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Research Article

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Hemoglobin Olympia (β20 Valine → Methionine): An Electrophoretically Silent Variant Associated with High Oxygen Affinity and Erythrocytosis

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ABSTRACT In a family with erythrocytosis, electrophoretic and chromatographic studies failed to demonstrate a hemoglobin variant. However, the oxygen dissociation curves of affected individuals were shifted to the left of normal and this shift persisted when oxygen equilibria were studied in 2,3-diphosphoglycerate-stripped hemolysates. A mutant hemoglobin was evidently present in the red blood cells of the affected persons and was responsible for the increased oxygen affinity and erythrocytosis. Specific staining of tryptic peptide maps of β-chains from the propositus showed that peptide βT_3 was positive for a sulfur-containing amino acid. Amino acid analysis yielded a composition identical to that of normal βT_3, except that there were 2.6 residues of valine and 0.4 residues of methionine (normal composition: Val = 3.0, Met = 0). This suggested that the β-chains of affected individuals consisted of a mixture of two kinds of chains, 40% of which had a methionyl residue in βT_3. Structural studies of isolated cyanogen bromide fragments demonstrated unequivocally that, in the abnormal β-chains, valine in position 20 is replaced by methionine. The new hemoglobin mutant is designated hemoglobin Olympia (β20 (B2) valine → methionine).

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INTRODUCTION

Several hemoglobin mutants have been detected because of associated erythrocytosis in heterozygotes. The delineation of the structural and functional aberrations of these hemoglobins and the elucidation of the pathophysiological basis of erythrocytosis have contributed significantly to the understanding of molecular disease in man (1–4). Abnormal electrophoretic or chromatographic patterns are characteristic of all the described mutants (4–9). We report a new variant associated with abnormal oxygen equilibria and erythrocytosis which was defined only after detailed structural studies on isolated globin chains. This electrophoretically and chromatographically silent variant is designated hemoglobin Olympia.

METHODS

Hematologic techniques and studies of erythropoietin. Routine hematologic data were obtained using standard techniques. Hemoglobin heat stability tests were performed as previously described (4). The red blood cell mass in the propositus was determined using ^{51}Cr-labeled autologous red cells (10). Urine specimens for erythropoietin (ESF)[1] determination were collected, processed, and assayed in exhypoxic polycythemic mice as previously described (11). The biological activity in the urine concentrates was quantitated by direct comparison with simultaneously assayed ESF Std B dose/response curve.

[1] *Abbreviations used in this paper:* bis-Tris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane); CM, carboxymethyl; CNBr, cyanogen bromide; DPG, diphosphoglycerate; ESF, erythropoietin; IRP, International Reference Preparation; PMB, para-mercuribenzoate; TPCK, L-(tosylamido 2-phenyl)ethyl chloromethyl ketone.

Electrophoretic studies. Hemolysates were prepared according to Drabkin (12). Hemoglobin electrophoresis in starch gels was performed using Tris-EDTA-borate buffers at pH 8.2 and 8.6, Tris-HCl buffers at pH's ranging from 8.6 to 9.3, and 0.05 M phosphate buffers in the pH range of 6.2–7.1. Agar gel electrophoresis was done using 0.05 M citrate buffers over a pH range of 6.0–7.0. Carboxy-hemoglobin was treated with para-mercuribenzoate (PMB) and electrophoresed as described by Rosenmeyer and Huehns (13). Electrophoresis of hemoglobin chains in 6 M urea was done as described by Chernoff and Pettit (14).

Chromatographic studies. Chromatography was performed on carboxymethyl (CM) Sephadex (C-50) columns using phosphate or Tris-maleate (15) buffer systems of various pH's, on DEAE Sephadex columns using Tris-HCl buffer systems (16), and on Amberlite CG-50 columns (Rohm and Haas Co., Philadelphia, Pa.) using 0.1 M potassium phosphate buffer, pH 7.0. Chromatography of PMB treated carboxyhemoglobin was carried out as described by Bucci and Fronticelli (17).

Globin and chain separation. Globin was prepared from whole lysates by acid acetone precipitation (18) and chains were separated following the application of globin (19) or hemoglobin (20) to 2 × 10 cm columns of Whatman CM-52 cellulose equilibrated with 8 M urea buffers made 0.05 M in 2-mercaptoethanol.

Cleavage with cyanogen bromide (CNBr). 58 mg of β-chains were cleaved by CNBr (21). The cleavage products were dissolved in 4 ml of 1% formic acid and eluted from a 1.8 × 294 cm column of Sephadex G-50 (fine) with 1% formic acid at a flow rate of 6 ml/cm² per h. Each fraction contained 9 ml.

Fingerprinting. Aminoethylated (22) chains and selected CNBr fragments were digested with trypsin (trypsin-TPCK, Worthington Biochemical Corp., Freehold, N. J.) (19). 2-mg portions of tryptic digests were subjected to paper electrophoresis in pyridine, acetic acid, and water buffer (10:0.4:90 by volume) pH 6.4 (23) followed by descending chromatography using a solvent of pyridine, isoamyl alcohol, and water (35:35:30 by volume). Peptides were identified by staining with 0.2% ninhydrin as well as by application of platinic iodide (sulfur-containing amino acids), Pauly (histidine), Sakaguchi (arginine), tyrosine, and Ehrlich (tryptophan) reagents in various sequences as outlined by Easley (24).

For preparative purposes, peptide maps were made using 5 mg of the tryptic digest of the aminoethylated chains, and 3.5 mg of the digest of the CNBr fragments. These peptide maps were lightly stained with 0.02% ninhydrin in acetone, after which selected peptides were eluted (25), hydrolyzed under vacuum in 6 N HCl at 108°C for 24 or 72 h, and subjected to amino acid analysis on a Beckman 120B amino acid analyzer.

Oxygen-hemoglobin equilibria. Oxygen dissociation curves of whole blood were obtained at 37°C with the mixing technique of Lenfant, Ways, Aucutt, and Cruz (26). Oxygen equilibria were determined using dialyzed or 2,3-diphosphoglycerate (DPG)-free (27, 28) hemolysates by the automated procedure of Imai et al. (29). Equilibrium curves were measured at 20°C using 0.2% solutions of hemoglobin in 0.1 M phosphate buffers at pH's ranging from 6.13 to 7.86. Alkaline Bohr effects, defined as $\Delta \log P_{50}/\Delta$ pH from pH 7.0 to 7.4 were determined from graphs relating log P_{50} to pH.

RESULTS

Propositus. The propositus, a 21 yr old Coast Guard mechanic, on entering the service at the age of 19, was told that he had a high hematocrit. Two years later he was admitted to the U. S. Public Health Service Hospital, Seattle, because of intermittent swelling of his lower lip and periorbital area. On admission the patient appeared plethoric. Physical examination was normal as was an extensive cardiac and pulmonary investigation. Arterial blood gas values were normal (P_{O_2} 97 mm Hg, P_{CO_2} 35 mm Hg, oxygen saturation 97.4%). Hematological examination showed an hematocrit of 62% and persistently normal leucocyte and platelet counts. The reticulocyte count was 2% (uncorrected). Total blood volume, red cell volume, and plasma volume were 79.5 ml, 42.0 ml, and 37.5 ml/kg body weight, documenting as absolute erythrocytosis. The hemoglobin-oxygen dissociation curve was shifted to the left of the normal curve. Hemoglobin heat stability did not differ from normal.

After discharge from the hospital, on the advice of his private physician, the patient was phlebotomized every 2–3 wk. On re-examination one year later he was

TABLE I

Data on Hematology and Hemoglobin Function for Members of the Family with Hb Olympia

Individual	Age	Hb Olympia	P_{50}*	Hill's n	Hb	Erythrocytes × 10⁶/mm³	Hematocrit	MCH	MCV	MCHC	Serum Fe/TIBC	ESF excretion‡
			mmHg		*g/100 ml*		%	*pg*	*μm³*	%	*μg/100 ml*	
H. E. (father)	52	+	6.86	2.5	17.8	5.74	56.1	30.9	99	31.1		
B. E. (mother)	48	−	8.54	2.8	15.2	4.96	47.2	30.5	96	31.5		
R. E. (brother)	27	+	6.77	2.7	19.7	6.25	61.2	31.5	99	31.6		
N. L. (sister)	23	−	9.13	2.7	14.1	4.54	44.6	31.0	100	31.0		
L. E. (propositus)	21											
(Jan. 1970)		+	6.76	2.6	20.7	6.95	62.0	30.0	90.2	33.5	90/320	5.5(±1.1)
(Mar. 1971)					13.8	5.89	42.0	23.5	72	32.9	20/491	16.0(±1.6)

* P_{50} = oxygen tension of hemolysates at 50% saturation. Measured at pH 7.09, 20°C.

‡ Mean (±SEM) as International Reference Preparation (IRP) units/day.

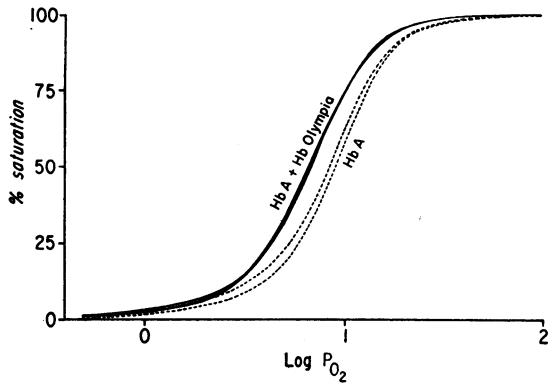

FIGURE 1 Oxygen dissociation curves determined at pH 7.09, 20°C, on solutions of unfractionated hemoglobins from the propositus, his father and brother (Hb A + Hb Olympia; solid lines) and from his mother and sister (Hb A; broken lines). The P_{50} values for the hemolysates containing Hb A + Hb Olympia range from 6.76 to 6.86 mm Hg while those for samples containing only Hb A are 8.54 and 9.12 mm Hg, illustrating the increased oxygen affinity attributable to the presence of Hb Olympia.

iron-deficient (serum iron 20 μg/100 ml, TIBC 491 μg/100 ml, saturation 6%) with a normal hematocrit (42%).

Family study. There was no history of polycythemia in the propositus' family. Hematological investigation, however, revealed that the father and a brother of the propositus also had erythrocytosis; furthermore, oxygen dissociation curves measured using hemolysates from the three affected members of the family were shifted to the left (Table I, Fig. 1). The father of the propositus had suffered a myocardial infarction at the age of 51.

Hemoglobin studies. Electrophoresis of hemoglobin in starch and agar gels at a variety of pH's, as well as electrophoresis of globin chains and PMB-treated hemoglobins, produced no evidence of an abnormal hemoglobin in the red cells of the propositus. In addition, isoelectric focusing of the propositus' hemoglobin (performed by Dr. H. F. Bunn) failed to separate Hb Olympia from Hb A. Elution profiles indistinguishable from those of the normal controls were produced by chromatography in DEAE Sephadex, CM-Sephadex, and Amberlite CG-50 columns. The pattern of hemoglobin chain elution from a CM-cellulose column in the presence of 8 M urea was normal as was the electrophoretic and chromatographic behavior of PMB hemoglobin chains.

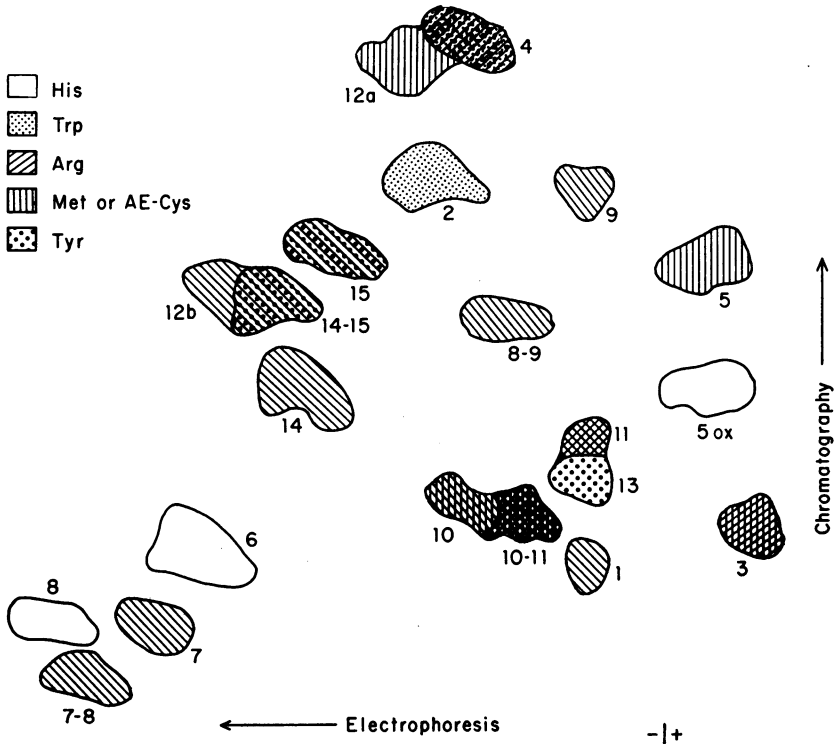

FIGURE 2 Map of tryptic peptides from aminoethylated β-chains of the propositus. Specific peptides are identified by specific stains and by comparison with maps of tryptic peptides from aminoethylated β^A-chains. The peptide pattern produced by the β-chains of the propositus is indistinguishable from that of normal β^A-chains; however, the zone normally occupied by $\beta^A T_3$ stains strongly for sulfur.

TABLE II

*Amino Acid Compositions of Tryptic Peptides from the β-Chain of Hemoglobin Olympia**

	Tryptic peptides from CNBr fragments βºCB-Ia and βºCB-Ib						βT₃ from mixture of β^A- and βº-chains
	T_1	T_2	T_{3a}	T_{3b}	T_4	T_{5a}	
Asp			1.03(1)	0.99(1)		1.89(2)	2.08(2)
Thr	1.01(1)	0.96(1)			1.00(1)	1.03(1)	
Ser		0.89(1)				1.97(2)	
HSer‡			0.41(0)			0.44	
Glu	2.03(2)			2.05(2)	1.03(1)	1.06(1)	2.17(2)
Pro	1.07(1)				1.07(1)	1.02(1)	
Gly		0.99(1)		3.02(3)		1.11(1)	3.05(3)
Ala		2.01(2)		0.99(1)		1.07(1)	1.00(1)
Val	0.85(1)	1.01(1)	0.97(2)	0.98(1)	1.83§(2)	0.99(1)	2.61(3)
Met						(1)	0.37(0)
Leu	1.05(1)	0.99(1)		1.00(1)	1.97(2)	0.94(1)	1.00(1)
Tyr					0.81(1)		
Phe						2.95(3)	
Try‖		1.00(1)			1.00(1)		
Lys	1.05(1)	0.97(1)					
His	0.87(1)						
HS Lac‡			0.60(0)			0.53	
Arg				0.96(1)	0.97(1)		0.93(1)

* Values in parenthesis refer to the amino acid compositions of the corresponding peptides in the human β^A-chain.

‡ After exposure to cyanogen bromide, methionine is detected as homoserine and homoserine lactone.

§ Valine determined after 72 h hydrolysis.

‖ Tryptophan detected on peptide maps by staining with Ehrlich reagent and in eluate from short column of amino acid analyzer.

Elucidation of the hemoglobin abnormality. Peptide maps prepared from tryptic hydrolysates of aminoethylated β-chains from the propositus were identical to those of aminoethylated β^A-chains.

Specific stains for tyrosine, tryptophan, arginine, and histidine produced identical patterns in both preparations (Fig. 2). However, stains for sulfur-containing amino acids indicated that, in addition to the expected sulfur-positive peptides βT_5 (methionine), βT_{10}, βT_{10-11}, and βT_{12a} (aminoethylcysteine), the βT_3 zone on the propositus' map was sulfur-positive. In the absence of additional peptides (which might indicate that the abnormal chain contained a third residue of aminoethylcysteine), it was tentatively concluded that a portion of the β-chains of the propositus contained a residue of methionine in βT_3.

Amino acid analysis (Table II) of βT_3 yielded non-integral values for valine (2.61) and methionine (0.37). For all other amino acids the composition was identical to that of normal βT_3. These finding indicated that one valyl residue is replaced by one methionyl residue in approximately 40% of the βT_3 peptides from the β-chains of the propositus.

The sequence of normal βT_3 is

18 20 23 30
Val-Asn-Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-Arg.

Valine occupies positions 18, 20, and 23; thus, in the abnormal β-chain of the propositus, a methionyl residue was expected in one of these three positions. The site of the substitution was identified as follows. In the normal β-chain there is only one methionine, in position β55. Cleavage by CNBr at this methionine residue produces two fragments: βCB-I, containing residues 1-55, and βCB-II, containing residues 56-146. Similar treatment of the abnormal β-chain of the propositus was expected to produce three fragments:

βCB-Ia (residues 1-18, 1-20, or 1-23),
βCB-Ib (residues 19-55, 21-55, or 24-55), and
βCB-II (residues 56-146).

The small sizes of βCB-Ia and βCB-Ib permitted their isolation by gel filtration (Fig. 3). Peak 1 contains undigested β-chains and fragments of molecular weight in excess of 10,000; peak 2 contains the βCB-II frag-

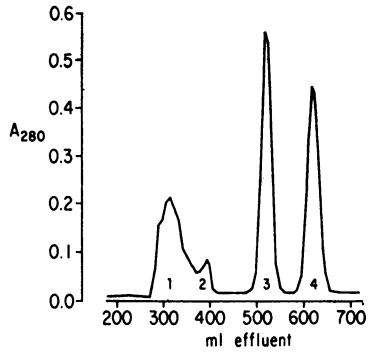

FIGURE 3 Separation of the products of CNBr digestion of β-chains from the propositus. Peak 1: uncleaved material in excess of 10,000 molecular weight; peak 2: βCB-II from β^A and β-Olympia chains; peak 3: βCB-I from β^A chain; peak 4: βCB-Ia and βCB-Ib from β-Olympia chain (see text).

ments, i.e., residues 56–146 from both normal and abnormal chains; peak 3 contains βCB-I fragments from the normal chains; peak 4 contains the βCB-Ia and βCB-Ib fragments from the abnormal β-chains.

Peptide maps prepared from the material in peak 4 produced the pattern shown in Fig. 4. In these maps, instead of βT_3, two new peptides appeared, marked βT_{3a} and βT_{3b}. Amino acid analyses of all peptides yielded the results shown in Table II. βT_1, βT_2, βT_4, and βT_{5a} are clearly identified. βT_{3a} contained 0.97 valyl, 1.03 aspartyl, and 1.01 methionyl (as homoserine plus homoserine lactone) residues, corresponding to a sequence Val-Asn-Met. βT_{3b}, on the other hand, had exactly the amino acid composition expected for a peptide containing the last 10 residues of βT_3. Thus, the specific splitting at methionyl residues by CNBr and the amino acid compositions of βT_{3a} and βT_{3b} unequivocally located the abnormality at residue β20. Hemoglobin Olympia thus differs from hemoglobin A by the substitution of methionine for valine at position β20.

Functional studies. The P_{50} value using whole blood from the propositus was 18.6 mm Hg (normal: 26.8± 0.6 mm Hg at pH 7.4); the alkaline Bohr effect was −0.57. P_{50} values for dialyzed hemolysates from the propositus and his affected relatives differed from normal to the same extent as did those for whole blood (Table I; Fig. 1). The Bohr effect in hemolysates was in the normal range (Fig. 5). Hill plots and coefficients (n) were similar for all hemolysates from normal and affected members of the family (Table I).

Removal of 2,3-DPG from lysates produced the expected shifts in oxygen dissociation curves. In 0.1 M bis-Tris, pH 7.08, the P_{50} for stripped Hb A was 2.02 mm Hg at 20°C. Under the same conditions, the hemoglobin from the propositus showed an average P_{50} of 1.46 mm Hg (1.38–1.64 mm Hg from four independent determinations). Thus the relative affinities of Hb A and the propositus' hemoglobin stand in much the same relationship to one another both before and after DPG stripping, indicating that there is no detectable difference in DPG effect between mixtures of Hb A and Hb Olympia and normal hemolysates.

Regulation of erythropoiesis. Prior to the phlebotomies, ESF excretion in the propositus average 5.5 International Reference Preparation (IRP) units/day, within the normal range but slightly above the mean in this laboratory for males of similar age (11). 1 yr later, at a hematocrit of 42, ESF excretion averaged 16.0 IRP units/day, four times the normal mean (Fig. 6).

DISCUSSION

Familial occurrence of erythrocytosis was noted at the beginning of this century and several pedigrees with this defect have been reported (30). In a number of families, transmission patterns and proportions of affected to nonaffected individuals are compatible with a recessively inherited defect; in others, erythrocytosis shows a dominant pattern of inheritance (30). In recent years, studies of families with dominantly inherited

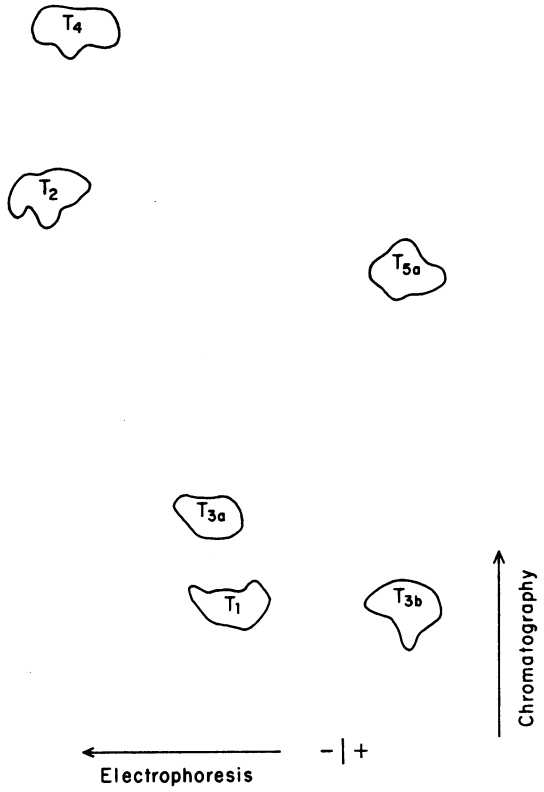

FIGURE 4 Peptide map of tryptic digest of material eluted in peak 4, Fig. 3. Peptides are numbered according to their position in the β-Olympia chain, based on analogy with the known sequence of peptides in the β^A-chain.

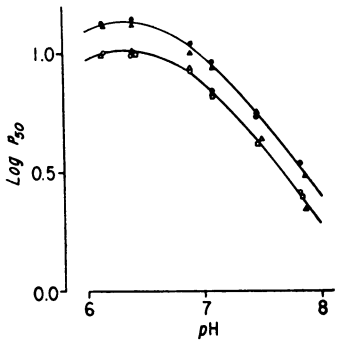

FIGURE 5 Bohr effects in hemolysates containing only Hb A (upper curve; ● subject N. L., ▲ subject B. E.) and Hb A + Hb Olympia (lower curve; ○ subject H. E., □ subject R. E., △ propositus). The extent to which the two curves are congruent is a strong indication that there are no differences in Bohr effect between the two kinds of hemoglobin solutions. In the case of Hb A alone the Bohr effect between pH 7.0 and 7.4 is −0.52 while that for Hb A + Hb Olympia is −0.53.

erythrocytosis have established hemoglobinopathy as the main etiologic possibility (4–9). Thus far, in all the families with hemoglobinopathic erythrocytosis, an abnormal hemoglobin was readily demonstrable with electrophoretic or chromatographic techniques (4–9). This, however, is not necessary for diagnosis. It has been estimated that of the 2,200 possible amino acid substitutions in adult hemoglobin, only about one-third will produce alterations in the electrical charge of the protein (31). Thus, when a mutant hemoglobin is ascertained through abnormal function, there is a high probability that the underlying molecular lesions will not be associated with abnormal electrophoretic or chromatographic behavior. This is amply demonstrated in the case of hemoglobin Olympia where a battery of chromatographic and electrophoretic methods failed to demonstrate a hemoglobin abnormality. The clue to hemoglobinopathy in this family was the increase in oxygen affinity of blood from the propositus and lysates from the propositus and members of his immediate family. Two types of defects may produce such functional abnormalities in the red cells: a mutant hemoglobin or an alteration in the intracellular level of DPG. The latter possibility was rendered unlikely in this instance, since the relative differences between the oxygen dissociation curves of the hemolysates of the propositus and normal individuals persisted after stripping of DPG. Thus, the presence of an abnormal hemoglobin remained as the only possible explanation of the abnormal red cell function and this was documented by the structural study of the β-hemoglobin chains.

From the Perutz model of cooperative interactions in hemoglobin (32) it can be deduced that the oxygen affinity of a mutant hemoglobin will be altered as a result of the amino acid substitution if the conformation around the oxygen binding sites is distorted, the ability of specific residues to bind hydrogen ions or DPG is abolished, or the allosteric equilibrium between the tertiary or quaternary oxy- and deoxyhemoglobin structures is affected. Such effects have been substantiated by model building (1, 2) or documented by X-ray crystallographic analysis (33–36) of high or low affinity mutants. In the case of Hb Olympia, it is not possible to deduce similar relationships between structure and function, for there is no special function attributable to residue 20β (B2 in the helical notation [37]) in normal hemoglobin (32, 38–40). The structural change involves a residue located on the surface of the polypeptide (41), which is not implicated in the allosteric interactions between subunits. It is possible, however, that the hydrophobic side chain of methionine at 20β may protrude into the interior of the subunit or that the bulk of this side chain, containing a relatively large sulfur atom, may distort the overall configuration of this portion of helix B thus affecting other sites having a role in subunit interactions.

The physiological effects of the Olympia mutation closely resemble the established pattern of other abnormalities with increased oxygen affinity. ESF production in the propositus was at least normal at an ele-

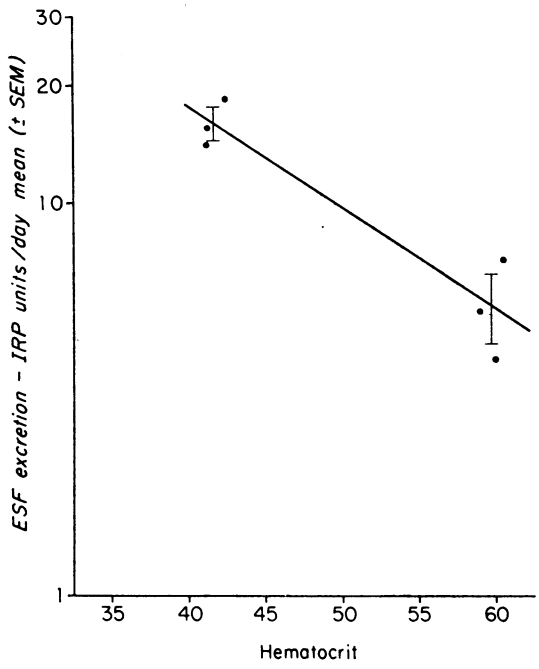

FIGURE 6 ESF/hematocrit relationship derived from data obtained from the propositus. ESF excretion data are shown as the mean (±SEM) plotted against the mean hematocrit. The 3 days of observations which comprise the average values of high and low hematocrits are indicated. Hormone excretion is expressed as IRP units/day.

vated hematocrit and hormone excretion was shown to be reciprocally related to alterations in the oxygen carrying capacity of the blood. In normal subjects made anemic through phlebotomy, ESF excretion of 16 U/day would be expected at a hematocrit of 25–30% (11). The patient with Hb Olympia excreted this amount of ESF at a hematocrit of 42%, thus demonstrating that he was functionally anemic. The findings in the propositus of this family demonstrate that persons having a hemoglobin with increased oxygen affinity may present with normal red cell mass when they are anemic and indicate that careful hematological study and assessment of ESF production are necessary before the absence of erythrocytosis in a carrier of a hemoglobin with increased oxygen affinity is attributed to unknown causes (42).

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