A QUANTITATIVE FRACTIONATION OF TISSUE FERRITIN AND HEMOSIDERIN*

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Ferritin and hemosiderin are two forms of storage iron of strikingly similar chemical and physical characteristics (1). Ferritin is present in dispersed form within the cell, while hemosiderin is present as insoluble particles which are visible microscopically. The separation of these substances has not been heretofore performed on a quantitative scale, although methods for the extraction of ferritin (1-4) and a qualitative separation of the two iron-protein compounds (5) have been reported.

The present study was designed to separate and distinguish quantitatively rabbit and human ferritin, hemosiderin, and hemoglobin and to determine quantitatively rabbit ferritin by an immunochemical technique.

Methods

Iron was determined with sulfosalicylic acid by the method of Lorber (6), and nitrogen by the micro-Kjeldahl procedure, with the use of a copper-selenium catalyst. Radioiron analyses were performed on tissue fractions which had been wet ashed, precipitated, and electroplated according to the method of Peacock et al. (7). Fe$^{55}$ was counted with an argon-filled Geiger-Müller tube having a counting efficiency of approximately 3 per cent.

The hemoglobin content was measured as pyridine hemochromogen by a modification of the method of Flink and Watson (8), and the solution containing 1 ml. of sample, 5 ml. of 10 per cent NH$_4$OH, 2 ml. of pyridine, and 2 ml. of fresh Na$_2$S$_2$O$_4$ was read at 500 and 555 m$\mu$ in the Beckman model DU spectrophotometer.

In measurements of hemoglobin in tissue extracts it was necessary to correct for the absorption by ferritin.\(^1\) Hemoglobin was estimated as the pyridine hemochromogen at 555 m$\mu$ (molecular weight of hemoglobin taken

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\(^1\) Analyses of tissue extracts of rabbit liver extremely low in iron content showed linear but negligible contribution of compounds other than ferritin under the conditions of hemochromogen determination in the wave-length range of 500 to 555 m$\mu$. Thus no correction, other than that for ferritin absorption, was considered necessary.
as 68,000; iron content 0.34 per cent). The absorption spectra of ferritin and of the pyridine hemochromogen differ markedly; this fact permitted the determination of the concentration of one in the presence of the other from the optical densities of the solutions at several wave-lengths and from the ratio of extinction coefficients of isolated ferritin and the molar extinction coefficients of hemoglobin at these wave-lengths. The equation derived\(^2\) for the mg. of hemoglobin iron is

\[
2.13 \times 10^{-3} (E_{555} - 0.468E_{500}) = \text{mg. hemoglobin iron per ml. tissue extract}
\]

where \(E_{555}\) and \(E_{500}\) = log \(I_0/I\) at 555 \(\mu\)m and 500 \(\mu\)m, respectively.

Recovery studies indicated that with a hemoglobin iron and ferritin iron ratio of about 1:180 the error in hemoglobin determination and calculation was 1.9 per cent. In a ratio of 1:10, hemoglobin recovery was 100.5 per cent, while a ratio of 1:1000, corresponding to a perfused, iron-heavy tissue, gave a hemoglobin recovery of only 81 per cent. In the latter case, however, because of the large amount of ferritin iron present, the error from the residual hemoglobin resulted in an error in the ferritin iron estimation of only 0.3 per cent.

Rabbit ferritin was measured quantitatively by an immunochemical technique. Ferritin, prepared from rabbit livers according to the method of Mazur and Shorr (2), was found to be antigenic when injected into young adult goats. A total of 100 mg. of alum-precipitated antigen was injected intravenously in increasing dosages over a period of 3 weeks, and blood was withdrawn 5 days after the last injection for the preparation of antiserum. "Total ferritin"\(^3\) nitrogen was estimated immunochemically in the region of antibody excess, with appropriate standards. The quantitative precipitin reaction was performed (9) by the addition of the antigen solutions to 2 ml. portions of antiserum containing enough 0.9 per cent NaCl to produce a final volume of 4 ml. The mixtures were incubated at 37°.
for 1 hour and refrigerated for 5 days. The centrifuged and washed specific precipitates were analyzed for total nitrogen.

Isotopically tagged ferritin, hemosiderin, and hemoglobin were prepared as follows:

**Ferritin and Hemosiderin**—Saccharated iron oxide (350 mg. of iron) was administered intravenously to rabbits to increase their iron stores. Fe$^{55}$ (0.9 mc.) was injected intravenously, followed by 150 mg. of iron as the saccharated oxide in divided doses over the subsequent 3 days. 1 week later the animals were sacrificed, and labeled ferritin was isolated from the livers by the method of Mazur and Shorr (2). The specific activity of the ferritin thus prepared was 1.3 μc. per mg. of ferritin iron. Hemosiderin, containing Fe$^{55}$, was prepared from tissue residue. The residue was washed with 10 times its volume of water and centrifuged at 1400 × g for 30 minutes. This was repeated twice. The washed residue was suspended in 5 times its volume of water, heated to 75° in a water bath, and the suspension was filtered three times through ten layers of 20/12 gage gauze. The granules passing through the gauze were washed twice with 10 volumes of 2 per cent (NH$_4$)$_2$SO$_4$, followed by two washes with 5 volumes of water to remove the (NH$_4$)$_2$SO$_4$. The final water suspension contained only brown hemosiderin granules as observed under the microscope. The specific activity of the purified hemosiderin thus prepared was 0.6 μc. per mg. of hemosiderin iron.

**Hemoglobin**—Rabbits were injected intravenously with 0.9 mc. of Fe$^{55}$. Blood was withdrawn by cardiac puncture, and the red cells were isolated by centrifugation, lysed in distilled water, and the stroma fraction was then separated by centrifugation. The specific activity of the hemoglobin was 4.2 μc. per mg. of iron.

**Tissue Fractionation for Ferritin, Hemosiderin, and Hemoglobin**

Fractionation of rabbit livers for the iron compounds was carried out after perfusion of the liver in situ through the portal vein with 0.9 per cent NaCl; fractionations of human liver were performed on non-perfused tissue. In both species, spleen, kidney, lung, and other organs were equally satisfactory for fractionation.

The tissue was cut into small pieces and homogenized in a Waring blender for approximately 3 minutes. A sample was taken for dry weight and for iron analysis. Aliquots of 8 to 20 gm. of blended tissue were fractionated.

The tissue aliquot (e.g., 10 gm.) was extracted with twice its weight of distilled water for 5 minutes in the semimicro Waring blender. The sus-

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4 2500 r.p.m.; radius from center of tube, 20 cm.; International No. 2 centrifuge, No. 240 head.
pension was transferred to a 100 ml. Lusteroid tube, and the blender was washed three times with a total of about 7 ml. of distilled water. The suspension was centrifuged for 30 minutes at 1400 × g and the supernatant fluid was decanted. The residue was washed with the same amount of water as in the initial extraction, the centrifugation repeated, and the extracts were combined.

For the determination of hemosiderin iron, the tissue residue was hydrolyzed for 2 hours in a boiling water bath with 5 times its weight of 1 per cent NaOH.

Ferritin and hemoglobin iron may be differentiated directly in the extract by the hemochromogen method, since ferritin iron equals the total iron content of the extract minus the hemoglobin iron content.

In general, fractionation experiments for tissue ferritin, hemosiderin, and hemoglobin iron were carried out adequately with the above method. However, if it was desirable to separate ferritin from hemoglobin, the original extract was transferred to a 150 ml. beaker and heated to 75° with mechanical stirring in a water bath. The heating process required 5 minutes. (It was necessary to standardize these conditions carefully.) The beaker was then placed in an ice bath for 30 minutes, and the contents were subsequently transferred to a 100 ml. Lusteroid tube and centrifuged at 1400 × g for 30 minutes. The supernatant fluid was decanted; the heat coagulum was washed with 10 ml. of water, recentrifuged for 15 minutes, and the wash liquid was added to the first supernatant fluid. This soluble fraction from the heated extract will be referred to hereafter as heat supernatant fluid.

The heat coagulum, containing hemoglobin and small amounts of other hemoproteins, was subjected to alkaline hydrolysis identical to that applied to the tissue residue fraction, and iron analysis was performed.

The heat supernatant fluid, containing ferritin, was subjected to direct iron analysis and immunochemical measurements.

To purify and compare the ferritin fraction further, ammonium sulfate to 50 per cent saturation (35 gm. of (NH₄)₂SO₄ per 100 ml.) was added to the heat supernatant fluid, and this was allowed to stand at room temperature for about 20 hours. The precipitate was dissolved in water, and the ferritin was further purified from this fraction by crystallization with cadmium sulfate (10).

Results

The results of a complete fractionation of rabbit liver are given in Table I. It was found that in two water extractions of tissue, with either low or high iron content, about 98 per cent of the extractable iron was recovered. Increasing the amount of water did not affect recovery, and a cloudy ex-
tract did not interfere with the results for total iron or pyridine hemochromogen. While the water extract was usually unsatisfactory for the immunochemical determination of ferritin, the clear soluble fraction, obtained after heating the extract, gave consistently accurate results by using

**TABLE I**

Fractionation of Rabbit Livers for Ferritin, Hemosiderin, and Hemoglobin Iron

8 gm. of non-perfused, iron-heavy liver samples were extracted twice with varying amounts of water and fractionated. All values represent duplicate fractionations.

<table>
<thead>
<tr>
<th>Water for each extraction</th>
<th>Extract</th>
<th>Residue</th>
<th>Heat supernatant</th>
<th>Heat coagulum</th>
<th>Ammonium sulfate ppt.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total iron</td>
<td>Total iron</td>
<td>Ferritin iron*</td>
<td>Total ferritin N*</td>
<td>Ferritin Fe:N</td>
</tr>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>2 × tissue weight weight</td>
<td>17.0</td>
<td>19.8</td>
<td>15.0</td>
<td>15.2</td>
<td>6.8</td>
</tr>
<tr>
<td>3 × tissue weight weight</td>
<td>16.5</td>
<td>19.5</td>
<td>14.6</td>
<td>15.1</td>
<td>6.6</td>
</tr>
<tr>
<td>4 × tissue weight weight</td>
<td>16.8</td>
<td>19.6</td>
<td>14.7</td>
<td>14.8</td>
<td>6.6</td>
</tr>
</tbody>
</table>

* Determined immunochemically.

**TABLE II**

Recovery of Labeled Hemoglobin, Ferritin, and Hemosiderin from Rabbit Livers

To 20 gm. samples of perfused, iron-heavy rabbit livers was added hemoglobin, ferritin, or hemosiderin containing Fe\(^{55}\), and the samples were partitioned for the iron compounds. All fractionations were run in duplicate.

<table>
<thead>
<tr>
<th>Radioactive compound added</th>
<th>Extract</th>
<th>Residue</th>
<th>Heat supernatant</th>
<th>Heat coagulum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m. Fe(^{55}) × 10(^3)</td>
<td>per cent</td>
<td>c.p.m. Fe(^{55}) × 10(^3)</td>
<td>per cent</td>
</tr>
<tr>
<td>Hemoglobin.............</td>
<td>555</td>
<td>94</td>
<td>33.8</td>
<td>6</td>
</tr>
<tr>
<td>Ferritin.............</td>
<td>144</td>
<td>98</td>
<td>2.7</td>
<td>2</td>
</tr>
<tr>
<td>Hemosiderin.............</td>
<td>28</td>
<td>6</td>
<td>420</td>
<td>94</td>
</tr>
</tbody>
</table>

an appropriate dilution of this fraction with 0.9 per cent NaCl. The heat supernatant fluids were found to be free of hemoglobin, and the iron content was essentially the same as that of ferritin determined immunochemically. Although there was a 7 per cent loss of ferritin after precipitation with ammonium sulfate, the average ferritin iron to nitrogen ratio of 2.29 in this fraction agreed well with average ratios of 2.26 obtained in the heat supernatant fraction and 2.29 obtained in the crystallized ferritin sample.
In Table II a recovery experiment is summarized in which tracer amounts of isotopically labeled ferritin, hemosiderin, and hemoglobin were added to rabbit livers. It may be seen that there is localization of the added radioiron in the appropriate fraction. While it would appear with the radioactive technique that there was a loss of about 6 per cent of ferritin iron into the heat coagulum, immunochemical measurements indicated a more complete recovery (98 per cent) in the heat supernatant fraction.

From the recovery data on human tissue, presented in Table III, it is evident that the fractionation method may be employed for a quantitative separation of hemoglobin and ferritin in one fraction and hemosiderin in the other, and the analytical methods allow the determination of the three iron compounds. Studies involving heat coagulation on human tissue are

### Table III

<table>
<thead>
<tr>
<th>Added compound</th>
<th>Extract</th>
<th>Per cent recovery</th>
<th>Residue</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.58</td>
<td></td>
<td>3.17</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (0.56 mg. iron)</td>
<td>4.28</td>
<td>103.4</td>
<td>3.14</td>
<td></td>
</tr>
<tr>
<td>Ferritin (2.15 mg. iron)</td>
<td>5.62</td>
<td>98.1</td>
<td>3.29</td>
<td></td>
</tr>
<tr>
<td>Hemosiderin (2.87 mg. iron)</td>
<td>3.76</td>
<td></td>
<td>5.87</td>
<td>97.2</td>
</tr>
</tbody>
</table>

not reported; while storage in a frozen state does not affect the initial extraction, this has been found to interfere occasionally with the separation of hemoglobin and ferritin by heat coagulation.

### DISCUSSION

A method has been described for the quantitative fractionation of tissue ferritin, hemosiderin, and hemoglobin iron. It has been demonstrated by immunochemical and radioactive techniques that the linkage between iron and its protein complexes is sufficiently strong so that the measurement of one may be taken to represent the other in this fractionation procedure.

It is not unlikely that a graded series of molecular aggregates exists from ferritin to the visible particles of hemosiderin. By varying the speed of centrifugation it is possible to change the proportion of these two fractions. The purpose of the present study has been to devise a reproducible method of separating the hemosiderin and ferritin iron into two fractions, each one containing the predominant characteristics of one of these compounds.
The method presented disregards other iron complexes of tissue. These would seem so small in amount as to be of no concern in the measurements (11). With the variation in the iron content from one portion of an organ to another, there seems to be little purpose in a method with an accuracy of greater than ±5 per cent.

The authors wish to acknowledge the valuable technical assistance of Mrs. Alice Rupen.

SUMMARY

The quantitative fractionation of rabbit and human tissues for ferritin, hemosiderin, and hemoglobin has been accomplished with an over-all accuracy of approximately ±5 per cent. Rabbit ferritin was determined quantitatively by an immunochemical technique with goat antiserum.

Results on fractionated tissues of iron analyses, immunochemical measurements, and recovery of radioiron-labeled compounds have established the validity of the procedure.

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