Erythrocyte Free Globin Levels in Some Anemic States[1] (34744)

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Free globin as identified by hemin binding capacity has been found in hemolysates of normal erythrocytes (1). It is probably a mixture of $\alpha\beta$ apoprotein and intermediate α_2 β_2 tetramers partially saturated with heme. *In vitro* studies of hemoglobin synthesis indicate that this free globin is a precursor to hemoglobin in the nucleated red cell whereas in the mature cell it constitutes a residuum of excess globin production (2). The latter is supported by the findings of similar amounts of free globin in reticulocytes and adult erythrocytes (2). We report here the amount of erythrocyte free globin found in conditions affecting hemoglobin synthesis.

Materials and Methods. Hemoglobin, hematocrit, red cell count, and reticulocyte counts were done on venous blood by standard methods (3). The serum iron was done by the method of Bothwell and Mallett (4) and the total iron binding capacity by the method of Morgan and Carter (5). Hemoglobin F was measured by the alkali denaturation procedure of Betke *et al.* (6). Hemoglobin A_2 was determined by the DEAE-cellulose[3] chromatographic method as described by Chernoff (7). Hemoglobin electrophoresis was done in a horizontal starch gel with Tris–citrate–boric acid buffer, pH 8.6 (8).

The erythrocyte free globin assay procedure is briefly summarized since it has been described in detail elsewhere (1). ^{59}Fe-hemin was isolated from rabbit blood labeled *in vivo* (9) or by an *in vitro* technic (10). Fresh saline-washed red cells were hemolyzed at 4° in a solution of 0.01 *M* sodium phosphate, 0.01 *M* potassium cyanide, pH 7.5, containing 50 to 100 μg of ^{59}Fe-hemin/ml. The hemolysate was passed through a column of DEAE-cellulose to remove free hemin. The column effluent was reconcentrated with concurrent dialysis against 0.01 *M* sodium phosphate, pH 6.8, containing potassium cyanide in a concentration of 100 mg/liter. Approximately 200 mg of hemoglobin were applied to a 2 × 20-cm column of CM-Sephadex (carboxymethyl-Sephadex C-50, Pharmacia, Uppsala, Sweden) equilibrated with the pH 6.8 buffer and eluted with a nonlinear gradient of 0.02 *M* sodium phosphate, pH 8.5, into a mixing chamber containing 250 ml of pH 6.8 buffer. All buffers contained potassium cyanide in a concentration of 100 mg/liter. For the separation of hemoglobins S and C, C and A, and S and A, a linear gradient was employed with 1 liter of buffer in each chamber. For hemoglobin SC and CA separations, the end buffer was pH 8.5; and for hemoglobin SA separations, the end buffer was pH 8.0. For the separation of hemoglobin F from hemoglobin A, a linear gradient was employed with 0.05 *M* sodium phosphate, pH 6.0, in the mixing chamber, and 0.05 *M* sodium phosphate, pH 6.0, containing 0.2 *M* sodium chloride, in the end chamber (11).

Each hemoglobin chromatographic fraction was reconcentrated and the hemoglobin concentration was determined in phosphate–cyanide buffer at 540 mμ. The radioactivity of 4-ml aliquots of each hemoglobin along with appropriate standards was determined. For these data the amount of free globin as newly

[1] This investigation supported by U.S. Public Health Service Research Grant 5-RO1-HE-06242, 5-RO1-AM-10327, National Institutes of Health Special Fellowship 1-F3CA23, 076-01A1, and State of Washington Initiative 171 Funds for Research in Biology and Medicine.

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[3] Diethylaminoethyl cellulose, Selectacel, Brown and Company, Keene, New Hampshire.

formed hemoglobin was calculated and expressed as the percentage of the total hemoglobin in that fraction. The value of 3.85% was used for the weight percent of heme in hemoglobin.

A few studies of the recombination of hemin with globin prepared from heterogeneous hemoglobin hemolysates were performed. Globin was prepared from stroma-free hemolysates and brought to pH 7.5 in phosphate buffer as described by Winterhalter and Huehns (12). ^{59}Fe-hemin in phosphate buffer with cynanide, pH 7.5, was gradually added with stirring to the globin solution. Excess hemin was removed by passing the mixture through a column of DEAE-cellulose. The resultant recombined hemoglobin solution was reconcentrated with concurrent dialysis against the pH 6.8 buffer and chromatographed on CM-Sephadex as described above.

Results. Free globin in the red cells of two individuals with homozygous β-thalassemia was markedly depressed when expressed either as percentage of hemoglobin A or per red cell (Table I). Four subjects with heterozygous β-thalassemia showed a normal concentration of free globin when related to hemoglobin A but a decreased amount per cell in keeping with the decreased cell hemoglobin. In one subject heterozygous for β-thalassemia and hemoglobin C, the free globin C level was low while the free globin A, although reduced in total amount, was present in an increased concentration when compared with the hemoglobin A present. In one patient with α-thalassemia, the free globin concentration was normal but the amount per cell was reduced. ^{59}Fe-hemin showed a high binding (9 times normal) to hemoglobin H in this patient; this is probably a function of a high degree of heme interchange, even in the presence of cyanide (12), and not due to the presence of free β globin.

Erythrocyte free globin assays were performed on hemolysates from individuals with homozygous hemoglobin S, hemoglobin SC,

TABLE I. RBC Free Globin Level in Thalassemia.

Subject	Diagnosis	Retic. ct. (%)	HCT (%)	Hb A_2 (%)	Hb F (%)	MCH (pg.)	Free globin A (% of Hb A)	Free globin A Per RBC (pg.)
—	Normal	1.0 ± 0.5[a]	44 ± 7[a]	<3.3[b]	<0.8[c]	30 ± 4[a]	0.22 ± 0.027[d]	0.058 ± 0.007[d]
1	β-Thalassemia, homozygous	7.6[e]	26	11.1	86.7	33	0.08[f]	0.026
2	β-Thalassemia, homozygous	18.0[e]	30	7.0	10.0	—	0.13	—
3	β-Thalassemia, heterozygous	0.6	45	4.3	1.7	19	0.23	0.044
4	β-Thalassemia, heterozygous	13.6	30	6.2	1.1	22	0.20	0.045
5	β-Thalassemia, heterozygous	2.7	35	5.7	1.1	18	0.25	0.045
6	β-Thalassemia, heterozygous	0.7	36	3.5	1.0	—	0.28	—
7	Hb C,	1.1	31	—	1.1	20	0.28	0.015
	β-thalassemia						0.09[g]	0.013[g]
8	α-Thalassemia	6.0	44	2.8	—	22	0.20	0.040
	with Hb H (10%)						1.80[h]	0.040[h]

[a] Normal ± 2 standard deviations; data from Hematology Division laboratories, University of Washington School of Medicine.

[b] Ref. (7).

[c] Ref. (6).

[d] Ref. (1); mean ± 1 standard deviation.

[e] Blood contained 145 normoblasts/100 WBC.

[f] Value represents free globin in Hb A_2 + Hb F; no detectable Hb A.

[g] Free globin C. The hemolysate contained 36% Hb A, 74% Hb C.

[h] Free globin H, but high degree of heme–heme exchange demonstrated. The hemolysate contained 10% Hb H.

hemoglobin SA, and hemoglobin CA (Table II). Analysis of chromatographic fractions in each of these experiments showed a symmetrical peak of radioactivity associated with each hemoglobin peak (Fig. la–c). This indicates the presence of a free globin fraction for each of the hemoglobin species. In individuals with homozygous hemoglobin S (subjects 9–13) the free globin S values were significantly lower than the corresponding normal free globin A values in normal subjects. The individual with hemoglobin SC (subject 14) had a free globin C level considerably below the free globin S level. In two individuals with hemoglobin SA (subjects 15, 16), the free globin levels were at or slightly below the normal free globin A value. Variable results were obtained in individuals heterozygous for hemoglobins C and A (subjects 17–20). Two of the four had high free globin A levels (subjects 17, 19) and one of the four had a free globin C level below the normal free globin A range (subject 20). In hemolysates containing hemoglobins SC, SA, and CA, the proportional amounts of the two hemoglobins present as compared to the two types of globin present were calculated. In five of the seven instances (subjects 15–18, 20) the proportion of free globins agreed within 10% of the proportion of the hemoglobins.

Experiments were carried out to determine if hemin would recombine with the abnormal globins S and C as readily as with globin A. Globin was prepared from hemolysates of blood containing hemoglobin SA (subject 15), hemoglobin CA (subject 18), and hemoglobin C-thalassemia (subject 7). After mixing with hemin and removal of excess hemin, the resultant reconstituted hemoglobins were separated by CM-Sephadex chromatography. The relative amounts of the recombined hemoglobins were compared with the relative amounts in the respective native hemolysates (Table III). Excellent agreement was observed especially with the hemoglobin SA and CA samples. The results indicate that hemin recombined with mutant globins as readily

TABLE II. RBC Free Globin Level in Hemoglobins SS, SC, SA, and CA.

Subject	Hb phenotype and % of each	Retic. ct. (%)	HCT (%)	MCH (pg)	Free globin A		Free globin S		Free globin C	
					% of Hb A	Per RBC (pg)	% of Hb S	Per RBC (pg)	% of Hb C	Per RBC (pg)
9	SS	12.7	24	34			0.12	0.039		
10	SS	18.9	19	28			0.11	0.031		
11	SS	16.3	20	30			0.13	0.031		
12	SS	3.6	28	—			0.08	—		
13	SS	22.0	23	—			0.11	—		
14	SC 53/47	1.6	31	26			0.19	0.026	0.09	0.011
15	SA 46/54	0.3	35	—	0.20	—	0.16	—		
16	SA 36/64	0.6	33	33	0.17	0.035	0.17	0.020		
17	CA 37/63	2.6	36	26	0.33	0.053			0.22	0.021
18	CA 42/58	0.5	36	29	0.26	0.044			0.21	0.026
19	CA 41/59	1.2	30	29	0.35	0.059			0.22	0.026
20	CA 41/59	2.9	36	30	0.18	0.032			0.14	0.017

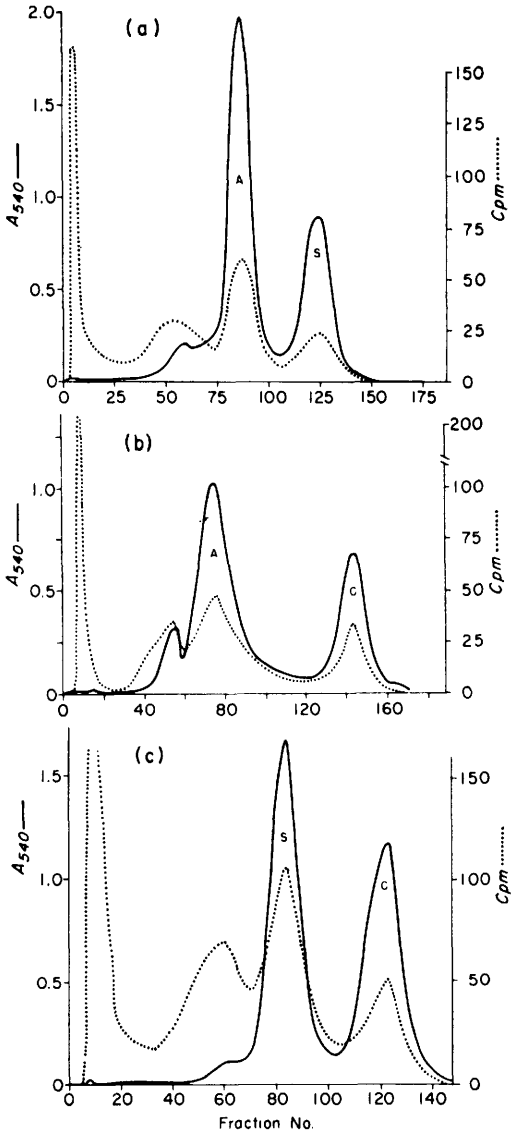

FIG. 1. Plots of typical CM-Sephadex chromatograms of red cells hemolyzed in ^{59}Fe-hemin. The initial peak of radioactivity is associated with the unabsorbed nonhemoglobin protein and has a high absorption at 280 mμ. The small 540-mμ peak preceding the first major hemoglobin peak represents hemoglobin A_1 (a and b); and hemoglobin S_1 (c). These fractions correspond to electrophoretic fractions A_3 and S_3 respectively.

as with globin A under the conditions of the experiment.

The mean free globin level for a group of eight iron-deficient subjects was 0.15% (Table IV), significantly less than the normal mean value of 0.22% previously obtained ($p < 0.001$). Because of the marked reduction in mean corpuscular hemoglobin in iron deficiency, the free globin per cell was less than one-half of the normal mean value in six of seven subjects. Only subject 25 had a globin level in the normal range. The most severely iron-deficient subject, as judged by cell indices (subject 26), had the lowest free globin level of 0.08 to 0.09% or only 0.01 pg/erythrocyte.

TABLE III. Comparison of Percentage of Hemoglobins in Hemolysate with Percentage after Hemin and Globin Recombination.

Subject	Hemolysate[a]				After recombination[a]			
	A_1	A	S	C	A_1	A	S	C
7[b]		26.2[c]		73.8		34.5		65.5
15[b]	5.9	52.6	41.5		5.2	51.4	43.4	
18	5.2	57.8		37.0	5.2	58.5		36.3

[a] Percentage of each hemoglobin present.

[b] Average values from duplicate chromatograms of same preparation.

[c] Hb A + A_1.

The free globin level in various other anemias, including sideroblastic anemia (subjects 29–31) were normal except for subject 31, whose free globin was slightly depressed (Table V). Free globin F and A were normal or slightly depressed in two studies of newborn blood (Table VI). The ratio of total amount of hemoglobin F and A was similar to the ratio of the amount of free globin in the two studies.

Discussion. Kruh and Borsook (13) in 1956 provided evidence for the coordinate synthesis of the heme and globin portions of hemoglobin. Other investigators since then have provided support for this concept. Heme biosynthesis in reticulocytes is regulated through a feedback inhibition by hemin of further synthesis of the porphyrin precursor, Δ-amino levulinic acid (14). Heme stimulates globin synthesis (15–17) and globin chains may modulate their own synthesis (18), although the mechanisms of these controls are not well established. The subject of hemoglobin biosynthesis has recently been reviewed by London *et al.* (19).

TABLE IV. RBC Free Globin A Level in Iron Deficiency.

Subject	Retic. ct. (%)	HCT (%)	MCH (pg)	SI[a]/TIBC (μg %)	Free globin A	
					% of Hb A	Per RBC (pg)
21	1.8	25	15	14/283	0.15	0.022
22	9.8	26	17	30/492	0.14	0.025
23	4.0	25	15	27/474	0.15	0.023
24	1.6	28	17	20/363	0.14	0.024
25	1.2	18	18	23/383	0.22	0.041
26	1.3	18	13	18/421	0.08	0.011
27	1.8	15	—	15/394	0.17	
28	1.1	32	20	34/444	0.16	0.032
	Mean ± SD				0.15 ± 0.028	0.025 ± 0.009
	Range				0.08 – 0.22	0.011 – 0.041
	Normal mean[b]				0.22 ± 0.027	0.058 ± 0.007
	Normal range[b]				0.17 – 0.26	0.047 – 0.069

[a] Serum iron ($N = 60$–200 μg %) and total iron binding capacity ($N = 270$–370 μg %); normal values from laboratories of the Hematology Division, University of Washington School of Medicine.

[b] Ref. 1.

In previous studies, a fraction of hemin binding globin has been found in normal cells, amounting to 0.22% ± 0.027 (mean ± 1 SD) of the total hemoglobin present. It was felt that quantitative studies in various anemias might provide insight into the degree to which globin synthesis was integrated with iron supply and porphyrin synthesis. It also seemed possible that the deficit or accumulation might provide information as to the disturbance in the synthesis of hemoglobin in certain obscure disorders.

In the present study, low free globin concentrations were found in homozygous β-thalassemia, sickle cell disease, and iron-deficiency anemia, and low to normal levels in sideroblastic anemia, even though hemoglobin synthesis in these various conditions is decreased for different reasons. Since in thalassemia there is a quantitative defect in

TABLE V. RBC Free Globin A Level in Various Anemic States.

Subject	Diagnosis	Retic. ct. (%)	HCT (%)	MCH (pg)	Free globin A	
					% of Hb A	Per RBC (pg)
29	Refractory dimorphic anemia with "ring" sideroblasts in marrow	2.0	34	—	0.19	—
30	Rheumatoid arthritis, porphyria cutanea tarda, and subacute "heme block" anemia with "ring" sideroblasts in marrow, partially responsive to pyridoxine therapy	5.6	34	34	0.18	0.063
31	Acute alcoholic liver disease, "ring" sideroblasts in marrow disappeared after 3 days in hospital	5.2	22	31	0.15	0.047
32	Erythropoietic protoporphyria	1.5	37	—	0.21	
33	Pernicious anemia, untreated	2.6	18	42	0.23	0.099
34	Pernicious anemia, untreated	1.8	22	34	0.21	0.071
35	Systemic lupus erythematosus with autoimmune hemolytic anemia	45.0	27	35	0.23	0.081

TABLE VI. RBC Free Globin Level in Cord Blood.

Subject	% of Hb		Ratio, F/A (%)	
	Free globin F	Free globin A	Hemoglobin	Free globin
36	0.13	0.12	84/16	85/15
37	0.25	0.22	79/21	81/19

globin synthesis (20–22) the decreased amount of free globin found in homozygous thalassemia was expected. In the heterozygous thalassemic states free globin was normal when expressed as percentage of hemoglobin A, but was decreased when expressed as free globin per red cell. One might expect a variable reduction in free globin levels in proportion to the degree of suppression of globin synthesis produced by the thalassemia gene.

Studies of hemoglobin synthesis in sickle cell anemia and sickle cell trait have shown that hemoglobin A is synthesized more rapidly than hemoglobin S (23, 24). The low free globin S level in the hemoglobin SS state and the similar ratios of free globin S to free globin A and hemoglobin S to hemoglobin A in the SA states parallel the decrease in hemoglobin S synthesis. The similar ratio of hemoglobin A to S and A to C in native hemolysates versus the ratio after hemin–globin recombination indicates that the mutant globins have the same affinity as normal globin for hemin and hence supports the conclusion that the differences in the amount of the two free globins is due to a variation in the synthetic rate.

The finding of low free globin levels in iron deficiency was not entirely expected. With the deficiency of heme which results from the lack of iron, it might be anticipated that free globin or globin not completely saturated with heme would be increased. Possible explanations for the low globin levels observed include the following:

1. In iron deficiency, free protoporphyrin in the red cells is increased (25). It is possible that some of the protoporphyrin binds to accumulated free globin, thereby decreasing the number of available heme binding sites. Preparations of globin can be shown to bind protoporphyrin to form protoglobin (26).

2. Heme deficiency might lead to premature activation of reticulocyte ribonuclease which would result in earlier breakdown of ribosomes and cessation of globin synthesis. Hemin has been shown to be an inhibitor of reticulocyte ribonuclease (27).

3. Heme may act in some other unknown way at a very early step in globin chain synthesis, such that a deficiency of heme would rapidly curtail further chain synthesis.

4. A deficiency of heme could have the effect of decreasing the amount of tetramers partially saturated with heme and increasing the amount of $\alpha\beta$ dimer apoprotein. The latter compound, known to be relatively unstable (11), could become denatured in the iron-deficient cell and therefore not bind hemin upon lysis of the cells in a solution containing hemin. Against this last possibility is the observation that reticulocytes and mature erythrocytes contain similar amounts of free globin (2), indicating stability of free globin in the red cell.

In sideroblastic anemias where there is also a deficiency of heme secondary to a probable defect in porphyrin synthesis, one would anticipate the red cell free globin to be altered in amount in the same direction as in iron deficiency. Only one case of chronic idiopathic sideroblastic anemia was studied here and the fact that the free globin level was not below normal may be because only some 20% of the red cells were hypochromic and hence affected by the block in heme synthesis. The majority of the cells, being normochromic, were presumably unaffected and hence might have a normal amount of free globin. The normal free globin levels found in the various other types of anemia were anticipated, since in these states the primary defect is not one of defective heme or globin synthesis.

Summary. The erythrocyte free globin level was determined in a variety of anemic states. Low free globin levels were found in homozygous β-thalassemia, sickle cell disease and iron-deficiency anemia, even though in these conditions hemoglobin synthesis is de-

creased for different reasons. Normal amounts of free globin were observed in various other anemias not characterized by a primary defect in hemoglobin synthesis. Therefore, a remarkable degree of coordination exists between globin synthesis and either iron supply or protoporphyrin production. Because of this it is not possible to obtain information about the nature of a defect in hemoglobin synthesis by the amount of free globin present.

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Received Nov. 5, 1969. P.S.E.B.M., 1970, Vol. 134.