Hemolysis and Erythropoiesis. VI. A Comparative Study of the Utilization of Hemoglobin Iron and Transferrin Iron by the Erythropoietic Tissue

By L. SANCHEZ-MEDAL, LORENZO DUARTE AND JUAN LABARDINI

Our previous observations have shown that intraperitoneal administration of autohemolysates accelerates the recovery from anemia in bled dogs.1-3 On the basis of this and other experimental data4-6 we reconsidered the hypothesis7,8 that red cell constituents have an erythropoietic stimulatory effect.1,6 However, it has been suggested that the above findings are due, instead, to the availability of iron derived from hemoglobin catabolism,9 or to the reutilization of hemoglobin constituents by erythropoietic tissue.10,11 Data indicating that these alternative explanations are unlikely are presented in this report.

Material and Methods

EDTA was used as an anticoagulant for all the laboratory determinations. Hemoglobin was estimated by the cyanmethemoglobin method. The packed cell volume was determined in Wintrobe tubes centrifuged at 2200 g. for 30 minutes. Radioactivity was measured in a well scintillation counter for a minimum of either 10,000 counts or 15 minutes. Ferric citrate labeled with 59Fe with a specific activity of 10-30 μCi./μGm. was used.

Inbred male Wistar rats weighing 250-350 Gm. were used. The rats were kept in collective cages and were fed a Purina diet. Animals of uniform age and weight were randomly divided in groups for each experiment. Groups N1-N6 were formed by normal animals; groups A1 and A2 were formed by anemic rats which were bled 2 ml. per 100 Gm., 48 hours after being intramuscularly injected with 5 mg. of iron-dextran* to prevent the occurrence of iron deficiency. The animals of each group were intravenously injected through the tail with 1 ml. of isotonic saline solution containing the following substances:

- Groups N1 and A1: 1 μCi. of 59Fe.
- Groups N2 and A2: hemolysate containing 3 mg. of 59Fe-labeled hemoglobin (equal to 10.4 μGm. of Fe).
- Group N3: hemolysate containing 150 mg. of 59Fe-labeled hemoglobin (equal to 520 μGm. of Fe).
- Group N4: 1 μCi. of 59Fe and hemolysate containing 3 mg. of unlabeled hemoglobin.
- Group N5: 1 μCi. of 59Fe and hemolysate containing 150 mg. of unlabeled hemoglobin.
- Group N6: 1 μCi. of 59Fe and 500 μGm. of iron as iron-dextran.

The 3-mg. dose of hemoglobin was below the binding capacity of circulating haptoglobin, which was estimated by starch-gel electrophoresis.24

The anemic rats were injected one hour after the bleeding. At different times after the injection of the tracer, blood was obtained from 12 rats of each group by heart puncture for duplicate determinations of hemoglobin, hematocrit and radioactivity in 1 ml. of whole

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*Iron-dextran (Imferon) was kindly supplied by Farmacéuticos Lakeside, S.A.
blood and in 1 ml. of plasma. For the calculation of per cent of $^{59}$Fe incorporated into the red cells, a blood volume of 5 ml./100 Gm. body weight was assumed and the plasma radioactivity (RA) was subtracted from the whole blood:

$$\text{RBC RA} = \text{whole blood RA - plasma RA} \left[ 1 - \left( \frac{\text{Ht}}{100} \right) \right].$$

Some rats of group N-3, i.e., animals injected with 150 mg. of $^{59}$Fe-labeled hemoglobin, were sacrificed 30 minutes after the injection, by bleeding to white. The femurs were dissected cleanly, rinsed in tap water and dried with gauze. Each femur was divided in two equal portions for radioactivity counting. Total skeletal radioactivity was calculated using the factor of Keene and Jandl (12.7 times the RA of one femur).

The unlabeled hemolysate was prepared with red cells obtained from rats of the same colony that were bled by heart puncture. The red cells were washed three times with isotonic saline and were frozen and thawed several times. Tetracycline, 30 $\mu$Gm. per ml. was added. The hemolysates were kept frozen until use. The $^{59}$Fe-labeled hemolysate was obtained from rats intravenously injected with 200 $\mu$Ci. of $^{59}$Fe immediately after they were bled 2 ml. per 100 Gm. Six days after the isotope injection, red cells were harvested and treated as described above.

### RESULTS

Thirty minutes after the injection of 150 mg. of $^{59}$Fe-labeled hemoglobin the femurs contained 0.553 per cent of the radioactivity; thus it was estimated that the entire skeleton contained 3.51 per cent of the injected hemoglobin.

In normal rats (Table 1), RBC $^{59}$Fe incorporation at all times after the injection of transferrin-bound $^{59}$Fe (group N1) was more than twice that found after the injection of 3 mg. of $^{59}$Fe-labeled hemoglobin (group N2). When a large dose (150 mg.) of $^{59}$Fe-labeled hemoglobin was given (group N3), a very small incorporation was obtained. After transferrin-bound $^{59}$Fe administration, a significant incorporation was observed at six hours, whereas with $^{59}$Fe-labeled hemoglobin, this was not observed until 24 hours after its administration.

In acutely bled rats, the results were very similar. The utilization by erythropoietic tissue of iron derived from a low dose of hemoglobin (3 mg.) was slower and less than the utilization of transferrin-bound $^{59}$Fe (Table 2). The utilization by erythropoietic tissue of transferrin-bound $^{59}$Fe was not

<table>
<thead>
<tr>
<th>Group</th>
<th>0.25</th>
<th>Time in Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>N 1</td>
<td>6.35±0.84</td>
<td>36.4 ± 1.17</td>
</tr>
<tr>
<td>N 2</td>
<td>0.57±0.12</td>
<td>4.8 ± 0.65</td>
</tr>
<tr>
<td>N 3</td>
<td>0.31±0.04</td>
<td>1.6 ± 0.09</td>
</tr>
<tr>
<td>N 4</td>
<td></td>
<td>3.1 ± 0.41</td>
</tr>
<tr>
<td>N 5</td>
<td>6.86±1.13</td>
<td>33.28±4.70</td>
</tr>
<tr>
<td>N 6</td>
<td></td>
<td>31.02±1.41</td>
</tr>
</tbody>
</table>

All values of N2, N3 and N6 are significantly different ($p < 0.0001$) from those of N1.
modified significantly (Fig. 1, Table 1) by the simultaneous intravenous administration of 150 mg. of unlabeled hemoglobin (group N5). A lower dose of unlabeled hemoglobin (3 mg.) (group N4) had no depressing effect on the RBC $^{59}$Fe incorporation, and 10 days after the radioiron injection the RBC $^{59}$Fe incorporation was even higher than in the controls. By contrast, 500 μgm. of iron-dextran (group N6) did decrease significantly ($p < 0.0001$) the RBC $^{59}$Fe incorporation (Fig. 1, Table 1).

**DISCUSSION**

It has been considered that the great erythrocytic production observed in hemolytic anemias is due to the availability of large amounts of iron. Hemo-
globin iron, it is assumed, is preferentially used for the production of new erythrocytes. This assumption is based on the observations that hemoglobin iron is reutilized for the synthesis of new heme pigment, whether it enters the...
body as free hemoglobin, as nonviable red cells or as normal compatible and viable red cells. In addition, it has been stated that "iron of injected hemoglobin solutions is available for erythropoiesis at the same rate as iron attached to the plasma iron-binding protein," and that "the rate of utilization of iron is the same regardless of mode of administration (injected inorganic iron or hemoglobin solutions)."

Results of the present investigation indicate that the availability of hemoglobin-bound Fe for erythropoiesis is less than that of transferrin-bound Fe in the normal state and in anemia. When transferrin-bound 59Fe was injected to normal (group N1) and acutely bled anemic rats (group A1), a significant amount of labeled iron was incorporated into the red cells within six hours, whereas 24 hours were required to reach a comparable utilization of iron after hemoglobin-59Fe injections (groups N2 and A2). Furthermore, at all the time intervals studied, utilization of transferrin-bound 59Fe by erythroid tissue was at least double that of hemoglobin-59Fe. A similar utilization of hemoglobin-bound iron has been observed in humans and dogs. As the amount of iron provided by the hemoglobin injection to the rats of groups N2 and A2 (10 μGm.) was about two thirds of that present in the plasma, the result we obtained might be attributed to iron overload. However, the observation that a significant amount of unlabeled hemoglobin did not decrease the utilization of transferrin-bound 59Fe rules out the possibility that the small, delayed utilization of hemoglobin. 59Fe is due to the presence in the circulation of excessive amounts of iron. However, 500 μGm. of intravenously given iron–dextran (group N6) decreased significantly the RBC incorporation of transferrin-bound 59Fe.

In view of these findings the validity of the concept that iron released from catabolized hemoglobin is preferentially utilized for hemoglobin synthesis is questionable. It has been reported that in dogs and rabbits with 59Fe labeled red cells in their blood stream, the repeated parenteral administration of iron is ultimately followed by a marked drop in the amount of circulating labeled red cells. This indicates that reutilization of the 59Fe released by catabolism of the labeled red cells is blocked by the parenteral iron. A similar observation, i.e., a marked drop in circulating 59Fe labeled red cells, has been reported in patients with hemochromatosis. This indicates that storage iron also competes with the iron derived from hemoglobin catabolism, and that in the presence of large stores of iron, reutilization of hemoglobin iron is inhibited.

We have observed that during recovery from anemia induced by bleeding, two dogs given daily intraperitoneal injections of 59Fe-labeled hemoglobin reutilized only 20.8 per cent of the 59Fe in the 14 days study period. This accounted for only 12.9 per cent of the total iron present in the hemoglobin produced during this period. Consequently, most of the iron (87 per cent) utilized for de novo hemoglobin synthesis by these dogs was derived from iron stores, and from the limited amount of intramuscular iron–dextran given in the first quarter of the experiment.

Ross has suggested that, notwithstanding its mode of entry, once iron has been introduced into the body it enters a metabolic pool and is metabolized in...
a uniform fashion. There is some evidence that this assumption is correct and applies to hemoglobin iron. It has been reported that after the injection of \(^{56}\)Fe-labeled hemoglobin, radioactive transferrin appears in the plasma in 30 minutes\(^{15,16,22}\); after three hours about 30 per cent of the injected activity is in the plasma,\(^ {12}\) and after six hours all the circulating radioactivity is due to transferrin-bound \(^{56}\)Fe.\(^ {22}\) It is also of interest that within 30 minutes after administration of \(^{14}\)C labeled nonviable red cells or hemoglobin, labeled bilirubin appears in the bile.\(^ {23}\) The conversion to \(^{14}\)C-labeled bilirubin was "nearly complete" when the amount of injected hemoglobin did not exceed the haptoglobin binding capacity of the plasma.\(^ {22}\) All these observations indicate that plasma hemoglobin is rapidly catabolized, mainly in the liver, and iron is released and handled in the same manner as nondepot iron. When large amounts of hemoglobin or nonheme iron are present, a significant portion of the hemoglobin iron goes into relatively inaccessible iron pools.\(^ {12}\) Evidence that this occurs even with hemoglobin released in or taken up by the bone marrow has been reported by Hughes-Jones.\(^ {20}\)

REFERENCES
