Hemoglobin Okaloosa (β48 (CD7) Leucine → Arginine)

AN UNSTABLE VARIANT WITH LOW OXYGEN AFFINITY

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ABSTRACT A slow-moving hemoglobin with electrophoretic mobility similar to that of hemoglobin S was discovered in a white laboratory technologist. She had an elevated reticulocyte count, as did several members of her family. Her red cell survival was shortened. Amino acid analysis indicated that leucine at position β48 (CD7) had been replaced by arginine. The abnormal hemoglobin, called Okaloosa, was heat-precipitable and had decreased oxygen affinity. It exhibited a greater change in oxygen affinity than hemoglobin A when 2,3 DPG was added to "stripped" hemolysates. These findings cannot be readily explained by current views of structure-function relationships in the hemoglobin molecule. However, it is of interest that the amino acid in position CD7 is normally leucine in the α, β, δ, and γ-hemoglobin chains and in the myoglobin chain of man and a wide variety of other vertebrates.

INTRODUCTION

Theories that relate the structure of the hemoglobin molecule to its function have, in general, followed upon clinical observations. Since most of the abnormal hemoglobins have been discovered in asymptomatic heterozygotes, most of the discrete structural changes that produce these hemoglobins are considered "neutral." We report here an abnormal hemoglobin that is associated with slight reticulocytosis. It exhibits increased heat precipitability and decreased oxygen affinity. Neither finding appears to be of clinical significance and could easily be overlooked, but it seems clear that the abnormal structure cannot be considered functionally equivalent to that of hemoglobin A.

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METHODS

Case report. The proband, a white medical technologist from Okaloosa County, Fla., discovered that she was a carrier of an abnormal hemoglobin when she used herself as a control for electrophoresis studies. She considered herself in good health, had never been anemic or jaundiced, and knew of no hematologic abnormalities in her family aside from ovalocytosis in a cousin. There was no history of cholelithiasis in family members. Physical examination was normal except for congenital ptosis of one eye. Serum bilirubin, haptoglobin, and red cell morphology were normal. Other hematologic data and those of available family members (Fig. 1), are presented in Table I. Hematocrit values of the proposita and one uncle were at the lower limit of normal; four of the five carriers of Hb Okaloosa had elevated reticulocyte counts.

Methods. Hematologic studies were carried out by routine methods. Samples from persons other than the proband were mailed from Florida and Alabama. In the past, we have obtained erroneously low values for hemoglobin A_2 in such samples, and that measurement was not carried out here. Microhematocrits were measured, and blood smears were prepared before mailing. Proportions of hemoglobin components were measured spectrophotometrically at 415 nm after elution from strips of cellulose acetate.[1] Ferrohemoglobin solubility was measured by the method of Itano (1). Heat precipitability was measured by a previously described technique (2) and as described by Keeling et al. (3). Precipitability by *p*-hydroxymercuribenzoate (PMB)[2] at pH 6 was estimated after incubation at 4°C for 18 h (3), and precipitability by 17% isopropanol was measured by a minor modification of the method of Carrell and Kay (4).

For studies of structure, Hb Okaloosa was purified on DEAE Sephadex (5), concentrated by ultrafiltration and converted to globin by mixing with 2% HCl-acetone at −20°C. The abnormal β-chain was separated and aminoethylated by the procedure of Clegg, Naughton, and Wea-

[1] Gelman Instrument Co., Ann Arbor, Mich.

[2] *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; 2,3-DPG, 2,3-diphosphoglycerate; MLS, mean life span; P_{50}, half saturation; PMB, *p*-hydroxymercuribenzoate.

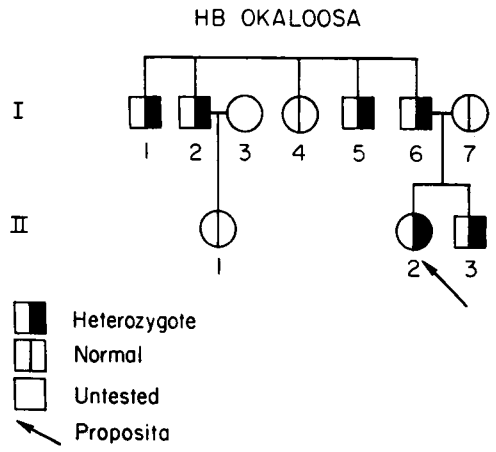

FIGURE 1 Pedigree of the proband.

TABLE I
Hematologic Data

	Sex	Hemoglobin	Hematocrit value	Reticulocyte count	Hb Okaloosa
		g/100 ml	%	%	%
I-1	M	15.9	43	2.4	32
I-2	M	14.9	45	3.1	33
I-4	F	—	—	1.6	0
I-5	M	17.9	49	1.8	34
I-6	M	15.6	49	4.3	32
I-7	F	—	41	1.1	0
I-1	F	13.4	40	1.1	0
II-2	F	13.0	38	3.6	36
II-3	M	15.8	49	3.4	32

therall (6), dialyzed against 1% formic acid, and lyophilized, and a portion was hydrolyzed with trypsin (7). The tryptic peptides were separated by automatic peptide chromatography on a column of Aminex A-5 resin[3] (0.9 × 16 cm) with a linear gradient of pyridine-acetic acid developers (8). The peaks that did not yield pure peptides were rechromatographed under the same conditions on a column of Aminex 50W-X2 resin (0.9 × 60 cm). After hydrolysis with 6N HCl at 100° in *vacuo*, amino acid analyses were carried out on each peptide using a Beckman-Spinco 120A amino acid analyzer.[4] Hydrolysis times were 48 h for βT-1, 72 h for βT-4 and βT-14, and 22 h for all the others.

Red cell survival was measured with diisopropylfluorophosphate (DFP) in propylene glycol[5] (9): [^{3}H]DFP (7 μCi/kg, sp act 1 Ci/mM or [^{14}C]DFP (50 mCi, sp act > 100 μCi/mM) was dissolved in 5 ml of sterile 0.9% sodium chloride solution and immediately injected intravenously through a 0.22 μm Millipore filter. The first blood samples were obtained 3 days after injection of DFP when plasma counts were negligible and for 4–8 wk thereafter. The hemoglobin content was measured, plasma was removed after centrifugation, 0.1 ml of Triton X-100[6] was added to the pellet, and hemoglobin content was again measured. Aliquots (0.5 ml) of the lysate were pipetted onto 7-cm disks of Whatman 43 filter paper, air dried, wrapped in another disk, and compressed into pellets weighing approximately 0.3 g. These were burned in a Tri-Carb model 305 Sample Oxidizer, the [^{3}H]H_2O being collected in 8 ml of Insta-Gel.[7] Samples were counted to 2% accuracy in a Beckman LS 130 liquid scintillation counter and were corrected for quenching and expressed as distintegrations per minute per milliliter blood in each day's sample.

With the aid of a Monroe 1785 calculator, linear equations and correlation coefficients were calculated for the regression of both dpm and log dpm on time. For semilogarithmic plots the mean cell life was calculated from 0.4343/slope. For linear plots, mean cell life was calculated from (calculated counts on day 0)/slope.

[3] Bio-Rad Laboratories, Richmond, Calif.
[4] Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.
[5] New England Nuclear Corp., Boston, Mass.
[6] Roehm and Haas Co., Philadelphia, Pa.
[7] Packard Instrument Co., Inc., Downers Grove, Ill.

Oxygen affinity of whole blood was measured by a minor modification of the method of mixing (10). Oxygen affinity of purified hemoglobins was measured after chromatography in 0.05 M Tris-HCl on refrigerated 1 × 7 in columns of DEAE Sephadex. Eluates containing hemoglobin A had a pH of 7.8, while those containing Okaloosa had a pH of 8.1. Acetate buffer (0.05 M, pH 5.2) was added to these eluates (approximately 1 ml/10 ml) to adjust their pH. In all cases affinity of hemoglobin A was measured in samples eluted from the same column as Okaloosa or from a column run under identical conditions. Hemoglobin fractions prepared in this manner have been shown to be "stripped" of 2,3-diphosphoglycerate (11). Samples were studied at 10°C by previously described methods (12). Methemoglobin content before and after analysis was estimated from the optical density at 630 nm.

RESULTS

In some studies 0.02–0.08 ml of 0.028 M 2,3-diphosphoglycerate (2,3-DPG)[8] in water was added to 7.0-ml samples of column eluate, and the pH was adjusted to approximately 7.1 (7.06–7.13). Content of 2,3-DPG was assayed by the method of Erickson and deVerdier (13).[9]

Structure. The electrophoretic pattern of the proband's hemolysate resembled that of persons with sickle-cell trait, and hemoglobin Okaloosa had the same electrophoretic mobility as hemoglobin S on starch gel and cellulose acetate (Fig. 2). The abnormal hemoglobin did not separate from Hb-A on agar, cells containing it did not sickle in sodium metabisulfite, and the solubility of deoxygenated whole hemolysate and that of the purified deoxyhemoglobin were only slightly lower than that of Hb-A in 2.58 M phosphate buffer (Table II).

Proportions of the abnormal hemoglobin in hemolysates (Table I) suggested that it was a β-chain variant, and this was confirmed by chromatography of globin. Samples of the proband's blood collected in Baltimore

[8] Pentacyclohexylammonium salt, tetrahydrate; Calbiochem, La Jolla, Calif.
[9] Kit No. 35-UV; Sigma Chemical Co., St. Louis, Mo.

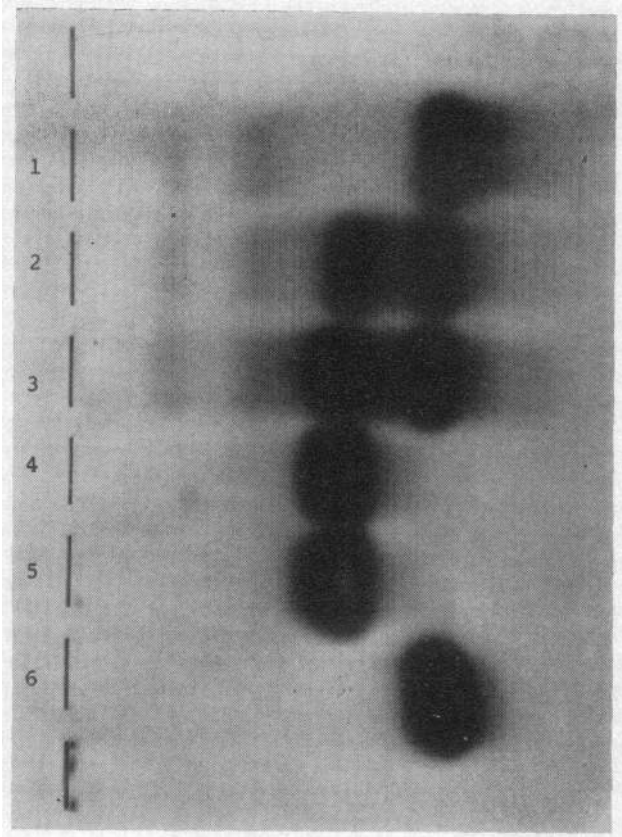

FIGURE 2 Starch gel electrophoresis at pH 8.6, Tris-EDTA-borate buffer: (*1*) normal hemolysate, (*2*) proposita's hemolysate, (*3*) sickle trait hemolysate, (*4*) purified Hb Okaloosa, (*5*) purified Hb S, (*6*) purified Hb A. Hemoglobin S moved somewhat more slowly than Okaloosa in hemolysates, but purified hemoglobins had the same mobility. After electrophoresis at pH 7 in phosphate buffer, both hemolysates and purified hemoglobins had the same mobility.

contained more Okaloosa than samples mailed from Alabama, suggesting that some denaturation occurred in transit. The chromatographic pattern from the tryptic hydrolysate of the abnormal aminoethylated β-chain is shown in Fig. 3, and amino acid compositions of all purified peptides are given in Table III. It will be seen that all the peptides expected from a normal human β-chain were found except for βT-5 and that two extra peptides were present. One of these was found along with βT-3 and βT-13 upon rechromatography of Zone I. The other was found along with βT-1 and βT-8 after rechromatography of Zone VI.

Using the nomenclature of Dayhoff (14) the residues of these two abnormal peptides can be aligned with those of the missing peptide, βT-5. It is apparent that leucyl residue no. 48 has been replaced by arginine in Hb Okaloosa.

Residue number	41	48	59
Normal βT-5	F F E S F G D *L* S T P D A V M G N P K		
Abnormal peptides from Okaloosa β-chain	F F E S F G D *R*:S T P D A V M G N P K βT-5A		βT-5B

Function. Reticulocyte counts were somewhat elevated in several carriers (Table I). The proband's red cell survival data were equally well fitted by a linear or an exponential decay curve (Fig. 4, Table IV). Data from the normal control, which were collected over a much longer period of time, were somewhat better fitted by an exponential decay curve. His mean cell life fell within accepted normal limits only when calculated by the linear model. The mean life span of the proband cells calculated by either model was 60% of that of the control.

Pure hemoglobin Okaloosa was more readily precipitated by heat than Hb A or S at temperatures of 55°C and higher (Fig. 5). When fresh hemolysate was heated at 65°C for 10 min, 13% of the hemoglobin precipitated; 2% of normal hemolysates and 22% of a hemolysate from a carrier of Hb Zurich precipitated under these conditions. Some but not all of the Hb Okaloosa in the proband hemolysate was precipitated by incubation with PMB. After incubation in isopropanol, Okaloosa precipitated faster than Hb Hasharon (15) but not as fast as hemoglobin Zurich. Heinz bodies were not formed after incubating the proband's red cells with brilliant cresyl blue for 2 h at 37°C.

Oxygen affinity of the proband's whole blood was slightly decreased (P_{50}, pH 7.4, 30 mm Hg). Her red cells contained a slightly decreased amount of 2,3-DPG (4.7 μmol/ml RBC; normal female 4.9–5.7 μmol/ml RBC). Oxygen affinity of the purified hemoglobin was slightly decreased (Fig. 6); the Bohr effect did not differ from that of Hb A (Hb A, $\Delta \log P_{50}/\Delta \text{ pH} = 0.75$; Hb Okaloosa, $\Delta \log P_{50}/\Delta \text{ pH} = 0.75$). Hill's constant was normal (Hb A $n = 2.79 \pm 0.19$ [SD]; Hb Okaloosa $n = 2.85 \pm 0.27$ [SD]). Methemoglobin content of samples of purified hemoglobins before oxygen affinity was measured was negligible; afterwards the average content

TABLE II
Ferrohemoglobin Solubility (g/liter) in 2.58 M Phosphate pH 7.2

Normal hemolysate	1.59
A-Okaloosa hemolysate	1.02
A-S hemolysate	0.63
Purified hemoglobin A	1.56
Purified Hb Okaloosa	0.97
Purified hemoglobin S	0

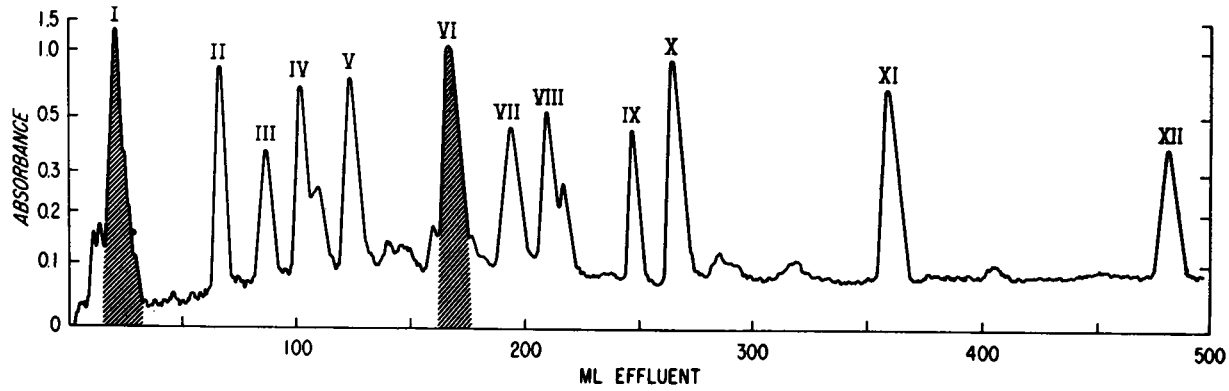

FIGURE 3 Peptide pattern of the trypic hydrolysate of aminoethylated β-chains from the proposita. Reaction of ninhydrin with peptides was followed at 570 nm. Shaded zones show position of abnormal peptides 5A and 5B. Gradient: pyridine-acetic acid buffers starting at pH 3.1 and ending at pH 5.0.

was 8% with a range of 2–20%. When 2,3-DPG was added to "stripped" hemolysates, P_{50} increased more with the Okaloosa samples than with hemoglobin A (Fig. 6).

DISCUSSION

Two explanations are available for the borderline hematocrit and hemoglobin levels of the proband. Hemoglobin Okaloosa has decreased oxygen affinity; carriers of such hemoglobins may have a decreased red cell mass because their blood releases oxygen to tissues more readily than normal (17). Okaloosa is unstable, and red cells containing it have a shortened life span; carriers of such hemoglobins may be anemic because of uncompensated hemolytic anemia (18). Other carriers in the family are not anemic, and one must discard both hypotheses. Some carriers exhibit reticulocytosis, and the life span of the proband's red cells is shortened, suggesting that compensated hemolytic anemia is present and that it is related to instability of the abnormal hemoglobin.

Because of the instability of Hb Okaloosa, it was necessary to purify samples for measurement of oxygen affinity on refrigerated columns. Eluates contained little or no DPG. Their pH's were 7.8 and 8.1, and these were adjusted by addition of 0.05 M acetate buffer. Different amounts of acetate were added to the two sets of column fractions, and the final ionic strengths differed. These differences were most marked in the samples adjusted to pH 6.8. Buffer composition (19) and ionic strength

TABLE III

Amino Acid Composition of Tryptic Peptides of Aminoethylated β-Chain of Hemoglobin Okaloosa

Amino acid	βT-1 VI	βT-1 V	βT-3 I	βT-4 IV	βT-5A VI abnormal	βT-5B I abnormal	βT-6 IX	βT-7 XI	βT-8 VI	βT-9 III	βT-10 VIII	βT-11 VII	βT-12A II	βT-12B XII	βT-13 I	βT-14 IV	βT-15 X
Lysine	1.33	0.98		0.97		1.18	0.98	0.98	1.00	1.13	0.96			1.12	1.03	0.98	
AE-cysteine											0.73		0.90				
Histidine	0.95							0.91		0.99	0.91	0.96		1.88		0.90	0.98
Tryptophan		0.20		0.29													
Arginine			1.00		1.03							0.98					
Aspartic acid			2.11		1.09	2.00				3.28	1.05	2.00	1.04			1.09	
Threonine	1.03	1.02		0.93		1.00					1.80				0.94		
Serine		0.97			0.99	1.00				1.05	0.98						
Glutamic acid	2.04		2.02	1.03	1.00						1.07	1.02			2.87		
Proline	1.13			1.08		2.15						1.17			2.00		
Glycine	0.16	1.05	2.96		1.08	0.91		1.17		2.16	1.10		1.10	1.12		1.17	
Alanine		2.00	1.03			1.02		1.08		2.16	1.09			1.09	2.10	4.00	
Valine	0.96	1.03	2.95	1.96		0.91	1.04			1.05		0.99	1.86	0.90	1.03	2.90	
Methionine						0.50											
Leucine	1.07	1.07	1.03	2.26						3.70	1.96	1.02	3.00	0.88		1.10	
Tyrosine				1.03											1.01		1.01
Phenylalanine					2.85					0.95	0.98	1.06		1.03	0.98		

Ratios of amino acids recovered following acid hydrolysis (see Methods). Roman numerals designate zone from chromatogram in Fig. 2.

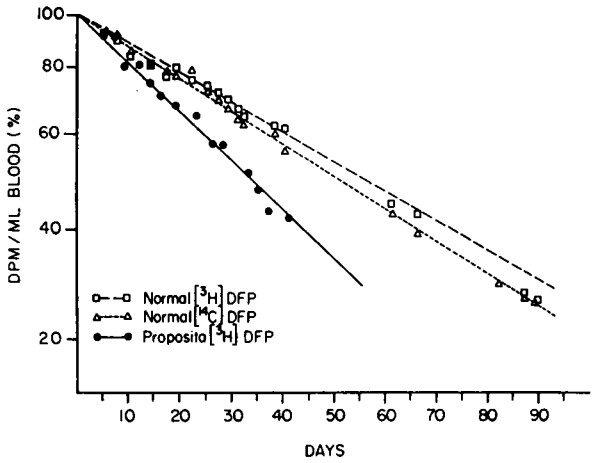

FIGURE 4 Survival of red cells from the proband and a normal control, after labeling with DFP.

TABLE IV
Survival Data-DFP Labeled Red Blood Cells

	Exponential decay		Linear decay	
	r	MLS	*r*	MLS
Proband-^{3}H	−0.993	47.8	−0.995	72.5
Normal control-^{3}H	−0.986	79.5	−0.923	116
Normal control-^{14}C	−0.999	72.5	−0.982	122
Normal-^{32}P				123
Sickle cell anemia-^{32}P		16.4		
Hemoglobin C disease		37.1		

Data from the proband and a normal control were fitted to exponential and linear decay curves. Correlation coefficient (*r*), and mean red cell life span (MLS) were calculated as outlined in the text. For reference, data for normals (15) and patients with hemoglobinopathies (16) have been recalculated from the literature.

both affect oxygen affinity, but this suboptimal technique for adjustment of pH was chosen to avoid denaturation of the dilute solutions of unstable hemoglobin. The fact that differences in P_{50} between the two hemoglobins were as great at pH 7.5 as at 6.8 suggests that the specific-ion and salt effects were not large.

The buffering effect of Tris is small at pH values below 7, and it is possible that the pH within the tonometers changed during oxygenation as Bohr protons were liberated. If that were the case, Hill plots might be expected to change shape or slope as a function of pH; they did not.

Addition of DPG to "stripped" hemolysates decreased oxygen affinity, but the effect on Hb Okaloosa was somewhat greater than that on Hb A. Bunn and Briehl have carried out similar experiments with a variety of normal and abnormal hemoglobins (20). Our data are not

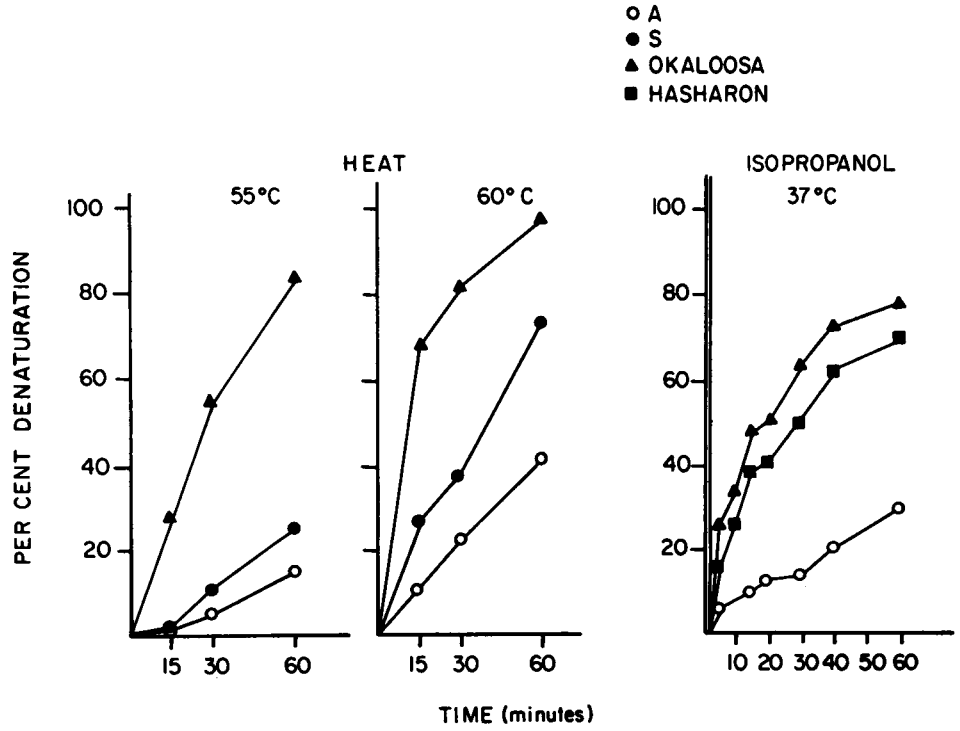

FIGURE 5 Denaturation by heat, and by 17% isopropanol.

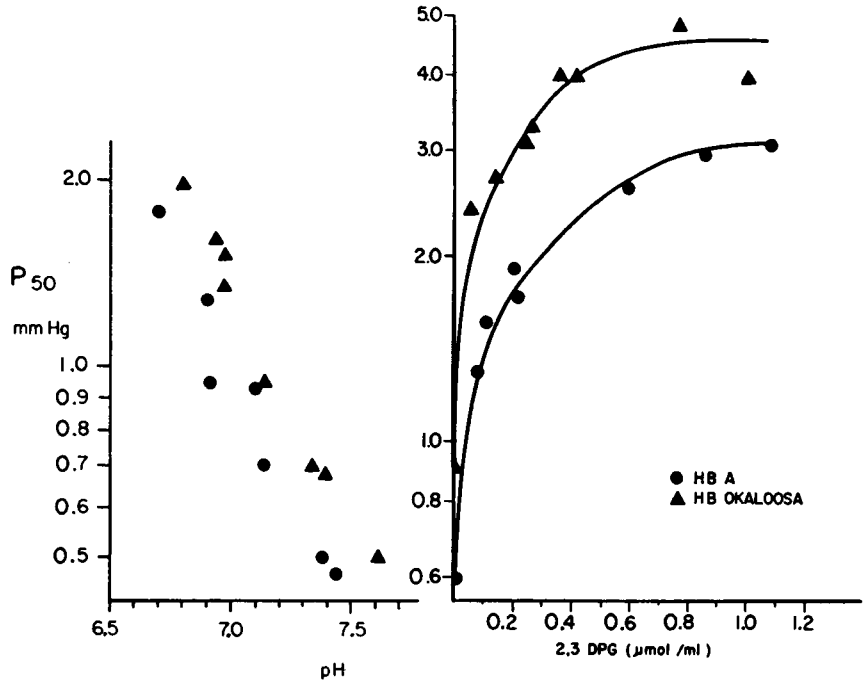

FIGURE 6 Oxygen pressure required for half saturation (P_{50}) of chromatographically purified hemoglobins A and Okaloosa, 0.05 M Tris-HCl, 10°C. *Left:* as a function of pH; samples contained no 2,3-DPG. *Right:* as a function of 2,3-DPG concentration; pH was 7.06–7.13.

entirely comparable to theirs, perhaps because we used the pentacyclohexylammonium salt of DPG, but the difference between Okaloosa and Hb A is at least equivalent to the most significant difference they noted with hemoglobin $M_{Milwaukee}$:

$$\left(\frac{P_{50}\ DPG}{P_{50}\ Stripped}\right)_{Mil} \div \left(\frac{P_{50}\ DPG}{P_{50}\ Stripped}\right)_{A} = \frac{2.35}{2.02} = 1.17$$

at DPG = 0.2 μmol/ml

$$\left(\frac{P_{50}\ DPG}{P_{50}\ Stripped}\right)_{Oka} \div \left(\frac{P_{50}\ DPG}{P_{50}\ Stripped}\right)_{A} = \frac{3.16}{2.65} = 1.19$$

It is not apparent why substitution of arginine for leucine at position CD7 produces decreased oxygen affinity and increased heat precipitability, but similar abnormalities have been observed in hemoglobins produced by neighboring mutations. Four unstable hemoglobins with decreased oxygen affinity are produced by substitutions at position CD1 (Hammersmith [21], Louisville [3], Bucuresti [22], and Torino [23]). These hemoglobins are quite unstable, and carriers have clinically significant hemolytic anemia. Substitutions at CD5 (Ferrara [24], Hasharon [2, 25]) produce mild heat instability, but hemolysis is barely detectable in most carriers, and oxygen affinity is normal. The CD1 substitutions are thought to interfere with anchoring of the heme ring (18, 26, 28). No mechanism has been suggested for abnormalities produced by substitution at CD5, which is on the surface of the molecule (18).

The properties of hemoglobin Okaloosa seem to fall between these two groups. Leucine at position CD7 is an "invariant" residue in α, β, δ, and γ-hemoglobin chains and in myoglobin chains of many species of animals (reference 14, p. D-369). It is an important spacer at the corner between helices C and D and touches heme-contacts CD1 and CD4 (29).[10] Clearly, the substitution of arginine for leucine at βCD7 is *not* a "neutral" mutation. As other abnormal hemoglobins are studied in the clinic and in the laboratory, a reason for the behavior of Hb Okaloosa may emerge.

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