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Research Article



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# The Metabolism of the Individual $C^{14}$-labeled Hemoglobins in Patients with H-Thalassemia, with Observations on Radiochromate Binding to the Hemoglobins during Red Cell Survival *

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Patients with thalassemia may have abnormal alterations in the proportions of the structurally normal hemoglobins F and $A_2$, or, in more rare circumstances, new hemoglobins of abnormal structure may appear. Such abnormal hemoglobins have not been shown to have substitutions of single amino acids in the polypeptide chain, as exemplified by hemoglobins S and C. Rather, they occur as unusual combinations of subunits of the hemoglobin molecule ($\alpha_2\beta_2$). Thus, tetramer formation with normal polypeptide units is seen. Examples of tetramer formation in thalassemia are hemoglobins H ($\beta_4$) (1), Bart's ($\gamma_4$) (2), and possibly $\alpha_4$ (3). There is also evidence that a delta chain tetramer may be present in very small quantities (4). Fusion of pieces of the beta and delta polypeptide chains into one polypeptide unit occurs in hemoglobin Lepore (5).

Investigations in this laboratory have been concerned with the manner in which the various hemoglobins are distributed among the red cells in thalassemia. Previous reports have dealt with the behavior of hemoglobins A, F, and $A_2$ (6, 7). The present study describes the distribution of hemoglobins A and H among the red cells and the relationship of this cellular distribution to red cell survival.

## Methods

*1) Patients.* The three unrelated H-thalassemia patients who were studied are described in Table I. They were all mature adults of Italian ancestry with mild to moderate anemia lacking transfusion requirement. Hemoglobin concentrations remained unchanged during the study period. Splenomegaly was present in all three patients and two had slight icterus. Only patient A.R. underwent splenectomy before the investigations. Patient S.S. had moderately advanced rheumatic heart disease with chronic congestive heart failure. Her medications included a digitalis preparation, sulfasuxazole (1.0 g

TABLE I

*Laboratory data of patients with H-thalassemia*

| | Patient S.S. | Patient A.R. | Patient J.L. |
|---|---|---|---|
| Age and sex* | 42, F | 29, M | 40, F |
| Hemoglobin, *g/100 ml* | 11.6 | 10.8 | 8.6 |
| Hematocrit, % | 44.5 | 42.0 | 32.0 |
| Red blood cells, */mm³ × 10⁶* | 6.9 | 5.8 | 4.9 |
| MCV,* *μ³* | 65 | 73 | 65 |
| MCH,* *μμg* | 17 | 19 | 17 |
| Reticulocyte count, % | 2–10 | 2–4 | 4–8 |
| Hemoglobin H, *% of total* | 11–15 | 17–22 | 6–9 |
| Hemoglobin $A_2$, *% of total* | 0.9 | 1.0 | 1.4 |
| Alkali-resistant hemoglobin, *% of total* | 2.2 | 2.7 | 1.1 |
| Serum iron, *μg/100 ml* | 122 | 50 | 100 |
| Serum iron-binding capacity, *μg/100 ml* | 300 | 300 | 285 |
| Serum bilirubin, *mg/100 ml* | 1.5–3.4 | 1.7–3.0 | 0.8 |
| $Cr^{51}$ red cell lifespan, *$t_{\frac{1}{2}}$ days* | 18 | 18 | 13 |
| Ratio of $Cr^{51}$ body surface cpm† (spleen/liver at 5–10 days) | 620/290 | 700/180 | 750/180 |
| Spleen | Intact | Removed | Intact |

* Abbreviations used: F = female, M = male; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin.
† Counts per minute are corrected for blood flow. Patient A.R. was scanned before splenectomy.

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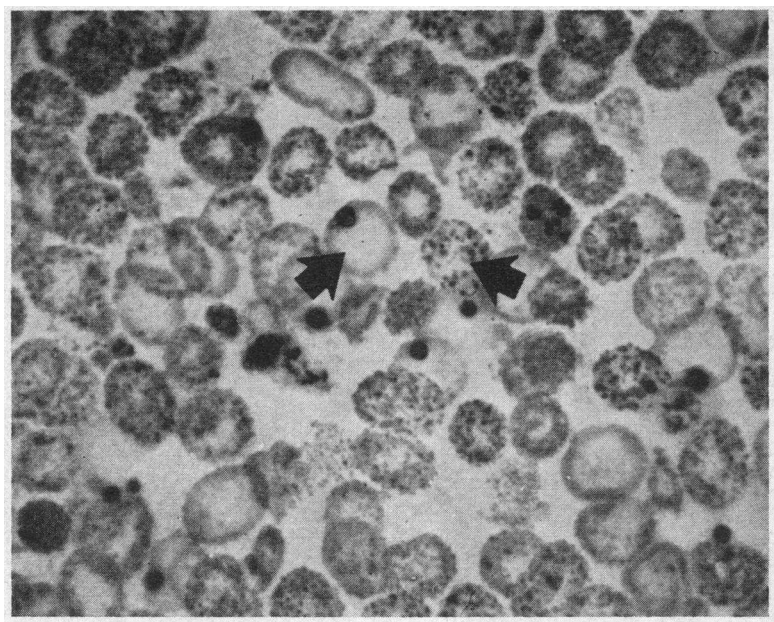

FIG. 1. PERIPHERAL BLOOD FILM OF THE SPLENECTOMIZED PATIENT A.R., MADE AFTER INCUBATION OF WHOLE BLOOD AT 37° C WITH BRILLIANT CRESYL BLUE (BCB) FOR 1 HOUR. The left arrow indicates a red cell containing a "postsplenectomy inclusion." The right arrow indicates a red cell containing "BCB inclusions." (Postsplenectomy inclusions stain with BCB as well as with methyl violet.)

daily), hydrochlorothiazide (50 mg daily), KCl, and a chelated cobalt compound.[1]

*2) Red cell inclusions.* Two types of red cell inclusions must be distinguished in H-thalassemia: *1)* Small uniform inclusions that are found after incubation of blood with brilliant cresyl blue (BCB) for 1 hour at 37° C (8). They represent *in vitro* precipitation of soluble hemoglobin H and shall be termed "BCB inclusions." *2)* Larger inclusions, varying somewhat in size and bearing close resemblance to Heinz bodies, which are found *in vivo* in a large fraction of the red cells only after splenectomy in H-thalassemia as well as in some other types of hemolytic anemia (9–13). They stain readily after incubation of blood with methyl violet for 10 minutes at room temperature (13). They shall be termed "postsplenectomy inclusions" (Figure 1).

*3) Centrifugal separation.* Populations of young and old red cells were separated by centrifugation (7). Measurements of soluble hemoglobin H, as described below, and of alkali-resistant hemoglobin (14) were made on the various layers. Reticulocytes, red cells containing BCB inclusions, and red cells containing postsplenectomy inclusions were enumerated.

*4) In vitro incubations.* Studies were made in heparinized whole blood or marrow aspirate of the *in vitro* incorporation of labeled amino acid into the hemoglobins. Five-ml portions of each sample containing 1.25 μc leucine-U-$C^{14}$ (SA, 246 mc per mmole) [2] were placed in 50-ml Erlenmeyer flasks with an air atmosphere and incubated in a Dubnoff shaker at 37° C. Individual flasks were removed periodically. The maximal incubation time was 4 hours. Reactions were stopped by cooling to 4° C. After hemolysis of the washed red cells with a dilute buffer, nonradioactive *l*-leucine was added to a concentration of 50 μg per ml. The numbers of reticulocytes and of nucleated red cells per flask were estimated. The separation of the hemoglobins is described below. Equal portions of autologous chilled unlabeled blood were added as carrier after the incubation of the marrow aspirate of patient J.L. This was done to increase the amount of hemoglobin H available for analysis.

*5) Carbon$^{14}$ hemoglobin survival times in vivo.* Patients S.S. and A.R. were given intravenous injections of 200 μc glycine-2-$C^{14}$ (SA, 3.06 mc per mmole).[2] Serial heparinized blood samples, usually 20 ml, were collected for 1½ to 3 months. The specific activity of hemoglobins A and H was determined as noted below, and the mean survival time of the individual hemoglobins in the circulation was estimated (15).

*6) Hemoglobin separation.* Hemolysates were prepared as previously described (7). Hemoglobins H and A were separated by starch block electrophoresis at pH 7.0 (0.044 M phosphate) (16). The proportion of hemoglobin H was determined from the volumes and optical

[1] Copoietin A, Lloyd Brothers, Cincinnati, Ohio.

[2] New England Nuclear Corp., Boston, Mass.

densities of the hemoglobin H and A eluates. Optical density was measured at 540 mμ, 560 mμ, and 576 mμ in the Beckman DU spectrophotometer to confirm that the samples were in the oxyhemoglobin form. Some determinations were made in the cyanmethemoglobin form, which yielded slightly lower values for hemoglobin H per cent, probably due to precipitation of hemoglobin during conversion. The separation of the hemoglobins in the carbon$^{14}$ survival time study of patient S.S. was performed chromatographically on columns of Amberlite CG 50 [3] using developer 6 (17), followed by purification of hemoglobin H by starch block electrophoresis or on columns of carboxymethylcellulose (CMC) [4] with adsorption and elution pH values of 6.0 and 7.2, respectively, for hemoglobin H (18). This method, while removing most of the nonheme proteins, gave relatively low yields of hemoglobin H. There was no difference in specific activity between starch block and CMC purified hemoglobin H.

*7) Carbon$^{14}$ radioactivity determinations.* Dilute hemoglobin solutions were concentrated by ultrafiltration and lyophilized after dialysis overnight against running tap water. Carbon$^{14}$ specific activity was determined by combustion of weighed samples in oxygen-filled flasks, as previously described (19). The maximal counting error (excluding day 1 and days beyond 60 in hemoglobin survival studies) was 6% (2 coefficients of variation). Allowance was made for the higher glycine content of hemoglobin H by reducing its specific activity relative to that of hemoglobin A by the molar ratio of the glycine content of the two hemoglobins, 72/84 (20).

*8) Radiochromate studies.* Four chromium$^{51}$ survival studies were performed in three patients. Two patients with hemoglobin H thalassemia (S.S. and J.L.) were studied after injection of chromium$^{51}$-labeled autologous red cells. One patient (H.J.) with chronic leukemic reticuloendotheliosis was studied after injection of autologous labeled red cells to observe the behavior of the chromium$^{51}$ label in red cells lacking hemoglobin H. In the fourth study, normal compatible type O Rh negative red cells were labeled with $Cr^{51}O_4^=$ and injected into patient S.S. to determine whether the phenomenon observed in the study of autologous hemoglobin H cells could be duplicated when normal red cells were used.

Freshly drawn blood (20 to 25 ml) was labeled with 200 to 250 μc of $Na_2Cr^{51}O_4$ containing a total of 1.3 to 2.4 μg chromium (21, 22). The mixture was incubated for 20 minutes at 37° C, after which 100 mg of sodium ascorbate was added. Twenty ml of the labeled whole blood was then immediately injected intravenously. Methyl violet stains of the labeled blood indicated that the labeling procedure did not cause formation of Heinz bodies.

The $Cr^{51}$ activity in the recipient was evaluated by periodic samples of heparinized blood for as long as 24 days. Samples were washed 3 times in 5 vol of saline and hemolyzed with 1 vol of a dilute buffer (pH 7.1, 0.009 M phosphate and 0.002 M KCN) and ½ vol of toluene. Two-ml portions of the centrifuged hemolysate were separated by starch block electrophoresis, as described above. Most of the separations were done within 4 days of sampling; the maximal delay was 8 days. The anodal zone, containing the hemoglobin H and its associated radioactivity, and the cathodal zone, containing hemoglobin A and its associated radioactivity, were separately eluted with phosphate buffer (pH 7.1, 0.041 M phosphate and 0.01 M KCN). The $Cr^{51}$ activity of 2.0-ml portions of the whole blood, the hemoglobin solutions, and of the separated eluates of hemoglobin A and H was measured in a well-type scintillation counter with a background of 8 cpm. The maximal counting error during the first 10 days of the two studies of autologous red cells in hemoglobin H thalassemia was 5% (2 coefficients of variation). Specific activity of the whole blood and hemolysate hemoglobin was obtained by dividing the counts per minute by the optical density at 540 mμ. Hemoglobin A and H radioactivity was calculated by multiplying the counts per minute by the total volume of the eluate.

[3] Rohm and Haas Co., Philadelphia, Pa.

[4] Whatman powder CM 70.

## Results

*1) Studies of centrifugally separated red cell populations.* Morphologic observations and measurements of soluble hemoglobin H content were made on populations of young and old red cells separated by centrifugation. The results are summarized in Table II. As Rigas and Koler have previously described (9), the postsplenectomy inclusions, when they are present, are found chiefly in the old red cells, which are found in the bottom layer and have less soluble hemoglobin H. Many of the red cells that contain postsplenectomy inclusions are devoid of BCB inclusions (Figure 1). However, patients S.S. and J. L. were not splenectomized, and therefore their red cells lacked postsplenectomy inclusions. Their erythrocytes had the same distribution of soluble hemoglobin H and of BCB inclusions as the splenectomized patient A.R. Thus the difference in soluble hemoglobin H content between young and old red cells is not due to simple failure to recover a proportion of the hemoglobin H that had been rendered insoluble in the form of an intracellular hemoglobin precipitate in old red cells.

In two subjects there was a significant elevation of alkali-resistant hemoglobin above 2.0%. This alkali-resistant hemoglobin was found to have anodal migration in starch block electrophoresis at pH 7.0 and probably represented hemoglobin

TABLE II

*Distribution of hemoglobins and of inclusions among young (top layer) and old (bottom layer) red cells separated by centrifugation of whole blood*

| Layer | Hemoglobin H | Alkali-resistant hemoglobin | % of red cells containing BCB inclusions* | % of red cells containing post-splenectomy inclusions* | Reticulocyte count |
|---|---|---|---|---|---|
| | *% of total* | *% of total* | | | % |
| | | Patient S.S. | | | |
| Whole blood | 13 | 2.2 | 87 | 0 | 2.7 |
| Top | 20 | 1.1 | 90 | 0 | 11.2 |
| Middle | 14 | 1.5 | 70 | 0 | 1.0 |
| Bottom | 10 | 2.3 | 62 | 0 | 0.1 |
| | | Patient A.R. | | | |
| Whole blood | 17 | 2.7 | 91 | 11 | 4.2 |
| Top | 22 | 1.7 | 99 | 3 | 10.5 |
| Middle | 20 | 2.4 | 95 | 3 | 4.1 |
| Bottom | 10 | 3.6 | 85 | 24 | 0.1 |
| | | Patient J.L. | | | |
| Whole blood | 7.0 | 1.1 | 56 | 0 | 4.9 |
| Top | 12.8 | | 86 | 0 | 17.1 |
| Middle | 8.8 | | 80 | 0 | 7.8 |
| Bottom | 3.6 | | 48 | 0 | 0.7 |

* Inclusions are as defined in the text and in Figure 1.

Bart's. It was consistently found in higher percentage in old red cells and thus achieved a cellular distribution which contrasted with that of hemoglobin H.

2) *In vitro and in vivo studies using $C^{14}$-labeled amino acids.* The incorporation of *l*-leucine-U-$C^{14}$ into hemoglobins A and H was measured during *in vitro* incubations of whole peripheral blood or marrow aspirate for as long as 4 hours (Table III). The proportions of hemoglobin H and the numbers of reticulocytes and normoblasts in the incubation mixtures are set out in Table IV. The hemoglobin H specific activity was at all times higher than that of hemoglobin A and at the conclusion of the incubations was 4 to 7.5 times that of hemoglobin A. In patient S.S. studies of both peripheral blood and marrow aspirate were done. The marrow aspirate, incubated in duplicate flasks for ½ hour, yielded specific activities that were 14 times

TABLE III

*Specific activity of hemoglobins H and A after incubation in vitro with l-leucine-U-$C^{14}$**

| Incubation time | Patient S.S. Peripheral blood | | | Patient S.S. Marrow aspirate† | | | Patient A.R. Peripheral blood | | | Patient J.L. Marrow aspirate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HbH | HbA | H/A | HbH | HbA | H/A | HbH | HbA | H/A | HbH | HbA | H/A |
| *min* | | | | | | | | | | | | |
| 5 | 13.9 | 5.1 | 2.7 | | | | 7.4 | 6.5 | 1.1 | 26.4 | 9.4 | 2.8 |
| 10 | 19.2 | 7.0 | 2.7 | | | | 10.4 | 5.6 | 1.9 | 34.8 | 11.4 | 3.1 |
| 15 | 21.4 | 6.0 | 3.6 | | | | 10.7 | 4.4 | 2.4 | 66.2 | 14.6 | 4.5 |
| 20 | 30.2 | 7.8 | 3.9 | | | | 12.1 | 4.6 | 2.6 | 75.0 | 16.8 | 4.5 |
| 30 | 43.5 | 9.8 | 4.4 | 601 | 143 | 4.2 | 28.7 | | | 142.0 | 28.0 | 5.1 |
| | | | | 601 | 149 | 4.0 | | | | | | |
| 60 | 69.0 | 17.9 | 3.9 | | | | | | | 236.0 | 54.0 | 4.4 |
| 120 | 128.0 | 33.1 | 3.9 | | | | 45.8 | 5.7 | 8.0 | 462.0 | 87.8 | 5.3 |
| 240 | 200.0 | 46.0 | 4.3 | | | | 53.7 | 7.2 | 7.5 | 732.0 | 142.0 | 5.2 |

* For each incubation the specific activity of hemoglobin H (HbH) and hemoglobin A (HbA) in counts per minute per milligram and the ratio of the two specific activities (H/A) are given.

† Duplicate flasks incubated for 30 minutes only.

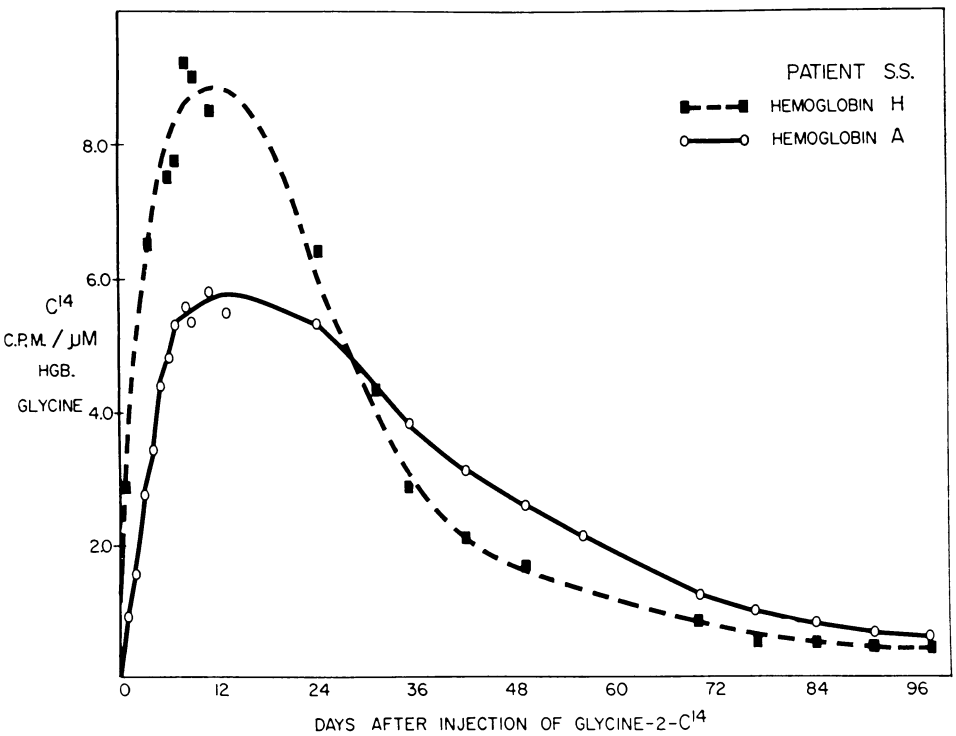


FIG. 2. SPECIFIC ACTIVITY OF HEMOGLOBINS H AND A GLYCINE AFTER INTRAVENOUS INJECTION OF GLYCINE-2-$C^{14}$ IN PATIENT S.S.

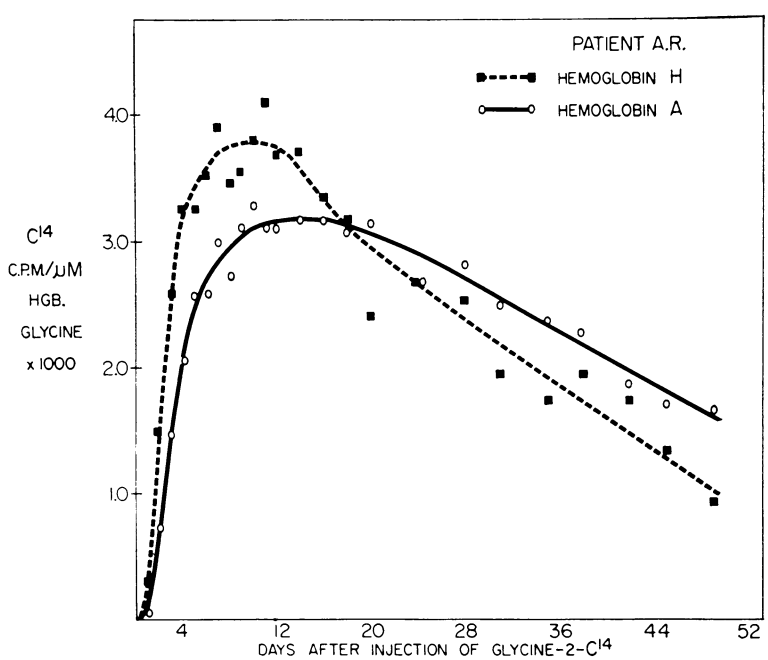


FIG. 3. SPECIFIC ACTIVITY OF HEMOGLOBINS H AND A GLYCINE AFTER INTRAVENOUS INJECTION OF GLYCINE-2-$C^{14}$ IN PATIENT A.R.

TABLE IV

*Hemoglobin H proportions and estimates of total numbers of synthesizing cells in incubations described in Table III*

| | | Hemoglobin H | Reticulocytes Total number per incubation flask | Normoblasts Total number per incubation flask |
|---|---|---|---|---|
| | | *% of total* | | |
| Patient S.S. | Peripheral blood | 14–15 | $2.5 \times 10^9$ | 0 |
| Patient S.S. | Marrow aspirate | 13–13 | $1.1 \times 10^9$ | $3.0 \times 10^7$ |
| Patient A.R. | Peripheral blood | 17–22 | $1.0 \times 10^9$ | 0 |
| Patient J.L. | Marrow aspirate | 3–6 | $0.8 \times 10^9$ | $1.2 \times 10^7$ |

higher than those of the corresponding peripheral blood incubation, yet the ratio of specific activity of the two hemoglobins was the same. This demonstrates that the qualitative synthesis of the hemoglobins was the same in the reticulocytes as in the normoblasts in this patient (S.S.), even though the quantitative synthetic activities were markedly different.

*In vivo* studies of the survival times of hemoglobins A and H in the circulation were performed in two subjects, using glycine-2-$C^{14}$. The results of the two studies were similar (Figures 2 and 3). Peak specific activities were attained on day 12 and were followed promptly by negative slopes. All curves had the characteristic pattern of predominantly random destruction (23), with a shortening of red cell survival time to about one-third the normal period of 120 days. The hemoglobin H specific activity peaks were higher than those of hemoglobin A, and the hemoglobin H negative slopes were steeper. Thus, although both hemoglobins exhibited curves of random destruction, hemoglobin H had a more rapid fractional turnover rate than hemoglobin A. The mean survival time of hemoglobin A was 45 days and that of hemoglobin H 33 days in patient A.R. The corresponding values in patient S.S. were 42 days and 28 days.

*3) Radiochromate binding to the hemoglobins during labeled red cell survival.* The two patients with hemoglobin H-thalassemia given autologous $Na_2Cr^{51}O_4$-labeled red cells showed a disappearance of the radioactive label from their blood with exponential negative slopes of $t_{\frac{1}{2}}$ 18 days (S.S.) and 13 days (J.L.) (Figures 4 and 5). The whole blood and whole hemoglobin followed curves that were almost identical. The chromium $^{51}$ radioactivity (counts per minute) of hemoglobins A and H, however, did not follow slopes parallel to those of the whole hemoglobin during these survival studies. The hemoglobin H

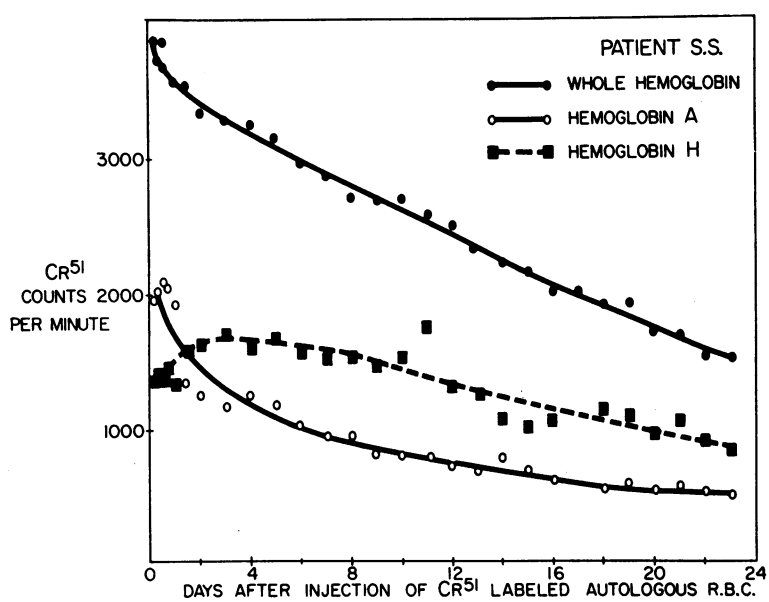


FIG. 4. BINDING OF RADIOCHROMATE TO HEMOGLOBINS H AND A IN BLOOD SAMPLES TAKEN OVER A PERIOD OF 23 DAYS AFTER INJECTION OF AUTOLOGOUS CHROMIUM$^{51}$-LABELED RED CELLS IN PATIENT S.S.

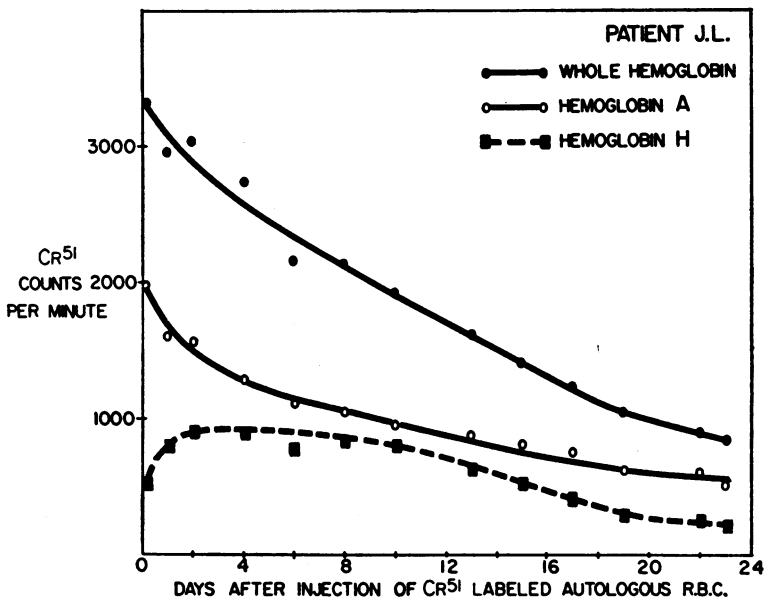


FIG. 5. BINDING OF RADIOCHROMATE TO HEMOGLOBINS H AND A IN BLOOD SAMPLES TAKEN OVER A PERIOD OF 23 DAYS AFTER INJECTION OF AUTOLOGOUS CHROMIUM$^{51}$-LABELED RED CELLS IN PATIENT J.L.

counts per minute actually increased for a few days to reach a plateau, followed ultimately by a decline. As the hemoglobin H counts per minute were rising, the hemoglobin A counts per minute fell more rapidly than those of the whole hemoglobin. The results in both patients were quite similar. Five separate samples were taken during the first 24 hours in the study of S.S. The close reproducibility of these multiple samples gave added confidence in the technique (Figure 4).

Electrophoretic separations on starch block were cut into 0.5-cm sections for localization studies of the hemoglobin and of the radioactivity. As described by others, the attachment of the $Cr^{51}O_4^=$ label to hemoglobin alters its electrophoretic mobility slightly in an anodal direction (24–28). In spite of this alteration, hemoglobin A and H radioactivities were well separated into cathodal and anodal zones, respectively, at neutral pH, as Malcolm, Ranney, and Jacobs have found (26). Localization studies were performed on the initial samples and then again on samples taken on day 7 and day 13 from patients S.S. and J. L., respectively, during studies of the survival of labeled autologous red cells. The second of the two samples studied confirmed in both patients that the hemoglobin H radioactivity peak had indeed grown at the expense of the hemoglobin A peak. In Figure 6 it is demonstrated that the hemoglobin H peak, which contained only 14% of the total hemoglobin, had acquired 62% of the total remaining radioactivity on day 7 of the survival study. Conversely, hemoglobin A contained 86% of the hemoglobin and only 38% of the radioactivity.

A control study performed in a patient without hemoglobin H thalassemia showed no anodal peaks of radioactivity in hemoglobin samples taken up to 10 days after injection of labeled autologous red cells. This excludes an alteration of labeled hemoglobin A during aging *in vivo* as an explanation for the phenomenon observed in the hemoglobin H thalassemia patients. A second control study of normal compatible red cells labeled with $Cr^{51}O_4^=$ and given to a hemoglobin H thalassemia patient also failed to show radioactivity peaks in the anodal zone in samples taken during 6 days following the injection. This finding demonstrates that the exchange of the chromium label from hemoglobin A to hemoglobin H is an intracellular phenomenon. This study also demonstrates the stability of the label in solution; hemoglobin H does not "steal" the label when labeled hemo-

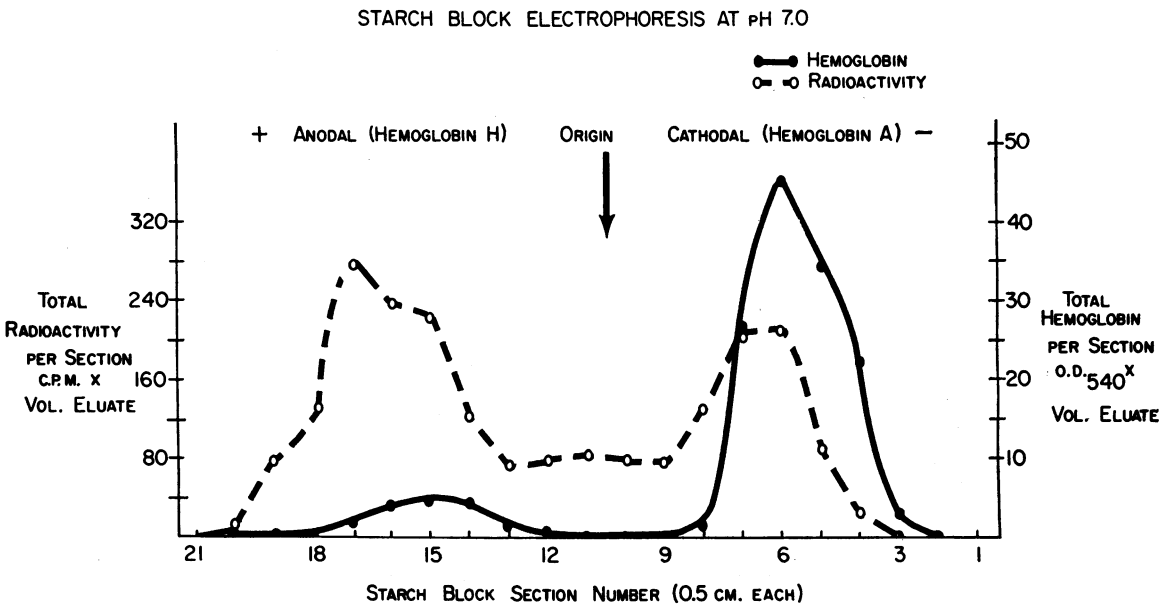


FIG. 6. ELECTROPHORETIC LOCALIZATION OF $Cr^{51}$ RADIOACTIVITY AND OF HEMOGLOBIN IN A BLOOD SAMPLE TAKEN ON DAY 7 OF THE STUDY SHOWN IN FIGURE 4. The hemoglobin solution was separated by starch block electrophoresis at pH 7.0 and then eluted from 0.5-cm sections.

globin A is added to it in solution under the conditions used. Localization studies on starch block electrophoresis of hemoglobin and radioactivity were done on samples taken on day 6 and day 9 of these two control investigations. Only one radioactivity peak was seen; the small amounts of radioactivity found in the anodal zone (Table V) occurred as tailing of the main hemoglobin A peak over the mid-line point of origin.

Repeat determinations that were done on samples stored for 2, 7, 9, and 11 days showed no significant alteration in $Cr^{51}O_4^=$ counts per minute in hemoglobins A or H. The average recovery of the radioactivity from the starch block

TABLE V

*Control studies of radiochromate binding to hemoglobin A during the lifespan of labeled red cells that do not contain hemoglobin H*

| Time after injection of $Cr^{51}$-labeled red cells | Patient H.J., control given labeled autologous red cells | | | | Patient S.S., hemoglobin H-thalassemia patient given labeled compatible normal red cells | | | |
|---|---|---|---|---|---|---|---|---|
| | Cathodal (Hemoglobin A) | | Anodal | | Cathodal (Hemoglobin A) | | Anodal (Hemoglobin H) | |
| | Total cpm | %* of total counts recovered | Total cpm | %* of total counts recovered | Total cpm | %* of total counts recovered | Total cpm | %* of total counts recovered |
| 20–60 minutes | 2,590 | 92 | 208 | 8 | 2,237 | 87 | 342 | 13 |
| 2–4 hours | 2,690 | 89 | 319 | 11 | 2,053 | 87 | 319 | 13 |
| 1 day | 2,399 | 87 | 348 | 13 | 1,902 | 85 | 343 | 15 |
| 2 days | 2,328 | 91 | 217 | 9 | 1,864 | 86 | 312 | 14 |
| 3 days | 2,225 | 91 | 224 | 9 | 1,881 | 85 | 338 | 15 |
| 4 days | 2,384 | 91 | 237 | 9 | 1,838 | 83 | 373 | 17 |
| 5 days | 1,892 | 91 | 184 | 9 | 1,873 | 85 | 325 | 15 |
| 6 days | 1,980 | 93 | 148 | 7 | 1,874 | 86 | 312 | 14 |
| 7 days | 1,897 | 92 | 160 | 8 | | | | |
| 8 days | 1,776 | 90 | 192 | 10 | | | | |
| 10 days | 1,644 | 91 | 156 | 9 | | | | |

* Expressed in terms of per cent of the total counts recovered from the starch block.

was 86%; the lowest recovery was 78%. There was no consistent alteration of radioactivity recovery during the course of the survival studies. The average recovery of the hemoglobin added to the starch block was 92%. During the studies of autologous radiochromate-labeled red cells the percentage of hemoglobin H ranged from 12.3 to 15.5 and from 6.1 to 9.1 for patients S.S. and J.L., respectively.

## Discussion

Rigas and Koler first observed that the young red cells found in the top layer of centrifuged columns of blood contained more soluble hemoglobin H than the bottom layer of old red cells (9). Their patients had postsplenectomy erythrocyte inclusions that were found in greatest frequency in the bottom layer. These authors concluded that although the cellular distribution of the hemoglobins was equal, less hemoglobin H was recovered from the old red cells because a proportion of it was present in the form of insoluble inclusions. However, morphologic observations by Fessas (29) and by Minnich and associates (10) indicated that the distribution of the hemoglobins was unequal in the absence of postsplenectomy inclusions. Fessas has stated that in certain individuals with alpha thalassemia trait in whom hemoglobin H is not electrophoretically demonstrable, occasional to rare cells may be observed that apparently contain hemoglobin H, as evidenced by the characteristic pattern of hemoglobin precipitation upon incubation with brilliant cresyl blue (29). In the present investigation, utilizing centrifugal separation in addition to *in vitro* and *in vivo* incorporation of labeled amino acids, the conclusion is justified that regardless of splenectomy, hemoglobins A and H are unequally distributed among the circulating red cells. Hemoglobin H also has a more rapid fractional turnover rate than hemoglobin A.

In two patients whose erythrocytes probably contained a small amount of hemoglobin Bart's, more of this hemoglobin was found in the old cells than in the young. Thus it would appear that hemoglobins H and Bart's achieve a cellular distribution analogous to that of hemoglobins A and F in patients with beta thalassemia (6, 7). Those cell precursors with higher rates of beta chain synthesis simultaneously have lower rates of gamma chain synthesis and in both syndromes have a shorter survival in the peripheral blood.

After splenectomy in H-thalassemia, the appearance of prominent intracorpuscular Heinz bodylike inclusions in high frequency is a predictable phenomenon (9, 10, 29). These postsplenectomy inclusions undoubtedly represent precipitation in the red cells of the labile hemoglobin H (9). Selective removal ("pitting") by the spleen of these particulate aggregates of denatured hemoglobin H from the surviving red cells is one possible explanation for the heterogeneous cellular distribution of the hemoglobins. Although no studies of splenic "pitting" have been made in H-thalassemia, indirect evidence is available that in some circumstances this mechanism is utilized in removing insoluble particulate matter from the red cells (11, 30). On the contrary, if hemoglobin precipitates are not removed from the red cells without simultaneous red cell destruction, then the shorter survival time in the circulation of erythrocytes containing more hemoglobin H offers the only alternative explanation.

The carbon$^{14}$ hemoglobin survival times performed in two patients demonstrate predominantly random-type red cell destruction in H-thalassemia. The random pattern pertains not only to hemoglobin H but to hemoglobin A as well. Thus the presence of a labile hemoglobin confers an element of random destruction upon the entire red cell population. In H-thalassemia this is superimposed on a moderate impairment of hemoglobin synthesis associated with thalassemia trait. The result is a mild anemia due to incompletely compensated hemolysis (31). On the other hand, when the labile hemoglobin is not superimposed on impaired hemoglobin synthesis, as in the case of hemoglobin Zürich (32), affected individuals show completely compensated hemolysis without anemia. In these patients anemia occurs only as the result of an aggravating factor, such as an oxidant drug.

Homogeneous labeling is an important requirement of any nonphysiological label such as $Na_2Cr^{51}O_4$. Hemoglobin H binds about twice as much $Cr^{51}O_4^=$ as hemoglobin A in the course of red cell labeling, possibly because the chromate ion becomes attached only to the beta polypeptide chain (9, 25, 26, 33). This lack of uniformity

casts some doubt on the theoretical acceptability of this label in hemoglobin H thalassemia. It appears that the heterogeneity of the label is still further augmented within a few days after the initiation of a chromium$^{51}$ red cell lifespan due to a net transfer of a substantial fraction of the label within the red cells from hemoglobin A to hemoglobin H. Hence hemoglobin H may become 13 times as radioactive as an equal quantity of hemoglobin A. Even though hemoglobin H may constitute a minor hemoglobin component, it may acquire most of the radioactive counts in the red cell.

Since hemoglobin H is catabolized more rapidly than hemoglobin A, erroneous conclusions may be drawn from routine chromium$^{51}$ survival and surface scanning studies. If intracorpuscular hemoglobin H precipitates are indeed removed selectively by the spleen, surface scanning during the course of chromium$^{51}$ survival studies may produce a false pattern of splenic sequestration because of the disproportionate amount of the radioactivity that hemoglobin H may acquire and leave in the spleen. In two of our patients (A.R. and J.L.), splenectomy failed to produce any subjective clinical improvement. There were no clearly significant increases of hemoglobin concentration, and reticulocyte percentages remained unchanged. In both of these cases patterns of splenic sequestration had been observed on body surface scanning after injection of autologous $Cr^{51}$-labeled red cells (Table I).

## Summary

From studies of three patients with H-thalassemia, it is concluded that the distribution of hemoglobins H and A among the red cells is unequal and that hemoglobin H has a more rapid fractional turnover rate than hemoglobin A. These conclusions are justified by the consistency of the results obtained from centrifugal separation of red cells, *in vitro* incorporation of labeled amino acid, and *in vivo* hemoglobin survival times measured with glycine-2-$C^{14}$. The latter studies showed patterns of random destruction with shortening of the red cell survival time to about one-third of normal.

During the study of the lifespan of $Na_2Cr^{51}O_4$-labeled autologous red cells in two patients with hemoglobin H-thalassemia, a substantial fraction of the label showed a net transfer from hemoglobin A to hemoglobin H. Control studies failed to demonstrate a similar phenomenon in red cells lacking hemoglobin H, either when given to the autogenous subject or to an H-thalassemia patient. Hemoglobin H, even though a minor hemoglobin component, may acquire a very large proportion of the $Cr^{51}$ label. Such heterogeneous trace labeling warrants caution in the interpretation of routine chromium$^{51}$ studies in hemoglobin H patients, particularly with respect to splenic sequestration patterns.

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