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Research Article

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Studies on the Formation of Ferritin in Red Cell Precursors *

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The mechanisms involved in the uptake and utilization of iron by red cell precursors have been studied by a number of investigators. Initially iron is released from the plasma protein, transferrin, onto the cell membrane and passes rapidly into the cell interior (1, 2). The results of several studies indicate that it is then taken up by a nonhemoglobin protein before being incorporated into hemoglobin (2–4). The nature of the precursor complex is, however, still not clear. In a series of *in vitro* studies on human and dog marrow suspensions, Greenough, Peters, and Thomas (4) found that it could be distinguished from ferritin but had properties in common with globin. They therefore suggested that the iron combines with a protein, which is possibly a part of the globin molecule, and that this complex (sideroglobin) then unites with protoporphyrin to form hemoglobin. Although such a concept could well account for one pathway taken by iron within the red cell precursor, it does not elucidate the significance of the appreciable quantities of ferritin that can be seen in erythroid precursors by electron microscopy (5). Theoretically it is possible that a proportion of the iron entering the cell is temporarily stored in ferritin before being used for hemoglobin synthesis, and recent evidence has been presented in support of this thesis (6). Alternatively, the ferritin iron may represent a surplus that is excreted when the cell has matured.

The present studies were undertaken to try to elucidate the possible role of ferritin in hemoglobin synthesis and to define the relationship, if any, between it and the hemoglobin precursor (Fraction 1) described by Greenough and coworkers (4).

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Methods

Marrow was obtained either from the ribs of subjects undergoing thoracotomy or by aspiration from the posterior iliac crest. All the marrows were obtained from hematologically normal subjects, except for two specimens that were obtained from patients with proven iron-deficiency anemia. Venous blood containing between 15 and 20% reticulocytes was obtained from two patients with pernicious anemia in early remission, and from one subject with idiopathic autoimmune hemolytic anemia. Reticulocyte-rich blood was also obtained from rats 3 to 4 days after the parenteral administration of an ethanolic solution of phenylhydrazine (30 mg per kg). All these samples were treated the same as the marrow. In addition, *in vivo* studies were carried out in one normal, splenectomized human subject and in both normal and splenectomized rats.

Specimens of marrow were suspended in balanced salt solution (B.S.S.) (7) containing 200 U penicillin, 100 μg streptomycin, and 100 μg neomycin per ml, and were processed and used within 1 hour after removal from the patient. Marrow obtained from ribs was shaken vigorously and then syringed repeatedly through a 20-gauge Luer lock needle until adequate dispersion of the cells was obtained. In certain studies splenic suspensions were also prepared in this way. The marrow was washed once in cold B.S.S. to remove the supernatant fat and was then suspended in B.S.S. containing 10 to 30% autologous serum.

$Fe^{59}Cl_3$ (SA, 20 to 30 μc per μg) bound to autologous serum was added to the marrow suspension at the start of the incubation. The amount of $Fe^{59}Cl_3$ added was always less than the binding capacity of the serum present. Serum iron levels were estimated by a modification of the method of Bothwell and Mallett (8) and iron binding capacities by the method of Bothwell, Jacobs, and Kamener (9). Equal samples of marrow suspension were incubated at 37° C in air in 25-ml Erlenmeyer flasks placed on top of a slowly revolving drum inclined at 10° to the horizontal. Samples of marrow were removed at varying times, and the reaction was stopped by the addition of 0.5% potassium cyanide in saline and rapid cooling to 0° C. In some experiments, further Fe^{59} labeling of the marrow was stopped after 5 to 10 minutes'

incubation by cooling the marrow rapidly to 4° C, washing it free of external radioactivity, and reincubating it in unlabeled incubation medium. After incubation the marrow cells were washed 3 times with saline and were then lysed by the addition of 4 vol of distilled water. The samples were then shaken vigorously and kept at 4° C for 30 minutes. The lysate was centrifuged for 20 minutes at $1,500 \times g$. The resulting clear red supernatant solution was siphoned off and kept. The precipitate ("stromal fraction") was washed twice in 10 vol of cold distilled water and centrifuged at $1,500 \times g$ for 20 minutes.

Isolation of Fraction 1 and main hemoglobin. A sample of the supernatant solution was chromatographed on Amberlite CG-50 resin, Type II,[1] prepared according to the method of Hirs, Moore, and Stein (10) as modified by Greenough and co-workers (4). The fraction eluting with the solvent front (Fraction 1) was separated with the starting buffer (0.0067 M sodium phosphate with 0.0033 M KCN at pH 7.0), and all other components (main hemoglobin) were eluted from the column by changing directly to the limiting buffer (0.5 M NaCl dissolved in the starting buffer). Recoveries of radioactivity were between 85 and 95%. To ascertain whether the main hemoglobin fraction represented an accurate measure of hemin synthesis, the percentages of counts of Fe^{59} incorporated into main hemoglobin and into hemin in a sample of supernatant solution were compared. Hemin was crystallized by the method of Labbe and Nishida (11). To correct for the incomplete recoveries obtained with this technique, a solution of pure hemoglobin, labeled with Fe^{55}, was added to the Fe^{59} supernatant solution at the beginning of the procedure, and the washed hemin crystals were finally digested, electroplated onto copper discs, and counted for Fe^{55} and Fe^{59} with methods previously described (12). In this way it was possible to calculate the percentage of Fe^{59}-labeled hemin in the original supernatant solution. With this approach the mean percentage of Fe^{59} incorporated into hemin was 83.4% (range, 77% to 90%) of the value for main hemoglobin.

Isolation of ferritin. Human liver ferritin was prepared from siderotic Bantu livers by the method of Mazur and Shorr (13). An antiserum was prepared by bleeding rabbits immunized with samples of alum-precipitated human ferritin by a modification of the method of Proom (14). (One ml of the pooled antiserum precipitated approximately 1 mg ferritin). In studies on rats an antiserum to purified horse spleen ferritin was used, since a cross-immunity has been shown between these two species (15).

To 1 vol of the solution to be tested was added an equal volume of 0.2 M sodium ethylenediaminetetraacetic acid (EDTA) buffered to pH 7.0. One ml antiserum and 0.2 ml carrier ferritin solution containing approximately 500 μg ferritin were then added, and the mixture was incubated for 1 hour at 37° C. The ferritin-antiferritin complex, which appeared as a visible precipitate, was washed 3 times with distilled water after centrifugation at $1,500 \times g$ for 10 minutes. The Fe^{59} activity of the precipitate was determined.

In this method carrier ferritin was added to the solution for two reasons: *1)* Precipitation could be verified by chemical methods, and *2)* it allowed for the measurement of minute amounts of labeled ferritin. The theoretical possibility that free Fe^{59} or some other labeled small molecule in the supernatant solution might attach to the carrier ferritin and be precipitated with the ferritin-antiferritin complex was avoided by the addition of EDTA to the solution, since preliminary studies showed that it prevented nonspecific attachment of free Fe^{59} to carrier ferritin. In a further experiment, antiserum was added to a marrow hemolysate containing EDTA and ferritin labeled physiologically with Fe^{59}. Under these circumstances 96% of the radioactivity was precipitated. The specificity of the antiserum was confirmed by the demonstration that when the antiserum was incubated with solutions of hemoglobin and transferrin labeled with Fe^{59} no activity was precipitated. In addition, negative results were also obtained with Fe^{59}-labeled main hemoglobin, obtained after chromatography of marrow supernatant solutions.

Ferritin was also identified using the Ouchterlony agar gel technique as described by Bodman (16). Antiferritin serum was placed in the center well while human liver ferritin, labeled *in vitro* with Fe^{59}, and marrow supernatant solutions were placed in the peripheral wells. After the bands of precipitation had formed, the gels were washed for 3 days with frequent changes of distilled water. They were dried until paper-thin and were then placed in apposition with X-ray films for several days to obtain radioautographs.

Results

Demonstration of physiologically labeled ferritin in marrow suspensions at various times of incubation. The incorporation of Fe^{59} into the "stromal fraction," Fraction 1, ferritin, and main hemoglobin was followed over several hours in six experiments. Since the major changes in the pattern of incorporation of Fe^{59} into the various fractions occurred in the first hour of incubation, most samples were analyzed during this period. To permit comparison between results obtained in the different experiments, the amounts of radioactivity present in the various fractions at different times were expressed as percentages of the total cellular uptake at 1 hour.

Radioiron was rapidly taken up by the marrow suspension. After 5 minutes between 23 and 37% was already present in cells, and this rose to between 71 and 79% by 30 minutes. Approximately half of this uptake was present in the

[1] Manufactured by the Rohm & Haas Co., Philadelphia, Pa.

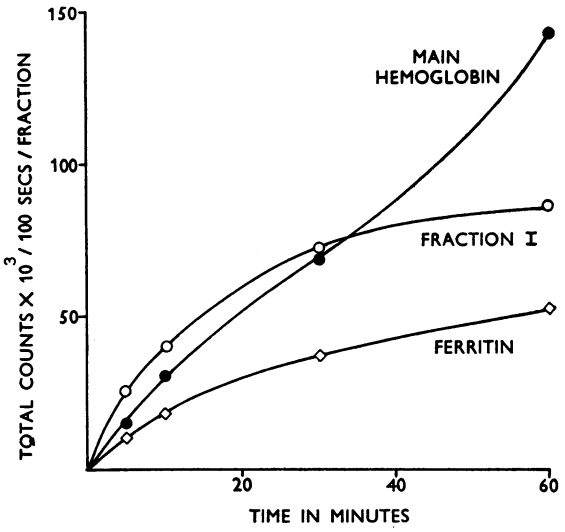

FIG. 1. INCORPORATION OF Fe^{59} INTO FRACTION 1, MAIN HEMOGLOBIN, AND FERRITIN. Analysis of samples of human rib marrow culture at various times up to 1 hour.

"stromal fraction." Between 5 and 30 minutes the mean percentage present in Fraction 1 rose from 5.5 (range, 3.7 to 9.9%) to 16.8 (range, 12.7 to 22.0%). The rate of increase over the next 30 minutes was much slower, the mean figure at 1 hour being 18.5% (range, 15 to 28%). Between 2.0 and 4.6% of the Fe^{59} was incorporated into ferritin by 10 minutes, and thereafter the radioactivity in ferritin increased in linear fashion up to 1 hour, when between 8.3 and 16.4% was present in this fraction. It was noteworthy that the amount of radioactivity in ferritin was always less than in Fraction 1. Only small amounts of Fe^{59} (between 2.0 and 5.9%) were incorporated into main hemoglobin in the first 10 minutes, but by 60 minutes between 19.0 and 36% was present in this compound. The pattern of changes occurring in one of the experiments is shown in Figure 1.

Between 1 and 12 hours there were no marked changes in the distribution of Fe^{59} between the various fractions. The rate of uptake of radioactivity by the cells was not as rapid as over the first hour, the figures after 4 hours ranging between 1½ and 3 times the 1-hour uptakes. At this stage between 17.6 and 32.5% of the activity taken up was in Fraction 1, from 10.2 to 23.0% in ferritin, and between 22 and 46% in main hemoglobin.

Site of formation of ferritin. As marrow is a heterogeneous tissue it was necessary to determine whether the ferritin isolated in the marrow supernatant solution had been labeled in red cell precursors or in reticuloendothelial cells. Since preliminary studies indicated that bone marrow suspensions obtained from the femurs of rats metabolized iron in similar fashion to human bone marrow suspensions, three *in vitro* studies were done in which the uptake of Fe^{59} from labeled autologous plasma by marrow suspensions was compared with that of splenic suspensions prepared in the same way (see Methods). After 3 hours' incubation the samples were washed 3 times with normal saline and were then counted for radioactivity. The total uptakes by the splenic suspensions were less than 10% of those obtained with equivalent wet weights of marrow. After homogenization with 4 vol of water, samples of the supernatant solution from the splenic suspensions were set up with antiferritin serum. It was found that 0, 2, and 14% of the activity taken up by splenic cells was precipitated as ferritin. The comparable figures obtained with the marrow suspensions were 10, 23, and 16%, respectively. When these figures were related to the total uptakes of the two tissues, the spleen's capacity to incorporate Fe^{59} into ferritin was between 0 and 10% of a comparable weight of marrow. Although it is possible that reticuloendothelial cells in the marrow may behave differently from those in the spleen, this seems unlikely, and the results obtained therefore suggest that the labeling of ferritin observed in marrow cultures was occurring predominantly in red cell precursors and not in reticuloendothelial cells.

Demonstration of physiologically labeled ferritin in Fraction 1. Addition of antiferritin serum to Fraction 1 resulted in the precipitation of a significant number of counts as ferritin. When a solution of Fraction 1 was placed opposite antiferritin serum on an agar gel diffusion plate no visible band resulted, but a radioautograph of the washed plate showed a line of radioactivity opposite Fraction 1 that coalesced with the lines of radioactivity corresponding to the bands of labeled pure ferritin (Figure 2). A similar band was noted when a sample of the whole supernatant solution was treated in the same way. These results therefore confirmed the presence of

ferritin in both the supernatant solution and Fraction 1.

When the percentage of counts incorporated into ferritin at various time intervals was measured directly on the supernatant solution, the results were in close agreement with those obtained when the same estimation was carried out on Fraction 1 (Table I). These findings indicate that all the ferritin present in the marrow supernatant solution was in Fraction 1. In addition, the percentage of radioactive ferritin present in Fraction 1 increased with time (Table II).

Effects of stopping labeling on different fractions. Four experiments were performed in which the marrow was incubated with Fe^{59}-labeled plasma for a period of 5 to 10 minutes, and further labeling of the marrow with Fe^{59} was then stopped. In this way it was possible to follow the fate of a "cohort" of radioiron at various time intervals. Figure 3 illustrates the pattern obtained in one experiment. The radioactivity in Fraction 1 and the "stromal fraction" fell with time up to 6 hours, whereas the activity in hemoglobin rose rapidly at first and then reached a plateau. In contrast, the radioactivity in ferritin remained the same throughout the time of incubation. When the counts present in Fraction 1 were corrected for the ferritin present, the nonferritin fraction fell rapidly to a very low level. During this period there was a reciprocal increase in the activity present in main hemoglobin.

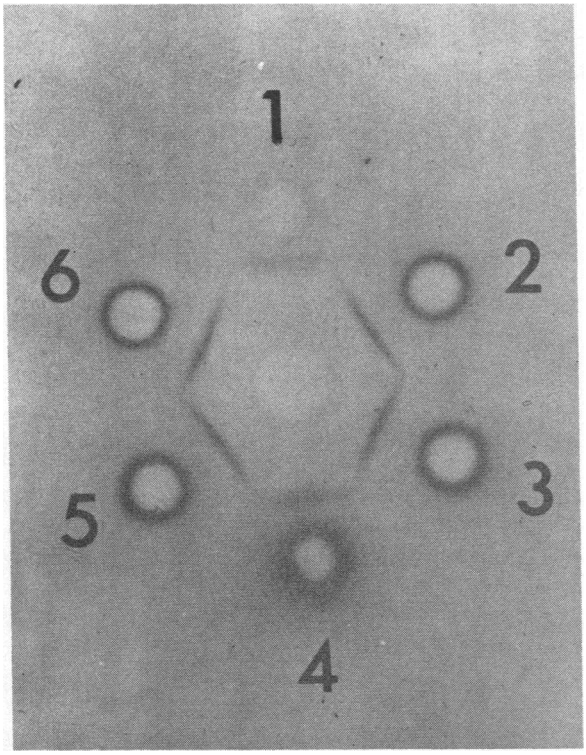

FIG. 2. RADIOAUTOGRAPH OF AGAR-GEL DIFFUSION PLATE. Center well, human liver ferritin antiserum; 1, Fe^{59}-labeled Fraction 1; 4, Fe^{59}-labeled marrow supernatant solution; 2, 3, 5, and 6, Fe^{59}-labeled human liver ferritin. Although no visible precipitate could be seen opposite the wells containing Fraction 1 and the marrow supernatant solution, bands of radioactivity coalescing with the liver ferritin bands are demonstrated.

TABLE I

A comparison of radioactivity in ferritin (expressed as a percentage of total marrow uptake) determined in marrow supernatant solution and in Fraction 1 after various periods of incubation in vitro

	Minutes			Hours				
Exp't no.	5	10	30	1	2	3	4	6
I. Supernatant solution	9.7	7.0	8.3		7.9		16.8	
Fraction 1	7.4	8.0	8.9		13.5		15.3	
II. Supernatant solution	9.9	8.4	9.8	9.4		15.1		
Fraction 1	8.4	9.2	9.9	10.0		16.1		
III. Supernatant solution	7.2	9.9	10.1	10.3	11.0			
Fraction 1	4.2	6.6	8.3	8.3	10.2			
IV. Supernatant solution		10.5	11.4	11.5	11.8		11.3	
Fraction 1		8.5	10.5	8.4	10.5		10.7	
V. Supernatant solution	11.1		9.2	11.2			11.2	8.9
Fraction 1	10.0		9.6	10.2			8.1	8.9

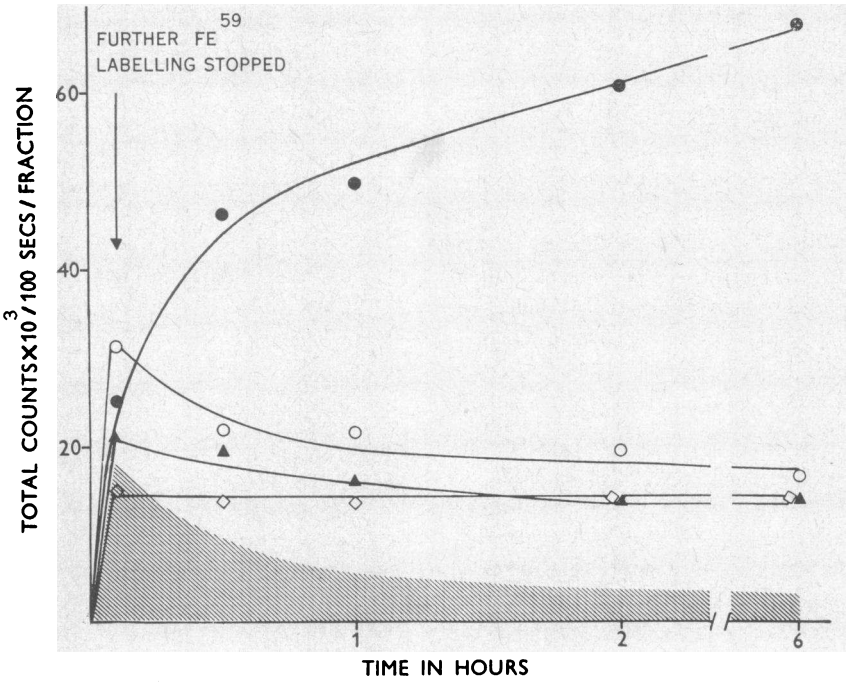

FIG. 3. INCORPORATION OF Fe^{59} INTO "STROMAL FRACTION," FRACTION 1, MAIN HEMOGLOBIN, AND FERRITIN. Analysis of samples of human rib marrow culture at various times up to 6 hours. Further labeling with Fe^{59} was stopped after 5 minutes by washing the cells and resuspending them in unlabeled medium. Closed triangles, "stromal fraction"; open circles, Fraction 1; closed circles, main hemoglobin; open diamonds, ferritin; hatched area, hemoglobin precursor, i.e., Fraction 1 minus ferritin.

In the three other experiments the results were very similar. The percentages of radioactivity precipitable as ferritin when labeling was stopped were 9.9, 5.1, and 6.5 as compared with figures of 9.9, 6.5, and 8.9, respectively, at 6 hours. During this period the nonferritin activity in Fraction 1 fell from 9.6 to 2.7, from 11.1 to 2.2, and from 11.3 to 6.1. The increase in main hemoglobin was from 26.3 to 64.6%, from 19.8 to 44.3%, and from 37.6 to 67.3% in the three experiments. It thus appears that the portion of Fraction 1 not precipitable by antiferritin serum is the active intermediate for iron incorporation into hemoglobin (hemoglobin precursor). Since in control experiments in which labeling was not stopped the activity in hemoglobin continued to rise to between 12 and 25 times the figure at 10 minutes, these data also suggest that ferritin does not act as an active intermediate for the incorporation of iron into hemoglobin in marrow cultures over 6 hours.

TABLE II

The percentage radioactivity precipitable as ferritin in Fraction 1 after various periods of incubation of marrow in vitro (labeling with Fe^{59} not stopped)

Exp't no.	Minutes			Hours				
	5	10	30	1	2	3	4	6
I	43	51	51	59	69		74	
II	41	46	51	61		67		
III	30	43	49	50	58			
IV	31	33	55	58		71		78

Kinetics of Fe^{59} incorporation into ferritin in marrow cells in vivo. Since it was not possible to show any drop in ferritin activity during the incubation of marrow *in vitro,* further studies were carried out *in vivo* in rats to find out whether labeled ferritin was still present in the reticulocytes entering the circulation. Since it seemed possible that the ferritin might be removed by the spleen, studies were done in both normal and splenectomized animals. Twelve splenectomized rats and 12 controls were given an intravenous injection of Fe^{59}-labeled plasma,

and batches were killed at intervals thereafter. Four hours after injection the pooled marrows from three splenectomized and three control animals contained 9.6 and 12.0%, respectively, of the radioactivity in the ferritin fraction. At 24 hours the corresponding figures were 1.8 and 2.2%. However, it was not possible to show radioactive ferritin in the circulating red cells of the remaining animals at any time up to 4 days after the administration of the radioiron. In an extension of the study, plasma (labeled with 25 μc Fe^{59}) was injected into a normal splenectomized human subject, and blood samples were collected at daily intervals for 7 days thereafter. At no time was labeled ferritin demonstrated. These findings suggest that the ferritin present in red cell precursors is either ultimately used for hemoglobin synthesis or is extruded before the cells leave the marrow.

Iron uptake by reticulocyte suspensions. Reticulocyte suspensions, obtained from the blood of two patients with pernicious anemia in early remission, were incubated with labeled plasma for 4 hours. In both cases hemoglobin and Fraction 1 formation was normal. No label was detectable in ferritin in one experiment, but small and possibly significant amounts of Fe^{59} were incorporated into ferritin in the other. In a third experiment, a reticulocyte suspension from a patient with idiopathic autoimmune hemolytic anemia was incubated for 1 hour in labeled plasma. At this time 10.3% of the activity taken up by the reticulocytes was in ferritin. Several samples of the suspension were then reincubated in unlabeled medium after the cells had been washed 4 times in cold saline. After a further 3 hours' incubation 8.5% of the radioactivity was still present in the ferritin fraction.

Two similar experiments were carried out on reticulocyte suspensions obtained from rats that had been given phenylhydrazine 3 to 4 days previously. After 1 hour's incubation 2.0 and 0.9%, respectively, of the Fe^{59} were present in ferritin. After 3 further hours of incubation in unlabeled

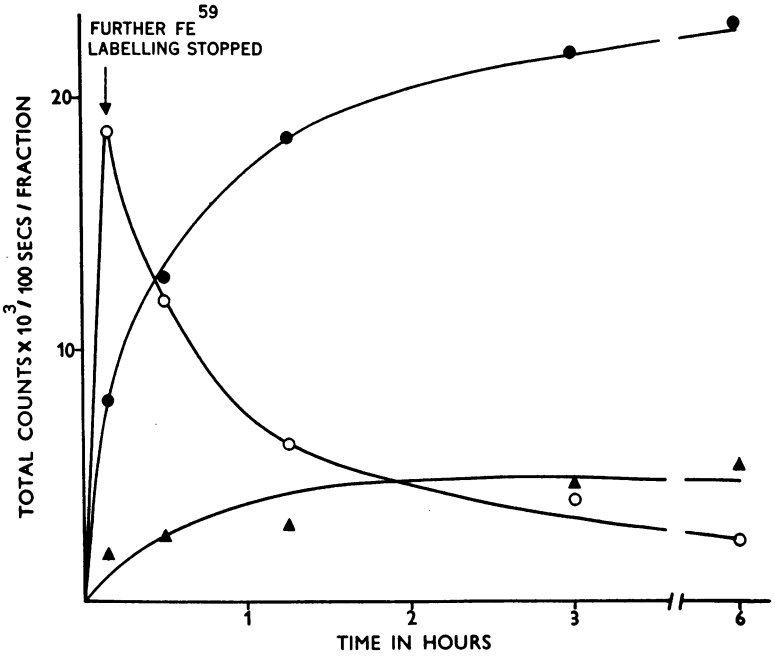

FIG. 4. IRON-DEFICIENT HUMAN ILIAC CREST MARROW CULTURE. INCORPORATION OF Fe^{59} INTO "STROMAL FRACTION," FRACTION 1, AND MAIN HEMOGLOBIN. Analysis of samples at various times up to 6 hours. Further labeling was stopped after 8 minutes by washing the cells and resuspending them in unlabeled medium. Closed triangles, "stromal fraction"; open circles, Fraction 1; closed circles, main hemoglobin. No ferritin could be identified at any stage.

substrate the comparable figures were 3.2 and 0.6%.

Iron uptake by iron-deficient marrows. Marrow from a case of iron deficiency was incubated with labeled plasma, and samples were taken at progressive times of incubation up to 4 hours. The incorporation of Fe^{59} into hemoglobin and Fraction 1 was similar to that observed in normal marrows, but no labeling of ferritin could be detected.

In an experiment using another severely iron-deficient marrow in which further labeling with Fe^{59} was stopped after 8 minutes' incubation (Figure 4), the activity in Fraction 1 fell to much lower levels relative to the total uptake than in normal marrows. This can be ascribed to the fact that it contained only the hemoglobin precursor, since no labeled ferritin was demonstrable at any time.

Effect of lead on the incorporation of Fe^{59} into ferritin, Fraction 1, and main hemoglobin. In five experiments 10^{-4} M lead acetate was added to the marrow suspension at the start of incubation. There was little effect upon total iron uptake, but radioactivity in the stromal fraction increased by a mean of 95% (range, 40 to 154%) compared with the control tubes. Both Fraction 1 and ferritin activity were greater by mean percentages of 38 (range, 12 to 97) and 40 (range, 12 to 90), respectively. The effect upon Fe^{59} in hemoglobin precursor (i.e., Fraction 1 minus ferritin) was variable, ranging from a decrease of 46% to increases of 2, 34, 36, and 125%. Incorporation of radioactivity into main hemoglobin was inhibited by from 38 to 80% (mean, 55%). The results in one experiment are illustrated in Figure 5.

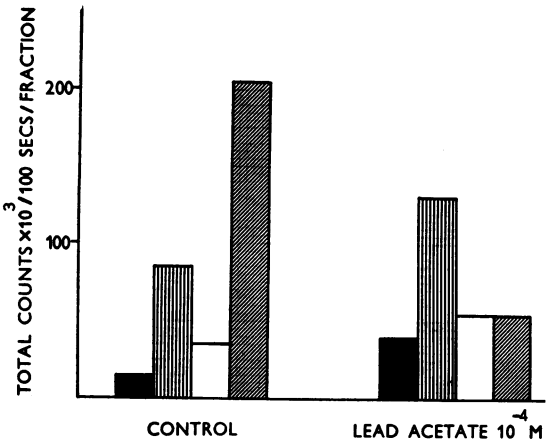

FIG. 5. EFFECT OF 10^{-4} M LEAD ACETATE ON HUMAN RIB MARROW CULTURE. INCORPORATION OF Fe^{59} INTO "STROMAL FRACTION," HEMOGLOBIN PRECURSOR, MAIN HEMOGLOBIN, AND FERRITIN AFTER 4 HOURS. Black, "stromal fraction"; vertical lines, ferritin; unshaded, hemoglobin precursor, i.e., Fraction 1 minus ferritin; diagonal lines, main hemoglobin. There is diminished radioactivity in hemoglobin and increases in "stromal fraction," ferritin, and hemoglobin precursor in the lead-poisoned culture as compared with the control.

Effect of varying saturations of transferrin on ferritin and main hemoglobin synthesis. A comparison was made between the uptake of iron into ferritin and main hemoglobin, respectively, at various saturations of transferrin. At high saturations the amount of iron incorporated into main hemoglobin did not increase, whereas incorporation into ferritin continued to rise progressively. The results in one experiment are shown in Figure 6. In five similar experiments the general pattern was confirmed, with a mean drop in the ratio of the Fe^{59} main hemoglobin to Fe^{59} ferritin from 4.1 at saturations between 30 and 40% to 2.8 at saturations between 80 and 100%.

Discussion

Greenough and his collaborators have shown that iron in red cell precursors passes through a nonhemoglobin iron-protein complex (Fraction 1) before being incorporated into hemoglobin (4). Although these workers were not able to identify radioactive ferritin in Fraction 1, the present studies indicate that it contains an appreciable amount and that the proportion present rises progressively with time. There are two possible reasons for these discrepant findings. In the first place experiments done in the current study revealed that approximately 50% of the radioactive ferritin in Fraction 1 is removed by the high-speed centrifugation (100,000 × g for 30 minutes) to which Greenough and co-workers subjected the marrow lysate before chromatography, and secondly there were differences in the immunochemical techniques used in the two studies. As a result, the kinetic patterns for Fraction 1 reported by Greenough and co-workers correspond more closely to those obtained for the hemoglobin precursor (Fraction 1 minus ferritin) in the present investigation. The current findings

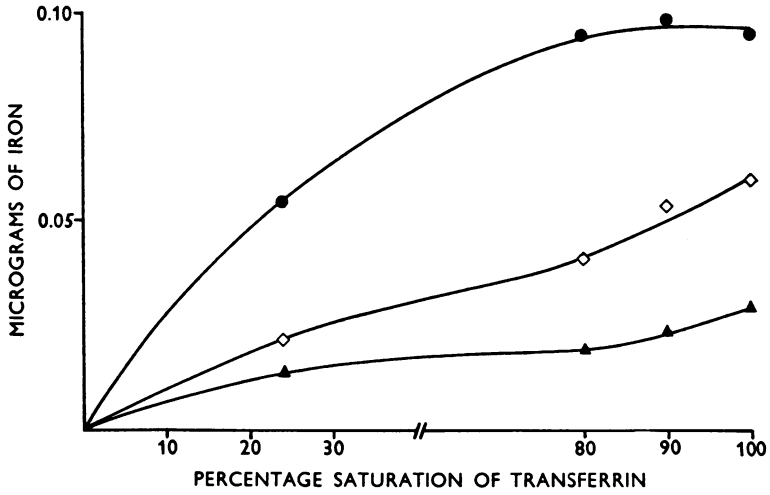

FIG. 6. RELATIONSHIP BETWEEN TRANSFERRIN SATURATION AND THE INCORPORATION OF IRON INTO "STROMAL FRACTION," FERRITIN, AND MAIN HEMOGLOBIN IN HUMAN MARROW CULTURE. After the addition of 10 μc Fe^{59} to 20 ml plasma, unlabeled iron as ferrous ammonium sulfate was added to equal samples in amounts calculated to produce the desired saturation of the transferrin. These samples were then dialyzed against running iron-free water for 16 hours before use. Closed triangles, "stromal fraction"; open diamonds, ferritin; closed circles, main hemoglobin. At high saturations of transferrin more iron was incorporated into ferritin.

lend further support to the suggestion (4) that this substance is an important precursor of hemoglobin. In experiments where labeling was allowed to continue for several hours radioactivity appeared in this fraction at an earlier stage than in hemoglobin and then leveled off. In addition, in experiments where further uptake of Fe^{59} was stopped after 5 minutes' incubation, the radioactivity present in the fraction dropped while that in hemoglobin rose reciprocally. The fact that the fraction only fell to a limited extent is compatible with the suggestion that it was being fed from a large stromal pool, or alternatively, it is possible that some other active precursor substance was present.

Significant amounts of iron taken up by marrow cultures were rapidly incorporated into ferritin. However, since ferritin is visible on electron microscopy both in red cell precursors and in reticuloendothelial cells of the marrow (5) it was important to establish the cellular location of the labeled ferritin in the various experiments. In an attempt to resolve this problem the uptake of plasma-bound radioiron and its incorporation into ferritin were compared in bone marrow and splenic suspensions. Since only negligible amounts of radioiron were incorporated into ferritin in the splenic suspensions, it seemed likely that the reticuloendothelial cells of the bone marrow were not responsible for the degree of ferritin labeling observed in marrow cultures. These findings are in agreement with the results of other workers (17–19) who have shown in several different ways that reticuloendothelial cells have an extremely limited capacity to take up iron bound to transferrin. Although it therefore seems probable that the incorporation of radioiron into ferritin observed in the present study was taking place predominantly in developing red cell precursors, there is still some doubt as to its significance. Recently Mazur and Carleton (6) have suggested on the basis of both *in vivo* and *in vitro* studies that most of the iron derived from plasma is incorporated into ferritin before its incorporation into heme. They based this conclusion on several observations. When rats were given Fe^{59} intravenously, the activity in marrow ferritin dropped between 5 and 12 hours while the activity in hemoglobin was still rising. This relationship was not shown in animals in which radioiron uptake had been stimulated by phenylhydrazine or depressed by endotoxin. Under

these circumstances the rise and fall of radioactivity in ferritin and hemoglobin fractions ran parallel. In further experiments Mazur and Carleton incubated reticulocytes *in vitro* with Fe^{59}-labeled plasma. Further labeling was then stopped after 20 minutes. At this time the specific activity of the ferritin iron was much lower than that of the iron in heme. However, since ferritin contains approximately 2,400 atoms of iron per molecule as compared with only 4 in heme, they calculated that each ferritin molecule had accumulated many more atoms of iron than each heme molecule. They therefore interpreted these observations as indicating a precursor relationship between ferritin iron and heme iron. At the same time these authors pointed out that when incubation of reticulocytes was continued for 120 minutes after further labeling had been stopped, the calculated loss of Fe^{59} per micromole of ferritin was many times greater than the gain by hemoglobin. These results suggested that the major portion of the labeled iron in ferritin might eventually leave the cell. These interpretations depend on the assumption that Fe^{59} taken up by ferritin is uniformly dispersed in a large iron pool. It is, however, doubtful whether such an assumption is justifiable, since it has been shown that plasma-bound Fe^{59} is incorporated to a greater extent into the low density, iron-poor molecules of hepatic ferritin than into the high density ferritin molecules of high iron content (15). This lack of homogeneity of hepatic ferritin indicates the difficulties associated with any quantitative interpretation of the uptake and release of Fe^{59} by ferritin.

In the present study an attempt was made to elucidate the significance of the ferritin formed in red cell precursors by studying the incorporation of Fe^{59} into this fraction under various experimental conditions. At high saturations of transferrin more iron was taken up by red cell precursors, but this additional uptake was not incorporated into heme. These results are in agreement with those obtained by other workers (20). In the present investigation the bulk of the extra iron was found to be incorporated into ferritin. A similar build-up in ferritin activity was noted when hemoglobin synthesis was depressed by lead. In contrast, when marrow from an iron-deficient subject was incubated in radioactive plasma, there was a rapid movement of iron through the hemoglobin precursor into hemoglobin, but no ferritin was formed at any time. These observations suggest that iron is only incorporated into ferritin when more iron enters the cell than can be immediately utilized for hemoglobin synthesis. Since this storage form of iron could not be identified in circulating red cells, it is either utilized for the synthesis of heme at some later stage, or is removed from the red cell precursors. Although it seems entirely possible that ferritin iron is available for hemoglobin formation if required, no evidence in support of this thesis was obtained in the present investigation. In the experiments in which isotope labeling was stopped, the activity in ferritin rapidly reached a plateau, and there was no significant fall in activity for several hours afterwards. In contrast, there was a sharp decline in the activity present in the hemoglobin precursor while the activity in hemoglobin rose. In addition, in studies where labeling was allowed to continue for as long as 12 hours there was a steady build-up in ferritin over the whole period. Although these results in no way exclude the possibility that iron taken up and stored in ferritin is slowly released for hemoglobin synthesis, they afford no positive evidence that this occurs.

If ferritin iron is not utilized for heme synthesis, it must leave the red cell precursors, since labeled ferritin could not be identified in circulating erythrocytes. This could theoretically occur either within the bone marrow or after the reticulocyte enters the circulation. Since it has been established that the spleen has the capacity to remove hemosiderin granules from young red cells (21), *in vivo* experiments were carried out in splenectomized rats to find out whether labeled ferritin could be identified in circulation at any time after the intravenous administration of radioiron. Although appreciable quantities of radioactive ferritin were present in the marrows of these animals, none was found in the circulation. Although these observations appear incompatible with the demonstration that reticulocytes incubated *in vitro* can incorporate radioiron into ferritin, the explanation for this apparent discrepancy may lie in the limited capacity of the reticulocyte to take up iron from the plasma under normal circumstances (17). If Fe^{59} incorporated into fer-

ritin within red cell precursors is not utilized for heme synthesis, then it seems probable that it leaves the cell before it enters the circulation. This possibility would fit in well with previous electron microscopic findings in which it has been demonstrated that ferritin can move freely between the red cell precursors and the reticuloendothelial cells of the marrow (5). Although it was originally suggested by Bessis that the movement was in the opposite direction, several workers have questioned the validity of this interpretation (12, 22). If the present proposal is a valid one it might explain, in part at least, ferrokinetic data which indicate that some of the iron delivered to the marrow enters a labile pool which may feed it back into the plasma again (23). Although evidence has been produced to show that this pool is located on the stroma of red cell precursors, the present findings are compatible with another short circuit involving the movement of iron from plasma into ferritin in red cell precursors, and thence to reticuloendothelial cells that release the iron back into the plasma.

Summary

In vitro and *in vivo* studies were carried out in human subjects and in rats on the uptake and utilization of transferrin-bound iron by red cell precursors. Iron rapidly appeared in a fraction, isolated on column chromatography, which consisted of ferritin and an active hemoglobin precursor. Further experiments were then carried out to elucidate the significance of the ferritin component.

With increasing percentage saturation of transferrin more iron was incorporated into ferritin, while no ferritin labeling was detected when marrow from an iron-deficient subject was cultured. These observations suggest that the iron not immediately required for hemoglobin synthesis in red cell precursors is incorporated into ferritin. No evidence of the later utilization of this ferritin iron for the formation of hemoglobin was obtained, although this possibility could not be excluded. Since labeled ferritin could not be identified in circulating erythrocytes, ferritin iron not utilized for heme synthesis must leave the red cell precursors. The possibility that the spleen might remove ferritin from reticulocytes was investigated in splenectomized rats. Although labeled ferritin was demonstrated in the marrow of these animals after the intravenous injection of Fe^{59}, at no stage could it be detected in circulating red cells. It was concluded that iron incorporated into ferritin in red cell precursors may leave these cells before they enter the circulation. It is possible that this removal is effected by reticuloendothelial cells within the marrow.

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