LIVER SINUSOIDAL CELLS

Identification of a Subpopulation for Erythrocyte Catabolism

D. MONTGOMERY BISSELL, LYDIA HAMMAKER, and RUDI SCHMID

From the Department of Medicine, University of California, San Francisco, California 94122

ABSTRACT

Sequestration and degradation of red blood cells (RBC) are believed to occur in part in the liver, but the magnitude and cellular localization of this process remain uncertain. This problem was studied in rats by investigating isolated parenchymal and sinusoidal cell populations of the liver. After digesting the perfused liver with pronase, hepatic sinusoidal cells were isolated free of RBC and debris. Of the isolated cells, 90% were phagocytic, as judged by their uptake of colloidal 198Au or of aggregated albumin-211I administered in vivo. After administration of spherocytic (heat-treated) RBC, however, only about one quarter of the isolated cells were found to contain phagocytized RBC. This apparently distinct population of RBC-phagocytizing cells is designated as "erythrophagocytic (EP)" cells. The EP cell population was further characterized functionally by its specific phagocytosis of colloidal carbon and of 99mtechnetium-sulfur colloid and histochemically by its peroxidase activity. The role of the EP population in the catabolism of RBC-hemoglobin was studied in isolated hepatic sinusoidal cells by assay of microsomal heme oxygenase (MHO), which is the inducible enzyme system that converts heme to bilirubin. The MHO activity of individual sinusoidal isolates was related directly to their content of EP cells. Assay of the MHO activity of the whole spleen and of the total EP cell population of the liver suggested that these two tissues may be of comparable importance in their ability to degrade RBC-hemoglobin.

Senescent red blood cells (RBC) disappear from the circulation of the rat at a rate of about 0.67% per day (2). Sequestration is thought to take place primarily in the spleen, liver, and bone marrow (3-5) by virtue of the relatively large number of reticuloendothelial cells in these tissues. The removal of RBC is believed to occur by a process of phagocytosis followed by intracellular digestion of the red cell matrix and hemoglobin (6). The protein moiety of hemoglobin and the RBC matrix appear to be degraded primarily by acid hydrolases (7), while fission of the protoporphyrin ring of the hemoglobin-heme is catalyzed by microsomal heme oxygenase (MHO). This microsomal enzyme system was recently shown to cleave ferriprotoporphyrin IX at its α-methene bridge and thereby convert the heme to bilirubin IX-α (8, 9). The enzyme is present in relatively high specific activity in spleen, liver, and bone marrow (10) and is inducible by substrate (10-12).

Within the liver, it is believed that RBC are sequestered and degraded by sinusoidal cells with phagocytic properties, frequently referred to as Kupffer cells (5). Recent light and electron microscopy studies (13, 14) suggested the possibility that only a fraction of the total sinusoidal cells may be concerned with RBC phagocytosis. If this concept is correct, the RBC-phagocytizing cell ought to be...
identifiable by its high MHO activity and by morphological evidence of RBC sequestration.

In the present experiments, it was possible to separate hepatic parenchymal and sinusoidal cells into pure and viable fractions and to identify a population of sinusoidal cells that is specifically concerned with RBC phagocytosis. These so-called erythropagocytic (EP) cells differ from other sinusoidal cells in their phagocytic and histochemical properties. Moreover, indirect evidence indicates that EP cells contain most, if not all, of the MHO activity present in isolates of hepatic sinusoidal cells. Part of this work has been presented in abstract form (1).

MATERIALS AND METHODS

Isolation of Cells

SINUSOIDAL CELLS: The procedure of Roser (15) was followed with modifications as noted below. All manipulations with the isolated cells were carried out in polyethylene or siliconized glass, to minimize loss due to adherence of cells to untreated glass surfaces. In fed male Sprague-Dawley rats, 225–400 g, under ether anesthesia, the splenic pedicle was ligated, and a loose silk was placed around the inferior vena cava above the renal veins, which was tied as the perfusion started. The liver was perfused in situ through the portal vein with 0.04% pronase (Calbiochem, Los Angeles, Calif.) dissolved in Eisen's buffer, pH 7.50 (16), by gravity flow from a reservoir 30 cm above the operative field. On perfusion, the liver rapidly blanched. The thoracic cavity was entered, the superior vena cava was briefly clamped to distend the liver and then cut, and the liver was gently massaged to free it from blood. The excised liver was blotted dry, weighed, and minced with scissors. The minced tissue was suspended in 75 ml of the same pronase solution at 34°C and agitated with a magnetic stir-bar at approximately 30°C for 45 min. The pH of the mixture was monitored frequently (Radiometer Co., Copenhagen, Denmark) and adjusted to 7.35 as needed with 0.5 N NaOH. After 15 min of pronase digestion, as the suspension became visibly viscous, 0.5 mg of DNase (type I, Sigma Chemical Co., St. Louis, Mo.) was added, and this was repeated towards the end of the 45 min incubation. At this point, fibrous strands were the only material grossly identifiable in the mixture, but since microscopically the preparation still contained intact hepatic parenchymal cells, the mixture was homogenized briefly (ten strokes) in a standard glass-Teflon Potter-Elvehjem apparatus. This maneuver not only disrupted virtually all remaining parenchymal cells but also yielded an appreciable improvement in the recovery of sinusoidal cells. Isolation of sinusoidal cells from the pronase digest was carried out with centrifugation of the mixture and albumin flotation of the pellet, as described by Roser (15), except that 6 ml of a 21% albumin solution (w/v) was used. The final cell isolate was washed, suspended in serum-free medium 199 (Grand Island Biological Co., Grand Island, N. Y.), and counted in a hemacytometer.

HEPATIC PARENCHYMAL CELLS: The method of Berry and Friend (17) was employed, except that the perfusion medium contained only 0.05% collagenase (Worthington Biochemical Corp., Freehold, N. J.), hyaluronidase being omitted. This modification did not seem to lower appreciably the yield of cells and resulted in preparations with more reproducible microsomal hemoxygenase activity. It was possible to recover a sinusoidal cell isolate from collagenase-dispersed hepatic tissue by combining the supernatant fractions obtained from the initial sedimentation and first wash of the parenchymal cells and adding 0.04% pronase (final concentration). This mixture was stirred for 30 min at 30°C. Disruption of parenchymal cells and isolation of sinusoidal cells then proceeded as above. Since sinusoidal cell fractions prepared in this manner generally contained fewer EP cells than cell isolates prepared solely with pronase, the latter method of preparation was preferred except as noted specifically under Results.

The viability of all cell preparations was ascertained by the exclusion of trypan blue dye in vitro (18). Only cells completely free of dye were considered viable.

Identification of EP Cells in Sinusoidal Cell Isolates

ERYTHROCYTES: Washed normal rat RBC were rendered spherocytic by heating for 1 hr at 45°C (19). Approximately 8 X 10⁹ heated RBC per kilogram of rat weight, suspended in an equal volume of isotonic saline, were injected into a tail vein 2–4 hr before the perfusion of the liver. This interval was chosen because the fraction of cells in the sinusoidal isolate containing RBC was maximal at that time. Sinusoidal cells containing phagocytized RBC were easily recognizable in a hemacytometer chamber under bright-field illumination, and the number of these cells was quantitated and expressed as a percentage of the total number of sinusoidal cells in the isolate.

COLLOIDAL CARBON: "Higgins India Ink" (A. W. Faber-Castell Pencil Co., Inc., Newark, N. J.), 20 mg per kilogram of rat weight, suspended in isotonic saline, was given by tail vein 5 min before perfusion of the liver. With intervals of up to 2 hr between injection of carbon and perfusion of the liver, the results were comparable. Cells in the sinus-
colloidal isotope containing aggregates of cytoplasmic carbon were identified in a hemacytometer and quantitated as above.

Radioabeled Colloid Particles: Colloidal $^{198}$Au (Abbott Laboratories, North Chicago, Ill.), 100 $\mu$Ci, was injected 1 hr before perfusion of the liver, to allow for complete clearing of the colloid from the blood (30); its cellular localization was determined by radioautography (see below).

Aggregated human albumin-$^{198}$I (E. R. Squibb & Sons, New Brunswick, N. J.) of the type used for clinical liver scanning, 10–20 $\mu$Ci, was given intravenously 13 min before perfusion of the liver. In order to minimize the enzymatic degradation of the ingested, labeled albumin during the period required for the sinusoidal cell isolation, with subsequent release of $^{198}$I to the medium (20, 21), neutral buffered formalin was added to the pronase solution in a final concentration of 0.1%. While the formalin reduced the final cell yield by approximately 40%, presumably by a partial inhibition of pronase, the recovery of $^{198}$I in sinusoidal cells, expressed as percentage of whole liver radioactivity, was doubled. The sinusoidal cells containing aggregated albumin-$^{198}$I were identified by radioautographic techniques. Although the formalin treatment killed the sinusoidal cells, they retained sufficient peroxidase activity to remain identifiable.

$^{99m}$Technetium-sulfur colloid ($^{99m}$Tc-S) was prepared daily from $^{99m}$pertechnetate generator eluate (New England Nuclear Corp., Boston, Mass., or Amersham/Scarle, Arlington Heights, Ill.) by the method of Stern et al. (22), with human serum albumin (3.6 mg/ml, final concentration) in place of gelatin. Approximately 20 $\mu$Ci $^{99m}$Tc-S, suspended in 0.5 ml isotonic saline, was given to unanesthetized rats by tail-vein injection 30 min before perfusion of the liver; this time interval was chosen arbitrarily. Longer time intervals between isotope administration and liver perfusion gave quantitatively and qualitatively comparable results.

RBC, labeled with $^{59}$Fe (23), were heated and injected as above; labeled cells, containing 1–3 $\mu$Ci of the isotope and suspended in an equal volume of isotonic saline, were injected 90 min before perfusion of the liver. In some instances, both $^{99m}$Tc-S and $^{59}$Fe-labeled RBC were injected in the same animal 30 min and 90 min, respectively, before perfusion of the liver. Although 90 min is a suboptimal interval for obtaining maximum sequestration of RBC by sinusoidal cells, with $^{59}$Fe-labeled RBC this shorter than usual interval was chosen to minimize potential loss or redistribution of radioactive iron liberated by intracellular breakdown of ingested RBC-hemoglobin.

Quantitation of gamma-emitting isotopes in cell isolates and in whole liver was carried out in a Nuclear-Chicago well-type scintillation counter (Nuclear-Chicago, Des Plaines, Ill.) after administration of the various radioactive particles and sacrifice of the animals at the times indicated, the total uptake of radioactivity in the liver was determined by estimating the isotope content of a small sample of the liver. For each type of radiolabeled particles, appropriate controls were carried out to ascertain that virtually all radioactivity in the liver was contained in sinusoidal cells. In all instances, isolated hepatic parenchymal cells exhibited less than 3% of the total radioactivity in the liver, for this purpose, total hepatic parenchymal cell volume was calculated by the formula of Weibel et al. (24). Moreover, perfusion of the liver in situ for 30 min with hyaluronidase and collagenase, a treatment that digests mainly the intersitial matrix of the liver (17), reduced the total hepatic radioactivity less than 10%, as compared to perfusion with buffer alone. These findings indicated that few, if any, of the administered radioactive particles were taken up by hepatic parenchymal cells or were lodged in the interstitial matrix. The recovery of sinusoidal cells from each liver, therefore, could be calculated by comparing the isotope content of the cell isolate with that of the total radioactivity in the intact liver.

Radioautography: Samples of the sinusoidal isolates were spread on glass slides, allowed to dry in air, fixed with 5% glutaraldehyde, and stained for peroxidase (see below). The stained preparations were washed for 1 hr in 0.05 M Tris-HCl buffer, pH 7.60. Washing appeared to free the slides of unreacted stain, which by its presence caused spurious silver grains to appear in the emulsion used in radioautography. Ilford K-5 nuclear emulsion was applied to the slides (25) and exposed for 18 hr–2 wk at room temperature. The emulsion was processed with Kodak D-19 developer and fixer. Positive cells were identified by a cluster of silver grains overlaying the cell proper and extending in radial tracks into the immediate vicinity of the cell.

Histochemical Identification of Peroxidase: Sinusoidal cell isolates, spread and dried on glass slides, were fixed for 1 min in 5% glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.60, at 4°C and washed at 4°C for 1–3 min in the same buffer containing 5% sucrose. The staining method of Graham and Karnovsky (26) was employed, using 0.1% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) and 0.003% hydrogen peroxide. In general, 2–4 hr of immersion in this solution was adequate for identification of peroxidase-positive cells in the isolates.

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1 We are indebted to the Nuclear Medicine Laboratory, University of California, San Francisco, for a generous supply of this material.
Assay of Microsomal Heme Oxygenase

The sinusoidal isolate, either collagenase- or pronase-prepared, was suspended in 0.1 M potassium phosphate buffer, pH 7.40, at 4°C and sonicated for 30 sec at the lowest output of a Heat Systems Ultrasonic Sonifier, Model W 185 D (Plainview, Long Island, N. Y.). All of the cells were disrupted by this treatment with no measurable loss of MHO activity as compared to similar cell preparations homogenized mechanically. The sonicate was centrifuged at 20,000 g for 10 min, and the enzyme assay was carried out in the supernatant as described previously (8), except that all constituents of the assay system were reduced proportionately to yield a final volume of 0.5 ml. The final assay mixture contained 0.5–2.0 mg protein per ml (27). The MHO activity of whole liver and spleen was measured as previously described (8). Results were expressed as nmoles of bilirubin formed per minute, per 10 mg supernatant protein, except as noted otherwise.

The millimolar extinction coefficient of bilirubin in the various assay systems was determined by the addition of measured amounts of pure bilirubin (Pfanstiehl Labs., Inc., Waukegan, Ill.) bound to human albumin. Bilirubin, dissolved in a small amount of 0.05 N NaOH, was added rapidly to an equimolar amount of 1% human albumin in isotonic saline. Under these conditions, peak absorption was at 468 nm and the millimolar extinction coefficient of the pigment was 59. Various amounts of this solution were added to the supernatant obtained from spleen, whole liver, or sinusoidal cell isolates. In all instances, the extinction coefficient ranged from 58 to 63. In the present study, 60 was chosen as the millimolar extinction coefficient in all enzyme assays.

RESULTS

Isolation of Sinusoidal Cells

The pronase method produced a cell isolate that was essentially free of RBC and debris and in all instances contained less than 1% of intact parenchymal liver cells (Fig. 1). By the trypan blue exclusion method, more than 99% of the
isolated sinusoidal cells appeared to be viable. Further evidence of cell viability was the observation that 15 min after seeding into a tissue culture dish, over 60% of the cells adhered to the plastic surface.

Staining of the sinusoidal cell isolates with hematoxylin and cosin revealed cells of varying size and tinctorial properties. The largest cells, approximately 30 μ in diameter, frequently had a kidney-shaped nucleus and a relatively high ratio of cytoplasm to nucleus, whereas other cells had scant cytoplasm and were the size of small lymphocytes. However, by their staining characteristics, these smaller cells could readily be distinguished from lymphocytes that had been harvested from rat blood, incubated with pronase, and carried through the isolation procedures outlined for the sinusoidal cells. The larger cells accounted for about 15-25% and the smaller cells for 50-70% of the total cell isolate, the remainder being cells of intermediate size. With Oil Red O stain, 3-5% of the cells were seen to contain small lipid bodies, these may represent "fat-storing" cells (13, 14).

After injection of colloidal 198Au or of aggregated albumin-131I, 85-95% of the isolated cells contained radioactivity, as determined by radioautography. It was apparent, therefore, that by these criteria, most of the cells in the isolate were phagocytic. Thus, on morphological and functional grounds, most of the isolated cells appeared to have been derived from the hepatic sinusoids, with minimal, if any, contamination by peripheral lymphocytes. The average yield of sinusoidal cells from a 300 g rat was 1.5 × 10⁶ cells.

The EP Cells

When 8 × 10⁹ heat-treated RBC per kilogram of rat weight were administered intravenously and hepatic sinusoidal cells were harvested 2-4 hr after the infusion, it was observed that only 15-25% of the isolated cells contained detectable RBC. An even smaller percentage of the cells contained RBC when the interval between the erythrocyte infusion and the isolation of the sinusoidal cells was shorter than 2 hr. On the other hand, if this interval was appreciably longer than 4 hr, intracellular degradation of the ingested RBC often had proceeded to a point where morphological recognition of the intracellular RBC became difficult. It was found, however, that regardless of the time interval, the percentage of isolated sinusoidal cells containing RBC could not be increased by infusion of up to four times the standard amount of heat-treated RBC. Thus, the sinusoidal isolate appeared to contain a population of cells that was functionally distinct with respect to RBC phagocytosis.

Though RBC-phagocytizing cells seemed to be a functionally separate population within the sinusoidal cells, the difficulty of accurately quantitating this subgroup using degradable particles such as RBC was apparent. Attempts were made, therefore, to further characterize this population of sinusoidal cells with nondegradable particles and with histochemical methods. It was observed that uptake of carbon particles was confined to a quantitatively similar population of cells. Positive cells exhibited large cytoplasmic aggregates of carbon, while the remainder of the sinusoidal cells showed either no carbon or occasionally a single carbon granule. The proportion of sinusoidal cells ingesting carbon was similar (23 ± 5%, 15 rats) to that ingesting RBC. Moreover, when both substances were given to the same animal, all cells containing RBC also exhibited large cytoplasmic aggregates of carbon (Fig. 2). The reverse correlation was 90-95%, in that a few sinusoidal cells appeared to contain carbon aggregates in which no RBC could be detected. While some of these cells may have ingested carbon particles only, we could not rule out the possibility that the large carbon aggregates obscured ingested RBC or that digestion of the intracellular RBC had progressed to a stage at which the RBC were no longer detectable by morphological means.

When the sinusoidal isolate was stained for peroxidase activity, approximately 30 ± 8% of the cells were found to be positive, confirming earlier observations in rat liver sections by Fahimi (14). Morphologically the peroxidase-positive cells were reasonably uniform in size and generally comprised the larger cells of the isolate. Typical staining was diffusely cytoplasmic, though many positive cells in addition had darkly stained cytoplasmic granules (Fig. 3). Studies with inhibitors of peroxidase served to verify the enzymatic nature of the reaction (14, 34). No peroxidase activity was detectable after fixation of the cells for 2 hr (see Materials and Methods) or in the absence of hydrogen peroxide. Sodium azide (10⁻³ M) or potassium cyanide (10⁻⁴ M) in the reaction medium markedly reduced the intensity of staining, particularly with respect to the diffuse cytoplasmic staining normally observed.

When cell isolates, obtained from rats previously injected with carbon, were stained for peroxidase
activity, it was found that virtually all of the peroxidase-positive cells contained carbon. Moreover, in the sinusoidal isolates of animals injected with RBC, the proportion of peroxidase-positive cells and that of cells containing RBC were identical. Since cells with ingested RBC become peroxidase-positive by virtue of the peroxidase activity of the phagocytized hemoglobin (14), the discriminatory value of the peroxidase stain under these circumstances is limited. However, the finding that in

Figure 2  Hepatic sinusoidal cells isolated by the direct pronase method from a rat pretreated with carbon particles and spherocytic RBC. The larger cells are distended with ingested RBC, which on direct inspection under bright-field illumination are easily recognizable by the red color of their hemoglobin; the larger cells also contain carbon. The remaining smaller cells contain neither carbon nor RBC. × 440.

Figure 3  Hepatic sinusoidal cells, isolated by the direct pronase method and stained for peroxidase, without counterstain. The larger, darker cells are peroxidase positive and are easily distinguished from the smaller unstained sinusoidal cells. The preparation is free of erythrocytes. × 440.
normal rats and in animals injected with RBC a similar proportion of sinusoidal cells was peroxidase-positive indicated that it was the cell population with endogenous, enzymatic peroxidase activity (14) that was involved in RBC phagocytosis.

The peroxidase-positive population of sinusoidal cells was found to be responsible also for the hepatic uptake of $^{99}$Tc-S. On radioautography, $^{99}$Tc-S radioactivity was demonstrated exclusively in peroxidase-positive cells (Fig. 4). Moreover, in the sinusoidal isolates, recovery of the radioactive tracer was found to reflect closely the number of peroxidase-positive cells (correlation coefficient,
$r = 0.789$) (Fig. 5), whereas correlation with the total number of sinusoidal cells in the isolate was relatively poor ($r = 0.372$). Finally, after injection of $^{59}$Fe-labeled RBC and of $^{99}$Tc-S to the same animal, recovery of the two isotopes in the sinusoidal cell isolate, expressed as a fraction of the total $^{59}$Fe or $^{99}$Tc-S activity in the intact liver, was similar despite variable cell yields from animal to animal. These data provided, therefore, additional evidence that $^{99}$Tc-S and RBC are sequestered by the same sinusoidal cell.

Thus, the population of sinusoidal cells concerned with erythrophagocytosis appeared to be characterized by three additional properties, namely, histochemically demonstrable peroxidase activity, phagocytosis of carbon particles and uptake of $^{99m}$technetium-sulfur colloid.

QUANTITATION OF THE EP CELL FRACTION.

These observations permitted estimation of the relative proportion of EP cells in individual sinusoidal isolates and calculation of the absolute number of these cells in the whole liver. The use of $^{99}$Tc-S was found to be the most convenient way of estimating the per cent recovery of EP cells from whole liver, because its gamma emission can easily be measured. For similar reasons, the percentage of EP cells in individual isolates was estimated by means of the peroxidase reaction, rather than by radioautography after $^{99}$Tc-S injection. On the basis of these parameters, the absolute number of EP cells in whole liver was calculated as follows:

1. \[
\% \text{ recovery of EP cells} = \frac{\text{cpm} \ ^{99}\text{Tc-S in isolate}}{\text{cpm} \ ^{99}\text{Tc-S in whole liver}} \times 100
\]

2. \[
\text{Total EP cells in whole liver} = \text{total cells in isolate} \times \% \text{ peroxidase-positive cells in isolate} \div \% \text{ recovery of EP cells}
\]

3. \[
\text{Total EP cells in whole liver/100 g rat} = \frac{\text{total EP cells in whole liver} \times 10^6}{\text{rat weight in g}}
\]

Results for a large series of individual experiments are given in Table I. Since in rats of this size, liver weight was shown to be proportional to body weight (28), absolute numbers of EP cells are expressed per 100 g rat weight. The liver weight itself was deemed not to be a suitable reference base inasmuch as the liver was distended to a variable degree by the perfusion with pronase solution.

COLLAGENASE-PREPARED CELL ISOLATES:
The collagenase method was utilized primarily for the preparation of parenchymal cell fractions, and hepatocytes isolated in this manner had the morphological appearance and viability reported by Berry and Friend (17). When sinusoidal cells were recovered from these preparations, the total number of cells obtained was equal to, or greater than, the number of cells procured by the direct pronase method. However, sinusoidal cell isolates prepared by the collagenase method differed significantly in composition from those obtained by the direct pronase method, in that they exhibited only 1/5-1/4 of the $^{99}$Tc-S recovery of the latter. Since this finding suggested that the isolates prepared with collagenase may be relatively poor in EP cells, the percentage of cells was determined that took up carbon particles or gave a positive peroxidase reaction. In all instances, the three parameters ($^{99}$Tc-S uptake, carbon phagocytosis, and peroxidase reaction) yielded similarly low proportions of EP cells in individual collagenase-prepared cell isolates (Table II, column 3). Because of their lower EP cell content, these preparations thus were less suitable for study of microsomal heme oxygenase activity than were pronase-prepared isolates.

MICROSOMAL HEME OXYGENASE: The specific activity of MHO in the supernatant of entire sinusoidal isolates exhibited a considerable range.

### Table I

**Characterization of Sinusoidal Cells Isolated from Rat Liver by the Pronase Method**

| Characteristic                        | Range* | Mean* ± sd |
|--------------------------------------|--------|------------|
| Total isolated sinusoidal cells X 10^6, per 100 g rat weight | 23-129 | 53 ± 23    |
| % EP cells (peroxidase method) in sinusoidal isolates | 13-40 | 30 ± 8     |
| % recovery of EP cells ($^{99}$Tc-S method) from the liver | 5-25 | 15 ± 6     |
| Calculated number of EP cells X 10^6 in liver, per 100 g rat | 62-154 | 104 ± 24   |

* 36 animals.  † Equation (1) in Results.  § Equations (2) and (3) in Results.
Table II

Microsomal Heme Oxygenase Activity in Sinusoidal Isolates and in EP Cells

| Method of isolate preparation | No of experiments | % EP cells in isolate (peroxidase method) | MHO* of isolate per 10 mg supernatant protein | MHO* of isolate per 10⁶ EP cells |
|------------------------------|-------------------|------------------------------------------|-----------------------------------------------|---------------------------------|
|                              | ± 10             | ± 10                                      | ± 10                                          | ± 10                                       |
| Collagenase                  | 5                | 11 ± 1                                    | 0.07 ± 0.05                                   | 0.32 ± 0.15                               |
| Pronase                      | 17               | 31 ± 7                                    | 0.26 ± 0.05                                   | 0.39 ± 0.08                               |
|                              | p < 0.01         | p < 0.01                                  | p = 0.2                                       | p = 0.2                                   |

*MHO*, microsomal heme oxygenase activity in the 20,000 g supernatant fraction of sonicated sinusoidal cells, as moles of bilirubin formed per minute, under standard conditions (8).

Figure 6. Comparison of microsomal heme oxygenase activity of sinusoidal isolates with the per cent fraction of EP cells in the isolates. The sinusoidal isolates were prepared from normal rats, and the EP cells were identified by their positive peroxidase reaction. The line was drawn by the method of least squares, showing a high degree of correlation (r = 0.833).

Discussion

Although extensive previous observations indicated that RBC are phagocytized by sinusoidal cells of the liver (5, 6, 13), the present findings indicate that this function is restricted to a population of these cells, comprising approximately 30% of all hepatic sinusoidal cells. Moreover, the same population of cells appears to be concerned with the phagocytosis of colloidal carbon and ⁹⁹Tc-S particles and to be identifiable by a positive peroxidase reaction. The finding that in a large number of rats the liver contained a relatively constant fraction of sinusoidal cells exhibiting these characteristics suggests that these cells represent a distinct and separate population of the hepatic sinusoidal cells. This concept is in agreement with the results of recent electron microscope studies on...
These observations suggest that the sinusoidal cells of the liver are functionally heterogeneous. While this is not a novel concept, the evidence from morphological studies has been conflicting (13, 14, 29). Particle size may be an important determinant in the apparent functional specificity of the EP cell. In addition to RBC, carbon and 99Tc-S particles were phagocytized by the EP cell population only, whereas colloidal 198Au and aggregated albumin-131I were ingested by virtually all cells of the sinusoidal isolate. The particle size of the latter two colloids, as used in this study (21, 30), was in the 5-50 nm range. In contrast, 99Tc-S, as prepared here (22) and in a commercial kit (31), was a suspension of particles in the 800-1500 nm range, which approaches the size of an average erythrocyte (6-8 μm). The particle size of the colloidal carbon was similar to that of 99Tc-S, as judged by the fact that 90% of the carbon particles of a dilute suspension were retained by a Millipore filter of 450 nm pore size. Thus, the particles that were ingested by EP cells were 10- to 100-fold larger than those phagocytized by all sinusoidal cells. In a very recent publication, Widmann et al. (32) presented similar observations on the phagocytosis of carbon and other large particles by peroxidase-negative cells in the hepatic sinusoids. In addition, these investigators observed by electron microscopy very small amounts of carbon also in peroxidase-negative cells. In the present study by light microscopy, this was rarely observed, probably due to differences in resolving power. In analogy to the uptake of other small particles (colloidal gold and aggregated albumin) by peroxidase-negative cells, one may anticipate that small carbon particles will appear in these cells.

Although the peroxidase reaction paralleled the uptake of carbon and 99Tc-S particles as a cell marker for EP cells, the physiological significance of this enzymatic activity is not clear. The peroxidase activity of EP cells is located largely in the cisternae of the endoplasmic reticulum and appears to be an intrinsic component of this organelle (14). The sensitivity of this enzyme activity to extrinsic inhibitors distinguishes it from the peroxidatic activity of hemoglobin (14). It is unknown whether the peroxidase of the EP cells participates in the catabolism of heme (11) or may play a bactericidal role, as has been postulated for the peroxidase of polymorphonuclear leukocytes (33). The present findings, together with the observation that peritoneal macrophages of the guinea pig contain peroxidase activity (34), indicate that the previous findings that macrophages generally lack this enzyme activity require revision (35). The discrepancy between present and earlier results may be ascribed largely to differences in histochemical technique.

Since the fractional recovery of sinusoidal cells from the liver and the number of EP cells in the isolate could be determined, it was possible to estimate the total number of sinusoidal and of EP cells in the liver per 100 g rat weight. A prerequisite for this calculation is the assumption that the ratio of EP cells to total sinusoidal cells in an individual cell isolate reflects the respective ratio in the intact liver; this assumption seems reasonable in view of Fahimi’s finding (14) that the hepatic sinusoids in rat liver sections contain the same proportion (25-40%) of peroxidase-positive cells as do the sinusoidal isolates of the present study. Thus, if rat liver has an average of 1 × 10⁸ EP cells per 100 g rat weight and if these represent 30% of the total sinusoidal cells, then the absolute number of sinusoidal cells is 3.3 × 10⁹ per 100 g rat weight. This figure is in good agreement with those of other studies, carried out by different methods. In a morphometric study of liver sections, Weibel et al. (24) estimated 3.09 × 10⁹ sinusoidal cells per 100 g rat weight, and Jandl et al. (19), using chemical measurement of hepatic DNA, calculated 1.18 × 10⁹ sinusoidal cells per 400 g rat, which corresponds to 3 × 10⁸ per 100 g.

Though the present experiments demonstrated phagocytosis of exogenously administered spherocytic RBC by hepatic EP cells, this did not necessarily permit the inference that EP cells function in this manner in normal rats under physiological conditions. A possible approach to this problem was the measurement of MHO activity in EP cells. If these cells normally play a role in the removal and digestion of senescent erythrocytes, they should exhibit intrinsic MHO activity, in contradistinction to other phagocytic cells, such as peritoneal...
macrophages, which are not in direct contact with erythrocytes and consequently contain very low native MHO activity (11). This was indeed observed, in that EP cells isolated from untreated rats contained relatively high levels of MHO activity (Table II). The presence of this enzyme activity implies that EP cells possess an operational mechanism for the conversion of heme to bilirubin. It could be argued that in addition to the heme of ingested RBC, this enzyme system could also be stimulated by the turnover of hemoproteins intrinsic to the EP cells. However, in contrast to hepatic parenchymal cells, sinusoidal cells exhibit relatively little smooth endoplasmic reticulum, which is the cell organelle containing most of the intracellular hemoproteins (36). Plasma hemoglobin is another potential substrate for the MHO activity of EP cells. Data to be presented elsewhere, however, suggest that free hemoglobin and haptoglobin-bound hemoglobin are taken up and degraded almost entirely by hepatic parenchymal cells. Furthermore, an infusion of dissolved hemoglobin stimulates MHO largely in hepatic parenchymal cells, whereas infused spherocytic RBC stimulate MHO only in sinusoidal cells. It is likely, therefore, that the native MHO activity of EP cells reflects continuous low-grade catabolism of the hemoglobin of ingested RBC.

The total MHO activity of the EP cells in the liver of untreated rats is approximately 0.4 nmoles bilirubin formed per minute per 100 g rat weight. This is about 12% of the total MHO activity of the liver, as calculated from assay of the enzyme activity in a 20,000 g supernatant fraction of whole liver homogenates (8). If results obtained with enzyme assay in vitro may be extrapolated to the in vivo situation, this figure indicates that hepatic parenchymal cells possess the bulk of the MHO activity of the whole organ. This enzyme activity presumably is related to turnover of the heme-containing enzymes in the parenchymal cells (36, 37), and, in addition, may reflect degradation of hemoglobin that has gained access to the parenchymal cells from the plasma.8

In a variety of red cell disorders in man (4, 38, 39) and in experimental conditions in the rat (19, 39, 40), the spleen is considered the principal site of RBC sequestration. Under physiological conditions, on the other hand, it has been difficult to evaluate the role of the spleen in this process, relative to the importance of the sinusoidal cells of the liver and the mesenchymal phagocytes of the bone marrow (41). Since the total MHO activity of the EP cell population of the liver was found to be approximately 75% of the total enzyme activity in the spleen, it is possible that the hepatic EP cells play a role that is quantitatively comparable to that of the spleen in the removal and breakdown of senescent RBC.

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REFERENCES

1. Bissell, D. M., L. Hammaker, and R. Schmid. 1971. Cellular sites of erythrocyte and hemoglobin catabolism in the liver. Blood 38:789.
2. Landaw, S. A., and H. S. Windish. 1970. Endogenous production of 14CO a method for calculation of RBC lifespan in vivo. Blood 36:562.
3. Wintrobe, M. M. 1967. Clinical Hematology. Lea & Febiger, Philadelphia 6th edition 166.
4. Jandl, J. H., A. R. Jones, and W. B. Castle. 1957. The destruction of red cells by antibodies in man I Observations on the sequestration and lysis of red cells altered by immune mechanisms. J Clin Invest. 36:1418.
5. Jandl, J. H. 1967. The spleen and reticuloendothelial system. In Pathologic Physiology: Mechanisms of Disease W. A. Sodeman and W. A. Sodeman, Jr., editors. W. B. Saunders Company, Philadelphia 4th edition. 897.
6. Harris, J. W., and R. W. Kellermayer. 1970. The Red Cell. Harvard University Press, Cambridge, Massachusetts Revised edition 526.
7. Axline, S. G., and Z. A. Cohn. 1970. In vitro induction of lysosomal enzymes by phagocytosis. J Exp Med 131:1239.
8. Tenhunen, R., H. S. Marver, and R. Schmid. 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc. Nat. Acad. Sci. U. S. A. 61:748.
9. Tenhunen, R., H. S. Marver, and R. Schmid. 1969. Microsomal heme oxygenase. Characterization of the enzyme. J Biol. Chem. 244: 6386.
10. Tenhunen, R., H. S. Marver, and R. Schmid. 1970. The enzymatic catabolism of hemo-

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8 Bissell, D. M., L. Hammaker, and R. Schmid. In preparation.
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globin stimulation of microsomal heme oxygenase by hemin. J. Lab. Clin. Med. 75:410.
11. PIMSTONE, N. R., R. TENHUNEN, P. T. SEITZ, H. S. MARVER, and R. SCHMID. 1971. The enzymatic degradation of hemoglobin to bile pigments by macrophages. J. Exp. Med. 133:1264.
12. PIMSTONE, N. R., P. ENGEL, R. TENHUNEN, P. T. SEITZ, H. S. MARVER, and R. SCHMID. 1971. Inducible heme oxygenase in the kidney: a model for the homeostatic control of hemoglobin catabolism. J. Clin. Invest. 50:2042.
13. ATERMAN, K. 1963. The structure of the liver sinusoids and the sinusoidal cells. In The Liver. Ch. Rouiller, editor. Academic Press Inc., New York. 1:251.
14. FAHMI, H. D. 1970. The fine structural localization of endogenous and exogenous peroxidase activity in Kupffer cells of rat liver. J. Cell Biol. 47:247.
15. ROSER, B. 1968. The distribution of intravenously injected Kupffer cells in the mouse. J. Reticuloendothel Soc. 5:453.
16. KERN, M., and H. N. ESERN. 1959. The effect of antigenic stimulation on incorporation of phosphate and methionine into proteins of isolated lymph node cells. J. Exp. Med. 110:207.
17. BERRY, M. N., and D. S. FRIEND. 1969. High yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. J. Cell Biol. 43:506.
18. GIRARDI, A. J., H. McMICHAEL, JR., and W. HENLE. 1956. The use of HeLa cells in suspension for the quantitative study of virus propagation. Virology. 2:532.
19. JANDL, J. H., N. M. FILES, S. B. BARNETT, and R. A. MACDONALD. 1966. Proliferative response of the spleen and liver to hemolysis. J. Exp. Med. 122:209.
20. EIKENREICH, B. A., and Z. A. Cohn. 1967. The uptake and digestion of iodinated human serum albumin by macrophages in vitro. J. Exp. Med. 126:941.
21. PALMER, D. L., D. RIFKIN, and D. W. BROWN. 1971. 3H-labelled colloidal human serum albumin in the study of reticuloendothelial system function. II. Phagocytosis and catabolism of a test colloid in normal subjects. J. Infect. Dis. 123:437.
22. STERN, H. S., J. G. McAFREE, and G. SCHRUMAN. 1956. Preparation, distribution and utilization of technetium 99m-sulfur colloid. J. Nucl. Med. 7:665.
23. OSTROW, J. D., J. H. JANDL, and R. SCHMID. 1962. The formation of bilirubin from hemoglobin in vivo. J. Clin. Invest. 41:1628.
24. WEIR, E. R., W. STAUBLI, H. R. GNAGI, and F. A. HESS. 1969. Correlated morphometric and biochemical studies on the liver cell. I. Morphometric model, stereologic methods, and normal morphometric data for rat liver. J. Cell Biol. 42:68.
25. CARO, L. G., and R. P. VAN TUREGGEN. 1962. High resolution autoradiography. I. Methods. J. Cell Biol. 15:173.
26. GRAHAM, R. C., Jr., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14:291.
27. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARRE, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.
28. ADDIS, T., and H. GRAY. 1950. Body size and organ weight. Growth. 14:49.
29. WISE, E., and W. TH. DAREM. 1970. Fine structural study on the sinusoidal lining cells of rat liver. In Mononuclear Phagocytes. R. van Furth, editor. F. A. Davis Co., Philadelphia. 200.
30. Abbott Laboratories, North Chicago, Illinois. Literature accompanying Autrasean-198®.
31. SACERBRUNN, B. J. L., C. TOCKE, and W. EDWARDS. 1970. Evaluation of commercial 99mTc-sulfur colloid kit. J. Nucl. Med. 11:399.
32. WIDMANN, J. J., R. S. COTRAN, and H. D. FAREM. 1972. Mononuclear phagocytes (Kupffer cells) and endothelial cells. Identification of two functional cell types in rat liver sinusoids by endogenous peroxidase activity. J. Cell Biol. 52:159.
33. PENCS, S. H., and J. S. KLEBANOFF. 1971. Quantitative leukocyte iodination. New Engl. J. Med. 284:744.
34. COTRAN, R. S., and M. LEY. 1970. Ultrastructural localization of horseradish peroxidase and endogenous peroxidase activity in guinea pig peritoneal macrophages. J. Immunol. 105:1396.
35. VAN FURTH, R., J. G. HIRSCH, and M. E. FEDORKO. 1970. Morphology and peroxidase cytochemistry of mouse mononuclear, monocytes and macrophages. J. Exp. Med. 132:794.
36. MARVER, H. S., and R. SCHMID. 1972. The porphyrias. In The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. R. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Company, New York. 3rd edition.
37. BAKKEN, A. F., M. M. TULLER, and R. SCHMID. 1972. Metabolic regulation of heme catabolism and bilirubin production. Hormonal control of hepatic heme oxygenase activity. J. Clin. Invest. 51:530.
38 Harris, I. M., J. M. McAlister, and T. A. J. Prankerd 1957. The relationship of abnormal red cells to the normal spleen. Clin. Sci. (London). 16:223
39. Jandl, J. H., and M. E. Kaplan 1960 The destruction of red cells by antibodies in man. III. Quantitative factors influencing the patterns of hemolysis in vivo. J. Clin. Invest. 39:1145.
40. Kaplan, M. E., and J. H. Jandl. 1961. Inhibition of red cell sequestration by cortisone. J. Exp. Med. 114:921.
41. Keene, W. R., and J. H. Jandl. 1965 Studies of the reticuloendothelial mass and sequestering function of rat bone marrow. Blood. 26:137.
42. Stieffel, C., D. Mouton, and G. Bozzi. 1970. Kinetics of the phagocytic function of reticuloendothelial macrophages in vivo. In Mononuclear Phagocytes. R. van Furth, editor F. A. Davis Co, Philadelphia. 336.