Proc. Nat. Acad. Sci. USA
Vol. 72, No. 10, pp. 3853–3857, October 1975
Biochemistry

Imbalanced globin chain synthesis in heterozygous β-thalassemic bone marrow

(β thalassemia/proteolytic degradation/white cell proteins)

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Communicated by H. A. Krebs, July 3, 1975

ABSTRACT **Globin synthesis was studied in the bone marrow of seven heterozygous β-thalassemic subjects. We found evidence of significant imbalance of α- and β-chain production particularly at short times of incubation. There was a progressive decrease in α/β-chain production ratio with increasing incubation time which was due to a decreased rate of net α-chain production, indicating that a large proportion of the newly synthesized α chains are degraded, particularly in bone marrow, within a few minutes of synthesis, leading to relatively low α/β ratios if these are measured solely at incubation times greater than 10 min. The significant degradation of excess α chains explains why inclusion body formation and ineffective erythropoiesis, notable in β-thalassemia homozygotes where there is gross chain imbalance, are not observed to any marked degree in heterozygotes.**

It is now well established that imbalanced globin-chain synthesis in red-cell precursors is the basic cause of the extensive intramedullary red-cell destruction which leads to the ineffective erythropoiesis characteristic of homozygous β thalassemia (1). It follows, therefore, that imbalanced globin-chain production should also occur, albeit to a lesser degree, in heterozygous β thalassemia. However, Schwartz (2, 3) has claimed that although imbalanced α- and β-chain synthesis can be demonstrated in reticulocytes of affected individuals, this is not so in bone marrow. Subsequently a number of other groups have also showed balanced α- and β-chain synthesis in the bone marrow of β-thalassemia heterozygotes (4–8).

To explain these findings it has been proposed (5, 7) that in β-thalassemia heterozygotes there is compensation for defective β-chain synthesis by the β-chain locus on the unaffected chromosome. Alternatively, it has been suggested that there is a compensatory reduction in α-chain synthesis or that possibly both these mechanisms may be operative (9). However, if α- and β-chain synthesis is balanced due to compensating β-chain synthesis in the bone marrow of β-thalassemia heterozygotes (where the bulk of hemoglobin synthesis takes place) it is not clear why red cells should be poorly hemoglobinized or why occasional red cell inclusions (10) or ineffective erythropoiesis (11) should occur. Furthermore, assay of mRNA in cell-free systems clearly shows a deficiency of β-chain mRNA in heterozygous β-thalassemic bone marrow (12). Clegg and Weatherall (13) studied hemoglobin synthesis in β-thalassemia heterozygotes and demonstrated imbalanced globin-chain synthesis in bone marrow, though to a lesser extent than in reticulocytes. They suggested that contamination of β chains by non-heme proteins and a more rapid destruction of α chains in bone marrow than in reticulocytes might explain the findings of apparent balanced globin-chain synthesis in bone marrow.

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This paper describes the results of studies designed to distinguish between these possibilities. We have obtained clear evidence that there is indeed imbalance between α- and β-chain synthesis in bone marrow in this condition. Furthermore, unless suitable precautions are observed, this imbalance can be largely masked under the normal conditions of assay by white-cell proteins which contaminate β chains, and by rapid and extensive proteolytic destruction of excess free α chains.

MATERIALS AND METHODS

Collection and Incubation of Samples. Seven individuals with heterozygous β thalassemia and 12 patients in whom hemoglobin synthesis was normal were studied. All reticulocyte and marrow samples were obtained at diagnostic venepuncture and iliac-crest bone marrow aspiration. Peripheral blood samples were collected into heparin or acid–citrate–dextrose (14), the plasma was removed by centrifugation, and the cells were then washed three times in balanced saline (RS: 0.13 M NaCl, 7.4 mM $MgCl_2$, 5 mM KCl).

Bone-marrow samples were obtained by aspiration and collected into bottles containing 500 units of heparin in saline. Usually samples containing not more than 0.2–0.5 ml of cells were taken since larger aspirates were grossly contaminated with peripheral blood. The cells were then washed three times in RS at 4°, as for reticulocytes.

Incubations were performed as described previously (15) and stopped after 2 hr (unless stated otherwise) by addition of a large volume of ice-cold RS The cells were then washed a further four times in the same solution at 4°. If the volume of incubated cells was very small, approximately 0.1–0.2 ml of unincubated peripheral blood cells from the same patient were then added to provide carrier hemoglobin.

Lysates were prepared by freezing the cells in acetone/dry ice followed by thawing in warm water and repeating the procedure twice. The lysates were then diluted with distilled water (4 volumes/volume of cells for reticulocytes; 10 volumes/volume of cells for marrow) and "whole-cell globin" (15) was prepared by the acid-acetone method. Generous dilution of bone-marrow lysates with water before addition of acid-acetone is important to obtain whole-cell bone-marrow globin which gives satisfactory chromatographic separations. Furthermore, lysates should be converted to globin without delay. If samples have to be stored it is *essential* that they are in the form of globin and not as lysates (see *Discussion*).

Separation of Globin Chains on CM-Cellulose. Method A was that described by Clegg *et al.* (16). Method B was similar but used a modified concave gradient made in a three-chamber apparatus (17) and gave greatly improved

Table 1. Total counts and specific activity α/β ratios in heterozygous β-thalassemic reticulocytes and bone marrow

Case no.	Reticulocytes method A		Bone marrow							
			Method A		Sephadex G-100/method A			Method B		
						Specific activity			Specific activity	
	Total counts	Specific activity	Total counts	Specific activity	Total counts	by A_{280}	by $^3H/^{14}C$	Total counts	by A_{280}	by $^3H/^{14}C$
1	1.79	1.86	1.52	1.47	—	—	—	1.76	1.68	—
2	2.29	2.23	1.54	1.39	1.75	1.80	1.80	1.60	1.46	—
3	2.19	2.10	1.51	1.26	1.67	1.46	1.62	1.69	1.60	1.64
4	—	—	—	—	1.55	1.49	1.55			
5	1.84	1.66	1.60	1.56	—	—	—			
6	1.99	2.00	1.26	1.28	1.64	1.48	1.48			
7	1.89	1.76	1.25	1.19	1.46	1.39	1.35			
Mean ± SD	2.00 ± 0.20	1.93 ± 0.21	1.45 ± 0.15	1.36 ± 0.14	1.61 ± 0.11	1.52 ± 0.16	1.56 ± 0.17	1.68 ± 0.08	1.58 ± 0.11	

resolution in the pre-β- and β-chain region of the chromatogram. The first two chambers were each filled with 200 ml of the usual starting buffer while the third chamber held 200 ml of modified limit buffer (8 M urea, 0.025 M Na_2HPO_4, 0.05 M 2-mercaptoethanol, pH 6.8).

Determination of radioactivity incorporated into globin chains followed previously described methods (15).

Gel Filtration of Sephadex G-100. Globin samples (30–50 mg) were chromatographed, together with blue dextran and ϵ-2,4-Dnp-lysine as markers, on a 2.5 × 80 cm column of Sephadex G-100 in 20% HCOOH at room temperature. Uniformly ^{14}C-labeled globin of known α/β ratio was added as internal standard for subsequent specific activity determinations. After chromatography, globin fractions were recovered by freeze drying.

RESULTS

The α/β-chain production ratios (α/β ratios) of the 12 nonthalassemic controls were determined by method A. In normal reticulocytes these ratios averaged 1.03 ± 0.14 and 0.99 ± 0.12 for total counts and specific activity respectively, while in bone marrow the corresponding ratios were 0.89 ± 0.08 and 0.84 ± 0.05.

Corresponding data for the β-thalassemia heterozygotes are given in Table 1. Using method A the mean α/β ratios in reticulocyte whole-cell globin were 2.00 and 1.99 for total counts and specific activity, respectively, while for whole-cell bone-marrow the corresponding mean ratios were 1.45 and 1.36.

Preliminary experiments with some 60 bone-marrow samples from non-thalassemic individuals suggested that nonheme proteins present in bone-marrow whole-cell "globin" may, under some conditions, chromatograph in the β-chain region during chain separation on CM-cellulose. It was found that much of this non-globin protein can be removed from globin by passage through Sephadex G-100 in 20% formic acid, a strong dissociating solvent. In addition, a considerable improvement in the resolution in the β-chain region made by modifying the conditions of gradient elution during chain separation on CM-cellulose also has the effect of reducing considerably the amount of non-globin protein associated with β chains; using these methods, α/β ratios from normal aspirated marrow samples averaged approximately unity.

Five β-thalassemic bone-marrow whole-cell globin samples (aliquots of the samples used for the α/β ratios determined by method A in Table 1) were chromatographed on Sephadex G-100 in 20% formic acid and then the globin chains were separated by method A. The α/β ratios ranged from 1.46 to 1.75 (mean 1.61) for total counts and from 1.35 to 1.80 (mean 1.52) for specific activity (Table 1). Comparison of these values with those obtained from the same globin samples determined by method A alone (Table 1) shows that there is a significant increase in α/β ratio after Sephadex G-100 purification.

Prolonged exposure of globin to 20% formic acid at room temperature may give erroneous A_{280} values which affect the subsequent specific activity determinations. We, therefore, added a small amount of highly radioactive ^{14}C-labeled globin of known α/β ratio to all globin samples before purification on Sephadex G-100, as an internal standard for specific activity determinations. Determined by this method the α/β specific activity ratios ranged from 1.35 to 1.80 (mean 1.56), values similar to those determined by optical methods (Table 1).

Three samples of β-thalassemic whole-cell globin were fractionated into α and β chains using the modified three-chamber elution gradient (method B). Again, a significant increase in α/β ratio was obtained compared with those values obtained on the same globin samples by method A (Table 1). In one case specific activity ratios were checked by the ^{14}C internal standard to ensure that prolonged exposure to 8 M urea (method B takes approximately 12 hr) did not affect the specific activity determinations.

There is no doubt that procedures aimed at giving better resolution of α and β chains on chromatography, or prior purification of globin, both result in α/β ratios which are increased significantly over those obtained by the simple linear gradient method for chain separation.

These results suggest, but do not prove, that contamination of β-chains is at least one reason for the relatively low α/β ratios seen sometimes when method A alone is used for their determination. The improvement in the elution profiles (smaller pre-β chain peak, better separation of pre-β from β chain, and lower radioactivity background) of bone-marrow whole-cell globin on CM-cellulose after Sephadex G-100 purification or on elution with the shallow concave gradient produced by the three-chamber device also provide indirect evidence that this is the case. The experiments with ^{14}C-labeled globin are particularly relevant since they rule out any possibility of preferential loss of β chains on Sepha-

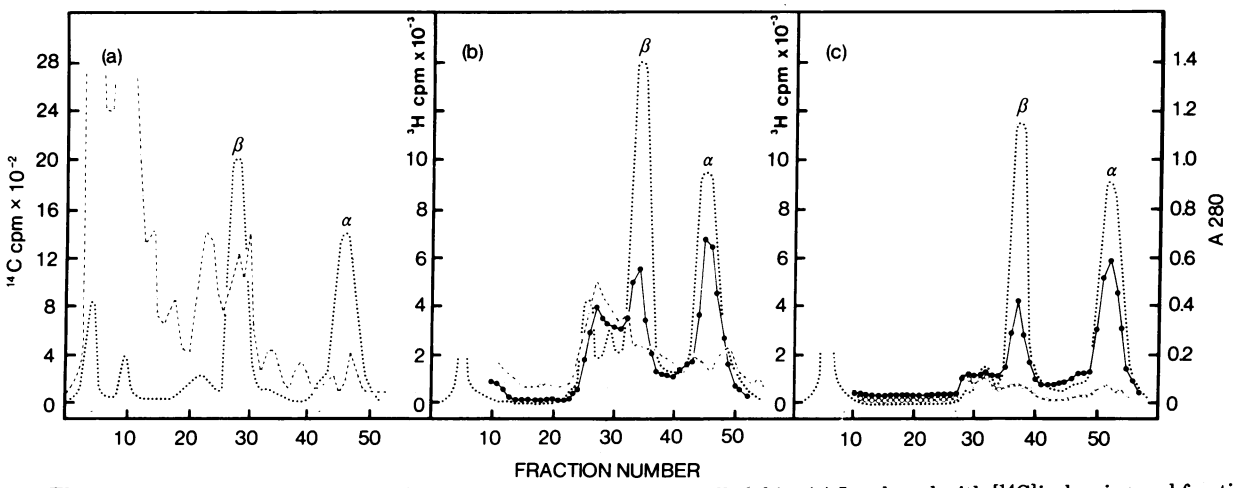

FIG. 1. Elution profiles of heterozygous β-thalassemic bone-marrow whole-cell globin. (a) Incubated with [^{14}C]isoleucine and fractionated by method A. (b) Incubated with [^{3}H]leucine and [^{14}C]isoleucine and fractionated by method A. (c) The same globin sample as in (b) but fractionated by Sephadex G-100 and method A. Absorbance, · · · ·; [^{14}C]isoleucine radioactivity, O----O; [^{3}H]leucine radioactivity, ●——●.

dex G-100 in 20% formic acid or during prolonged chain separations as causing the increased α/β ratios.

Direct evidence that β chains in bone-marrow whole-cell globin are contaminated with non-heme proteins was obtained from the experiment shown in Fig. 1. Incubation of heterozygous β-thalassemic bone-marrow with [^{14}C]isoleucine followed by chain separation of whole-cell globin resulted in the elution profile shown in Fig. 1a. (Normal α and β chains contain no isoleucine.) This resembles elution profiles made from normal bone marrow similarly treated and suggests that radioactive non-heme proteins chromatograph over the entire elution range, and particularly in the β-chain region. (Addition of nonradioactive cord-blood globin does not alter the elution pattern, showing that the [^{14}C]isoleucine counts do not represent γ-chains which have smeared over the chromatogram in the absence of homologous carrier protein.) In another experiment a heterozygous β-thalassemic bone-marrow sample was incubated with both [^{3}H]leucine and [^{14}C]isoleucine and whole-cell globin was then separated by method A before (Fig. 1b) and after (Fig. 1c) chromatography of globin on Sephadex G-100 in 20% formic acid. The nonpurified sample gave a high [^{14}C]isoleucine incorporation into the pre-β chain region of the CM-cellulose chromatogram, whereas after Sephadex G-100 purification almost all the [^{14}C]isoleucine counts had been removed. There was also a concomitant increase in α/β ratio from 1.28 to 1.48.

There thus seems little doubt from these experiments that whole-cell "globin" prepared from heterozygous β-thalassemic bone marrow does contain significant amounts of contaminating proteins which can substantially affect the determination of α/β ratios when these are measured by the conventional linear gradient method of CM-cellulose chromatography.

A number of observations suggest that the non-heme proteins which contaminate bone-marrow globin are derived from white cells. Preliminary experiments with non-thalassemic samples showed that consistently higher α/β ratios are obtained if lysates are prepared under conditions which minimize white-cell lysis. Furthermore, bone marrow samples derived from ribs gave consistently lower α/β ratios compared with samples from the iliac crest, and rib samples invariably have a higher proportion of non-erythroid cells. We investigated the effect of dextran sedimentation (18) on heterozygous β-thalassemic bone-marrow cells, a procedure which has been used by some investigators selectively to concentrate nucleated red-cell precursors from erythrocytes (2, 18).

An aliquot of cells from the incubation performed on Case 2 was allowed to sediment through 2% dextran for 45 min at 4° (18). The top (i.e., unsettled cells) and bottom fractions were removed and the α/β ratios were determined and white-cell counts were performed on each fraction. The α/β ratio of the unfractionated cells was 1.54 for total counts and 1.39 for specific activity, with a myeloid (M) to erythroid (E) ratio of 1/1. In the top (unsettled cells) fraction, the corresponding figures were 1.19 and 1.24, M/E ratio 3/1, and in the bottom (settled cells) fraction 1.54 and 1.62 with no detectable white cells present. Thus a selective concentration of white cells relative to nucleated red cells in the top fraction leads to a reduction in α/β ratio. In a second experiment three cell aliquots from the incubation on Case 3 were settled in 2% dextran for 15, 30, and 45 min, respectively, the top and bottom fractions were then collected, and the α/β ratios were determined by method A. The α/β specific activity ratios of the top fractions were 1.46 at 15 min, 1.38 at 30 min, and 1.29 at 45 min, whereas in the settled cells the α/β ratio remained constant at 1.50.

These two experiments demonstrate that the presence of white cells in bone-marrow samples can significantly affect the determination of α/β ratios unless precautions are taken to minimize contamination of β chains during chromatography.

Effect of incubation time on α/β ratios

Even after globin purification on Sephadex G-100 or chain separation by method B, the α/β ratio in heterozygous β thalassemic bone-marrow is much less than in reticulocytes. We previously suggested (13) that this might be due to an increased rate of free-α-chain degradation by proteolysis in bone marrow, and experiments done in this laboratory have shown this to be the case in homozygous β thalassemia. Furthermore, other experiments with homozygous β-thalassemic cells showed that the rate of increase in α-chain specific

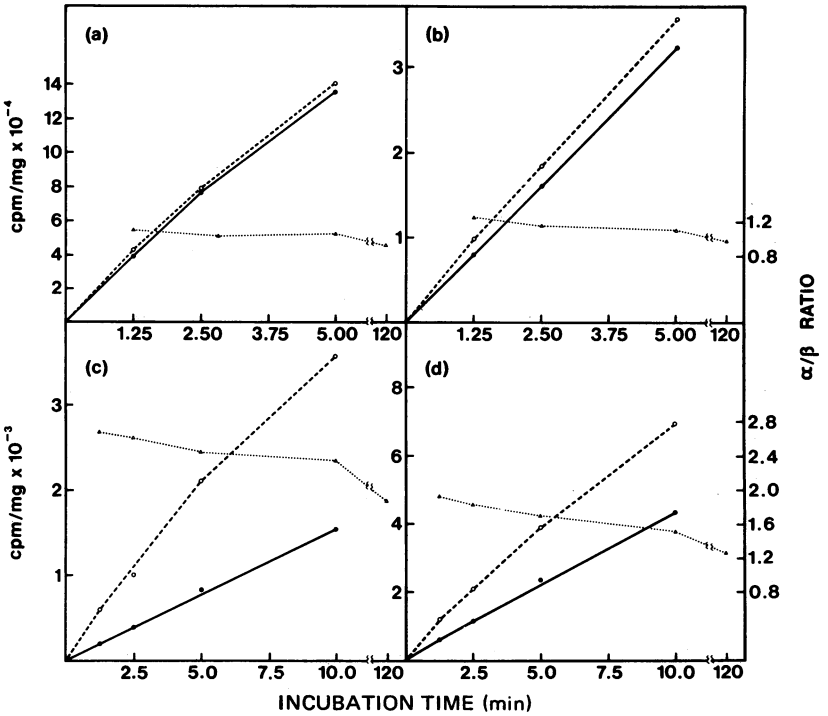

FIG. 2. Synthesis of α (O----O) and β (●——●) chains from normal reticulocytes (a) and bone marrow (b), and from heterozygous β-thalassemic reticulocytes (c) and bone marrow (d). α/β ratio, Δ····Δ.

activity with incubation time declines as the incubation time increases, whereas the γ-chain specific activity increases linearly for at least 3 hr (19). Also this difference is more evident in bone marrow than in reticulocytes.

Here we have studied the effects of incubation time on α- and β-chain synthesis in heterozygous β-thalassemic bone marrow and reticulocytes over periods ranging from 1.25 min to 2 hr. The experimental conditions were as described in the *Materials and Methods* section except that 2 mCi of [^{3}H]leucine (dissolved in 0.1 ml of incubation medium) was used for 0.2 ml of cells incubated. Normal bone-marrow and reticulocyte samples were also incubated as a parallel control. The results are given in Fig. 2a and b. It is evident that in both normal marrow and reticulocytes α- and β-chain synthesis are synchronous and follow essentially a parallel time course. At all but the earliest times the α/β ratios remain constant; during the first 5 min there is some evidence of an increased rate of α-chain synthesis since α/β ratios of approximately 1.2 are seen in both marrow and reticulocytes.

The results obtained with incubations of heterozygous β-thalassemic bone-marrow and reticulocytes are strikingly different. The α- and β-chain specific activities (determined by method A after globin purification on Sephadex G-100) from a 2 hr incubation are shown in Fig. 2c and d and it can be seen that at early times there is a strong indication of an increased rate of α-chain synthesis compared to later times, whereas β-chain synthesis is linear. The effect of this apparent fall-off in the rate of α-chain synthesis on the α/β ratio is also dramatic. Thus in reticulocytes it dropped from 2.66 at 1.25 min to 1.86 at 2 hr, and in bone marrow from 1.93 at 1.25 min to 1.26 at 2 hr.

DISCUSSION

The experiments described here extend our previous findings of imbalanced globin chain synthesis in heterozygous β-thalassemic bone marrow (13). Furthermore, there seems little doubt that white-cell proteins in bone-marrow whole-cell globin may chromatograph with β chains on CM-cellulose unless precautions are taken to exclude them.

In our previous paper (13) we also produced evidence, since confirmed by others (20, 21), of a pool of radioactive free α chains in β-thalassemic bone-marrow cells. A similar situation also exists in reticulocytes, where globin chain imbalance is not disputed, but not in normal reticulocytes or bone marrow. Nevertheless, it has been claimed (21) that such a pool in marrow is not necessarily evidence of chain imbalance, since although large in radioactive terms it contains negligible amounts of protein. The short-term incubations described here suggest that excess free α chains are mostly degraded by proteolysis within a few minutes of synthesis, making it highly unlikely that a substantial pool of globin chains could exist in bone-marrow cells for any length of time. The absence of any marked degree of inclusion-body formation and ineffective erythropoiesis [notable in homozygous β thalassemia where α/non α ratios may be as high as 20/1 (1)] also suggests that it is within the proteolytic capacity of the cells of heterozygotes to cope with a 2-fold α-chain excess.

Many authors claim to have observed balanced globin-chain synthesis in the bone marrow of heterozygous β thalassemics (2–8). From our experiments it is evident that the method used for isolating α and β chains from bone-marrow globin is crucial if reliable α/β ratios are to be obtained. Unless the bulk of contaminating non-heme proteins are removed prior to chromatography or a high resolution chromatographic system is employed, it is impossible to obtain pure enough α and β chains for their specific activities to be determined reliably. Furthermore, it is very important to ensure that globin is precipitated from dilute lysates of bone-marrow cells (see *Materials and Methods*). Neglect of this simple point can result in chain separations in which the

resolution of α and β chains is quite inadequate for the reliable determination of specific activities.

The conditions under which samples are stored are also very important. We routinely converted lysates to globin at the end of each experiment. On a few occasions this was not done with the entire sample and we noticed a consistent drop, progressive with time, in the α/β ratio when this was determined subsequently on a stored lysate. Thus the α/β ratio of whole-cell globin prepared from a lysate (Case 3) stored for a few weeks at $-20°$, fell from 1.26 to 1.10, whereas, it remained unchanged in globin stored under identical conditions. Similar though less marked effects take place with peripheral blood lysates and have also been noted by other workers (W. G. Wood, personal communication). We do not yet have a satisfactory explanation for this behavior but clearly it should be considered when planning this type of experiment.

Finally, the use of dextran sedimentation to enrich for nucleated cells is not recommended unless it can be shown that the ratio of nucleated myeloid to erythroid cells is unaltered by the particular procedure employed. In our hands this was not the case, for there was a definite and progressive loss of nucleated red cells relative to white cells during the sedimentation process, thus exacerbating an already incipient problem.

It seems likely that failure to observe globin-chain imbalance in heterozygous β-thalassemic bone marrow is largely a technical problem which can be overcome if precautions are taken to avoid the effects of white-cell protein contamination of "globin" and the deterioration which takes place in stored lysates. Either of these phenomena, if sufficiently pronounced, may augment the effects of proteolytic destruction of α chains to such an extent that globin synthesis appears to be balanced. Prolonged incubations are also to be avoided, for similar reasons.

It still appears, however, that there is apparently greater chain imbalance in reticulocytes than in bone marrow in heterozygous β thalassemia; possibly more active proteolysis in marrow is a sufficient explanation but it would need even shorter time incubations (less than 1 min) to establish this with certainty.

An interesting point which does emerge from the short-term reticulocyte incubations, however, is that indirectly they suggest that in normal reticulocytes there must be a considerable excess of α chains synthesized which are immediately broken down by proteolysis. It is difficult to see how there can be an α/β ratio of 2.7 at short times in β-thalassemia reticulocytes where one β-chain locus is inactive, unless the early-time α/β ratio in normal reticulocytes is of the order of 1.3 to 1.4. In fact, there is a hint of this in the 1.25 min incubations where the α/β ratio is 1.2, and it would be consistent with the findings in rabbit reticulocytes that α-chain synthesis exceeds that of β chains by a factor of 1.24 to 1 at very early times (22).

The experiments described here indicate that there is indeed a significant degree of imbalanced globin-chain synthesis in heterozygous β-thalassemic bone marrow, commensurate with what would be expected from that observed in the marrow of homozygotes. They suggest that a compensating increased output of β-chains from the normal locus is limited and this would explain the low mean cell hemoglobin which is always found in this disorder. Finally, there is no evidence that a mechanism for the suppression of α-chain synthesis exists in β-thalassemia heterozygotes.

G.C. thanks the Greek government for a personal grant. J.B.C. and D.J.W. thank the Medical Research Council and Wellcome Trust for financial support.

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