotomy may be necessary to reduce blood viscosity.

Reduced oxygen affinity

Only a handful of abnormal hemoglobins have been reported in which a reduced oxygen affinity is the sole abnormality *(Table 2)*. Because of the increased oxygen delivery resulting from the low oxygen affinity, it might be expected that the erythropoietin response would be reduced and these variants would be associated with mild anemia. While this response occurs in most of these variants, it is not so with Hb Kansas carriers. With Hb Kansas, the oxygen affinity is so low that even at normal arterial oxygen tensions there is sufficient desaturation to give rise to clinical cyanosis. The possibility of low-affinity hemoglobins should be considered in patients with low hematocrit or cyanosis with no other cause apparent after evaluation. The p50 is usually elevated. Despite these findings, patients usually require no specific treatment once the correct diagnosis is established.

THE UNSTABLE HEMOGLOBINS

At the molecular level, considering the three-dimensional model of the hemoglobin molecule, it would appear that the stability of the hemoglobin tetramer is dependent on both the internal molecular positioning of nonpolar amino acids and the stability of the large $\alpha_1\beta_1$ contacts. These properties serve to hold the four chains together. In most unstable hemoglobins one or more of these properties have been disrupted.

Unstable hemoglobins are hemoglobins that, because of the nature of the substitution, deletion, or insertion of amino acids *(Table 3),* tend to undergo spontaneous oxidation within the red cell and precipitate to form insoluble inclusions called Heinz bodies. Their presence results in the so-called congenital Heinz body hemolytic anemia. Most patients with this condition are found to have a nonspherocytic hemolytic anemia. The anemia is exacerbated by infections and oxidative drugs such as sulfonamides, pyridium, and antimalarials. It must be remembered that the normal red cell is undergoing continual

Table 3. Examples of unstable hemoglobins

Hemoglobin	Substitution	Reference
Hb Köln	β98 (Val→Met)	Carrell et al, 1966 (42)
Hb Hammersmith	β42 (Phe→Ser)	Dacie et al, 1967 (43)
Hb Bristol	β67 (Val→Asp)	Sakuragawa et al, 1984 (44)
Hb Gun Hill	β91→95 deleted	Murari et al, 1977 (45)
Hb Montreal	β73→75 deletion, insertion	Plaseska et al, 1991 (46)

Figure 3. Diagrammatic representation of the heme pocket formed by amino acids. Oxygenation can occur only between the non–heme-linked histidine and iron.

Table 4. Examples of hemoglobin M variants

Hemoglobin	Substitution	Clinical presentation	Reference
HbM Boston	α58 (His→Tyr)	Cyanosis at birth	Gerald and Efron, 1961 (50)
HbM Saskatoon	β63 (His→Tyr)	Cyanosis	Stavem et al, 1972 (51)
HbM Iwate	α87 (His→Tyr)	Cyanosis at birth	Hayashi et al, 1966 (52)
HbM Hyde Park	β92 (His→Tyr)	Cyanosis	Hutt et al, 1998 (53)
HbFM Fort Ripley	α92 (His→Tyr)	Cyanosis at birth	Hain et al, 1994 (54)

physical stress and has to be able to deform in arterioles in order to travel through the microcirculation. The insoluble Heinz bodies are torn out of the red cell during passage in the microcirculation of the spleen, which is ≤3 microns across (47). In such circumstances, Heinz bodies are pitted out of the red cell along with some membrane, leading to the presence of "bite cells" in the peripheral smear. Other disturbances such as K^+ and Ca^{++} changes are secondary to the physical damage caused by Heinz bodies.
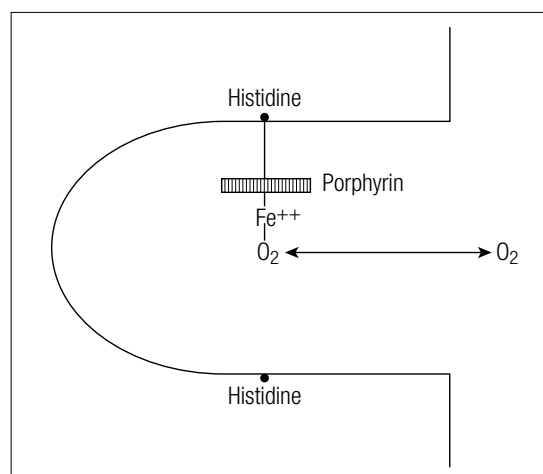
The first report of a child with idiopathic congenital non-spherocytic hemolytic anemia associated with cyanosis and splenomegaly is attributed to Cathie (48). The patient was a small boy. His spleen was removed and, several months later, the red cells were found to contain numerous Heinz bodies. In 1966 Carrell et al described the amino acid substitution giving rise to an unstable hemoglobin (Hb Köln) as the cause of this anemia (42).

The clinical findings in patients suffering from unstable hemoglobin disease include neonatal jaundice, anemia, cyanosis, pigmenturia, splenomegaly, and drug intolerance. The severity of the disease is highly dependent on the degree of instability of the abnormal hemoglobins. The disorder is clearly expressed in heterozygotes, and it seems likely that with most substitutions or deletions, homozygosity would be lethal. Heinz bodies are usually not seen until the spleen has been removed; they can be detected in the peripheral smear by supravital staining. Unstable hemoglobins are detected by their precipitation in isopropanol or after heating to 50°C. HbA₂ and HbF may be increased. Hemoglobin electrophoresis reveals that most unstable hemoglobins migrate like HbA or HbS. Complete characterization includes amino acid sequencing and gene cloning and sequencing.

Not for the first time, observations made on patients suffering from certain abnormal hemoglobin have provided the stimulus for basic scientific work.

HEMOGLOBIN M AND METHEMOGLOBINEMIA

For hemoglobin to combine with oxygen, its iron atoms must be in the ferrous state. Should oxidation (or de-electronation) of the hemoglobin molecule occur, the ferrous iron is converted to ferric iron and methemoglobin is formed. Methemoglobin is valueless as a respiratory pigment. Every day about 1% of the total circulating hemoglobin concentration is converted into methemoglobin. The iron is itself attached on one side of the

"heme pocket" to the amino acid residue histidine—the proximal histidine. Another histidine is situated on the other side of the pocket. This second histidine is not directly linked to the ferrous atom and is called the distal histidine. Normally oxygen is able to slip freely between the distal histidine and the ferrous atom during oxygenation and deoxygenation *(Figure 3)*. In the normal individual there is a balance between the spontaneous process of methemoglobin formation and a series of protective mechanisms that reconvert the pigment back to hemoglobin.

Methemoglobinemia may be caused by the ingestion of nitrites and nitrobenzenes, enzyme deficiencies such as methemoglobin reductase or diaphorases, and certain abnormal hemoglobins. In 1948 Hörlein and Weber (49) described a German family in which some members had been cyanotic from birth and found that the abnormality was associated with the globin and not with the heme. Hemoglobin M has subsequently been recognized as a perfect example of a molecular abnormality. Such abnormal hemoglobins, collectively called hemoglobin M, all have amino acid substitutions involving either the histidyls themselves or amino acids lining the heme pocket *(Table 4)*. Carriers of hemoglobin M are often cyanotic and suffer from anemia. The anemia is more significant than the hemoglobin

level suggests because some 25% of circulating hemoglobin is in the ferric form and therefore is not functional. No effective treatment exists for the cyanosis that is present in patients with hemoglobin M.

POSSIBLE CLINICAL DEVELOPMENTS IN HEMOGLOBIN RESEARCH

It is well known that crocodiles kill their prey by drowning them. Crocodiles are capable of remaining under water without surfacing to breathe for more than an hour. It has been shown that when crocodiles hold their breath, bicarbonate ions, the final product of respiration, accumulate and markedly reduce the oxygen affinity of their hemoglobin. This releases a large fraction of hemoglobin-bound oxygen to the tissues (55). Hence, the oxygen affinity of crocodile hemoglobin is markedly reduced by the physiologic concentration of carbon dioxide. The bicarbonate ions thus formed bind to deoxyhemoglobin and facilitate the giving up of oxygen (Figure 2).

Amino acid sequence identity between crocodile and human hemoglobin is 68% for the alpha subunit and 51% for the beta subunit. In crocodile hemoglobin, the amino acid residues involved in bicarbonate ion binding are located at the $\alpha_1\beta_2$ contact. This junction acts as a flexible joint during hemoglobin respiration.

In collaboration with Osaka University in Japan, Jeremy Tame at the MRC Laboratory of Molecular Biology in Cambridge, England, was able to transplant this unique allosteric effect from the Nile crocodile *(Crocodylus niloticus)* into human hemoglobin by replacing a total of 12 amino acids at critical positions in the alpha and beta chains. This new engineered hemoglobin was named Hb Scuba (56). The clinical implication of this work for transfusion medicine is mind-boggling!

Acknowledgment

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