Effect of Iron Loading on Non-Heme Iron Compounds in Different Liver Cell Populations*

C. P. Van Wyk,‡ M. Linder-Horowitz, and H. N. Munro

From the Physiological Chemistry Laboratories, Department of Nutrition and Food Science, Cambridge, Massachusetts 02139

SUMMARY

Concentrations of total non-heme iron and ferritin iron were determined for whole livers and isolated Kupffer cells of rats before and after iron treatment. Rats were injected either once, with iron dextran (25 mg of iron), or multiple times, with hemoglobin (totaling 10 mg of iron) or iron dextran (totaling 140 mg of iron), 1 or 16 to 20 weeks before use. Particularly at the highest iron dosage, the liver contained a large proportion of the dose administered as non-heme iron, and in control rats or rats treated with 16 to 25 mg of iron, 70 to 80% of this iron was in the form of ferritin. A nonhepatocyte fraction, containing mostly Kupffer cells, was isolated from each liver after differential digestion of the hepatocytes and Kupffer cells. In all cases, very little ferritin could be shown in the Kupffer cell fraction by antibody precipitation or disc electrophoresis following extraction of the cells with heat treatment, sonic oscillation, and detergents, in various combinations. The Kupffer cell fraction did, however, contain at least two other forms of non-heme iron visualizable by electrophoresis as a very slow migrating band and as large brown granules which did not enter the gel. These granules were isolated in the case of the rats treated with 140 mg of iron and had an iron-protein ratio of 1.9. Based on known values for the percentage of nonhepatocyte nuclei in liver, the total content of non-heme and ferritin iron in hepatocytes and nonhepatocytes were calculated. The hepatocytes invariably contained almost all of the ferritin iron while the Kupffer cell fraction contained almost all of the nonferritin, non-heme iron. The protein content of the Kupffer cell fraction was commensurate with its estimated contribution to liver volume.

Apart from iron circulating as hemoglobin in the blood, a significant part of the body iron is normally present in the liver in the form of the non-heme compounds, ferritin and hemosiderin (1, 2). Following administration of iron, a considerable part of the total dose is deposited in the liver, and ferritin protein accumulates (1, 3, 4).

The liver consists of several distinct cell types, of which the parenchymal (hepatocyte) and the reticuloendothelial (Kupffer) cells are more than 95% of the cell population and volume (5, 6). Non-heme iron has been identified in both of these cell types by histochemical reactions (7-10), and ferritin by electron microscopy (8, 11). The iron present in the liver after oral or parenteral administration is deposited in both cell types, but the relative amounts and form of the iron in the hepatocytes and Kupffer cells have not been established with certainty.

In the present investigation, we have measured the non-heme iron and ferritin content of livers and isolated Kupffer cells obtained from normal rats and iron rats iron-loaded by injection of iron dextran or hemoglobin. The non-heme iron content of the liver and of the isolated Kupffer cells was much increased by iron administration. The ferritin content of the liver was also elevated, but the Kupffer cells yielded only traces of ferritin in the case of both normal and iron-treated animals. Consequently, iron retained by the Kupffer cell appears to be in some other form.

METHODS

Animals and Treatments—Female Fisher rats (140 to 225 g body weight) were used. They were maintained on Red Label Rat Chow under thermostatic conditions. Iron was administered intraperitoneally as iron dextran (Imferon, Lakeside Laboratories, Inc., Milwaukee, Wisconsin) or as bovine hemoglobin (Pentex Biochemicals, Kankakee, Illinois). The iron dextran was given either (a) as a single dose of 25 mg of iron to each rat 1 week before killing, (b) as a single dose of 25 mg of iron to each rat 18 to 20 weeks before killing, or (c) as repeated doses of 10 mg of iron per 100 g of body weight twice weekly for 4 weeks, and the animals were killed 15 weeks later. The hemoglobin (0.8 g per rat) was injected every 2nd day for 10 days, and the rats were killed between the 2nd and 10th day after the last injection.

In the study of Kupffer cell isolation, animals were injected intraperitoneally with trypan blue (4 mg/100 g, body weight) in Hanks' balanced salt solution (Grand Island Biological Company, Grand Island, New York) every 2nd day on three occasions. These animals were killed about 1 week after the last injection.

Liver Analysis and Kupffer Cell Isolation—The rats were anesthetized with 0.2 ml/100 g of body weight of Equithesin (chloralhydrate-pentobarbital; Jensen-Salsbery Laboratories, Kansas City, Missouri) and the liver was perfused through the portal...
vein with isotonic 0.9% NaCl solution. In most cases the livers of two rats were combined and minced with scissors, and a 1-g portion was homogenized with 9 volumes of ice-cold Gey's balanced salt solution (12) and set aside for measurement of total nuclear population, total iron, heme iron, ferritin iron, and, where indicated, protein and trypan blue content. The remainder of the minced livers was used for Kupffer cell isolation.

Our procedure was a modification of that of Mills and Zucker-Franklin (13). Minced liver samples were incubated with 5 volumes of 0.25% Pronase (Calbiochem) in Gey's solution for 1 hour at 25° with continuous stirring. Tissue suspensions were then filtered through a nylon screen (108 mesh) and the retained masses of undissociated liver were dried briefly on filter paper and weighed. Of the original liver mass, 25 to 50% was not dissociated. The filtrate from the screening was centrifuged in 15-ml conical tubes in an International refrigerated centrifuge at 1200 × g for 10 min. The supernatant fluid was aspirated and the pellet was resuspended in a volume of Gey's solution equivalent to 7 times the original weight of liver dissociated. This washing procedure was repeated a further five times. After the third washing, fibrous material was removed by passage through the same kind of nylon screen. During the washing procedure, the upper part of the pellet was cream-colored and the lower part was bright red as a result of trapped erythrocytes. The upper part consisted of cell debris and was removed and discarded; the lower part was suspended in Gey's solution and subjected to the same analyses as the whole homogenate.

**Cell and Nuclear Counting**—To enumerate the cell population of the whole liver, the nuclei in the homogenate were counted as described by Knox, Horowitz, and Friedell (14). After 10-fold dilution of the homogenate in Gey's solution, a portion was suspended in 9 volumes of a solution containing 0.13% orcein and 45% acetic acid. The stained nuclei were counted in a hemacytometer chamber. Kupffer cells were counted by diluting the final cell suspension directly with 9 volumes of the orcein reagent.

In order to measure the frequency of multinucleation in parenchymal cells, liver cells were dissociated by the method of George, Friedman, and Byers (16) in which minced liver is forced through a tissue press (Harvard Apparatus Company, Dover, Massachussetts) into saline free Locke's solution containing EDTA. The suspension, containing all liver cell types, was diluted with 1 volume of orcein solution, which did not rupture the cells, and the frequency of multinucleate hepatocytes was enumerated in a hemacytometer.

**Iron Determinations**—Total iron was determined on 0.5-ml samples of liver homogenate and on 3-ml samples of Kupffer cell suspension. The samples were digested with 2 vol of 72% (w/v) HClO4 and 1 ml of concentrated HNO3 over a microburner until the solution cleared and then for a further 20 min. The digests were diluted with deionized water to a known volume and samples were analyzed for iron by the bipyridyl procedure (16) following adjustment of the pH to above 3 with sodium acetate.

To determine ferritin iron in the liver homogenate and in the Kupffer cell preparations, samples were heated to 70° for 10 min to release ferritin from whole cells and vesicles and to conglutinate most of the nonferritin protein (16). This procedure is not subject to loss of ferritin, as judged by recovery of added ferritin. When indicated, samples were previously treated with 1 to 1.5% deoxycholate, 0.3% sodium lauryl sulfate or with sonic oscillation (at 5 periods of 10 sec, 20 kc per sec, with a Branson sonic oscillator). After heat treatment and removal of the bulk of liver protein by centrifugation, the supernatant was titrated to maximum iron precipitation with specific ferritin antiserum and the iron in the precipitates was determined after washing them twice with isotonic NaCl solution (4).

Heme iron was measured on the samples by the pyridine-hemochromogen procedure (17), with bovine hemoglobin, which contains 0.34% iron, as a standard. Absorption was read at 417 my.

**Trypan Blue Determination**—Trypan blue was extracted from the liver homogenates and Kupffer cells of trypan blue-injected animals with 1.5 volumes of dimethylformamide. Samples were mixed, and, after standing overnight, were centrifuged at 8000 rpm for 10 min in the Sorvall RC-2B centrifuge to remove coagulated protein. The supernatant fluid was measured for optical density at 610 my (1 × 10⁸ OD unit per µg of trypan blue). The absorbance of liver samples prepared from uninjected rats was subtracted. Trypan blue absorbance, in a mixture of dimethylformamide and Gey's solution, was not reduced by adsorption to the liver homogenate.

**Protein Determination**—The protein content of the whole homogenate and of the Kupffer cell suspension was measured by a modification of the method of Lowry (18), with bovine serum albumin as the standard.

**Electrophoresis** Disc electrophoresis on acrylamide gels was used to identify different iron compounds present in the isolated Kupffer cell preparations. Kupffer cells were concentrated by sedimentation at 1200 × g for 10 min and resuspended in a small volume of water. Following lysis with sodium lauryl sulfate (0.3%, w/v), the samples were subjected to electrophoresis as previously described (4). In this procedure gels were stained for iron by the Prussian blue reaction and for protein with Amido black, and the migration of bands relative to the migration of the tracking dye was measured. The staining procedures detect less than 1 µg of ferritin iron and 2 µg of ferritin protein. In addition, total iron content was determined chemically on selected parts of some of the gels.

**RESULTS**

**Liver Nuclear Population**—As judged by nuclear counts, the livers of the female rats used had a constant population of cells per g of wet weight, except in the case of animals chronically treated with large doses of iron. As shown in the legend to Table I, the total nuclear population in the livers of untreated rats was 2.5 × 10⁸ per g, which is close to the nuclear density reported in previous investigations (6, 19). Iron treatment did not alter this except in the case of repeated dosage with iron dextran, which tended to increase the number to 2.8 to 2.9 × 10⁸ per g. Under these same conditions Golberg, Smith, and Martin (7) noted that multinucleated, syncytial cells had replaced the usual Kupffer cells, and this may explain the increase in the number of nuclei per g which we observed.

**Kupffer Cell Isolation Procedure**—In our hands, the Pronase procedure of Mills and Zucker-Franklin (13) yielded Kupffer cell preparations which appeared on microscopic examination to be free of contamination by parenchymal cells. On light microscopy, the morphology of the isolated cells differed markedly from that of parenchymal cells and, in the case of rats injected with trypan blue, most cells contained phagocytosed, blue granules. This substantiates the observation of Mills and Zucker-Franklin (13) that Pronase treatment preferentially digests parenchymal cells and leaves Kupffer cells intact.
Rats were treated with iron as hemoglobin or iron dextran in the total doses indicated. Total non-heme iron recovered from the liver, in micrograms, is shown, as well as the total non-heme iron, ferritin iron, and heme iron contents (micrograms per 10^7 nuclei) in whole livers and in Kupffer cells isolated from them. The mean values (± S.D.) are shown, with the number of determinations in parentheses. Control livers contained 2.5 ± 0.2 × 10^7 nuclei per g, while livers from hemoglobin- and Imferon-treated rats contained 2.5 ± 0.2 (6), 2.8 ± 0.1 (4), 2.6 ± 0.2 (6) and 2.0 ± 0.3 (6) × 10^7 nuclei per g, respectively, in the order given below.

### Table I

<table>
<thead>
<tr>
<th>Treatment of rats</th>
<th>Total iron given</th>
<th>Total non-heme iron in Liver</th>
<th>Whole liver</th>
<th>Kupffer cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>µg iron/10^7 nuclei</td>
<td>µg iron/10^7 nuclei</td>
</tr>
<tr>
<td>Control</td>
<td>1.0</td>
<td>6.4 ± 1.3 (4)</td>
<td>5.3 ± 1.2 (4)</td>
<td>0.23 ± 0.33 (3)</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>16</td>
<td>17.3 ± 2.6 (5)</td>
<td>12.9 ± 2.5 (5)</td>
<td>7.1 ± 4.4 (4)</td>
</tr>
<tr>
<td>Iron dextran (once)</td>
<td>25</td>
<td>77.9 ± 7.2 (4)</td>
<td>25.3 ± 3.7 (4)</td>
<td>27.8 ± 13.9 (4)</td>
</tr>
<tr>
<td>1 week</td>
<td>25</td>
<td>7.8 ± 4.9 (4)</td>
<td>34.9 ± 22.2 (4)</td>
<td>34.8 ± 13.9 (4)</td>
</tr>
<tr>
<td>18-20 weeks</td>
<td>25</td>
<td>8.3 ± 5.6 (6)</td>
<td>73 ± 5 (6)</td>
<td>1.7 ± 0.8 (1)</td>
</tr>
<tr>
<td>Iron dextran (multiple)</td>
<td>140</td>
<td>58.6 ± 290 ± 33 (6)</td>
<td>73 ± 5 (6)</td>
<td>1.7 ± 0.8 (1)</td>
</tr>
</tbody>
</table>

In order to remove debris from the Kupffer cells following Pronase treatment, repeated washing in Gey's solution was required. The effect of repeated washing was evaluated by measuring the content per Kupffer cell of trypan blue or iron after different numbers of washings. No significant change in cell content of either was observed by washing 12 times as compared with six times. Accordingly, the cells were routinely washed six times, a procedure which also left no visible debris.

The yield of Kupffer cells following Pronase digestion for 1 hour was determined by counting the number of cell nuclei in the final Kupffer cell preparation and comparing it to the total number of cells in the weight of tissue digested. The latter value was obtained by recovering undigested liver at the end of Pronase treatment, blotting it, and subtracting the weight of this residue from the original liver weight. The yield of Kupffer cells measured in this way averaged 9% of the total cell population. This is about a quarter of the percentage of nonparenchymal cells estimated to be present in liver tissue (5).

Since trypan blue has long been used to identify Kupffer cells (20), an attempt was made to determine the number of these cells in the liver cell population by extraction of the trypan blue. The concentration of trypan blue in isolated Kupffer cells was compared with the total trypan blue content of whole liver homogenate, and the Kupffer cell population was calculated on the assumption that all of the dye recovered from the homogenate derived from ruptured Kupffer cells. Since a significant proportion of hepatocytes is multinucleate (21), it was necessary to correct the total nuclear counts obtained from the liver homogenate in order to express the data as cell population. Direct measurement on cell suspensions from livers of three rats indicated that 27% (26 to 31%) of the hepatocytes were binucleate and 0.1 to 0.3% had more than two nuclei. This is in close agreement with data obtained by other workers (22, 23). With the use of these data in a series of five rats, the trypan blue method yielded a Kupffer cell population of 73 ± 14% of the total cells in the liver. This is about twice the frequency of Kupffer cells reported by others who used histological techniques (5, 24) and suggests that trypan blue is not confined to Kupffer cells but also enters the parenchymal cells of the liver.

**Non-Heme Iron Compounds in Liver and Kupffer Cells**—The concentrations of total non-heme iron and ferritin iron were measured in the whole liver and in the separated Kupffer cells. The results are expressed in Table I as iron per 10^7 nuclei. In the whole liver of untreated animals, 83% of the non-heme iron was present as ferritin. After iron administration in any of the forms used there was a 3- to 50-fold increase in non-heme iron. In the case of single doses of iron dextran or several doses of hemoglobin, ferritin was responsible for about 70% of the additional iron deposited, and thus the proportion of ferritin to total non-heme iron remained essentially unaltered. When repeated massive doses of iron dextran were administered, the deposition of iron in the liver was very large and the proportion present as ferritin fell to 25%. Consequently, although the non-heme iron content of the liver rose 50 fold above the control level, the ferritin increased only 5-fold, which was nevertheless more than twice the amount observed after the other iron treatments. The data also show that hemoglobin iron was a less efficient source of liver iron. This was not due to retention of unchanged hemoglobin in the liver, since the concentration of heme iron remaining in the liver after perfusion was not affected by this or any other treatment (Table I).

The non-heme iron and ferritin iron contents of the Kupffer cell preparations were quite different from those of whole liver (Table I). Very little non-heme iron was found in the cells isolated from untreated rats. Following hemoglobin or iron dextran administration, large amounts of non-heme iron were observed in the Kupffer cells, but only a small proportion of it was ferritin iron. This observation was true irrespective of the time after iron dextran administration (1 week or 18 weeks). In the case of rats given multiple injections of iron dextran, attempts to isolate Kupffer cells were frustrated by the presence of abundant brown granules which simulated nuclei under the microscope and made nuclear counting impossible. Similar but less abundant brown granules were visible within the Kupffer cells 1 week after a single dose of iron dextran. The nature of these granules is discussed further below.

Since the yield of ferritin from the Kupffer cell preparations was persistently low even in iron-treated rats, attempts were made to see whether additional ferritin could be released from them by other treatments. Richter (8, 11) has described membrane-bound ferritin masses in Kupffer cells following iron treatment. Accordingly, we treated the cell suspensions by sonic
oscillation or by lysis in water containing 1 to 1.5% deoxycholate. The deoxycholate was diluted to 0.2 to 0.3% with 0.05 M NaCl solution and the preparation was heated to 70°, followed by antibody precipitation of ferritin as described under "Methods." In addition, treatment of the Kupffer cell preparation with 0.3% sodium lauryl sulfate followed by centrifugation yielded a supernatant fraction from which ferritin was precipitated by using the specific antibody. No additional ferritin was released by sonic oscillation or by the detergents; all treatments released less than 5% of the non-heme iron as ferritin iron in the case of cells isolated from the livers of rats given one 25-mg dose of iron as iron dextran.

**Distribution of Non-Heme Iron Compounds among Liver Cell Populations**—In order to assess the contribution of Kupffer cell non-heme iron and ferritin iron to the total iron content of the liver, a figure of 39% for Kupffer and other nonhepatocyte nuclei per g of liver (5) was accepted, since our attempts to determine liver, a figure of 39 ci, for Kupffer and other nonhepatocyte nuclei to give the total distributions of non-heme iron and ferritin iron in this cell distribution, the data from Table I were recalculated with trypan blue had been unsuccessful. By using this cell distribution, the data from Table I were recalculated to give the total distributions of non-heme iron and ferritin iron in the Kupffer cells and the hepatocytes of 1 g of liver (Table II). As shown for the control series of rats, 99% of the non-heme iron of the liver was present in the hepatocytes, mainly as ferritin.

**Table II**

Distribution of non-heme and ferritin iron in 1 g of liver after various iron treatments

Micrograms of non-heme and ferritin iron present in 1 g of whole liver and in the Kupffer cell and hepatocyte populations of that gram of liver. The content of non-heme iron, ferritin iron, and, by difference, the nonferritin, non-heme iron was calculated for whole liver from the data in Table I. The amounts of these three forms of iron in the Kupffer cells and other nonhepatocytes were also calculated from the data on the basis that 39% of the cells of the liver are nonhepatocytes (5). The amounts of these three iron forms in hepatocytes were obtained by the difference between the quantities calculated for total liver and for the Kupffer cell fraction.

<table>
<thead>
<tr>
<th>Treatment of rats</th>
<th>Total non-heme iron</th>
<th>Ferritin iron</th>
<th>Nonferritin, non-heme iron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole liver</td>
<td>Kupffer cell fraction</td>
<td>Hepatocytes</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin injected</td>
<td>163</td>
<td>2.3</td>
<td>161</td>
</tr>
<tr>
<td>Iron dextran injected</td>
<td>431</td>
<td>69</td>
<td>362</td>
</tr>
<tr>
<td>1 week</td>
<td>1076</td>
<td>308</td>
<td>768</td>
</tr>
<tr>
<td>18-20 weeks</td>
<td>1282</td>
<td>351</td>
<td>931</td>
</tr>
</tbody>
</table>

**Table III**

Protein content and ratio of iron to protein in liver cell populations

Protein content, either as milligrams per 10⁷ nuclei or as milligrams of total protein per cell population of 1 g of liver, and the ratio of non-heme content to protein content after various iron injections are given for the average liver cell (value for whole liver in the mixed cell population), for nonhepatocytes (Kupffer cell fraction) isolated from whole liver, and, by calculation, for hepatocytes by using a figure of 39 for the percentage of nonhepatocyte nuclei (5). Values given are the means (±S.D.) with the number of determinations in parentheses where indicated.

<table>
<thead>
<tr>
<th>Protein content</th>
<th>Ratio of non-heme iron to protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein/10⁷ nuclei</td>
<td>Total protein/cell population</td>
</tr>
<tr>
<td>Average liver cell</td>
<td>7.4 ± 0.0 (14)</td>
</tr>
<tr>
<td>Kupffer cell fraction</td>
<td>0.97 ± 0.12 (14)</td>
</tr>
<tr>
<td>Hepatocyte fraction</td>
<td>11.5</td>
</tr>
</tbody>
</table>
Kupffer cell preparations isolated from the livers of untreated rats or from rats given iron as iron dextran or hemoglobin were treated with sodium lauryl sulfate and applied to disc electrophoresis. The number of preparations of each kind analyzed is shown, as is the estimated number of Kupffer cells (as lysate) applied to each gel. The \( R_P \) values of migration (mean ± S.D., with number of determinations in parentheses) and estimated abundance of the three iron bands present are given. The \( R_P \) of whole rat liver ferritin was 0.17 ± 0.01 (14) and of interferon was 0.09 ± 0.01 (4). Band 3 represents material remaining at the border between sample and stacking gel.

The amount of protein per cell in both cell populations was not appreciably altered by iron treatment, so that the iron-protein ratio was increased by iron administration in proportion to the changes in iron deposition recorded earlier (Table I). Since the protein content of the Kupffer cell is low, consistent with its smaller size, the effect of iron deposition on the iron-protein ratio was much more striking than in the case of the hepatocytes (Table III).

Electrophoresis of Iron Compounds in Kupffer Cells—Lysates of the Kupffer cells were applied to polyacrylamide gels of disc electrophoresis and were stained for iron and protein bands in order to substantiate the quantitative findings and further identify the types of iron present (Table IV). Three kinds of iron compounds could be distinguished by their \( R_P \) values. One was identical in migration with liver ferritin (\( R_P \) of 0.13 to 0.17) and stained positively for protein. A second band ran more slowly than ferritin (\( R_P \) of 0.04 to 0.05) and did not stain for protein; however, the \( R_P \) of iron dextran was distinguishably faster (0.09). The third form of iron remained at the top of the stacking gel; it also did not stain for protein.

An attempt was made to determine quantitatively the relative distribution of iron in these three bands after the various treatments given the animals. Comparison of chemical iron in each band with the intensity of staining in the Prussian blue reaction showed that the latter is not a reliable guide to the relative amounts in the three bands. Kupffer cells from untreated rats showed only a trace of Band 2, but no Band 1, although some ferritin was found by chemical analysis (Table I). Kupffer cells from hemoglobin-treated rats showed only a ferritin band, although much larger amounts of nonferritin, non-heme iron were found by chemical analysis (Table I), and, on analyzing the gel for iron at the locations of Bands 2 and 3, considerable amounts of iron were recovered in spite of the negative staining reaction. The Kupffer cells from iron dextran-treated rats showed the presence of all three types of bands on the gel; chemical analysis of the gels showed the same proportions of total non-heme iron (Bands 2 plus 3) to ferritin iron as were obtained by direct chemical analysis of the cells (Table I).

In the case of one preparation of Kupffer cells obtained after multiple iron dextran injections, no ferritin was seen in the gel, Band 2 stained weakly, and enormous quantities of iron were retained in Band 3 at the top of the gel. Since Band 3 material was the only material present in abundance, it was surmised that it was the same material visualize under the light microscope as large brown granules. Further evidence of this was obtained by isolating these granules from the Kupffer cell preparation. The cell suspension was treated with 0.3% sodium lauryl sulfate and the granules were harvested by centrifugation and washing with water. The isolated granules appeared under the light microscope to be unchanged by this treatment. They were very rich in iron, the iron-protein ratio of the granules being 1.9. This is a ratio of iron-protein far above that of liver ferritin, which has an average ratio of 0.27, but similar to that of hemosiderin granules isolated from human liver (25).

**DISCUSSION**

Liver Cell Populations—The original objective of this study was to examine separately iron compounds present in preparations of liver parenchymal cells (hepatocytes) and of Kupffer cells. Attempts to separate out the parenchymal cells by published procedures (15, 21, 26, 27) were not successful in our hands. With animals injected with trypan blue to identify Kupffer cells, it was invariably found that such procedures yield hepatocyte preparations heavily contaminated with Kupffer cells. These latter were readily distinguished from the hepatocytes by their size and appearance and contained blue granules. Further treatment of the preparations by differential centrifugation or sucrose gradients failed to provide better resolution. Consequently, we turned to the preparation of Kupffer cells free of hepatocytes and computed the data for hepatocytes by difference from whole liver. The procedure of digestion of hepatocytes with Pronase, which leaves the more resistant Kupffer cells intact (13), proved to be successful. Mills and Zucker-Franklin (13) demonstrated by electron microscopy that the majority of the cells surviving this treatment have the characteristics of Kupffer cells and that they retain their morphological integrity.
Our studies on trypan blue-treated rats show that the Pronase method yields almost exclusively cells that have taken up and concentrated this colloidal dye and are thus of reticuloendothelial type. The purity of the product obtained by this method can be assessed from the micrographs provided by Mills and Zucker-Franklin (13). In one such micrograph, 43 Kupffer cells, no hepatocytes, four biliary epithelial cells, and 15 neutrophils and plasma cells are seen. In our Kupffer cell preparations, we also found no hepatocytes, and only occasional neutrophils.

Since trypan blue has been used as a specific dye for Kupffer cell identification with the light microscope (20), it was thought that the uptake of this dye by the liver would provide a measure of the Kupffer cell population. Based on this assumption, the proportion of Kupffer cells in the liver was estimated to be more than twice that established by careful histological and stereological studies (5, 6, 24). These latter authors all agree that 56 to 65% of liver nuclei represent hepatocytes and 30 to 34% are Kupffer cells, the remaining 10% of nuclei being derived from bile duct cells, blood vessels, and connective tissue. Since the trypan blue method exaggerates the number of Kupffer cells in the liver, it must be concluded that other cells take up this dye but fail to concentrate it as recognizable granules.

Although Kupffer cells account for one-third of the cell population of the liver, they contribute a much smaller proportion to the mass of the liver because of their smaller dimensions. Weibel et al. (6) found that 83% of the entire volume of the liver could be ascribed to hepatocytes and 4% to connective tissue. Consequently, 13% of the mass includes the remaining liver cell types and the extracellular space. This agrees with our calculations (Table III) that the protein content per Kupffer cell is less than one-tenth that of the hepatocyte.

Iron Deposition in Liver—Our results are in general agreement with the observations of others that the liver is a major site of iron deposition, particularly under conditions of extreme iron loading. Hahn et al. (28) and Golberg et al. (7), using massive doses of hemoglobin and iron dextran, respectively, found that about half of the injected material could be recovered in the livers of the animals that they used. We recovered 42% of the administered dose in the livers of rats treated with a total of 140 mg of iron as iron dextran. However, at more moderate doses, particularly of hemoglobin, we found that a much lower percentage of the dose was present in the liver 2 to 10 days after the last of a series of injections. This may occur because there are well developed physiological mechanisms for dealing with hemoglobin degradation and reutilization of its iron (29, 30). It is also possible that some of the iron entering the liver may have been lost through excretion into the bile (7, 31).

Our biochemical studies showed that, in the normal rat, most of the non-heme iron in the liver was confined to the hepatocytes where it was present almost exclusively as ferritin. Following single doses of 16 to 25 mg of iron as hemoglobin or iron dextran, a large proportion of the extra iron in the liver was deposited in the hepatocytes as ferritin, but the remainder was found in the Kupffer cells where it took some other form. After repeated dosage with iron dextran totaling 140 mg, there appeared to be a limit to the capacity of the liver to convert this to ferritin, so that three-quarters of the non-heme iron was now present in other forms. Since we were unable to isolate the Kupffer cells from such livers, we could not tell whether this non-heme, non-ferritin iron, the bulk of which seemed to be in the form of brown granules, was again primarily in the Kupffer cells. However, the light microscopic studies of Golberg et al. (7) on rats treated in the same way showed that much of this iron was present in the Kupffer cells as brown "sideroid pigment" granules and that the Kupffer cells had been transformed into multinucleated syncytial cells. Furthermore, we observed that similar although less abundant granules were visible in the Kupffer cells of animals given a single dose of iron dextran.

The capacity of rat or mouse liver cells to accumulate non-heme iron in response to iron dextran or hemoglobin has been examined histochemically by Richter (8, 11), MacDonald, Enlo, and Pochet (9), Glomasi (10), and Pochet (32). All agree in showing by electron microscopy or by the Prussian blue reaction that both the hepatocytes and Kupffer cells contain iron granules some weeks after iron dextran administration. The quantitative interpretation of such data however suffers from several defects. First, the detection of these granules by light microscopy depends on the iron being present in aggregates of sufficient size for detection; ferritin, which usually represents the major form of nonheme iron, is commonly present in dispersed form in the cytoplasm (8, 33, 34). Second, the cytoplasmic volumes of the hepatocyte and the Kupffer cell differ by a factor of more than 10, so that results expressed per cell may give a totally different picture from results expressed per unit of liver weight. Thus, when Richter (8) states that Prussian blue granules were found almost exclusively in the Kupffer cells following hemoglobin injection, it is possible that significant amounts of similar granules passed undetected in the larger hepatocytes. His finding may therefore be incompatible with our observation of considerable amounts of nonferritin, non-heme iron in the hepatocyte population following hemoglobin injection (Table II). In this connection, it has been recently shown that, at the large dose levels used by us, large amounts of hemoglobin are taken up directly by the parenchymal liver cells (35).

Of additional interest is the electron microscopic study of Richter (11) on the livers of mice injected with iron dextran and killed at various times thereafter. At six days after injection, iron was present in the Kupffer cells within membrane-delimited granules; some of this deposited iron was morphologically similar to iron dextran and some was identified by electron microscopy as ferritin. After 2 to 3 weeks, granulated inclusions containing ferritin but not iron dextran were also observed in the hepatocytes. These latter particles showed cristae and double membranes characteristic of mitochondria and Richter gives them the name of "siderosomes." Although Richter identified ferritin by electron microscopy as a component of the Kupffer cell granules deposited after iron dextran administration, we were unable by a variety of procedures to extract significant amounts of ferritin from isolated Kupffer cells of animals receiving the same treatment with iron. Chemical studies of iron-containing "hemosiderin" granules extracted from different sources have been attempted by several investigators. Richter himself (25) isolated granules from livers of human subjects with hemochromatosis and McKay and Fineberg (36) isolated similar structures from horse spleen. Both groups, however, failed to extract significant amounts of ferritin from the granules, although Richter claimed to see ferritin within them under the electron microscope. We also failed to extract ferritin from the isolated granules that we obtained from livers of rats chronically treated with iron dextran, and we were generally unable to extract more than traces of ferritin from any of our Kupffer cell preparations. Indeed, the granules that we isolated, as well as those described...
by Richter (35), could not have contained more than one-eighth of their iron in ferritin form, since there was very little protein present relative to the amount of iron, the iron-protein ratio being 1.9.

In conclusion, all of the evidence suggests that there is little, if any, ferritin present in the Kupffer cells of normal or iron-treated rats. In fact, we cannot exclude the possibility that the traces of ferritin that we found were due to contamination from released hepatocyte ferritin, especially since it migrates on gels similarly to hepatocyte ferritin. However, if Kupffer cells contain any ferritin of their own, it must represent only a small proportion of the non-heme iron and it must be embedded in some highly impenetrable material, perhaps mucopolysaccharide (37).

As a consequence, while both Kupffer cells and hepatocytes may contain non-heme iron, our studies show that that portion of the non-heme iron and it must be embedded in some highly impenetrable material, perhaps mucopolysaccharide (37).

Acknowledgment—We gratefully acknowledge the assistance of Mrs. Joan R. Moor with some of the techniques and determinations.

REFERENCES
Effect of Iron Loading on Non-Heme Iron Compounds in Different Liver Cell Populations
C. P. Van Wyk, M. Linder-Horowitz and H. N. Munro


Access the most updated version of this article at http://www.jbc.org/content/246/4/1025

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/4/1025.full.html#ref-list-1