Increased Formation of Early-Labeled Bilirubin in Rats with Iron Deficiency Anemia: Evidence for Ineffective Erythropoiesis

By Stephen H. Robinson

Iron deficiency anemia is usually characterized by erythroid hyperplasia of the bone marrow, but impaired net synthesis of hemoglobin. This association suggests that the anemia may be due in part to ineffective erythropoiesis, i.e., destruction of immature red cells in the bone marrow or soon after their release into the circulation. Morphologic observations are also compatible with ineffective erythropoiesis. The red cell precursors in iron deficiency often have ragged, poorly hemoglobinized cytoplasm. Similar cytologic abnormalities are found in the adult erythrocytes, and recent studies have shown that these cells have a shortened survival in the circulation.1,2

Ineffective erythropoiesis is most readily demonstrable by ferrokinetic measurements,3 but such studies in patients with iron deficiency have been subject to conflicting interpretations.4,5 This pathophysiologic process is also associated with a characteristic abnormality of bile pigment metabolism, consisting of increased formation of the early-labeled fraction of bilirubin, despite impaired production of circulating red blood cells.6,7 In order to assess ineffective erythropoiesis, the formation of early-labeled bilirubin and hemoglobin heme was investigated in rats with iron deficiency anemia.

Materials and Methods

Mothers, with litters of 7–10 day old rats, were placed on an iron deficient diet. Drinking water was demineralized by passage through an ion exchange resin. The male babies were weaned and maintained on the same diet when they were about three weeks old. These animals gained weight slowly as compared to rats fed standard laboratory chow, reaching 200–300 grams at 14–16 weeks of age when the experiments were performed. Two rats were maintained on the same deficient diet but received intramuscular injections of iron-dextran, 1 mg. per day for ten days when they were 5–6 weeks old, and then 5 mg. per day for 5 days when they reached larger size at 10–11 weeks of age. These animals gained weight normally and showed complete normalization of...
their hematologic values (Table 1). Control measurements were made in rats which had been maintained on standard food pellets* and were comparable in weight to the experimental animals.

The animals were anesthetized with ether, and the common bile duct was cannulated with polyethylene tubing.† A tapered polyethylene catheter was also placed in a lateral tail vein. Two to three hours after surgery, the rats received 50 μg glycine-2-14C through the tail vein catheter, and bile samples were collected in the dark over ice at 1, 2, 3.5, 8, 24, 48, and 72 hours. The volume and bilirubin concentration‡ of each bile sample were measured, and the bilirubin was then crystallized for radioassay.‡‡ Nonradioactive bile containing a measured amount of “carrier” bilirubin was added to samples with insufficient pigment for direct crystallization.‡‡ The rate of excretion of labeled pigment (disintegrations per minute in bilirubin-14C excreted per hour) was calculated from the total rate of excretion and the specific activity of bilirubin during each period of bile collection. Values were plotted at the median times of each collection period and smooth curves were drawn through these points. In addition, the per cent of administered glycine-14C incorporated into bilirubin-14C was calculated for two broad intervals, 0–3.5 hours and 3.5–60 hours after glycine administration. These somewhat arbitrary divisions conform to the initial sharp peak and the plateau component of early-labeled pigment formation in the rat, and are useful in quantitating contributions from nonerythropoietic sources.‡‡

Blood samples were removed from the tail at about 18 and 36 hours, and from the heart at the conclusion of the experiment at 72 hours. Hemoglobin hemin was crystallized for radioassay‡‡ from the separated red cells, and the incorporation of glycine-14C was calculated from the specific activity of hemin and the total hemin equivalent of the rat’s blood volume; it was estimated that in the anemic animals blood volume fell 5 per cent for each 10 per cent decrease in hemoglobin concentration.‡‡‡

Similar experiments were performed in three additional rats during the acute response to iron therapy. Intramuscular injections of 5 mg iron-dextran were given daily for two days before cannulation of the bile duct and administration of glycine-14C. Injections of iron were continued on each of the three days of experimental observation.

**RESULTS**

Hematologic data are given in Table 1. Iron deficient rats were severely anemic, with an average hemoglobin concentration of 6.9 g. per 100 ml. Peripheral blood smears were typical of iron deficiency. In addition, all of these animals displayed modest reticulocytosis, reticulocytes averaging 7.3 per cent. Reticulocyte counts were stable over several weeks of observation, and were not associated with any change in hemoglobin concentration. These hematologic changes all reverted to normal after treatment with iron-dextran (Table 1), demonstrating that the anemia was due specifically to iron deficiency.

Figure 1 shows representative curves of labeled hemoglobin heme and bilirubin production in rats given glycine-2-14C. In untreated iron deficient rats, synthesis of circulating hemoglobin was below the normal range. Glycine-14C incorporation into hemoglobin heme averaged 0.28 per cent in five anemic rats, as compared to 0.52 per cent in control animals (Table 2). The initial sharp peak of early bilirubin formation was slightly higher than the

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* Purina Laboratory Chow, Purina Co., St. Louis, Missouri.
‡ Glycine-2-14C, 15–25 mc per mM, New England Nuclear Corporation, Boston, Massachusetts.
Table 1.—Hematologic Values in Experimental Animals

<table>
<thead>
<tr>
<th>Condition</th>
<th>Hemoglobin (Gm./100 ml.)</th>
<th>Hematocrit (%)</th>
<th>MCHC * (%)</th>
<th>Reticulocytes (%)</th>
<th>Total Bilirubin Excretion μg./hr./100 Gm. body weight</th>
<th>Bilirubin in Bile μg./hr./Gm. circulating hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, 10 rats</td>
<td>14.9 ± 0.3</td>
<td>44.6 ± 0.5</td>
<td>33.0 ± 0.5</td>
<td>1.2 ± 0.3</td>
<td>29.0 ± 2.0</td>
<td>33.2 ± 1.8</td>
</tr>
<tr>
<td>Iron deficiency, 5 rats</td>
<td>6.9 ± 1.0</td>
<td>24.4 ± 3.2</td>
<td>27.7 ± 0.7</td>
<td>7.3 ± 2.1</td>
<td>24.7 ± 3.1</td>
<td>72.6 ± 8.1</td>
</tr>
<tr>
<td>Acute treatment of iron deficiency,†</td>
<td>6.9 ± 1.5</td>
<td>25.3 ± 5.2</td>
<td>27.0 ± 0.4</td>
<td>9.9 ± 1.6</td>
<td>25.0 ± 3.3</td>
<td>60.0 ± 11.9</td>
</tr>
<tr>
<td>Iron deficiency in remission †</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat 1</td>
<td>14.3</td>
<td>43</td>
<td>33</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat 2</td>
<td></td>
<td>45</td>
<td>—</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means ± SE are given for each experimental group.
* Mean corpuscular hemoglobin concentration.
† Given iron dextran, 5 mg. per day, starting 2 days before experiment.
‡ Given iron dextran, 1 mg. per day for 10 days 9–10 weeks earlier, and 5 mg. per day for 5 days 4–5 weeks earlier.
Fig. 1.—The production of early-labeled bilirubin and hemoglobin heme from glycine-2-¹⁴C in a rat with iron deficiency anemia. Stippled areas show the means ± 2SD in a group of 10 control animals. The scale is adjusted to correspond to a dose of 1 mc. glycine-¹⁴C.

Table 2.—Per Cent Incorporation of Glycine-2-¹⁴C into Hemoglobin Heme-¹⁴C and Bilirubin-¹⁴C in Rats with Iron Deficiency

<table>
<thead>
<tr>
<th>Condition</th>
<th>Maximal Isotope in Circulating Hemoglobin Heme (3 days)</th>
<th>Isotope in Early-Labeled Bilirubin</th>
<th>Ratio of Late Phase Bilirubin-¹⁴C to Hemoglobin-heme-¹⁴C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Normal, 10 rats</td>
<td>0.52 ± 0.04</td>
<td>0.015 ± 0.001</td>
<td>0.080 ± 0.003</td>
</tr>
<tr>
<td>Iron deficiency, 5 rats</td>
<td>0.28 ± 0.02</td>
<td>0.015 ± 0.001</td>
<td>0.118 ± 0.007</td>
</tr>
<tr>
<td>Acute treatment of iron deficiency, 3 rats</td>
<td>1.67 ± 0.17</td>
<td>0.036 ± 0.005</td>
<td>0.190 ± 0.034</td>
</tr>
</tbody>
</table>

Means ± SE are given for each experimental group.

control curve in the iron deficient rat represented in Figure 1, but was within the normal range in each of the other four animals in this experiment. Thus, the mean per cent incorporation of glycine-¹⁴C into early-labeled bilirubin between 0 and 3.5 hours was similar in anemic and normal rats (Table 2). By contrast, the later or plateau phase of the early peak was significantly elevated in all of the deficient rats (Fig. 1), and glycine-¹⁴C incorporation
Fig. 2.—The production of early-labeled bilirubin and hemoglobin heme in an iron deficient rat in which iron therapy was begun 2 days earlier. The scale is adjusted to a dose of 1 mc. glycine-2-14C.

into bilirubin during the 3.5-60 hour interval was approximately 50 per cent higher than the control value (Table 2). The disparity between the production of late phase bilirubin and circulating hemoglobin heme in iron deficient rats is underlined when the ratios of isotope incorporation are examined (Table 2). Relative to the synthesis of hemoglobin heme-14C, the formation of late phase bilirubin-14C is almost three times higher in anemic than in normal rats.

Quite different results were obtained in rats treated with iron for two days before the administration of glycine-14C. As anticipated, response to therapy was associated with an increase in the synthesis of hemoglobin-14C to levels well above normal (Fig. 2, Table 2). Unexpectedly, however, the earliest (nonerythroid) component of the early bilirubin fraction was strikingly elevated (Fig. 2); glycine incorporation into bilirubin during the 0-3.5 hour interval was approximately two and one half times greater than in control animals, 0.036 as compared to 0.015 per cent (Table 2). In addition, the plateau component remained elevated during the response to iron. Indeed, glycine incorporation into bilirubin from 3.5-60 hours was higher than before therapy, largely because the marked elevation of the initial sharp component continued beyond the arbitrarily defined limit of 3.5 hours (Fig. 2).
DISCUSSION

The total excretion of bilirubin is nearly normal in rats with iron deficiency anemia, when calculated on the basis of body weight, although the quantity of circulating hemoglobin is markedly diminished (Table 1). Therefore, much of this bilirubin must be derived from sources other than normal erythrocyte senescence. Indeed, Table 1 indicates that iron deficient rats produce over twice as much bilirubin per gram of circulating hemoglobin as normal rats. This is probably due in part to shortened survival of iron deficient erythrocytes, but the present experiments demonstrate that an increase in the early-labeled fraction of bile pigment accounts for some of this excess.

The early-labeled peak of bile pigment is observed within a short time after the administration of an isotopic precursor such as glycine-¹⁴C, and accounts for 10–20 per cent of the total labeled pigment produced under normal conditions. This bilirubin fraction is formed before significant numbers of erythrocytes containing labeled hemoglobin heme gain access to the circulation, and unlike the major portion of bile pigment, is derived from sources other than the hemoglobin of senescent erythrocytes. Recent investigations have shown that the early peak originates from several different sources. The initial sharp component, which in rats is maximal 1–2 hours after glycine-¹⁴C administration (Fig. 1), appears to arise exclusively from non-erythroid sources, for the most part in the liver. The later or plateau phase, which is formed over the ensuing two and one half to three days, also is largely independent of erythroid sources in rats under normal conditions, but contains a small erythropoietic component which is augmented with increased rates of red cell production.

Enlargement of the erythropoietic component occurs both with the physiologic bone marrow response to such stimuli as hemorrhage, hypoxia, or erythropoietin administration, and with the pathophysiologic derangement called ineffective erythropoiesis that is found in diseases such as pernicious anemia, thalassemia, and refractory normoblastic (sideroblastic) anemia. The increase in early pigment formation with physiologically regulated erythroid hyperplasia is associated with a more or less proportionate rise in the production of labeled hemoglobin. In ineffective erythropoiesis, on the other hand, the formation of erythrocyte hemoglobin is disproportionately low, suggesting that immature red blood cells are destroyed without gaining access to the circulation and their hemoglobin degraded to early-labeled bilirubin.

In iron deficient rats there is enlargement of the plateau portion of the early-labeled peak, which contains the erythropoietic component, whereas the production of labeled hemoglobin is depressed—a relationship typical of ineffective erythropoiesis. Ferrokinetic data had at first seemed inconsistent with ineffective erythropoiesis, since incorporation into red cell hemoglobin is both rapid and high in iron deficiency anemia. However, Pollycove and Lawrence found that the lag phase between incorporation into bone marrow cells and its appearance in circulating erythrocytes is prolonged, and that plasma iron turnover is normal or increased in iron deficient subjects. These observations, together with recent studies of chelatable iron...
EARLY-LABELED BILIRUBIN

before and after iron therapy,\textsuperscript{28} suggested that iron is released during hemolysis of erythroid precursors and then reutilized by successive cohorts of maturing cells. The present finding that incorporation of glycine-\textsuperscript{14}C into erythrocyte hemoglobin, unlike that of \textsuperscript{59}Fe, is below normal in iron deficiency (Fig. 1, Table 2) is consistent with this interpretation, since the heme moiety labeled by glycine is irreversibly degraded to bilirubin and is not available for reutilization.

McKee et al. have recently reported evidence for preferential shortening of the reticulocyte life span in rats with iron deficiency anemia.\textsuperscript{29} In preliminary experiments from this laboratory, based on a previous study of reticulocyte hemolysis,\textsuperscript{30} the excretion of labeled bilirubin was greatly increased in rats transfused with iron deficient as compared to normal reticulocytes containing \textsuperscript{14}C-labeled hemoglobin heme.\textsuperscript{31} Since reticulocytes are increased in rats with iron deficiency anemia\textsuperscript{29} (Table 1) but are at most only moderately elevated\textsuperscript{29} in man, the pathophysiology of this disorder may differ somewhat in the two species. The investigations of iron metabolism in patients\textsuperscript{6,28} however, are consistent with the present findings in rats, although the relative importance of intramedullary hemolysis and reticulocyte hemolysis in the two species remains to be ascertained.

The pattern of early-labeled pigment formation during the response to iron therapy was different from that observed in untreated rats, notably with regard to the marked rise in the initial bilirubin component (Fig. 2). This finding is consistent with increased bilirubin production from hepatic rather than erythropoietic sources, since it has been demonstrated that the initial sharp peak of early pigment formation is independent of either the production or destruction of red blood cells\textsuperscript{14,23,25} and originates largely from the turnover of nonhemoglobin hemes in the liver.\textsuperscript{26,27,32} Heme synthesis is primarily regulated by end-product repression of ALA-synthetase,\textsuperscript{35} and it would not be surprising if the activity of this enzyme were elevated in the liver in iron deficiency. Sudden availability of iron might then lead to a transient excess of total heme synthesis, thus accounting for the rise in hepatic bilirubin formation. Similar increases in the hepatic component of the early-labeled peak have recently been reported in a variety of experimental conditions.\textsuperscript{32,33,36} Although the quantitative implications remain to be fully assessed, these observations suggest that alterations of heme metabolism in the liver may be a relatively common cause of increased bilirubin production.

These experiments thus illustrate two distinct patterns of increased formation of early-labeled bile pigment. Ineffective erythropoiesis, with disordered heme metabolism in the bone marrow, leads to exaggeration of the plateau phase of early pigment production, as found in rats with untreated iron deficiency anemia. Altered heme metabolism in the liver, on the other hand, may be associated with a rise in the initial bilirubin peak, as observed in iron deficient rats during repletion with iron.

**SUMMARY**

The production of early-labeled bilirubin and erythrocyte hemoglobin heme was measured in rats with iron deficiency anemia, using glycine-\textsuperscript{2,14}C as
precursor. The erythropoietic component of the early pigment fraction was significantly augmented and the formation of labeled hemoglobin depressed in the anemic animals, findings characteristic of ineffective erythropoiesis. By contrast, the hepatic component of early-labeled bilirubin was substantially enlarged during the acute response to iron therapy. These experiments illustrate that overproduction of bilirubin may originate from both erythropoietic and hepatic sources of the early-labeled fraction of bile pigment, as well as from hemolysis of circulating red blood cells.

SUMMARIO IN INTERLINGUA
Le production de bilirubina a marcation precoce e de hem de hemoglobina erythrocytic esseva mesurate in rattos con anemia a carentia de ferro con le utilisation de glycina-2-14C como precursor. Le componente erythropoietic del precoce fraction de pigmento esseva significativamente augmentate, e le formation de marcate hemoglobina esseva deprimite in le animales anemic—constatationes le qual es characteristic de un inefficace erythropoiese. Per contrasto, le componente hepatic de bilirubina a marcation precoce esseva significativmente augmentate durante le responsa acute a therapia con ferro. Iste experimentos illustra que le superproduction de bilirubina pote haber su origine fontes si ben erythropoietic como etiam hepatic del prococemente marcate fraction de pigmento biliari e, in plus, in le hemolyse de circulante erythrocytos.

ACKNOWLEDGMENT
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REFERENCES
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