

Hemoglobin Louisville (β 42 (CD1) Phe \rightarrow Leu): an Unstable Variant Causing Mild Hemolytic Anemia

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ABSTRACT An unstable hemoglobin variant termed Hb Louisville, was found in four members of a Caucasian family, who were suffering from a mild hemolytic anemia. The variant showed a decreased stability upon warming at 65°C and an increased tendency to dissociate in the presence of sulfhydryl group-blocking agents. The structural abnormality was identified as a replacement of phenylalanyl residue in position 42 (CD1) by a leucyl residue. Substitution of this phenylalanyl residue, which participates in the contact with heme, by a nonpolar leucyl residue has apparently less severe consequences than a replacement of the same residue by a polar seryl residue as in Hb Hammersmith.

Oxygen equilibrium studies of total hemolysate from one Hb Louisville heterozygote indicated a decreased oxygen affinity, a marked decrease in heme-heme interaction, and a normal Bohr effect. Studies with isolated Hb Louisville were not made because it was not possible to separate the variant from normal Hb A.

INTRODUCTION

Familial occurrence of unstable hemoglobin variants has been reported on numerous occasions, and the structural abnormalities in many of these have been determined (summarized in references 1-4). The present report describes a new variant which occurred in a father and three of his sons causing a relatively mild hemolytic anemia.

METHODS

Hematological studies. Routine hematological examinations were made by standard techniques (5). Freshly collected blood samples were mailed in ice airmail special

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delivery from Louisville, Ky., to Augusta, Ga. The level of 2,3-diphosphoglycerate (2,3-DPG)¹ was determined on two occasions; the method used was that described by Grisolia, Moore, Luque, and Grady (6). Hemolysates were prepared usually within 24 hr after collection by mixing 1 vol of washed, packed, red cells, 1 volume of distilled water, and 0.2 vol of carbon tetrachloride for 10 min. Stroma and other debris were removed by centrifugation at 10,000 *g* for 20 min at 4°C.

Hemoglobin studies. Horizontal starch gel hemoglobin electrophoresis at pH 9.0 was done by the method described before (7). Chromatographic analyses were made using columns of DEAE-Sephadex (8, 9). The per cent alkali-resistant hemoglobin (% F_{AD}) was determined by the method of Betke, Marti, and Schlicht (10). The heat stability of the hemoglobin in red cell hemolysate was tested using a modification of the method described by Grimes, Meisler, and Dacie (11). Freshly prepared red cell hemolysate was diluted with distilled water until a final hemoglobin concentration of 200-250 mg/100 ml was reached. 5-ml portions of this diluted hemolysate were mixed with 0.5 ml 2.5 M sodium phosphate buffer, pH 6.9, and incubated at 65°C for 2, 4, 6, 8, 10, and 15 min. After cooling in ice, the precipitate was removed by centrifugation for 15 min at 5000 rpm, and the extinctions of the supernates were determined at 523 m μ using a Zeiss PMQII spectrophotometer (523 m μ is the approximate isobestic point for oxy-, deoxy-, and ferrihemoglobin). The presence of the abnormal hemoglobin was also investigated by starch gel electrophoresis of the *p*-hydroxymercuribenzoate (PMB) derivatives of the hemoglobins from various members of the family. The procedure is the same as used in a previous study (12).

Structural analyses. Isolation of the abnormal β -chain from the hemoglobin of subject He. E. (Fig. 1) was possible after treatment with *p*-chloromercuribenzoate (PCMB) following the technique described in detail previously (12). The precipitate, i.e. the PMB derivative of the β -chain from the unstable hemoglobin variant, formed within 1 hr and was isolated by centrifugation at 8000 rpm for

¹Abbreviations used in this paper: 2,3-DPG, 2,3-diphosphoglycerate; PCMB, *p*-chloromercuribenzoate; PMB, *p*-hydroxymercuribenzoate; PTH, phenylthiohydantoin.

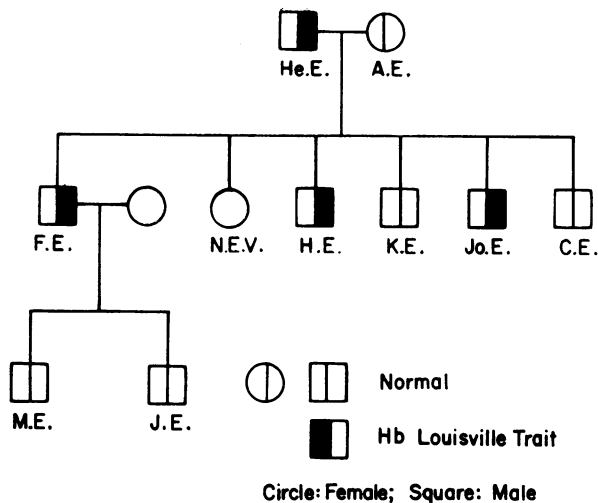


FIGURE 1 The pedigree of family E.

20 min at 4°C. The material was dissolved in about 50 ml 2% KCN and dialyzed overnight against distilled water at 4°C. Heme was removed using the acid-acetone procedure of Anson and Mirsky (13). The recovery was 207 mg dehemed chain from 4200 mg hemoglobin.

The isolated β -chain was digested with trypsin (Worthington Biochemical Corp., crystallized three times, salt-free) at room temperature for 2 hr at pH 9.0 in a pH-stat. Trypsin equal to 0.5% of the weight of the material was added at zero time and again 30 min later. The insoluble material formed at pH 6.5 was removed by centrifugation. The pH of the supernate was lowered to 2.5 whereafter the soluble peptides were taken to dryness by flash evaporation at 37°C. The peptides were separated by a chromatographic procedure using a column of Dowex 50-X2 (14,15). Rechromatography of the Dowex 50 zones was made on columns of Dowex 1-X2 (16). Amino acid analyses were made with a Spinco model 121 automated amino acid analyzer equipped with high sensitivity cuvettes and an Infotronics integrator (Infotronics, Inc., Houston, Tex.). Samples were hydrolyzed at 110°C under reduced pressure with 6 N HCl for 24 hr.

Sequential data on one peptide were obtained by use of the modified Edman phenylthiohydantoin (PTH) procedure (17). Approximately 0.5 μ mole of this peptide was distributed over four strips of Whatman filter paper No. 1. The PTH amino acid which resulted from the degradation procedure was identified by paper chromatography using solvent systems described by Schroeder, Shelton, Shelton, Cormick, and Jones (17). The procedure has continued to give satisfactory results in many degradations with the possible exception of degradations involving a histidyl residue. The insoluble core was not analyzed further.

Oxygen equilibrium analyses. These studies were made on red cell hemolysates from the Hb Louisville heterozygote He. E. and his normal wife A. E. (Fig. 1). The hemolysates were stripped from 2,3-DPG by dialysis against 0.2 M sodium chloride for 24 hr at 4°C, followed by dialysis against 0.1 M sodium phosphate buffers, pH 6.8–8.2, for an additional 24 hr. Oxygen affinity curves were measured at 37°C by the procedures of Astrup and coworkers (18, 19) and Siggaard-Andersen, Jørgenson, and Naeraa (20).

Spectral analyses were made with a Zeiss spectrophotometer, model PMQII.

RESULTS

Case report. The propositus (Fig. 1, H.E.) is a 21-year-old Caucasian male with parents having English and Irish ancestry (the father) and English and American Indian ancestry (the mother). At the age of 16, the propositus was diagnosed as anemic by his family physician and was unsuccessfully treated with various hematinics including iron.

Physical examination revealed a slender well developed youth. Skin and mucous membranes were quite pale, and a slight scleral icterus was noted. The liver was palpable and was percussed 4 cm below the right costal margin. A nontender spleen was felt 3–4 cm below the left costal margin. Cholecystogram, upper G.I. series, and chest X-ray were normal; the abdominal film revealed hepatosplenomegaly.

The peripheral blood on admission showed: Hb 11 g/100 ml; packed cell volume (PCV) 34%; reticulocytes 13.1%; platelets 106,000/mm³; WBC 3700/mm³ (subsequently rose to normal and remained so on later studies) with a normal differential. The peripheral smear revealed aniso- and poikilocytosis of moderate degree, and a mild hypochromia. Occasional teardrop forms, burr cells, target cells, and schistocytes were seen. A rare red cell showed basophilic stippling. The half-life of the ⁵¹Cr-labeled red cells was approximately 9 days (28–30 days in normal subjects); there was significant hypersplenism and sequestration.

Routine biochemical data were within normal limits except for a low cholesterol (140 mg/100 ml), an elevated serum lactate dehydrogenase (LDH) (1670 U, 100–350 U normal value), and an elevated bilirubin (total 2.2 mg/100 ml, with direct 0.5 mg/100 ml). The total serum vitamin B₁₂ level was 248 μ g/ml (normal 100–500 μ g/ml) and that of serum folic acid 1.8 μ g/ml (normal over 5 μ g/ml). No clinical evidence for a folic acid deficiency was apparent. Plasma haptoglobin was less than 1 mg/100 ml (expressed as milligrams of hemoglobin bound with normal values ranging from 20 to 200 mg/100 ml). Serum iron was 100 μ g/100 ml. Direct Coombs was negative as were the cold and warm agglutinins. Stool was negative for blood and helminths. The urinary and fecal urobilinogen excretion was within normal limits; urinary porphobilinogen was negative, but hemosiderin was present.

Family study. Patient's father (Fig. 1, He. E.) was found to be anemic in 1943 while serving in the U. S. Armed Forces. He was splenectomized in 1944. In our studies he was never found to be severely anemic; his white blood count was increased, and characteristic post-splenectomy changes were present.

TABLE I
Hematological Data

Name	Hb Louisville heterozygotes				Normals				
	He. E.	F. E.	H. E.	Jo. E.	A. E.	K. E.	C. E.	M. E.	J. E.
Age, yr	47	26	20	11	52	13	11	2	1
Hb, g/100 ml	13.5	13.2	13.5	11.5	13.5	13.4	12.4	12.3	12.4
PCV, %	40.2	40.5	39.3	34.3	40.9	41.3	37.6	37.5	37.3
RBC, 10 ⁶ /mm ³	4.17	4.37	3.96	3.84	4.62	5.08	4.64	4.76	5.48
MCV, μ ³	96	93	99	89	90	83	82	78	68
MCH, μμg	31.7	30.2	33.3	29.3	30.6	27.5	28.0	25.3	21.9
MCHC, %	32.3	32.6	33.1	32.2	33.8	33.2	33.9	32.6	32.7
WBC, 10 ³ /mm ³	29	11.9	7.4	7.4	6.2	7.2	6.2	12.5	13.0
Reticulocytes, %	—	8.6	9.5	6.8	—	—	—	—	—
2,3-DPG, μM/g Hb	17.6	16.3	12.4	11.8	18.3	—	—	—	—
F _{AD} , %	1.4	—	3.9	—	—	—	—	—	—

PCV, packed cell volume; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells; 2,3-DPG, 2,3-diphosphoglycerate; F_{AD}, alkali-resistant hemoglobin.

The older brother (F.E.), aged 26 yr, had a palpable nontender spleen, 4 cm below the left costal margin. He was not anemic, and no icterus was found. His two sons are normal.

Another brother (Jo. E.), aged 11 yr, was hospitalized in 1967 at Children's Hospital, Louisville, Ky., with a diagnosis of iron deficiency anemia, probably nutritional. Since treated with iron, he has shown no response. Our studies revealed a mild to moderately severe anemia, resembling that of the propositus. His spleen was not palpable.

The mother and two additional brothers are normal.

Hematological studies. Table I lists hematological data on nine members of family E. No striking differences were seen between the data of the four subjects, who are heterozygous for the abnormal variant, and those of the normal relatives. The levels of 2,3-DPG fell within our normal limits of 12–18 μM/g of Hb. Fetal hemoglobin was not elevated as judged by starch gel electrophoresis except in the subjects He.E. and H.E. The per cent Hb F by alkali denaturation (% F_{AD}) was slightly elevated in these patients considering that our normal values are usually below 1%.

Heinz bodies were easily demonstrated in the peripheral blood of He. E., the only member of the family who had been splenectomized (Fig. 2). They were not present in the other affected members until after incubation with a redox dye. Even then, only small numbers were perceptible.

Hemoglobin studies. Starch gel electrophoresis at pH 9.0 of hemolysate from nine members of family E. showed no abnormal hemoglobin band. When the gel was overloaded with hemoglobin from the propositus a faint, benzidine-positive, zone was noted in the position of

α-chains. A DEAE-Sephadex chromatogram of hemoglobin from He. E. showed normal hemoglobin zones except for a small α-chain zone (0.6%); the Hb A₂ level was 2.5%. Addition of PMB to hemolysate from He. E. (4 mmoles or 12 mmoles/mole of hemoglobin) resulted in a partial precipitation of hemoglobin. Starch gel electrophoresis of the supernate revealed normal Hb A and Hb A₂ (as their PMB derivatives) and an increased amount of a band which has been identified as the PMB derivative of free α-chains (12, 21).

The heat stability curves of the hemoglobin of hemolysates from the nine members of the E. family are presented in Fig. 3. Some 35–50% of the hemoglobin from He. E. and his three sons F.E., H.E., and Jo. E. precipitated after 8–10 min of incubation at 65°C. The hemoglobins from the other five subjects appear to behave normally; the small differences are likely due to slight variations in the technique. It is noteworthy that precipitation of the unstable hemoglobin was not observed when the temperature of incubation was lowered to 60°C.

Structural studies. The amino acid composition of the precipitate isolated after treatment of the hemoglobin from hemolysate of He.E. with PCMB (see Methods) gave these results (values between parentheses represent the theoretical values for the normal β-chain): Lys 11.0 (11); His 8.7 (9); Arg 3.4 (3); Asp 13.6 (13); Thr 6.8 (7); Ser 5.7 (5); Glu 11.4 (11); Pro 6.5 (7); Gly 12.6 (13); Ala 15.8 (15); Val 15.6 (18); Met 0.8 (1); Ile 0.9 (0); Leu 18.5 (18); Tyr 2.4 (3); Phe 7.5 (8). These data indicate that this precipitate consists mainly of hemoglobin β-chains. The soluble peptides in a tryptic digest of this precipitate were separated by Dowex 50-X2 chromatography, and each Dowex 50 zone was rechromatographed on a column of Dowex 1-X2. The amino

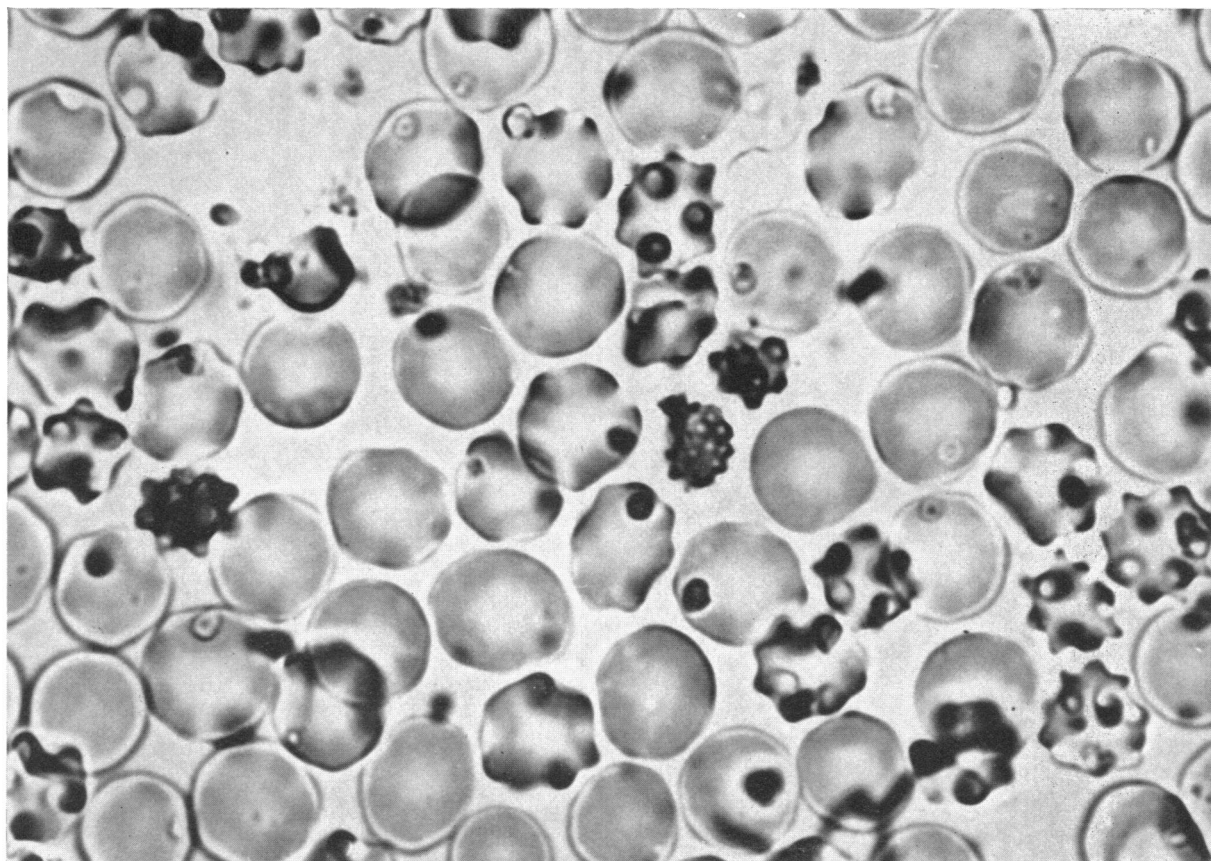


FIGURE 2 Heinz body formation in the peripheral blood of subject He. E. after exposure to brilliant cresyl blue. The photograph was taken with an ordinary optical microscope. $\times 970$.

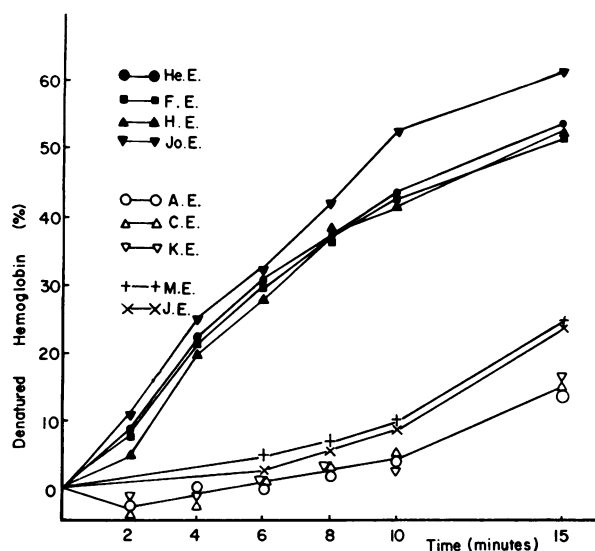


FIGURE 3 The instability of the hemoglobin from four members of the E. family on warming to 65°C . See text for details. The subjects are identified in Fig. 1 and Table I.

acid compositions of these peptides are listed in Table II; all β -chain peptides were recovered except the core peptides T-10, T-11, and T-12, and the $\beta\text{T-2}$ peptide, which was not obtained sufficiently pure to allow an accurate determination. The compositions of all peptides are consistent with those of the corresponding peptides of the β -chain of normal Hb A, except that of $\beta\text{T-5}$. This peptide contained two phenylalanyl residues (three in the β^{A} -chain) and two leucyl residues (one in the β^{A} -chain) indicating a Phe \rightarrow Leu replacement. Analysis of a $\beta\text{T-5}$ peptide from a normal β -chain, made at approximately the same time and under the same conditions, gave these results: Lys 1.02; Asp 3.12; Thr 1.01; Ser 1.98; Glu 1.08; Pro 1.87; Gly 2.01; Ala 1.03; Val 1.01; Met 0.65; Leu 1.10; Phe 2.72. These data indicate that the low recovery of phenylalanine in the 24 hr hydrolysate of the abnormal $\beta\text{T-5}$ peptide is not due to an incomplete hydrolysis of a possible Phe-Phe peptide bond. Degradation of this peptide (Fig. 4) with the PTH procedure was successful for four residues and showed a Phe-Leu-Glu-Ser- amino terminal sequence.

Helix C	Interhelical region CD								Helix D							Helix E			
(17)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(1)	(2)	(3)
41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	
—Phe	—Leu	—Glu	—Ser	(Phe,	Gly,	Asp,	Leu,	Ser,	Thr,	Pro,	Asp,	Ala,	Val,	Met,	Gly,	Asn,	Pro,	Lys)	

FIGURE 4 The amino acid sequence of the amino terminus of the β T-5 peptide of Hb Louisville. The numbers refer to the positions of the residues in the chain and those between parentheses to the positions of the residues in the different helices or the interhelical region.

This result indicates that the phenylalanyl residue in position 42 (interhelical position CD1) is replaced by a leucyl residue. Fig. 5 presents a photograph of the chromatogram which identifies the amino terminal residue as phenylalanine and the residue in second position as leucine. It is noteworthy that the position of the abnormal β T-5 in the Dowex 50 chromatogram is about the same as that of the normal β T-5.

Oxygen equilibria. The oxygen affinity curves of the stripped red cell hemolysates from subjects He. E. and his normal wife, A.E., were determined in paired experiments at pH values varying between 6.68 and 7.28 (at 37°C) and at a P_{CO_2} value of 38 mm Hg. Fig. 6 A presents plots of the log P_{O_2} values vs. the log $Y/(100-Y)$ values according to Hill's equation: $KX^n = Y/(100-Y)$, where X is the P_{O_2} (in mm/Hg), Y the oxygen saturation (in per cent), and K and n are constants. A minimum of five oxygen equilibria analyses were made on each sample. Hemolysate of subject He. E. showed a slight decrease in oxygen affinity and a considerable de-

crease in the value n which is a measure of the heme-heme interaction. No significant difference was observed between the Bohr effects of the two samples (Fig. 6 B). The mean value of factor n in Hill's equation was 2.85 for normal subject A. E. and 1.85 for subject He. E. with the Hb Louisville heterozygosity. This large difference likely indicates that the decreased heme-heme interaction is a significant property of this hemoglobin variant. It was unfortunate indeed that oxygen equilibria analyses of pure Hb Louisville were not made because of the inability to separate the variant from normal Hb A. The visible spectra of the hemolysates from the two subjects were identical, and no significant formation of ferrihemoglobin occurred during the oxygen equilibria analyses.

DISCUSSION

This study shows the presence of an abnormal hemoglobin in four members of a Caucasian family, which could readily be identified as a β -chain variant with a phenylalanine to leucine substitution in position 42. The

TABLE II
*Amino Acid Composition of Soluble Peptides from 2 hr Digest of β -Chain of Hemoglobin Louisville**

Amino acid	T-1	T-3	T-4	T-5	T-6	T-7	T-8	T-9	T-13	T-14	T-15
Lysine	1.08			0.93	1.00	1.08	1.00	1.07	1.01	1.04	
Histidine	0.95					0.97		1.02		0.84	1.00
Arginine		0.93	1.01								
Aspartic acid		2.18		3.10				3.04		1.04	
Threonine	0.96		0.91	0.96					1.03		
Serine				1.71				0.98			
Glutamic acid	1.96	2.07	0.99	1.09					2.89		
Proline	1.03		1.11	2.05					2.02		
Glycine		2.87		1.96		1.05		2.10		1.11	
Alanine		1.06		1.01		0.90		2.03	1.93	3.77	
Valine	0.93	2.73	1.35‡	1.01	0.98			1.05	1.10	2.62§	
Methionine				0.71							
Leucine	0.98	1.03	1.89	1.96				3.83		1.14	
Tyrosine			0.76						0.88		0.87
Phenylalanine				1.85				1.01	0.90		
Tryptophane§			+								
Yield, %	32	54	16	32	20	66	28	24	22	32	40

* Data are presented as residues per molecule of peptide. Amino acids present in less than 0.1 residue are omitted.

‡ Low recovery due to the presence of a Val-Val bond.

§ Detected by color reactions on paper (22).

|| The percentage yield was calculated on the basis of recovery after chromatography on Dowex 50 and subsequently on Dowex 1, assuming 100% hydrolysis of the appropriate cleavage points during tryptic digestion.

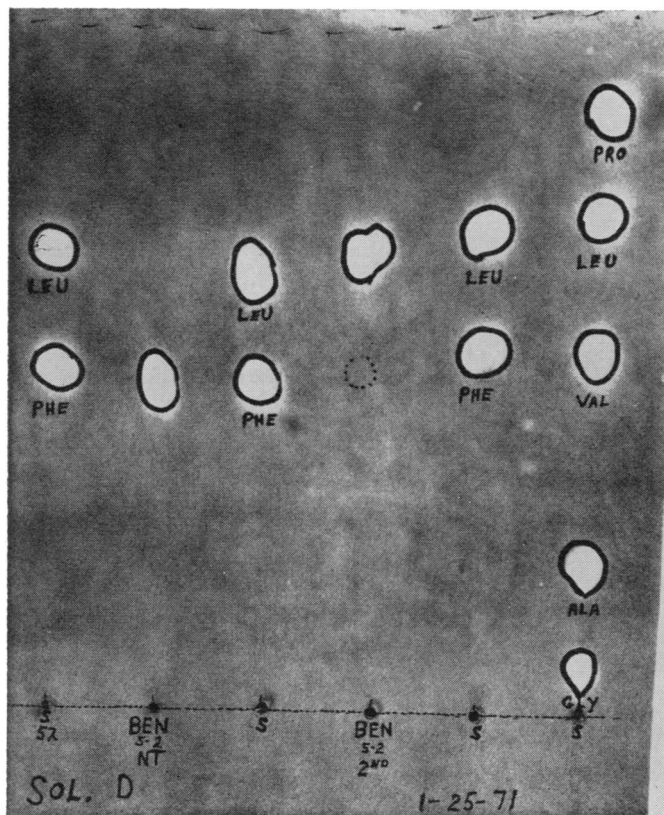


FIGURE 5 Results of PTH degradations at the amino terminal residue and at residue 2 of the abnormal β T-5 peptide. Samples from left to right are: Leu and Phe standards; amino terminal residue of β T-5; Leu and Phe standards; residue in second position of β T-5, Leu and Phe standards; Pro, Leu, Val, Ala, and Gly standards. Solvent D was used in this experiment; results from chromatograms developed with other solvents gave comparable data. Photographed with MP-3 Polaroid camera.

abnormal hemoglobin could not be separated from normal Hb A by electrophoretic and chromatographic procedures. The isolation of the abnormal β -chain was only possible because of an increased dissociation of the hemoglobin in the presence of the PCMB reagent, which is known to be attached to the reactive sulfhydryl groups of the protein (23). The results from the heat lability test also indicate the instability of the variant and suggest that 30–35% of the hemoglobin of the heterozygous subjects are of the abnormal type.

Phenylalanyl residue in position 42 or in the first position of the region between the C and D helices of the β -chain participates in the contacts with heme (24–26). Substitution of this phenylalanyl residue removes a contact with heme and “leaves a gap at the surface of the heme pocket” (3). Replacement by the noncharged but polar seryl residue, as in Hb Hammersmith (27), results in a severe instability of the hemoglobin probably be-

cause the presence of the hydrophobic OH group opens the heme pocket to water (3). The substitution in Hb Hammersmith causes also a reduction in oxygen affinity; moreover, the heme group may easily be lost from its β -chain (28–30). These observations are consistent with the clinical condition of the Hb Hammersmith hemoglobinopathy, which is characterized by red blood cells with large and numerous Heinz bodies and a severe hemolytic anemia that is unrelieved by splenectomy (27, 29).

It is interesting that replacement of the phenylalanyl residue by the noncharged and nonpolar leucyl residue, as in Hb Louisville, has less severe consequences. The Hb Louisville heterozygotes suffer from a mild anemia, and it is difficult to produce Heinz bodies in their red cells with redox dyes except for the subject who was splenectomized. In the heat stability test the hemoglobin variant does not precipitate at 60°C, but precipitation

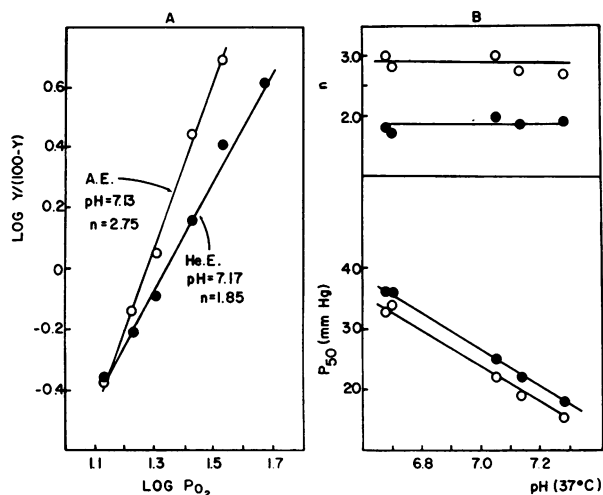


FIGURE 6 Oxygen equilibria of red cell hemolysates from a subject with a heterozygosity for Hb Louisville (He. E.) and from a normal control (A. E.). (A) Examples of oxygen dissociation curves; (B) Bohr effects and values of factor n . Open circles: normal hemolysate; closed circles: hemolysate of Hb Louisville heterozygote. The levels of 2,3-DPG in these hemolysates were 3.5 μ moles/g Hb (normal hemolysate) and 2.5 μ moles/g Hb (hemolysate of Hb Louisville heterozygote) which indicate the removal of 80–85% of 2,3-DPG by the dialysis procedure.

occurs only at 65°C. Hb Louisville exhibits a considerable tendency to dissociate in the presence of sulfhydryl group-blocking agents. Apparently the phenylalanyl to leucyl substitution at position 42 (CD1) causes an increased dissociation of the tetramer into dimers as well as a weakening of the α_1 - β_2 contact to the extent that an increased monomer formation will occur. The dramatic decrease in the heme-heme interaction and the slight change in oxygen affinity of hemoglobin from a heterozygous Hb Louisville carrier suggest that the structural alteration influences the integrity of the α_1 - β_2 contact, which is known to be important in relation to the effect of the oxygenation state of one heme group on the affinity for molecular oxygen of a second heme group in the molecule (24, 25). The apparent loss of this heme-heme interaction of Hb Louisville is probably not due to a complete dissociation of the molecule into smaller subunits because ultracentrifugation analyses of total hemolysate failed to show a peak with a lower s_{20} value in addition to the normal tetramer with a s_{20} value of 4.36.

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