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Research Article

STUDIES OF THE INCORPORATION OF Fe^{59} INTO NORMAL AND ABNORMAL HEMOGLOBINS *

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This study was undertaken to ascertain whether different normal and abnormal hemoglobin components exhibit different rates of Fe^{59} incorporation in human subjects and in experimental animals. The hemoglobin of normal man contains minor components, hemoglobins A_2 and A_3, in addition to the principal component, hemoglobin A_1. Patients with hereditary hemoglobin abnormalities have relatively large amounts of abnormal hemoglobins; electrophoretic analysis of the hemoglobin of many experimental animals reveals a small amount of an electrophoretically rapid component, comparable to hemoglobin A_3 in human subjects. The present study was designed to observe the rate of Fe^{59} appearance in at least two hemoglobin components of a subject following the intravenous administration of Fe^{59}.

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MATERIALS AND METHODS

Except for L.B., who had thalassemia major, all the patients in this study had disseminated neoplastic disease in addition to their hemoglobin abnormalities. These patients were selected for study because 40 to 100 $\mu c.$ of Fe^{59} could be given, and accurate measurement of Fe^{59} activity in small amounts of hemoglobin components was thereby facilitated. Most of the patients received radiotherapy before or during the study period. Two patients (J. B. and L. B.) had received blood transfusions within

TABLE I
Experimental subjects

Pt.	Sex	Age	Diagnosis	Hemoglobin abnormality	Fe^{59} given	Transfusion within 4 mos. of study period	Hematocrit (before injection of Fe^{59})
		yrs.			$\mu c.$		%
D. S.	F	80	Chronic-lymphatic leukemia	Sickle cell trait	80	No	30
R. P.	M	21	Osteogenic sarcoma with pulmonary metastases	Sickle cell trait	40	No	45
J. B.	M	49	Carcinoma of the lung	Hemoglobin C trait	100	Yes, 1,500 ml. one month prior to study	34
J. R.	M	65	Multiple myeloma	Alkali resistant hemoglobin (40%)	90	No	26
L. B.	M	30	Cooley's anemia	Alkali resistant hemoglobin	100	Yes	25
S. P.	M	56	Carcinoma of the lung	Rapid abnormal hemoglobin component	100	No	34
O. G.	F	43	Disseminated carcinoma	Sickle cell trait	80	No	32

six weeks before the administration of Fe^{59}. Relevant data concerning these patients are recorded in Table I.

The isotopic iron was administered intravenously as either Fe^{59} citrate or Fe^{59} bound to human β_1 globulin. Blood samples were obtained at intervals after the administration of the isotope and hemoglobin was prepared by the method of Drabkin except that $AlCl_3$ was omitted from the saline washings of the erythrocytes. The final hemoglobin solutions were centrifuged at 15,000 × G for 30 minutes and converted to carbonmonoxyhemoglobin before electrophoretic or chromatographic separation of components. The hemoglobin of L.B. was separated by alkali denaturation of oxyhemoglobin.

Electrophoretic separation of hemoglobin components was carried out in starch blocks by the method of Kunkel, Ceppellini, Müller-Eberhard and Wolf (1). After a 16 hour run the dense mid-portion of each component was cut from the block, and the carbonmonoxyhemoglobin was eluted from the starch with water. The hemoglobin concentration and isotopic activity were determined on the eluates. The hemoglobin concentration was determined in a Coleman Universal Spectrophotometer and the radioactivity of a 2 ml. aliquot was measured in a well-type scintillation counter. Sufficient counts were recorded to secure an accuracy of at least 3 per cent.

For the separation of fetal hemoglobin, the alkali denaturation technique of Singer, Chernoff and Singer (2) was used in the case of L.B.; the only modification was the use of 0.5 ml. hemoglobin specimens. Alkali denaturation was not entirely satisfactory for the purpose of this study because of the dilution of hemoglobin F in the alkali resistant fraction. Consequently for the second patient with alkali resistant hemoglobin, a modification of the chromatographic technique of Morrison and Cook (3) was utilized for separating fetal hemoglobin. The fetal hemoglobin sample selected for assay was the most

TABLE II

Studies of Fe^{59} incorporation in hemoglobin components of patients with sickle cell trait

Patient	Days after Fe^{59}	Hemoglobin fraction	Radio-activity	Ratio of specific activities of components where total = 100
			cpm/mg. Hgb.	
D. S.	2	Total hemolysate	28	100
		A	27	96
		S	28	100
D. S.	6	Total hemolysate	152	100
		A	159	105
		S	154	101
D. S.	11	Total hemolysate	174	100
		A	176	101
		S	179	103
D. S.	18	Total hemolysate	182	100
		A	182	100
		S	170	93
D. S.	34	Total hemolysate	194	100
		A	196	101
		S	194	100
D. S.	62	Total hemolysate	172	100
		A	169	98
		S	182	106
D. S.	76	Total hemolysate	147	100
		A	170	116
		S	156	106
D. S.	101	Total hemolysate	162	100
		A	161	99
		S	151	93
R. P.	6	Total hemolysate	11.7	100
		A	11.9	102
		S	13.4	115
O. G.	2.5	Total hemolysate	91	100
		A_1	89	98
		S	102	112
		A_2	80	88
		A_3	50	55
O. G.	5	Total hemolysate	129	100
		A_1	127	98
		S	125	97
		A_2	114	88
		A_3	82	64
O. G.	9	Total hemolysate	144	100
		A_1	143	99
		S	146	101
		A_2	122	85
		A_3	85	59
O. G.	10	Total hemolysate	143	100
		A_1	142	99
		S	146	102
		A_2	128	90
		A_3	92	64
O. G.	12	Total hemolysate	142	100
		A_1	148	104
		S	139	98
		A_2	138	97
		A_3	107	75
O. G.	24	Total hemolysate	153	100
		A_1	145	95
		S	167	109
		A_2	114	75
		A_3	111	73
O. G.	39	Total hemolysate	150	100
		A_1	159	106
		S	158	105
		A_2	148	99
		A_3	116	77
O. G.	49	Total hemolysate	150	100
		A_1	145	97
		S	145	97
		A_2	160	107
		A_3	129	86

concentrated 5 ml. fraction eluted with phosphate buffer, pH 6.3, and sodium concentration, 65 mEq. per L. The remainder of the hemoglobin was represented by the most concentrated fraction eluted with a buffer of sodium concentration, 425 mEq. per L. The hemoglobin concentration and radioactivity were determined in the same manner as for components separated by starch electrophoresis.

RESULTS

1. *Major fractions of hemoglobin*

a). Sickle cell trait. Three patients with sickle cell trait were studied over periods ranging from 10 to 101 days. No significant difference in Fe^{59} incorporation between sickle cell hemoglobin and hemoglobin A_1 was observed in any of the three patients who were studied. Results in these patients are recorded in Table II.

b). Hemoglobin C trait. The patient with hemoglobin C trait who was studied had been transfused six weeks prior to the administration of Fe^{59}. The results of the study are recorded in Table III. Since the isotopic activity of hemoglobin A and C differed in the initial specimens obtained from this patient, the reliability of the observation was checked by separating larger amounts of hemoglobin A and C on successive electrophoretic analyses and subsequently preparing recrystallized hemin from each component. The radioactivity of the preparations of hemin recrystallized from the A and C hemoglobin components showed a C:A ratio of 1.25; the pooled hemoglobin components used for the crystallization had a radioactivity ratio for C and A hemoglobins of 1.4.[1] In this patient the relatively higher radioactivity of the hemoglobin C fraction was attributed to dilution of the hemoglobin A fraction by the hemoglobin of previous transfusions. This hypothesis was sup-

TABLE III

Studies of Fe^{59} incorporation in hemoglobin components of a previously transfused patient with hemoglobin C trait

Days after Fe^{59}	Hemoglobin fraction	Radioactivity	Ratio of specific activities of components where total = 100
		cpm/mg. Hgb.	
3	Total hemolysate	36	100
	A	30	83
	C	55	153
4	Total hemolysate	57	100
	A	49	86
	C	84	147
5	Total hemolysate	63	100
	A	58	92
	C	100	159
6	Total hemolysate	77	100
	A	66	86
	C	111	144
14	Total hemolysate	87	100
	A	87	100
	C	134	154
18	Total hemolysate	84	100
	A	76	90
	C	122	145
21	Total hemolysate	87	100
	A	81	93
	C	120	138
25	Total hemolysate	86	100
	A	83	97
	C	107	124
69	Total hemolysate	63	100
	A	62	98
	C	61	97

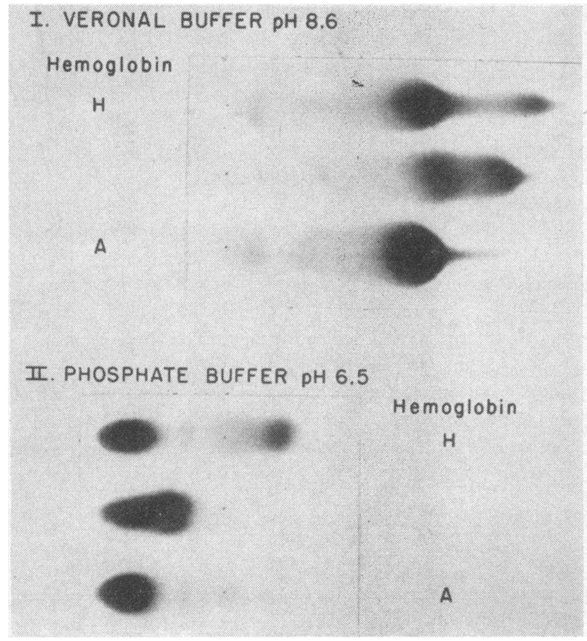

FIG. 1. FILTER PAPER ELECTROPHORETIC PATTERNS IN DIFFERENT BUFFERS OF NORMAL ADULT AND H HEMOGLOBIN TOGETHER WITH THE HEMOGLOBIN OF PATIENT S.P. WHO HAD AN ELECTROPHORETICALLY RAPID ABNORMAL HEMOGLOBIN

This hemoglobin is designated as "J group hemoglobin" (5). Electrophoresis for six hours at 450 volts; paper stained with bromphenol blue.

[1] The difference in radioactivity in the recrystallized hemin of C and A hemoglobins may be within the error of the methods since only a small amount of hemin from hemoglobin C was recovered.

TABLE IV

*Studies of Fe^{59} incorporation in hemoglobin components of patient with an electrophoretically abnormal rapid hemoglobin **

Days after Fe^{59}	Hemoglobin fraction	Radio-activity	Ratio of specific activities of components where total = 100
		cpm/mg. Hgb.	
1	Total hemolysate	55	100
	A	50	91
	Rapid component*	53	96
3	Total hemolysate	163	100
	A	150	92
	Rapid component	163	100
6	Total hemolysate	237	100
	A_2	185	78
	A	250	105
	Rapid component	234	99
9	Total hemolysate	239	100
	A	235	98
	Rapid component	242	101
13	Total hemolysate	263	100
	A	252	96
	Rapid component	253	96
16	Total hemolysate	269	100
	A	266	99
	Rapid component	260	97

* This component may have represented hemoglobin J or another rapid component (see text).

ported by the finding of approximately equal Fe^{59} activity in the hemoglobin A and C components isolated from the final blood specimen obtained 69 days after the administration of the isotope and more than three months after the last transfusion. At that time hemoglobin A from previous transfusions had disappeared.

c). Electrophoretically rapid abnormal hemoglobin. Patient S.P. who had an electrophoretically rapid abnormal hemoglobin was an American Negro; approximately 50 per cent of his hemoglobin had an electrophoretic mobility faster than hemoglobin A at pH 8.6. He was presumed to have hemoglobin J on the basis of electrophoretic mobility of hemoglobin specimens in acid and alkaline buffers (see Figure 1) (4), but no family studies could be carried out. Since this abnormal hemoglobin might also have been another electrophoretically rapid abnormal hemoglobin, *e.g.*, hemoglobin N, rather than hemoglobin J, it is designated as "electrophoretically rapid hemoglobin component" or "J group hemoglobin" (5) in this study. The results of the studies of Fe^{59} incorporation in hemoglobin A and the rapid major component are recorded in Table IV. Again no significant difference in Fe^{59} incorporation was noted between hemoglobin A and the major abnormal fraction.

d). Fetal hemoglobin. Patient J.R. had a large amount of fetal hemoglobin in association with multiple myeloma. By the method of alkali denaturation, the fetal hemoglobin amounted to 40 per cent of the total hemoglobin, and similar values were obtained by chromatographic techniques. The patient was known to have had a normal hemoglobin concentration during an unrelated brief

TABLE V

Studies of Fe^{59} incorporation in various hemoglobin components in patients with alkali resistant hemoglobin

Days after Fe^{59}	Hemoglobin fraction*	Radio-activity	Ratio of specific activities of components where total = 100
		cpm/mg. Hgb.	
a. Patient J. R.			
1	Total hemolysate	10.3	100
	Fetal	9.8	95
	Remainder	9.4	91
3	Total hemolysate	101	100
	Fetal	106	105
	Remainder	87	86
4	Total hemolysate	151	100
	Fetal	144	95
	Remainder	142	94
6	Total hemolysate	191	100
	Fetal	178	93
	Remainder	172	90
8	Total hemolysate	203	100
	Fetal	196	96
	Remainder	193	95
13	Total hemolysate	243	100
	Fetal	225	93
	Remainder	205	84
b. Patient L. B.			
5	Total hemolysate	7.3	100
	Fetal	15.2	208
6	Total hemolysate	7.9	100
	Fetal	14.4	182
7	Total hemolysate	7.4	100
	Fetal	15.8	214
13	Total hemolysate	7.0	100
	Fetal	14.4	206
17	Total hemolysate	6.7	100
	Fetal	16.5	246

* See text for methods employed in separation of fetal hemoglobin.

TABLE VI

Studies of Fe^{59} incorporation in hemoglobin components of healthy rabbits

Rabbit I				Rabbit II			
Days after Fe^{59}	Hb fraction	Radio-activity	Ratio of specific activities of components where total = 100	Days after Fe^{59}	Hb fraction	Radio-activity	Ratio of specific activities of components where total = 100
		cpm/mg. Hgb.				*cpm/mg. Hgb.*	
3	Total hemolysate	4,352	100	6	Total hemolysate	3,065	100
	Fast	1,940	45		Fast	1,137	37
	Main	4,692	108		Main	3,002	98
16	Total hemolysate	3,881	100	22	Total hemolysate	2,887	100
	Fast	2,324	60		Fast	2,218	77
	Main	4,198	108		Main	2,945	102
48	Total hemolysate	2,591	100	48	Total hemolysate	2,072	100
	Fast	2,985	115		Fast	1,951	94
	Main	2,547	98		Main	2,040	98
71	Total hemolysate	1,770	100	71	Total hemolysate	1,382	100
	Fast	1,671	94		Fast	1,047	76
	Main	1,553	93		Main	1,400	101

illness three years earlier; he did not have microcytosis and the values for hemoglobin A_2 were normal. Consequently he probably belonged to the group of patients with normal hemoglobin concentration and "hereditary persistence of fetal hemoglobin production" recently summarized by Jacob and Raper (6). In this patient the hemoglobin components were separated chromatographically for the Fe^{59} incorporation studies; the results (see Table V, a) showed the alkali resistant hemoglobin to have the same radioactivity as the remainder of the hemoglobin.

Patient L.B. who had thalassemia major had required whole blood transfusions two to three times monthly for several years. He had been transfused three weeks before Fe^{59} incorporation studies were done. As a result of multiple transfusions, he had extensive hemosiderosis; decreased hemoglobin production and dilution of the isotope by increased storage iron probably accounted for the low levels of radioactivity observed in the hemoglobin. In this patient, the fetal hemoglobin was separated by alkali; the greater isotopic activity of the fetal component (see Table V, b) was attributed to dilution of hemoglobin A by previous transfusion.

2. *Minor components of human hemoglobin*

On starch electrophoresis, two minor components of hemoglobin, hemoglobin A_2 and hemoglobin A_3, described by Kunkel and Bearn (7), were isolated during studies of Patient O.G. (Table II), and a few observations on hemoglobin A_2 were made during the study of Patient S.P. (Table IV). In Patient O.G. the isotopic activity of the electrophoretically rapidly moving hemoglobin A_3 component was significantly lower than the activity of the major fractions in the early period after the administration of Fe^{59}. However, the relative isotopic activity of the A_3 component appeared to increase somewhat in the later specimens, although Fe^{59} activity never reached the values observed in the principal components. A less striking initial decrease in the isotopic activity of the minor basic component, hemoglobin A_2, was also noted in O.G. and in S.P. Storage of the hemoglobin specimens in the cold and repeating the separations after a period of 10 days did not significantly alter the relative isotopic activities of the components.

3. *Rapid hemoglobin component in experimental animals*

Preliminary experiments showed that shortly after the administration of Fe^{59}, the "spear" or electrophoretically most rapid component of the hemoglobin of dogs, rabbits and mice exhibited Fe^{59} activity which was significantly lower than the isotopic activity of the main hemoglobin component. To test the effect of erythrocyte aging upon the relative activity of the slow component, two rabbits were given, respectively, 40 and 60 μc. of Fe^{59} and hemoglobin separations were carried out at intervals thereafter. The results of the experiments in these rabbits are recorded in Table VI. If the life span of the rabbit erythrocyte is taken to be about 60 days (8, 9), the increase in isotopic activity of the rapid hemoglobin component correlated well with cell aging.

DISCUSSION

Major and minor components of human hemoglobin

Because hemoglobin S and hemoglobin A coexist in most of the erythrocytes of subjects with sickle cell trait, it is reasonable to suppose that both hemoglobins are synthesized simultaneously before maturation of the cell. If this is the case both hemoglobins would be expected to exhibit the same isotopic activity although the total amount of each might show considerable variability in different patients with the trait. In the present study Fe^{59} activity of the hemoglobins of the circulating erythrocytes was measured; this technique probably would not yield evidence distinguishing between simultaneous, alternating or sequential synthesis of different hemoglobins in the immature erythrocytes of the bone marrow. However, the study of Kunkel and Bearn (7) of minor human hemoglobin components and the data on Fe^{59} incorporation into minor hemoglobin components in this study indicate that the interrelationships of various hemoglobins are not necessarily simple since Fe^{59} activity of the hemoglobins may change with time. The fact that minor components exhibit lower isotopic activities than the normal major component has been interpreted as evidence of change in hemoglobin with erythrocyte aging (7), although other explanations are possible. Following the administration of Fe^{59} the minor rapid hemoglobin component, hemoglobin A_3, showed low Fe^{59} activity which subsequently increased; in contrast, in the absence of previous transfusions, the two major components of hemoglobin in patients with sickle cell trait, hemoglobin C trait, alkali resistant hemoglobin, and "group J hemoglobin" trait both showed comparable Fe^{59} activity throughout the period of study. These observations provide some evidence against changes in *major* hemoglobin components with erythrocyte aging.

Studies in patients who had been previously transfused (L.B. and J.B.) demonstrated the effect of previous transfusions in diluting hemoglobin A, with resultant higher isotopic activity in the fetal and hemoglobin C components.

From the data obtained by recrystallization of heme from the hemoglobin components isolated by starch electrophoresis, most of the radioactivity appeared to be due to Fe^{59} in the heme of hemoglobin, although some (nonspecific) adsorption of the isotope elsewhere or incorporation into heme enzymes may have occurred.

In view of the decreased Fe^{59} activity of the fast component (A_3) of normal adult hemoglobin, the data concerning Fe^{59} incorporation in the patient with an electrophoretically rapid *major* component are of particular interest. In that patient, S.P., Fe^{59} incorporation into the electrophoretically rapid hemoglobin did not differ significantly from the incorporation into hemoglobin A_1. However, lower radioactivity of small amounts of hemoglobin A_3 might not be detected in the presence of the large amount of rapid hemoglobin. The findings of the present study concerning major components of hemoglobin are in agreement with those of Motulsky who found no significant differences in Fe^{59} incorporation in major hemoglobin components in patients with hemoglobin H-thalassemia and sickle cell-hemoglobin C disease (10).

The diminished radioactivity of the electrophoretically rapid (A_3) component of human hemoglobin, when compared with the isotopic activity of the lower main (A_1) component, was first observed by Kunkel and Bearn (7); these investigators also noted that this difference appeared to be related to erythrocyte aging. In the present study, a gradual increase with time in the Fe^{59} activity of the electrophoretically rapid A_3 component was again noted. Precise correlation of this effect with erythrocyte aging was not possible because of the probable occurrence of accelerated erythrocyte destruction in patients with disseminated neoplastic disease. With accelerated erythrocyte destruction, particularly of the random variety, reincorporation of Fe^{59} into newly synthesized hemoglobin might result in consistently higher isotopic activity in the A_1 hemoglobin component.

Electrophoretically rapid hemoglobin component in experimental animals

Studies of Fe^{59} incorporation into the electrophoretically rapid component of hemoglobin in two normal rabbits yielded data which could be better correlated with erythrocyte aging than the data obtained in man. In these rabbits a gradual increase in the isotopic activity of the fast component was noted during the first 48 days of the study. The decline which was observed at 70 days in the relative Fe^{59} activity of the most rapid hemoglobin component in one of these animals may have been related to reincorporation of the isotopic iron into the newly formed main hemoglobin component.

These observations in experimental animals indicate that an electrophoretically rapid hemoglobin may be formed in aging erythrocytes as suggested by Kunkel and Bearn (7). However, our preliminary studies of young and old erythrocytes separated by osmotic hemolysis (11, 12) have failed to demonstrate differences in relative isotopic activity between the hemoglobin components in the young resistant cells as contrasted with the older more fragile cells. Furthermore, the hypothesis that erythrocyte aging is responsible for the formation of this electrophoretically rapid component fails to explain the appearance of significant isotopic activity in the rapid component within a few days after the administration of Fe^{59} when Fe^{59} labeled hemoglobin should be present only in young red blood cells.

Further studies of this electrophoretically rapid hemoglobin component may disclose a correlation with decreasing enzyme levels in aging erythrocytes. However, the influence of factors other than cell aging (for example, hemoglobin distribution within the erythrocyte) on the formation of this electrophoretically rapid hemoglobin component should also be studied.

SUMMARY

1. The incorporation of Fe^{59} into the major components of hemoglobin in individuals with sickle cell trait, hemoglobin C trait, electrophoretically rapid major hemoglobin component of the "J group," probable hereditary persistence of fetal hemoglobin production and thalassemia major revealed no difference in Fe^{59} incorporation in the major components.

2. Observations on the minor electrophoretically rapid components of hemoglobin in a patient with sickle cell trait confirmed the observations of Kunkel and Bearn (7) that the isotopic activity of this hemoglobin A_3 component gradually increased during the 50 day period of study.

3. The studies of the incorporation of Fe^{59} into the electrophoretically rapid component of the hemoglobin of rabbits also supported the hypothesis that the formation of an electrophoretically rapid hemoglobin component in experimental animals is correlated with erythrocyte aging. The possibility that factors other than cell aging may influence the formation of this electrophoretically rapid normal component was discussed.

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