

Hemoglobin Abraham Lincoln, β 32 (B14) Leucine \rightarrow Proline

AN UNSTABLE VARIANT PRODUCING SEVERE HEMOLYTIC DISEASE

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ABSTRACT An unstable hemoglobin variant was identified in a Negro woman with hemolytic anemia since infancy. A splenectomy had been performed when the patient was a child. The anemia was accompanied by erythrocyte inclusion bodies and excretion of darkly pigmented urine. Neither parent of the *proposita* demonstrated any hematologic abnormality, and it appeared that this hemoglobin variant arose as a new mutation. Erythrocyte survival in the patient was greatly reduced: the erythrocyte $t_{1/2}$ using radiochromium as a tag was 2.4 days, and a reticulocyte survival study performed after labeling the cells with L-[14 C]leucine indicated a $t_{1/2}$ of 7.2 days. When stroma-free hemolysates were heated at 50°C, 16–20% of the hemoglobin precipitated. The thermal lability was prevented by the addition of hemin, carbon monoxide, or dithionite, suggesting an abnormality of heme binding. An increased rate of methemoglobin formation was also observed after incubation of erythrocytes at 37°C. The abnormal hemoglobin could not be separated from hemoglobin A by electrophoresis or chromatography, but it was possible to isolate the variant β -chain by precipitation with *p*-hydroxymercuribenzoate. Purification of the β -chain by column chromatography followed by peptide mapping and amino acid analysis demonstrated a substitution of proline for β 32 leucine. It appears likely that a major effect of this substitution is a disruption of the normal orientation of the

adjacent leucine residue at β 31 to impair heme stabilization.

INTRODUCTION

Structural abnormalities of the hemoglobin molecule are known to bring about a variety of adverse clinical consequences in the affected individual (1). A group of these abnormalities is characterized by instability of the hemoglobin molecule and produces the clinical picture of congenital Heinz body anemia. This syndrome has its onset in infancy and consists of hemolytic anemia, with inclusion bodies demonstrable in the erythrocytes after incubation and supravital staining or in the unincubated cells of splenectomized individuals, and excretion of abnormal heme degradation products in the urine (2). In this report we present the findings in a woman with a severe form of this syndrome, in whom a new hemoglobin β -chain variant was identified.

METHODS

Hematological studies. Hematologic measurements were performed by standard methods (3). Autohemolysis and osmotic fragility tests were carried out with sterile defibrinated blood as described by Dacie (4). Erythrocyte glutathione was determined by the method of Beutler, Duron, and Kelly (5). The ascorbate cyanide screening test was performed as described by Jacob and Jandl (6). The glucose-6-phosphate dehydrogenase assay employed the method of Zinkham (7).

Erythrocyte survival measurements using radiochromium were performed as previously described (8). For determination of the reticulocyte survival time [14 C]L-leucine was used as a radioactive label as described by Alter, Kan, and Nathan (9).

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Oxygen affinity measurements were made using samples of fresh whole blood to which heparin was added as an anti-coagulant. The procedure described by Edwards and Martin (10) was used in these determinations. Erythrocyte 2,3-diphosphoglycerate (2,3-DPG)¹ was measured as previously described (11).

Hemoglobin studies. Blood samples were collected in heparin and transported in melting ice. The cells were washed three times in cold isotonic saline and lysed with 3–5 vol of cold water. The cell stroma were removed by centrifugation.

Hemoglobin electrophoresis was carried out in starch gel at pH 8.6 (12) and agar gel at pH 6.2 (13). Alkali-resistant hemoglobin was quantitated as described by Betke, Marti, and Schlicht (14). Hemoglobin A₂ determinations were performed by DEAE-Sephadex chromatography (15). Methemoglobin was determined by the method of Evelyn and Malloy (16). These determinations were made with fresh defibrinated blood or defibrinated blood samples incubated aseptically at 37°C for 24 or 48 h.

For determination of the percentage of thermolabile hemoglobin, stroma-free erythrocyte lysates were incubated for varying time periods at 50°C under conditions described by Grimes, Meisler, and Dacie (17). The samples were cooled, and the precipitates were collected by centrifugation and washed free of soluble hemoglobin with 0.1 M phosphate buffer, pH 7.4. The precipitates were suspended in a solution of 1% HCl in acetone to remove heme, collected by centrifugation, and dissolved in water. Protein determinations of these solutions and of material similarly prepared from an aliquot of the original lysate were performed by the method of Lowry, Rosebrough, Farr, and Randall (18).

Structural analysis. Trypsin digestion and peptide mapping procedures were adapted from methods described by Clegg, Naughton, and Weatherall (19). High voltage electrophoresis of the tryptic digests was carried out at pH 4.7 (pyridine:acetic acid:water, 5:5:390) on 3-cm strips of Whatman 3 MM paper. The strips were dried and sewn to sheets of paper, and descending chromatography was performed with butanol:acetic acid:water:pyridine (15:3:12:10). Peptides were identified by staining with ninhydrin or stains specific for individual amino acids.

Peptides were eluted from the papers with distilled water and were hydrolyzed in 6 N HCl under reduced pressure at 110°C for 40 h. Phenol was added before the hydrolysis to reduce tyrosine oxidation. Amino acid analyses were performed with a Spinco model 120C amino acid analyzer (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.).

Materials. [¹⁴C]L-leucine was obtained as a sterile aqueous solution from New England Nuclear, Boston, Mass. Radiopharmaceutical ⁵¹Cr was a product of Abbott Laboratories, North Chicago, Ill. The sodium salt of *p*-hydroxymercuribenzoate (*p*MB) was obtained from Sigma Chemical Co., St. Louis, Mo., and was further purified as described by Boyer (20). Other chemicals were of reagent grade.

RESULTS

Case report. The *proposita* is a 26-yr-old Negro woman, born in New Orleans, La., who was known to have anemia since early childhood. She was hospitalized

¹ Abbreviations used in this paper: 2,3-DPG, 2,3-diphosphoglycerate; MCV, mean corpuscular volume; *p*MB, *p*-hydroxymercuribenzoate.

TABLE I
Representative Hematologic Values of the Proposita

Hemoglobin concentration	8.5 gm/100 ml
Hematocrit	24%
Erythrocyte count	$1.67 \times 10^6/\text{mm}^3$
Mean corpuscular volume (MCV)	122 μm^3
Mean corpuscular hemoglobin	40 pg
Mean corpuscular hemoglobin concentration	35%
Leukocyte count	13,000/mm ³
Nucleated erythrocytes	4/100 leukocytes
Reticulocyte count	25%
Serum bilirubin concentration	2.3 mg/100 ml
Serum lactate dehydrogenase	840 IU
Serum iron concentration	127 $\mu\text{g}/100 \text{ ml}$
Total serum iron-binding capacity	317 $\mu\text{g}/100 \text{ ml}$
Platelet count	350,000/mm ³

several times between 2 and 5 yr of age because of severe anemia, and received transfusions on a number of occasions. A splenectomy was done when she was 5 yr old but apparently had little effect on the course of her anemia. She has had a variable degree of jaundice and anemia accompanied intermittently by dark urine. These manifestations have worsened strikingly with infections, sometimes requiring supportive transfusions. She is not known to have been exposed to oxidant drugs or chemicals but has reported an increase of jaundice and pigmenturia after intake of alcohol. A cholecystectomy was performed when she was 23 yr of age because of cholecystitis and gall stone formation. She has no children.

Neither parent of the *proposita* has been known to have anemia or jaundice. Blood samples from the parents showed normal hematologic findings, and neither showed evidence of anemia, erythrocyte inclusion body formation, or a heat-unstable hemoglobin fraction. Blood group analysis (A,B,D,C,E,c,e,C^w, V,K,k,Fy^a,Fy^b,JK.^aS.s,M,N,P,Lu^a, and Lu^b) of the patient and her parents gave no indication of nonpaternity. The parents have no other children.

Hematological studies. Representative hematologic values of the patient are presented in Table I. Hemoglobin values varied widely depending on her clinical state and, during the course of our experience over a 5-yr period, varied from 4.8 to 11.0 g/100 ml. Reticulocyte counts have ranged from 20 to 50%. The erythrocytes were very large having an average mean corpuscular volume (MCV) of 122 μm^3 from eight determinations over a 3-yr period. The macrocytosis was unchanged after administration of 5 mg of folic acid daily for 2 mo, suggesting that this finding was not a result of folate deficiency. The erythrocyte morphology reflected an active hemolytic process and postsplenectomy changes as well as the usual features which accompany hemoglobin in-

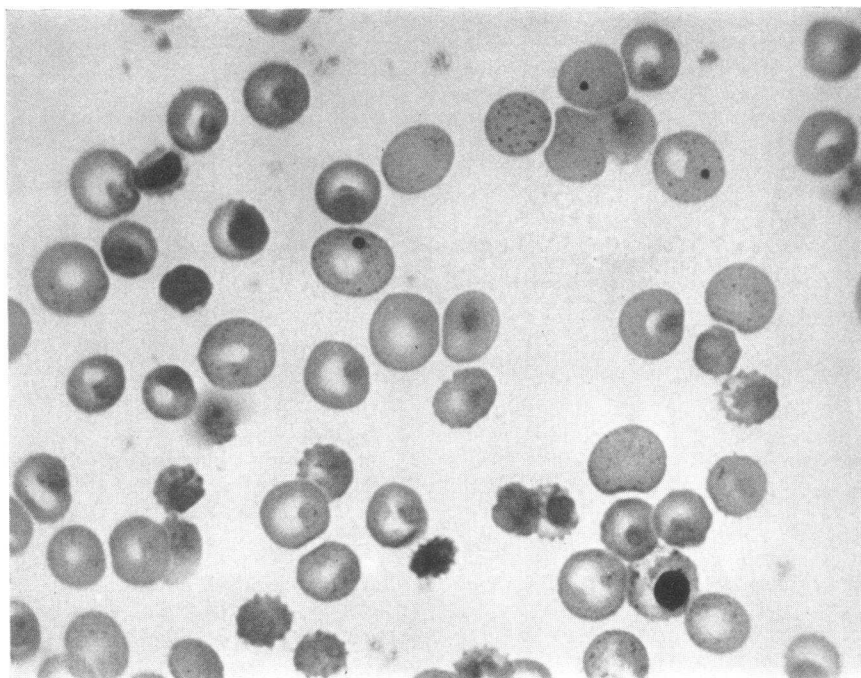


FIGURE 1 Photomicrograph of a peripheral blood film from the propoita (Wright's stain).

stability. Nucleated erythrocyte forms and Howell-Jolly bodies were invariably present in significant numbers, and many of the cells in freshly prepared smears stained

with Wright's stain showed inclusion bodies (Fig. 1). Irregularly contracted erythrocyte forms were also regularly observed. Supravital staining produced typical

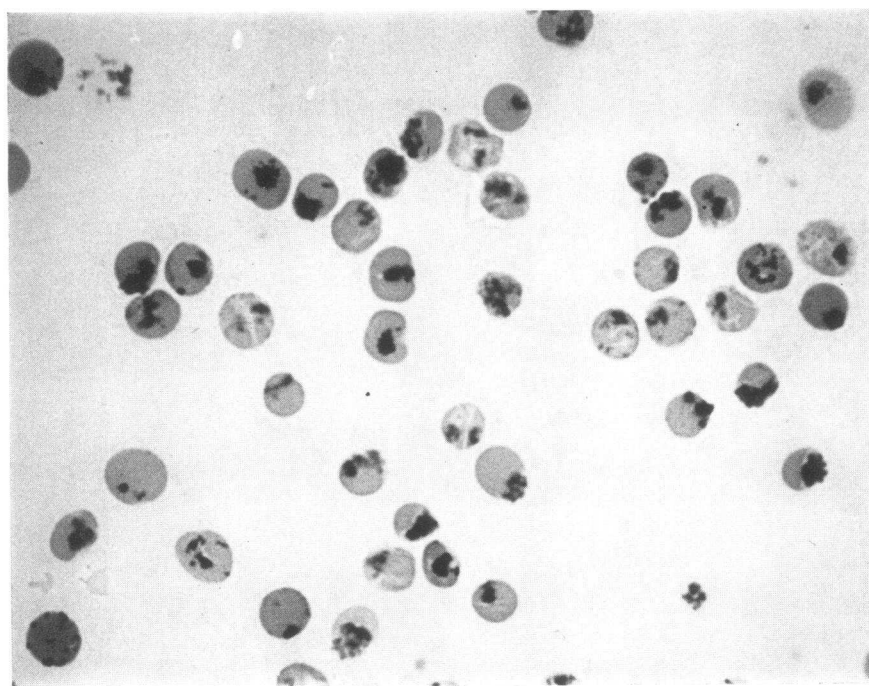


FIGURE 2 Erythrocytes from the propoita after staining for 30 min with new methylene blue. Reticulocytes and erythrocytes containing inclusion bodies make up a large percentage of the cells.

TABLE II
Erythrocyte and Hemoglobin₂ Values

Autohemolysis (with and without glucose)	<2.0%
Osmotic fragility (fresh sample)	Normal
Reduced glutathione	44 mg/100 ml (N 60-90)
After incubation with acetylphenylhydrazine	32 mg/100 ml
Glucose-6-phosphate dehydrogenase	15.8 U/g Hb (N 5-10)
Alkali-resistant hemoglobin	2.1%
Hemoglobin A ₂	5.8%
Methemoglobin (fresh sample)	<1% (normal control <1%)
After 24-h incubation	6.7% (normal control <1%)
After 48-h incubation	10.1% (normal control <1%)
Half-saturation Po ₂ (37°C, pH 7.4)	25.3 mm (<i>n</i> = 26)
Bohr effect ($\Delta \log P_{50}/\Delta \text{pH}$)	0.55
2,3 DPG	3.77 $\mu\text{mol/ml}$ (N 3.4 ± 1.3)

Heinz body inclusions in virtually every cell (Fig. 2). Bone marrow preparations from the patient exhibited erythroid hyperplasia with increased quantities of stainable iron.

Erythrocyte survival studies demonstrated a greatly accelerated rate of erythrocyte destruction. In our initial study using the radiochromium tagging procedure, a half-survival time of 2.4 days was obtained (Fig. 3). The normal range for this determination is 25-35 days. In the second study we employed a form of radioactive label which was incorporated into the hemoglobin to avoid possible inaccuracy that might result from elution of the radioactive tag. For this purpose a sample of blood from the patient was incubated under sterile conditions with L-[¹⁴C]leucine as described by Alter and co-workers (9). The reticulocytes labeled in this manner demonstrated a half-survival time of 7.2 days (Fig. 3), confirming the greatly shortened erythrocyte survival in this patient.

Autohemolysis and osmotic fragility studies were normal. A strongly positive result was obtained in the as-

corbate cyanide screening test for methemoglobin formation (6), a finding reported to occur with high frequency in unstable hemoglobin disorders (21). Glucose-6-phosphate dehydrogenase activity was increased which is consistent with the elevated reticulocyte count. A significantly low level of reduced glutathione was present (Table II) but was not greatly affected by incubation of the cells with acetylphenylhydrazine.

The percentage of methemoglobin was normal in fresh blood samples but increased to abnormal levels after incubation of the blood at 37°C for 24 or 48 h. The concentration of hemoglobin A₂ was substantially elevated and the alkali-resistant hemoglobin fraction was slightly increased (Table II). Electrophoresis of freshly prepared hemolysates in starch gel at pH 8.6 demonstrated a diffuse band migrating more slowly than the major hemoglobin fraction, which corresponded to hemoglobin A. Uncombined α -chains formed a prominent band near the origin (Fig. 4). Electrophoresis in agar gel at pH 6.2 produced a normal pattern.

Heat lability of the hemoglobin was demonstrated by the appearance of a flocculent precipitate when stroma-free hemolysates were heated at 50°C. The precipitated hemoglobin after heating for 4 h accounted for 16-20% of the total. Heat stability of the hemoglobin was also examined under conditions in which methemoglobin formation was prevented. When the hemolysate was saturated with carbon monoxide or maintained in a nitrogen

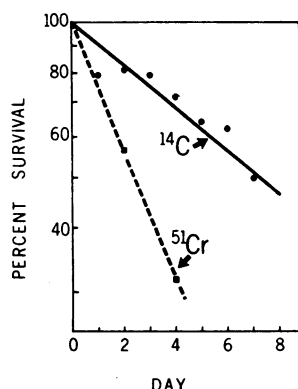


FIGURE 3 Erythrocyte survival of the proposita. The ⁵¹Cr study used whole blood. The [¹⁴C]leucine result indicates survival of reticulocytes.

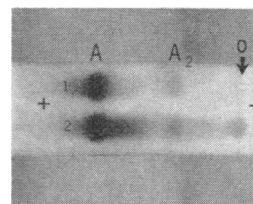


FIGURE 4 Starch gel electrophoresis, pH 8.6, Tris-EDTA-borate buffer. 0, origin; 1, normal control; 2, patient.

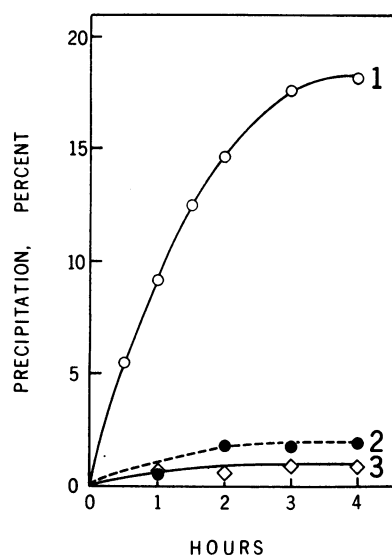


FIGURE 5 Heat stability test. 1, untreated hemolysate; 2, with added dithionite; 3, hemolysate saturated with carbon monoxide.

atmosphere together with the reducing agent sodium dithionite, hemoglobin precipitation was effectively prevented (Fig. 5). Heat instability in this test was also prevented by addition of an excess of hemin, but cyanide produced only a small and inconsistent effect. The 540/280 nm ratio of an unheated hemolysate prepared from erythrocytes from the patient was 0.411, indicating a partial depletion of heme of the variant hemoglobin (22). The corresponding value from a hemolysate prepared from erythrocytes from a normal individual was 0.428.

Hemoglobin studies. Chromatography of a stroma-free hemolysate on DEAE-Sephadex (15) demonstrated a small peak of free α -chains emerging immediately before hemoglobin A₂. The major hemoglobin components were eluted as a single peak. Globin prepared from an unfractionated hemolysate was chromatographed on carboxymethylcellulose as described by Clegg and coworkers (19) and yielded only globin fractions corresponding to normal α - and β -chains. A heat-unstable protein fraction was prepared by heating a freshly made hemolysate at 45°C for 1 h. The precipitate was washed with 0.1 M

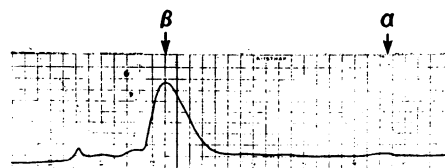


FIGURE 6 Elution pattern from carboxymethylcellulose (19) of β MB-precipitated globin from stroma-free hemolysates prepared from the patient's erythrocytes. The normal elution positions of α - and β -chains are indicated.

TABLE III
Amino Acid Composition of the Variant β -Chain

	Amino acid residues/ β -Chain		
	24-h hydrolysate	72-h hydrolysate	Normal β -chain
Lysine	11.0	11.0	11
Histidine	6.9	7.8	9
Arginine	2.7	2.6	3
Aspartic acid	13.2	13.2	13
Threonine	7.2	6.4	7
Serine	5.0	3.9	5
Glutamic acid	11.4	11.4	11
Proline	8.0	8.3	7
Glycine	13.5	13.4	13
Alanine	14.4	13.9	15
Valine	17.7	18.1	18
Methionine	1.0	>0.2	1
Isoleucine	0.0	0.0	0
Leucine	17.0	16.9	18
Tyrosine	2.0	2.0	3
Phenylalanine	7.9	7.7	8

phosphate buffer to remove soluble hemoglobin, and the material was dissolved in a small volume of 0.1 N HCl. Heme was removed by precipitation in acetone-HCl (23) and the protein was subjected to carboxymethylcellulose chromatography (19). The eluted fractions again appeared indistinguishable from those of normal α - and β -chains. Approximately equal quantities of α - and β -chain globin were recovered.

An abnormal globin chain was successfully isolated by application of a β MB precipitation procedure (24, 25). A stroma-free lysate of the patient's erythrocytes was allowed to react with β MB under conditions described by Rosemeyer and Huehns (26). After the mixture was stirred in the cold for 90 min, the precipitate formed was recovered by centrifugation and washed three times with a solution made up of 100 ml of 0.1 M sodium phosphate buffer, pH 6.0, 20 ml of 2 M sodium chloride, and 60 ml of water. The washed precipitate was prepared as a fine suspension in water and added in drops to a 1% solution of HCl in acetone at -20°C (23) to remove heme. The precipitate was recovered by centrifugation and washed successively with acetone-HCl, acetone, and ether, and finally dried in a stream of nitrogen. The dried protein was dissolved in 8 M urea containing 0.3 M 2-mercaptoethanol. The solution was saturated with nitrogen, allowed to stand at room temperature for 3 h, and then dialyzed twice against 50 vol of starting buffer for the carboxymethylcellulose chromatography procedure of Clegg et al. (19). The material was applied to the column which was then eluted with a linear phosphate buffer gradient at pH 6.7 (19). A major protein peak

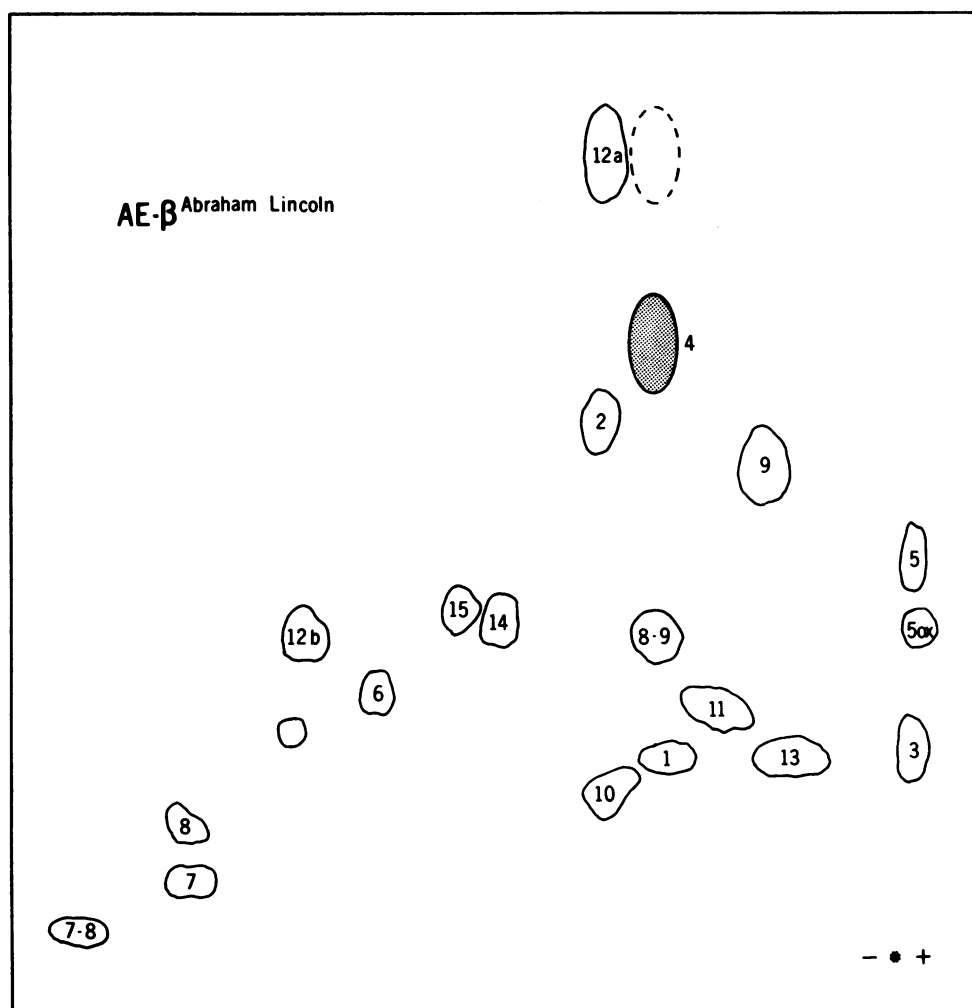


FIGURE 7 Peptide map of the aminoethylated β -chain. The abnormal peptide is indicated by the shaded area. The position of normal β T4 is shown by the interrupted outline.

emerged at the position at which normal β -chains were eluted, with only traces of other eluted material detected corresponding to α - or to other non- β -globin species (Fig. 6). Fractions corresponding to the β -peak were combined and subjected to gel filtration on a column of Biogel P-2 (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 0.5 M formic acid. The protein-containing effluent fractions were pooled and lyophilized.

The amino acid composition of the purified globin chain is presented in Table III. These data demonstrate that the protein material consists almost exclusively of β -chains, in which there is a probable substitution of a leucine residue by proline.

The purified β -chain was aminoethylated and trypsin digested (19) followed by peptide mapping. All of the normal β -chain peptides were identified in their normal position except for β T4 (Fig. 7). The β T4 peptide displayed

normal electrophoretic mobility but a lesser degree of migration in the chromatography step.

The abnormal peptide was identified on unstained maps with the aid of a long-wave ultraviolet lamp, and the peptide material was eluted. The amino acid composition of the β T4 peptide is shown in Table IV and demonstrates that a leucine is replaced by proline in this peptide. Two leucine residues are normally present in β T4, one in the amino terminal position and the other in the position at β 32. It seemed unlikely that the amino terminal leucine was replaced in this peptide, because the arginine-proline bond formed would be resistant to the action of trypsin (27, 28), and would result in the formation of a hybrid peptide composed of β T3 and β T4. A change of this kind was not observed, both β T3 and β T4 having been identified as individual spots in the peptide map. To confirm this assumption, a subtractive Edman degra-

TABLE IV
Amino Acid Composition of β T4

	Amino acid residues	Normal β T4
Arginine	0.80	1
Threonine	0.92	1
Glutamic acid	1.11	1
Proline	1.94	1
Valine	1.92	2
Leucine	1.00	2
Tyrosine	0.86	1
Tryptophan*	+	1

* Tryptophan was determined qualitatively by staining on paper.

dation (29) of isolated β T4 peptide material was performed. This study demonstrated that leucine was the amino terminal residue of the peptide. We conclude that the leucine \rightarrow proline substitution in this hemoglobin variant occurs at β 32. The β T4 peptide therefore appears to have the following structure:

Leu-Pro-Val-Val-Tyr-Pro-Try-Thr-Gln-Arg.
31 32 33 34 35 36 37 38 39 40

DISCUSSION

The hemoglobin abnormality identified in this patient is one of a number of variant hemoglobin forms that produce hemolytic anemia accompanied by erythrocyte inclusion body formation and excretion of darkly pigmented heme degradation products in the urine. In each of these conditions the abnormal hemoglobin form is characteristically unstable in solution and undergoes intracellular precipitation as the apparent basis for the hemolytic process (2).

A number of pathologic mechanisms have been identified to account for the hemoglobin instability of these variant forms. In one group of these abnormalities, of which Hb Philly (24) is an example, the amino acid substitution occurs at an α - β -contact point in the hemoglobin molecule. Disruption of the normal configuration of the β -chains at this contact point appears to cause the hemoglobin to dissociate readily into subunits. The latter are relatively unstable in solution and are subject to precipitation within the erythrocytes.

In another group of unstable hemoglobin variants, including Hb Riverdale-Bronx (30), Shepherd's Bush (31), and Wien (32), the substitution of a polar amino acid for a nonpolar residue results in molecular instability apparently by producing distortion of the subunit configuration (2).

In a larger group of unstable hemoglobin variants, including several that produce the most severe clinical consequences, disturbances affecting the binding of heme

to the globin chains appear to be the major cause of molecular instability. Included in this group are Hb Hamersmith (33), Bristol (34), and Köln (35). In these abnormal hemoglobin forms, the amino acid substitutions have been shown to occur at positions in the protein chain that are in close contact with the heme groups (2). Hydrophobic amino acids normally present in these positions, which form the heme "pocket," appear to be largely invariant (36), and are of critical importance in maintaining globin-heme stability; when substitution of any of these residues occurs, heme binding is almost always affected (2).

Based on experimental evidence obtained with a group of unstable hemoglobins having altered heme binding of the β -chains, Jacob, Brain, Dacie, Carrell, and Lehmann (22, 37) have proposed a series of events to account for the formation of Heinz bodies in these disorders. Loss of heme is assumed to occur as a consequence of altered heme affinity of the abnormal β -chains. The heme released is degraded in a poorly understood manner and is excreted as pigmented products in the urine. The unstable heme-depleted hemoglobin appears to undergo dissociation into heme-containing α -chains and poorly soluble β -globin. The latter, primarily as a consequence of heme loss but possibly also because of inherent configurational changes, exhibits increased reactivity with glutathione to form mixed disulfides (37). Ultimately the globin forms insoluble precipitates which become bound to the cell membrane by disulfide linkages with membrane sulfhydryl groups. These changes are thought to alter the erythrocyte cell membrane to produce adverse osmotic and permeability changes leading to premature destruction of the cell (37).

Based on observations of Bunn and Jandl (38), who demonstrated that methemoglobin formation is an apparent requisite for heme loss from normal hemoglobin, Jacob and coworkers also examined a group of unstable hemoglobins with decreased heme affinity for evidence of this property (37). It was shown by these studies that heme oxidation to the met-form also may be required before the heme group can be released from these unstable hemoglobin variants. Thus when methemoglobin formation was prevented by the addition of heme ligands that interfered with heme oxidation or when excess hemin was added to maintain the globin chains in a heme-replete form (22), heat instability was markedly reduced or prevented. Similarly, Grimes and Meisler (39) have shown that thermolability of an unstable hemoglobin was increased after its conversion to the methemoglobin form and was substantially reduced when the hemoglobin was heated in the presence of a reducing agent.

Many of the findings in the patient described in this report point toward altered heme affinity as a major factor in the development of intracellular hemoglobin pre-

cipitation. The increased rate of methemoglobin formation in the patient's erythrocytes, the reduced level of erythrocyte glutathione, and the heat lability of the hemoglobin preventable by the addition of dithionite, carbon monoxide, or hemin, all suggest a mechanism leading to Heinz body formation and hemolytic disease similar to the proposed scheme summarized above. The substitution of proline for leucine at β 32 can be expected to disrupt the structure of the B helix of the β -subunits (36), and although β 32 leucine is apparently not in close contact with the heme (40), distortion of the tertiary structure of the β -chain could readily affect the orientation of the adjacent heme-contact residue, β 31 leucine, to impair heme stabilization.

Other findings, however, appear not to support this mechanism for Heinz body formation in the erythrocytes of this patient. The failure of cyanide to protect the hemoglobin against heat precipitation is in contrast to findings in other unstable hemoglobins with altered heme affinity (22). Normal autohemolysis and osmotic fragility values obtained with erythrocytes from the patient also appear to be inconsistent with erythrocyte membrane changes that characteristically result from the attachment of the Heinz bodies to the erythrocyte membrane (22). Our finding of approximately equal amounts of α - and β -chain protein in the heat-insoluble precipitates may indicate a mechanism for Heinz body formation similar to that recently proposed by Winterbourn and Carrell (41). These investigators studied the protein composition of Heinz bodies obtained from erythrocytes of several patients with unstable hemoglobins, and found both α - and β -chain globin present. They have suggested that simultaneous precipitation of both α - and β -chains is likely to have occurred in the formation of the Heinz body inclusions in these hemoglobinopathies. It is possible, however, that our findings of α - and β -chain globin in heat-precipitated protein may have resulted from the initial precipitation of the abnormal β -chain followed by precipitation of the normal α subunits, which are known to be heat labile (41).

Hb Abraham Lincoln is the sixth reported example of a leucine \rightarrow proline substitution. In each of these abnormalities (Table V), a marked degree of hemoglobin instability has been demonstrated.

In Hb Genova (43, 44) the proline substitution appears to produce hemoglobin instability primarily as a result of disturbances of molecular conformation (2). In all of the other hemoglobin variants included in this group, direct or indirect evidence has been presented demonstrating that heme-binding is impaired. Each of these abnormalities is accompanied by a severe degree of hemolytic disease. From these examples it appears that the coexistence of reduced heme affinity and altered heli-

TABLE V
Unstable Hemoglobin Variants Resulting from
Leucine \rightarrow Proline Substitutions

	^{51}Cr $t_{1/2}$	Hemoglobin concentration	Reticu- locyte count	Reference
	days	g/100 ml	%	
Bibba (α 136)	—	6.5–7.5	6–16	42
Genova (β 28)	12	11	4	43, 44
Abraham Lincoln (β 32)	2.4	8–10	20–30	—
Santa Ana (β 88)	5	9	16	45, 46
Sabine (β 91)	4	9	50	47
Southampton (β 106)	—	7–8.6	30–49	48

cal structure produces the most severe forms of hemoglobin instability.

The family studies of the patient described in this report suggest that the abnormality arose as a new mutation. It is of interest that among the other leucine \rightarrow proline variants in which heme binding is defective, in only one instance (45) has the abnormality occurred in more than a single family member.

Measurements of the half-saturation Po_2 , Bohr effect, and 2,3-DPG in whole blood from the patient (Table II) produced normal values. More detailed studies will be required to fully define the oxygen affinity properties of this hemoglobin. Studies of hemoglobin synthesis by erythrocytes of the patient will be described in a later report.

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