Globin Synthesis in Fractionated Normoblasts

of β-Thalassemia Heterozygotes

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ABSTRACT Globin chain synthesis was examined in erythroid cells of increasing maturity, fractionated from the whole bone marrow of β-thalassemia heterozygotes by a density gradient centrifugation procedure. In experiments using total cell "globin," a gradient of α/β chain ratios was observed, increasing with erythroid cell maturation from unity in the basophilic cells up to 2.0 in reticulocytes. Gel filtration of the lysates from these marrow fractions revealed the presence of free α chains even in the most immature cells, the amount of which increased with erythroid cell age; the total α/β ratio derived from gel filtration experiments showed a gradient similar to that observed in the total globin experiments. However, the α/β ratio of the hemoglobin fraction obtained by gel filtration remained constant throughout maturation at an average of 0.65. This latter finding is incompatible with balanced synthesis at any stage of red cell development and excludes the possibility that total β chain production is higher in the early cells than in the later cells or that α chain production in the early cells is reduced to the level of β chain synthesis. Furthermore, in a Hb S/β-thalassemia marrow examined, the β^A/β^S ratio remained constant throughout maturation while the $\alpha/\text{non-}\alpha$ ratio showed an increase like that observed in the simple β-thalassemia heterozygotes. This argues strongly against increased synthesis from either the thalassemic or nonthalassemic β chain gene being responsible for the balanced synthesis in the immature cells. These findings lead us to suggest that, in β-thalassemia heterozygotes, a large α chain pool is present throughout erythroid cell maturation and that the observed increase in α/β ratios is a function of the ability of those cells to degrade the excess α chains.

INTRODUCTION

An increasing body of evidence has been presented recently that suggests that the molecular defect in at least some forms of β-thalassemia is a deficiency of translatable β messenger (m) RNA (1–8). This deficiency has been demonstrated in cell-free assays of mRNA from both bone marrow and reticulocytes obtained from patients either homozygous or heterozygous for β-thalassemia. However, several studies on intact cells have confirmed the findings of Schwartz (9), that α and β chain synthesis of β-thalassemia heterozygotes appears to be more balanced in their bone marrow than in the peripheral blood. This difference in globin chain synthetic ratios between bone marrow and blood has been attributed to one or more of the following: (a) instability of β mRNA from the thalassemia chromosome (9); (b) higher synthesis of β chains in the earlier cells due to compensation from the normal β^A gene (10, 11); (c) reduction of α chain synthesis in the more immature cells (10, 11); (d) contamination of the β chain by a nonglobin protein (12); and (e) removal of the α chain excess in the more immature cells, probably through the action of proteolytic enzymes (12).

An understanding of the mechanism responsible for this pattern of globin synthesis is of importance not only to understand the pathophysiology of β-thalassemia but also to elucidate the control mechanisms of protein synthesis. Therefore, we examined globin chain synthesis in whole bone marrow and peripheral blood, as well as in populations of marrow erythroid cells fractionated according to their degree of maturity. Carboxymethyl (CM)[1] cellulose chromatography of the ^{3}H-labeled whole cell "globin" and gel filtration experiments of the fractionated marrow lysates allowed the determination of the total chain synthetic ratios and measurements of the *detectable* free α chain pool. From determination of α/β chain ratios in the hemoglobin peak obtained from gel filtration experiments, the size of the *total* free α chain pool was inferred.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CM, carboxymethyl.

Patients	Age	Sex	Diagnosis	Hemoglobin	Packed cell volume	Red blood cells	MCV	MCH	MCHC	Hb A$_2$	Hb F	Hb S
				$g/100\ ml$	$\%$	$\times 10^6/mm^3$	μm^3	pg	$\%$	$\%$	$\%$	$\%$
J. B.	25	M	Normal	—	—	—	—	—	—	1.85	0.85	—
W. S.	26	M	Normal	—	—	—	—	—	—	1.95	1.45	—
J. C.	44	M	β-thal heterozygote	12.8	38.0	5.72	67	22.3	33.6	5.50	1.73	—
D. M.	23	M	β-thal heterozygote	13.3	40.5	6.79	59	19.7	32.7	5.13	0.83	—
S. M.	45	M	β-thal heterozygote	12.5	37.3	5.52	68	22.7	33.9	4.16	1.50	—
H. T.	50	M	β-thal heterozygote	11.7	35.0	5.56	63	21.0	33.4	5.65	1.58	—
J. W.	22	M	HbS/β-thal	12.4	37.6	5.24	71	23.7	33.1	5.05	6.25	62.3

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

The results of these experiments indicate that the proportion of β chain synthesis from the β-thalassemia gene is constant throughout maturation and that the size of the total α chain pool also remains constant. However, since the amount of detectable free α chains is much less in the immature cells than in the mature, we suggest that removal of the excess chains from the early cells by proteolytic digestion is the most likely explanation of the observed increase in α/β ratios with erythroid cell maturation.

METHODS

Marrow samples. Bone marrow samples, aspirated from the iliac crest, were obtained from two clinically normal
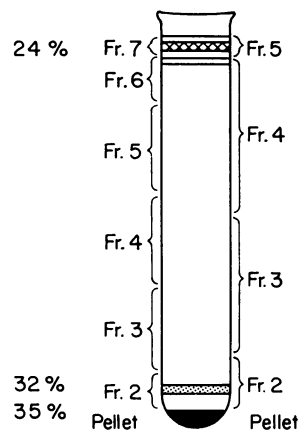

FIGURE 1 The appearance of the BSA gradient after centrifugation, showing on the right the fractions collected in the majority of the samples and on the left those collected in the sample from patient H. T.

volunteers (Caucasian), four patients with heterozygous β-thalassemia of Mediterranean origin, and one black patient with HbS/β-thalassemia. The marrow samples were collected in acid citrate dextrose. Peripheral blood samples, collected in EDTA, were obtained from all the above patients. Hematological data documenting the diagnoses are given in Table I.

Experimental approach. The general outline of the experiments described here is as follows. Blood and bone marrow samples were incubated with [^{3}H]leucine for 1 h, after which the washed bone marrow cells were applied to a density gradient of bovine serum albumin (BSA). After centrifugation for 40 min, the gradient was fractionated and samples were removed for the preparation of slides. The remainder of each fraction was lysed by freezing and thawing and either converted to globin for measurement of the total α/β ratios on CM-cellulose columns or centrifuged for the removal of the stroma before gel filtration on Sephadex G-75. Hemoglobin and "α chain" fractions obtained from the gel filtration columns were also converted to globin, and the incorporated radioactivity was measured.

Cell incubations. Bone marrow (0.5–0.8 ml packed cells) and peripheral blood samples were washed three times in NKM saline (NaCl 0.13 M, KCl 0.005 M, MgCl$_2 \cdot 6$ H$_2$O 0.0074 M) and preincubated in 1 ml of the medium of Lingrel and Borsook (13) at 37°C. The reticulocyte proportion in the blood samples was increased by centrifuging the cells at 23,000 g for 45 min and the top 1 ml of cells was used for the incubation. After 15 min, 50 μCi [^{3}H]-leucine (54 Ci/mmol, Amersham/Searle Corp., Arlington Heights, Ill.) was added, and the incubation was continued for exactly 60 min. Hemoglobin synthesis was stopped at the end of the incubation by the addition of ice-cold NKM saline, and the cells were washed three times. Approximately one-fifth of the bone marrow sample was removed for measurement of synthesis in the whole marrow and kept with the incubated blood sample at 4°C until conversion to globin at the same time as the remainder of the marrow cells.

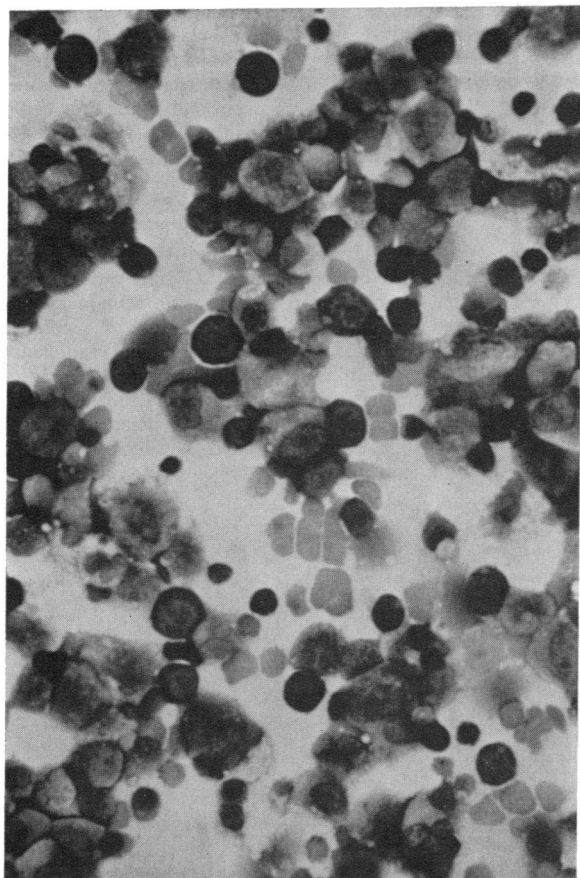*Cell separation on BSA gradients.* Density gradients were prepared from sterile, 35% BSA containing 0.1%

FIGURE 2 The appearance of the cells in J. S., fraction 5, after separation of the bone marrow by density gradient centrifugation, demonstrating the high proportion of basophilic normoblasts.

Differential cell counts. On each cell fraction obtained from the gradient, the erythroid proportion of the nucleated cells was calculated, and a differential count of the erythroid cells was performed. At least 2,000 cells were counted per fraction. The erythroid cells were subdivided in order of increasing maturity into pronormoblasts, and basophilic, polychromatophilic, and orthochromatic normoblasts according to standard criteria. The polychromatophilic compartment was further subdivided into "polychromatophilic normoblasts I" and "polychromatophilic normoblasts II", by the degree of nuclear condensation and the amount of cytoplasmic hemoglobinization; polychromatophilic normoblasts I were the younger cells. This was necessary to tabulate the clearly observed differences between fractions consisting largely of "early" and "late" polychromatophilic normoblasts.

Separation of globin chains on CM-cellulose. For studies of total chain synthesis, cells were lysed by freezing and thawing three times after the addition of carrier cells from the unincubated blood sample of the same individual. "Globin" was precipitated from the lysate plus membranes with acid acetone, and the chains were separated on CM-cellulose columns (14). The radioactive profile of each separation was monitored in Bray's solution (15) in a liquid scintilla-

sodium azide (Sigma Chemical Corp., St. Louis, Mo.). The albumin was diluted with NKM saline to 24% and 32% solutions, 7.5 ml of each being added to each side of a gradient maker (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N. J.). The gradient was poured from the 32% side of the gradient maker and allowed to flow under gravity at 10 ml/h down the side of a cellulose nitrate tube containing a cushion of 1.5 ml 35% BSA. The incubated marrow cells (0.4–0.7 ml of packed cells in NKM saline to a total volume of 1 ml) were layered onto the top of the gradient and accelerated gradually up to 20,000 *g* in a swing bucket rotor (Sorvall HB-4, Ivan Sorvall, Inc., Newtown, Conn.). Centrifugation was carried out at 5°C for 40 min.

The separated cells were collected in drops after piercing the bottom of the tube with a needle that passed through the pellet of red cells. The first few drops, containing the pellet cells displaced by the needle's entry, were discarded, and unequal fractions were collected as shown in Fig. 1. Careful collection of cells by aspiration from the top with a Pasteur pipette was equally satisfactory. The albumin was removed from the fractions by washing three times with saline, after which a small sample was obtained from each fraction for smears.
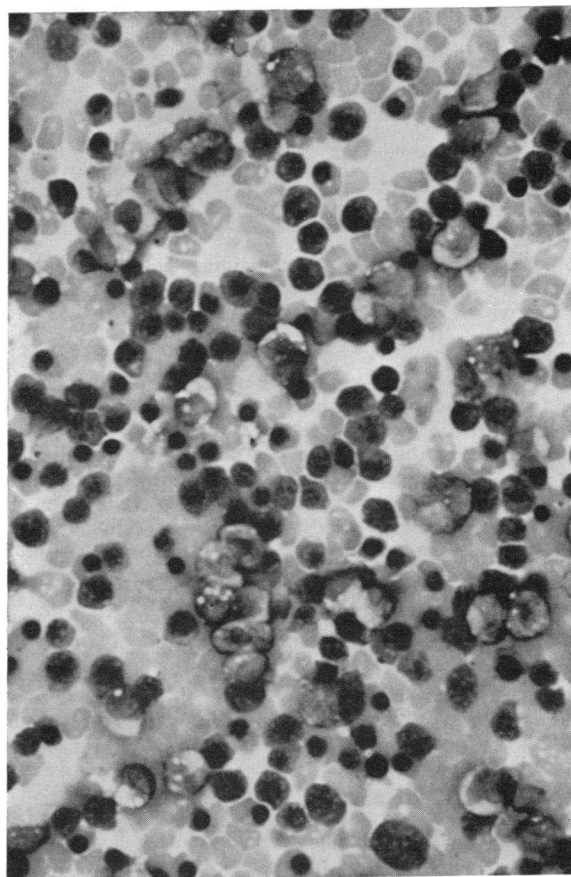

FIGURE 3 J. S., fraction 4, consisting largely of polychromatophilic normoblasts, the majority of which are of the younger "polychromatophilic I" type.
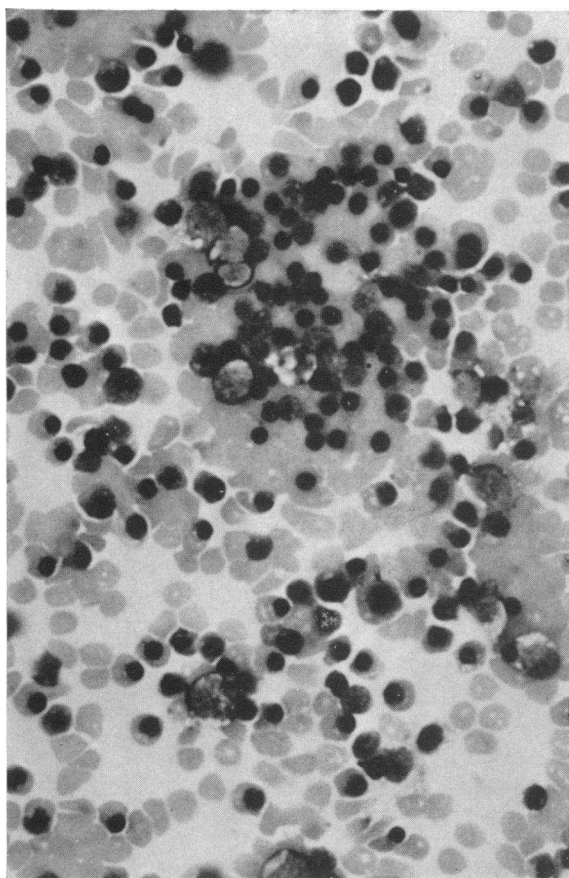
FIGURE 4 J. S., fraction 2, containing polychromatophilic normoblasts of the older "polychromatophilic II" type and orthochromatic normoblasts.

free α chains, therefore, the Sephadex G-75 α chain radioactivity was multiplied by the proportion of that radioactivity that migrated with the α chains on CM-cellulose. The Sephadex G-75 total α/β ratios were then obtained from the total radioactivity incorporated into the hemoglobin peak, the hemoglobin α/β ratio, and the corrected value for the radioactivity incorporated into free α chains (12).

Gel filtration of the isolated β chains. The CM-cellulose-separated β chains were dialyzed against 0.5% formic acid to remove the urea, freeze-dried, and dissolved in 5% formic acid. One-fifth of the sample was removed for measurement of the specific activity and the remainder was chromatographed on a 100×2-cm column of Sephadex G-100 in 5% formic acid. Optical density and radioactive profiles of the column eluate were prepared, and the globin peak was pooled and freeze-dried. After it was redissolved in a small volume of 5% formic acid, the specific activity of the globin was calculated and compared with the same sample before gel filtration.

RESULTS

Erythroid cell separation. The appearance of the gradient after centrifugation is illustrated in Fig. 1. Basophilic cells are restricted to the band at the top of

tion counter, and the α/β ratios were calculated from the total counts incorporated into each peak.

Gel filtration of fractionated cell lysates on Sephadex G-75. To determine the size of the α chain pool in the different cell fractions, several samples were subjected to gel filtration on Sephadex G-75 columns. Carrier cells from the unincubated peripheral blood of the same individual were added to the marrow cell fractions and, after lysis by freezing and thawing, membranes were removed by centrifugation at 40,000 g for 60 min. Separation of the free chains from the hemoglobin was carried out in 0.05 M Tris-HCl, pH 7.4 (12, 16). Liquid scintillation counting of the fractions followed a modification of the method of Mahin and Lofberg (17). To 0.2 ml of each fraction was added 0.2 ml 60% perchloric acid and 0.4 ml hydrogen peroxide. The capped vials were heated at 70°C for 60 min before 15 ml of scintillant was added. This consisted of 4 vol toluene containing 6 g 2,5 diphenyloxazole (PPO)/ liter to 3 vol 2-ethoxyethanol.

After the addition of approximately 40 mg hemoglobin A to the Sephadex G-75 α chain peak, conversion to globin, and separation of the chains on CM-cellulose, it was observed that not all of the radioactivity migrated with the α chains, particularly in samples from the immature cell fractions. To obtain a more reliable estimate of the amount of
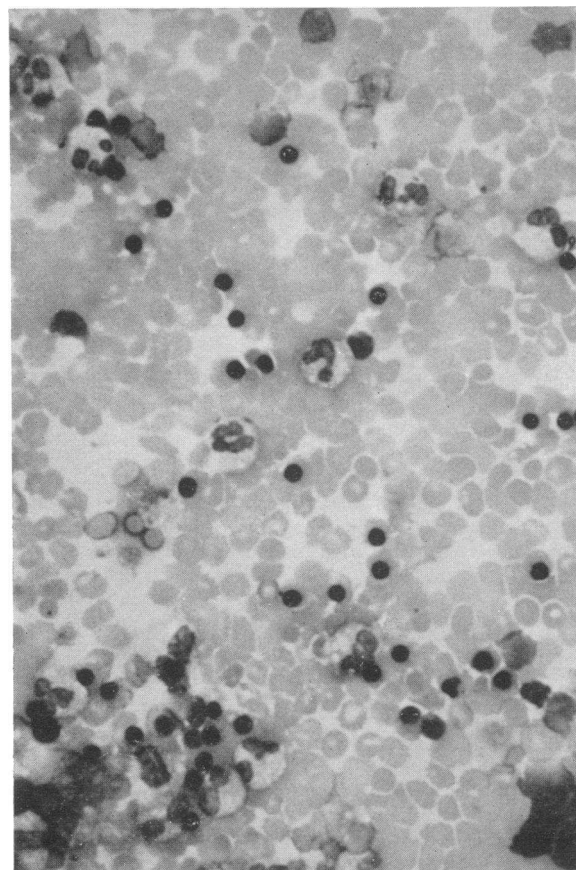

FIGURE 5 J. S., pellet fraction. The nucleated erythroid cells are almost entirely orthochromatic normoblasts.

TABLE II

Differential Erythroid Cell Counts and Globin Synthesis Ratios in the Fractionated Bone Marrows of Two Normal Individuals

| Patient | Fraction* | Percentage erythroid‡ | Normoblasts§ | | | | | Sephadex G-75 chromatography | | CM-cellulose α/β ratio** |
			Pro-normo-blasts	Baso-philic	Poly-chromato-philic I	Poly-chromato-philic II	Ortho-chromatic	Hb α/β ratio‖	Total α/β ratio¶	
J. B.	5	8	18	36	34	11	2			0.77
	4	22		7	26	60	7			0.89
	3	60		3	21	66	13			0.84
	2	60			6	65	29			1.01
	Pellet	12			4	22	74			0.99
	Blood									1.17
	Whole marrow	22	3	8	14	47	28			1.00
W. S.	6	5	21	38	23	12	6	0.86	0.88	
	5	9	3	39	46	37	4			0.86
	4	16		4	49	42	5	0.93	0.98	
	3	17		1	24	70	5			0.99
	2	10			2	82	18	0.79	0.89	
	Pellet	11			1	19	80			1.05
	Blood									0.91
	Whole marrow	22	2	5	17	50	27	0.67	0.79	

* Defined in text and Fig. 1.
‡ Nucleated erythroid cells as a percentage of the total nucleated cells.
§ Expressed as the percentage of the total nucleated erythroid cells.
‖ Ratio of α to β chain radioactivity in the hemoglobin fraction obtained after Sephadex G-75 chromatography.
¶ Calculated from total counts incorporated into the hemoglobin and α chain peaks obtained after Sephadex G-75 chromatography and from α/β ratio of the Sephadex G-75 hemoglobin fraction.
** α to β chain radioactivity calculated after chromatography of whole cell globin on CM-cellulose.

the gradient, where they constitute from 48 to 69% of the erythroid cells (Fig. 2). Some of the early poly-chromatophilic cells (polychromatophilic I in Tables II–V) are also found at the top of the gradient, while orthochromatic cells are rare, their presence probably explained by entrapment by or adhesion to other cells. When large samples were separated, two faint bands could be discerned close to the top of the gradient, but no homogeneous cell type could be ascribed to either of them. The majority of the polychromatophilic cells are dispersed throughout the bulk of the gradient, and fractions in which the erythroid content was 77–95% poly-chromatophilic were routinely observed (Figs. 3 and 4). Orthochromatic cells largely passed right through

TABLE III

Differential Erythroid Cell Counts and Globin Synthesis Ratios in the Fractionated Bone Marrows of Two β-Thalassemia Heterozygotes

| Patient | Fraction* | Percentage erythroid‡ | Normoblasts§ | | | | | CM-cellulose α/β ratio** |
			Pro-normo-blasts	Baso-philic	Poly-chromato-philic I	Poly-chromato-philic II	Ortho-chromatic	
J. C.	5	10	20	49	22	9	—	1.14
	2 + 3 + 4	29		2	12	55	30	1.62
	Pellet	24			7	41	52	1.82
	Blood							1.95
	Whole marrow	31	2	7	26	53	12	1.67
D. M.	5	24	19	43	27	9	2	0.94
	3 + 4	24		2	60	35	3	1.32
	2	53		1	25	68	7	1.35
	Pellet	13			3	26	71	1.55
	Blood							1.85
	Whole marrow	33	3	6	30	51	10	1.21

*, ‡, §, and ** as in Table II.
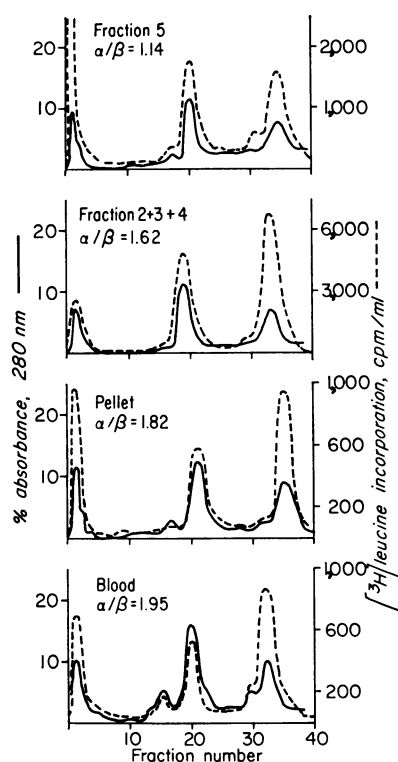
FIGURE 6 Chain separations of the whole cell globin of four marrow fractions from the β-thalassemia heterozygote, J. C., demonstrating the increasing disparity of the α/β ratio with erythroid cell maturation.

the gradient to be collected in the pellet of red cells found in the cushion of 35% albumin (Fig. 5).

Differential erythroid cell counts after centrifugation are listed in Table II for the normal controls and Tables
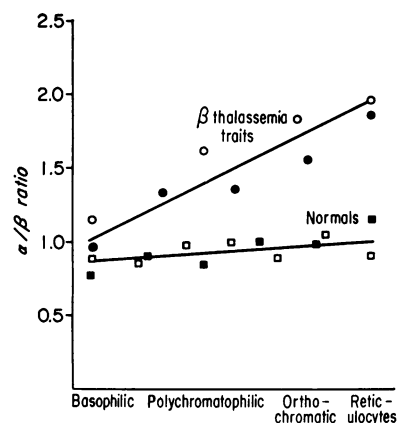

FIGURE 7 Diagrammatic representation of the α/β ratios during erythroid cell maturation in two normals (■——■ J. B., □——□ W. S.) and two β-thalassemia heterozygotes (●——● D. M., O——O J. C.).
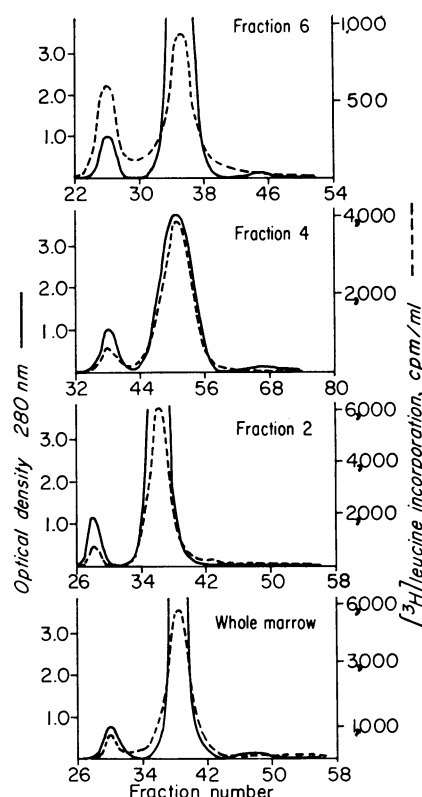
FIGURE 8 Gel filtration of the fractionated marrow lysates from a normal individual, W. S., showing the virtual absence of a free α chain peak in any of the fractions.

III–V for the β-thalassemia heterozygotes. In the initial experiments (D. M. and J. C., Table III) fewer fractions were collected, and the intermediate fractions were pooled. However, it became clear that radioactive incorporation into each fraction was high enough to allow the collection of more fractions, each containing fewer cells.

This simple technique gave reliable results on routine marrow aspirates with varying degrees of erythrocyte contamination. In general it was noted that the lower the peripheral blood contamination and the higher the erythroid content of the marrow, the better the separation. Since the albumin gradient can be poured during the cell incubation, the total time involved, from receiving the sample to obtaining washed, fractionated cells, was approximately 3 h.

Patterns of globin chain synthesis during erythroid cell maturation. The α/β chain ratios obtained by CM-cellulose separation of the whole cell globin are listed alongside the differential erythroid cell counts in Tables II and III.

In the two normal samples (Table II), the α/β ratios in the fractions containing the more mature cells are
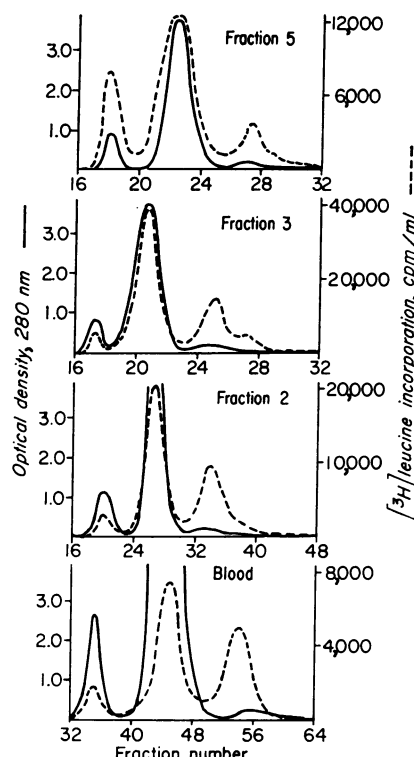
FIGURE 9 Gel filtration of the fractionated marrow lysates from a β-thalassemia heterozygote, S. M., showing an increase in the size of the detectable free α chain peak with erythroid cell maturation.

very close to 1.0, but in the less mature cells the ratio is somewhat lower than this, and the ratio appears to increase with increasing maturity of the erythroid cells.

In the β-thalassemia heterozygotes (Table III, Fig. 6) there is clear evidence of a "gradient" of α/β ratios, showing good correlation with the increasing maturity of the nucleated erythroid cell content of each fraction. Thus, the α/β chain ratio approaches unity in the fraction consisting mostly of basophilic cells, increasing to almost 2.0 in peripheral blood reticulocytes.

A comparison of the results from the normal and β-thalassemia patterns is presented diagrammatically in Fig. 7. These data, in contrast to previous observations, suggest that α/β chain synthesis in the bone marrow of β-thalassemia heterozygotes is "balanced" only in the very early erythroid cells, but do not shed light on the mechanism responsible for this pattern. Further investigation was carried out to determine whether an α chain pool was detectable in any of these fractions.

The size of the α chain pool during erythroid maturation. Stroma-free lysates from the various marrow cell fractions obtained from one normal individual (W. S., Table II) and two patients with thalassemia traits (Table IV) were subjected to gel filtration

on Sephadex G-75. Virtually no free α chains could be detected in any of the fractions from the normal individual (Fig. 8). In the thalassemic samples, however, a peak of free α chains after the hemoglobin peak could be detected in all fractions (Fig. 9), including the immature fraction, where the total α/β chain synthesis appeared completely balanced. The small OD²⁸⁰ peak, observed behind the hemoglobin peak, is probably a nonglobin protein and is not produced by the free α chains, which must be extremely small in amount relative to the carrier hemoglobin added to each fraction (18). In none of these gel filtrations did the OD²⁸⁰ peak coincide with the α chain radioactivity, but eluted one or two tubes later.

Addition of carrier hemoglobin to the α chain peak and separation of the globin chains demonstrated that the incorporated radioactivity was found in α chains but not in β chains (Fig. 10). However, not all the radioactivity eluted with the α chains on these columns and in samples from the immature cell fractions up to 40% of the radioactivity eluted in the breakthrough peak. This decreased to less than 5% in the pellet fraction and peripheral blood samples.

The ratio of radioactivity in the hemoglobin peak to that in the free α chains decreased with increasing red cell maturity, indicating a large pool of free α chains in the more mature cells at the end of the incubation and separation procedures.

The Sephadex G-75 hemoglobin peak was converted to globin, and the chains were separated to calculate the α/β ratio in the purified hemoglobin and the total α/β ratio. If, in β-thalassemia heterozygotes, the α chain
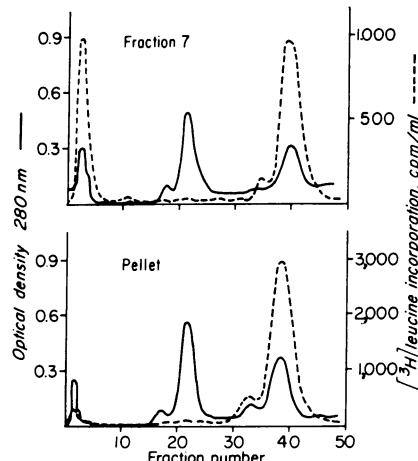

FIGURE 10 Chain separation of the Sephadex G-75 low mol wt peak from fraction 7 and the pellet of H. T. after the addition of carrier hemoglobin and conversion to globin. Note the absence of any radioactivity in the β chain and the difference in the proportion of radioactivity in the breakthrough peaks between the two samples.

Table IV

Differential Erythroid Cell Counts and Results of Gel Filtration Experiments on the Fractionated Bone Marrows of Two β-Thalassemia Heterozygotes

Patient	Fraction*	Percentage erythroid‡	Normoblasts§ Pro-normo-blasts	Baso-philic	Poly-chromato-philic I	Poly-chromato-philic II	Ortho-chromatic	Sephadex G-75 chromatography Hb/free α chain‡‡	Hb α/β ratio‖	Total α/β ratio¶	CM-cellulose α/β ratio**
S. M.	5	16	13	35	32	18	2	4.75	0.66	1.01	
	4	22		6	54	37	2	3.41	—	—	
	3	46		1	18	73	9	2.76	0.67	1.27	
	2	34		1	4	22	74	1.92	0.69	1.57	
	Pellet	24				17	83	—	—		1.87
	Blood							1.34	0.58	1.76	2.02
	Whole marrow	38	8	6	21	40	24	—	—	—	1.13
H. T.	7	12	11	31	34	19	5	4.15	0.55	0.92	
	6	31	3	14	60	21	2	—	—	—	—
	5	50		8	62	29	1	2.61	0.73	1.39	
	4	43		1	46	45	8	—	—	—	1.55
	3	44			9	80	11	1.98	0.60	1.41	
	2	11			9	66	25	—	—	—	1.77
	Pellet	15				27	73	1.57	0.68	1.75	1.86
	Blood							—	—	—	2.01
	Whole marrow	39	1	9	26	50	14	—	—	—	1.60

*, ‡, ‖, ¶, and ** same as in Table II.
‡‡ Calculated after the proportion of the Sephadex G-75 α chain radioactivity migrating with α chains on CM-cellulose had been measured.

pool increases in size with red cell maturity, as suggested by the hemoglobin/α chain ratios, then the α/β ratio of the Sephadex G-75 hemoglobin fractions should show a concomitant decrease, while the Sephadex G-75 total α/β ratio should increase in the same way as the whole cell α/β ratios. However, the results in Table IV show clearly that while the total α/β ratios obtained by gel filtration increase from unity in the immature cells to 2.0 in the reticulocytes, no decrease was observed in the α/β ratio of the purified hemoglobin fractions as the
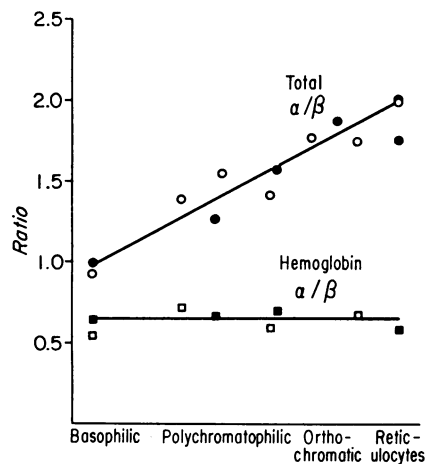

Figure 11 Diagrammatic representation of the total α/β ratios and the α/β ratios in the hemoglobin fraction after gel filtration of the fractionated marrow lysates of two β-thalassemic heterozygotes. ●——●, ■——■, S. M.; ○——○, □——□, H. T.

erythroid cells matured (Fig. 11). This suggests that there is a relatively large α chain pool in all fractions, and the closeness of the hemoglobin α/β ratios in each fraction indicates that the real size of the pool is similar throughout maturation.

Evidence for the continued synthesis of β^A from the β-thalassemia gene during erythroid cell maturation. To determine if the "balanced" chain ratios observed in the immature cells could be due to higher synthesis of β chains from either the thalassemic gene or the nonthalassemic β gene, the marrow fractionation procedure was also applied to a sample from a patient with HbS/β thalassemia. For the increase in α/β ratios with red cell maturity observed in the β-thalassemia heterozygotes to be due to instability of the β^A mRNA from the thalassemic chromosome would require a concomitant *decrease* in the β^A/β^S ratio. Similarly, greater compensation from the β^S gene in the early cells than in the late cells would produce an *increase* in the β^A/β^S ratio with maturation.

Table V lists the α/non-α and β^A/β^S ratios in the fractionated marrow of a hemoglobin S/β-thalassemia double heterozygote. As in the β-thalassemia heterozygotes, the ratio of α/non-α chains increases with maturation from unity in the youngest fraction to over two in reticulocytes (Fig. 12). The β^A/β^S ratios show clearly that there is no increase or decrease in the ratio, which remains *constant* throughout maturation (Fig. 13). This experiment provides evidence neither for an unstable β^A mRNA, at least in this subject, nor for differentially

TABLE V

Differential Erythroid Cell Counts and Globin Synthesis Ratios in the Fractionated
Bone Marrow of a HbS/β-Thalassemia Heterozygote (J. W.)

Fraction*	Percentage erythroid‡	Normoblasts§					CM-cellulose chromatography	
		Pro-normo-blasts	Baso-philic	Poly-chromato-philic I	Poly-chromato-philic II	Ortho-chromatic	β^A/β^S ratio	$\alpha/\gamma + \beta^A + \beta^S$ ratio**
5	21	16	33	33	16	4	0.42	0.97
4	28	1	15	53	24	7	0.48	1.32
3	62		2	59	35	3	0.43	1.43
2	91		1	8	80	12	0.38	1.60
Pellet	37			3	31	66	0.45	2.02
Blood							0.46	2.37
Whole marrow	53	1	8	20	54	16	0.55	1.13

*, ‡, §, ** Same as in Table II.

increased synthesis from either of the β chain genes in the immature cells.

Evidence against the possible contamination of the β chain by a nonglobin protein. The results in Tables III and IV demonstrate that whether the total α/β ratio in the fractionated marrow of β-thalassemia heterozygotes

is determined directly from the whole cell globin or after gel filtration, the results are virtually identical. Thus if the more balanced ratios observed in the immature fractions were the result of a nonglobin protein contaminating the β chain, the contaminant must also chromatograph with the hemoglobin fraction during gel filtration on Sephadex G-75. To examine this possibility, β chains, obtained from the earliest cell fractions of S. M. and H. T., after gel filtration on Sephadex G-75 and separation of the hemoglobin fraction on CM-cellulose, were subjected to a third chromatography by passage through a column of Sephadex G-100, in 5% formic acid. Any protein with the same molecular weight as hemoglobin should be well separated from the free β globin chains by this procedure. The optical density and radioactive profiles from this separation (Fig. 14) demonstrate that virtually all the radioactivity eluates with
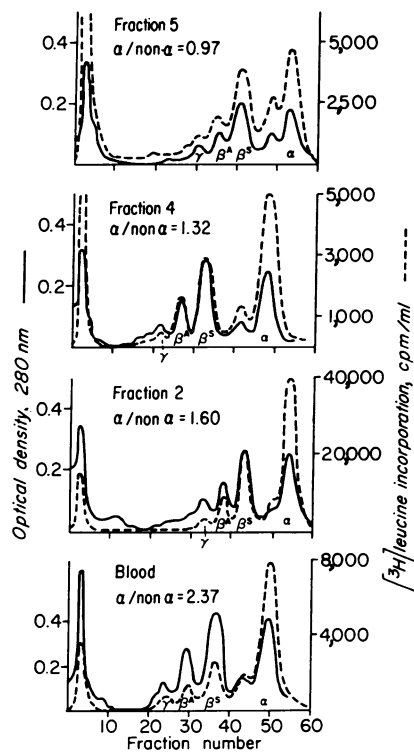

FIGURE 12 Chain separations of the whole cell globin in the fractionated marrow of a HbS/β-thalassemia patient, J. W., showing an increase in the α/non-α ratio similar to that observed in the β-thalassemia heterozygotes.
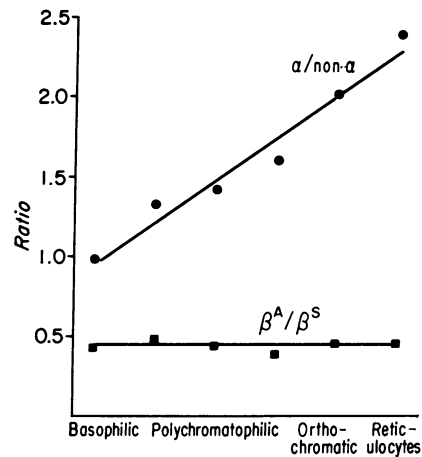

FIGURE 13 Diagrammatic representation of the α/non-α and β^A/β^S ratios during erythroid cell maturation in a HbS/β-thalassemia double heterozygote.
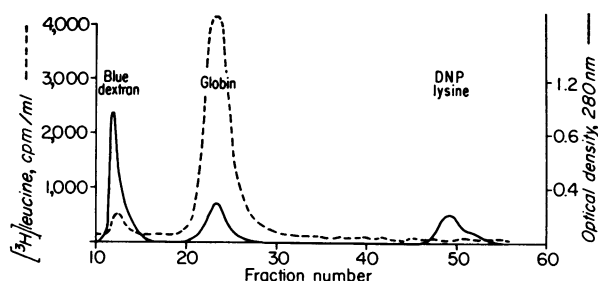
Figure 14 Optical density and radioactive profile of the Sephadex G-100 chromatography of β chains obtained from patient H. T., fraction 7, Sephadex G-75 hemoglobin peak.

the globin, while Table VI shows that the specific activities of these β chains were not diminished by passage through such a column.

DISCUSSION

Globin synthesis studies in the reticulocytes of β-thalassemia heterozygotes have shown conclusively that this disorder is characterized by deficient β chain synthesis. Thus, in the majority of β-thalassemia heterozygotes, the synthesis of α chains is twice that of the β chains. Similar studies in bone marrow samples from the same patients, however, have shown balanced α/β chain synthesis (4, 7, 9, 10, 19) or less imbalance than is observed in the peripheral blood (12). The data presented in this paper confirm that the excess α chain production in the bone marrow appears less than that in the peripheral blood but, by density gradient separation of the erythroid cell population, it has been possible to examine this situation in greater detail. Thus, the α/β ratios obtained from the whole cell chain separations on CM-cellulose appear balanced only in the cell cohorts consisting largely of pronormoblasts and basophilic normoblasts. With increasing erythroid cell maturity, increasing imbalance in the ratios is observed until in the reticulocyte an α/β ratio of 2.0 is achieved.

There are several possible mechanisms that could be responsible for the apparently balanced synthesis in the early erythroid cell precursors and the gradual increase in the imbalance observed with maturation. The observed pattern might be an artifact, due to contamination of the β chain by a radioactive, nonglobin protein (12). Or, it could be due to higher β chain synthesis in early cells, in cis and/or trans to the β-thalassemia determinant (10, 11). Similarly, a decrease in the amount of available α chains in the earlier cells, due either to decreased synthesis (10, 11) or increased destruction (12), could produce this effect. The experiments described here provide information on all of these points.

Two lines of evidence suggests that contamination of the β chains of the early cells by a nonglobin protein,

contamination that decreases with maturation, is not a major cause of the observed differences in the α/β ratios of immature and mature erythroid cells. First, the constancy of the β^A/β^S ratio in the fractionated marrow and the peripheral blood of the HbS/β-thalassemia heterozygote is inconsistent with such contamination, unless both chains were equally contaminated. The mean β^A/β^S ratio of these fractions was 0.45, which compares well with the HbA/HbS ratio of 0.42 measured by column chromatography of the hemolysate. Second, for the total α/β ratios from both the whole cell chain separations on CM-cellulose and those obtained after gel filtration on Sephadex G-75 to correspond so closely would require that any β chain contaminant also chromatograph with the hemoglobin fraction on Sephadex G-75. That the specific activity of the β chains obtained from this fraction was not diminished by passage through a column of Sephadex G-100 (Table VI) is evidence against such a contaminant. The possibility that the contaminating protein is also a tetramer that dissociates into monomers of 16,000 mol wt, which then migrate with the β chain on CM-cellulose, still cannot explain the constancy of the β^A/β^S ratio.

The second possible explanation of balanced ratios in the marrows of β-thalassemia heterozygotes, that of increased synthesis from either or both of the β chain genes in the immature cells, can be examined in the situation where the β-thalassemia gene is combined with a β chain structural mutation in trans, such that the products of the two β chain genes can be differentiated.

Examination of globin synthesis in the HbS/β-thalassemia heterozygote provides direct evidence as to the relative synthesis directed by the thalassemic and nonthalassemic β chain genes. Production of an unstable β mRNA by the β-thalassemia gene would result in a

TABLE VI
The Effect of Gel Filtration through Sephadex G-100 on the Specific Activities of the β Chains Obtained from the Most Immature Cell Fractions of β-Thalassemia Heterozygotes S. M. and H. T.

Patient	Fraction	Counts in Sephadex G-100 globin peak	Specific activity	
			Before G-100	After G-100
		%	cpm/mg	cpm/mg
S. M.	Fraction 5 G-75 Hb β chain	92	7,880	7,720
H. T.	Fraction 7 G-75 Hb β chain	92	24,478	22,791

higher β^A/β^S ratio in the early cells compared to the more mature ones, while higher compensatory synthesis in the early cells by the nonthalassemic β gene would result in a lower β^A/β^S ratio in those cells than in the later cells. The β^A/β^S ratios from the fractionated marrow of the HbS/β-thalassemia heterozygote, however, remained constant through maturation, even though the α/non-α ratio showed the same increase as in the β-thalassemia heterozygotes. This confirms previous reports that the mRNA produced by the β-thalassemia gene is at least as stable as that from the β^S gene (11, 12, 20) and also demonstrates that although the β gene in *trans* increases its output to compensate for the deficit from the β-thalassemia gene (11), the degree of compensation does not decrease as the erythroid cell matures.

If the observed balanced synthesis in immature erythroid cells of β-thalassemia heterozygotes cannot be accounted for by increased β chain synthesis from either the thalassemic or nonthalassemic genes, then reduced numbers of radioactive α chains at the end of the experimental procedure must be responsible for the observed ratio of 1.0 in the early cells. A reduction in the level of α chain synthesis in the immature cells to match the reduced amount of β chains has been suggested as an explanation for the differing ratios from marrow to blood (10, 11). It is feasible that such a mechanism could exist in the most immature cells by feedback inhibition of α mRNA synthesis, since it is in these cells that hemoglobin and mRNA synthesis overlap. However, if synthesis were truly balanced in the early cells, then no free α chain peak should be observed upon gel filtration, and the α/β ratio of the hemoglobin peak so obtained should be close to 1.0. This ratio should then decline with erythroid maturation as the total α/β ratio increases. The results from the two β-thalassemia heterozygotes described here (S. M. and H. T.), plus two others to be discussed in a separate paper (in preparation) show a constant hemoglobin α/β ratio throughout maturation with a mean of 0.65, indicating that in fact there is a relatively large pool of free α chains, the size of which is also constant throughout maturation.

The difference between the size of the total pool inferred from the constancy of the hemoglobin α/β ratios with maturation and the detectable pool measured by gel filtration can be reconciled by assuming that the imbalance in chain synthesis in β-thalassemia heterozygotes is constant throughout maturation, generating a large α chain pool, even in the early cells. Once the pool is established, the entry of further α chains into the pool is matched by their proteolytic digestion. When protein synthesis is stopped by the addition of ice-cold saline and the washing of the cells, proteolytic activity continues, its effectiveness being greatest in the immature

cells and gradually decreasing as the cells become more fully hemoglobinized. For the α chain pool to remain constant in size during maturation, while proteolysis of the excess α chains declines, the overall rate of globin synthesis must also decline. This supposition is supported by the fact that the α/β ratio in the whole bone marrow tends to be much lower than expected from the α/β ratios of the individual cell fractions.

Evidence for proteolytic activity in erythroid cells, including reticulocytes, has been reviewed and expanded by Hunter and Jackson (21). Degradation of excess α chains has been demonstrated in the peripheral blood of β-thalassemia homozygotes (22, 23), and evidence for the greater destruction of free α chains in the bone marrow of β-thalassemia heterozygotes compared with peripheral blood has been reported by Clegg and Weatherall (12). In direct contradiction to our conclusions, Kan, Nathan, and Lodish (10) demonstrated that in β-thalassemia heterozygotes the α/β ratios of nascent chains was 1.0 in bone marrow and 2.0 in peripheral blood. However, in homozygotes, greater imbalance was detected in nascent chains than in the supernate (24). Further investigation is required to explain this contradiction and direct evidence is needed to confirm the destruction of α chains in the early erythroid cells. Cell separation before a "puromycin" experiment, as performed by Bank and O'Donnell (23) and Clegg and Weatherall (12), might solve this problem, but uncertainty as to the effect of cell fractionation procedures on globin chain synthesis have so far prevented this.

ACKNOWLEDGMENTS

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