Hemoglobin Synthesis in β-Thalassemia: the Properties of the Free α-Chains

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ABSTRACT The decrease in hemoglobin A (HbA, $\alpha_2\beta_2$) synthesis in the erythroid cells of patients with β-thalassemia is due to a selective defect in β-chain synthesis. Since α-chains continue to be formed at a normal rate in these cells, this results in a marked relative excess of α-chain synthesis over β- and γ-chain synthesis. The α-chains uncombined with β- or β-like–chains (δ, γ) will be referred to as free α-chains. The experiments presented in this paper show that these free α-chains are capable of combining with β-chains to form HbA and are, therefore, structurally normal. Alternatively, in the absence of added β-chains, α-chain aggregates of various sizes are formed.

Peripheral blood from patients with β-thalassemia was incubated with radioactive amino acids and hemolysates were prepared. Column chromatography demonstrates that a majority of the free α-chains are not present in HbA. They are strongly bound to carboxymethylcellulose resin at pHs from 7.0 to 10.0, and do not elute with HbA. However, when chemically prepared hemoglobin H ($Hb\beta_4$) is added to the fresh hemolysates, the free α-chains are readily recovered in the HbA peak. This indicates that the free α-chains are able to combine normally with β-chains to form HbA. Freshly labeled hemolysates were also subjected to Sephadex G-100 chromatography. The free α-chains eluted as a broad peak migrating between myoglobin and hemoglobin, consistent with their forming α-chain aggregates of various mol wt between 16,000 and 64,000.

It is suggested that the chromatographic behavior of the free α-chains reported herein simply reflects the chemical properties of normal α-chains in the absence of adequate numbers of β- or γ-chains. The tendency of these free α-chains to aggregate may lead to their intracellular precipitation and the subsequent destruction of the cells containing them.

Dr. Arthur Bank is a Leukemia Society Scholar.

Received for publication 6 October 1967 and in revised form 8 November 1967.

INTRODUCTION

The thalassemia syndromes, a group of hereditary anemias of man, are characterized by a decrease in the level of HbA (1). Normally, the synthesis of the α- and β-globin polypeptide chains which form HbA ($\alpha_2\beta_2$) is approximately equal (2–4). In the cells of patients with thalassemia, unbalanced globin chain synthesis occurs, and in β-thalassemia a relative excess of α-chain synthesis over β- and γ-chain synthesis has been documented (2–5). These free α-chains have been shown not to be present in HbA (4, 6, 7). This report demonstrates that the free α-chains produced by the cells of patients with β-thalassemia are capable of combining with added β-chains ($Hb\beta_4$) to form HbA and are, therefore, structurally intact. On the other hand, in the absence of β- or β-like–chains with which to combine, the free α-chains form aggregates of various molecular weights.

METHODS

Preparation of Hemolysates. Heparinized blood was obtained from patients with thalassemia major, thalassemia minor, and hemolytic anemias other than thalassemia. The washed cells were incubated with Krebs-Ringer bicarbonate solution and with either leucine-^{3}H, leucine-^{14}C, or ^{14}C-amino acid mixture, as described previ-

ously (4, 8). Incorporation into globin was always linear during the hour of incubation. After 1 hr, the incubation mixture was centrifuged, the supernate was removed, and the cells lysed with 4 volumes of a solution containing 0.002 M Mg for 1 min at 4°C, and returned to isotonicity with either 1 volume of 1.5 M sucrose or 1.5 M KCl. After the stroma and white cells were removed by centrifugation at 15,000 *g* for 15 min, carbon monoxide (CO) was bubbled through the hemolysate or the cyanomethemoglobin (CN) derivative was prepared (9).

Assays for radioactivity. Aliquots of hemolysates and column eluates were assayed for radioactivity as follows: 50 μl of a cold hemoglobin solution (15 mg/ml) was mixed with up to 0.8 ml of the column eluate. This was mixed with 10 ml of 3% acid acetone at −20°C and the precipitate was washed once with acetone, and dissolved in 0.2 ml of formic acid; 0.8 ml of water and 1.0 ml of 20% trichloroacetic acid (TCA) were then added, and the tube was heated at 90°C for 30 min. The precipitates were collected on Millipore filters, 2.5 cm diameter, and washed with 5% TCA. 0.2 ml of formic acid was added to the vial to dissolve the precipitate. 12 ml of Bray's solution was then added (10) and the vials counted in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Carboxymethylcellulose (CMC) chromatography. CMC resin (0.79 meq/g)[1] was equilibrated with 0.01 M potassium dihydrogen phosphate (KH_2PO_4) and the pH of the resin was adjusted to 6.0 before the column was packed. When less than 30 mg of hemoglobin was added, columns measuring 1.0 × 30 cm were used. After application of the sample, the hemoglobin eluted with a two-chamber gradient in which the first chamber contained 500 ml of 0.01 M KH_2PO_4 at pH 6.7, and the final chamber 500 ml of unbuffered 0.02 M K_2HPO_4 (11). For larger amounts of hemoglobin, a 2.5 × 30 cm column was used with each chamber containing 1000 ml of eluting fluid. The hemolysates added to these columns were dialyzed overnight against 0.01 M KH_2PO_4 at pH 6.7 unless otherwise specified. Optical density (OD) measurements were performed on the eluates and hemolysates at 280 and 415 mμ using a Beckman spectrophotometer. Radioactivity was measured as described previously.

Sephadex G-100 chromatography. Sephadex G-100 was equilibrated at 4°C with either 0.1 M Tris buffer (pH 7.5) or a buffer that contained 0.1 M Na_2HPO_4, 0.1 M NaCl, and 0.01 M KCN adjusted to pH 6.9 (7). 0.4–1.0 ml of fresh hemolysates (less than 4 hr old) with hemoglobin concentrations of 5–20 mg/ml was added to columns measuring 1.1 × 150 cm. 0.05–0.10 ml of myoglobin solutions that contained 50 mg/ml was added as a marker where indicated. 0.4–1.2-ml aliquots of eluate were collected. The eluates were assayed for their optical density and radioactivity content as described above.

Globin-chain chromatography. Globin was prepared from hemolysates and the α, β, and γ chains identified and quantitated as described previously (4). The total radioactivity in individual globin-chain peaks was determined by precipitation of the protein of all fractions of each peak and by totaling the radioactivity present in these fractions.

[1] Serva CM, Gallard-Schlesinger Chemical Mfg. Corp., L. I., New York.

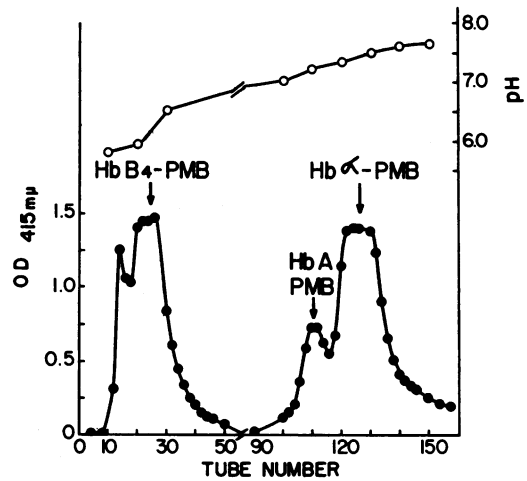

FIGURE 1 Elution pattern from CMC-chromatography of PMB-treated hemolysate of a normal subject. Heparinized blood from a normal subject was treated with PMB and chromatographed with CMC-chromatography as described in Methods. (●—●) represents the OD 415, and (○—○) the pH of the eluted fractions.

Preparation of Hbβ_4 and Hbα. Fresh CO-treated hemolysates were mixed with *para*-hydroxy-mercuribenzoate (PMB) overnight, as described in detail by Bucci and Fronticelli (11). CMC-chromatography with the 2.5 × 30 cm column was then performed (Fig. 1). The PMB derivatives of Hbβ_4 and Hbα eluted and were identified by globin-chain chromatography. The Hbα PMB derivative was dialyzed overnight against 0.05% thioglycollate in 0.01 M KH_2PO_4 buffer at pH 7.4 to remove the mercury groups (11) and was then rechromatographed on the large-sized CMC column described. The Hbβ_4 PMB peak was concentrated and chromatographed with a mercaptoethanol-treated Sephadex G-10 column (12) in order to remove the mercury groups.

RESULTS

Free α-chains on CMC-chromatography of hemolysates

(*1*) *In the absence of added β-chains.* The elution pattern of the PMB derivatives of HbA, Hbβ_4, and Hbα is shown in Fig. 1. HbA and Hbβ_4 eluted in the same position as their PMB derivatives with this chromatographic system (12). Dialyzed hemolysates were prepared from the radioactively labeled peripheral blood of thalassemic subjects (thal hemolysates) and chromatographed, using the 0.01 M–0.02 M phosphate gradient de-

scribed in Methods. 80–90% of the OD 415 added to the CMC column was recovered in the HbA region. This recovery is comparable to that of lysates prepared from the cells of non thalassemic subjects (non-thal hemolysates). On the other hand, only 20–30% of the radioactivity added to the column was recovered from the thal hemolysates; 80–90% of the added radioactivity was recovered when non-thal hemolysates were used. In addition, no additional peak of radioactivity in the area of the Hbα PMB (Fig. 1) was seen when thal hemolysates were used. Attempts to remove more radioactivity from the CMC resin with 0.1 M K_2HPO_4 as an eluent were also unsuccessful. The majority of the radioactivity present in the thal hemolysates remained bound to the CMC resin at pH 10.0 at a 0.1 M phosphate buffer concentration (Table I).

In order to investigate the possibility that the poor recovery of radioactivity when thal hemolysates were used was due to a loss of α-chains, the properties of Hbα on CMC resin were studied. Hbα was prepared by PMB treatment of a normal adult hemolysate, and CMC-chromatography, and subsequent removal of the mercury from the isolated Hbα PMB peak were performed as described in Methods. This Hbα fraction was then reapplied to a CMC column at pH 6.7 and rechromatographed with the 0.01 M KH_2PO_4 at pH 6.7 to 0.02 M K_2HPO_4 gradient. More than 90% of the OD 415 remained bound to the CMC resin. This experiment indicated that the radioactivity bound to the CMC resin using the radioactive thal hemolysates could represent normal Hbα or free α-chains.

Globin-chain analysis was then performed on the HbA peaks obtained from CMC-chromatography of two thal hemolysates. The $\alpha/(\beta$ and $\gamma)$ ratio of radioactivity in these two thal hemolysates before CMC chromatography was 4.8 and 3.3 whereas that of the HbA peak eluted from the CMC resin was 1.2 and 1.3 (Table I, Fig. 2). In the two experiments, 84 and 90% of the γ- and β-chain radioactivity present in the initial thal hemolysate was recovered in the HbA peak, whereas only 19 and 33% of the α-chain radioactivity was recovered (Table I). These data indicate a marked preferential loss of α-chains from the total hemolysate during the chromatography, presumably due to irreversible binding of the chains to the CMC resin.

(2) In the presence of Hbβ_4. In two separate experiments, one-half of a labeled thal hemolysate was mixed with Hbβ_4, prepared as described in Methods, immediately after lysis of the thal peripheral blood. The other halves of the same thal he-

TABLE I

Results of Globin-Chain Chromatography of Total Hemolysates and HbA Peaks Isolated from CMC Columns

	Experiment No.	cpms		
		β and γ	α	$\alpha/(\beta$ and $\gamma)$
I	Total hemolysate	1202	5756	4.8
	HbA (no Hbβ_4)	896	1112	1.2
	HbA (added Hbβ_4)	1088	5540	5.1
II	Total hemolysate	540	1800	3.3
	HbA (no Hbβ_4)	473	604	1.3
	HbA (added Hbβ_4)	456	1740	3.8

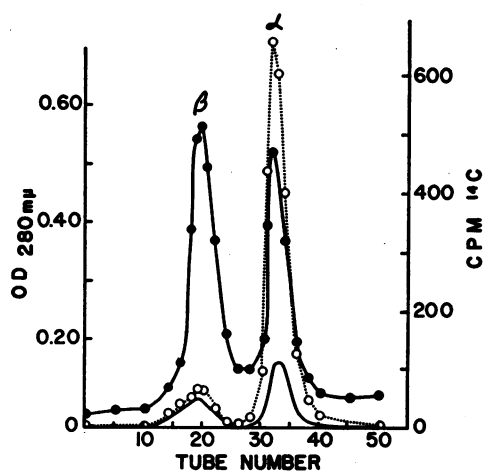

FIGURE 2 Elution pattern from globin chain chromatography of HbA-isolated from radioactively labeled thal hemolysates in the presence and absence of added Hbβ_4. One-half of a radioactively labeled hemolysate of a patient with thalassemia major was mixed with Hbβ_4 immediately after lysis of the cells, whereas the other half was not. After a 2 hr dialysis, as described in Methods, each half of the hemolysate was subjected to CMC-chromatography and the HbA peaks eluted, were pooled separately, and subjected to globin-chain chromatography separately. The figure represents the superimposition of the elution patterns of the two globin chain columns. (●—●) is the OD 280, (—) the ^{14}C radioactivity in globin chains when no Hbβ_4 had been added, and (○—○) the ^{14}C radioactivity in the presence of Hbβ_4. The numerical data corresponding to that presented in this figure are shown in Table I, Experiment I.

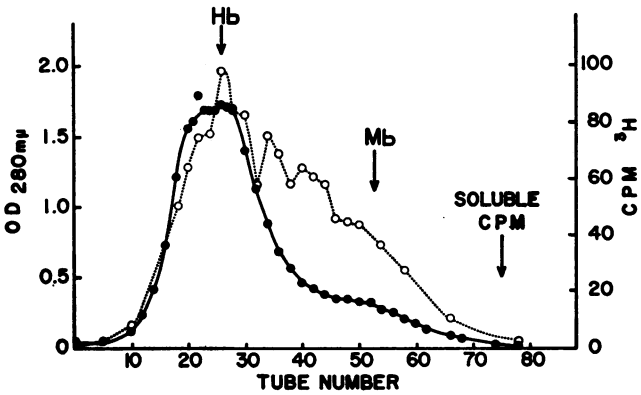

FIGURE 3 Elution pattern from Sephadex G-100 chromatography of a radioactively labeled thal hemolysate. 0.5 ml of the fresh hemolysate of patient D. G. (Table II) was applied to the column along with 0.1 ml of a myoglobin solution (50 mg/ml). Sephadex G-100 chromatography was performed as described in Methods. (●—●) represents the OD 280, and (○—○) the ^3H radioactivity. The arrow with Hb denotes the area at which hemoglobin eluted, the arrow with Mb the area at which myoglobin eluted, and the arrow with soluble cpms the area at which radioactivity of free amino acids eluted.

molysates were analyzed without Hbβ_4 addition and have been discussed. In Experiment I (Table I, Fig. 2), the hemolysates (both with and without Hbβ_4) were dialyzed against 0.01 M KH_2PO_4 at pH 6.7 for 2 hr, whereas in Experiment II overnight dialysis was performed. The HbA peaks were isolated by CMC-chromatography and subjected to globin-chain chromatography. The isolated HbA of the portions of the thal hemolysates which were mixed with Hbβ_4 now revealed 96% recovery of the α-chains present initially in the total hemolysate, whereas β-chain recovery was similar to that obtained without Hbβ_4 (Table I, Fig. 2). This almost complete recovery of radioactive α-chains in HbA on the addition of Hbβ_4 demonstrates that the free α-chains of thal hemolysates are capable of combining with β-chains.

Free α-chains on Sephadex G-100 chromatography

A single hemoglobin(Hb) peak identified at OD 415 eluted from the Sephadex G-100 columns (Fig. 3). 60–80% of the radioactivity applied to the column was recovered in the Hb peak when non-thal hemolysates were applied, whereas only 10–30% of the radioactivity of thal hemolysates was recovered in this region. 40–50% of the radioactivity was recovered in the Hb area when hemolysates from the cells of patients with thalassemia minor were used. Additional radioactive peaks of molecular weight lighter than that of Hb and intermediate between that of Hb and myoglobin were seen only when thal major or thal minor hemolysates were used (Table II, Fig. 3). No such additional lighter peaks were seen with non-thal hemolysates. The amount of radioactivity recovered in the region lighter than Hb varied between 10 and 40% of the total radioactivity added to the columns. No single symmetrical peak lighter than Hb eluted from these columns despite the use of fresh hemolysates in either the oxy, CO, or CN form.

Globin-chain chromatography revealed that the lighter peaks of radioactivity in the thal major and minor hemolysates contained predominantly radioactive α-chains (Table II, Fig. 4C). In each of the patients studied, the $\alpha/(\beta$ and $\gamma)$ ratio of radioactivity in these lighter regions was significantly higher than that of the total hemolysate (Table II, Fig. 4). Globin-chain analysis of the isolated Hb region revealed an $\alpha/(\beta$ and $\gamma)$ ratio of radioactivity of 1.0 or less, which shows a preferential decrease in α-chains (Table II, Fig. 4B).

TABLE II

Results of Globin-Chain Chromatography of Hemolysates and Peaks Isolated from Sephadex G-100 Columns

	$\alpha/(\beta$ and $\gamma)$ Ratios of radioactivity		
	Total hemolysate	Hb region	Lighter region
Thalassemia major			
D.G.	2.8	0.94	7.8
I.J.	3.3	1.0	4.1
A.A.	4.8	0.5	ND*
Thalassemia minor			
D.D.	2.0	1.0	4.5
M.L.	2.2	1.9	3.0
G.G.	2.4	1.2	ND
Normal			
E.G.	0.9	1.0	0‡

* ND, not done.
‡ 0, none present.

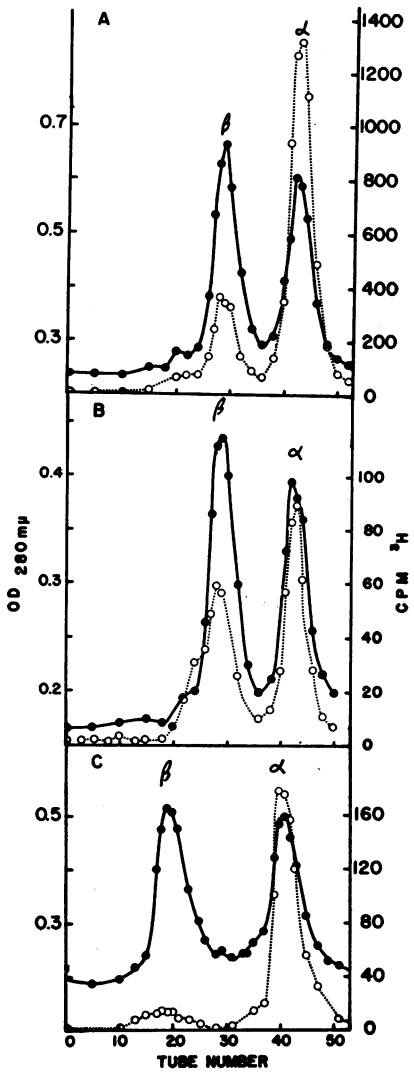

FIGURE 4 Elution pattern from globin-chain chromatography of a radioactively labeled thal hemolysate and the radioactively labeled peaks separated from it by Sephadex G-100 chromatography. (A) The total hemolysate of patient D. G. was subjected to globin-chain chromatography. (B) Tubes 15–30 eluted from Sephadex G-100 as shown in Fig. 3 were pooled and subjected to globin-chain chromatography. (C) Tubes 31–50 eluted from Sephadex G-100, as shown in Fig. 3, were pooled and subjected to globin-chain chromatography. (●—●) represents the OD 280 and (○—○) the ^3H radioactivity. The numerical data corresponding to that presented in this figure are shown in Table II, D. G. 2 ml of a cold non-thal hemolysate containing 15 mg/ml of Hb was added as OD 280 marker to pooled peaks (B) and (C) before globin-chain chromatography.

DISCUSSION

The underlying defect in the erythroid cells of patients with β-thalassemia is believed to be a defect in the gene for β-chain synthesis (1, 4). This leads to either decreased or defective synthesis of mRNA for β-chains, which in turn results in the decreased synthesis of β-chains (13). α-Chain synthesis and release from the ribosome proceed at a normal rate. This results in a relative excess of α-chains (4, 14). The studies presented here suggest that these excess or free α-chains are structurally intact, since they are capable of combining normally with added β-chains to form HbA.

The synthesized α-chains have been shown previously (4) and in the present experiments to migrate indistinguishably from the α-chains of HbA when subjected to globin-chain CMC-chromatography. Using the CMC-chromatographic system for separating HbA described in this paper, the majority of the α-chains do not migrate with HbA in the absence of added β-chains (Table I, Fig. 2). The addition of $Hb\beta_4$ leads to an alteration in the migration of the newly synthesized α-chains into a peak that is chromatographically identical with HbA. These results strongly suggest that the synthesized α-chains are capable of forming HbA when adequate numbers of β-chains are present.

It is possible that some other molecule containing β-chains and the synthesized α-chains could migrate in the HbA peak obtained from CMC and not be HbA. Using starch gel electrophoresis, compounds that contained α- and β-chains have been isolated wherein it is believed that only two of the four globin chains of the $\alpha_2\beta_2$ tetramer are linked to heme groups (15). This method of separation was not used with radioactive hemolysates in the experiments reported here because elution of material from these gels is difficult and variable; this problem makes it impossible to accurately determine the per cent of radioactive α-chains recovered from different parts of the gel in such experiments.

The experiments with Sephadex G-100 chromatography demonstrate that the free α-chains have no single, predominant molecular configuration. Rather, they form aggregates ranging from monomers to tetramers migrating between myoglobin and Hb. The irreversible binding of the free

α-chains to CMC resin may also reflect the tendency of α-chains to aggregate. A similar state of α-chain aggregation has been noted by other authors when these chains are prepared by chemical means (12, 16). In these latter studies, the average mol wt determined for the α-chains were between 21,000 and 30,000, which indicates the presence primarily of monomers and dimers. In addition, it has been suggested that the size of the α-chain aggregates increases with the concentration of α-chains present (12).

Chemically prepared α-chains have also been shown to be capable of combining with added β-chains to form HbA (12, 16). In addition, the chemically reconstituted HbA has been shown to have a normal oxygen dissociation curve, indicating that these α-chains are functionally intact (12). The similar properties of the free α-chains described here with those of chemically prepared α-chains suggest that the free α-chains produced in the cells of patients with β-thalassemia may be functionally, as well as structurally, normal when adequate numbers of β-chains are present. Only oxygen dissociation curves of reconstituted HbA formed by the addition of $Hb\beta_4$ to fresh thal hemolysates would confirm the functional integrity of the excess α-chains of the thal hemolysates. However, such studies are not possible, since the minute amount of reconstituted HbA containing the excess α-chains is inseparable from the bulk of the unlabeled, preformed HbA present in these hemolysates.

In studies similar to those described, using radioactively labeled thal major hemolysates subjected to Sephadex G-100 chromatography, Huehns and Modell have reported finding two peaks of radioactivity lighter than Hb consistent with Hbα monomers and dimers (6, 7). On the addition of the $Hb\beta_4$ to these peaks, they were found to migrate on starch gel electrophoresis in the region of HbA (7). Experimental details of the methods of elution and the percentage of recovery of α-chains in HbA on the starch gels were not presented in this report (7). In addition, the radioactive peaks were not characterized with regard to their globin-chain composition, and were identified only by their migration on the starch gels. Other authors (5), using sucrose-density gradient centrifugation, have reported that the α-chains in thal hemolysates have a molecular weight similar to that of Hb, which suggests an α-chain tetramer as the predominant form.

The $\alpha/(\beta \text{ and } \gamma)$ ratio of radioactivity in globin prepared from the Hb region eluted from Sephadex G-100 chromatography was less than 1.0 in one of three thal major hemolysates (Table II, A. A.). This result is consistent with the suggestion that in the cells of these patients, a pool of free, unlabeled α-chains is present which dilutes the α-chain radioactivity in newly synthesized HbA (7). However, the $\alpha/(\beta \text{ and } \gamma)$ ratios of the HbA of the two other thal major hemolysates, as well as of each of the three thal minor hemolysates, were equal to, or exceeded, 1.0 and, therefore, provide no evidence of a significant pool of α-chains in these hemolysates. One explanation for the $\alpha/(\beta \text{ and } \gamma)$ ratios of greater than 1.0 in the HbA obtained from thal major hemolysates from CMC-chromatography (Table I) is that it results from the exchange of free α-chains into HbA during the dialysis and preceding this chromatography (4, 7).

The relationship between the in vitro properties of free α-chains and their fate in vivo remains unclear. Fessas, Loukopoulos, and Kaltsoya have shown that the inclusion bodies found in the erythroid cells of patients with thalassemia major are composed of aggregates of α-chain peptides (17). After splenectomy, more inclusions are present in the circulating erythroid cells, which suggests that the spleen preferentially destroys cells containing these inclusions (18). The soluble α-chain aggregates described in this report may represent an intermediate stage between free α-chains released from the ribosomes, and insoluble α-chain aggregates. α-Chain aggregation may simply be due to the unique chemical properties of normal α-chains when adequate numbers of β or β-like-chains with which to combine are absent.

It has been proposed on the basis of studies which used a rabbit reticulocyte system that, α-chains are normally required for the release of β-chains from the ribosomes, and that this provides a mechanism for the coordination of α- and β-chain synthesis (19). An alternative suggestion for the synchronization of α- and β-chain synthesis has been made by others (20), and is supported by the data presented here. This hypothesis involves the

destruction of cells containing significant amounts of excessive α- or β-chains. Since α- and β-chains are of approximately the same size, and serve the same function, it would not be surprising if they were synthesized at approximately the same rate in most erythroid precursors under normal conditions. Red cell precursors in which unbalanced α- and β-chain synthesis occurred would be preferentially destroyed. There is evidence that in patients with HbH disease, the cells containing the most $Hb\beta_4$ have the shortest half-life (21). The α-chain aggregation reported here in the cells of patients with β-thalassemia and the proposed preferential destruction of excess α-peptide-containing cells (4, 16, 17) are also consistent with this idea.

ACKNOWLEDGMENTS

I am especially indebted to Doctor Paul A. Marks for his advice, support, and encouragement during the course of this research. I am grateful to Doctors Reinhold and Ruth Benesch for several useful discussions. I also wish to thank Doctors James Wolff and Marion Erlandson and their colleagues for the use of their patients.

This work was supported in part by the U. S. Public Health Service grant GM-14552, National Science Foundation grant GB-4631, and the Cooley's Anemia Foundation.

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