Retinoids, retinoid-binding proteins, and retinyl palmitate hydrolase distributions in different types of rat liver cells

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Abstract A study was conducted to determine the levels and distributions of retinoids, retinol-binding protein (RBP), retinyl palmitate hydrolase (RPH), cellular retinol-binding protein (CRBP), and cellular retinoic acid-binding protein (CRABP) in different types of isolated liver cells. Highly purified fractions of parenchymal, fat-storing (stellate), endothelial, and Kupffer cells were isolated in high yield from rat livers. The retinoid content of each fraction was measured by HPLC analysis. RBP, CRBP, and CRABP were measured by sensitive and specific radioimmunoassays, and RPH activity was measured by a sensitive microassay. The concentrations of each parameter expressed per 10^6 parenchymal or fat-storing cells were, respectively: retinoids, 1.5 and 83.9 μg of retinol equivalents; RBP, 138 and 7.4 ng; RPH, 826 and 1152 pmol FFA formed hr^{-1}; CRBP, 470 and 236 ng; and CRABP, 5.6 and 8.7 ng. When these data were expressed on the basis of per unit mass of cellular protein, the concentrations of RPH, CRBP, and CRABP in the fat-storing cells, which contain 10-fold less protein than the large parenchymal cells, were seen to be greatly enriched over parenchymal cells. The parenchymal cells contained approximately 9% of the total retinoids, 98% of the total RBP, 90% of the total RPH activity, 91% of the total CRBP, and 71% of the total CRABP found in the liver. The fat-storing cells accounted for approximately 88% of the total retinoids, 0.7% of the total RBP, 10% of the RPH activity, 8% of the total CRBP, and 21% of the CRABP in the liver. The endothelial and Kupffer cell fractions contained very low levels of all of these parameters. Thus, the large and abundant parenchymal cells account for >70% of the liver's RBP, RPH, CRBP, and CRABP; but the much smaller and less abundant fat-storing cells contain the majority of hepatic retinoids and greatly enriched concentrations of RPH, CRBP, and CRABP. — **Blaner, W. S., H. F. J. Hendriks, A. Brouwer, A. M. de Leeuw, D. L. Knook, and D. S. Goodman.** Retinoids, retinoid-binding proteins, and retinyl palmitate hydrolase distributions in different types of rat liver cells. *J. Lipid Res.* 1985. **26:** 1241–1251.

Supplementary key words fat-storing cells • parenchymal cells • endothelial cells • Kupffer cells

The liver plays a central role in the uptake, storage, and mobilization of retinol (vitamin A) in the body. The metabolism of retinoids in the liver, where over 95% of the body's retinoid reserves are found (1, 2), is both complex and highly regulated. The liver takes up dietary retinoid as chylomicron retinyl ester (3–5); hydrolases convert the retinyl ester to retinol (2, 6), which subsequently is reesterified to retinyl esters and stored in hepatic lipid droplets (2, 3, 7–9). When retinoid is required to meet body needs, the stored retinyl ester is enzymatically hydrolyzed by retinyl palmitate hydrolase (RPH) to free retinol (10–12). In a highly regulated process, the retinol is secreted into the circulation bound to retinol-binding protein (RBP) (13, 14).

The liver comprises several different types of cells, including the hepatic parenchymal cells (hepatocytes), the sinusoidal cells, and epithelial cells that line the bile ductules and ducts. The nonparenchymal sinusoidal and perisinusoidal cells include the Kupffer cells, endothelial cells, and the fat-storing cells (which are also known in the literature as perisinusoidal stellate cells (15), lipocytes (16), vitamin A-storing cells (17), and Ito cells (18)). The parenchymal cells are large and abundant, composing much of the liver in terms of cell number, cell volume, and cellular protein. The sinusoidal cells account for about 30% of the total number of cells in the liver but they constitute <10% of hepatic protein, because they are much smaller than the parenchymal cells (19).

The cellular localization of the processes involved in retinoid uptake, storage, and mobilization from the liver has been a matter of much interest and some controversy. The parenchymal cells of the liver are known to be primarily involved in chylomicron remnant uptake (20, 21), to be the site of localization and synthesis of RBP (13, 14, 22), and probably to store some retinyl ester (23–25).

Abbreviations: CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; FFA, free fatty acids; GBSS, Gey's balanced salt solution; HPLC, high performance liquid chromatography; RBP, retinol-binding protein; RPH, retinyl palmitate hydrolase.

Among the nonparenchymal cell types both the Kupffer (26) and fat-storing cells (see (15) for review) have been suggested to be important in retinoid storage and metabolism. However, convincing evidence now exists that Kupffer cells are not involved in these processes (27–29). In contrast, a growing body of evidence exists which suggests that the fat-storing (stellate) cells play an important role in hepatic retinoid storage and metabolism. Retinoid, mainly in the form of retinyl ester, has been localized in the fat-storing cells by fluorescence (15, 30) and electron microscopy (31). High levels of retinoid have been found in isolated populations of these cells by Knook and coworkers (32–35), who have reported that these cells contain the majority of the retinoid found in liver. Evidence for the transfer of retinoid, after its hepatic uptake, from parenchymal to fat-storing cells has been reported by Blomhoff et al. (21, 36). Additionally, cellular retinol-binding protein (CRBP), a protein thought to be important in the intracellular transport of retinol between the sites of these metabolic events (2), has been shown to be highly localized in the fat-storing cells by immunocytochemical studies (22, 37).

We now report the results of a study designed to determine the localization and concentrations of retinoids and of several important retinoid-binding proteins and a metabolizing enzyme activity, namely RBP, RPH, CRBP, and cellular retinoic acid-binding protein (CRABP) in highly purified populations of parenchymal, fat-storing, endothelial, and Kupffer cells isolated from 12-month-old female BN/BiRij rats. The underlying aim of this study was to extend the knowledge available about the involvement of each liver cell type in retinoid storage and metabolism.

MATERIALS AND METHODS

Animals and diet

All liver cell isolations were carried out using healthy 12-month-old female BN/BiRij rats. The rats had mean body weights of 180 ± 10 g. All rats were maintained on standard laboratory chow (Diet AM II, Hope Farms, Woerden, The Netherlands). This diet contained 5.5 mg of retinyl acetate per kg diet. The total dietary protein consisted of 95% animal-derived protein that contained no vitamin A. The remaining protein in the diet was derived from alfalfa (3%) and dried grasses (2%). These plant proteins contributed approximately 1.5 mg of mixed carotenoids per kg of diet. Each rat consumed about 15 g of the diet per day. These animals were obtained from the aging colonies in Rijswijk and were maintained under clean conventional conditions (38).

Cell isolations

Parenchymal and nonparenchymal cell preparations were isolated (in Rijswijk) from different animals. The three nonparenchymal cell types (fat-storing, endothelial, and Kupffer cells) were isolated from the same livers.

Nonparenchymal cell suspensions were prepared essentially as described previously (35). Briefly, livers were perfused through the portal vein in situ with Gey's balanced salt solution (GBSS) at 37°C for 6 min at a constant flow rate of 10 ml/min, followed by perfusion for 6 min with 0.2% pronase E (Merck, Darmstadt, West Germany) in GBSS. The liver was carefully excised, connected to a circulating perfusion system, and perfused with 0.05% pronase E and 0.05% collagenase type I (Sigma, St. Louis, MO) in GBSS for 30 min at 37°C with a flow rate of 10 ml/min. The liver capsule was removed and the partially digested contents were incubated for 30 min at 37°C in 100 ml of GBSS containing 0.02% pronase E and 0.05% collagenase. The pH of the medium was monitored throughout the incubation and maintained at pH 7.4 with 1 N NaOH. To separate fat-storing from endothelial and Kupffer cells, the nonparenchymal cell suspension was centrifuged on a discontinuous two-layer Nycodenz (a registered trademark of Nyegaard and Co., Oslo, Norway) density gradient. The cell suspension was mixed with a Nycodenz stock solution of 28.7% (w/v) to yield a final concentration of 11.5% (w/v) Nycodenz in GBSS. This suspension was underlayered by a solution of 17.2% (w/v) Nycodenz in GBSS and covered with 1 ml of GBSS. After centrifugation at 1400 *g* for 17 min at 4°C, the top of the 11.5% Nycodenz layer was highly enriched with fat-storing cells and the top of the 17.2% Nycodenz layer with endothelial and Kupffer cells, as described elsewhere (35). The Kupffer and endothelial cells were separated by centrifugal elutriation in a J-21 Beckman centrifuge equipped with a JE-6 elutriator rotor and a Sanderson separation chamber (Beckman Instruments, Palo Alto, CA), as has been described previously (32).

Parenchymal cells were isolated by two different procedures. The first isolation procedure was carried out at 8°C in the absence of Ca^{2+} and dissociating enzymes as has been previously described (35). The second procedure involves perfusion of the liver in situ at 37°C with Ca^{2+}-free medium followed by enzymatic digestion in a medium containing collagenase, as has been previously described (39, 40). Parenchymal cells isolated at 8°C and 37°C were further purified by centrifugal elutriation as described previously (41).

The purity of each cell fraction from every isolation was assessed by both light and electron microscopy in Rijswijk as has been described previously (34). Protein content of each cell fraction from every isolation was estimated by the method of Lowry et al. (42).

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Electron microscopy

Cell samples were fixed by adding samples of at least 10^6 cells in GBSS to an excess of 2% glutaraldehyde in 0.15 M Na cacodylate buffer (pH 7.4) for 15 min, followed by post-fixation in 1% OsO_4 in the same buffer for 20 min, both at 4°C. After the first dehydration step in 70% ethanol, aliquots of the cell suspensions were put into microfuge tubes and pelleted at 7969 *g* for 5 min in a Microfuge B (Beckman Instruments, Palo Alto, CA). The cell pellets (∅ 1 mm) were further dehydrated in a graded ethanol series and embedded in LX 112 (Ladd Research Industry Inc., Burlington, VT). Ultrathin sections were mounted on uncoated copper grids, contrasted with uranyl acetate and lead citrate, and examined under an EM 410 electron microscope (Philips, Eindhoven, The Netherlands).

Retinoid analysis

Samples of minced whole liver, perfused with GBSS for 6 min, or of purified liver cell suspensions were lyophilized and retinoids were extracted overnight at 4°C with methanol–diisopropyl ether 1:2 (v/v) containing internal standard retinyl acetate, 50 mM EDTA, and 50 μg/ml butylhydroxytoluene. After the overnight extraction, 5% H_2O (v/v) was added to yield a H_2O concentration equal to the HPLC eluents. Extracts were filtered through Millex-HV_4 0.45 μM filters (Millipore, Yonezawa, Japan). Retinoids were determined by reverse phase high performance liquid chromatography (HPLC) using a Radial-PAK C_{18} analytical column (Waters Associates, Milford, MA) as has been described previously (34). All-*trans* retinol, retinyl palmitate, retinyl stearate, and retinyl oleate were identified using authentic samples of each compound as standards; and quantitated using retinyl acetate as an internal standard, as has been described elsewhere (35).

Shipment and storage of samples

Duplicate samples of each liver mince and each liver cell preparation were sent on Dry Ice from Rijswijk to New York by overnight air freight. Cell preparations were shipped either as pellets or suspended in a small volume of GBSS. All samples arrived in the frozen state. In New York the preparations were assayed for RBP, RPH activity, CRBP, and CRABP. After arrival in New York the cell fraction preparations were stored at −20°C prior to assay. The samples were assayed within 2 weeks after arrival.

Assay of RBP

For RBP analysis, one of the duplicate samples of each cell preparation or liver mince was thawed and suspended in a small volume of 10 mM Tris HCl, pH 7.0, so that the final concentration of cells was greater than 2 × 10^6 cells per ml. Each cell suspension was homogenized for 1 min at setting 6 with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). RBP levels in the cell homogenates were determined by a sensitive and specific radioimmunoassay (43, 44), using an LKB-Wallace RiaGamma 1274 counter (LKB Instruments, Gaithersburg, MD).

Assay for RPH

RPH activity levels were determined with aliquots taken from the homogenates made for RBP determinations. RPH activity was measured using a sensitive microassay that measures the formation of [1-^{14}C]palmitate from retinyl [1-^{14}C]palmitate in 50 mM Tris-maleate, pH 8.0, containing 0.75% sodium cholate, as has been described previously (11).

Assays for CRBP and CRABP

CRBP and CRABP were measured by specific radioimmunoassays. These assays were developed using antibodies raised in turkeys, as described by Adachi et al. (45), and using CRBP and CRABP isolated from rat testis homogenates. Purified monospecific IgG against CRBP and against CRABP were each obtained by immunosorbent affinity chromatography of the whole turkey IgG fractions with CRBP or with CRABP linked to Sepharose, respectively (46, 47). The use of such purified antibodies and other modifications (46, 47) increased the sensitivity of the assays by an order of magnitude compared to that previously reported (45). No immunological cross-reactivity between CRBP and CRABP was observed in either radioimmunoassay. Cell samples were thawed and suspended in a small volume of 1.0% Triton X-100 in 50 mM imidazole, pH 7.4, containing 0.03% bovine serum albumin, 0.01% leupeptin, 0.1% Thimerosal, and 0.15 M NaCl so that the final concentration of cells was greater than 2 × 10^6 cells per ml. Samples were homogenized using a Polytron homogenizer for 1 min at setting 6 and centrifuged at 100,000 *g* for 1 hr. After centrifugation the supernatants were removed and immediately assayed for CRBP and CRABP. The radioimmunoassays for CRBP and CRABP employed identical protocols and were carried out as described elsewhere (46, 47). These assays were carried out in the homogenization buffer, and ^{125}I levels were determined in an LKB-Wallace RiaGamma 1274 counter.

RESULTS

Characterization of isolated liver cell preparations

Different types of liver cells were purified and isolated by the procedures described in Materials and Methods. These separation procedures allowed highly purified populations of the nonparenchymal fat-storing (stellate),

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endothelial, and Kupffer cells to be isolated from the same liver preparation. However, other livers were required for the isolations of parenchymal cells, because these cells (parenchymal) are selectively destroyed during the procedure used for the nonparenchymal cell isolations. Parenchymal cells were isolated using two procedures. In one procedure cells were constantly maintained at 8°C; in the second procedure the cells were maintained constantly at 37°C during perfusion and incubation of the liver tissue. The two procedures for parenchymal cell isolation were used in order to explore, and to try to minimize, the possibility that ongoing metabolism at 37°C might affect levels of the parameters being measured.

Characteristics of the isolated liver cell populations (fat-storing, endothelial, Kupffer, and parenchymal 37°C isolation) are given in **Table 1.** The percent yields (as calculated from Table 1) for the fat-storing cells (20.7%), endothelial cells (53.6%), and Kupffer cells (34.7%) are as high as has been previously reported (35), and the percent yield of parenchymal cells isolated at 37°C (47.0%) is in good agreement with previously reported recoveries (39, 40). Although the 8°C and 37°C parenchymal cell isolation procedures (data not given for the 8°C procedure) resulted in nearly homogenous populations of this cell type (>99% purity), the 37°C procedure resulted in the isolation of a 13-fold greater number of cells than were obtained by the 8°C procedure. In addition, the parenchymal cells isolated at 8°C did not appear to be viable as judged by trypan blue dye exclusion. The mean percent viability values (as judged by ability to exclude trypan blue) for the isolated fat-storing cells, endothelial cells, Kupffer cells, and 37°C parenchymal cells were 82%, 96%, 92%, and 91%, respectively.

Fig. 1 shows low magnification electron micrographs of representative liver cell preparations. The fat-storing cell preparations (Fig. 1a) were observed to be contaminated with endothelial cells (16% of all cells present) (see arrow), with Kupffer cells (2%), and with lymphocytes (2%). The isolated endothelial cell populations (Fig. 1b) were found to contain fat-storing cells (3%), Kupffer cells (3%) (see arrow), and lymphocytes (9%). The Kupffer cell preparations (Fig. 1c) included some endothelial cells (12%) (see arrow), fat-storing cells (1%), and lymphocytes (3%). The isolated parenchymal cell populations, using the 8°C isolation procedure (Fig. 1d) and the 37°C isolation procedure (Fig. 1e), were found to contain less than 1% contaminating cell types.

Micrographs of the isolated cell preparations at higher magnifications are shown in **Fig. 2.** A representative micrograph of fat-storing cells (Fig. 2a) shows that these cells were intact and laden with lipid droplets. These droplets, which are known to serve as storage depots for retinyl esters (16, 23, 31), are numerous and appear to fill much of the cytosolic contents of this cell. Endothelial cells (Fig. 2b) and Kupffer cells (Fig. 2c) are shown to have been isolated intact and to be void of this type of lipid droplet. Parenchymal cells isolated at 8°C (Fig. 2d) seem to possess greatly reduced quantities of cytosolic contents when compared with parenchymal cells isolated at 37°C (Fig. 2e).

Distribution of retinoid, RBP, RPH, CRBP, CRABP in isolated liver cell fractions

The retinoid content of each cell preparation was determined by HPLC analysis (**Table 2**). The main retinoids found were retinyl esters. All parenchymal cell fractions,

TABLE 1. Characteristics of the different hepatic cell type isolates

Cell Type Isolate	Number of Isolations	Yield[a,b]	Purity[a]	Protein Content[a]
		total cells × *10*^{6}	%	*μg/10*^{6} *cells*
Fat-storing	6	18.5 ± 5.5	79.3 ± 14.9[c]	188.0 ± 80.2
Endothelial	6	56.4 ± 19.4	84.0 ± 4.3[d]	97.5 ± 42.8
Kupffer	6	17.4 ± 4.8	83.2 ± 6.4[e]	128.8 ± 51.8
Parenchymal, 37°C	4	283 ± 33	>99	1934 ± 161

[a]The values are expressed as the mean ± SD.

[b]The best estimates for the numbers of each cell type present in a gram of liver from a 12-month-old female BN/BiRij rat, as determined from histological data from the literature and electron microscopic cell counts, are: 108 × 10^6 parenchymal cells, 16 × 10^6 fat-storing cells, 19 × 10^6 endothelial cells, and 9.0 × 10^6 Kupffer cells (49-53). For the 12-month-old female BN/BiRij rats used in this study, we estimate 602 × 10^6 parenchymal cells, 89 × 10^6 fat-storing cells, 106 × 10^6 endothelial cells, and 50 × 10^6 Kupffer cells to be present in each perfused liver.

[c]The composition of contaminating cells expressed as percent of total cells present was: 16.3 ± 12.3% endothelial cells, 1.7 ± 1.6% Kupffer cells, 1.7 ± 1.7% lymphocytes, and <1% other cell types.

[d]The composition of contaminating cells expressed as percent of total cells present was: 3.2 ± 3.1% fat-storing cells, 3.0 ± 2.8% Kupffer cells, 9.0 ± 9.0% lymphocytes, and <1% other cell types.

[e]The composition of contaminating cells expressed as percent of total cells present was: 1.0 ± 1.5% fat-storing cells, 11.7 ± 5.7% endothelial cells, 2.7 ± 2.6% lymphocytes, and <1% other cell types.

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but not all nonparenchymal cell fractions, contained detectable amounts of free retinol. The mean parenchymal cell level was found to be less than 0.1 μg per 10^6 cells and, taking into account the detection limit, the average retinol level was calculated to be less than 0.1 μg retinol per 10^6 cells for each cell type. Retinyl palmitate was found to be the predominant retinyl ester present in all liver cell fractions, comprising 80–90% of the total retinyl ester in each cell fraction. In addition, retinyl oleate and retinyl stearate were consistently found in smaller quantities. Both total liver and fat-storing cell extracts contained several additional very small peaks that eluted with retention times close to that of retinyl palmitate. These peaks were 5% or less of the total identifiable retinyl ester peaks and were probably retinyl linoleate, retinyl myristate, or other retinyl esters known to be present in whole livers (2, 48). Very high concentrations of retinyl esters (Total Retinoid) were found in the isolated fat-storing cells. The levels of retinyl esters in the fat-storing cells were approximately 50-fold the levels found in the isolated parenchymal cells, when expressed as μg of retinyl esters per 10^6 cells.

RBP was detected in the isolated cell fractions at the levels given in **Table 3.** Serial dilutions prepared from cell fraction homogenates gave identical displacement curves of ^{125}I-labeled RBP, in the radioimmunoassay, to that obtained with purified rat serum RBP. High levels of RBP were found in the isolated parenchymal cells. Only low levels of RBP were found in the fat-storing cells or in the other isolated nonparenchymal cells. When expressed per 10^6 cells, the level of RBP in parenchymal cells was approximately 25-fold that in fat-storing cells, however, the level of RBP in parenchymal cells was only 2–3-fold that in fat-storing cells when expressed as per mg cell protein.

Table 4 gives the RPH activity levels measured in the homogenates of the isolated liver cell fractions. RPH activity was found to be highly enriched in the fat-storing cells. When expressed as a level of activity per mg cell protein, the level of RPH in the fat-storing cells was more than 10-fold that of the parenchymal cells.

Measurable levels of CRBP and of CRABP were detected in all cell fractions. These levels are shown in **Table 5.** As is true for whole liver homogenates, serial dilutions prepared from the cell fraction homogenates displaced ^{125}I-labeled CRABP in its radioimmunoassay in a manner that was identical to that observed with purified rat testis CRABP (47). Similarly, purified rat testis CRBP and serial dilutions prepared from the cell fraction homogenates resulted in identical displacement curves of ^{125}I-labeled CRBP in the radioimmunoassay of CRBP.

For CRBP, high levels were found in the fat-storing cells and in the parenchymal cells isolated at 37°C. Only low levels of CRBP were found in parenchymal cells isolated at 8°C. Since the parenchymal cells isolated at 8°C did not exclude trypan blue dye and appeared to have lost cytosolic contents (see above, Figs. 1 and 2), we presume that the low CRBP levels found in these cells reflected the loss of CRBP from the intracellular compartment during the isolation procedure. When expressed per mg cell protein, the concentration of CRBP in the fat-storing cells was 5-fold that in the 37°C parenchymal cells.

Endothelial and Kupffer cells contained very small quantities of each retinoid-related parameter studied. For CRABP, levels were found to be low in comparison with CRBP levels in all cell samples tested.

Our best estimates for the numbers of each cell type present in a gram of liver from a 12-month-old female BN/BiRij rat, as determined from histological data from the literature and electron microscopic cell counts, are: 108×10^6 parenchymal cells, 16×10^6 fat-storing cells, 19×10^6 endothelial cells, and 9.0×10^6 Kupffer cells (49–53). From these estimates and the measured cellular levels of each parameter, it is possible to estimate the total amount of each component that was examined in this study, which should be expected in a whole liver (per g of liver). The actual total liver levels of retinyl ester, RBP, RPH, CRBP, and CRABP were measured in liver minces or liver homogenates prepared from six 12-month-old female BN/BiRij rats. From these estimated and measured values it is possible to calculate both an approximate recovery and the relative distribution of the total liver content of each parameter within the four types of liver cells. **Table 6** shows the measured liver levels, the calculated approximate recovery, and the estimated relative distribution within the liver cell types for each parameter.

DISCUSSION

The recent work of Knook and coworkers (32–35) has shown that highly purified populations of perisinusoidal fat-storing (stellate) cells isolated from rat liver contain a majority (about 80%) of the total stores of hepatic retinoids. Moreover, Blomhoff et al. (21, 36) observed that within 2 hr after uptake of chylomicron retinyl esters by normal intact rat liver much of the retinol moiety was transferred to the fat-storing cells. These observations strongly suggest that the fat-storing cells are important in retinoid storage and metabolism. To further explore the role played by fat-storing cells in these processes, we have examined the distribution and levels of retinoids and of the significant parameters of retinoid metabolism, RBP, RPH, CRBP, and CRABP, in highly purified preparations of the four major types of liver cells isolated from

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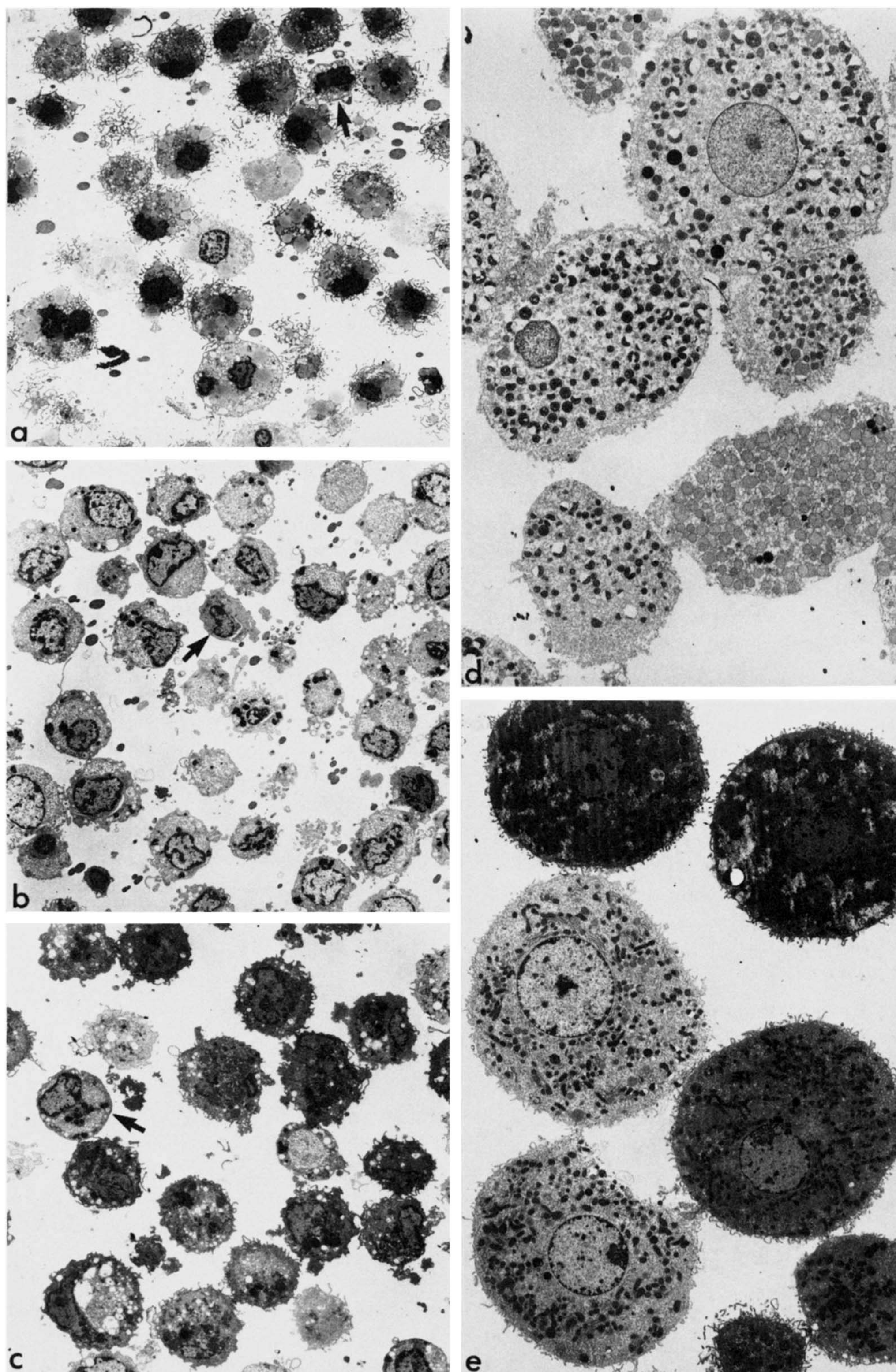

Fig. 1. Low magnification electron micrographs of representative liver cell fractions. (a) Fat-storing cells; the arrow points to a contaminating endothelial cell; (b) endothelial cells, the arrow points to a contaminating Kupffer cell; (c) Kupffer cells, the arrow points to a contaminating endothelial cell; (d) parenchymal cells prepared using the 8°C isolation procedure; (e) parenchymal cells prepared using the 37°C isolation procedure. (a) × 1210; (b and c) × 1540; (d and e) × 2030.

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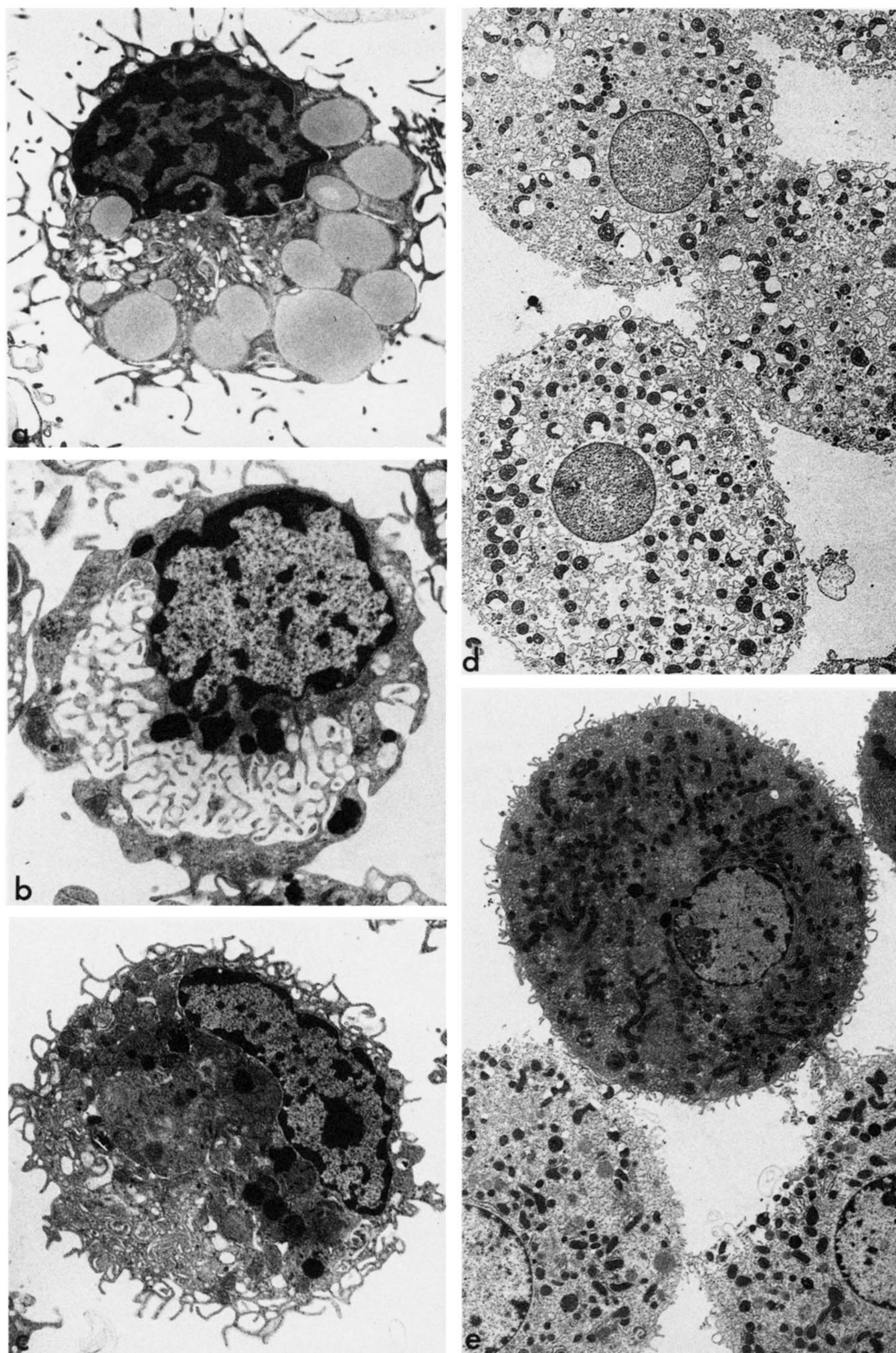

Fig. 2. High magnification electron micrographs of representative liver cell fractions. (a) Fat-storing cell; (b) endothelial cell; (c) Kupffer cell; (d) parenchymal cells prepared using the 8°C isolation procedure; (e) parenchymal cells prepared using the 37°C isolation procedure. (a and b) × 7680; (c) × 6840; (d) × 2750; (e) × 3500.

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TABLE 2. Retinoid concentrations in hepatic cell fractions[a]

Cell Fraction	Number of Isolations	Retinyl Palmitate	Retinyl Oleate	Retinyl Stearate	Total Retinoid
Fat-storing	6	71.0 ± 26.1	2.5 ± 0.9	10.4 ± 3.7	83.9 ± 30.7
Endothelial	6	1.0 ± 0.3	<0.1	<0.1	1.1 ± 0.3
Kupffer	6	1.1 ± 0.4	<0.1	<0.1	1.2 ± 0.4
Parenchymal, 37°C	4	1.3 ± 0.6	<0.1	0.1 ± 0.1	1.5 ± 0.7

[a]The values are expressed as μg of retinol equivalents per 10^6 cells (mean ± SD). The mean free retinol levels in all fractions were <0.1 μg of retinol per 10^6 cells.

12-month-old female BN/BiRij rats. Although it is possible that our findings may not be generalized to all strains of rats under all nutritional conditions, the data reported in this manuscript strongly indicate that fat-storing cells are both important and active in hepatic retinoid storage and metabolism.

The retinoid concentrations measured in the purified cell fractions prepared for this study are very similar to those reported recently for liver cells prepared from similar 12-month-old female BN/BiRij rats (35). The parenchymal cell concentrations of retinoid were within the range reported by Olson and Gunning (23), as determined from cells isolated from male Sprague-Dawley rats. In our study, most (approximately 88%) of the total retinoid in liver was found in the fat-storing cell fraction and 9% of the parenchymal cell fraction (see Table 6). Only a small portion (<5%) of the total retinoid measured in purified cell fractions was in the form of free retinol. Retinyl palmitate constituted 85-90% of the total retinyl ester present in the different cell fractions. Although the exact proportion of total hepatic retinoid present in fat-storing cells should be considered as a tentative estimate, since it depends on a number of assumptions used here (see results), it is clear that the large majority of hepatic retinyl esters was present in these cells.

Unlike the retinoid distribution, 98% of the hepatic RBP was found in the parenchymal cell fraction. This finding is consistent with immunocytochemical (22, 37) and immunofluorescence (54) studies that reported the localization of RBP within these cells.

The RPH activity was found to be distributed in both the parenchymal (90% of total RPH activity) and fat-storing (10%) cell fractions. If the levels of this activity are expressed on the basis of cellular protein content (Table 4), it is obvious that RPH activity is very enriched in the fat-storing cells. This finding indicates that fat-storing cells not only store retinoid but are also very active in the metabolism of retinyl esters. It should be noted that the RPH activity measured in the fat-storing cells is thought to be involved in the hydrolysis of stored retinyl ester (2, 11-13). As indicated above, most of the hepatic retinyl ester stores are found in these nonparenchymal cells.

The data reported in this study indicate that the per cell levels of CRBP are similar in parenchymal and fat-storing cells. However, if these levels are expressed on the basis of cellular protein concentration, the fat-storing cells are seen to be highly enriched in CRBP. The present quantitative radioimmunoassay data are thus consistent with the results of recent immunocytochemical studies (22) that have shown localization of CRBP in parenchymal and in fat-storing cells, with the strongest localization in the fat-storing cells.

Parenchymal cells isolated using the 8°C isolation procedure contained less that 5% of the CRBP found to be present in cells isolated using the 37°C procedure. Similarly the cells isolated at 8°C contained 18% less protein and appeared in micrographs (Figs. 1 and 2) to have less cytosolic contents that the cells isolated at 37°C. These observations, taken together with the inability of the cells isolated with 8°C procedure to exclude trypan blue dye, strongly suggest that these cells have lost cytosolic contents and can not be used as a reliable preparation to determine the true parenchymal cell content of CRBP. Interestingly, the levels of RBP, RPH, and CRABP were as high in parenchymal cells isolated at 8°C as in those isolated at 37°C. For RBP and RPH, this can readily be explained by their compartmentalization in membranous organelles (10, 55), which would not be expected to be lost during the cold isolation procedure, inasmuch as most cell organelles were retained within the cells. The finding that parenchymal cells isolated at 8°C contain more CRABP

TABLE 3. Retinol-binding protein concentrations in hepatic cell fractions

Cell Fraction	Number of Isolations	Retinol-Binding Protein[a]	
		ng/10⁶ cells	*ng/mg protein*
Fat-storing	6	7.4 ± 3.5	39.0 ± 19.3
Endothelial	6	3.2 ± 1.4	32.8 ± 13.1
Kupffer	6	5.7 ± 0.9	44.0 ± 6.9
Parenchymal, 37°C	4	138 ± 67.9	71.4 ± 35.1

[a]The values are given as the mean ± SD.

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TABLE 4. Retinyl palmitate hydrolase concentrations in hepatic cell fractions

Cell Fraction	Number of Isolations	Retinyl Palmitate Hydrolase[a]	
		pmol FFA formed/hr per 10^6 cells	*pmol FFA formed/hr per mg protein*
Fat-storing	6	1152 ± 144	6129 ± 768
Endothelial	6	78 ± 9	803 ± 94
Kupffer	6	140 ± 15	1092 ± 113
Parenchymal, 37°C	4	826 ± 47	427 ± 24

[a]The values are given as the mean ± SD.

than those isolated at 37°C may either reflect some previously unreported and not understood compartmentalization of CRABP, or may arise from biological variability in the values measured for the cells isolated by the two procedures. It should be noted that liver contains only low levels of CRABP, in contrast to its high content of CRBP.

In order to interpret the data presented in this report, it is essential to ask whether these values adequately reflect concentrations as they actually exist in whole liver. Were the isolated liver cell fractions composed of truly representative samples of each of the different types of liver cells, or were they nonrepresentative subpopulations of the cells found in the whole liver? To address this concern, we have carefully evaluated our isolated cell fractions and the data obtained from these fractions. Every cell isolate was of high purity (as determined by both light and electron microscopy) and high yield. Electron micrographs (see Figs. 1 and 2) of each cell fraction indicated that the fractions contained intact cells which qualitatively (morphologically) appeared to be representative of the cells seen in the intact liver. To evaluate quantitatively the validity of our measurements, we have estimated the relative numbers of each cell type present in an intact liver of a 12-month-old female BN/BiRij rat (see Results) and measured retinoid content and RBP, RPH, CRBP, and CRABP concentrations from whole liver minces or homogenates (see Table 6). From these values and the measured level of each parameter found in the cell fractions it is possible to estimate an approximate recovery for these parameters. As seen in Table 6, the estimated approximate recoveries ranged from 56% (for CRABP) to 82% (for CRBP). The recoveries of RBP and RPH activity in the isolated cell fractions were, respectively, 74% and 71%. Considering the inherent limitations of these estimations, the approximate recoveries of the parameters are very good. Based on these criteria, our isolated liver cell fractions appear to be truly representative of the whole liver population of cells. Thus, we conclude that the concentration of each parameter detected in these fractions was an accurate and valid measure of the cellular concentration found in the liver.

The findings reported here considerably extend the information available about the role of the fat-storing cell (and the other types of liver cells) in retinoid storage and metabolism. It is known that dietary retinoid is taken up by the parenchymal cells and is either secreted into the circulation as holo-RBP, stored as retinyl ester in the parenchymal cells, or transferred to the fat-storing cells (36). Most of the liver's stores of retinyl ester are found in the fat-storing cells. The fat-storing cells are highly enriched in CRBP and in RPH activity, and thus have the metabolic capabilities for hydrolysis of stored retinyl esters (a necessary process for retinol mobilization from the liver) and for directed transport of retinol (bound to CRBP) within or possibly outside the cell. For retinol mobilization, a molecule of stored retinoid must be transferred back to the parenchymal cells, for secretion as the retinol-RBP complex. Almost all of the RBP in liver is found in parenchymal cells.

The mechanisms involved in the transfer of retinol between parenchymal and fat-storing cells, for retinoid storage, and for its mobilization are not known. Although both RBP (21) and CRBP (37) have been suggested as possible transport proteins in this process, there are no data available supporting either of these possibilities. Moreover, it has not been demonstrated that it is retinol, rather than retinyl ester, that is transferred between cells. Considering the high RPH activity we have observed in fat-storing cells, it is tempting to speculate that the trans-

TABLE 5. Cellular retinol- and cellular retinoic acid-binding protein concentrations in hepatic cell fractions

Cell Fraction	Number of Isolates	CRBP[a]		CRABP[a]	
		ng/10^6 cells	*ng/mg protein*	*ng/10^6 cells*	*ng/mg protein*
Fat-storing	6	236 ± 89	1256 ± 477	8.7 ± 5.7	44 ± 30
Endothelial	6	6.5 ± 2.5	35 ± 13	1.8 ± 1.4	18 ± 14
Kupffer	6	13 ± 4.8	101 ± 37	3.8 ± 2.2	29 ± 17
Parenchymal, 37°C	4	470 ± 238	243 ± 124	5.6 ± 3.9	2.9 ± 3.3

[a]The values are given as the mean ± SD.

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TABLE 6. Estimated recovery and distribution of retinyl ester, retinol-binding protein, retinyl palmitate hydrolase, cellular retinol-, and cellular retinoic acid-binding protein in hepatic cell fractions

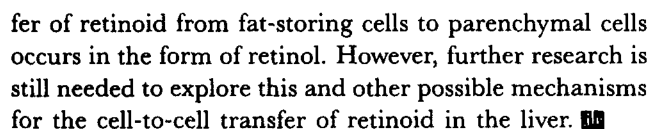
	Liver Level[a]	Estimated Recovery[b]	Relative Distribution within Each Hepatic Cell Fraction[c] (%)			
			FS[d]	E[d]	K[d]	P[d]
		%				
Retinyl ester	2215 ± 593	63.4	88	1	1	9
RBP	20.6 ± 1.4	73.8	0.7	0.7	0.7	98
RPH	14.7 ± 5.6	71.2	10	0.2	0.2	90
CRBP	67.5 ± 1.3	82.4	8	0.2	0.2	91
CRABP	1.6 ± 0.3	56.4	21	4	5	71

[a]Retinyl esters, RBP, RPH, CRBP, and CRABP were measured in homogenates prepared from six livers of 12-month-old female BN/BiRij rats. Liver levels of retinyl esters are expressed as μg retinol equivalents/g wet weight; RBP, CRBP, and CRABP as μg/g wet weight; RPH as pmol FFA formed/min per mg protein. All values are given as the mean ± SD.

[b]Recovery was calculated for each parameter using the estimates of the number of each cell type present in a gram of liver as given in the text. The calculations of recovery were made by multiplying the total number of each cell type present in a gram of liver by the level of each parameter (expressed per 10^6 cells), summing the values obtained for the four cell types, and dividing by the mean measured liver level.

[c]The values represent the estimated percentage distribution of the total amount of each parameter in each of the four types of isolated liver cells, per g liver (or in the total liver).

[d]Abbreviations are: FS, fat storing cells; E, endothelial cells; K, Kupffer cells; and P, parenchymal cells (37°C isolation).



fer of retinoid from fat-storing cells to parenchymal cells occurs in the form of retinol. However, further research is still needed to explore this and other possible mechanisms for the cell-to-cell transfer of retinoid in the liver.

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