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Polycythemia Associated with a Hemoglobinopathy *

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The efficiency of hemoglobin as an oxygen-carrying substance is dependent upon certain features of its molecular configuration. The functional integrity of the molecule depends not only on spatial relationships between the heme and globin moieties, but on interrelations between the four globin chains (1, 2). The functional significance of portions of the molecule can be investigated by a study of hemoglobin with known abnormalities of structure. Many structural abnormalities have been reported, but functional abnormalities are extremely rare.

Despite considerable differences in the amino acid composition of the β, δ, and γ chains, the oxygen dissociation curves of purified solutions of hemoglobin A ($\alpha_2\beta_2$), hemoglobin F ($\alpha_2\gamma_2$), and hemoglobin A_2 ($\alpha_2\delta_2$) are quite similar (3–5). In contrast, hemoglobins H (β_4), Bart's (γ_4), and α^A, in which interaction between α and β chains is impossible, have very high oxygen affinities (1, 2, 6). A Bohr effect is not present, and oxygen dissociation curves are hyperbolic rather than sigmoid in shape, indicating that heme-heme interactions are absent.

Hemoglobin A treated with carboxypeptidase A also exhibits increased oxygen affinity and absence of heme-heme interactions (7). Altered oxygen affinity is thought to be a nonspecific result of inability of this modified hemoglobin to undergo a conformational change on deoxygenation.

Other abnormal hemoglobins with altered oxygen affinity are characterized by substitutions of a single amino acid. Oxygen affinity of the hemoglobins M appears to be reduced because of their tendency to become oxidized to methemoglobin (8), and the amino acid substitutions responsible for these changes are thought to stabilize the iron of the heme moiety in the trivalent state (9). Hemoglobin D_{Punjab} has been reported to have increased oxygen affinity (10, 11), and blood containing hemoglobin Zurich has increased oxygen affinity (12). Hypotheses have not been advanced to explain the mechanism of altered oxygen affinity, but alterations in both interchain relationships and electrostatic interactions are undoubtedly involved.

In all of the hemoglobinopathies described, clinical consequences of altered oxygen affinity are either overshadowed by other deleterious effects of the disorder (hemoglobins H, Zurich, M, Bart's, and Lepore) or the magnitude of the alteration is so small that no significant clinical alteration is observed (hemoglobin D_{Punjab}). The only hemoglobinopathy in which alteration of oxygen affinity has been clinically significant is that described by Reissmann, Ruth, and Nomura, in which a hemoglobin of low oxygen affinity produced impressive cyanosis (13). In the present investigation a new hemoglobin, hemoglobin Chesapeake, was found to be associated with significantly elevated hematocrit levels in heterozygous carriers. Blood oxygen affinity was increased, and increased oxygen affinity was shown to be a property of the isolated abnormal hemoglobin.

Methods

Hematologic and cardiorespiratory studies were performed by standard methods. Blood oxygen dissociation curves were determined by the manometric method of Hellegers and his associates (14).[1] Details of these analyses will be published elsewhere (12). Electropho-

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[1] Blood oxygen dissociation curves were determined by Dr. Andre Hellegers and his co-workers.

resis, alkali denaturation, dissociation and recombination experiments, and incubation with oxidant dyes were performed as previously described (15). Hemoglobin-glutathione complex was prepared for comparison of electrophoretic mobility by the method of Huisman and Dozy (16).

For studies of the oxygen affinity of purified hemoglobin Chesapeake, blood was obtained with heparin as anticoagulant. Red cells were washed four times, hemolyzed by addition of 6 vol of 0.01 M phosphate buffer, pH 6.8, and centrifuged for 30 minutes in a refrigerated centrifuge at 22,000 *g*. Carboxymethyl cellulose (CMC) chromatography was performed at 4° C on a 4- × 20-cm column by a modification of the technique of Huisman and Meyering (17). A pH gradient from 6.8 to 8.3 was established with 0.01 M phosphate buffers that did not contain KCN. Flow rates were 3.5 to 4 ml per minute, and the chromatographic procedure was completed within 4 hours. Dialysis against 0.1 M phosphate buffer was carried out overnight. Samples were diluted with 0.1 M phosphate, and oxygen affinity was measured by the method of Allen, Guthe, and Wyman (18) with minor modifications of Ranney, Briehl, and Jacobs (2). The entire procedure was completed within 30 hours. Purity of samples used for these measurements was determined by electrophoresis.

Partial pressure of oxygen in millimeters mercury (Po_2) was plotted against proportion of oxyhemoglobin (y) according to the logarithmic form of Hill's equation (7, 19): $\log [y/(1-y)] = n \log Po_2 + k$. Linear regression of $y/(1-y)$ on Po_2 was calculated by the method of least squares. The slope of the line obtained, n in the above equation, is a constant that reflects the magnitude of heme-heme interactions. Covariance analyses of these data were performed by the method of Snedecor (20).

Specimens of hemoglobin A and hemoglobin Chesapeake prepared by the chromatographic procedure were converted to their methemoglobin derivatives by overnight dialysis against 0.067 M phosphate buffer, pH 6.5, or 0.067 M Tris buffer, pH 8.5, each containing 0.025 M $K_3Fe(CN)_6$. Absorption spectra were measured with a Cary recording spectrophotometer. Heat precipitability was determined by the method of Dacie and his associates (21), using unaltered hemolysates.

For globin analysis, hemoglobin Chesapeake was purified by starch block electrophoresis, and globin was isolated with cold acetone that contained 2 ml of concentrated HCl per 100 ml. Alpha and β peptide chains were separated on CMC by gradient elution chromatography in buffers containing urea and mercaptoethanol, and α chains were converted to aminoethyl derivatives (AE-α chains) with ethyleneimine. After desalting and tryptic hydrolysis, peptide mapping and amino acid analyses were carried out. Details of these procedures are published elsewhere (22).

Results

Propositus. An 81-year-old white man (F. M.) of German-Irish extraction sought medical advice because of mild angina pectoris. His past health had been good, except for an episode of melena at age 76 thought to be due to atrophic gastritis. At that time the hematocrit value had been 39%, rising to 46% 1 month after discharge.

TABLE I

*Hematologic data obtained from the family of the propositus, F. M.**

Pedigree no.	Sex	Hb Ches carrier	Hb	Hct	RBC	Retics	MCV	MCH	MCHC	Starch block electrophoresis		
			g/100 ml	%	*/mm³*	%	*μ³*	*μμg*	%	*% A*	*% Ches*	*% A_2*
I_1	M	+	19.9	58.0	6.35		91.0	31.5	34.0	63.2	34.9	2.0
I_2	F	+	16.2	48.0	5.13	0.8	94.0	32.0	34.0			
I_3	F	+	15.5	48.1	4.71	0.7	102.0	33.0	32.0	70.3	27.7	2.0
I_5	F	+	18.0	53.5	5.54	0.9	97.0	33.0	34.0			
I_7	F	+	16.8	50.8	5.44	0.6	94.0	31.0	33.0			
I_{10}	F	+	17.6	52.5	5.59	0.6	94.0	32.0	33.0			
I_{14}	F	−		34.2								
II_1	M	+	18.7	51.8	5.78		90.0	32.0	36.0	73.0	25.1	1.9
II_3	M	−	17.0	48.0	5.23	2.0	92.0	32.0	35.0			
II_5	M	−	15.3	43.3	4.70	1.3	92.0	32.6	35.0			
II_8	F	−	15.3	45.5	4.67	1.1	97.0	33.0	33.6			
II_{10}	F	−		44.7		1.0						
II_{12}	M	−	16.4	47.6	5.55	0.6	86.0	29.5	34.5			
II_{14}	F	−	15.7	47.0	5.02	0.2	94.0	31.5	33.5			
II_{16}	F	+	15.9	49.7	4.86	0.7	102.0	33.0	32.0	72.5	25.1	2.4
II_{18}	M	+	17.2	52.1	5.41	0.4	96.0	32.0	33.0	70.0	28.0	2.0
II_{20}	F	+	14.9	48.6	5.09	0.8	96.0	29.0	30.5	71.0	26.2	2.8
II_{26}	F	+	17.0	52.7	5.66	0.2	93.0	30.0	32.0	68.4	29.8	1.8
III_{28}	F	−	14.5	45.3	4.81	0.4	93.5	30.0	32.0			
III_{30}	M	+	16.8	53.8	6.00	0.6	90.0	28.0	31.0	74.6	23.3	2.1
III_{31}	F	+	15.0									
III_{33}	M	+	17.4	54.2	5.58	0.7	92.0	31.0	32.0	69.7	27.7	2.6
III_{35}	F	+	15.9	48.8	5.20	0.8	94.0	30.6	32.0			
III_{36}	M	−	14.5	43.6	4.88	0.4	89.0	30.0	33.5			
III_{37}	M	−	16.0	47.8	5.74	1.2	83.5	28.0	33.0			
III_{39}	M	+	16.8	50.0	5.59	0.6	90.0	31.0	34.0	74.6	23.0	2.4

* Abbreviations: Hb Ches = hemoglobin Chesapeake; Hct = hematocrit; RBC = red blood cells; retics = reticulocytes; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.

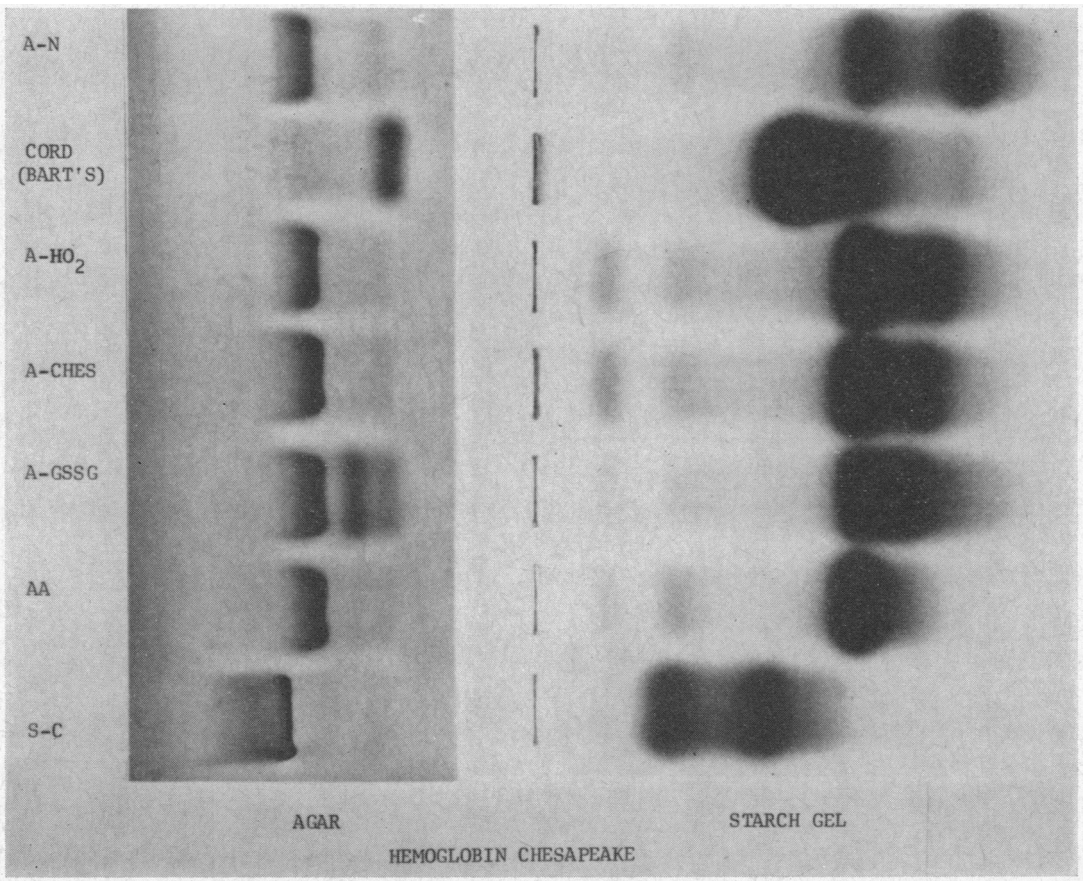

FIG. 1. HEMOGLOBIN ELECTROPHORESIS ON AGAR (pH 6.0) AND STARCH GEL (pH 8.6) (BROMOPHENOL BLUE STAIN). Hemoglobin Chesapeake is compared with hemoglobins N, Bart's (and F), Hopkins-2, synthetic hemoglobin-glutathione complex, S, and C.

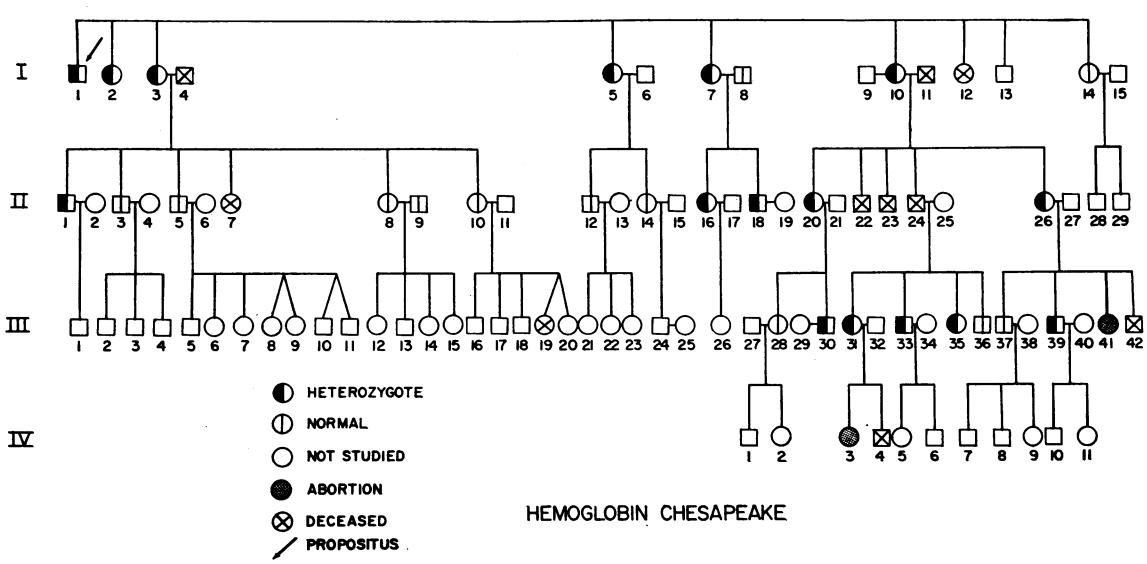

FIG. 2. PEDIGREE OF THE PROPOSITUS, F. M. ○, female; □, male.

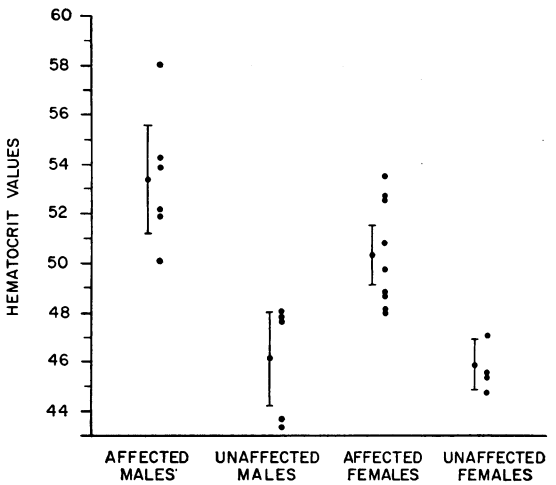

FIG. 3. COMPARISON OF HEMATOCRIT VALUES IN AFFECTED AND UNAFFECTED FAMILY MEMBERS. The vertical bars indicate mean ± 2 SE.

On examination he appeared much younger than his stated age and was quite plethoric; no other significant physical abnormalities were present.

His hematocrit was 58%, leukocyte count 5,100 per mm^3, and platelets 251,000 per mm^3. Hematologic studies of members of his family are listed in Table I. No cardiopulmonary abnormalities sufficient to produce secondary polycythemia could be detected: arterial oxygen tension was 72 mm Hg (normal, 85 to 95 mm Hg), and cardiac output and oxygen consumption were normal. A chest

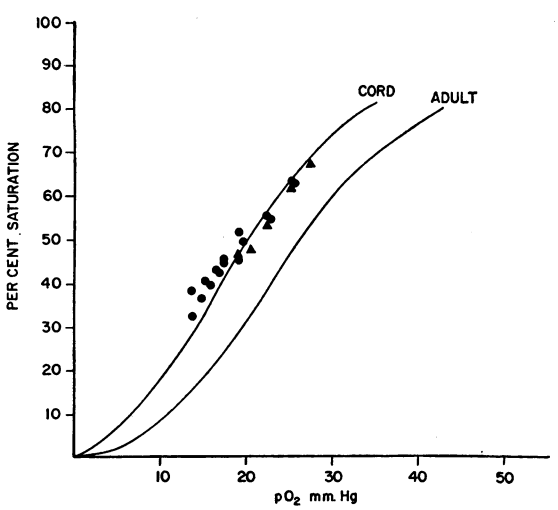

FIG. 4. OXYGEN AFFINITY AT 38° C OF BLOOD FROM THE PROPOSITUS (●) AND A NIECE (▲). Data are compared with data of Hellegers and Schruefer for maternal and fetal blood (24).

X ray showed cardiomegaly of hypertensive configuration. Plasma erythropoietic activity was less than 0.03 U per ml (23). An abnormal hemoglobin, designated hemoglobin Chesapeake, was detected by starch gel electrophoresis (Figure 1).

Treatment consisted of phlebotomies, to maintain the patient's hematocrit value at less than 50%, and vasodilators. Angina disappeared, but recurred 6 months later with its original severity.

Family study. Hemoglobin Chesapeake was detected in the blood of 16 of 26 members of F. M.'s

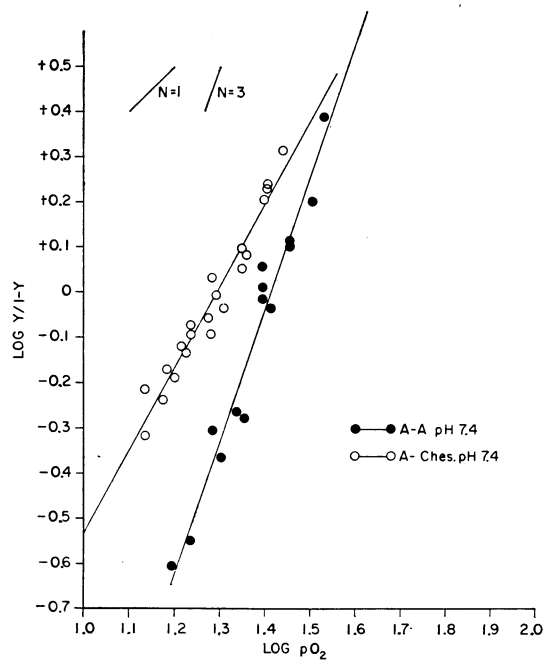

FIG. 5. OXYGEN AFFINITY AT 38° C OF BLOOD FROM THE PROPOSITUS AND A NIECE (○). Data are compared with data from four controls (●) and plotted according to Hill's equation, in which y = fraction of hemoglobin present as oxyhemoglobin and n = slope of the line. Reference lines with slopes of 1 and 3 are shown in the upper left-hand corner of the Figure.

family (Figure 2). Inheritance followed an autosomal dominant pattern. The hematocrit values of heterozygous carriers were significantly higher than those of their normal siblings ($p < 0.005$ for men, $p < 0.001$ for women) (Table I, Figure 3). Red cell morphology was normal in all family members. Leukocytes and platelets appeared normal on blood smears. The arterial oxygen tension of one polycythemic woman (II_{16}) was 99 mm Hg. No illness was associated with the pres-

ence of hemoglobin Chesapeake. Two female carriers (II_{26}, III_{31}) had each had one spontaneous abortion. Each of these women subsequently had normal children, and one had had a child who died while undergoing surgery for multiple congenital cardiac defects.

Oxygen affinity of blood and purified hemoglobin. Blood samples from F. M. and from a niece (II_{16}) had oxygen affinities approximately as great as that of umbilical cord blood (Figure 4). Data from these subjects and data from four normal controls are plotted according to Hill's equation in Figure 5. For hemoglobin Chesa-

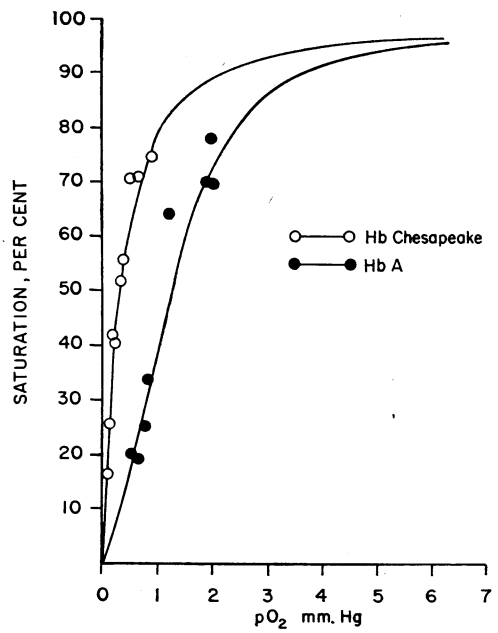

FIG. 6. COMPARISON OF OXYGEN AFFINITY AT 10° C OF HEMOGLOBIN CHESAPEAKE (○) AND HEMOGLOBIN A (●) ISOLATED FROM THE SAME BLOOD SAMPLES. The "hemoglobin A" samples contained 30% hemoglobin Chesapeake.

peake carriers, the slope (n) is 1.8; for normal blood n is 2.9. Covariance analysis indicated that the difference in slopes was significant at the 1% level.

The results of three experiments with purified hemoglobin at 10° C and pH 7.4 are plotted in Figure 6. Samples of hemoglobin Chesapeake prepared by CMC chromatography contained less than 5% of hemoglobin A, whereas hemoglobin A samples contained approximately 30% hemoglobin Chesapeake. No methemoglobin absorption band

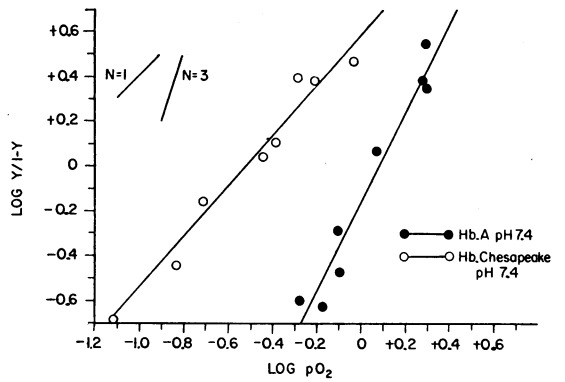

FIG. 7. COMPARISON OF OXYGEN AFFINITY AT 10° C OF HEMOGLOBIN CHESAPEAKE (○) AND HEMOGLOBIN A (●), PLOTTED ACCORDING TO HILL'S EQUATION. The "hemoglobin A" samples contained 30% hemoglobin Chesapeake.

(between 610 and 650 mμ) was detected in any sample after dialysis. The oxygen tension required for half saturation of hemoglobin Chesapeake ($P_{\frac{1}{2}} = 0.3$ mm Hg) was higher than that of hemoglobin A isolated from the same blood sample ($P_{\frac{1}{2}} = 1.2$ mm Hg). The data in Figure 6 are plotted according to Hill's equation in Figure 7. The slope of the regression line for hemoglobin Chesapeake is 1.1; that of hemoglobin A is 2.0. Covariance analysis indicated that the difference in slopes was significant at the 1% level.

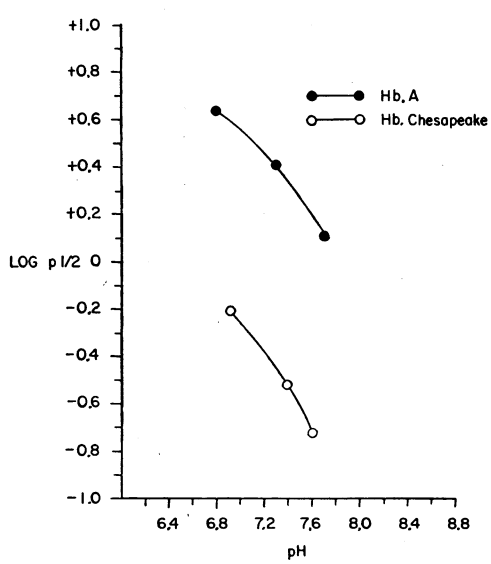

FIG. 8. VARIATION OF THE OXYGEN PRESSURE AT 10° C REQUIRED FOR HALF SATURATION OF HEMOGLOBIN ($P_{\frac{1}{2}}$) WITH pH. Data for hemoglobin Chesapeake (○) are compared with data of Ranney and co-workers (2) for hemoglobin A (●).

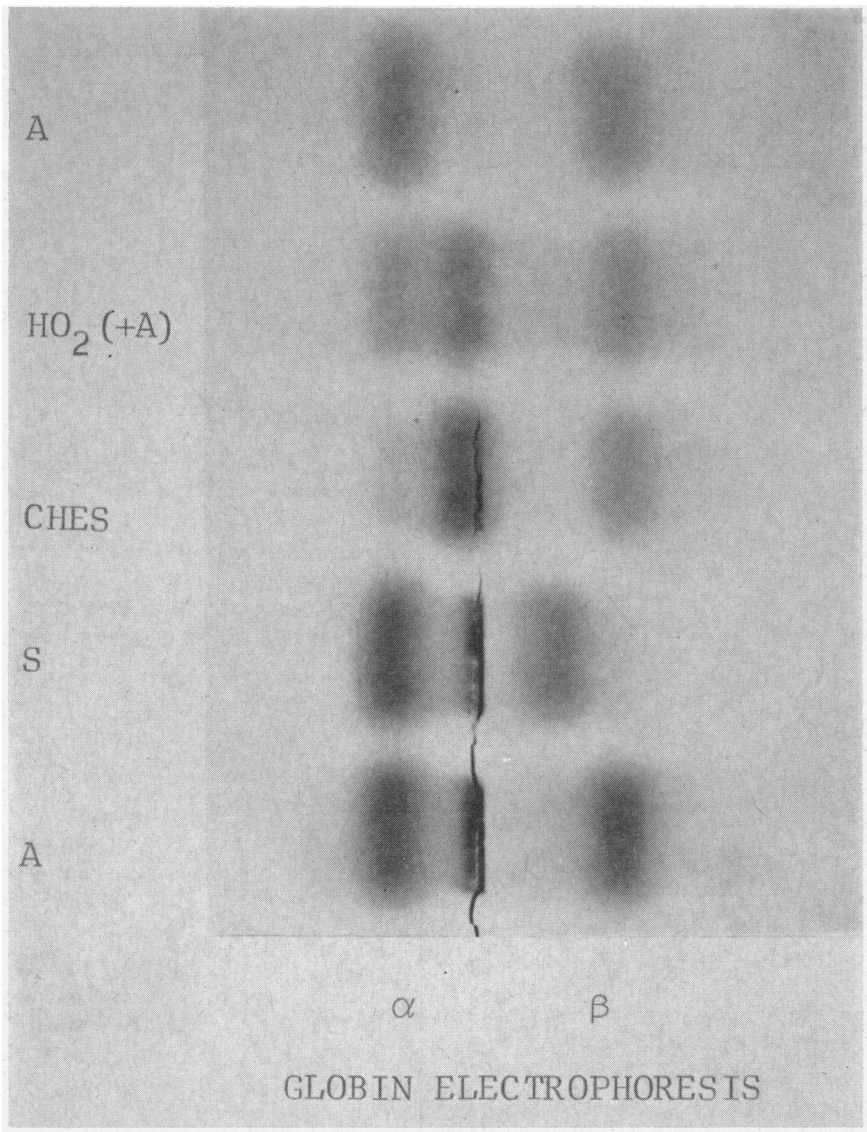

FIG. 9. GLOBIN ELECTROPHORESIS IN STARCH GEL CONTAINING 6 M UREA. Globin from purified hemoglobin Chesapeake is compared with globin from hemoglobins A and S and semipurified hemoglobin Hopkins-2 (amido black stain).

Similar experiments were carried out at pH 6.8 and 7.6 with samples of hemoglobin Chesapeake. No methemoglobin absorption band (between 610 and 650 mμ) was detected in these samples after dialysis. The two regression lines had slopes of 1.1 and 1.2. The oxygen tension required for half saturation of hemoglobin is plotted against pH in Figure 8. Included are data of Ranney and her associates for hemoglobin A, obtained under the same conditions (2). A Bohr effect of approximately normal magnitude was present.

Physicochemical characteristics of hemoglobin Chesapeake. On starch gel at pH 8.6, hemoglobin Chesapeake migrated more rapidly than either hemoglobin A or the synthetic hemoglobin-glutathione complex. Its mobility was approximately the same as that of hemoglobins Hopkins-2 or J, and less than that of hemoglobins Bart's, N, or I, suggesting that it had one less positive charge than hemoglobin A at pH 8.6. Hemoglobin Chesapeake did not separate from hemoglobin A during citrate agar electrochromatography at pH 6.0. It was not alkali resistant, it was not pre-

cipitated by incubation at 50° C for 2 hours, and no inclusion bodies formed in red cells after incubation with brilliant cresyl blue or new methylene blue.

Dissociation and recombination with hemoglobin C, and electrophoresis of globin (Figure 9), indicated that altered electrophoretic mobility was due to an abnormality of the α chain. Peptide mapping of tryptic digests of the AE-α chain of hemoglobin Chesapeake showed that peptides α^{A}T10 and α^{A}T11 were missing and that a new peptide was present. The arginine stain revealed only two (α^{A}T4 and α^{A}T14) of the three major arginine positive peptides normally found on AE-α chain peptide maps. These observations suggested that the C-terminal arginine in peptide α^{A}T10 had been replaced by a neutral amino acid, that the bond to peptide α^{A}T11 was no longer susceptible to trypsin, and that the new peptide was a conjugate, $\alpha^{\text{Chesapeake}}$T10-11.

TABLE II

Mole ratios of amino acids in "new" peptide of hemoglobin Chesapeake, compared with data for hemoglobin A (25)

	α^{A}T10	α^{A}T11	α^{A}T10 + α^{A}T11	$\alpha^{\text{Chesapeake}}$ T10–11
Lysine		1	1	1.2 (1)
Histidine				
Arginine	1		1	
Asparagine		2	2	2.0 (2)
Threonine				
Serine				
Glutamine				
Proline		1	1	1.0 (1)
Glycine				
Alanine				
Cysteine				
Valine		2	2	1.9 (2)
Methionine				
Isoleucine				
Leucine	1		1	2.0 (2)
Tyrosine				
Phenylalanine		1	1	0.9 (1)
Tryptophan*				

* By staining reaction on paper.

Analysis of the new peptide (Table II) confirmed this conclusion. With two exceptions, the amino acids found in the new peptide were those expected from a combination of peptides α^{A}T10 and α^{A}T11 (25). No arginine residues were detected, and the mole ratio of leucine was 2 instead of the anticipated 1. Substitution of leucine for the only arginine in peptide α^{A}T10 (position 92 of the α chain) satisfactorily accounts for the charge differences seen on starch gel electrophoresis of hemoglobin Chesapeake. Full details of these analyses will be published elsewhere (26).

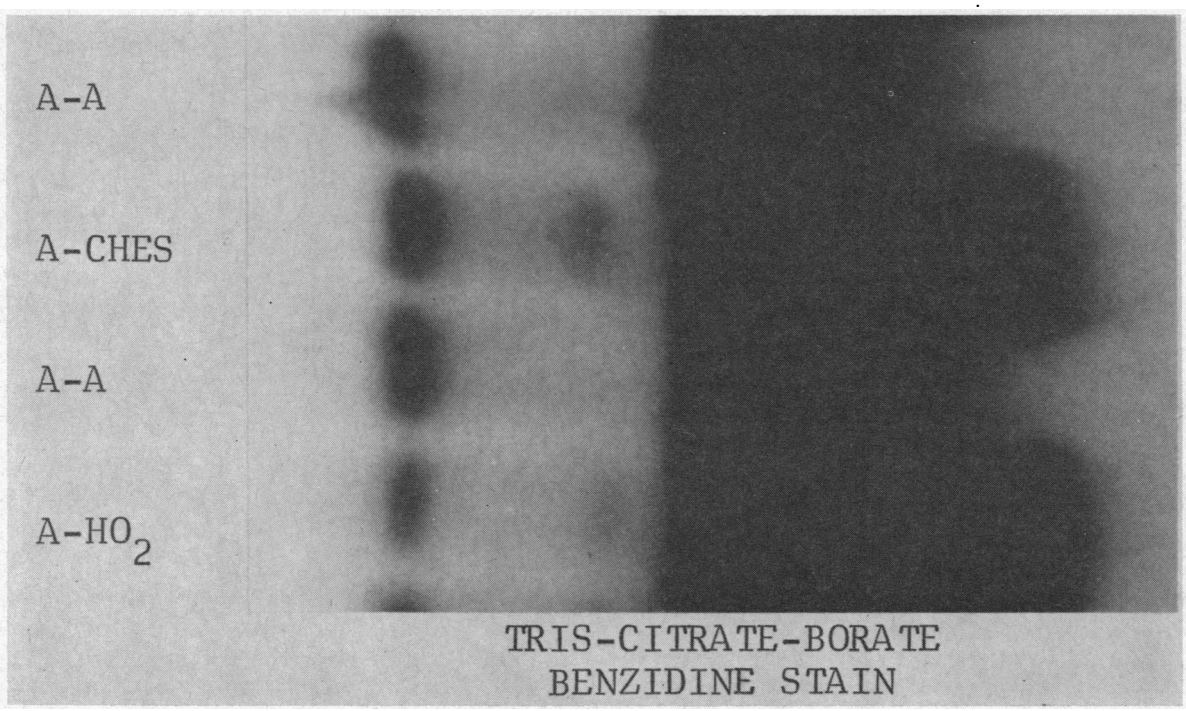

FIG. 10. STARCH GEL ELECTROPHORESIS AT pH 8.6. The gel was overloaded and stained with benzidine in order to demonstrate variants of hemoglobin A_2.

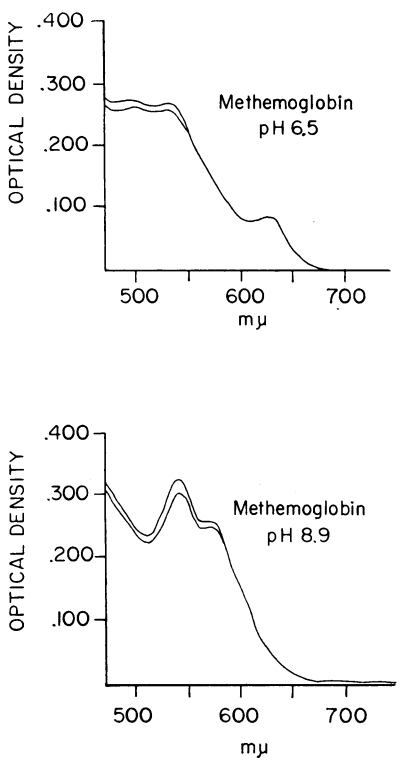

FIG. 11. COMPARISON OF VISIBLE ABSORPTION SPECTRA OF HEMOGLOBIN CHESAPEAKE AND HEMOGLOBIN A AS ACID AND ALKALINE METHEMOGLOBIN DERIVATIVES. The upper line represents hemoglobin Chesapeake.

A small amount of a hemoglobin with the electrophoretic mobility of hemoglobin S could be detected by starch gel electrophoresis with a Tris-citrate-borate buffer system, but not with other systems (Figure 10). This fraction contained about 1% of the total hemoglobin present. No structural analyses of it were carried out.

Oxygenated solutions of hemoglobin Chesapeake had normal visible and ultraviolet absorption spectra, and deoxygenated solutions of hemoglobin Chesapeake had normal visible absorption spectra. After conversion to acid or alkaline methemoglobin, the optical density of solutions of hemoglobin Chesapeake was somewhat greater than that of hemoglobin A between wavelengths of 470 and 550 mμ (Figure 11).

Discussion

Mild polycythemia in the propositus and in members of his family was associated with the presence of an abnormal hemoglobin with increased oxygen affinity. Blood containing 25 to 35% of the abnormal hemoglobin had an oxygen affinity approximately as great as that of umbilical cord blood. Presumably, heterozygous carriers had an increased number of red cells because their blood delivered less oxygen per red cell than did the blood of their normal siblings. Increased amounts of erythropoietin could not be demonstrated in the propositus's serum, a result similar to that found in polycythemic persons who have lived at high altitudes for long periods of time (23).

Carriers of hemoglobin Chesapeake were quite similar to some cases of "benign familial polycythemia" (27), particularly to individuals with mildly elevated hematocrit values and without splenomegaly. Hemoglobin, studied by paper electrophoresis, was normal in the patients described by Auerback, Wolff, and Mettier (28). Blood from two of the patients reported by Nadler and Cohn (29) has been restudied [2]; both hemolysates were normal after starch gel electrophoresis at pH 8.6.

The eight normal offspring of carrier females were of particular interest. There was no suggestion that fetal oxygenation had been deficient during the eight pregnancies in question, despite absence of the usual low oxygen affinity of maternal blood relative to that of the fetus. The presence of high affinity for oxygen in the mother was found to be compatible with the production of term infants who developed normally, for there had been only two spontaneous abortions among 21 pregnancies. Similar observations have been made in another hemoglobinopathy: a female carrier of hemoglobin Zurich was capable of bearing normal offspring, although the affinity of her blood for oxygen was almost as great as that of carriers of hemoglobin Chesapeake (11). Adequacy of fetal oxygenation is the result of many interrelated factors, including rates of uterine and umbilical blood flow, size of placental capillary bed, and fetal blood volume. No such data are available in this family.

The increased oxygen affinity of umbilical cord blood is not a property of the fetal hemoglobin it contains (4). Heterozygous carriers of hemoglobin Chesapeake may have had an abnormal fetal hemoglobin while *in utero,* of composition

[2] Through the courtesy of Dr. Nadler.

$\alpha_2^{Ches}\gamma_2$. Unfortunately, we were unable to study any blood samples that might have contained this hemoglobin. Such studies would be of considerable interest, for the fetal analogue of hemoglobin Chesapeake might have increased oxygen affinity, and the oxygen affinity of blood containing it might be very high.

The faint band of hemoglobin demonstrated only by discontinuous starch gel electrophoresis presumably had the composition $\alpha_2^{Ches}\delta_2$. This "second A_2 hemoglobin" contained about 1% of the total hemoglobin present. Carriers of hemoglobin Chesapeake had relatively low proportions of hemoglobin A_2 ($\alpha_2\delta_2$) in their hemolysates (1.8 to 2.6%). Since approximately 30% of the major hemoglobin component was hemoglobin Chesapeake, one would expect about 1% of the second hemoglobin A_2, a prediction consistent with the observed data.

Blood from heterozygous carriers, containing about 30% hemoglobin Chesapeake, exhibited increased oxygen affinity and decreased heme-heme interaction ($n = 1.8$). Semipurified samples of hemoglobin Chesapeake showed the same findings, as well as a Bohr effect of approximately normal magnitude. Heme-heme interactions were diminished ($n = 1.1$), but not abolished ($n = 1.0$). In studies of hemoglobin Chesapeake, isolated by starch block electrophoresis in barbital buffer, pH 8.6, Nagel has obtained values for n of 1.3 on four separate measurements of oxygen equilibrium. He has also found lowered values for n in studies of the whole hemolysate; preliminary measurements have indicated an n of about 1.8 (30).

Analysis of electrophoretic studies indicated that hemoglobin Chesapeake had one less positive charge than hemoglobin A, at pH 8.6. The amino acid substitution responsible for altered electrophoretic mobility of hemoglobin Chesapeake was replacement of positively charged arginine by uncharged leucine in amino acid no. 92 of the α chain. This substitution corresponds to a one step mutation in the messenger ribonucleic acid codon for arginine, from cytosine-guanine-(adenine) to cytosine-uracil-(adenine), as outlined by Beale and Lehmann (31). A substitution of this type might produce increased oxygen affinity either by direct electrostatic interactions or by altering interchain relationships.

The effects of arginine and leucine would be expected to differ from each other because of the difference in their electric charges. The heme-linked histidine of the α chain is only five amino acid residues away from the site of substitution (32), in such a position that an alteration of charge might affect the avidity of the heme iron atom for electrons donated by oxygen.

Charged amino acid side chains, like that of arginine, tend to protrude outwards from the surface of the hemoglobin molecule in an aqueous environment, whereas nonpolar groups, like that of leucine, tend to be buried in the interior of the molecule (33). Amino acid no. 92 of the α chain is at the border between α and β chains (34), an area in which the α and β chains slide past each other during the spatial rearrangements of oxygenation and deoxygenation. Altered structural relationships produced by substitution of a nonpolar for a polar amino acid could mechanically impede free equilibrium between the oxygenated and deoxygenated forms of the molecule. This hypothesis is favored by demonstration of impaired heme-heme interactions, but data at present are insufficient to strongly indicate either alternative.

Summary

1. An abnormal hemoglobin, called Chesapeake, was associated with mild polycythemia in a Caucasian family.

2. Blood from heterozygous carriers had an oxygen affinity as great as that of umbilical cord blood, and increased oxygen affinity was shown to be a property of purified hemoglobin Chesapeake.

3. Hemoglobin Chesapeake differed from hemoglobin A in substitution of leucine for arginine in the ninety-second amino acid residue of the α chain.

4. Hypotheses are presented relating the altered function of hemoglobin Chesapeake to this discrete structural abnormality.

Acknowledgments

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