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Studies on Hemoglobin Biosynthesis: Asynchronous Synthesis of Hemoglobin A and Hemoglobin A₂ by Erythrocyte Precursors *

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Hemoglobin A₂ comprises less than 3.3% of the total hemoglobin of normal adults (1, 2). Elevated levels are found in many individuals with thalassemia minor (3, 4), in some patients with pernicious anemia in relapse (5), and in persons heterozygous for hemoglobin Zürich (6). Reduced levels are encountered in the newborn child (3), in some patients with anemia due to iron deficiency (5), and in persons with hereditary persistence of fetal hemoglobin (7, 8). In the study to be described, measurements were made of the relative rates of synthesis of hemoglobins A and A₂ by red cell precursors of patients with certain hematologic disorders.

Methods

Blood samples were drawn into tubes containing heparin, 0.2 mg per ml. Experiments were initiated within 2 hours except that blood from two patients with thalassemia major was transported in cooled containers and studied within 5 hours.

Measurement of hemoglobin. The concentrations of hemoglobin solutions were compared by measuring optical densities of oxyhemoglobin at 415 m μ of cyanmethemoglobin or cyanhematin (9) at 543 m μ .

Synthesis of hemoglobins A and A₂ by reticulocytes in vitro. Hemoglobin synthesis by reticulocytes *in vitro* was studied by incubating cells with Fe⁵⁹ citrate (1 μ g of Fe per ml; initial specific activity, approximately 30 μ c per mg) or uniformly labeled leucine-C¹⁴ (specific activity, 0.10 μ c per 0.0348 mg) and measuring the specific activities of the purified hemoglobin fractions.

In experiments employing radioactive iron, 1 ml of Fe⁵⁹ citrate (8 to 30 μ c) was incubated with 4 ml of a type AB human serum having a high iron-binding capacity. The final incubation medium was made by adding the Fe⁵⁹ serum to 5 ml of a modified Krebs-Henseleit

solution containing glucose, amino acids, penicillin G, and streptomycin sulfate as described by Borsook (10). Ferrous sulfate was omitted.

In experiments employing leucine-C¹⁴ the final incubation medium was made by drying samples (0.5 to 1 ml) of a solution of uniformly labeled leucine-C¹⁴ containing 100 μ c per ml (representing 348 μ g of leucine) in the incubation flask and then adding 4 to 8 ml of leucine-free modified Krebs-Henseleit solution without serum.

Reticulocyte-rich heparinized blood was centrifuged, and the plasma, leukocytes, and platelets were removed. The red cells were washed three times with 0.85% NaCl; then 1 vol of cells was added to 2 to 4 vol of incubation mixture. The cells were incubated at 37° C in a Dubnoff-type shaking water bath. The incubation was terminated at times ranging from 30 minutes to 20 hours. In some cases the rate of synthesis of each hemoglobin fraction was studied by removing samples from the incubation flask at various time intervals.

In experiments employing radioactive iron, the reaction was stopped by adding the mixture to $\frac{1}{2}$ vol of cold 0.1 N KCN. The red cells were washed five times with several volumes of 0.85% NaCl and then lysed by shaking with distilled water and carbon tetrachloride. Stromal material was removed by low speed centrifugation, and the cleared hemolysate was further centrifuged at 25,000 g for 1 hour. The hemolysate was dialyzed for several days against large volumes of 0.85% NaCl with many changes of the outside fluid.

In experiments employing leucine-C¹⁴ the incubation was stopped by freezing and thawing. Stromal material was removed by centrifugation, and then free leucine was removed by passing the hemoglobin solution through a column of Sephadex G-25¹ equilibrated with the chromatographic developer to be used in the subsequent separation of hemoglobin fractions. The complete removal of free leucine by this technique was confirmed by experiments in which, following passage through Sephadex, the hemolysate was dialyzed against large volumes of 0.85% NaCl with many changes of the outside fluid and no loss of radioactivity.

Preparation of hemoglobins A and A₂. In all experiments utilizing leucine-C¹⁴ and in some experiments utilizing Fe⁵⁹, hemoglobin fractionation was performed by

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¹ Pharmacia, Uppsala, Sweden.

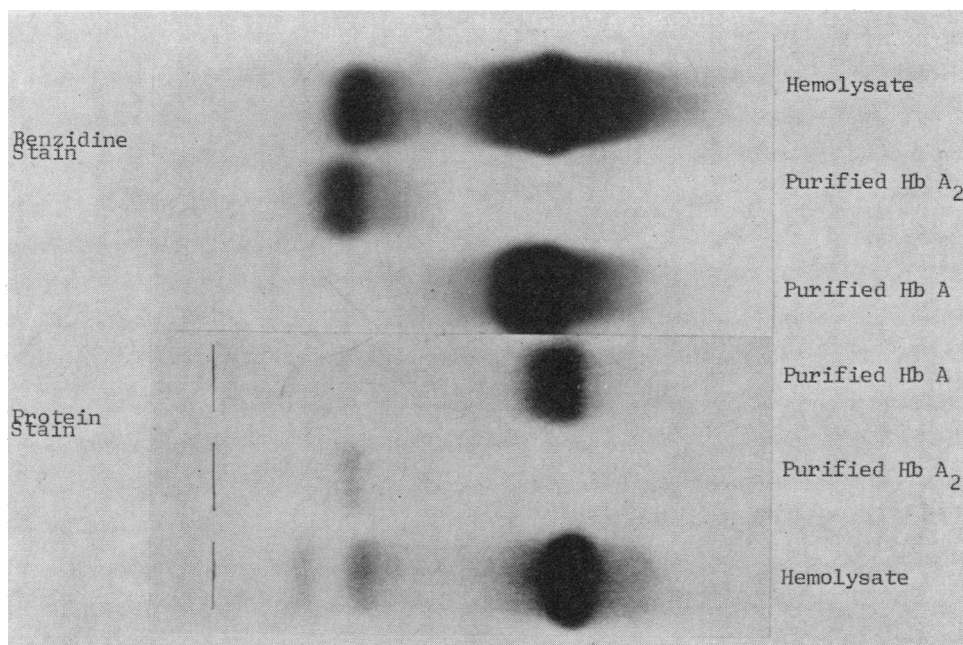


FIG. 1. STARCH GEL ELECTROPHORESIS AT PH 8.6. The Figure shows the results of electrophoresis of purified hemoglobins (Hb) A and A₂ and the cleared hemolysate from which the fractions were derived. The upper pattern was stained with benzidine and the lower pattern with amido black 10B.

initial column chromatography on Amberlite CG-50 followed by starch block and starch gel electrophoresis. In the remainder of the experiments utilizing Fe⁵⁹, separation was achieved by starch block electrophoresis alone.

Twenty- \times 1-cm columns were equilibrated at 4° C for several days against Developer IV or VI (11, 12), and 70 to 100 mg of hemoglobin in 1-ml vol was ap-

plied. After elution of the nonhemoglobin proteins and, in some cases, hemoglobin F I (11), the column was equilibrated at 28° and any hemoglobin F II eluted, followed by hemoglobins A and A₂. In some experiments hemoglobins A (or S) and A₂ were eluted as a single peak by the passage of 0.3 M sodium phosphate buffer, pH 7.6. After chromatography the fractions were dialyzed against water and concentrated by ultrafiltration.

TABLE I
Incorporation of Fe⁵⁹ into hemoglobin fractions by reticulocytes in vitro
(hemoglobins A and A₂ separated by starch block electrophoresis)*

Patient	Diagnosis	Hb g/100 ml	Hct %	Retic %	Hb A ₂ %	Incuba- tion time hours	Specific activity		
							Hb A cpm/mg	Hb A ₂	Ratio SA A/A ₂
Reticulocyte preparations									
A. B.	Ovalocytosis	11.4	36	5.1	2.7	6	81.6	38.6	2.1
R. R.	Spherocytosis	12.4	33.4	8.0	2.2	4.5	10.9	5.8	1.9
G. R.	Zieve's syndrome	8.2	28.2	12.2	2.5	21	86.4	40	2.2
L. Y.	Pyruvate kinase deficiency	10.6	34.3	15.6	3.0	5.3	16.0	8.4	1.9
J. Y.	Pyruvate kinase deficiency		28	42.2	2.5	7	257	141	1.8
M. W.	Iron deficiency	4.3	16.7	7.1	1.9	17.5	218	74	2.9
I. W.	Iron deficiency	7.8	27.2	13.2	2.3	21.3	362	179	2.0
O. P.	Iron deficiency	7.3	29	15.6	1.9	21.5	360	316	1.1
W. C.	Folic acid deficiency		22.6	22.5	2.5	4	123	72	1.7
P. K.	Pernicious anemia		17.2	28.6	3.2	20	244	103	2.4
F. S.	Pernicious anemia	4.9	17	19.3	3.2	21	526	211	2.5
D. W.	Myeloid metaplasia	8	25	8		5	673	325	2.1
M. J.	Sickle-cell anemia	9.8	31.8		3.6	15.3	15.5 (S)	6.9	2.2 (S/A ₂)

* Hb = hemoglobin, Hct = hematocrit, and retic = reticulocytes.

Hemoglobins A and A₂ were further purified by starch block electrophoresis using barbital buffer, pH 8.6 (3). Hemoglobins A, S, and A₂ were separated by starch gel electrophoresis (13, 14), individual bands being cut out and placed in slots in a starch block. After elution from starch block each fraction was concentrated, dialyzed against 0.001 M phosphate buffer, pH 7.0, and its purity checked by starch gel electrophoresis (Figure 1).

Measurement of radioactivity in hemoglobin fractions. Fe⁵⁹ radioactivity was measured in hemoglobin solutions of known volume and concentration with a 5- or 3-inch well-type NaI solid crystal scintillation counter. A 1.05-Mev base line with a 100-Kev window was employed.

For measurement of leucine-C¹⁴, known amounts of hemoglobin of approximately 0.5 mg in 0.1- or 0.2-ml vol were dried on aluminum planchets. The dried material was then redissolved and spread with a few drops of 91% formic acid and dried again as a thin spot in the center of the planchet. By this method the necessity for correction for self-absorption was avoided (15).

Enough sample and background counts were taken to assure a sample per cent error of less than 5%.

Results

Incorporation of Fe⁵⁹ into hemoglobins A and A₂ by reticulocytes in vitro. Table I indicates the results of 13 experiments in which blood from individuals with a variety of clinical conditions was incubated with radioactive iron for periods ranging from 4 to 21 hours. In each case hemoglobins A and A₂ were separated by starch block electrophoresis without initial column chromatography. In every experiment except one, the specific activity of hemoglobin A was approximately twice that of hemoglobin A₂. The relative specific activities of hemoglobins A and A₂ were not related to the number of reticulocytes in the incubation mixture.

To rule out the possibility that hemoglobin A

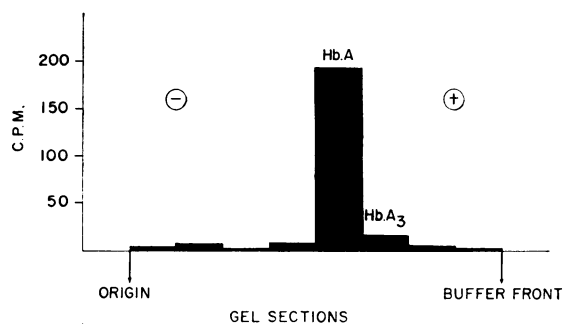


FIG. 2. DISTRIBUTION OF Fe⁵⁹ RADIOACTIVITY ALONG THE STARCH GEL. Radioactive hemoglobin A eluted from a starch block electrophoretic run was subjected to starch gel electrophoresis. The gel was cut in sections and the radioactivity measured in each section. Significant radioactivity was found only in the area of hemoglobin A.

separated by starch block was contaminated with a radioactive protein, two types of experiments were performed. Erythrocytes from a patient with pyruvate kinase deficiency (J. Y., Table I) were incubated with Fe⁵⁹, and hemoglobin A was separated by starch block electrophoresis. The eluted hemoglobin was subjected to starch gel electrophoresis, the gel sectioned, and the radioactivity of each section determined (Figure 2). No significant radioactivity was detected in any section other than that containing the hemoglobin A band. To exclude contamination of hemoglobin A by an iron-containing nonheme protein (16), the specific activities of hemoglobins A and A₂ were measured in four experiments in which the nonheme proteins were removed by initial column chromatography (Table II). In each case the specific activity of hemoglobin A was greater than that of hemoglobin A₂.

TABLE II

*Incorporation of Fe⁵⁹ into hemoglobin by reticulocytes (hemoglobins A and A₂ separated by column chromatography followed by starch block electrophoresis)**

Patient	Diagnosis	Hct	Retic	Hb A ₂	Incubation time	Specific activity		Ratio SA A/A ₂
						Hb A	Hb A ₂	
		%	%	%	hours	cpm/mg		
Reticulocyte preparations								
J. P.	Pernicious anemia	29.9	9		5	19.3	6.7	2.9
M. B.	Pernicious anemia	22.8	50	2.7	5	58	21	2.8
J. W.	Folic acid deficiency	25	28	3.8	4.5	55	30.9	1.8
J. N.	Sickle-cell anemia	25	16.8		5	112 (S)	51.8	2.2 (S/A ₂)

* See footnote to Table I for abbreviations.

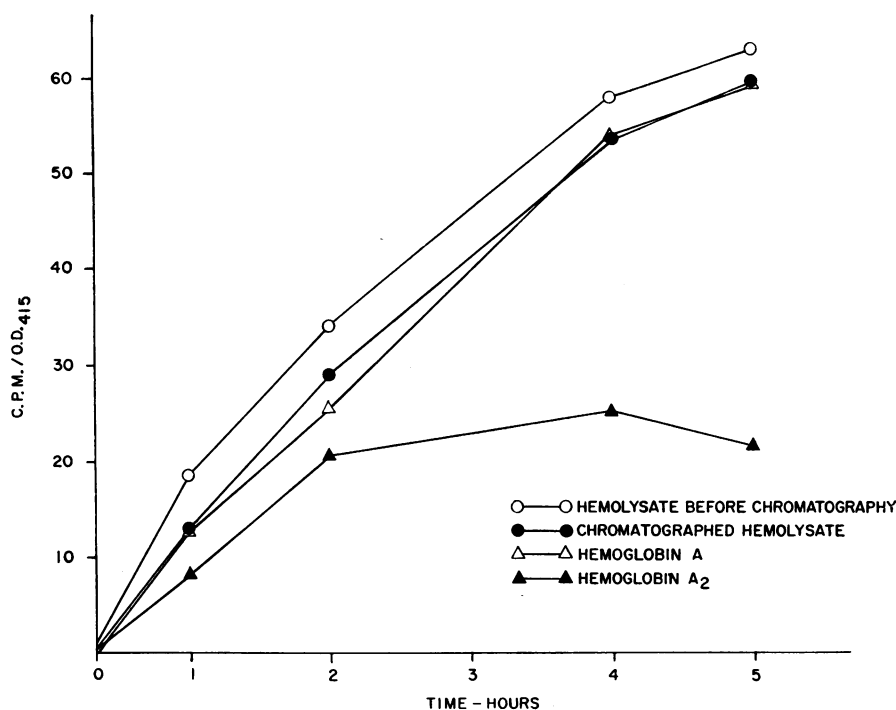


FIG. 3. INCORPORATION OF Fe^{59} BY RETICULOCYTES. The rise in the specific activities is shown for the hemolysate before and after chromatography and for the purified hemoglobins A and A₂ fractions.

Figure 3 shows the results of an experiment in which blood was incubated for 5 hours with radioactive iron, samples being withdrawn at various times and studied before and after column chromatography. In each case the specific activity of hemoglobin A₂ was lower than that of hemoglobin A or of the whole hemolysate, the differences in specific activities becoming more marked with time of incubation.

Incorporation of Fe^{59} into hemoglobins A and A₂ in vivo. Patient R. R. (Table I) was given an intravenous injection of 10 μ c of Fe^{59} . Blood was collected on the fifth and sixth days after administration. Hemoglobins A and A₂ were separated, and the radioactivity in each fraction was determined. The specific activities of hemoglobins A and A₂ on day 5 were 2.7 and 2.8 and on day 6, 2.6 and 2.6, cpm per mg, respectively. The specific activities of hemoglobins A and A₂ were almost identical, indicating that synthesis of these two fractions was proportional to the peripheral blood concentrations.

Incorporation of Fe^{59} into hemoglobins S and A₂ by reticulocytes in vitro. Two experiments

were done with the blood of patients having sickle-cell anemia. The results are shown in Tables I and II. Hemoglobin S had a higher specific activity than hemoglobin A₂ in both experiments.

In the experiments with the blood of patient J. N. (Table II), a portion of the cells was re-

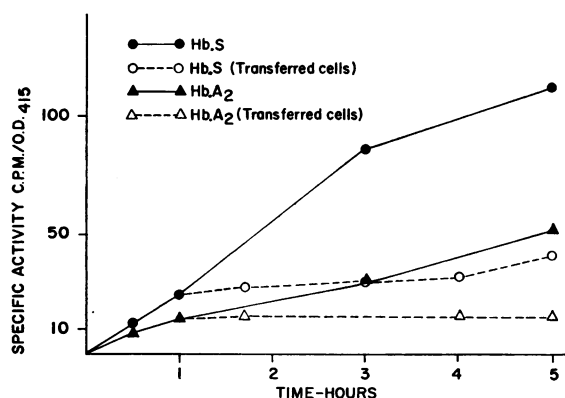


FIG. 4. INCORPORATION OF Fe^{59} INTO HEMOGLOBINS S AND A₂ BY RETICULOCYTES. The specific activities of hemoglobins S and A₂ are shown for cells incubated with Fe^{59} for 5 hours and for cells incubated with the radioactive isotope for 1 hour and then transferred to a non-radioactive medium.

TABLE III
Incorporation of Fe^{59} into hemoglobin by bone marrow suspensions

Patient	Diagnosis	Hb g/100 ml	Hct %	Retic %	Hb A ₂ %	Incubation time hours	Specific activity		Ratio SA A/A ₂
							Hb A	Hb A ₂	
Bone marrow suspensions									
J. W.	Folic acid deficiency	6.5	18.8	0.3	4.2	4.3	157	128	1.1
J. W.	After 7 days of therapy		25	28	3.8	4.3	538	398	1.4
M. B.	Pernicious anemia		16.1	1.9		5	72	58	1.2
J. H.	Pneumococcal pneumonia	1.11	34	8.1		5.5	1.6	1.2	1.3

moved after 1 hour of incubation, washed with 0.85% NaCl, and then placed in an incubation medium containing nonradioactive ferrous sulfate equilibrated with human serum. Incubation was then continued for 4 more hours. As seen in Figure 4 there was no drop in the specific activity of hemoglobins S or A₂. This indicates that no significant exchange of iron occurred between hemoglobin and the medium.

Incorporation of Fe^{59} into hemoglobins A and A₂ by bone marrow in vitro. The results of four experiments in which bone marrow samples were incubated with Fe^{59} are indicated in Table III. Concentration of the nucleated cells by centrifugation was not attempted. In each case the specific activity of hemoglobin A was slightly greater than that of hemoglobin A₂. However, the ratios of the specific activities were much closer to unity than those found for experiments employing reticulocytes.

Incorporation of leucine-C¹⁴ into hemoglobins A and A₂ by erythrocyte precursors in vitro. A

series of experiments was done with leucine-C¹⁴ as the radioactive tracer. In each case non-hemoglobin proteins were removed by column chromatography before separation of the hemoglobin fractions. Table IV shows the incorporation into hemoglobins A and A₂ by reticulocytes. The specific activity of hemoglobin A was two to three times that of hemoglobin A₂ at incubation times ranging from 2 to 5 hours. In each experiment, however, no difference in the specific activities of the two hemoglobins was seen after very short periods of incubation (Figure 5).

Table IV shows the results of an experiment in which reticulocytes were incubated in modified Krebs-Henseleit solution from which leucine had not been excluded. The ratios of the specific activities of hemoglobins A and A₂ were the same as in experiments in which leucine was excluded.

Table IV also shows the results of an experiment in which bone marrow was incubated with leucine-C¹⁴. The ratio of the specific activities of hemoglobins A and A₂ was much closer to unity

TABLE IV
Incorporation of leucine-C¹⁴ into hemoglobin by reticulocytes and bone marrow in vitro

Patient	Diagnosis	Hct %	Retic %	Hb A ₂ %	Incubation time min	Specific activity		Ratio SA A/A ₂	
						Hb A	Hb A ₂		
Reticulocyte preparations									
M. G.	Autoimmune hemolytic anemia	25	12.2	2.5	3	17.5	20.8	0.8	
					10	51.9	60.6	0.8	
					45	108.9	80.1	1.3	
					240	334.8	134.5	2.5	
W. C.	Hereditary spherocytosis	25	20.9	2.2	240	3,157	1,059	3.3	
Reticulocyte preparation with excess leucine									
A. C.	Pyruvate kinase deficiency	29	30.8	2.7	240	4,400	1,237	3.5	
Bone marrow suspension									
E. G.	Bone marrow	40	<1	3.0	240	67.7	43.9	1.5	

TABLE V
In vitro incorporation of leucine-C¹⁴ into various hemoglobin fractions by erythrocyte precursors in thalassemia

Patient	Diagnosis	Hb g/100 ml	Hct %	Retics %	Nu- cleated RBC/ 100/ WBC*	Hb				Specific activity			Ratio SA	
						A	S	A ₂	F	A	S	A ₂	A/A ₂	S/A ₂
S. B.R.	Sickle-cell thalassemia	13.2	43.4	3.6	0	31.1	1.9	63.9	3.1	86	88.1	53.0	1.6	1.7
S. B.	Sickle-cell thalassemia	8.5	25	9.5	4	6.7	6.4	81.4	5.5	36.8	36.0	35.1	1.1	1.0
A. C.	Thalassemia	8.6	26.3	2.2	19	93.8	5.0		1.2	6.0		8.5	0.71	
S. P.	Thalassemia		21.5	6.9	147	61.1	35.0		3.9	1,870		1,729	1.1	
A. S.	Thalassemia		30	12.8	305	15.9	80.6		3.4	1,900		2,600	0.73	
M. D.	Thalassemia		32	2.0	32	86.1	11.4		2.5	558		1,010	0.55	

* RBC = red blood cells; WBC = white blood cells.

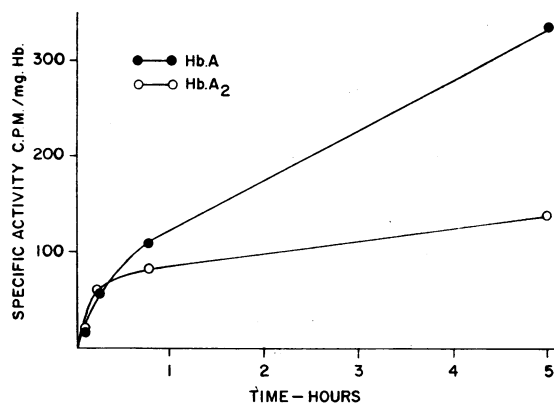


FIG. 5. INCORPORATION OF LEUCINE-C¹⁴ BY RETICULOCYTES IN VITRO. The specific activities of hemoglobin A and A₂ are equal during the first half hour of incubation.

than in the case of the experiments employing reticulocytes. This is analogous to the results of the experiments with Fe⁵⁹.

Synthesis of hemoglobins A and A₂ by erythrocyte precursors from patients with sickle-cell thalassemia and thalassemia. The blood of two patients with sickle-cell thalassemia was incubated with leucine-C¹⁴ and the specific activity of hemoglobins A, S, and A₂ determined. The results are shown in Table V. In the case of patient S. B.R., who had only mild disease, the ratios of the specific activities of hemoglobins A and A₂ and hemoglobins S and A₂ were 1.6 and 1.7. These results are at the lower limit of the range of the results obtained with reticulocytes in nonthalassemic disorders. However, in the case of patient S. B., who had a more severe form of the disease, the ratios were 1.1 and 1.0. These results are at the lower limit of the range of the results obtained with bone marrow in nonthalassemic disorders.

Experiments were performed using the blood of four patients with thalassemia major. The results are shown in Table V. In the case of patient A. C., Fe⁵⁹ was the isotope employed; for the other three experiments leucine-C¹⁴ was used. The results obtained were markedly different from those with reticulocytes in nonthalassemic disorders. The ratio of the specific activities of hemoglobins A and A₂ was 1.1 in one case and less than unity in the three other experiments.

Discussion

The relative percentage of hemoglobin A₂ in the peripheral blood depends on the relative rates

of synthesis and destruction of the major and minor hemoglobin components. Decreased synthesis of hemoglobin A₂ occurs in individuals with hereditary persistence of fetal hemoglobin (8). In patients with hemoglobin Zürich the elevated level of hemoglobin A₂ appears to be a reflection of loss of unstable hemoglobin Zürich (6). In thalassemia minor the increased percentage of hemoglobin A₂ in the peripheral blood is probably due to a combination of increased hemoglobin A₂ synthesis and decreased hemoglobin A production (17). The cause of the changes in hemoglobin A₂ levels in pernicious anemia and iron deficiency anemia is not known (5). Except for studies in thalassemic individuals (18), little is known about the relative survival times of hemoglobins A and A₂ in various hematologic disorders.

In the present study reticulocytes were obtained from individuals with a variety of hematologic disorders affecting erythrocyte production. Synthesis of hemoglobins A and A₂ by reticulocytes was measured *in vitro* by determining the rates of incorporation of Fe⁵⁹ or leucine-C¹⁴ into the two protein fractions. With reticulocytes from patients with disorders other than thalassemia, the results of all experiments except one were quite similar. The specific activity of hemoglobin A₂ was consistently less than that of hemoglobin A at periods of incubation ranging from 4 to 22 hours. At shorter incubation times this difference was much less marked. In experiments in which bone marrow was incubated under similar conditions the relative specific activities of hemoglobins A and A₂ were closer to unity, although that of hemoglobin A was slightly higher than that of hemoglobin A₂. In experiments utilizing blood from individuals with thalassemia major the ratio of the specific activity of hemoglobin A to hemoglobin A₂ was less than unity in three of four cases.

It is unlikely that these results are due to a technical artifact. There were no changes in the proportions of hemoglobins A and A₂ during incubation. Nonhemoglobin proteins were removed by column chromatography, and each fraction was further purified by starch block and starch gel electrophoresis. The contamination of hemoglobin A by a nonhemoglobin protein with identical electrophoretic and chromatographic charac-

teristics is possible but unlikely since, in experiments utilizing blood from individuals with sickle-cell anemia, the specific activity of hemoglobin A₂ was also less than that of hemoglobin S. No evidence of exchange between radioactive iron in hemoglobin A or A₂ and the incubation medium was found. Modifications of the relative rates of synthesis of hemoglobins A and A₂ due to relatively low concentrations of iron or leucine in the incubation mixture are unlikely, particularly in the experiments utilizing leucine, because of the large quantities of radioactive leucine that were added to the mixture. In addition similar results were obtained in an experiment utilizing excess leucine in the incubation mixtures.

Differences in the relative specific activities of hemoglobins A and A₂ could result from differences in their carrier pool sizes. A similar explanation has been invoked to explain the lower specific activity of hemoglobin F as compared with hemoglobin A in the blood of individuals with sickle-cell anemia and thalassemia major (18, 19). In these conditions the cells containing relatively high concentrations of hemoglobin F survive longer, and the turnover rate of hemoglobin F is consequently lower. Similar differences in specific activity of hemoglobins A and F in blood obtained from thalassemic individuals have been found after *in vitro* incubation of reticulocytes with radioactive amino acids (20, 21). In the present experiments, however, such variation in carrier pool is very unlikely, since at early times of incubation with both radioactive iron and amino acids, the specific activities of hemoglobins A and A₂ were similar. A difference in unlabeled carrier pool size would have resulted in differences in the specific activities at all times of incubation.

Similar specific activities of hemoglobins A and A₂ after administration of intravenous Fe⁵⁹ have been previously reported (22) and are confirmed in the present studies. These results indicate that the two hemoglobins are synthesized *in vivo* in the proportion found in the peripheral blood. Incubation of bone marrow suspensions with Fe⁵⁹ or leucine-C¹⁴ revealed specific activities for the two fractions that were much closer to unity than in experiments with reticulocytes. These results indicate that hemoglobin A₂ synthesis terminates before hemoglobin A synthesis in the reticulocyte. The hemoglobin A/A₂ ra-

tios of unity or less in blood samples obtained from individuals with thalassemia major probably reflect the large number of nucleated red cells present in the incubation mixture. However, even when bone marrow was diluted with blood containing less than 1% reticulocytes, the hemoglobin A/A₂ ratio was never less than unity, as was the case in three of four thalassemic blood samples studied. A fundamental difference in the relationship of hemoglobin A to A₂ synthesis in this disorder as compared with other hematologic states may be an alternative explanation for these findings.

Hemoglobin synthesis in the reticulocytes is almost complete, *in vitro* incorporation studies of the type presented in this study measuring only the last vestiges of protein synthesis. The results of these experiments indicate that, in reticulocytes, hemoglobin A₂ synthesis is retarded before that of hemoglobin A. The fact that similar results were not obtained in the presence of nucleated red cells suggests that these findings are real and not the result of an artifact of the *in vitro* incubation system. The mechanism by which hemoglobin synthesis is "turned off" in the reticulocyte has not been worked out. The *in vitro* differences in the retardation of hemoglobins A and A₂ synthesis may provide a model for the further study of these mechanisms.

Summary

The incorporation of both radioactive iron and amino acids into hemoglobins A and A₂ in an *in vitro* system has been studied. In reticulocyte preparations the specific activity of hemoglobin A always exceeds that of hemoglobin A₂ at incubation times of greater than 2 hours, whereas at earlier times, this difference is less marked. The difference is also less in experiments utilizing bone marrow and blood from individuals with thalassemia major. These results indicate that, in the reticulocyte, hemoglobin A₂ synthesis is retarded before that of hemoglobin A.

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