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Regulation of Hemoglobin β-Chain Synthesis in Bone Marrow Erythroid Cells by α Chains

(globin synthesis/reticulocytes/L-*O*-methylthreonine)

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ABSTRACT **Synthesis of α and β chains of hemoglobin was studied *in vitro* in intact reticulocytes and bone marrow cells. The cells were from rabbits having a variant form of hemoglobin in which L-isoleucine is in the α but not in the β chains. This characteristic permitted a selective inhibition of α-chain synthesis to be produced by addition to the incubation medium of L-*O*-methylthreonine, an inhibitor of protein synthesis that is a specific antagonist of L-isoleucine.**

In studies with reticulocytes, 25 mM L-*O*-methylthreonine produced a 60–70% inhibition of α-chain synthesis, but β-chain synthesis was unaffected even after incubation times for 4 hr. Because reticulocytes contain a pool of uncombined α chains which might have obscured the demonstration of an α chain-dependent mechanism for β-chain synthesis, subsequent studies were done with bone marrow cells. The latter had little or no detectable α-chain pool. A substantial inhibition of α-chain synthesis by the bone marrow cells was produced by the isoleucine antagonist but was also accompanied by a significantly decreased rate of β-chain synthesis.

These findings suggest that the coordinated synthesis of the complementary α- and β-globin chains of hemoglobin may reflect in part a modifying effect of α-chain synthesis on the synthesis of β chains.

The mammalian hemoglobin molecule is a tetramer composed of a pair of α subunits and a pair of β or other non-α-globin–heme subunits. The α and non-α globin components of hemoglobin are assembled on separate polyribosomes (1, 2), indicating that the synthesis of each of these protein units is potentially subject to independent regulation. Several studies have suggested that significant differences exist between the synthesis of the α and β chains in intact hemoglobin-synthesizing cells. These include differences in the size of polyribosomes that synthesize α and β chains (1, 3, 4), the presence of unequal quantities of α chain and β chain messenger RNA in association with ribosomes in the cells (5), and differences in the rates of translation of the two types of globin chains (6). Other experimental evidence has also suggested that initiation of β-chain translation may be considerably more rapid than that of α chains (4, 5).

In spite of these differences in the biosynthesis of the two globin chain types, nonpathologic hemoglobin-synthesizing cells characteristically synthesize α and non-α hemoglobin chains at very nearly equal rates (7, 8). To account for this apparent coordination of the synthesis of the complementary globin subunits, it has been proposed that regulatory mechanisms may exist within the hemoglobin-synthesizing cell whereby the rate of synthesis of one globin chain type may be influenced by the synthesis of the other (9, 10).

To investigate this question we previously looked for evidence of a possible role of the non-α hemoglobin subunit as a modifier of the rate of α-globin synthesis by human reticulocytes (11, 12). These studies suggested that α-chain synthesis proceeds independently of the synthesis of the complementary non-α chain.

Other evidence from studies of rabbit reticulocytes (13, 14) and human erythroid cells from individuals having an α-chain structural abnormality (15) has suggested that synthesis of the non-α component may depend in part on the presence or concomitant synthesis of α chains.

To examine this possibility we performed experiments with intact hemoglobin-synthesizing cells in a manner similar to our previous studies (11, 12). Erythroid cells were obtained from a strain of rabbits having hemoglobin in which there are three isoleucine residues per α chain but no isoleucine in the β chains. L-*O*-Methylthreonine, a specific antagonist of L-isoleucine, was used to produce a selective inhibition of α-chain synthesis, allowing an assessment to be made of any consequent effect on β-chain synthesis.

MATERIALS AND METHODS

Animals used in this study were of a mixed strain of rabbits, all of which were homozygous for a variant form of hemoglobin in which the single isoleucine residue normally present in the β chain at β-112 (16) is replaced by valine. The α chains of these animals contain three isoleucine residues. Incorporation of L-[^{14}C]-isoleucine into the hemoglobin of these rabbits was confined almost exclusively to the α chains (Fig. 1), which was a means for identification of the homozygous form of this hemoglobin.

Preparation of Cells. Reticulocytosis was induced in the rabbits by injections of phenylhydrazine using the injection schedule recommended by Rich (17). Reticulocyte counts of 90% or more were achieved after 7–12 days. Blood was drawn into heparinized syringes by cardiac puncture, the blood was centrifuged at 4°, and the plasma and buffy coat were removed by aspiration. The erythrocytes were washed three times by centrifugation and suspension in a cold isotonic saline solution (140 mM NaCl–5 mM KCl–1.5 mM $MgCl_2$).

For preparation of bone marrow cells, the animals were exsanguinated and the bone marrow was collected by flushing out the long bones (humerus, femur, and tibia) with cold saline. The marrow was kept at 4° and homogenized gently in a Dounce tissue homogenizer with a Teflon pestle. The suspension was centrifuged at 500 × *g* for 5 min and the

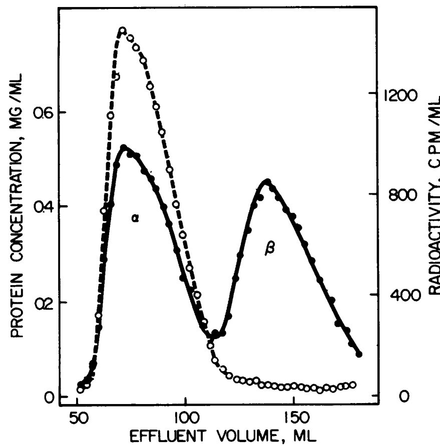

FIG. 1. Incorporation of L-[^{14}C]isoleucine into α and β chains of the variant rabbit hemoglobin. Globin was prepared from reticulocytes of the rabbit after incubation of the cells with radioactive L-isoleucine. Chromatography was done on a carboxylmethyl-cellulose column. (●——●) protein concentration; (O···O) radioactivity.

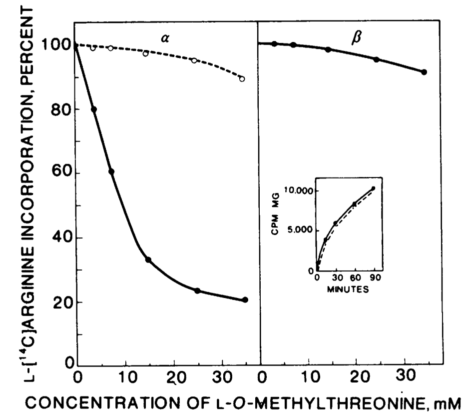

FIG. 2. Effect of various concentrations of L-O-methylthreonine on α- and β-chain synthesis by reticulocytes. The cells were incubated for 15 min with L-[guanido-^{14}C]arginine as the radioactive precursor. Values are expressed as percent of the specific activity of a control incubated without the inhibitor. The control values were 2415 cpm/mg for the α chain and 2335 cpm/mg for the β chain. (●)L-O-methylthreonine; (O)L-O-methylthreonine + L-isoleocine. *Insert* demonstrates the very nearly equal rates of synthesis of α (●) and β (O) chains for a 90-min incubation.

supernatant was poured off. The marrow, about 5 ml, was then taken up in cold saline, filtered once through two layers of gauze, centrifuged once more, and suspended in 1.5 volumes of saline. Carrier erythrocytes from a nonanemic rabbit homozygous for the variant hemoglobin were added to the marrow suspension in an equal amount. The carrier cells were prepared by washing three times with saline, each time discarding the upper third of the cells, thus eliminating most of the reticulocytes normally present in peripheral blood. These carrier cells did not incorporate radioactive amino acids into protein. In rabbits treated with phenylhydrazine, most of the bone marrow cells were erythroid and more than half were nucleated, as demonstrated in smears stained with Wright–Giemsa stain.

Cells Were Incubated at 37° in Erlenmeyer flasks open to room air in a metabolic shaker. The cells were suspended in the incubation medium in a final packed cell volume of 8%. The medium contained 140 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 5 mM Tris·HCl (pH 7.4), 1 mg/ml of glucose; 20 μM hemin, and a mixture of L-amino acids (18) with the exception of isoleucine. The samples were incubated for 15 min before addition of the radioactive amino acid. Each flask contained 2 μCi of a radioactive L-amino acid per ml of packed volume of cells. The nonradioactive form of the amino acid was omitted from the medium. *L-[guanido-^{14}C]arginine* (Amersham-Searle) had a specific activity of 50–54 Ci/mol. For some experiments non-radioactive L-arginine was added to reduce the specific activity to about 10 Ci/mol. Uniformly labeled L-[^{14}C]-leucine (240 Ci/mol) and L-[^{14}C]isoleucine (250 Ci/mol) were obtained from New England Nuclear Corp. L-O-Methylthreonine was purchased from Calbiochem.

Globin Preparation. Incubations were terminated after various times by pipetting aliquots of the incubation mixtures into chilled tubes. The cells were collected by centrifugation at 4°. Cells were lysed by addition of three volumes of cold distilled water and repeated freeze-thawing in dry ice–alcohol and a 37° waterbath. The stroma were sedimented by centrifugation at 20,000 × g for 10 min. The clarified lysates were used immediately or were stored at −20° after saturation with carbon monoxide and rapid freezing in a dry ice–alcohol mixture. Except when indicated, globin was prepared from the total unfractionated lysates by precipitation of the protein in acetone–HCl at −20° (19). Globin from hemoglobin as well as from uncombined chains was therefore included in the globin preparations.

Globin-Chain Fractionation and Determination of Radioactivity. Globin chromatography on carboxymethylcellulose columns was performed by the method of Dintzis (20) with the elution by a linear gradient of pyridine–formic acid buffer. Eluent fractions comprising the α- and β-chain protein were pooled, and the protein was precipitated by addition of trichloroacetic acid in a final concentration of 10% (w/v). The precipitates were washed twice in ethanol–ether (1:2, v/v) and twice in ether, and dried in a stream of nitrogen. For specific activity determinations, the globin fractions were dissolved in 0.2 M formic acid. Aliquots were taken for protein estimation (21), and other aliquots were pipetted onto stainless steel planchets for counting in a low-background gas-flow counter with a thin window.

Hemoglobin Chromatography. DEAE–Sephadex column chromatography was done at room temperature under described conditions (22). Sephadex G-75 gel filtration was performed as previously described (12).

RESULTS

Reticulocyte Studies. For most of the globin-chain synthesis studies, L-[^{14}C]arginine was used as the radioactive precursor. This amino acid is equally represented in rabbit α and β chains (16), which allows specific activity values for the two chains to be compared without need for correction.

In experiments with 15-min incubations α-chain synthesis was inhibited to a progressively greater degree with increasing concentrations of L-O-methylthreonine in the medium (Fig. 2). Synthesis of the β chains was inhibited by less than 10% at the highest concentration of the inhibitor used (35 mM).

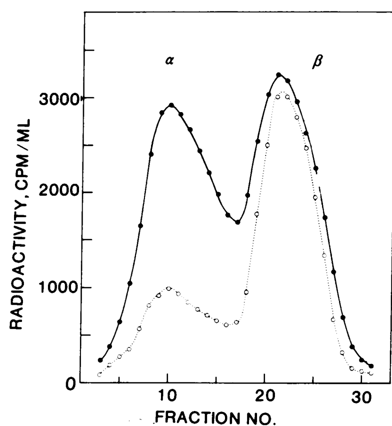

FIG. 3. Effect of 25 mM L-O-methylthreonine on incorporation of L-arginine into α and β chains by reticulocytes. The cells were incubated for 15 min with the radioactive precursor. About equal quantities of protein were subjected to carboxymethylcellulose chromatography. (○) with L-O-methylthreonine; (●) control.

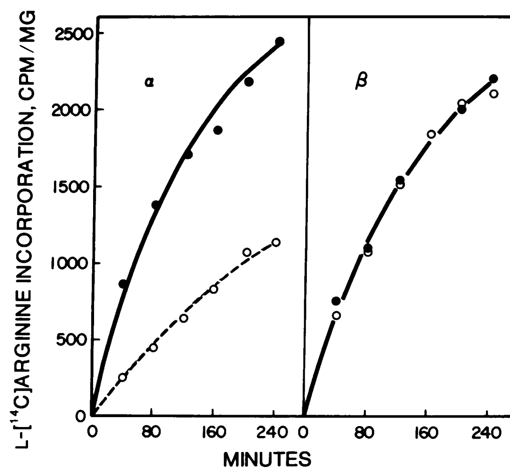

FIG. 4. Effect of L-O-methylthreonine on α- and β-chain synthesis by reticulocytes for a 4-hr incubation. (○) with L-O-methylthreonine; (●) control.

When nonradioactive L-isoleucine was added to the medium together with L-O-methylthreonine, the inhibitory effect on α-chain synthesis was virtually abolished, indicating that the inhibition resulted specifically from isoleucine antagonism. A similar study by Hori and Rabinovitz (23) used rabbits having hemoglobin in which isoleucine presumably was present in both the α and β chains. In this study L-O-methylthreonine produced an equal inhibitory effect on α-chain and β-chain synthesis.

In subsequent experiments 25 mM L-O-methylthreonine was used (Fig. 3). In several short-time incubation studies with L-[^{14}C]arginine as a precursor, the isoleucine antagonist consistently inhibited α-chain synthesis to less than 50% of the control value, whereas β-chain synthesis was inhibited by less than 5%. In a similar study with L-[^{14}C]leucine, α-chain synthesis was inhibited to 16% of the control value with an observed rate of β-chain synthesis of 104% of the control.

To seek evidence of a possible dependence of β-chain synthesis on the presence or concomitant synthesis of α chains, we incubated reticulocytes with the isoleucine antagonist for various times. Because reticulocytes contain a pool of uncombined α chains (24), an inhibition of β-chain synthesis under these conditions might be expected to be delayed until the α-chain pool in the cells becomes depleted. An experiment with a 4-hr incubation (Fig. 4) did not show any demonstrable effect on β-chain synthesis, in spite of a substantial suppression of α-chain synthesis.

Bone Marrow Studies. During these studies a report (25) appeared demonstrating that although free α chains can be found in rabbit reticulocytes, virtually no uncombined α chains are present in erythroid bone marrow cells of these animals. To determine if this difference existed in the variant rabbits we used, reticulocytes and bone marrow cells were incubated with L-[^{14}C]arginine to label the globin chains, and the stroma-free cell lysates were subjected to gel filtration on Sephadex G-75 columns.

The reticulocyte lysates demonstrated a prominent radioactivity peak after elution of the hemoglobin peak (Fig. 5). When the fractions comprising this peak were added to nonradioactive hemoglobin and the mixture was subjected to globin precipitation and chromatography, virtually all of the recovered radioactivity corresponded to the α-globin peak. Gel filtration of the bone marrow lysate yielded a much smaller peak of radioactivity eluted after the hemoglobin tetramer fraction. When this material was chromatographed on a carboxymethylcellulose column only a very small amount of radioactivity was eluted with the globin peaks, and about equal α- and β-chain radioactivity was recovered, indicating that uncombined α chains were either absent or present in a very low concentration.

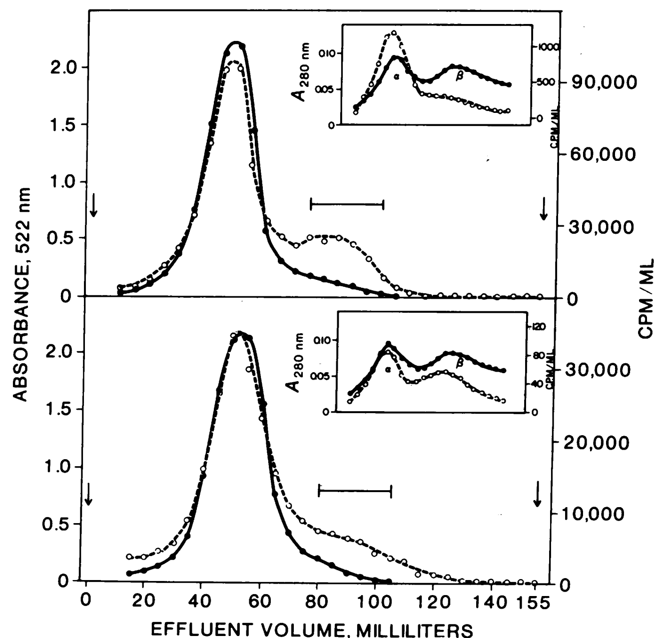

FIG. 5. Gel filtration of radioactive cell lysates after incubation of reticulocytes (*top*) and bone marrow cells (*bottom*) with L-[^{14}C]arginine. *Arrows* on the *left* indicate the elution volume of blue dextran; those on the *right*, the elution volume of myoglobin. Eluent fractions indicated by *brackets* were combined and added to nonradioactive carrier hemoglobin. These samples were subjected to carboxymethylcellulose chromatography (*inserts*). (●——●)Absorbance; (○· · ·○)radioactivity.

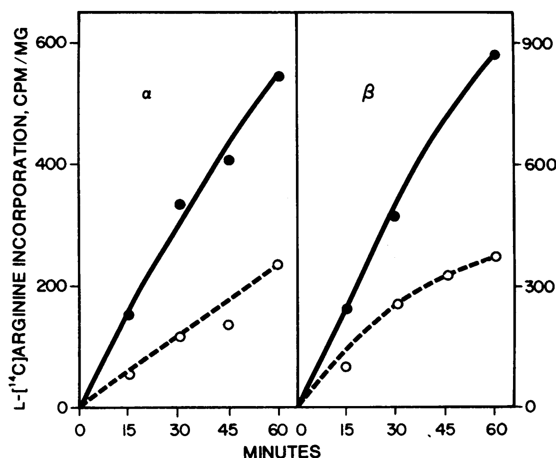

FIG. 6. Incorporation of L-[^{14}C]arginine into α- and β-globin fractions by bone marrow cells. At the termination of the incubations the cells were lysed and globin was prepared from the stroma-free lysates for carboxymethylcellulose chromatography. (●)Control; (○)L-O-methylthreonine.

In subsequent studies with L-O-methylthreonine, bone marrow cells from the variant rabbits were used. A time-course study of globin-chain synthesis by bone marrow cells is shown in Fig. 6. In contrast to the studies with reticulocytes, the addition of L-O-methylthreonine to the incubation medium resulted in an inhibition of radioactivity incorporation into both the α- and β-chain fractions.

Because of the possibility that nonglobin proteins also synthesized by the bone marrow might have had similar chromatographic properties as the globin proteins and thereby influenced these results, three additional experiments were done in which the bone marrow lysates were subjected to a series of purification steps before the carboxymethylcellulose chromatography. In these experiments the cells were chilled after the incubations and nonradioactive reticulocytes were added to the bone marrow cells as a source of free α chains before lysis. In this manner any uncombined β chains that might have been synthesized could be stablized by their incorporation into hemoglobin tetramers. The stroma-free lysates were subjected to column chromatography on DEAE-Sephadex and the eluent fractions comprising the hemoglobin peak were combined. After concentration of the hemoglobin, Sephadex G-75 gel filtration was performed and the material in the hemoglobin peak was again collected. Globin was prepared from the purified hemoglobin; this material was passed once more through a Sephadex G-75 column. Fig. 7 demonstrates the potential of this procedure for removal of impurities having gel filtration characteristics similar either to hemoglobin or to globin. In some experiments gel filtration of the globin was done in Sephadex G-100 equilibrated with 0.2 M formic acid-0.02 M pyridine. Under these conditions the eluted protein could be applied directly to the carboxymethylcellulose column for separation of the α- and β-globin chains.

In experiment 1 (Table 1), addition of 25 mM L-O-methylthreonine produced a substantial inhibition of α-chain synthesis and a smaller degree of inhibition of β-chain synthesis. To establish with greater certainty that the inhibitory effect on β-chain synthesis was not a nonspecific effect of the isoleucine antagonist, the experimental conditions were further modified in experiments 2 and 3. The following changes were

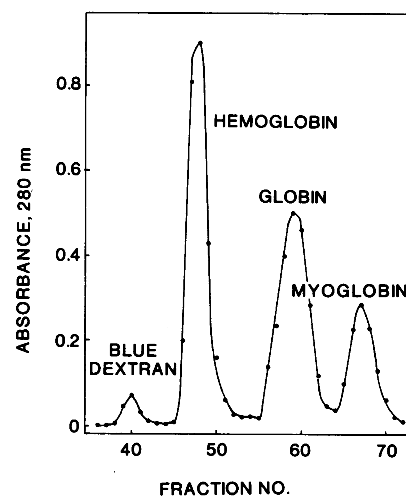

FIG. 7. Elution pattern of hemoglobin and globin from Sephadex G-75.

made in the procedure: (i) The cells were incubated for 1 hr before addition of the radioactive precursor to achieve maximal α-chain depletion; (ii) 20% dialyzed serum was added (26) to stimulate globin synthesis; and (iii) the concentration of L-O-methylthreonine was reduced to 10 mM. Under these conditions (Table 1), the inhibition of α-chain synthesis was decreased to about 50%, but the synthesis of β chains was again significantly suppressed.

In additional studies with bone marrow cells, the inhibitory effect of L-O-methylthreonine was also prevented by addition of L-isoleucine, again demonstrating the specificity of the inhibitor.

DISCUSSION

Several possible regulatory mechanisms have been proposed to account for the apparent coordinated control of globin-chain synthesis. Colombo and Baglioni (9, 27) initially suggested that β chains might be required for release of α chains from ribosomal protein-synthesizing sites, thus providing a mechanism whereby the availability or concomitant synthesis of β chains would regulate the use of α chains for hemoglobin formation, secondarily affecting α-chain synthesis. Baglioni and Campana later considered this proposed mechanism unlikely (13), mainly in view of evidence that reticulocytes con-

TABLE 1. *Effect of L-O-methylthreonine on globin synthesis by bone marrow cells**

Exp.	Addition	[^{14}C]Arginine incorporation (cpm/mg) α	β
1	None	805	906
	L-O-MeThr, 25 mM	181 (22.5%)	777 (85.9%)
2	None	2006	2284
	L-O-MeThr, 10 mM	1010 (50.5%)	1860 (81.5%)
3	None	934	1200
	L-O-MeThr, 10 mM	511 (54.7%)	1012 (84.1%)

* The globin preparations were subjected to a series of purification procedures before determination of specific activity. *L-O-MeThr*, L-O-methylthreonine.

tain a pool of uncombined α chains. Baglioni and Campana subsequently suggested (13) that β-chain synthesis might be regulated by means of an α chain-dependent process with the control mechanism affecting the release of completed β chains from ribosomes.

Blum, Schapira, and coworkers (28–30) studied globin-chain synthesis in a cell-free system derived from rabbit reticulocytes, and examined the effects of added globin chains or hemoglobin subunits. Their results suggested that α chains may specifically inhibit α-chain synthesis, but by a mechanism that does not affect the release of the completed chains from ribosomes. Addition of β chains to their cell-free incubations also produced a specific inhibition of β-chain synthesis, suggesting that this chain may also regulate its own rate of synthesis. They also observed that β-chain synthesis was stimulated by low concentrations of α chains, suggesting that overall regulation of globin-chain synthesis may be a function of the α chains.

Shaeffer *et al.* (10) also observed that addition of β subunits resulted in an inhibition of β-chain synthesis. They concluded that the β chains that were added combined with free α chains, thereby interfering with an α chain-dependent mechanism for β-chain synthesis. This mechanism appeared to function by facilitating release of completed β chains from ribosomal protein-synthesizing sites. When these experiments were performed with addition of hemin (14), it was observed that the inhibitory effect of added β chains was greatly reduced. It was thus concluded that heme may be important in the regulation of globin-chain synthesis. Tavill *et al.* (24, 25) performed other experiments that also support this conclusion.

A recent study by Adams *et al.* (15), using intact erythroid cells, also suggested that α chains may modify the rate of β-chain synthesis. In these experiments globin-chain synthesis was studied *in vitro* in reticulocytes from a patient having a hemoglobin variant in which the α chains are unstable. It appeared that the unstable chain underwent rapid breakdown within the cells, presumably releasing intact β chains which in turn could combine with any free α chains that were present. Synthesis of β chains in these cells was substantially less than the overall synthesis of α chains, which was interpreted to represent a possible α chain-dependent mechanism for the regulation of β-chain synthesis.

The results of the experiments described in this report provide further evidence that globin-chain synthesis in intact erythroid cells is regulated in a manner to promote nearly equal rates of synthesis of the complementary globin chains. Although the experimental evidence suggests that α chains exert a regulatory effect on β-chain synthesis, these findings do not necessarily imply an absolute requirement for α chains or for α-chain synthesis for β-chain synthesis to occur. A regulatory mechanism by which β-synthesis is only partially affected by the synthesis of its complementary chain might well provide an equally effective means of control of globin synthesis and, at the same time, be consistent with the excessive β-chain synthesis in erythrocytes in patients with α thalassemia (31) and other conditions (32) in which α-chain synthesis is impaired.

The presence of added hemin in all of the cell incubations in these experiments suggests that this apparent control mechanism exists apart from any regulatory influence of heme. Erythroid cells appear to be freely permeable to exogenous hemin, which is rapidly incorporated into hemoglobin by globin-synthesizing cells (26, 33).

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