The liver participates in the removal from the circulation of both damaged red blood cells (RBC) and plasma hemoglobin. The specific hepatic cell types involved in these processes have been identified by fractionation of rat liver into pure isolates of parenchymal and sinusoidal cells. After injection of $^{59}$Fe-labeled hemoglobin, $85\%-95\%$ of the radioactivity in the liver was associated with the parenchymal cells, regardless of whether the hemoglobin was bound to haptoglobin or was free in plasma. By contrast, $^{59}$Fe-labeled spherocytic RBC were sequestered entirely by the sinusoidal cell population. Stimulation of microsomal heme oxygenase by administered hemoglobin or RBC indicated that these cell fractions not only sequester but also degrade the ingested hemoglobin-heme. Infusion of doubly labeled $^{59}$Fe,$^{125}$I-hemoglobin indicated that the hepatic parenchymal cells remove the intact hemoglobin molecule without exchange or transfer of the heme moiety to other carrier proteins. By contrast, heme bound to albumin was detached from the albumin before its uptake by the parenchymal cells. These findings suggest that, contrary to previous belief, hepatic parenchymal cells play a key role in the metabolism of plasma hemoglobin.

The removal of senescent red blood cells (RBC) from the circulation is still incompletely understood. Possible mechanisms that have been proposed for this process include: (1) intravascular lysis with subsequent removal of the released hemoglobin from the circulation; (2) sequestration of intact RBC by phagocytosis, followed by intracellular digestion of the hemoglobin; and (3) fragmentation of senescent RBC, which may lead to both intravascular escape of some hemoglobin and intracellular digestion of the hemoglobin remaining within the RBC fragments. In terms of overall hemoglobin catabolism, distinguishing among these postulated degradative mechanisms may be unnecessary, because in vivo the heme of plasma hemoglobin and of intact RBC is converted to bilirubin at similar rates and with comparable efficiency. On the other hand, if significant differences should exist in the anatomic sites and cell types that are involved in the removal...
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and catabolism of plasma hemoglobin and of intact RBC, these differences may be of importance in the regulation of iron homeostasis, and they may play a role in the heme economy of a particular tissue or cell type. For example, it has been postulated that heme compounds dissolved in the plasma exert a regulatory effect on the rate of intrinsic heme synthesis in parenchymal cells of the liver.\textsuperscript{3}

The fate of normal intact senescent RBC is uncertain, though it has been found that RBC altered by immunologic, chemical, or physical means are removed mainly by the spleen and liver in experimental animals.\textsuperscript{4-8} Similar data have been reported in man.\textsuperscript{5-7} In recent studies in rats, we identified a subpopulation of hepatic sinusoidal cells that appear to be specifically responsible for the sequestration of intact damaged RBC in the liver.\textsuperscript{9} These erythrophagocytic (EP) cells, which comprise approximately 30\% of the total number of sinusoidal cells in the liver, are characterized by distinct functional, histochemical, and enzymatic properties.\textsuperscript{9} For example, they exhibit a relatively high activity of microsomal heme oxygenase (MHO), which is the substrate-inducible enzyme system responsible for the conversion of hemoglobin-heme to bilirubin.\textsuperscript{10} Since the total activity of this enzyme system in the EP cells of the liver and in the spleen of normal animals is similar, we suggested that the liver and spleen may be of comparable importance in the removal and catabolism of physiologically aged erythrocytes.\textsuperscript{9}

In contrast to intact RBC, hemoglobin dissolved in plasma is taken up largely by the liver,\textsuperscript{7,11-13} with the bone marrow accounting for only 10\%-15\% and the spleen for less than 5\% of the removal of an injected dose of labeled hemoglobin.\textsuperscript{11} This appears to hold true whether the hemoglobin is free in plasma or bound to haptoglobin,\textsuperscript{11} except that in the former instance a significant amount of the administered hemoglobin is sequestered by the kidneys\textsuperscript{11,14,15} or excreted in the urine. It is unclear, however, which cell type in the liver is responsible for hemoglobin uptake and catabolism. From the kinetics of plasma hemoglobin disappearance\textsuperscript{16,17} and on the evidence that macrophages in other organs appear to take up dissolved hemoglobin,\textsuperscript{11,18} it is widely held that plasma hemoglobin is taken up by sinusoidal macrophages in the liver.\textsuperscript{18} This concept is based on inferential evidence, however, and has been disputed.\textsuperscript{19}

We have studied the cellular sites of the uptake and degradation of intact RBC and of plasma hemoglobin in the liver by fractionation of the organ into virtually pure isolates of hepatic parenchymal and sinusoidal cells. Rats were injected intravenously either with \textsuperscript{59}Fe-labeled intact spherocytic RBC or with \textsuperscript{59}Fe-hemoglobin, and the relative distribution of the isotope in the two cell populations was estimated. The uptake of the label in the two cell types was compared also with the heme-mediated stimulation of MHO. This combined physical and enzymatic approach demonstrated that plasma hemoglobin, whether free or haptoglobin-bound, is taken up and catabolized almost exclusively in hepatic parenchymal cells, whereas intact RBC are phagocytosed and degraded solely in the EP cells of the hepatic sinusoids. A preliminary report of these observations has been published.\textsuperscript{20}
MATERIALS AND METHODS

Preparation of Isolated Hepatic Parenchymal and Sinusoidal Cells

Fed, male Sprague-Dawley rats, weighing 225–400 g, were sacrificed under ether anesthesia. Isolates of parenchymal or sinusoidal cells were obtained by perfusing the liver in situ either with collagenase or with Pronase solution, as described previously.9 On the average, 15% of the total sinusoidal cells and 20% of the parenchymal cells of the liver were recovered. Sinusoidal cells constituted less than 3% of the total cells in parenchymal cell preparations, and parenchymal cell contamination of sinusoidal cell isolates was 0%–1%. The EP subpopulation of the sinusoidal cell isolate was identified and quantitated by morphologic and functional criteria.9 Cell counts were carried out in a hemacytometer chamber under bright-field illumination.

Preparation of Spherocytic RBC and Hemoglobin

Rat RBC, washed in isotonic saline, were suspended in an equal volume of isotonic saline and rendered spherocytic by heating for 1 hr at 49°C. Each rat received approximately 10⁹ RBC/100 g weight by intravenous injection into a tail vein. Control animals were given a comparable volume of cell-free supernatant from a similar preparation of heated RBC.

Soluble hemoglobin was prepared from a portion of the washed RBC, which were lysed with 6 vol of 0.01 N NaOH. The resulting solution was made isotonic with sodium chloride and brought to pH 7.8 with sodium phosphate buffer. The slightly alkaline pH served to retard spontaneous crystallization of rat hemoglobin. Stroma was removed by centrifugation at 20,000 g for 10 min. Hemoglobin was measured with the Hycel reagent (Hycel, Houston, Tex.). The final hemoglobin solution had a concentration of 30–40 mg/ml and was injected intravenously by tail vein without anesthetizing the animal.

Preparation of Radiolabeled Hemoglobin, Methemalbumin, and Albumin

59Fe-hemoglobin was prepared in rats made anemic by bloodletting and given 59FeCl₃.² The specific activity of the labeled hemoglobin was 0.1 µCi/gm. 59Fe, 125I-hemoglobin was prepared by exposing 59Fe-hemoglobin to 100 µCi of Na125I (New England Nuclear, Boston, Mass.) in the presence of chloramine T.21 Unreacted 125I was removed by dialysis against several liters of isotonic saline in the cold. When 59Fe-heme was split from doubly labeled hemoglobin by acid acetone and crystallized,22 radioactivity due to 125I was undetectable in the recovered heme, which indicated that 125I labels exclusively the globin moiety. 59Fe, 125I-methemalbumin was prepared from crystallized 59Fe-hemin22 and rat albumin (Fraction V, Pentex, Kankakee, Ill.), as described elsewhere.10 The molar ratio of heme to albumin was 1:4. The 59Fe-methemalbumin was labeled with 125I as described above for hemoglobin. After dialysis against isotonic saline, the labeled material was chromatographed on Sephadex G-100 (Pharmacia, Piscataway, N.J.) to eliminate possible protein aggregates and labeled albumin fragments. The peak of radioactivity from the column corresponding to the peak of albumin was identified, and its volume was reduced with Lyphogel (Gelman Instrument, Ann Arbor, Mich.). In some instances, rat albumin alone was labeled with 125I under the conditions outlined above. The homogeneity of this material was evaluated by cellulose acetate electrophoresis, which was carried out at 175 V for 90 min in Tris-barbital buffer, pH 8.6. Of the total radioactivity applied to the strip, 96% migrated with the albumin, with the remaining 4% staying at the origin and possibly representing denatured material. With all labeled proteins, at least 95% of the total radioactivity was precipitable with 10% trichloroacetic acid.

Distribution of Radiolabeled Hemoglobin or Methemalbumin after Intravenous Injection

Rats were sacrificed 10–30 min after intravenous injection of radiolabeled material. After perfusing the liver in situ free of blood, its total radioactivity was estimated from
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the radioactivity of a weighed tissue aliquot. The remainder of the liver was then sub-
jected to Pronase digestion for isolation of the sinusoidal cells.9 The radioactivity con-
tained in the total sinusoidal cells of the whole liver was calculated from the radioactivity in
the sinusoidal cell isolate divided by the fractional recovery of this cell population.9
The total radioactivity in all parenchymal cells of the liver was computed by measuring the
radioactivity in a known number of isolated cells. This figure was then extrapolated to the
total number of hepatic parenchymal cells, based on the assumption that rat liver con-
tains an average of $5 \times 10^8$ parenchymal cells/100 g body weight.4,23 Uptake of labeled
material in the spleen and kidneys was determined without correction for contained blood.
Residual radioactivity remaining in the blood at the time of sacrifice was estimated from
the radioactivity in a blood aliquot extrapolated to the total blood volume.11 All radio-
active measurements were made in a Nuclear-Chicago well-type scintillation counter.
The $^{59}\text{Fe}$, $^{125}\text{I}$ doubly-labeled compounds were measured by gamma spectroscopy. None
of the $^{125}\text{I}$ activity appeared in the $^{59}\text{Fe}$ channel. An appropriate correction was made
for the 5% of the $^{59}\text{Fe}$ activity that appeared in the $^{125}\text{I}$ channel.

Assay of Microsomal Heme Oxygenase

The enzyme assay was carried out as previously described,9,10 except that hepatic
parenchymal cells were disrupted by 15 sec of sonication. Microsomal heme oxygenase
(MHO) activity in isolated sinusoidal cells was expressed as nmoles of bilirubin formed
per minute by the total sinusoidal cells, per 100 g rat weight, the total sinusoidal cells
being calculated as previously described.9 The MHO activity of the whole liver was
taken to reflect the enzyme activity of the parenchymal cells. This appeared justified,
since previous9 and present studies indicated that at least 90% of the enzyme activity of
the liver resides in the parenchymal cells whose total mass is about 20 times larger than
that of the sinusoidal cells.23 In the present experiments, isolated parenchymal cells were
studied in parallel with the whole liver to ensure that the changes in MHO activity
observed in whole liver were ascribable indeed to changes in parenchymal cell activity.

Miscellaneous Procedures

The haptoglobin-binding capacity of rat serum was determined by intravenous injection
of various amounts of hemoglobin to a group of rats and subsequent separation of the
haptoglobin-bound from free hemoglobin by chromatography on Sephadex G-100.24 The
haptoglobin-binding capacity was found to average 1.7 mg hemoglobin/100 g rat weight
(Fig. 1), which is in the range reported in the literature.11,17 Haptoglobin-bound hemo-
globin was quantitated in arbitrary units by integrating the area under the 415 nm peak
in the void volume of the Sephadex column.

Radioautography of sinusoidal cells was carried out as described previously.9 Rats
were given approximately 1.5 mg/100 g of $^{59}\text{Fe}$-hemoglobin, which is within the hapto-
globin-binding capacity of the plasma. After 30 min, the sinusoidal cells were isolated,
spread on glass slides, and stained for peroxidase activity.9 The slides were then coated
with photographic emulsion and exposed for 10 wk.

RESULTS

Hepatic Localization of Intravenously Administered
$^{59}\text{Fe}$-Hemoglobin and $^{59}\text{Fe}$-RBC

In preliminary studies, the half-life of haptoglobin-bound hemoglobin in the
circulation was found to be about 20 min, while that of free hemoglobin
was 10–15 min, as reported previously.2,11 Thirty minutes after the injection
of labeled hemoglobin, 70%–75% of the administered radioactivity had been
cleared from the circulation. When the amount of hemoglobin administered
was within the haptoglobin-binding capacity of rat serum, about two-thirds
of the amount removed from the circulation was recovered in the liver, in
agreement with previous findings. When the liver was fractionated into parenchymal and sinusoidal cells, isolated parenchymal cells contained, on the average, 85%–95% of the total radioactivity of the liver, whereas less than 15% of the total label was localized in the sinusoidal cell population (Fig. 2).

Although previous studies had indicated that intercellular transfer of hemoglobin iron is negligible during the first 30 min after hemoglobin uptake by the liver, specific steps were taken to establish that no label was lost from the sinusoidal cells during the isolation procedure. Accordingly, sinusoidal cells were isolated in the presence of 0.1% formalin, which renders the cells nonviable at the moment of sacrifice of the animal and consequently minimizes the degradation of hemoglobin that could take place during the isolation procedure. This maneuver yielded no increase in the recovery of 59Fe label in the sinusoidal cell preparations. In addition, isolated sinusoidal cells were examined by autoradiography to determine which of these cells contained detectable amounts of the 59Fe tracer. After 10 wk of exposure, the only radioactive label detectable by autoradiography was associated with the occasional hepatic parenchymal cells that contaminated the sinusoidal cell isolates.

When the amount of injected hemoglobin exceeded the haptoglobin-binding capacity of rat plasma, most of the labeled material was sequestered in the kidneys or excreted in the urine. The unbound hemoglobin that escaped renal removal was taken up preferentially by hepatic parenchymal cells to an
extent similar to the uptake of haptoglobin-bound hemoglobin (Fig. 3). By contrast, sequestration of 59Fe-labeled spherocytic RBC was a function solely of the EP subpopulation of the hepatic sinusoidal cells (Fig. 2), as reported previously.9

**Response of Hepatic MHO to Administered Hemoglobin and RBC**

Following intravenous infusion of dissolved hemoglobin, the MHO activity of the liver began to rise within 4 hr and, after 10-12 hr, reached values two to four times above baseline (Fig. 4). This rise in enzyme activity reflected almost entirely MHO activity in hepatic parenchymal cells, as discussed earlier. Sinusoidal cells responded to hemoglobin infusions also with a rise in MHO activity, which, though a constant finding, was always inconsequential compared to the total increase in hepatic MHO activity. In contrast, after infusion of spherocytic RBC containing an equivalent amount of hemoglobin, no increase in hepatic MHO activity was detectable, while the enzyme activity in the isolated sinusoidal cells doubled (Fig. 4). This increase could not be attributed to an influx of MHO-containing mononuclear cells from the circulation into the liver or to particle-mediated hyperplasia of sinusoidal cells,25 because during the time course of these experiments the absolute number of EP cells in the liver computed per 100 g rat weight9 did not differ from that in control animals.

**Hepatic Sequestration of Doubly Labeled Hemoglobin and Methemalbumin**

When hemoglobin, labeled with 59Fe in the heme and with 125I in the globin moiety, was injected in amounts within the haptoglobin-binding capacity of the serum, both labels were taken up initially by the liver in a ratio virtually identical to that of the administered material (Fig. 5). If more

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*Fig. 3. Uptake of dissolved labeled hemoglobin by hepatic sinusoidal cells, whole liver, and spleen. Sinusoidal cells were prepared 30 min after intravenous injection of hemoglobin in amounts within (shaded area) and in excess of haptoglobin-binding capacity of serum. Radioactivity in each sinusoidal cell isolate was corrected for recovery of sinusoidal cells from the whole liver. Results are expressed as mg hemoglobin taken up in 30 min by the respective tissues per 100 g rat weight.*
than 15 min were allowed to elapse between injection of the hemoglobin and sacrifice of the rat, the ratio of 125I activity to 59Fe activity progressively decreased, probably due to the more rapid degradation of the labeled globin with consequent loss of part of the 125I from the liver. Analysis of the sinusoidal cell population showed that it contained 10%-15% of either isotope taken up by the whole liver, regardless of the time that had elapsed between injection and removal of the liver. The similar uptake of the heme and the globin moieties of hemoglobin by the liver indicated that the hemoglobin molecule was sequestered intact by the hepatic parenchymal cells.

In contrast to these findings with hemoglobin, with methemalbumin labeled in the heme with 59Fe and in the albumin with 125I a marked dissociation in the hepatocellular uptake of the two labels was apparent (Fig. 5). While the uptake of labeled heme was at least as rapid as that of hemoglobin, only negligible amounts of the albumin label were found in the liver. As with hemoglobin, the sinusoidal cells exhibited less than 15% of the total 59Fe-heme activity of the whole liver.

**DISCUSSION**

The present findings confirm earlier observations that the sinusoidal cells are the principal site in the liver where intact, damaged erythrocytes are removed and degraded; hepatic parenchymal cells appear to play no role in this process (Fig. 2). For hemoglobin dissolved in plasma, the exact cellular site of removal in the liver has been less well understood. By analogy with the sequestration of intact RBC, it is frequently assumed that soluble hemoglobin also is removed by hepatic sinusoidal cells. Evidence supporting this assumption was the finding that bone marrow macrophages sequester hemoglobin. Direct studies of the site of hepatic hemoglobin uptake, employing labeled haptoglobin complexed with hemoglobin or
histochemical methods, further seemed to suggest a prominent role for hepatic sinusoidal cells in the removal of circulating hemoglobin. On the other hand, in an autoradiographic study Muller-Eberhard et al. noted that injected cyanomethemoglobin was associated primarily with the parenchymal cells of rabbit liver. These studies are difficult to evaluate, because they fail to provide quantitative information about the amount of hemoglobin contained in the different cell types in the liver. Since the sinusoidal cells constitute only 5% of the liver volume, a given amount of intracellular hemoglobin would be relatively concentrated in these cells, while a similar amount of hemoglobin within the parenchymal cells would be distributed in a much larger volume and consequently could escape detection.

The present findings indicate that most of the hemoglobin dissolved in plasma is taken up and degraded in the parenchymal cells of the liver. Over a wide range of plasma hemoglobin concentrations, at least 85% of the pigment taken up by the liver was contained in the parenchymal cells (Figs. 2 and 3). Moreover, this uptake was accompanied by a commensurate stimulation of MHO activity in the parenchymal cells (Fig. 4) that suggests the pigment was not only sequestered but also degraded in these cells. Haptoglobin-bound hemoglobin and free hemoglobin appeared to be handled by the liver in a similar manner, except that a large portion of the free hemoglobin was lost by way of the kidneys.

In comparison to the parenchymal cells, the sinusoidal cells of the liver appeared to play a subordinate role in the removal of plasma hemoglobin in that they contained in all instances less than 15% of the hemoglobin taken up by the whole organ (Fig. 3) and exhibited only a modest stimulation of MHO activity, as compared to the more pronounced increase in enzyme activity in the parenchymal cells (Fig. 4). Though occasional parenchymal cells were found in the sinusoidal cell isolates, their number was insufficient to account for these findings. It is possible that the relatively small amount of plasma hemoglobin taken up by sinusoidal cells of the liver, as well as by the spleen (Fig. 3) and presumably by the bone marrow, reflects continuous pinocytosis of plasma proteins, as has been described for macrophages in vitro. Another possible explanation is that the administered pigment load included a small fraction of denatured, aggregated, or crystalline hemoglobin that, like aggregated albumin, presumably would be removed rapidly by reticuloendothelial macrophages. Despite the precautions taken, this
possibility could not be excluded because rat hemoglobin readily crystallizes in aqueous solutions of physiologic pH.

The observations with doubly labeled hemoglobin indicated that the hemoglobin molecule, when bound to haptoglobin, is sequestered intact by hepatic parenchymal cells (Fig. 5), which suggests that hemoglobin is transported to the liver without the mediation of secondary heme carriers, such as hemopexin. This is in accord with the earlier finding that the binding of hemoglobin to haptoglobin is irreversible and involves dissociation of the hemoglobin molecule into dimers.32 Moreover, Bunn and Jandl33 found that, in vitro, binding to haptoglobin prevented both the exchange of hemoglobin-heme with the heme of unbound hemoglobin and its transfer onto albumin. Thus, despite the reported relatively high affinity of hemopexin for heme,18,30,34,35 hemopexin appears to function as an intermediate carrier for hemoglobin-heme only if haptoglobin is depleted18 and possibly only if methemoglobin is present.27 In marked contrast to these observations were the findings with doubly labeled methemalbumin (Fig. 5) which clearly demonstrated dissociation of the heme from its albumin carrier prior to uptake of the heme by the hepatic parenchymal cells. This dissociation could reflect transfer of the heme from albumin to hemopexin, since, in the rat, hemopexin has a higher affinity for heme than does albumin.18,35 The uptake by the liver of the albumin moiety of doubly labeled methemalbumin was minimal (Fig. 5), being similar in extent to that of labeled albumin without bound heme.

The stimulation of MHO activity in hepatic sinusoidal or parenchymal cells following infusion either of intact RBC or of hemoglobin, respectively, indicated that the heme moiety of the interiorized hemoglobin was degraded at the site of its uptake. The time course of this enzyme stimulation by intravenously administered hemoglobin resembled that reported previously for the kidney.15 In both hepatic parenchymal and sinusoidal cells, peak enzyme activity was reached 8–12 hr after the dose of hemoglobin (Fig. 4), which would be predicted from the similar rates at which labeled soluble hemoglobin and intact RBC are converted to bilirubin in vivo.5 The apparent quantitative discrepancy between net MHO stimulation in the two cell fractions after the administration of hemoglobin or of RBC (Fig. 4) probably reflects primarily the 20-times larger mass of parenchymal cells as compared to sinusoidal cells in rat liver.23

The present findings suggest the hepatic parenchymal cells may play a more immediate role in iron reutilization and transport than has generally been assumed; it was postulated previously that these cells receive iron primarily from transferrin and serve a storage function.37 For example, Lee et al.38 noted that copper-deficient pigs lack the means of mobilizing iron from sites where RBC and hemoglobin are sequestered and catabolized, and consequently iron accumulates in phagocytic cells of the reticuloendothelial system. Unexpectedly, hepatic parenchymal cells were also found to contain increased amounts of iron, which was difficult to ascribe to a mere storage function, since iron absorption and plasma iron concentration were markedly depressed. On the basis of the present observations, it seems likely that the
iron accumulation noted in the hepatic parenchymal cells of copper-deficient pigs reflects sequestration and catabolism of circulating hemoglobin. Similarly, in hemolytic states, accumulation of hemosiderin in hepatic parenchymal cells may be viewed as a direct result of increased levels of circulating hemoglobin, with heme-albumin and heme-hemopexin contributing to a variable extent in chronic hemolysis.38

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Hemoglobin and Erythrocyte Catabolism in Rat Liver: The Separate Roles of Parenchymal and Sinusoidal Cells

D. Montgomery Bissell, Lydia Hammaker and Rudi Schmid