Red Cells, Ferritin, and Iron Storage during Amphibian Development

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Both circulating red blood cells and the liver store iron in ferritin during larval (bullfrog) or embryonic (mouse) development. In contrast, circulating red cells in adults do not store iron and the liver is the main site of iron storage and ferritin. The structure of larval red cell and liver ferritin was compared to adult liver ferritin and the redistribution of iron was measured during amphibian metamorphosis when larval red cells are replaced by adult red cells. Liver ferritin from larvae and adults was very similar in terms of amino acid composition, subunit size, and microheterogeneity (observed by isoelectric focusing) with 80 to 90% of the ferritin composed of three subtypes with an apparent pi of 4.8 to 5.1; the remainder of ferritin (10 to 20%) was composed of four subtypes in the pi range 5.1 to 5.5. In contrast to liver ferritin, larval red cell ferritin had a higher serine and glycine content. Moreover, the microheterogeneity of red cell ferritin was quantitatively unique with all seven subtypes (pi range 4.8 to 5.5) present in approximately equal amounts. In Fe-labeled prometamorphic tadpoles, the specific activity (disintegrations per min per μg of iron) of red cell ferritin was 22,000, liver ferritin was 5,000, and hemoglobin was essentially unlabeled. At the end of metamorphosis the specific activity of liver ferritin was 4,000 and hepatic ferritin was 13,000; the liver and red cells accounted for 70% of the radioactivity, the remainder being in the carcass. Developmental changes in iron content included a slight increase in the liver, a decrease in the carcass and red cells, and a transient increase in the plasma (maximum when 50% of the red cells were replaced). No change was observed in the ferritin content of the liver or liver parenchymal cells. The data suggest that the iron in red cell ferritin was preferentially used for the hemoglobin of the first adult red cells. Although the reason for the storage of iron in red cells during early animal development is not known, its presence in both free-swimming larvae and the embryos of placental mammals suggests a special role such as accessibility. The relatively unique structure of red cell ferritin which was observed may be related to its role in development.

During the early embryonic development of mammals or larval development of amphibians, the circulating red blood cells have the dual functions of iron storage and oxygen transport (2-5). In the adult animal, the red cells which are released into the circulation are more specialized and store essentially no iron. The developmental difference in red cell iron storage is most easily observed by comparing the fully differentiated, mature, circulating red cells of embryos and adults, since partly differentiated red cells of both embryos and adults store iron for hemoglobin synthesis before release into the circulation.

Most of the iron in an animal is contained in hemoglobin. Since red cells are constantly being formed and degraded and because iron is poorly excreted, a large fraction of the body iron is stored for new red cell formation. The fraction of iron distributed among myoglobin, transport proteins, cytochromes, and other iron proteins is small (approximately 9% of the total) (6). The circulating red blood cells of adult animals do not store iron and the major iron storage sites in the adult are the liver, spleen, and bone marrow (6). Iron is stored in ferritin, a specialized protein that can contain up to 4500 iron atoms/molecule and hemosiderin, a poorly characterized, iron-rich compound which appears to be derived from ferritin (7, 8). Although all purified ferritins are similar in size, subunit number, and gross amino acid composition, there are tissue-specific minor variations in amino acid composition and iron content. In addition, ferritin preparations display tissue-specific variations in the range of apparent isoelectric points of tissue isoferritins (9). The physiological significance of the varieties of ferritin from different tissues is not known but may be related to the availability of stored iron for utilization (7, 9).

Iron storage in the animal changes during development, since the contribution of circulating red cells to iron storage changes. Iron storage in the embryo or larva is divided between the liver and the circulating red cells which pass through the liver. In contrast, the liver is quantitatively the...
major iron storage organ in the adult and iron storage does not involve the circulating red cells. For example, in the larval bullfrog, 29% of the ferritin is in the circulating red cells, whereas only 0.6% of the ferritin is in the circulating adult red cells (2). The results reported here compare the structure of ferritin from the liver of larvae and adults with ferritin from larval red cells and show that larval and adult liver ferritin are very similar and that red cell ferritin is relatively unique. In addition, the results show that the iron and ferritin content of the liver did not decrease when the iron and ferritin content of the circulating red cells declined, indicating that the iron stores in red cells were preferentially consumed and iron stores in the liver were conserved during early development. Although developmental changes in iron storage occur in both mammals and amphibians, bullfrogs were used in these experiments because of the large size and ease of experimental manipulation of the larvae (tadpoles).

**EXPERIMENTAL PROCEDURES**

**Animals** — Bullfrog tadpoles were obtained from Howe Brothers Minnow Farm, Atlanta, TX or Blue Ridge Fish Hatcheries, Kernersville, NC. Adult frogs were obtained from Mogul-Ed and Lemberger Associates, NC. Tadpoles were homogenized in 20 mM KC1 and fractionated into heat-soluble (ferritin) and heat-insoluble components (hemosiderin and heme) by heating to 70° for 10 min and separating by sedimentation at 18,000 g for 15 min at 4°. One-half was used to determine total liver radioactivity and the other was homogenized in 20 mM KC1 and fractionated into heat-soluble (ferritin) and heat-insoluble components (hemoglobin and hemosiderin) by heating to 70° for 10 min and separating by sedimentation at 10,000 × g for 10 min. Hemoglobin and hemosiderin radioactivity were distinguished by the extraction of heme from heat-insoluble material with ethyl acetate/acetic acid and HCl and by analyzing both the heme and hemosiderin fractions. The radioactivity recovered in the ferritin, heme, and hemosiderin fractions averaged 93% of the total radioactivity. All of the data were corrected to a specific activity of 1.0 mcg iron/100 g liver (in the absence of iron supplementation) and to a specific activity of 1.0 mcg iron/100 g liver (in the absence of iron supplementation).

**Measurement of Iron, Protein, and Ferritin Concentration** — All animals were thoroughly bled before the tissues were collected. The total iron content of the carcass, the liver, and liver fractions (heat-soluble, heat-insoluble, and ethyl acetate-extractable) was usually determined after complete digestion of the tissue or tissue fraction with HClO4/HNO3 (9). The digestive tract was removed before digestion of the carcass because the digestive tract contained approximately 1.0 mg of iron from small stage 20 and contained essentially no iron during metamorphosis when the animal stopped eating. In some experiments the iron content of the liver was determined on homogenized tissue digested for 24 h at room temperature with 10.8 N HCl according to Osaki et al. (12). The results of both digestion procedures with liver in the same samples were similar, showing no more than 1 N HCl for 30 min to release iron. Iron was measured as the o-phenanthroline complex as described previously (3). Plasma iron concentrations were determined as the 3,3-dipyridyl-5,5-dimethyl-(4-phenyl)-2,4-triazine (ferrozine) complex (13). Protein concentrations were determined by the method of Lowry (14).

**Exchangeable Iron** — To determine the ferritin concentration of aqueous extracts of amphibian liver during development, an antiserum to tadpole liver ferritin was used in double diffusion in agar as previously described (4). Livers were excised from animals which had been thoroughly perfused through the portal vein with amphibian Ringer's solution (15) without calcium and containing 0.02% EGTA. Liver parenchymal cells were isolated from cell suspensions prepared from perfused livers by continuing the perfusion with Ringer's solution containing CuCl2 (1.4 mM) and collagenase II (1 mg/ml). After the livers swelled, they were excised, shaken in the perfusate, and teased with forceps to free the cells (16). Parenchymal cells were collected by sedimentation from the suspension by sedimentation at 40 × g for 5 min, the preparations appeared to be 80 to 90% pure, as judged by hemocytometric analysis. Livers or parenchymal cells were homogenized in 0.5 ml of H2O and the supernatant solution obtained after sedimentation at 18,000 × g for 15 min at 4° was analyzed for ferritin, by immunodiffusion, as well as total protein.

**Electrophoresis and Isoelectric Focusing in Polyacrylamide Gels** — The gel concentrations for analytical and preparative electrophoresis in acrylamide gels were 4, 7, or 8%, and the buffers of Davis (17) were used as previously described (3). For preparative procedures, the monomers were recrystallized from chloroform (acrylamide) or acetone (N,N'-methylenebisacrylamide) before use (18).

**Amino Acid Composition** — Apoferritin was prepared from ferritin by diafiltration against 1% thiglycolic acid in 0.1 N sodium acetate at pH 5.2 followed by dialysis against multiple changes of distilled water (28:10:55) with 0.1% CuSO4, followed by dialysis against the same solvent without CuSO4, or dyes, or a modification of the procedure of the pyloric caeca of amphistome (27). Cysteic acid was determined on perfused intestines and liver samples hydrolyzed with 6 N HCl (27). Amino acid analysis was performed according to the procedure of Spackman et al. (28) as described previously (3).
**Isolation of Ferritin from Tadpole and Frog Liver—**Liver obtained from adult frogs or premetamorphic tadpoles (stages 10 to 13) which had been thoroughly bled were stored at −15 °C until use. All purification procedures were at 0 °C. Liver homogenates, prepared in 19 volumes of 50 mM imidazole-HCl, pH 7.0, were clarified by sedimentation at 12,000 x g for 30 min (Fraction I) and applied to a column of DE52 (4.0 x 9.2 cm) equilibrated with 50 mM imidazole-HCl, pH 7.0. After washing the column with 2 liters of starting buffer, ferritin was eluted with 500 ml of buffer containing 0.08 M KCl. The ferritin-rich fraction, concentrated by ultrafiltration (Fraction II), was applied to a column of Sepharose 6B (2.6 x 70 cm) in 20 mM potassium phosphate, pH 6.8. The column eluate was monitored for ferritin (280 and 340 nm) and heme proteins (540 nm); the separation of ferritin and heme proteins was complete. Further purification of the ferritin fraction after concentration by ultrafiltration (Fraction III) was effected by electrophoresis in 4% polyacrylamide gels containing sodium dodecyl sulfate with electrophoresis in 4% polyacrylamide gels followed by electrophoresis in 7% gels. Ferritin was removed from the gel segments by homogenization of the slices in 20 mM potassium phosphate buffer, pH 6.8, using a hand homogenizer, with approximately 80% recovery.

### RESULTS

**Isolation of Ferritin from Bullfrog Tadpole and Frog Liver—**Ferritin, which appeared to be homogeneous with respect to iron and protein components after electrophoresis in gels of three different acrylamide concentrations, was prepared by a procedure described under "Experimental Procedure" which avoided the high temperatures that can lead to degradation. Although the same procedure could be applied to both adult and larval tissue with similar final results, examination of the properties of Fraction III (Table I) shows that the ferritin preparation from adult liver was much less pure at this stage than the preparation from tadpole liver.

Because of the high iron content of ferritin, the iron to protein ratio can be used as a measure of purification. The ratio (mg/mg) of iron to protein for purified preparations from other sources ranges from 0.10 to 0.32 and reflects the degree of saturation with iron and/or the amount of apoferritin present (7, 8). The iron to protein ratio was 0.110 ± 0.018 for six preparations of frog liver ferritin and 0.196 ± 0.038 for four preparations of tadpole liver ferritin (Table I). The ratio for tadpole red cell ferritin was 0.13 (3) and for feritin from the liver of iron-loaded Rana catesbeiana, 0.23 (29). The results of six preparations of frog liver ferritin and 0.196 ± 0.038 for four preparations of tadpole liver ferritin (Table I). The ratio for tadpole liver ferritin was 0.19 (3) and for feritin from the liver of iron-loaded Rana catesbeiana, 0.23 (29). The results of a single experiment using sucrose gradient centrifugation to fractionate frog and tadpole liver ferritin on the basis of iron content suggested that the difference in the iron to protein ratio for tadpole and frog liver ferritin was due to differences in the degree of iron saturation rather than to differences in the amount of apoferritin present.

**Ferritin Purification**

Ferritin was prepared from amphibian liver as described under "Experimental Procedures." The average amount of tissue used was 18 g for frog liver and 7 g for larval liver.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Iron (mg)</th>
<th>Total Protein (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Larval Liver</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (Aqueous Extract)</td>
<td>4.59</td>
<td>725</td>
<td>0.63</td>
</tr>
<tr>
<td>II (GC-52)</td>
<td>1.25</td>
<td>91.5</td>
<td>0.27</td>
</tr>
<tr>
<td>III (Sepharose 6B)</td>
<td>0.551</td>
<td>2.08</td>
<td>15.9</td>
</tr>
<tr>
<td>IV (Polyacrylamide gel electrophoresis)</td>
<td>0.310</td>
<td>1.60</td>
<td>19.4</td>
</tr>
<tr>
<td><strong>Adult Liver</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (Aqueous Extract)</td>
<td>2.52</td>
<td>3040</td>
<td>0.083</td>
</tr>
<tr>
<td>II (GC-52)</td>
<td>1.02</td>
<td>216</td>
<td>0.469</td>
</tr>
<tr>
<td>III (Sepharose 6B)</td>
<td>0.449</td>
<td>55.2</td>
<td>0.833</td>
</tr>
<tr>
<td>IV (Polyacrylamide gel electrophoresis)</td>
<td>0.184</td>
<td>1.58</td>
<td>11.6</td>
</tr>
</tbody>
</table>

**Subunit Analysis of Apoferritin—**The electrophoretic mobility of liver apoferritin subunits was measured in 12% polyacrylamide gels containing sodium dodecyl sulfate with ovalbumin, lactate dehydrogenase, chymotrypsinogen, myoglobin, and cytochrome c used as standards for molecular weight. Using different preparations for each developmental stage, a subunit molecular weight of 20,200 ± 1,600 for frog liver apoferritin and 20,300 ± 1,100 for tadpole liver apoferritin was calculated. Such a subunit size is comparable to other ferritins such as those from tadpole red cell and horse spleen (3, 7, 8). Occasionally, protein components in the range of 10,000 to 16,000 daltons were observed; but, since they constituted only a small and variable (0 to 12%) amount of the total protein, they were considered to be artifacts.

Separation of horse spleen apoferritin into two components has been observed after dissociation with 67% acetic acid and electrophoresis in 15% polyacrylamide gels, containing 8 M urea and 0.9 M acetic acid (21). When tadpole or frog liver apoferritin was dissociated with acetic acid containing 10 mM mercaptoethanol and analyzed in the acid/urea gels as described, only one component was observed. In a single experiment, horse spleen apoferritin also produced only one band if mercaptoethanol were present during dissociation and analysis.

**Amino Acid Composition of Apoferritin—**The amino acid compositions of tadpole and frog liver apoferritin (Table II) were essentially identical and were very similar to that reported by Shinjo and Abe (29) on ferritin isolated by a different procedure from iron-loaded frogs. There appeared to be a difference between liver and red cell apoferritin in the amount of serine and glycine (Table II). Cystine and cysteic acid were virtually absent from HCl hydrolysates of tadpole and frog liver ferritin, as was previously reported for tadpole red cell ferritin (3).
However, cysteine could be detected either as S-sulfocysteine after reduction of methanesulfonic acid hydrolysates of liver apoferritins with sodium tetraphionate or as cysteic acid after HCl hydrolysis of performic acid-oxidized liver and red cell apoferritin (Table II).

Analysis of Ferritin Microheterogeneity—Purified preparations of ferritin, which appear to be homogeneous when analyzed electrophoretically on a series of components or subtypes by isoelectric focusing in a narrow pH range (7, 9). All of the subtypes are immunologically related and all contain iron, with the exception of the apoferritin that is present in some samples. The origin of ferritin microheterogeneity is not known, but there is no simple relationship between microheterogeneity and iron content, the multiplicity of cell type in the tissue used, cell age, or method of preparation (7, 9, 23). However, the microheterogeneity of ferritin is specific for different tissues and thus has been extensively used as an analytical tool (7, 9). Accordingly, the microheterogeneity of ferritin preparations from the livers of bullfrog larvae and adults and from the red cells of larvae was analyzed by isoelectric focusing (pH range of 4 to 6). Purified preparations from both liver and red cells were used which were homogeneous when analyzed by electrophoresis of the holoprotein or subunits. In addition, crude preparations (red cell lysates and heat-soluble fractions of aqueous liver extracts) were also analyzed in order to eliminate the possibility that microheterogeneity was introduced during isolation. Although the same seven subtypes could be observed after isoelectric focusing of ferritin from the red cells of larvae and the livers of larvae and adults, the distribution was markedly different (Fig. 1). For example, 81% of larval liver ferritin and 90% of adult liver ferritin were composed of three subtypes with apparent pI in the range of 4.80 to 5.10. In contrast, only 40% of tadpole red cell ferritin was composed of the three subtypes focusing in the pH range 4.80 to 5.10, and the remainder was composed of approximately equal amounts of the four subtypes which focused in the pH range 5.10 to 5.49 (Fig. 1). The distribution was the same for purified tadpole ferritin or red cell lysates (illustrated in Fig. 1) and for purified liver ferritins (illustrated in Fig. 1) or crude liver extracts. All of the ferritin subtypes contained iron and the distribution of iron was similar to that of protein. Ferritin from adult liver also contained a unique, acidic component (1 to 6% of the protein) which had an apparent pI of 4.7.

Distribution of Iron and Ferritin during Development—In bullfrogs, the replacement of larval red cells by adult red cells occurs during metamorphosis when eating ceases and there can be no exogenous source of iron for the new red cells. The liver is an important storage organ in adults. In order to test the possibility that the iron for adult red cells came from the iron stored in the liver, the iron and ferritin content of the liver were examined. The results in Table III show that the concentration of ferritin in the whole liver and in the liver parenchymal cells was essentially unchanged during metamorphosis, in contrast to the dramatic decrease in red cell.

![Figure 1](http://example.com/ferritin_focusing.png)

**Fig. 1.** The microheterogeneity of ferritin from larval red cells, larval liver, and adult liver after isoelectric focusing. Purified ferritin (electrophoretically homogeneous) from liver and red cells, or red cell lysates and liver extracts were analyzed for microheterogeneity by isoelectric focusing in slabs of polyacrylamide gel (pH 4 to 6). The gels were stained for protein or iron, photographed, and the photographs were scanned at 550 nm (see "Experimental Procedures"). Both the photographs and the traces of the scan are presented. The same seven ferritin subtypes focusing between pH 4.8 and 5.5 were observed in all preparations, although the distribution was markedly different for red cells and liver. The three ferritin subtypes which focused between pH 4.8 and 5.1 accounted for 80 to 90% of liver ferritin, whereas for red cell ferritin all seven subtypes were present in approximately equal amounts. The results were the same for purified ferritin or crude ferritin preparations. When red cell lysates were used, ferritin was identified by comparison of gels stained for iron and protein; only one nonferritin protein focused in the pH range used and is indicated by an arrow in the top plate. In contrast, larval red cell lysate, larval liver ferritin, and adult liver ferritin.

TABLE III

<table>
<thead>
<tr>
<th>Stage</th>
<th>Red Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Liver</th>
<th>Parenchymal Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ug/100 ug proteins</td>
<td>ug/100 ug proteins</td>
<td>ug/100 ug proteins</td>
</tr>
<tr>
<td>5</td>
<td>0.96</td>
<td>2.3</td>
<td>3.5</td>
</tr>
<tr>
<td>13</td>
<td>1.1</td>
<td>3.4</td>
<td>1.5</td>
</tr>
<tr>
<td>20</td>
<td>1.1</td>
<td>2.7</td>
<td>1.1</td>
</tr>
<tr>
<td>22</td>
<td>0.66</td>
<td>1.8</td>
<td>2.7</td>
</tr>
<tr>
<td>24</td>
<td>0.44</td>
<td>2.1</td>
<td>1.3</td>
</tr>
<tr>
<td>25</td>
<td>0.15</td>
<td>1.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data from reference (4).

The concentration of ferritin protein in aqueous tissue extracts was estimated using double diffusion in agar and purified tadpole liver ferritin as a standard (see "Experimental Procedures"). Extracts of perfused livers and liver parenchymal cells (80 to 90% pure) were prepared as described under "Experimental Procedures." The results are the average of the analysis of three to five perfused livers or parenchymal cells from two to three livers. The error is presented as the standard deviation.
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ferritin. Furthermore, the iron content of the liver was relatively constant during metamorphosis, increasing by the end of metamorphosis (Table IV). The observed iron content of the liver agrees with previously published values corrected for difference in animal size (12) with the exception of stage 20, an exception which might be attributed to the retention of iron-laden red cells if the animals were not bled before analysis. No change in the distribution of iron between ferritin and hemosiderin occurred until stage 25, when the percentage in ferritin decreased from 36 ± 2% to 23%. Developmental changes were observed in the iron content of the carcass (Table IV), which decreased probably due to gill and tail resorption, and in the concentration of iron in the plasma (Table IV) which showed a transient increase during the red cell replacement indicative of increased iron transport and new red cell formation.

Distribution of 

Iron during amphibian development

The liver and plasma were collected from animals undergoing spontaneous metamorphosis. For total iron, the pooled livers or carcasses of 2 to 6 animals were totally digested and the pooled plasma from 20 to 30 animals was deproteinized before analysis for iron (see "Experimental Procedures"). The results are the average of multiple analyses, 7 to 13 for liver iron, 3 to 13 for plasma iron and 3 to 4 for carcass iron. The error is the standard deviation. In a single set of analyses on the spleens pooled from 6 to 12 animals, the iron content during metamorphosis was 3 to 6 µg.

### Table IV

<table>
<thead>
<tr>
<th>Stage</th>
<th>Liver</th>
<th>Plasma</th>
<th>Carcass</th>
<th>Red Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/liver</td>
<td>µg/100 ml</td>
<td>µg/animal</td>
<td>pg/cell</td>
</tr>
<tr>
<td>13</td>
<td>146 ± 36</td>
<td>58 ± 11</td>
<td>152 ± 33</td>
<td>0.99</td>
</tr>
<tr>
<td>18</td>
<td>426 ± 138</td>
<td>75 ± 21</td>
<td>107 ± 30</td>
<td>0.69</td>
</tr>
<tr>
<td>20</td>
<td>401 ± 117</td>
<td>88 ± 8</td>
<td>110 ± 38</td>
<td>0.73</td>
</tr>
<tr>
<td>22</td>
<td>495 ± 71</td>
<td>98 ± 34</td>
<td>141 ± 28</td>
<td>0.63</td>
</tr>
<tr>
<td>23</td>
<td>627 ± 212</td>
<td>139 ± 78</td>
<td>115 ± 29</td>
<td>0.54</td>
</tr>
<tr>
<td>24</td>
<td>565 ± 50</td>
<td>64 ± 9</td>
<td>49 ± 10</td>
<td>0.50</td>
</tr>
<tr>
<td>25</td>
<td>604 ± 230</td>
<td>73 ± 21</td>
<td>107 ± 30</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Iron reference (30).

![Diagram](http://www.jbc.org/)

**Fig. 2.** The distribution of 

Fe in red cells and the liver during amphibian development. Prometamorphic bullfrog larvae (stage 18) were injected with [59Fe]NTA. At various stages of spontaneous metamorphosis when larval red cells containing large amounts of ferritin are replaced by adult red cells which contain little ferritin, two to four animals were killed and the distribution of radioactivity was determined (see "Experimental Procedures"). The results are the average of three or four experiments. Essentially all of the injected radioactivity was conserved, the whole body counts averaging 3.0 ± 0.2 x 10^6 dpm during the experiment. The radioactivity in the carcass declined slightly from 1.00 x 10^6 dpm (stage 18) to 0.78 x 10^6 dpm at the end of metamorphosis (stage 24). The plasma radioactivity dropped to ≤ 3% of the total by stage 20 at the beginning of metamorphosis. Most of the radioactivity (> 90%) was distributed between ferritin and hemoglobin in the developing animal is also making large numbers of new adult red cells. In adult animals, the iron for the continual formation of new red cells comes from iron removed from the hemoglobin of old red cells and stored in the reticuloendothelial cells of the liver, marrow, and spleen and in the parenchymal cells of the liver. In contrast, the results in Tables III and IV and Fig. 2 show that in the developing frog, the iron for hemoglobin in the first group of adult red cells came largely from the ferritin of the larval red cell ferritin.

**DISCUSSION**

The loss of storage iron from the circulating red cells occurs during the shift in red cell formation from the primitive red cell line containing embryonic or larval hemoglobin to the definitive cell line containing fetal and/or adult hemoglobin (4, 5). Thus, at the time iron stores are lost from the circulating red cells, the developing animal is also making large numbers of new adult red cells. In adult animals, the iron for the continual formation of new red cells comes from iron removed from the hemoglobin of old red cells and stored in the reticuloendothelial cells of the liver, marrow, and spleen and in the parenchymal cells of the liver (6).
cells; the iron and ferritin stores in the liver and the small iron stores in the spleen were conserved. A comparison of the specific activity of the iron in larval red cell ferritin (22,000 dpm/µg) and adult red cell heme (15,000 dpm/µg) suggests that some additional iron of a lower specific activity was also used for the new adult red cells. Such a hypothesis is supported by a comparison of the total amount of ferritin red cell iron (49 µg) in the circulation before the red cell replacement (stage 18) and the total amount of iron (86 µg) in red cell hemc after the cell replacement (stage 24). Some of the iron of low specific activity may have come from the carcass, which lost iron during the red cell replacement. Alternatively, some iron in the liver could have turned over, leaving in the new red cells and being replaced by iron from the carcass. The unusual feature of ferritin in the circulating larval red cells is the preferential utilization of its iron compared to larval liver ferritin (Fig. 2). The ferritin in larval red cells differs from the ferritin in larval liver in both amino acid composition and in microheterogeneity, whereas liver ferritin from both larval and adult stages is very similar (Table II, Fig. 1). Although the structural basis for the microheterogeneity of ferritin is not known definitely, it has been shown to be characteristic of the cell or tissue from which the ferritin is isolated and has been used extensively as an analytical tool (7, 9).

The reason for the storage of iron in circulating red cells early in development is not known. In the case of free-swimming larvae such as the bullfrog tadpole, it can be argued that extra iron is stored in red cells in preparation for the starvation and increased red cell formation that occur during metamorphosis. However, iron is also stored in the circulating red cells of the embryonic mouse (5), a placental mammal which presumably has a constant supply of iron. Furthermore, the iron content of embryonic mouse liver is conserved during the replacement of embryonic red cells by adult red cells (2), in analogy to the developing bullfrog. A possible explanation for the storage of iron in embryonic or larval red cells is that early in animal development, red cell ferritin is a more readily accessible source of iron than liver ferritin, either because of the unique structure of red cell ferritin (Table II, Fig. 1) and/or embryonic transferrin (31) or because the system for releasing iron from liver ferritin (7) has not yet been formed. A possible candidate for the release of iron from ferritin, the NADH-FMN-dependent ferritin reducing activity of amphibian liver, increases 3-fold during metamorphosis (12). The reduction of the ferritin content of circulating adult red blood cells and the shift in iron storage to the liver and other organs indicate that the expression of ferritin genes during red cell differentiation is altered as an animal develops.

In summary, the iron stored in the circulating red cells early in animal development appeared to be utilized in preference to liver iron stores for the formation of the first adult red cells. Comparison of ferritin from larval liver, adult liver, and larval red cells showed larval red cell ferritin to be unique, which suggests a specialized role for red cell ferritin during animal development.

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REFERENCES

   (Brown, E.B. et al. eds) pp. 90-106, Grune and Stratton, New York
   Enzymol. 26c, 3-27
   Chem. 251, 1936-1940
   30, 1280-1286
   Biochemistry and Medicine (Crichton, R. R., ed) pp. 371-376
   North Holland, Amsterdam
Red cells, ferritin, and iron storage during amphibian development.
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