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Research Article

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Erythrocyte Function and Marrow Regulation in Hemoglobin Bethesda (β145 Histidine)

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ABSTRACT Hemoglobin Bethesda (β145 histidine) is one of the two mutants known to affect the penultimate hemoglobin tyrosines. The result of this substitution is extreme disorganization of the oxygenation function of the molecule. Red cells containing 45% Hb Bethesda and 55% Hb A have increased oxygen affinity but, paradoxically, a normal Bohr effect. As is usually seen with other hemoglobins with increased oxygen affinity, Hb Bethesda clinically is manifest in heterozygotes by erythrocytosis. Red cell production in affected individuals is erythropoietin dependent. The reciprocal interdependence of oxygen delivery and effective erythropoiesis was documented by alterations in erythropoietin excretion, quantitative iron kinetics, and reticulocyte production in response to phlebotomy-induced reduction in the oxygen-carrying capacity.

INTRODUCTION

At the present time a number of abnormal hemoglobins have been detected because of their properties of abnormal oxygen affinity and the appearance of erythrocytosis in heterozygous carriers. The correlations between structural abnormalities and the aberrant properties in most of these hemoglobin variants have been clarified (1, 2) and the pathophysiological basis of the erythrocytosis elucidated (3). This report describes the clinical presentation and studies of erythrocyte function and erythropoiesis in a family having one of these variants, Hb Bethesda (β145 histidine) (4).

METHODS

Hematologic and hemoglobin studies. All studies were performed on blood less than 16 hr old at the time of initial processing. Routine hematologic studies were performed using standard techniques. Hemolysates were prepared by a modification of the method of Drabkin (5). Hemoglobin electrophoresis was performed on starch gel using a Tris-EDTA-borate buffer system of pH 8.6 (3), Tris-HCl buffers at pH's 8.8 and 9.1, and phosphate buffers over a range of pH's from 6.0 to 7.2. Hemoglobin electrophoresis on agar gel was performed using 0.05 M citrate buffer at pH 6.2 (3). Alkali-resistant hemoglobin was quantitated by the techniques of Jonxis and Visser (6) and Betke, Marti, and Schlicht (7). Separation and quantitation of hemoglobin fractions were carried out on Amberlite CG-50 columns (Rohm & Haas Co., Philadelphia, Pa.) using potassium phosphate buffers at pH 7.0 and on DEAE-Sephadex using 0.05 M Tris-HCl buffer at pH 7.9.

Studies of oxygen-hemoglobin equilibria. The oxygen-hemoglobin dissociation curves and Hill's n of heparinized whole blood samples were determined using the mixing techniques of Lenfant, Ways, Aucutt, and Cruz (8). Oxygen-hemoglobin equilibria characteristics were also examined in dilute red cell suspensions using a modification of the method of Imai et al. (9). For these studies, 1 vol of washed red cells was diluted in 300 vol of isotonic phosphate buffer solutions ranging in pH from 6.0 to 7.9. Continuous oxygen dissociation curves were determined at 37°C and 600 mμ. The degree of hemolysis and proportion of methemoglobin were measured after each experiment and found to be negligible. In addition, the O_2 tension at 50% hemoglobin saturation was determined in red cell suspensions of hematocrit 27% using the gasometric method described by Van Slyke and Neill (10). Oxygen equilibria in hemoglobin solutions were determined as previously described (9, 11).

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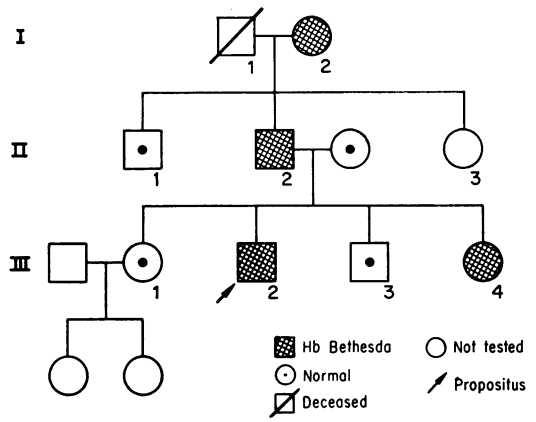

FIGURE 1 Pedigree of propositus (III-2) with Hb Bethesda.

Red cell levels of 2,3-diphosphoglycerate (DPG)[1] and ATP were measured using a modification of the method of Bartlett (12).

Studies of the regulation of erythropoiesis. Total and effective erythropoiesis, quantitated by iron kinetic studies and serial reticulocyte counts, and the urinary excretion of erythropoietin (ESF) were monitored before and after graded phlebotomies designed to reduce the oxygen-carrying capacity of the blood by approximately 15%. Hematocrits and reticulocyte counts were performed at least once daily. The reticulocyte number was first determined by screening the equivalent of 10^4 red cells using a Miller ocular (13) and then corrected for the concentration of red cells in circulation (14). The urine for ESF determination was collected and processed by previously described means and assayed in ex-hypoxic polycythemic mice using an international reference preparation (IRP) of urinary ESF as the standard by which quantitation was achieved (15). Ferrokinetic studies were performed using ^{59}Fe (as $FeCl_3$) bound to the transferrin in autologous plasma and injected intravenously. The plasma iron turnover (PIT), marrow transit time (MTT), and erythrocyte iron turnover (EIT) were determined as previously described (16).

RESULTS

The propositus. The propositus, III-2 (Fig. 1), a 23 year old Caucasian of northern European origins, was admitted to the Malcolm Grow Medical Center, Andrews Air Force Base, Washington, D. C., in March, 1968, for evaluation of unexplained erythrocytosis. Past medical history was unremarkable. On physical examination the propositus appeared healthy although plethoric. There were no abnormalities of the cardiovascular system and the spleen was not palpable. The remainder of the physical examination was entirely within normal limits. Routine hematologic examination revealed a persistently elevated hematocrit of 60–65%, normal leukocyte and platelet counts, and a normal absolute basophile count and leukocyte alkaline phosphatase value. The significance of the elevated hematocrit was confirmed by a standard measurement of red cell mass using ^{51}Cr-labeled autologous red cells (49 cm^3/kg). Arterial blood gases, intravenous pyelography, chest X-ray, and electrocardiogram were within normal limits. An alkali denaturation test revealed 0.6 g/100 ml resistant to alkali, within normal limits. To examine the possibility that a functionally abnormal hemoglobin accounted for the erythrocytosis in this otherwise healthy individual, a measurement of whole blood P_{50} (P_{O_2} in mm Hg at which 50% of the hemoglobin exists in the oxy form) was made which gave a value of 11.5 mm Hg at standard pH (7.4) and temperature (37°C). Normal values for this technique (8) are 26.8±0.6 mm Hg.

Electrophoretic and chromatographic studies. Hemoglobin electrophoresis on cellulose acetate at pH 8.6 or on starch gels using buffers over a wide pH range revealed no abnormally migrating band. However, electrophoresis on agar gel using a citrate buffer (pH 6.2) disclosed an abnormally migrating band with cathodal mobility (Fig. 2). In addition, chromatography on Amberlite GC-50 (Rohm & Haas Co.) disclosed the presence of an abnormal hemoglobin comprising 46% of the total hemoglobin in solution (Fig. 3). This abnormal fraction had the same electrophoretic and chromatographic characteristics as Hb Rainier (3, 4) but was clearly nonidentical because of its susceptibility to alkali denaturation. This finding prompted the detailed structural studies resulting in the definition of Hb Bethesda (4).

Family studies. The family history was positive in that the paternal grandmother was known to be poly-

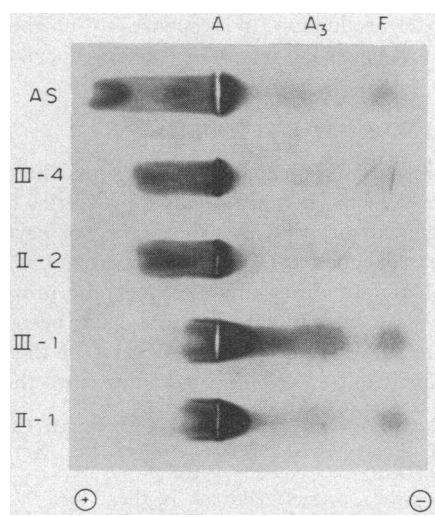

FIGURE 2 Citrate-agar gel electrophoresis (pH 6.2) of hemolysates with Hb Bethesda. An abnormal band with cathodal migration is seen in III-4 and II-2.

[1] *Abbreviations used in this paper:* DPG, 2,3-diphosphoglycerate; EIT, erythrocyte iron turnover; ESF, erythropoietin; IRP, international reference preparation; MTT, marrow transit time; PIT, plasma iron turnover.

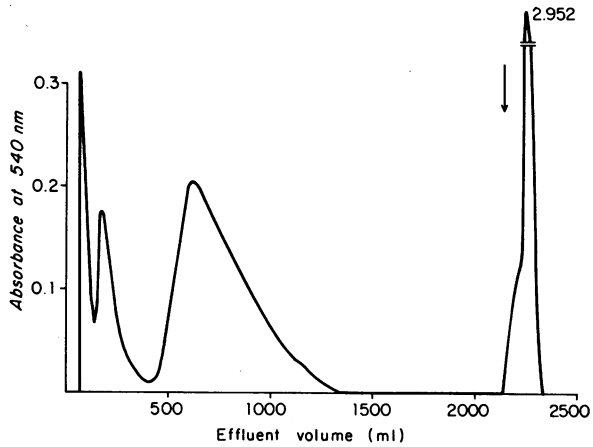

FIGURE 3 Elution profile of a hemolysate containing Hb's A and Bethesda. CG-50 Amberlite chromatography using a 2.5 × 25 cm column at 5°C. The flow rate was 25 ml/hr and fraction volume 8.0 ml. 430 mg of hemoglobin was applied on the column. The first three chromatographic peaks, A_3, $Bethesda_3$, and A, were eluted with 0.1 M potassium phosphate buffer in 10^{-3} M KCN (pH 7.0). The Bethesda peak was eluted with 0.1 M potassium phosphate in 10^{-3} M KCN and 5×10^{-1} M NaCl (pH 7.0).

cythemic for many years. Siblings, parents, and other available relatives were examined hematologically. Erythrocytosis due to Hb Bethesda was found in three additional family members (Table I) spanning three generations (Fig. 1). There was no history of spontaneous abortions in the affected females.

Red cell function. Oxygen-hemoglobin dissociation curves measured in red cell suspensions with the automated procedure are shown in Fig. 4. The curve obtained with cells from a normal sibling demonstrated the usual sigmoid shape with P_{50} values of 24.2 mm Hg (pH 7.4) and 35.0 mm Hg (pH 7.0). In contrast,

TABLE I
Hematologic Values—Hb Bethesda Family

Subject	Age	Sex	Hct	Hemoglobin	Hb Bethesda*	DPG‡
	yr			*g/100 ml*		
I-2	77	F	56.5	19.6	+	
II-1	55	M	49.0	17.0	−	
II-2	50	M	52.0	17.8	+	
III-1	25	F	44.0	ND§	−	
III-2‖	23	M	61.0	20.5	+	4.85
III-3	21	M	45.0	16.8	−	4.69
III-4	19	F	50.0	ND	+	

* Documented by citrate-agar gel electrophoresis.
‡ As micromoles per cubic centimeter packed red cells (normal range, males: 4.8±0.3) (19).
§ ND, Not determined.
‖ Propositus.

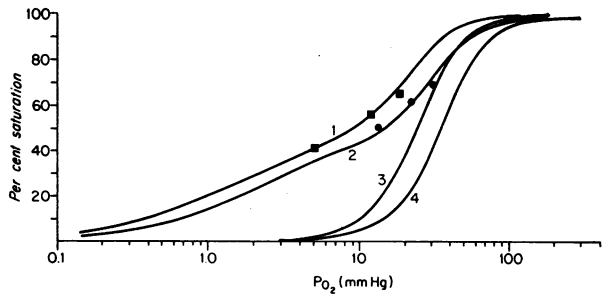

FIGURE 4 Continuous oxygen equilibrium curves of red cell suspensions. 1/10 ml volumes of washed red cells (hematocrit 27%) were diluted to 7.0 ml in physiologic phosphate buffer solutions, pH 7.4 and 7.0. Oxygen equilibria were determined at 37°C and at 600 mμ (9). Curves 1 and 2: cells containing Hb's A and Bethesda at pH 7.4 and 7.0, respectively. Curves 3 and 4: normal cells at the same pH's. Symbols represent measurements obtained using the gasometric technique (10) with the patient's red cells in physiological phosphate buffer at pH 7.4 (■) and 7.0 (●).

the dissociation curves of the propositus were biphasic. Measured P_{50}'s were 9.0 and 14.4 mm Hg at pH 7.4 and 7.0, respectively. The oxygen tensions determined around 50% saturation by the gasometric technique

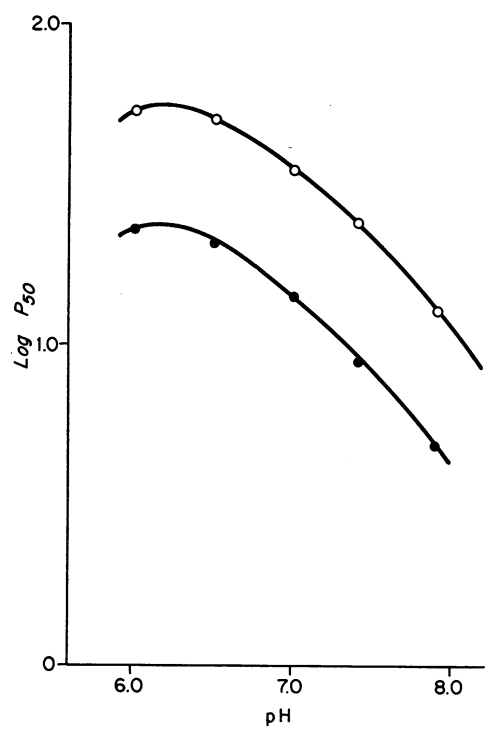

FIGURE 5 Comparison of the Bohr effect in normal (○) and Hb Bethesda (●) red cell suspensions. The Bohr effect was determined from the oxygen equilibrium curves obtained under the conditions defined in Fig. 4 and over a pH range of 6.0–7.9 ("r" values were calculated at pH 7.4).

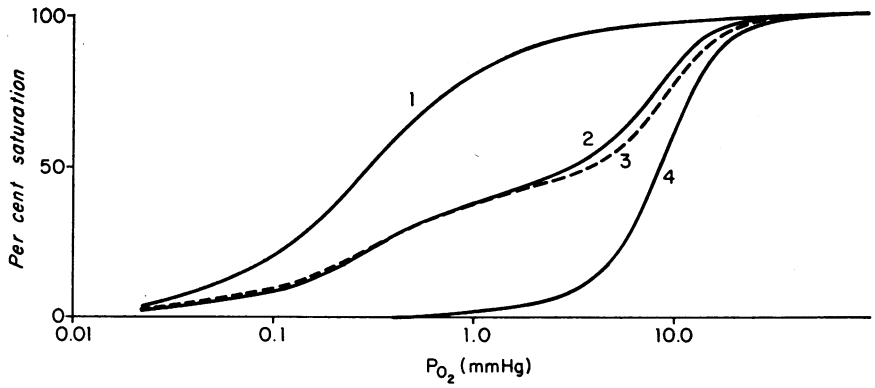

FIGURE 6 Oxygen equilibrium curves of (*1*) purified Hb Bethesda; (*2*) whole hemolysate containing Hb's A and Bethesda; (*4*) purified Hb A. The curves were obtained using the automated method of Imai, et al. (9), with 0.2% hemoglobin concentrations in phosphate buffer at pH 7.0, 20°C, and at 600 mμ. (*3*) is the curve constructed from data obtained in (*1*) and (*4*) and adjusted for a mixture containing 46% Hb Bethesda and 54% Hb A.

were in the same range as those obtained by the automated procedure (Fig. 4).

The Bohr effect factor in red cells containing Hb Bethesda was essentially normal (Fig. 5); "r" values ($\Delta \log P_{50}/\Delta$ pH) at pH 7.4 were 0.68 for the propositus and 0.64 in normal controls.

The oxygen-hemoglobin dissociation curve at pH 7.0 of the propositus's unfractionated lysate (Fig. 6) closely resembled that obtained in red cell suspensions with the exception that oxygen affinity was, as expected (9), greater in the lysate. A theoretical curve (Fig. 6) calculated on the basis of the oxygen dissociation characteristics of purified Hb's A and Bethesda, studied under identical conditions, had a slope strikingly similar to those of the hemolysate and the red cell suspension. The findings suggest that the dissociation curve is made up of two components having low (Hb A) and high (Hb Bethesda) oxygen affinity. The slope of the curve implies that there is little interaction between the component hemoglobins.

Finally, measured erythrocyte DPG concentrations were normal (Table I).

Studies of the regulation of erythropoiesis. Basal ESF excretion in the prephlebotomy state averaged 5.9 IRP U/day and ranged from 4.3 to 7.2 U. After the initial phlebotomy, mean excretion rose to 6.7 U/day (range: 4.6 to 9.0 U). After the second phlebotomy, at a hematocrit of 52.4, ESF excretion averaged 8.4 U/day (range: 6.4 to 12.3 U). Thus, as the oxygen-carrying capacity was reduced by 13%, average ESF excretion increased in a stepwise fashion (Fig. 7). Coincident with this was a rise in the mean reticulocyte count from a low of 0.7 to 1.7% (peak 2.2%), documenting an effective response to the phlebotomy. As the hematocrit was allowed to return toward prephlebotomy levels, the reticulocyte count fell.

Initial iron kinetic studies revealed a PIT of 0.94 mg Fe/100 ml whole blood per day and an EIT of

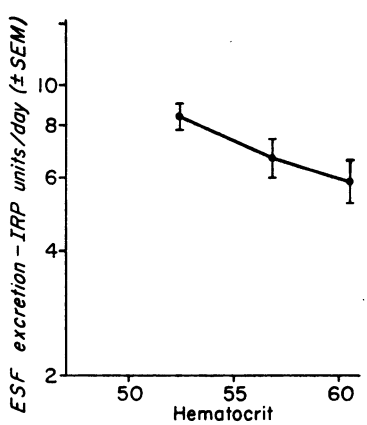

FIGURE 7 Erythropoietin excretion at various hematocrit levels for the propositus with Hb Bethesda.

TABLE II
Erythrokinetic Response to Phlebotomy

Subject	N	Hct (mean)	Reticulocyte count (mean)	PIT*	EIT*	MTT	ESF excretion
	(*days*)					(*days*)	(*IRP U/day*)
Propositus	4	60.5	0.7	0.94	0.85	3.0	5.9‡
	7	56.8	1.1	—	—	—	6.7
	9	52.4	1.7	1.52	1.35	2.75	8.4‡

* Plasma iron turnover (PIT) and erythroid iron turnover (EIT) as milligrams Fe/100 ml whole blood per day.
‡ Difference between groups significant with $P < 0.05$.

0.85 mg/100 ml per day. Both of these values represent an increase of nearly 50% above normal (16). In response to phlebotomy, the PIT rose to 1.52 and the EIT to 1.35 mg/100 ml per day. Coincident with increased ESF production was a reduction in the MTT from 3.0 days (hematocrit 64) to 2.75 days (hematocrit 52). Table II summarizes the erythrokinetic response to phlebotomy in the propositus.

DISCUSSION

Hemoglobin Bethesda is one of the two mutations known to affect the penultimate tyrosines of the hemoglobin beta chain (11). These residues have remained invariant during evolution and have a critical role in the cooperative interreactions of hemoglobin with ligands. In his comprehensive model of the relationship between hemoglobin structure and function, Perutz (17) indicated that in the deoxyhemoglobin form the penultimate tyrosines are wedged into pockets between helices F and H. In this conformation, the C-terminal residues form salt bridges with the neighboring subunits, thus stabilizing the deoxy form. Combination of heme Fe with oxygen triggers changes in the conformation of the subunits including a narrowing of the pocket between the F and H helices. The penultimate tyrosines are expelled from this pocket pulling with them the C-terminal residues and rupturing the salt bridges which stabilized the molecule in the deoxy form. Disruption of the salt bridges decreases the stability of the deoxyhemoglobin structure thus shifting the equilibrium between deoxy and oxyhemoglobin conformation in favor of the latter.

In the two mutants with tyrosines HC2 substituted, Hb's Bethesda and Rainier, the molecular lesion is accompanied by disorganization of oxygenation properties (2, 3, 4, 18), providing evidence for the correctness of the Perutz model. Thus, as would have been expected from stereochemical considerations, heme-heme interactions in isolated hemoglobins Rainier and Bethesda are almost abolished and the Bohr effect is halved (11). The high oxygen affinity of these mutants is not due to the fact that they are unable to achieve the deoxy configuration. X-ray crystallography of deoxygenated Hb Rainier (18) revealed a normal deoxy structure, with the exception of the C-terminal region, and, although similar studies have not been performed, it is reasonable to assume that this is also the case in Hb Bethesda. It would appear that the substitution for tyrosine in these two mutants leads to decreased stability of the deoxyhemoglobin form and shifts the thermodynamic equilibrium between the oxy and deoxy forms in favor of the former.

These abnormalities in structure are reflected in red cell function. The oxygen affinity of red cells containing Hb Bethesda is extremely high and the P_{50} of whole blood of heterozygotes is on the order of 10.0–11.5 mm Hg (pH 7.4). Tissue oxygen supply in Hb Bethesda carriers, estimated on the basis of the oxygen dissociation curves of Fig. 4, is only about half that of a normal subject. Heme-heme interaction in this hemoglobin is almost abolished and the hemoglobin is an inadequate carrier of Bohr protons and demonstrates about half the normal DPG effect (11). This mutant hemoglobin, which comprises almost 50% of the red cell hemoglobin in the carriers, contributes little to oxygen and hydrogen ion transport.

The shape of the oxygen dissociation curves of red cell suspensions and hemolysates indicates that red cell hemoglobin consists of two components, one with high and the other with normal oxygen affinity. The observed curves were identical with the theoretical curve constructed on the basis of findings using purified fractions of Hb A and Hb Bethesda.

It is interesting to note that the n values of the hemolysate and red cell curves at 50% saturation are abnormally low (n of approximately 0.5). This may be explained as follows. If, in the patient's red cells, Hb Bethesda (46%) and Hb A (54%) behave independently of one another, the P_{50} values of the Hb Bethesda fraction will correspond to 23% saturation while that for the Hb A fraction will correspond to 73% saturation of the composite oxygen dissociation curve of the red cells. In fact, the n value at 23% saturation is 1.1, very close to that of purified Hb Bethesda while the n value at 73% saturation is 2.6, similar to that of purified Hb A. The point of 50% saturation of the curve of red cells or hemolysates is thus very near to the inflection point of the curves of Hb's A and Bethesda. Thus it is clear that in situations where two noninteracting hemoglobins are present in the red cells calculations of n values at 50% saturation may be misleading.

An unsolved question is the value of the Bohr effect factor in the blood of Hb Bethesda patients. In isolated Hb Bethesda the Bohr effect is almost halved, similar to the abnormal value obtained with Hb Rainier (11). Red blood cells containing 40% Hb Rainier have a Bohr effect diminished by approximately 25% (3), as might be expected in the presence of two species of hemoglobins, one with normal and the other with halved Bohr effect. In Hb Bethesda, the Bohr effect in whole blood is slightly higher than normal; there is no apparent explanation for this finding. The data indicate, however, that it is improper to draw conclusions about the Bohr effect of an abnormal hemoglobin on the basis of measurements on red blood cells or unfractionated hemoglobin solutions.

The displacement of the oxygen-hemoglobin dissociation curve in Hb Bethesda is comparable to that of

Hb Rainier. However, the clinical manifestation of this functional abnormality, i.e. the appearance of erythrocytosis, is apparently dictated by more than simply the position of the curve. Thus, there is a wide discrepancy between the hematocrit of the propositus and that of his father, although both have similarly severe displacement in the dissociation curve.

Clinically it is still uncertain whether the hyperviscosity associated with hemoglobinopathic erythrocytosis confers an increased risk of cardiovascular accident or other vascular complications. The affected family members with Hb Bethesda have been healthy and are asymptomatic. Reduction of the oxygen-carrying capacity by 13% in the propositus was associated with no restriction in physical activity or loss of sense of well being. The final prospective analysis of the risk factors associated with these abnormal hemoglobins will only become evident over a period of years of clinical observation.

The studies in this patient confirm the previous observations of the ESF-dependence of erythropoiesis in this setting (3). Thus, basal ESF excretion was in the high-normal range and increased appropriately in response to phlebotomy, demonstrating the oxygen dependence of ESF production. The increase in hormone production was confirmed by the attendant increase in both total (PIT) and effective (reticulocyte index) erythropoiesis and the shortening of the MTT by 0.25 days. The reduction in MTT reflects the degree of premature release of marrow reticulocytes and correlates well with the degree of ESF stimulation and anemia in the normal subject (16). Thus, both a mean ESF excretion of 8–10 U/day and MTT of 66 hr are compatible with values obtained in normal man at a hematocrit of 33–35 (15, 16).

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