Biochemical and Molecular Roles of Nutrients

Lecithin:Retinol Acyltransferase and Retinyl Ester Hydrolase Activities Are Differentially Regulated by Retinoids and Have Distinct Distributions between Hepatocyte and Nonparenchymal Cell Fractions of Rat Liver

Tomokazu Matsuura,*3 Mohamed Z. Gad,*4 Earl H. Harrison* and A. Catharine Ross*5

*Division of Nutrition, Department of Biochemistry, MCP-Hahnemann School of Medicine, Allegheny University, Philadelphia, PA 19129 and ¹Department of Nutrition, Pennsylvania State University, University Park, PA 16802

ABSTRACT The cellular distribution of enzymes that esterify retinol and hydrolyze retinyl esters (RE) was studied in liver of vitamin A–sufficient,–deficient, and deficient rats treated with retinoic acid or N-(4-hydroxyphenyl)-retinamide. Livers were perfused and cell fractions enriched in hepatocytes, and nonparenchymal cells were obtained for assays of RE and enzyme activity. The specific activity of lecithin:retinol acyltransferase (LRAT) was approximately 10-fold greater in the nonparenchymal cell than the hepatocyte fraction from both vitamin A–sufficient and retinoid-treated rats. Total RE mass, newly synthesized [3H]RE and LRAT activity were positively correlated in liver and isolated cells of both normal (P < 0.0001) and retinoid-treated rats (P < 0.0002). In nonparenchymal cells, these three constituents were nearly equally enriched as evaluated by their relative specific activity values (RSA, defined as the percentage of recovered activity divided by the percentage of recovered protein), which were each significantly greater than 1.0, with values of 4.3 for total RE mass (P < 0.05), 3.6 for newly synthesized [3H]RE (P < 0.01) and 3.8 for LRAT activity (P < 0.01). In contrast, the specific activities of neutral and acid bile salt–independent retinyl ester hydrolases (REH) did not vary with vitamin A status, and their RSA values were close to 1.0 in both hepatocytes and nonparenchymal cells. These data show that LRAT and REH are differentially regulated by retinoids and that these enzymes also differ in their spatial distribution between liver parenchymal and nonparenchymal cells. J. Nutr. 127: 218–224, 1997.

KEY WORDS: • retinoic acid • retinamide • stellate cells • vitamin A • rats

Nearly 50–80% of the total body vitamin A is stored in liver as long-chain fatty acid esters of retinol (Goodman and Blaner 1984). Two types of liver cells, hepatocytes (parenchymal cells) and stellate cells, are known to play important roles in the assimilation, storage and mobilization of vitamin A (Blomhoff and Wake 1991, Blomhoff et al. 1991). Chylomicron remnants containing newly absorbed dietary retinol esters (RE) are taken into hepatocytes through receptor-mediated endocytosis. During or shortly after uptake, these RE are hydrolyzed and the unesterified retinol is re-esterified with long-chain fatty acids, predominantly palmitic, stearic and oleic acid, to form hepatic RE stores (Goodman et al. 1965). Within a few hours, vitamin A is transferred from hepatic parenchymal cells to the stellate cells (Blomhoff et al. 1991) by mechanisms that have not yet been elucidated. The stellate cells (also known as fat-storing cells, Ito cells, and lipocytes (Blomhoff and Wake 1991)) are nonparenchymal cells situated in the perisinusoidal space. In normal rat liver, these cells are characterized morphologically by numerous cytoplasmic lipid droplets and biochemically by a high concentration of esterified retinol. Hendriks et al. (1985) estimated that in normal rat liver greater than 75% of RE resides in stellate cells. Cell culture studies with isolated stellate cells have shown that the number and size of the lipid droplets vary directly with vitamin A uptake and storage (Blaner et al. 1985, Matsuura et al. 1993).

Research in several laboratories (see references in Blomhoff and Wake 1991) has demonstrated the importance of stellate cells in hepatic RE storage. However, there is still little information concerning the cellular location(s) of the enzymes responsible for RE formation and hydrolysis. Lecithin:retinol acyltransferase (LRAT), the principal activity involved in hepatic retinol esterification (MacDonald and Ong 1988, Randolph and Ross 1991, Randolph et al. 1991), catalyzes the transfer of the sn-1 fatty acid from membrane phosphatidyl choline to retinol bound to the cellular retinol-binding proteins, CRBP or CRBP-II (MacDonald and Ong 1988). This enzyme has been localized to the endoplasmic reticulum (microsomal) fraction of liver, and, in liver, its activity has been shown to be strongly regulated by vitamin A and related reti-
retinoic acid (Matsuura and Ross 1993, Matsuura et al. 1996, Randolph and Ross 1991). Whereas livers of vitamin A–deficient rats had negligible LRAT activity (Randolph and Ross 1991) and lacked the ability to esterify a test dose of [3H]retinol in vivo (Matsuura and Ross 1993), LRAT activity increased rapidly after these rats were treated with retinol (Randolph and Ross 1991), retinoic acid or 4-hydroxyphenyl retinamide (4HPR) (Matsuura and Ross 1993, Matsuura et al. 1996).

The processes of chylomycin clearance and the mobilization of stored RE from intracellular lipid droplets both require RE hydrolysis. Chylomycin RE clearance takes place in hepatocytes, whereas most stored RE is present in stellate cells. A number of biochemically distinct RE hydrolase (REH) activities have been described in various subcellular fractions of rat liver (Harrison 1993). A neutral, bile salt–dependent REH that closely resembles pancreatic carboxyl ester lipase is present in the cytoplasmic and nuclear fractions (Harrison and Gad 1989); this REH activity is distributed broadly among hepatocytes and stellate cells, with little activity in endothelial or Kupffer cells (Blaner et al. 1985). Membrane-associated REH also have been described that are maximally active at neutral or acid pH and that are capable of hydrolyzing RE in the absence of bile salts (Gad and Harrison 1991, Harrison 1993). These bile salt–independent REH activities were localized in plasma membranes and/or endosomes (Gad and Harrison 1991, Harrison and Gad 1989, Harrison et al. 1995), sites that suggest their involvement in the initial catabolism of chylomycin remnant RE. However, their distribution between hepatocytes and nonparenchymal cells has not yet been reported.

The present studies had four related goals: 1) to determine the distribution of LRAT among hepatocytes and nonparenchymal cells in the livers of normal, vitamin A–sufficient rats; 2) to determine whether the LRAT induced in the livers of vitamin A–deficient rats by either retinoic acid or 4-HPR has the same cellular distribution as LRAT in vitamin A–sufficient rats; 3) to examine the relationship between LRAT activity measured in vitro and the ability of parenchymal and nonparenchymal cells to synthesize and store RE in vivo; and 4) to compare these data with the cellular distribution of neutral and acid bile salt–independent REH activities in order to provide a comprehensive picture of the location of liver enzymes responsible for RE synthesis and hydrolysis in both vitamin A–sufficient, vitamin A–deficient and retinoid-treated rats.

**MATERIALS AND METHODS**

*Animals and diets.* Male and female Lewis rats were reared following procedures (Bowman et al. 1990) approved by the Institutional Animal Use and Care Committee of the Medical College of Pennsylvania. The control, vitamin A–sufficient diet was a nutritionally adequate semipurified diet, described previously (Bowman et al. 1990), that contained 4 mg retinol/kg. The same diet without vitamin A was used to induce vitamin A deficiency. The vitamin A–free diet was fed to the lactating mothers of the rats used in this study beginning at 8–10 d of lactation; their male and female offspring were fed either the control or vitamin A–free diet from the time of weaning (d 20–21). With this protocol, rats fed the latter diet became vitamin A deficient (assessed by a plasma retinol <0.2 μmol/L) by approximately 45 d (males) and 53 d of age (females) (Matsuura et al. 1996). The methods used to prepare 4-HPR and retinoic acid for administration in vivo were described by Matsuura et al. (1996). The amounts of retinoic acid (20 μg intraperitoneally) and 4-HPR (0.5 mg intragastrically) were chosen based on doses that produced maximum increases in LRAT in activity in previous experiments (Matsura and Ross 1993, Matsuura et al. 1996). To determine the esterification of [3H]retinol 20 h before liver perfusion. To prepare this dose, tritiated retinol (Du Pont NEN Products, Boston, MA) was mixed with unlabelled retinol and puriﬁed on a column of aluminum oxide (Ross 1982). The resulting [3H]retinol (approximately 1 μBq/0.4 μg) was stored in ethanol under nitrogen at −20°C. Just prior to administration, ethanol was evaporated, and the [3H]retinol was mixed with 100 μL of ethanol and 10 μL of Tween 80 and then diluted with PBS. The concentration was determined by spectrophotometry (Matsura and Ross 1993), and 200 μL (0.4 μg) was then injected into a caudal vein.

*Isolation and separation of liver cells.* Rats were anesthetized with diethyl ether, and cannulae were inserted into the portal vein and inferior vena cava. The preparation of liver cell fractions is outlined in Figure 1. The liver was perfused first without collagenase at 37°C at approximately 15 mL/min with 100 mL of calcium-free perfusate (0.14 mol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L NaH2PO4, 0.4 mmol/L Na2HPO4, 9 mmol/L HEPES, 0.5 mmol/L EGTA, 4 mmol/L NaHCO3, 0.09% glucose). Following this first perfusate, the perfusate was discarded, and perfusion continued in a circulating fashion for 5–7 min with 100 mL of collagenase solution [60,000 U/L ( Worthington Biochemicals, Freehold, NJ) containing 0.14 mol/L NaCl, 5 mmol/L KCl, 5 mmol/L CaCl2, 0.5 mmol/L MgCl2, 0.4 mmol/L MgSO4, 0.5 mmol/L NaH2PO4, 0.5 mmol/L NaHPO4, 0.3 mmol/L Na2HPO4, 9 mmol/L HEPES, 4 mmol/L NaHCO3, 0.05 g/L trypsin inhibitor and 10 g/L glucose (Seglen 1973)]. After perfusion, a small portion of liver was removed and quickly frozen in liquid nitrogen for later
determination of enzyme activities and RE mass in whole liver. The remaining tissue, which was very soft at this time, was dissociated by gentle filtration through a wire mesh (mesh size 500 μm) and a fine nylon sieve (mesh size 105 μm; Small Parts Inc., Miami Lakes, FL) to obtain a suspension of dispersed well-separated cells that was centrifuged at 60 × g for 1 min and then washed in PBS by recentrifugation under the same conditions. The washed cell pellet, termed the “first hepatocyte” fraction (HP-1), was highly enriched in viable parenchymal cells as judged by light microscopy (cells were approximately 20–25 μm in diameter and had distinct nuclei, and >80–90% excluded trypan blue dye). To maximize recovery of the nonparenchymal cells, the liver tissue that remained on the wire mesh was dissociated by shaking in PBS for 5 min with a vortex mixer and then centrifuged at 60 × g for 1 min. The resulting cell suspension was used for cell fractionation by collagenase perfusion. This fraction, which contained dispersed cells of the size of both hepatocytes and smaller, nonparenchymal cells, was centrifuged at 60 × g for 1 min and washed in PBS by suspension and recentrifugation; it is referred to as the “second hepatocyte” fraction (HP-2). The trypan blue dye exclusion of cells in this fraction was lower (30–40%) than in HP-1. For the purposes of accurately assessing the recoveries of constituents, tissue that did not pass through the filter as a suspension of dispersed cells was collected and termed the “liver remainder”; this fraction contained parts of liver not well digested by collagenase. Following this, the supernatants from the two hepatocyte fractions were combined and centrifuged at 300 × g for 3 min to obtain the nonparenchymal cell–enriched fraction; this fraction consisted of intact cells smaller than and easily distinguished from hepatocytes. The remaining fraction, termed “combined supernatants and washes,” consisted of broken cells, cell sap and membranes as judged by light microscopy, but was essentially free of intact cells. It was saved for analysis as the nonparenchymal cell fraction. Liver tissue and isolated cells were frozen rapidly in liquid nitrogen and stored at −70°C prior to analysis. The enzymes of interest were found in preliminary studies to be stable under these conditions, whereas, in another preliminary study, collagenase perfusion was shown to have no effect on the hepatic LRAT activity of normal rats.

Preparation of liver and cell homogenates. Portions of liver tissue and isolated liver cells were thawed and homogenized with a Potter-Elvejhem homogenizer in 2.5 volumes of ice-cold homogenization buffer (0.28 mol/L sucrose, 0.01 mol/L K2HPO4, 1 mmol/L dithiothreitol, pH 7.25). Protein concentrations were determined by the method of Markwell et al. (1978).

Assay of lecithin:retinol acyltransferase and retinyl ester hydrolytic activities. In most experiments, LRAT activity was assayed in homogenates of whole liver and cell fractions (Randolph and Ross 1991, Matsuura and Ross 1993) to avoid the loss of enzyme activity that occurs when tissues are frozen. Microsomes from the isolated cell fractions. Homogenates of each sample containing 1 mg of protein were incubated in duplicate for 4 min with 5 pmol/L of [3H]retinol-CRBp in 0.15 mol/L K2HPO4 buffer, pH 7.4, containing 2 mmol/L dithiothreitol at 37°C; a boiled sample of each preparation served as background, which was subtracted. [3H]Retinyl ester was separated from [3H]retinol on columns of aluminum oxide, and radioactivity was determined by liquid scintillation counting (Ross 1982). The activities of acid and neutral REH were determined using a radiometric assay previously described (Harrison and Gud 1989). All assays were conducted for 30 min in the absence of exogenous detergents or bile salts and contained 40 μmol/L retinyl palmitate as substrate. Neutral REH activity was assessed in reaction mixtures containing 50 mmol/L Tris-maleate, pH 8, and acid REH activity was assayed in reaction mixtures containing 50 mmol/L sodium acetate, pH 5. All enzyme assays were conducted under conditions where the extent of product formation was proportional to the amount of enzyme (protein) in the assay mixture and time of incubation.

The retinol concentrations of plasma, liver and cell homogenates were determined using HPLC as described previously (Ross 1986). Esterified retinol was estimated as the difference between total retinol determined after saponification and unesterified retinol determined in nonsaponified plasma or liver cell homogenates. After in vivo administration of [3H]retinol, the [3H]RE in liver cell extracts was isolated by column chromatography, as for the LRAT assay.

Statistics. Results are presented as the means ± SD for the number of rats reported. Statistical comparisons were made between two groups with an unpaired Student’s t-test. In some experiments, linear regression analysis was used to determine correlation coefficients using the InStat 1.11 program (GraphPad Software, Inc., San Diego, CA).

RESULTS

Distribution of lecithin:retinol acyltransferase activity in hepatocyte and nonparenchymal cell fractions. When the specific activity of LRAT was determined in intact liver and in the hepatocyte and nonparenchymal cell fractions prepared from vitamin A–sufficient rat liver, the specific activity of LRAT in whole liver averaged 15.6 pmol RE/(min · mg homogenate protein) (Table 1). In contrast, the LRAT activity in isolated hepatocytes was only one-fifth of this value. In contrast, the specific activity of LRAT in the nonparenchymal cell fraction was 100% greater than that of intact liver. As a ratio, the specific activity of LRAT in the nonparenchymal cell fraction exceeded that in the hepatocyte fraction by 10:1.

Table 1 also presents data from vitamin A–deficient rats before or at 18–20 h after they received 20 μg of retinoic acid or 0.5 mg of 4-HPR. Lecithin:retinol acyltransferase activity was not detected in either the intact liver or isolated cell fractions of vitamin A–deficient rats. However, LRAT activity was readily detected in both the hepatocyte and the nonparenchymal cell fractions of vitamin A–deficient rats treated with either retinoid. Although the absolute LRAT activities differed somewhat among these treatments, which were conducted separately, in each case the activity of LRAT was approximately 10- to 11-fold greater in the nonparenchymal cell fraction than in the corresponding hepatocyte fraction.

Recoveries of protein, retinyl esters and lecithin:retinol acyltransferase activity in liver cell fractions of vitamin A–sufficient rats given a small (0.4 μg) dose of [3H]retinol. The recoveries of total RE mass (shown previously to be a marker of the stellate cells), newly synthesized [3H]RE, LRAT activity and total protein, determined 20 h after normal rats received [3H]retinol, are presented in Table 2. In comparison to whole liver, taken as 100%, the recovery of these entities among the cell fractions averaged 91% for RE mass, 76% for newly synthesized [3H]RE and 80% for total protein. The recovery of LRAT activity was only 48%, suggesting that the activity of this enzyme decreased during the cell separation procedure. For this reason, further purification of the nonparenchymal cell fraction was not conducted, and all liver cell fractions were frozen immediately after preparation and assayed for LRAT activity immediately after thawing. The fractions enriched in parenchymal cells (HP-1 plus HP-2) contained approximately 45% of liver protein but only 30% of the LRAT activity, esterified retinol mass and newly synthesized [3H]RE as compared with whole liver. Only 3.1% of total liver protein was recovered in the nonparenchymal cell fraction, but this fraction contained more than 10% each of the RE mass, newly synthesized [3H]RE and LRAT activity. The remainder and supernatant and washes, consisting of debris and broken cells, contained equivalent percentages of protein, esterified retinol and LRAT activity (see footnote to Table 2).

Comparison of relative specific activities for retinyl ester and lecithin:retinol acyltransferase. Table 2 also presents the RSA values, defined as the percentage of a given constituent recovered in a fraction divided by the percentage of protein recovered in the same fraction, were determined for the mass of RE, newly synthesized [3H]RE and LRAT activity. These RSA values were compared with 1.0, the RSA for whole liver. In the hepatocyte (HP-1) fraction, the RSA values for the mass of RE, newly synthesized [3H]RE and LRAT activity were...
each significantly less than 1.0, averaging 0.75 for RE mass ($P < 0.01$), 0.69 for newly esterified [3H]retinol ($P = 0.01$) and 0.67 for LRAT activity ($P < 0.02$); these data indicate a significant “de-enrichment” of these constituents in hepatocytes. In the nonparenchymal cell fraction, the RSA values for RE mass, newly synthesized [3H]RE and LRAT activity were each significantly greater than 1.0, averaging 4.31 ($P < 0.01$) for total RE mass, 3.65 for newly synthesized [3H]RE ($P < 0.005$) and 3.82 for LRAT activity ($P < 0.005$); these data indicate a significant positive enrichment in this cell fraction. The RSA values for the remainder and supernatant fractions (see footnote 3 to Table 2) did not differ significantly from 1 ($P > 0.05$), indicating that there was no selective difference between whole liver and these fractions containing nondigested tissue (remainder) or non-intact cells, debris and washings (combined supernatant and washes).

**Relationship between lecithin:retinol acyltransferase activity and retinyl ester level among liver cells.** For each cell fraction prepared from liver of vitamin A–sufficient rats, the quantity of [3H]RE newly synthesized in vivo was plotted against LRAT activity measured in vitro (Fig. 2A). The relationship was significant and linear ($r = 0.949, P < 0.0001$). A similar linear relationship was obtained when the total mass of RE was plotted against LRAT activity ($r = 0.828, P < 0.0001$, data not shown).

After vitamin A–deficient rats were treated with 4-HPR to induce liver LRAT activity (Fig. 2B), the relationship between newly synthesized [3H]RE and induced LRAT activity was also significant and linear ($r = 0.823, P < 0.0002$), with the greatest [3H]RE level and LRAT activity in the nonparenchymal cell fraction. Lecithin:retinol acyltransferase activity was negligible in the cell fractions and whole liver of vitamin A–deficient rats (closed symbols, Fig. 2B).

**Levels and distributions of bile salt–independent, neutral and acid retinyl ester hydrolase activities.** In contrast to the striking effect of vitamin A deficiency on liver LRAT, the activities of either bile salt–independent acid or neutral REH were not different in liver homogenates from vitamin A–sufficient and –deficient rats. The specific activities of neutral REH in liver of vitamin A–sufficient and –deficient rats ($n$ values are means ± SD for the number of rat livers indicated. * Significantly different from LRAT activity in the hepatocyte fraction ($P < 0.001$). ** Significantly different from LRAT activity in the hepatocyte fraction ($P < 0.001$). *** Significantly different from LRAT activity in the hepatocyte fraction ($P < 0.001$).

### TABLE 1

<table>
<thead>
<tr>
<th>Vitamin A status of rats</th>
<th>$n$</th>
<th>Liver</th>
<th>Hepatocyte fraction</th>
<th>Nonparenchymal cell fraction</th>
<th>Ratio of nonparenchymal to parenchymal cell fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sufficient</td>
<td>6</td>
<td>15.6 ± 4.7</td>
<td>3.0 ± 1.1</td>
<td>30.9 ± 6.3*</td>
<td>10.3</td>
</tr>
<tr>
<td>Deficient</td>
<td>2</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>---</td>
</tr>
<tr>
<td>Retinol acid–treated</td>
<td>3</td>
<td>5.3 ± 0.9</td>
<td>1.2 ± 0.9</td>
<td>13.2 ± 2.1*</td>
<td>11.0</td>
</tr>
<tr>
<td>4-HPR-treated</td>
<td>4</td>
<td>18.6 ± 2.6</td>
<td>5.4 ± 1.9</td>
<td>55.3 ± 9.2*</td>
<td>10.2</td>
</tr>
</tbody>
</table>

### TABLE 2

<table>
<thead>
<tr>
<th>Constituent</th>
<th>HP-1</th>
<th>HP-2</th>
<th>NP</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, %</td>
<td>24.6 ± 7.6</td>
<td>19.8 ± 5.6</td>
<td>3.1 ± 1.2</td>
<td>80.3 ± 4.4</td>
</tr>
<tr>
<td>RSA</td>
<td>1.9</td>
<td>1.0</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Mass of RE, %</td>
<td>18.4 ± 5.5</td>
<td>13.1 ± 3.8</td>
<td>11.5 ± 4.7</td>
<td>91.1 ± 36.6</td>
</tr>
<tr>
<td>RSA</td>
<td>0.75 ± 0.11*</td>
<td>0.67 ± 0.11*</td>
<td>4.31 ± 2.02**</td>
<td>80.3 ± 4.4</td>
</tr>
<tr>
<td>New [3H]RE, %</td>
<td>16.8 ± 5.7</td>
<td>12.9 ± 5.5</td>
<td>10.4 ± 3.2</td>
<td>76.0 ± 8.0</td>
</tr>
<tr>
<td>RSA</td>
<td>0.94 ± 0.18*</td>
<td>0.65 ± 0.17*</td>
<td>3.65 ± 0.90***</td>
<td>76.0 ± 8.0</td>
</tr>
<tr>
<td>LRAT activity, %</td>
<td>16.4 ± 6.5</td>
<td>13.2 ± 2.9</td>
<td>10.6 ± 2.7</td>
<td>48.0 ± 8.3</td>
</tr>
<tr>
<td>RSA</td>
<td>0.67 ± 0.11*</td>
<td>0.69 ± 0.11*</td>
<td>3.82 ± 0.11***</td>
<td>76.0 ± 8.0</td>
</tr>
</tbody>
</table>

1 Values are means ± SD for $n = 4$ livers from control male rats. Abbreviations used: HP-1, first hepatocyte fraction; HP-2, second hepatocyte fraction; NP, nonparenchymal cell fraction (see Materials and Methods and Fig. 1); RSA, relative specific activity (enrichment).
2 Recovery represents the total amount of each constituent recovered in all five fractions (the three cell fractions indicated and the remainder and combined supernatants and washings); see Materials and Methods, Fig. 1 and footnote 3 below), relative to the amount in the whole liver homogenate.
3 Values are expressed as the percentage of the constituent recovered in each fraction relative to the amount recovered in all five fractions (taken as 100%). The RSA value for each fraction is defined as the percentage of RE or LRAT activity recovered divided by the percentage of protein recovered in the same cell fraction. The percentage and RSA values (in parentheses) for the liver remainder were as follows: protein, 15.0 ± 3.9% (1.0); mass of retinyl ester, 13.9 ± 4.4% (0.94 ± 0.28); newly synthesized [3H]RE, 16.1 ± 5.0% (1.07 ± 0.15); LRAT, 19.7 ± 6.5% (1.18 ± 0.47). These values for the combined supernatant and washes were as follows: protein, 37.6 ± 4.1% (1.0); 43.2 ± 8.7% (1.12 ± 0.19); newly synthesized [3H]RE, 43.8 ± 8.5% (1.13 ± 0.15); LRAT, 38.5 ± 7.7 (0.99 ± 0.11). None of these RSA values differed significantly from 1.0 ($P > 0.05$).
4 Significantly different from 1.0 ($P < 0.05$); ** significantly different from 1.0 ($P < 0.01$); *** significantly different from 1.0 ($P < 0.005$).

---

**RETINOL ESTERIFICATION AND HYDROLYSIS IN LIVER CELLS**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>HP-1</th>
<th>HP-2</th>
<th>NP</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, %</td>
<td>24.6 ± 7.6</td>
<td>19.8 ± 5.6</td>
<td>3.1 ± 1.2</td>
<td>80.3 ± 4.4</td>
</tr>
<tr>
<td>RSA</td>
<td>1.9</td>
<td>1.0</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Mass of RE, %</td>
<td>18.4 ± 5.5</td>
<td>13.1 ± 3.8</td>
<td>11.5 ± 4.7</td>
<td>91.1 ± 36.6</td>
</tr>
<tr>
<td>RSA</td>
<td>0.75 ± 0.11*</td>
<td>0.67 ± 0.11*</td>
<td>4.31 ± 2.02**</td>
<td>80.3 ± 4.4</td>
</tr>
<tr>
<td>New [3H]RE, %</td>
<td>16.8 ± 5.7</td>
<td>12.9 ± 5.5</td>
<td>10.4 ± 3.2</td>
<td>76.0 ± 8.0</td>
</tr>
<tr>
<td>RSA</td>
<td>0.94 ± 0.18*</td>
<td>0.65 ± 0.17*</td>
<td>3.65 ± 0.90***</td>
<td>76.0 ± 8.0</td>
</tr>
<tr>
<td>LRAT activity, %</td>
<td>16.4 ± 6.5</td>
<td>13.2 ± 2.9</td>
<td>10.6 ± 2.7</td>
<td>48.0 ± 8.3</td>
</tr>
<tr>
<td>RSA</td>
<td>0.67 ± 0.11*</td>
<td>0.69 ± 0.11*</td>
<td>3.82 ± 0.11***</td>
<td>76.0 ± 8.0</td>
</tr>
</tbody>
</table>
FIGURE 2  Comparison of the in vivo synthesis of [3H]retinyl esters (RE) and the in vitro assay of lecithin:retinol acyltransferase (LRAT) in whole liver and cell fractions. Panel A: Vitamin A-sufficient rats. Livers of four male rats were analyzed individually. Abbreviations used: Rem, remainder (see Fig. 1); HP-1, first hepatocyte fraction; HP-2, second hepatocyte fraction; NP, nonparenchymal cell fraction; SN, combined supernatants and washes. Panel B: Livers of three male vitamin A-deficient rats 18-20 h after the administration of 0.5 mg of 4-[(N-hydroxyphenyl)-retinamide (4-HPR). Because HP-1 and HP-2, and whole liver and the liver remainder, were similar to each other in the study in panel A, only whole liver, HP-1 and the NP fractions were analyzed for the livers shown in panel B. The dark, overlapping symbols near the origin represent whole liver, HP-1 and NP fractions from three vitamin A-deficient rats without 4-HPR treatment.

DISCUSSION

Previous studies have shown that hepatic stellate (fat-storing) cells, which represent less than 1% of liver protein mass (Blomhoff and Berg 1990, Hendriks et al. 1987), are the major cellular site of vitamin A storage. Using preparations of highly purified hepatocytes and sinusoidal cells (comprised of stellate cells, Kupffer and endothelial cells) Hendriks et al. (1985) found that, per milligram of protein, stellate cells of vitamin A-sufficient rats contained approximately 300 times as much RE as hepatocytes. The composition of stellate cell RE, mainly retinyl palmitate, stearate and oleate, was indistinguishable from that found in whole liver. In contrast to stellate cells, neither Kupffer nor endothelial cells, which also are constituents of the nonparenchymal cell fraction, 

TABLE 3

<table>
<thead>
<tr>
<th>Constituent</th>
<th>HP-1</th>
<th>HP-2</th>
<th>NP</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral REH, %</td>
<td>20.9 ± 3.5</td>
<td>23.4 ± 6.1</td>
<td>2.6 ± 1.2</td>
<td>106.5 ± 16.2</td>
</tr>
<tr>
<td>RSA</td>
<td>0.88 ± 0.15</td>
<td>1.20 ± 0.22</td>
<td>0.80 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Acid REH, %</td>
<td>21.2 ± 6.3</td>
<td>22.6 ± 4.4</td>
<td>3.2 ± 1.5</td>
<td>80.1 ± 6.6</td>
</tr>
<tr>
<td>RSA</td>
<td>0.87 ± 0.12</td>
<td>1.17 ± 0.15</td>
<td>1.04 ± 0.18</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are means ± SD for n = 4 livers from normal male rats. Abbreviations used: HP-1, first hepatocyte fraction; HP-2, second hepatocyte fraction; NP, nonparenchymal cell fraction (see Materials and Methods and Fig. 1); RSA (enrichment) is defined as the percentage of each REH in each cell fraction relative to protein (see Table 2) in the same fraction.

2 Recovery represents the total amount of each constituent recovered in all five fractions (the three cell fractions indicated and the remainder and combined supernatants and washings, see Materials and Methods, Fig. 1 and footnote 3 below), relative to the amount in the same whole liver homogenate.

3 Values are expressed as the percentage of the constituent recovered in each fraction relative to the amount recovered in all five fractions (taken as 100%). The RSA value for each fraction is defined as the percent of REH activity recovered divided by the percentage of protein in the same cell fraction. The specific activities of neutral and acid REH in whole liver homogenates were 167 ± 11.1 and 315.4 ± 37.9 pmol/(min·mg protein), respectively. The percentage and RSA values (in parentheses) for the remainder were as follows: neutral REH, 20.0 ± 7.7% (1.34 ± 0.45); acid REH, 17.3 ± 4.9% (1.14 ± 0.09). These values for the combined supernatants and washes were as follows: 33.1 ± 3.3% (0.89 ± 0.09); acid REH, 35.8 ± 3.0% (0.96 ± 0.06). None of these RSA differed significantly from 1.0 (P > 0.05).
have been shown to play an important role in RE storage or retinoid metabolism. Blaner et al. (1985) confirmed stellate cells to be the major site of RE deposition. These investigators calculated the quantity of liver retinoids and retinoid-related proteins in stellate cells of normal rat liver, taking into account the lower number and mass of nonparenchymal cells compared with hepatocytes. They estimated that stellate cells contain \( \sim 88\% \) of total retinoid, \(< 1\% \) of retinol-binding protein, \( 8\% \) of CRBP, \( 21\% \) of cellular retinoic acid-binding proteins, and \( 10\% \) of a bile salt–dependent retinyl palmitate hydrolase (which differs from the REH enzymes that we have measured).

The first two objectives of the present studies were to examine the cellular distribution of LRAT in liver cell fractions enriched in parenchymal and nonparenchymal cells (containing the stellate cells) of normal rats and to compare this with the distribution of LRAT in the liver of vitamin A–depleted rats in which LRAT activity was induced by retinoic acid or 4-HPR. During the fractionation process, all fractions and supernatants were saved so that the recovery, distribution, and RSA values for each constituent could be calculated. The semipurified, nonparenchymal cell–enriched fraction (depleted of hepatocytes) was analyzed due to a loss of LRAT localization and distribution of LRAT and REH enzymes being induced. We believe that the analytical approach used in the present study is preferable for drawing inferences about these constituents.

When RSA values were calculated for each of these constituents (Table 2), the enrichment in the nonparenchymal cell fraction of total RE, newly synthesized \([^{3}H]RE\) and LRAT, in comparison to total protein, was evident, with RSA values of 3.6–4.3 (compared with 1.0 in whole liver) for each entity. Concomitantly, the RSA values for these constituents in the hepatocyte fraction were each significantly \(< 1.0\), indicating that the contents of RE and LRAT, per amount of cell protein, were lower in the hepatocyte fraction than in liver as a whole. For the other materials collected for recovery analysis (tissue remainder, and combined supernatants and washes), the percentage recoveries for the entities of interest—protein, RE mass, new \([^{3}H]RE\), LRAT, acidic REH or neutral REH—did not differ significantly from one another \((P > 0.05)\) or from the recovery of protein, and therefore the RSA values for RE, LRAT and REH enzymes in these fractions also did not differ from 1.0. These data indicate that although these fractions contained a substantial amount of liver protein, they were neither depleted nor enriched in any of the constituents measured and therefore do not affect the calculations of distribution or RSA for hepatocytes or nonparenchymal cells. Thus, in comparison to the RSA values for these constituents in hepatocytes, their RSA values in nonparenchymal cells are approximately five- to sixfold greater. Previous work (Blaner et al. 1985, Hendriks et al. 1985) demonstrated that, among cells of the nonparenchymal fraction, only stellate cells have an important role in vitamin A metabolism. In agreement, we found that the LRAT specific activity of Kupffer cells purified by Percoll density gradient centrifugation (Matsuura et al. 1989) was very low (less than 6% of that in the stellate cells from the same preparation; T. Matsuura and A. C. Ross, unpublished results). Thus, even though endothelial and Kupffer cells are present in the nonparenchymal cell fraction, it is unlikely that they affected the results for retinoid content or LRAT activity other than by contributing protein and thereby lowering (by dilution) the calculated RSA values for LRAT activity and retinoids. If RE is present nearly entirely in stellate cells of liver, then the co-enrichment of LRAT activity and RE implies that nearly all LRAT activity in the nonparenchymal cell fraction is also present in stellate cells. Although the RSA for LRAT activity in nonparenchymal cells exceeds that in hepatocytes by five- to sixfold, this may be a conservative estimate of its enrichment, because if nearly all RE are found in stellate cells and LRAT and RE are equally co-enriched in the nonparenchymal cell fraction (Table 2), then, based on co-localization, LRAT also is located primarily in stellate cells. This conclusion differs from that of Blaner et al. (1990), who interpreted their data from pronase E-Nycodenz centrifugation studies as showing that approximately 85% of LRAT activity resides in parenchymal cells. These authors did not, however, apply methods of analytical differential centrifugation that included recoveries of protein and the various cell constituents assayed. We believe that the analytical approach used in the present study is preferable for drawing inferences about the localization and distribution of LRAT and REH enzymes between liver parenchymal and nonparenchymal cells.

A third objective of our study was to compare the enrichment of newly formed \([^{3}H]RE\) and LRAT activity in nonparenchymal cells from vitamin A–sufficient rats and –deficient rats treated with retinoic acid or 4-HPR. As is shown in Table 1 and Figure 2, similar results were obtained in each case, with the greatest \([^{3}H]RE\) content and LRAT being present in nonparenchymal cells. These data imply that the LRAT activity induced by acute retinoid treatment has the same cellular distribution as LRAT activity in vitamin A–sufficient liver in the normal steady state.

Numerous results have led to the suggestion that liver stellate cells play an important role in hepatic fibrogenesis, particularly in alcoholic liver disease (Hautekeete et al. 1993, Horn et al. 1986), in which the liver pathology includes a type of cytoskeletal intermediate filament, desmin, and a form of microfilament, alpha-smooth muscle actin, in stellate cells (Gressner 1995, Yokoi et al. 1984). However, these cytoskeletal elements are also present in fibroblasts and smooth muscle cells, and desmin could not be detected in stellate cells in human liver (Enzan et al. 1994, Nouchi et al. 1991). Therefore, the utility of these proteins as markers of stellate cells is questionable. The enrichment of LRAT activity in rat liver stellate cells and the ability to detect LRAT in human liver (McDonald and Ong 1988) suggest that, if LRAT in human liver also is enriched in stellate cells, then the assay of LRAT activity may prove useful as a marker of stellate cells in future studies of human liver disease.

A fourth goal of this work was to provide comparative data on the cellular location of enzymes that esterify and hydrolyze retinyl esters. The cellular distribution of bile salt–indepen dent REH activities has not been studied previously. A comparison of the neutral and acid REH activities measured in this study with LRAT revealed two major differences. First, in contrast to LRAT, neither the neutral or acid bile salt–independent REH activities differed between vitamin A–sufficient and –deficient rats (Table 3). Second, the cellular distributions and RSA values of the neutral and acid REH did not reveal any preferential enrichment in either the parenchymal or nonparenchymal cell fractions. In previous work, the cellular distribution of a neutral bile salt–dependent REH (retinyl palmitate hydrolase) was also shown to be uniform, on the basis of cell protein, throughout normal rat liver (Blaner et al.
(1985). Although the exact physiological roles of the bile salt–dependent and –independent REH have yet to be clarified, the subcellular location of the bile salt–independent REH in liver plasma membranes and endosomes suggests that these enzymes may be involved in the initial metabolism of chylomicron RE (Gad and Harrison 1991, Harrison and Gad 1989, Harrison et al. 1995). Chylomicron up take occurs almost exclusively in hepatocytes. The localization of both acid and neutral bile salt–independent REH in both parenchymal and nonparenchymal cell suggests that these enzymes also may have a role in mobilizing stored vitamin A esters.

In conclusion, the results of this study demonstrate that LRAT and REH activities are differentially regulated by vitamin A. They also provide evidence that the enzymes capable of esterifying CRBP-bound retinol and of hydrolyzing RE in the absence of bile salts have spatially distinct distributions in the livers of both normal and retinoid-treated rats.

LITERATURE CITED


