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### Research Article

Hemoglobin Gun Hill is an unstable mutant hemoglobin associated with mild compensated hemolysis. This abnormal protein has a deletion of five amino acids in the  $\beta$ -chains. The deletion includes the heme-binding proximal histidine at position 92. The  $\beta$ -chains of hemoglobin Gun Hill lack heme groups. Approximately 32% of the circulating hemoglobin in heterozygous subjects consists of the mutant hemoglobin. When reticulocytes were incubated with radioactive amino acid the specific activity of hemoglobin Gun Hill was three to six times that of hemoglobin A. Total incorporation of radioactivity into hemoglobin Gun Hill was two to three times that into hemoglobin A. There were 20-50% more total counts in  $\beta$ -Gun Hill ( $\beta^{\text{GH}}$ ) than in  $\beta^{\text{A}}$ . These results indicate that in reticulocytes there was greater synthesis of the abnormal  $\beta$ -chains than  $\beta^{\text{A}}$ -chains. The ratio of the specific activities of the  $\alpha$ -chains of hemoglobin Gun Hill to the  $\alpha$ -chains of hemoglobin A was 20: 1. There was evidence of a free pool of  $\alpha$ -chains in the reticulocytes containing hemoglobin Gun Hill. After 10 min of incubation approximately 40% of the total  $\alpha$ -chain radioactivity was in the free pool. When protein synthesis was blocked by incubation of reticulocytes with puromycin, the specific activity of the  $\alpha$ -chains of hemoglobin Gun Hill continued to increase due to direct exchange of  $\alpha$ -subunits between the free pool and preformed [...]

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# Synthesis of Hemoglobin Gun Hill: Increased Synthesis of the Heme-Free $\beta^{\text{GH}}$ Globin Chain and Subunit Exchange with a Free $\alpha$ -Chain Pool

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**ABSTRACT** Hemoglobin Gun Hill is an unstable mutant hemoglobin associated with mild compensated hemolysis. This abnormal protein has a deletion of five amino acids in the  $\beta$ -chains. The deletion includes the heme-binding proximal histidine at position 92. The  $\beta$ -chains of hemoglobin Gun Hill lack heme groups. Approximately 32% of the circulating hemoglobin in heterozygous subjects consists of the mutant hemoglobin. When reticulocytes were incubated with radioactive amino acid the specific activity of hemoglobin Gun Hill was three to six times that of hemoglobin A. Total incorporation of radioactivity into hemoglobin Gun Hill was two to three times that into hemoglobin A. There were 20–50% more total counts in  $\beta$ -Gun Hill ( $\beta^{\text{GH}}$ ) than in  $\beta^{\text{A}}$ . These results indicate that in reticulocytes there was greater synthesis of the abnormal  $\beta$ -chains than  $\beta^{\text{A}}$ -chains. The ratio of the specific activities of the  $\alpha$ -chains of hemoglobin Gun Hill to the  $\alpha$ -chains of hemoglobin A was 20:1. There was evidence of a free pool of  $\alpha$ -chains in the reticulocytes containing hemoglobin Gun Hill. After 10 min of incubation approximately 40% of the total  $\alpha$ -chain radioactivity was in the free pool. When protein synthesis was blocked by incubation of reticulocytes with puromycin, the specific activity of the  $\alpha$ -chains of hemoglobin Gun Hill continued to increase due to direct exchange of  $\alpha$ -subunits between the free pool and preformed hemoglobin Gun Hill. Studies of the assembly of  $\beta^{\text{A}}$  and  $\beta^{\text{GH}}$  revealed that the rates of translation of the two polypeptide chains were equal and uniform. No evidence was obtained for the existence of "slow points" in the process of globin chain assembly. The studies also suggest that lack of strong heme-globin binding does not hinder the synthesis of globin chains.

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## INTRODUCTION

Abnormal hemoglobins generally occur in smaller amounts than hemoglobin A in the blood of heterozygotes (1). The degree of preponderance of the normal form over the mutant form varies considerably among the different hemoglobins (2). Unstable hemoglobins are often present in only small quantities in affected patients (3). These variations in the quantities found may be due to differences in the rates of synthesis, or rates of destruction, of these structurally altered molecules (3, 4).

Several stages in the pathway of the synthesis of globin chains, including the process of translation, have been considered as possible points of regulation of hemoglobin production (1, 5, 6). Certain authors have presented evidence that heme stimulates the synthesis of globin (7–9). Other experiments have suggested that heme attaches to the growing globin polypeptide chains on the ribosomes, and at the point of heme attachment, there is a slowing down of the process of chain assembly or translation (10).

Hemoglobin Gun Hill is an unstable mutant having a deletion of five amino acids in the  $\beta$ -chains (11, 12). The deletion includes the heme-binding histidine at  $\beta 92$ . As a result of the deletion, the  $\beta$ -chains of hemoglobin Gun Hill lack heme. This abnormal hemoglobin, with its missing section of polypeptide chain and its deficiency of heme groups, provided a unique opportunity to examine certain aspects of hemoglobin synthesis.

## METHODS

*Handling of blood samples.* Venous blood samples were collected using heparin as the anticoagulant. The samples were packed in ice and transported to the laboratory within 30 min of collection.

*Hematologic data.* Standard methods were employed for the routine hematologic examinations (13). The concentrations of hemoglobin solutions were determined by comparing

the optical densities at 540  $\mu$  of the cyanmethemoglobin derivatives to a reference standard (Acuglobin, Ortho Pharmaceutical Corp., Raritan, N. J.). Because of the absence of heme groups on the  $\beta$ -chains of hemoglobin Gun Hill, the extinction coefficient at 540  $\mu$  of the mutant hemoglobin is half that of hemoglobin A. The concentrations of globin solutions were determined using the Kjeldahl protein nitrogen method or the biuret reaction (14) and a reference protein standard (Versatol, Warner-Chilcott, Morris Plains, N. J.).

*Hemoglobin synthesis in vitro.* Incubation of reticulocytes with radioactive amino acid was performed by a modification of the method of Lingrel and Borsook (15). Except for the incubation step all manipulations of the reticulocytes were done at 4°C. Plasma was removed by centrifugation of the anticoagulated blood at 8000 rpm for 5 min in a Sorvall RC2-B refrigerated centrifuge. In most experiments the cells from 20 ml of blood were transferred to 1 ml conical Pyrex tubes and centrifuged at 15,000 rpm for 50 min. The top 10% of the packed cells from each tube was removed, pooled, and resuspended in 0.13 M NaCl, 0.005 M KCl, 0.0074 M MgCl<sub>2</sub> (NKM). This procedure served to increase the reticulocyte percentage from 5 to 10% to approximately 20%.

The concentrated reticulocytes were washed twice with NKM and once with the incubation mixture of Lingrel and Borsook (15) lacking leucine. The cells were resuspended in incubation medium and incubated for 10 min at 37°C in a Dubnoff-type metabolic shaker. 250  $\mu$ Ci of leucine-<sup>3</sup>H or 5–15  $\mu$ Ci of leucine-<sup>14</sup>C was added to 1 ml of cell suspension and incubation continued for time periods varying from 3.5 min to 5.5 hr. Incubation was terminated by pipetting portions of the cell suspension into 10–20 volumes of ice-cold NKM. The cells were washed twice in NKM, frozen, and thawed.

*Analysis of radioactive hemoglobin.* Erythrocyte samples intended for starch-block electrophoresis of hemoglobin were hemolyzed by shaking with an equal volume of distilled water and one-half volume of carbon tetrachloride. Stroma was removed by centrifugation at 10,000 rpm. Hemoglobin A and hemoglobin Gun Hill were separated by starch-block electrophoresis according to the method of Kunkel, Ceppellini, Müller-Eberhard, and Wolf (16). Hemoglobin A<sub>2</sub> has the same electrophoretic mobility as hemoglobin Gun Hill and was thus included with the abnormal fraction during subsequent processing.

Samples of erythrocytes for column chromatography of hemoglobin were hemolyzed by the addition of four volumes of distilled water. Stroma was removed by centrifugation at 20,000 rpm for 30 min. In some experiments, employing unfractionated lysates, the entire mixture was processed without first attempting to remove cell membranes. Hemoglobin A, hemoglobin Gun Hill, and free  $\alpha$ -hemoglobin chains, were separated by chromatography on 1  $\times$  30 cm columns of diethylaminoethyl (DEAE) cellulose according to the method of Chernoff, Pettit, and Northrop (17). Hemoglobin A<sub>2</sub> did not separate from hemoglobin Gun Hill and the contribution of this minor normal hemoglobin to the experimental results was ignored. Before application to the DEAE columns, the hemoglobin samples were equilibrated with the starting buffer, by passage through a 2.5  $\times$  65 cm Sephadex G-25 column. The procedure also served to remove free radioactive leucine.

The  $\alpha$ ,  $\beta^A$ , and  $\beta$ -Gun Hill ( $\beta^{GH}$ ) globin polypeptide chains were prepared from hemolysates, purified hemoglobin A, and hemoglobin Gun Hill, by a modification of the method of Clegg, Naughton, and Weatherall (18). Heme was removed

by the acid-acetone method (19). 20–80 mg of lyophilized globin was dissolved in 5 ml of a pH 8.7 buffer containing 8 M urea, 0.1 M boric acid, 0.004 M ethylenediaminetetraacetic acid (EDTA), 0.04 M NaOH, and 7 mg/ml of 2,2'-dithiobisethylamine dihydrochloride (cystamine) (20).

After standing at room temperature for 1 hr, the cystamine-treated globin samples were dialyzed for 2.5 hr against three changes of 100 ml of 0.005 M Na<sub>2</sub>HPO<sub>4</sub>, pH 6.7, containing 8 M urea. The protein was then applied to a 1.5  $\times$  30 cm column of carboxymethyl cellulose equilibrated with the same urea buffer. The column was continuously monitored by a UV absorbance detector. After the initial peak of nonglobin protein was removed, the  $\alpha$ ,  $\beta^A$ , and/or  $\beta^{GH}$  chains were eluted with a linear gradient formed by mixing 275 ml of the starting buffer and 275 ml of an 8 M urea buffer containing 0.03 M Na<sub>2</sub>HPO<sub>4</sub>, pH 6.7. Mercaptoethanol was omitted from the buffers in this system.

*Measurement of radioactivity incorporated into hemoglobin and globin.* In the initial experiments, the <sup>14</sup>C radioactivity in hemoglobin A and hemoglobin Gun Hill fractions obtained by starch block electrophoresis was determined using a Nuclear-Chicago low background gas-flow counter. Radioactivity in pooled, dialyzed, globin prepared from hemolysates, purified hemoglobin A and hemoglobin Gun Hill, was measured in a Beckman LS-250 liquid scintillation counter, (Beckman Instruments, Inc., Fullerton, Calif.) using the scintillation mixture described by Bray (21). The radioactivity in the effluent of the 8M urea columns was monitored by adding 1 ml of each fraction to 10 ml of Bray's solution. Radioactivity in hemoglobin fractions from the DEAE columns was measured by adding 1 ml of each fraction to 15 ml of Bray's solution containing 60 g/liter of a thixotropic gelling agent (Cab-O-Sil, Beckman Instruments Inc., Fullerton, Calif.). The uniformity of quenching was monitored by the combination external standard-channels ratio method but no corrections were made.

*Studies of  $\beta$ -globin chain assembly.* The method was based on that of Dintzis (22) using the technique detailed by Clegg, Weatherall, Na-Nakorn, and Wasi (23). Globin was prepared from reticulocytes which had been incubated for 3.5 and 10 min with leucine-<sup>3</sup>H. The  $\beta^{GH}$ ,  $\beta^A$ , and  $\alpha$ -chains were separated by urea-CMC chromatography. The central portions of the  $\beta^A$  and  $\beta^{GH}$  peaks were pooled separately and the total radioactivity determined in each sample. Uniformly labeled  $\beta^A$  and  $\beta^{GH}$  chains, prepared by incubating reticulocytes 5.5 hr with leucine-<sup>14</sup>C, were added to the corresponding leucine-<sup>3</sup>H-labeled samples to give a final ratio of 2–3:1, <sup>3</sup>H to <sup>14</sup>C.

2-Mercaptoethanol was added to give a final concentration of 0.05 M, and the mixture allowed to stand at room temperature for 1 hr. After addition of solid tris(hydroxymethyl) amino methane to a concentration of 1 mole/liter, the pH was adjusted to 9.2 with concentrated HCl. 0.5 M ethylenimine was added and the mixtures allowed to stand 2.5 hr. The aminoethylated globin chains were dialyzed extensively against water, lyophilized, and digested with trypsin.

*Fingerprint analysis.* 2–5 mg of trypsin-digested globin was applied to an 18  $\times$  36 inch sheet of Whatman 3MM filter paper. The material was spotted in the middle of the long dimension of the paper, 3.5 inches from the edge. Electrophoresis was carried out at 2500 v for 3 hr in a Varsol-cooled lucite tank (Savant Instruments, Inc., Hicksville, N. Y.) using a 1.25% acetic acid, 1.25% pyridine, pH 4.7 buffer. After drying, the sheets were cut perpendicular to the direction of electrophoresis at points 3.5 inches, anodic, and 15.5 inches, cathodic, to the origin. By this tech-

TABLE I  
Incorporation of Leucine-<sup>14</sup>C into HbA and Hb  
Gun Hill by Reticulocytes

Experi- ment No.	Time	Specific activity		Total radioactivity*	
		HbA	Hb Gun Hill	HbA	Hb Gun Hill
	min	cpm/mg		cpm	
1	5	3.4	12.7	221	406
	30	21.4	88.8	1,391	2,842
	60	43.0	191.6	2,795	6,131
	240	109.0	453.0	7,085	14,496
2	15	23.4	124.0	1,521	3,968
3	30	15.0	56.0	975	1,792
	60	22.0	86.0	1,430	2,752
4	5	3.4	10.9	221	349
	60	19.7	69.7	1,281	2,230
	120	30.1	186.2	1,957	5,958
5	5	1.4	4.4	91	141
	60	12.7	70.9	826	2,269
	120	22.6	128.3	1,469	4,106

\* Total leucine-<sup>14</sup>C incorporated per 100 mg total hemolysate hemoglobin calculated on the basis of 65% hemoglobin A and 32% hemoglobin Gun Hill.

nique, all the tryptic peptides (Tp) of the  $\beta$ -chain, except the leucine-lacking Tp VII, Tp VIII, and Tp VII-VIII, were retained on the remaining  $19 \times 18$  inch sheet of paper. Descending chromatography was performed perpendicular to the direction of electrophoresis for 18 hr using a solvent composed of butanol:acetic acid:water:and pyridine, 15:3:12:10 v/v. The fingerprints were stained with ninhydrin and the peptide spots cut out and eluted as described by Clegg and associates (23). The two isotopes were counted simultaneously using the "<sup>3</sup>H" and "<sup>14</sup>C above <sup>3</sup>H" preset windows of the LS-250 scintillation counter. The gain setting was adjusted to give an efficiency of 16.5% for <sup>3</sup>H and 7.2% for <sup>14</sup>C in the lower channel and 0.46% for <sup>3</sup>H and 57.7% for <sup>14</sup>C in the upper channel.

**Materials.** L-leucine-(4,5-<sup>3</sup>H), 6 Ci/mmole, and L-leucine-<sup>14</sup>C, 250 mCi/mmole were purchased from Schwarz Bioresearch. Cystamine diHCl was obtained from Sigma Chemical Co., St. Louis, Mo.

## RESULTS

*Incorporation of radioactive amino acid into whole hemoglobin A and whole hemoglobin Gun Hill in vitro.* Reticulocytes were incubated in the presence of leucine-<sup>14</sup>C followed by separation of hemoglobin A and hemoglobin Gun Hill by starch-block electrophoresis. The contamination of hemoglobin Gun Hill by hemoglobin A<sub>2</sub> was ignored because of poor synthesis of the minor normal hemoglobin by reticulocytes (24). In all ex-

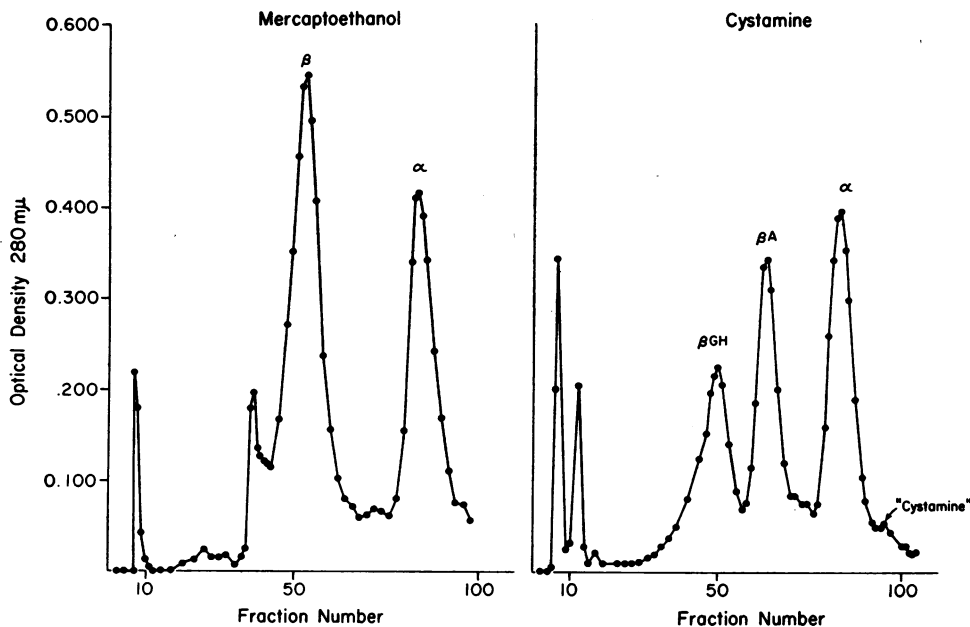


FIGURE 1 Column chromatography on CMC in 8 M urea of globin prepared from a hemolysate containing hemoglobin Gun Hill. The left panel shows no separation of  $\beta^A$  and  $\beta^{GH}$  when the standard technique of Clegg and associates (18) was employed using 2-mercaptoethanol. The right panel shows separation of the two  $\beta$ -chains after treatment of the globin with cystamine. Excess cystamine was frequently detected as a small peak with an optical density at 280  $m\mu$  of approximately 0.05 on the descending portion of the  $\alpha$ -chain peak. The  $\beta$ -chain peaks are larger than the corresponding  $\alpha$ -chain peaks because of the greater extinction coefficient of the  $\beta$ -chains at 280  $m\mu$ .

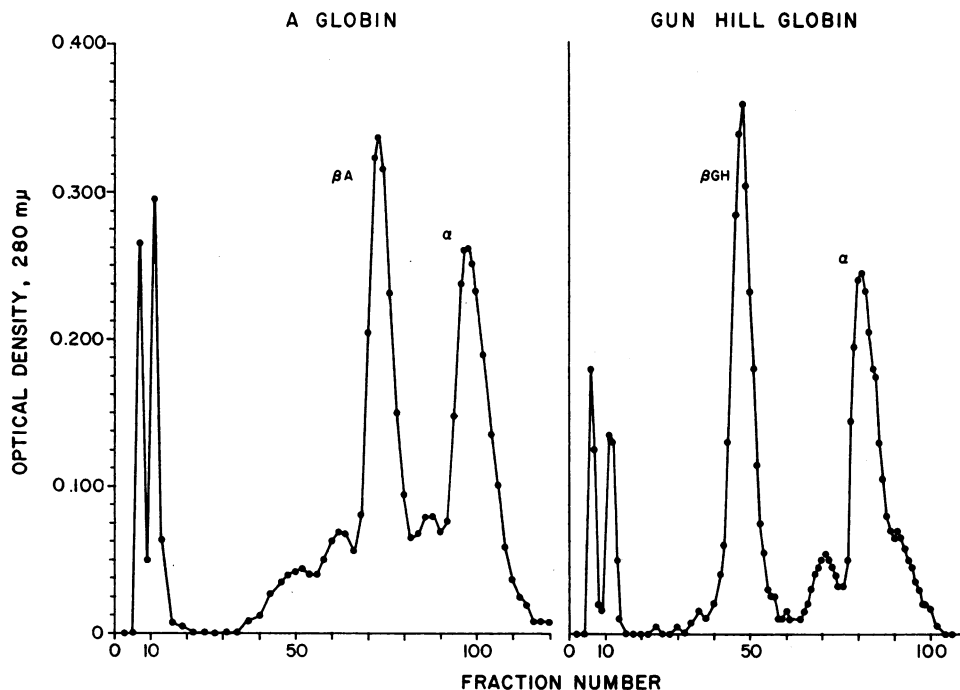


FIGURE 2 Urea-CMC chromatography of cystamine-treated globin prepared from purified hemoglobin A and hemoglobin Gun Hill. Only one  $\beta$ -peak was present in either purified hemoglobin.

periments, after 5 to 240 min of incubation, the specific activity of hemoglobin Gun Hill was 3–6 times greater than hemoglobin A (Table I).

When the specific activities were calculated from hemoglobin determinations based on either heme absorption (corrected for heme depletion in hemoglobin Gun Hill) or direct protein measurement no differences were noted in the results.

Starch-block electrophoresis indicated that approximately 32% of the hemoglobin in the hemolysates consisted of hemoglobin Gun Hill. From the specific activities and the relative proportions of the two hemoglobins, it was calculated that the total incorporation of radioactivity into hemoglobin Gun Hill was 2–3 times greater than into hemoglobin A (Table I).

*Separation of  $\beta^A$  and  $\beta^{GH}$  globin chains by column chromatography.* The measurements of incorporation of labeled amino acid into whole hemoglobin suggested that significantly more hemoglobin Gun Hill than hemoglobin A was synthesized by reticulocytes *in vitro*. A more direct comparison of the total radioactivity incorporated into  $\beta^A$  and  $\beta^{GH}$  was sought using the method of globin chain chromatography on CMC in 8 M urea developed by Clegg, Naughton, and Weatherall (18, 25). However,  $\beta^A$  and  $\beta^{GH}$  globin chains are identically charged (the differences in the electrophoretic mobilities of hemoglobin A and hemoglobin Gun Hill are due

to differences in heme content), and no separation was achieved by chromatography in the presence of 2-mercaptoethanol (Fig. 1). Therefore, advantage was taken of the fact that the reactive  $\beta 93$  cysteine is one of the

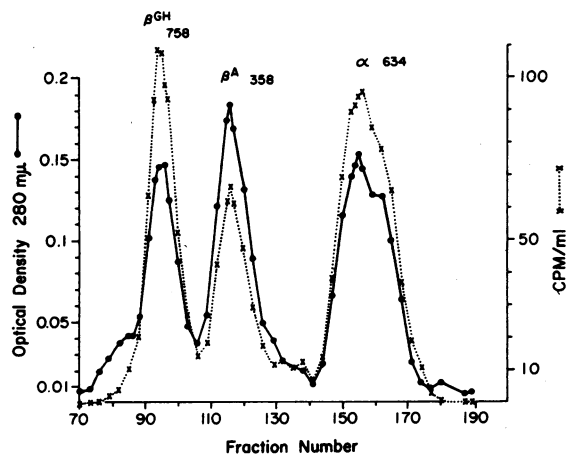


FIGURE 3 Urea-CMC chromatography of globin from a hemolysate prepared after incubation of reticulocytes with leucine- $^{14}\text{C}$  for 30 min. There was 50% more total radioactivity in  $\beta^{GH}$  than in  $\beta^A$ . Total incorporation of radioactivity into  $\alpha$ - and  $\beta$ -chains was equal. The specific activities of the peak fractions expressed as cpm/ml per OD 280 are indicated near the peaks. No correction is made for the differences in the extinction coefficients of  $\alpha$ - and  $\beta$ -chains.

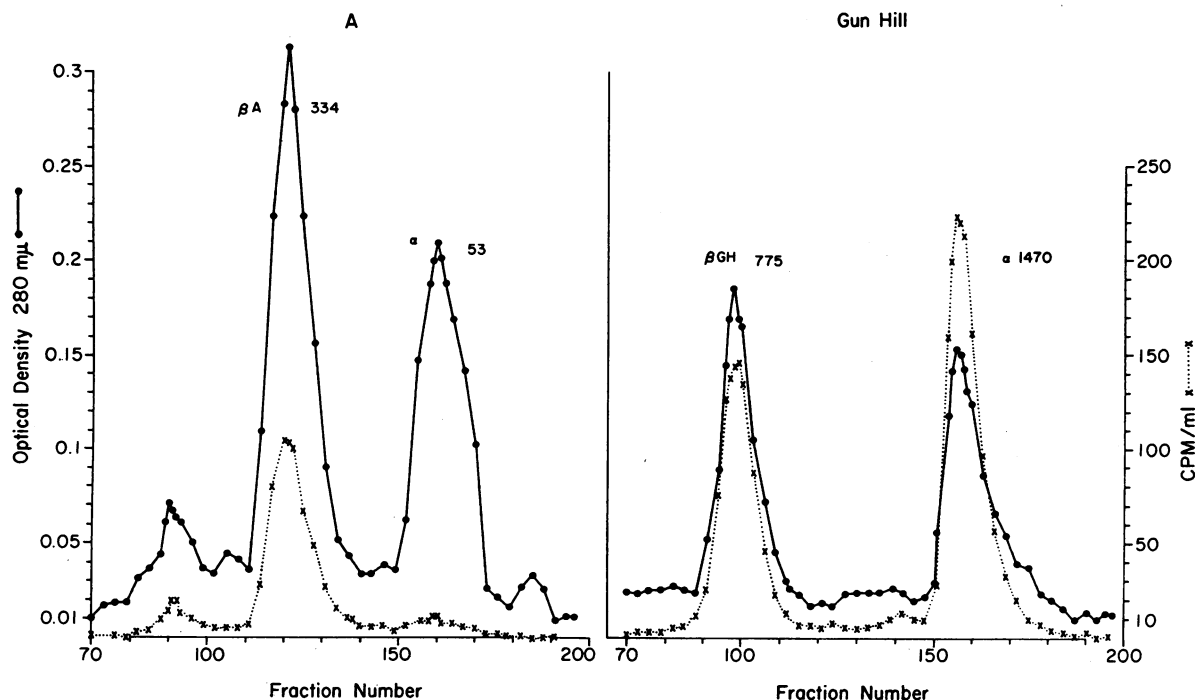


FIGURE 4 Urea-CMC chromatography of globin after separation of the two hemoglobins by starch-block electrophoresis. Very little radioactivity was found in the  $\alpha$ -chains of hemoglobin A.

five amino acid residues missing from  $\beta^{\text{GH}}$ . Cystamine (2,2'-dithiobisethylamine dihydrochloride) is known to attach to free sulfhydryl groups of proteins by means of the disulfide interchange reaction. The free amine group of cystamine adds an additional positive charge to the protein molecule to which it couples (20). This procedure was applied to whole globin prepared from a hemolysate containing hemoglobin Gun Hill. Cystamine imparted an additional positive charge to  $\beta^{\text{A}}$ , altered its mobility relative to  $\beta^{\text{GH}}$ , and allowed the

separation of the two  $\beta$ -chains on the urea CMC column (Fig. 1). The identities of the two  $\beta$ -peaks were confirmed by fingerprint analysis. Approximately 40–43% of the total  $\beta$ -chain was estimated to be  $\beta^{\text{GH}}$  on the basis of measurement of the area under the chromatography peaks. The appearance of the additional  $\beta$ -peak was specific for mixtures of hemoglobin A and Gun Hill and did not occur when globin from normal hemolysates, purified hemoglobin A, or purified hemoglobin Gun Hill was treated with cystamine (Fig. 2).

*Incorporation of radioactive amino acid into individual globin chains.* Reticulocytes were incubated for 30 min with leucine- $^{14}\text{C}$ . One portion of the hemolysate was subjected to starch block electrophoresis; the separated hemoglobins were converted to globin and the radioactivity determined. The specific activity of hemoglobin Gun Hill was over 5 times that of hemoglobin A.

The remainder of the hemolysate was converted directly to globin, treated with cystamine, and applied to a urea-CMC column. Measurement of radioactivity in the consecutive fractions from the CMC column and comparison of the areas under the graphed data showed there was 50% greater total incorporation of leucine- $^{14}\text{C}$  into  $\beta^{\text{GH}}$  than into  $\beta^{\text{A}}$  (Fig. 3). The specific activity of the peak fraction of  $\beta^{\text{GH}}$  was more than twice that of the peak fraction of  $\beta^{\text{A}}$  (Fig. 3).

TABLE II  
Incorporation of Leucine- $^{14}\text{C}$  into Whole Hemoglobin and Separated  $\alpha$ - and  $\beta$ -Globin Chains

Sample	Specific activity	Total radioactivity*
	<i>cpm/mg</i>	<i>cpm</i>
Hemoglobin A	116	7,540
Hemoglobin Gun Hill	605	19,360
$\alpha$ (A)	23	748
$\beta^{\text{A}}$	210	6,825
$\alpha$ (GH)	704	11,264
$\beta^{\text{GH}}$	513	8,208

\* Total leucine- $^{14}\text{C}$  incorporated per 100 mg of total hemolysate hemoglobin calculated on the basis of 65% hemoglobin A and 32% hemoglobin Gun Hill.

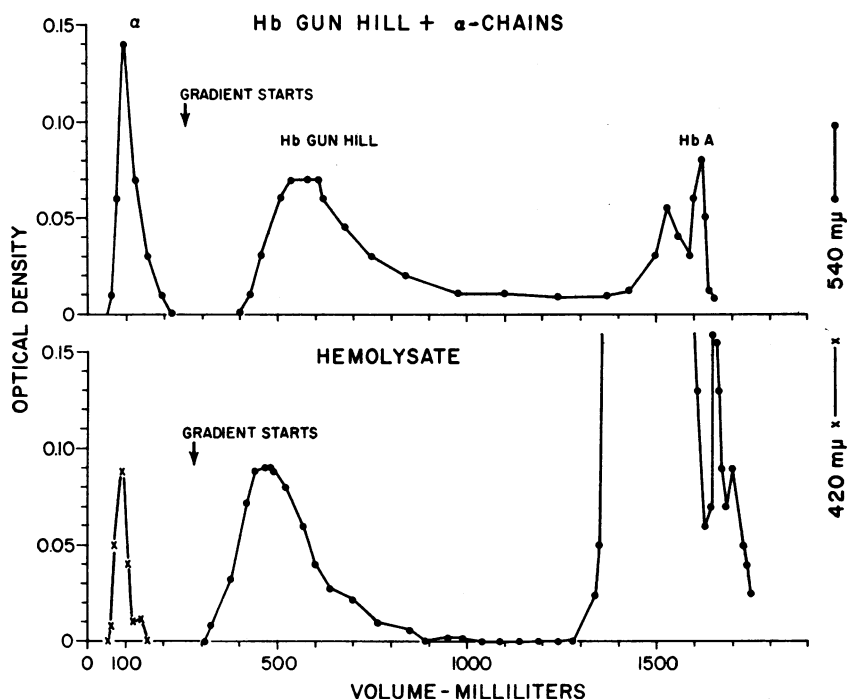


FIGURE 5 DEAE cellulose chromatography of  $\alpha$ -chains and hemoglobin Gun Hill. The upper panel shows the position of free  $\alpha$ -chains when an artificial mixture of  $\alpha$ -hemoglobin and hemoglobin Gun Hill was chromatographed. The lower panel shows the presence of a peak absorbing at 420  $m\mu$  and migrating in the position of free  $\alpha$ -chains, in a hemolysate containing hemoglobin Gun Hill.

These results indicated that more  $\beta^{\text{GH}}$  was synthesized than  $\beta^{\text{A}}$ . However, the 50% difference in synthesis of the two  $\beta$ -chains was too small to explain the fivefold greater specific activity of whole hemoglobin Gun Hill compared to whole hemoglobin A. Fig. 4 shows the distribution of radioactivity in the globin subunits of the hemoglobin A and hemoglobin Gun Hill fractions which had been separated by starch block electrophoresis before urea-CMC chromatography. The specific activities of  $\beta^{\text{A}}$  and  $\beta^{\text{GH}}$  were essentially the same as in the whole unfractionated hemolysate. However, the  $\alpha$ -chains of the abnormal hemoglobin had a specific activity almost 30 times greater than the  $\alpha$ -chains of hemoglobin A (Fig. 4). Thus almost all the  $\alpha$ -chain radioactivity in the hemolysate was in hemoglobin Gun Hill. Table II shows the specific activities of hemoglobin A and hemoglobin Gun Hill prepared by starch-block electrophoresis and the specific activities of the pooled globin subunits derived from CMC chromatography of the starch-block fractions. These data indicate there was 20% more total radioactivity in  $\beta^{\text{GH}}$  than in  $\beta^{\text{A}}$ .

There was equal total incorporation of radioactive amino acid into  $\alpha$ - and  $\beta$ -chains when unfractionated hemolysates were examined (Fig. 3). However when hemoglobin A and hemoglobin Gun Hill were purified

by starch-block electrophoresis the total radioactivity in  $\beta$ -chains was 24% greater than in  $\alpha$ -chains (Table II). Removal of a pool of free radioactive  $\alpha$ -chains by electrophoresis could explain this finding.

*Demonstration of a free  $\alpha$ -chain pool.* The markedly unequal distribution of radioactivity between the  $\alpha$ -chains of hemoglobin A and hemoglobin Gun Hill can be explained by the existence of a free pool of unlabeled  $\alpha$ -chains in the cells.

Dilution of newly synthesized radioactive  $\alpha$ -chains upon entrance into the pool would decrease the radioactivity in the  $\alpha$ -chains that combined with newly synthesized  $\beta^{\text{A}}$ . This would result in a greater specific activity of the  $\beta^{\text{A}}$ -chains compared to the  $\alpha$ -chains of hemoglobin A. Direct exchange of  $\alpha$ -subunits between the free  $\alpha$ -chain pool and preformed unstable hemoglobin Gun Hill would result in an increased specific activity of the  $\alpha$ -chains of the abnormal hemoglobin. Fig. 5 indicates that when a concentrated hemolysate containing hemoglobin Gun Hill was examined by DEAE column chromatography a fast moving, minor, heme-bearing peak was present in addition to the peaks for hemoglobin Gun Hill and hemoglobin A.

The minor peak had a mobility identical with that of authentic free  $\alpha$ -chains when an artificial mixture of

purified  $\alpha$ -chains (26) and partially purified hemoglobin Gun Hill was subjected to chromatography (Fig. 5).

A free  $\alpha$ -chain band was also demonstrated when concentrated hemolysates containing hemoglobin Gun Hill were subjected to starch-gel electrophoresis.

*Demonstration of  $\alpha$ -subunit exchange between hemoglobin Gun Hill and the free  $\alpha$ -chain pool.* Reticulocytes were incubated for 10 min in the presence of leucine- $^3\text{H}$  and a portion of the cells was frozen and hemolyzed (10 min sample). The remainder of the cells was transferred to nonradioactive medium containing 0.0025 M puromycin, an inhibitor of protein synthesis. Incubation was continued for 2 hr at which time the cells were frozen and hemolyzed (120 min sample). The 10 and 120 min specimens were examined by DEAE cellulose chromatography. Fig. 6 shows that in the 10 min sample there was a radioactive peak, just behind the initial peak of unincorporated leucine- $^3\text{H}$ , in a position identical with the previously demonstrated peak for free  $\alpha$ -chains. The radioactivity in this peak was reduced in the 120 min sample (Fig. 6). In contrast the specific activity of hemoglobin Gun Hill increased during the period of incubation with puromycin. The hemoglobin A and hemoglobin Gun Hill fractions from the 10 and 120

min samples were converted to globin and examined by urea-CMC chromatography.

Fig. 7 demonstrates that the specific activities of the  $\alpha$ - and  $\beta$ -chains of hemoglobin A did not change during the 2 hr incubation with puromycin, indicating that protein synthesis was blocked. Fig. 8 shows the subunits of hemoglobin Gun Hill. There was no increase in the specific activity of  $\beta^{\text{GH}}$  during exposure to puromycin, again demonstrating the absence of protein synthesis. In contrast there was a doubling of the radioactivity in the  $\alpha$ -chains. In the absence of protein synthesis, these findings suggest that subunit exchange occurred between the free  $\alpha$ -chain pool and hemoglobin Gun Hill.

An indication of the importance of the pool of  $\alpha$ -chains was obtained in an experiment in which reticulocytes containing hemoglobin Gun Hill were incubated for 10 min with leucine- $^3\text{H}$ . The cells were hemolyzed and one-half the hemolysate converted immediately to globin. The remainder of the hemolysate was placed on a DEAE cellulose column and starting buffer was passed to elute the material moving in front of hemoglobin Gun Hill. The fraction containing hemoglobin Gun Hill and hemoglobin A was then eluted as one peak with the final buffer of the usual nine chamber gradient (17) and converted to globin. The portion of

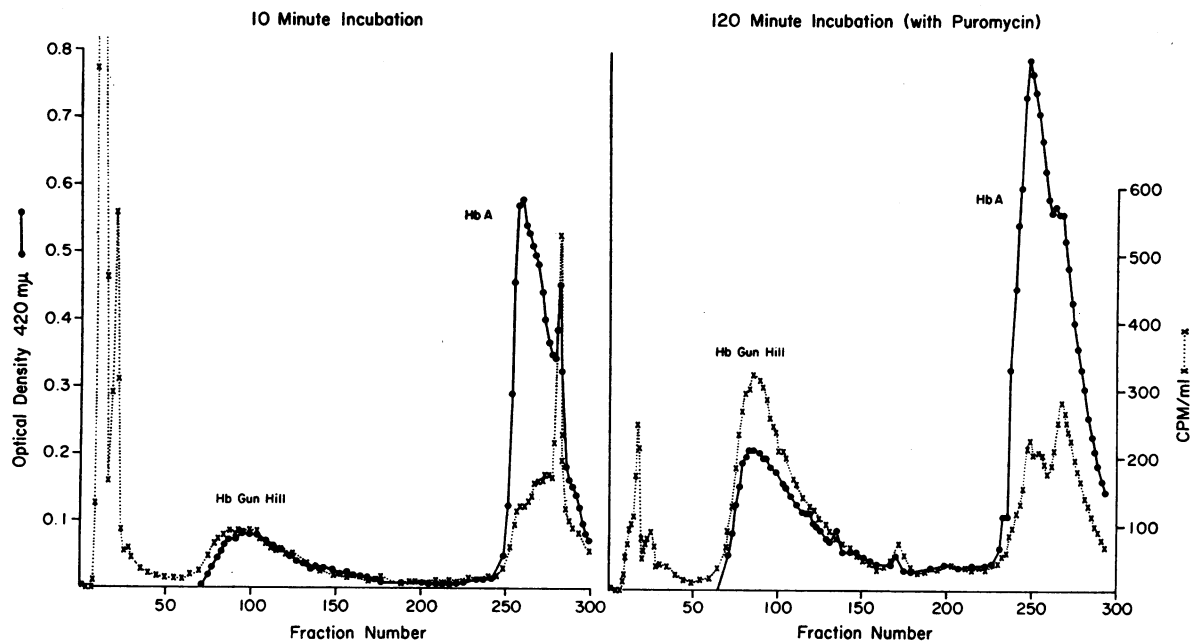


FIGURE 6 DEAE chromatography of radioactive hemolysates containing hemoglobin Gun Hill. The left panel shows the chromatography of a hemolysate prepared from cells after 10 min of incubation with leucine- $^3\text{H}$ . There is a peak of radioactivity migrating in the position of free  $\alpha$ -chains just behind the front running peak of unincorporated leucine. The right panel shows the chromatography of a hemolysate prepared after 2 hr of incubation in the presence of puromycin. The unincorporated leucine peak has been removed by prior passage through Sephadex G-25. The peak in the position of  $\alpha$ -hemoglobin is smaller than in the 10 min sample while the specific activity of hemoglobin Gun Hill has increased.



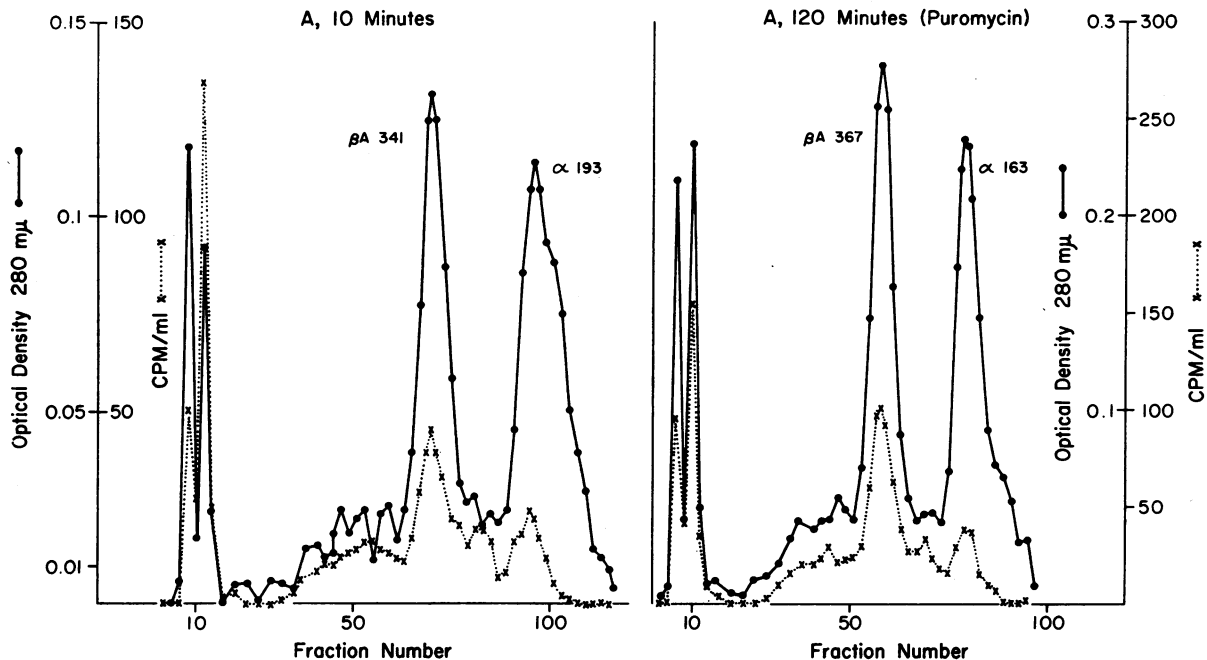


FIGURE 7 Globin chain chromatography on CMC in 8 M urea of hemoglobin A obtained from the DEAE chromatography of the hemolysates from the 10 and 120 min incubations. The specific activities of the  $\alpha$ - and  $\beta$ -chains are similar in both samples.

the incubation samples that had been converted directly to globin, and the globin prepared from material eluted from the DEAE column were both analyzed by urea-CMC chromatography. Fig. 9 shows that the specific activity of the  $\alpha$ -chain peak was reduced by 40% after passage of the hemolysate through the DEAE column. Thus approximately 40% of the newly synthesized  $\alpha$ -chains were found in the free pool after 10 min of incubation with leucine- $^3\text{H}$ .

*Assembly of  $\beta^A$  and  $\beta^{\text{GH}}$ -chains.* In an attempt to explain the greater synthesis of  $\beta^{\text{GH}}$  than  $\beta^A$ , the two chains were examined for differences in the rate of amino acid assembly or translation. The procedure, devised by Dintzis (22), is based on the fact that polypeptides are synthesized by sequential addition of amino acids from the N-terminal to the C-terminal end. When radioactive amino acid is added to reticulocytes which are actively synthesizing hemoglobin, the tracer is initially incorporated into the growing ends of partially completed chains on the polyribosomes. At first, radioactivity appears only near the C-terminal end of completed hemoglobin. Later, as chains are made with radioactive amino acid from almost the very beginning, a gradient of radioactivity appears in the finished hemoglobin and spreads toward, and finally reaches the N-terminal end. With further incubation, during which time all new chains are made with radioactive amino acid from N-terminal to C-terminal end, the slope of

the gradient of radioactivity decreases, until the polypeptide chains are found to be essentially uniformly labeled. The rate at which the spread of radioactivity approaches the N-terminal end, and the rate at which the slope of the gradient decreases, are dependent on the velocity of translation. Both rates are independent of chain initiation and the quantity of mRNA present. However, differences in growth rates along the polypeptide chain will affect the shape of the gradient of radioactivity (27).

Reticulocytes were incubated with leucine- $^3\text{H}$  for 3.5 and 10 min. The hemolysates were converted to globin and  $\beta^A$  and  $\beta^{\text{GH}}$  separated by urea-CMC chromatography.  $\beta^A$  and  $\beta^{\text{GH}}$  globin chains uniformly labeled with leucine- $^{14}\text{C}$  were added and the mixtures aminoethylated, digested with trypsin, and fingerprinted. The  $^3\text{H}:^{14}\text{C}$  ratio in each tryptic peptide was determined, plotted against the position of the leucine residue in the chain, and the curve extrapolated to the C-terminal position. The ratios were then normalized to the C-terminal value and the data replotted (Fig. 10). Straight line plots were obtained for  $\beta^A$  and  $\beta^{\text{GH}}$ . The slopes of the gradients of radioactivity from N-terminal to C-terminal were almost identical for the two chains at both time points. These results indicate that the rates of translation of  $\beta^A$  and  $\beta^{\text{GH}}$  are the same and are uniform throughout the process of chain assembly.

## DISCUSSION

It is now apparent that in human subjects, the turnover rates of heterogeneous hemoglobins may differ greatly. There is evidence that in patients with sickle cell anemia, hemoglobin S is more rapidly eliminated from the blood than hemoglobin F (28, 29). In thalassemia the turnover rate of hemoglobin F is slower than that of hemoglobin A since red cells containing greater amounts of hemoglobin F have a longer survival (30). In hemoglobin H disease there is a heterogeneous distribution of the abnormal hemoglobin and cells containing greater amounts of the unstable  $\beta_4$ -tetramer tend to be destroyed more rapidly (31).

Experiments bearing on turnover rates have also been performed on several mutant unstable hemoglobins (2-4, 12). Although no evidence has been found for heterogeneous distribution of these hemoglobins among erythrocytes, some of these variant proteins are destroyed more rapidly than hemoglobin A. Studies of the synthesis of hemoglobin Zurich suggested that that abnormal hemoglobin is made at a rate equal to hemoglobin A although found in amounts only half that of the normal protein in the peripheral blood of heterozygous persons (4). Recently, White and Brain have presented evidence that hemoglobin Koln, an unstable  $\beta$ -chain mutant, has a more rapid turnover rate than

hemoglobin A (3). The authors also found evidence of a free  $\alpha$ -chain pool and exchange of subunits between the pool and hemoglobin Koln in hemolysates.

Clegg and Weatherall (32) have shown that in cells containing hemoglobin H there can be direct exchange of  $\beta$ -chain subunits between hemoglobin H and hemoglobin A. These authors also examined the pattern of hemoglobin synthesis in reticulocytes from subjects with  $\beta$ -thalassemia (33). A large intracellular pool of  $\alpha$ -chains was present but no direct evidence of intracellular exchange between the pool and hemoglobin A could be demonstrated.

The present study of hemoglobin Gun Hill provided evidence of markedly unequal turnover rates of the mutant hemoglobin and hemoglobin A. Although the proportion of hemoglobin Gun Hill in the hemolysate was only 32%, over 50% of the  $\beta$ -chain radioactivity was incorporated into  $\beta^{GH}$ . Rapid destruction of unstable hemoglobin Gun Hill could explain this finding. The differences in the specific activities of the  $\alpha$ -chains of hemoglobin Gun Hill and hemoglobin A can best be explained by the presence of a free pool of  $\alpha$ -chains. Such a pool, if large in comparison with the amount of newly synthesized  $\alpha$ -chains, could greatly dilute the  $\alpha$ -chain radioactivity, and cause the low specific of the  $\alpha$ -chains of hemoglobin A. If the pool was small in

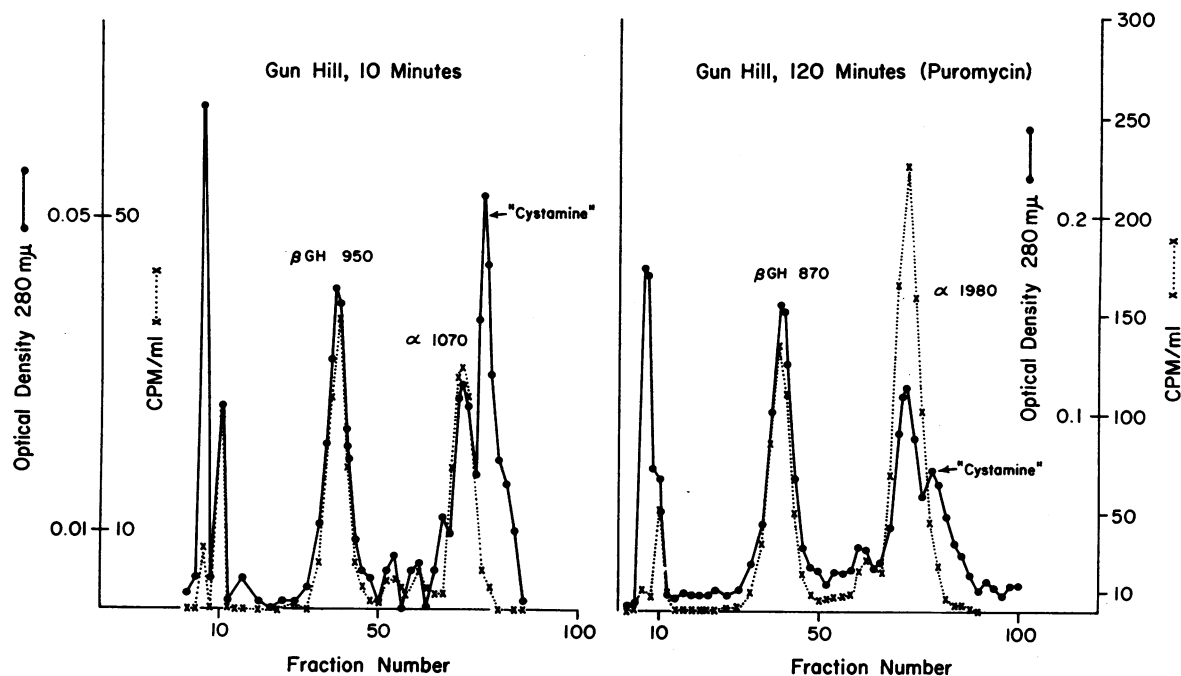


FIGURE 8 Globin chain chromatography of the DEAE peaks of hemoglobin Gun Hill from the 10 and 120 min incubations. The specific activity of  $\beta^{GH}$  did not change significantly after incubation in the presence of puromycin but there was a doubling of the radioactivity in the  $\alpha$ -chains. The relative prominence of the cystamine peak in the 10 min sample is due to the small amount of protein placed on the CMC column.

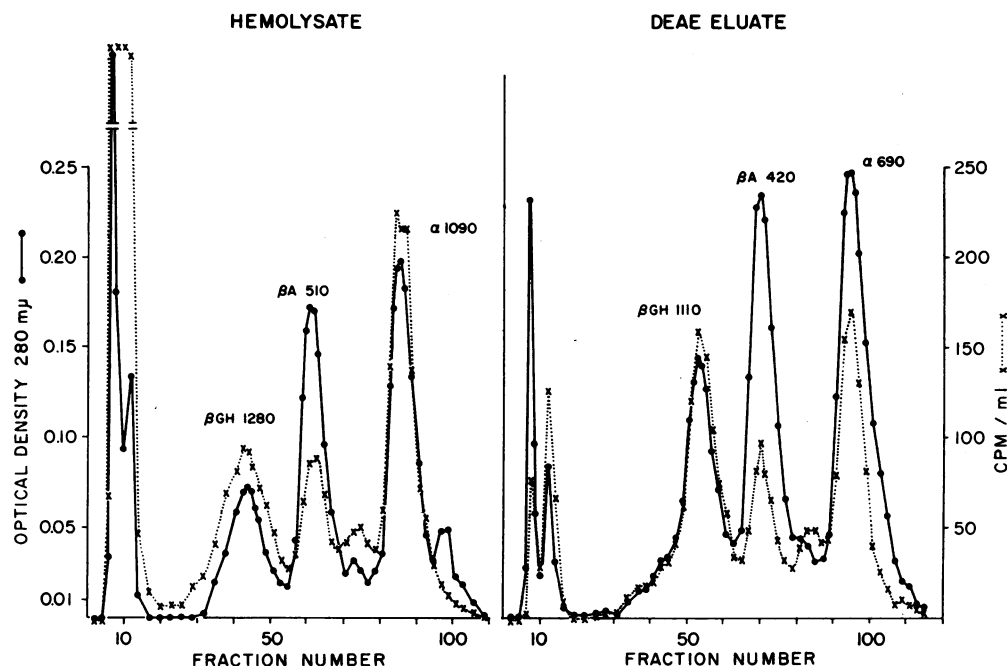


FIGURE 9 Globin chain chromatography after 10 min of incubation of reticulocytes with leucine- $^3\text{H}$ . The left panel shows the radioactivity in the  $\alpha$ - and  $\beta$ -chains of the unfractionated hemolysate. The right panel shows the radioactivity remaining in hemoglobin after free  $\alpha$ -chains were removed by DEAE chromatography. There was a 40% loss in  $\alpha$ -chain radioactivity after removal of the  $\alpha$ -chain pool.

comparison with the amount of hemoglobin Gun Hill present, and if the  $\alpha$ -chains in the pool were freely exchangeable with the  $\alpha$ -chains of preformed hemoglobin Gun Hill, then, as equilibrium was approached, most of the total  $\alpha$ -chain radioactivity would move into hemoglobin Gun Hill. This would cause the high specific activity of the  $\alpha$ -chains of hemoglobin Gun Hill.

Direct demonstration of the  $\alpha$ -chain pool was achieved by DEAE chromatography of a concentrated hemolysate and by starch gel electrophoresis. Entrance of newly synthesized  $\alpha$ -chains into the free pool was indicated by the finding that approximately 40% of the  $\alpha$ -chain radioactivity from a 10 min incubation was removed from an hemolysate when the  $\alpha$ -chain pool was separated from the whole hemoglobin fraction by DEAE chromatography. Purification of hemoglobin A and hemoglobin Gun Hill by starch-block electrophoresis resulted in a loss of 24% of the  $\alpha$ -chain radioactivity of the hemolysate. Presumably the free  $\alpha$ -chain radioactivity was separated from the hemoglobin fractions by electrophoresis. Intracellular exchange of radioactive  $\alpha$ -subunits between the  $\alpha$ -chain pool and hemoglobin Gun Hill was directly demonstrated when protein synthesis was blocked with puromycin.

The origin of the free  $\alpha$ -chains is probably not greater total synthesis of  $\alpha$ -chains than  $\beta$ -chains. Examina-

tion of the radioactivity of the fractions from CMC chromatography of globin indicated that total  $\alpha$ - and  $\beta$ -radioactivity were equal. It is likely that the origin of the pool is subunit dissociation of unstable hemoglobin Gun Hill. Gel filtration and ultracentrifuge studies have suggested that this unstable hemoglobin has an increased tendency to dissociate (34). Loss of the heme-free  $\beta$ -globin and persistence of the released  $\alpha$ -chains would result in excess of  $\alpha$ -chains. The tendency of hemoglobin Gun Hill to dissociate could explain the predominant exchange of  $\alpha$ -chains from the pool with the abnormal hemoglobin.

The present studies indicated that total synthesis of  $\beta^{\text{GH}}$  was 20-50% greater than total synthesis of  $\beta^{\text{A}}$ . In the absence of thalassemia, patients who are heterozygous for mutant hemoglobins generally possess more hemoglobin A than variant hemoglobin. The possibility was suggested by Itano (5) that, in heterozygotes for an abnormal hemoglobin, the altered structure of the variant is responsible for its diminished synthesis. A possible site for such an effect is the process of chain assembly or translation. In several in vitro studies interference with the process of translation by introduction of an amino acid analogue or removal of a specific tRNA fraction resulted in diminished synthesis of globin chains (35-38). There is some experimental evidence

that "slow points" in the process of translation may be responsible for the decreased synthesis of  $\delta$ -chains and  $\beta^S$  chains (1, 10, 39). A diminished velocity of translation in the vicinity of the heme-attachment point at the  $\beta 92$  histidine has also been suggested for  $\beta^A$ -chains (10). Because the five amino acid deletion in  $\beta^{GH}$  involves this heme-binding area, the loss of a slow point was initially considered a possible explanation for the more rapid synthesis of  $\beta^{GH}$  than  $\beta^A$ . Recently, however, precise experiments have indicated that the assembly of  $\beta^A$ -chains, in normal subjects, and in patients with thalassemia, is uniform along the length of the chain (23). The present study also failed to provide evidence for a "slow point" in the assembly of  $\beta^A$  globin chains. The rate of assembly of  $\beta^{GH}$  and  $\beta^A$  appeared to be almost identical. There is no explanation at present for the difference in the amounts of  $\beta^A$  and  $\beta^{GH}$  synthesized in these experiments.

The observation that the synthesis of  $\beta^{GH}$  is not retarded in comparison to  $\beta^A$  is of additional interest. Heme has been repeatedly shown to stimulate globin synthesis although the exact site of action is not known (7-9, 40, 41). If this effect is of physiological importance as a regulatory mechanism, the results of the present experiments would suggest that stimulation by heme is not dependent on strong heme-globin binding. It is possible, of course, that it is only heme in excess which exerts an effect to stimulate globin synthesis above basal levels. A more definitive statement in this regard awaits the appropriate experimental evidence.

Finally, it should be reemphasized that experiments employing reticulocytes must be interpreted with caution. Extrapolation of results obtained with cells which are approaching the end of the phase of protein synthesis may be invalid. Rates of synthesis of different hemoglobins have been shown to vary widely in bone marrow cells and reticulocytes (24).

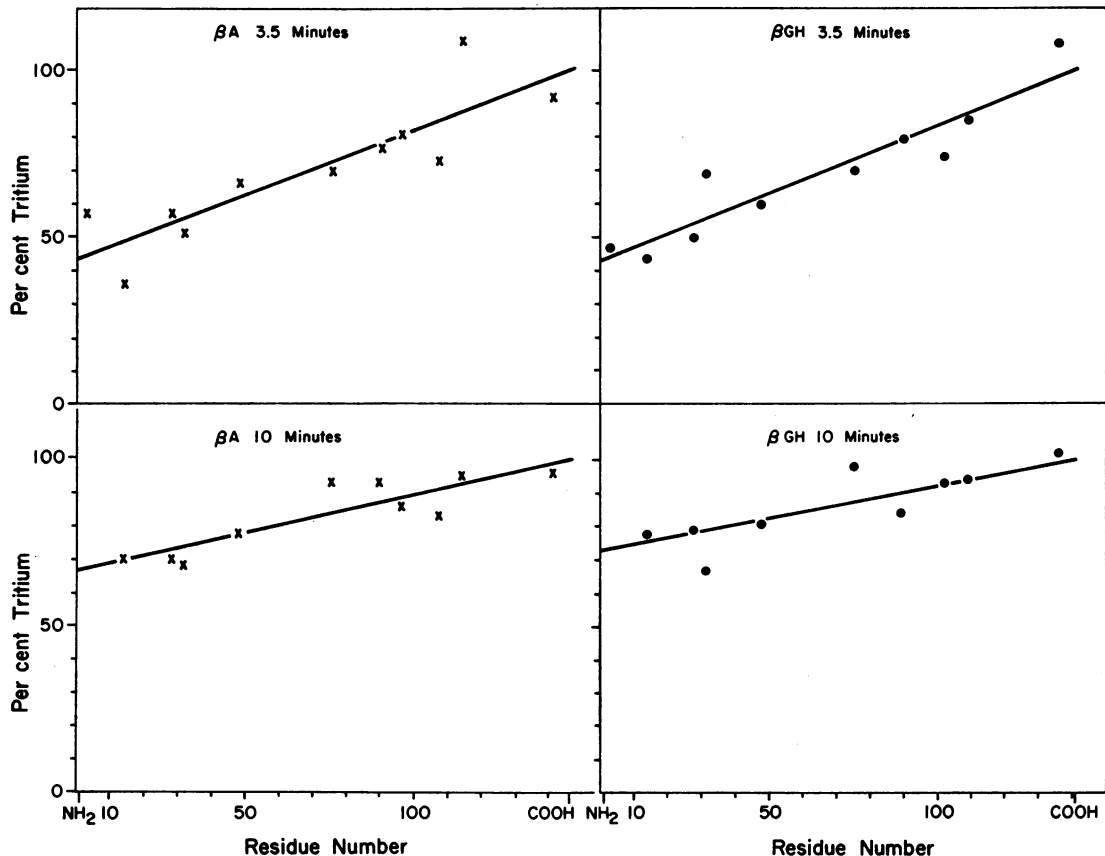


FIGURE 10 Assembly of  $\beta^A$  and  $\beta^{GH}$ . The radioactivity in the leucine residues along the polypeptide chains after 3.5 and 10 min of incubation with leucine- $^3\text{H}$  is expressed as a percentage of the extrapolated value for the C-terminal amino acid. The value for leucine at position 3 is not indicated for the 10 min specimens as recovery of  $\beta\text{TpI}$  from the chromatograms was poor in this experiment.

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