Hepatic uptake and metabolism of chylomicron retinyl esters: probable role of plasma membrane/endosomal retinyl ester hydrolases

Earl H. Harrison,[1] Mohamed Z. Gad,[2] and A. Catharine Ross[3]

Division of Nutrition, Department of Biochemistry, Medical College of Pennsylvania, 2900 Queen Lane, Philadelphia, PA 19129

Abstract Previous studies have indicated the presence of both neutral and acid, bile salt-independent retinyl ester hydrolases associated with plasma membrane and endosome fractions of rat liver homogenates. In the present studies, chylomicrons containing tritium-labeled retinyl esters were injected intravenously into rats in order to study the initial metabolism of retinyl esters during and after uptake into the liver. At various times after chylomicron injection, plasma was obtained and the liver was homogenized and subjected to analytical subcellular fractionation. Labeled retinyl esters were rapidly cleared from plasma (half-time ≈10 min) and appeared in the liver. Within the liver, label first appeared in plasma membrane/endosomal fractions that were also enriched in both neutral and acid, bile salt-independent retinyl ester hydrolase activities. At no time were the labeled esters significantly associated with fractions enriched in lysosomes. Rather, it appeared that the labeled esters were hydrolyzed and/or transferred to fractions enriched in endoplasmic reticulum. ■ These studies demonstrate the co-localization of newly delivered retinyl esters and bile salt-independent retinyl ester hydrolase enzyme activities and thus, suggest a probable role for these enzymes in the initial hepatic metabolism of chylomicron retinyl esters. This conclusion was further supported by the observation that plasma membrane/endosomal fractions were active in catalyzing the hydrolysis of chylomicron remnant retinyl esters in vitro.—Harrison, E. H., M. Z. Gad, and A. C. Ross. Hepatic uptake and metabolism of chylomicron retinyl esters: probable role of plasma membrane/endosomal retinyl ester hydrolases. *J. Lipid Res.* 1995. **36:** 1498–1506.

Supplementary key words retinoid · vitamin A · metabolism · rat

During intestinal absorption, dietary vitamin A is converted to retinyl esters, incorporated along with other neutral lipids into chylomicrons, and secreted into the lymphatics (1). In the vascular compartment much of the chylomicron triacylglycerol is hydrolyzed by lipoprotein lipase in extrahepatic tissues, resulting in the production of a chylomicron remnant (2). During this process, virtually all of the newly absorbed retinyl esters remain associated with the chylomicron remnant. In the rat, the chylomicron remnants are rapidly and almost quantitatively taken up by the liver, and there is evidence that the retinyl esters are rapidly hydrolyzed and reesterified during this process (3, 4). Chylomicron retinyl esters are initially taken up exclusively by the hepatocytes but are then largely transferred to the perisinusoidal stellate cells (4). During the initial uptake process the retinyl esters are associated with low density endosomes and then transferred to higher density vesicles, without being delivered to lysosomes (5).

As hydrolysis of retinyl esters occurs during both the hepatic uptake and the mobilization of retinyl ester stores from the liver, hepatic enzymes catalyzing the hydrolysis of retinyl esters are important in the body's economy of vitamin A. Earlier studies focused on a neutral, bile salt-dependent retinyl ester hydrolase activity that is now appreciated to be to the enzyme carboxylester lipase (6). This enzyme is secreted by the pancreas into the intestinal lumen where it can presumably hydrolyze dietary retinyl esters and other lipid esters (7–10). The enzyme is also secreted by the liver (11) and its role in hepatic retinol metabolism is under investigation, but is unclear at present. In addition to carboxylester lipase, a number of tissues contain membrane-bound, bile salt-independent retinyl ester hydrolase activities (12–14). We have previously demonstrated the occurrence of both acid and neutral retinyl ester hydrolase activities associated with rat liver preparations

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Abbreviations: REH, retinyl ester hydrolase; LRAT, Lecithin:retinol acyltransferase; ARAT, acyl-CoA:retinol acyltransferase.
[1] To whom correspondence should be addressed.
[2] Current address: Biochemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.
[3] Current address: Department of Nutrition, Pennsylvania State University, University Park, PA.

enriched in plasma membranes and/or endosomes (9, 15). We speculated that these activities might be important in hepatic metabolism of chylomicron remnant retinyl esters.

In the present investigations we have used quantitative subcellular fractionation techniques to characterize the hepatic uptake and intracellular fate of chylomicron retinyl esters and to further explore the localization of the membrane-associated retinyl ester hydrolases. The results demonstrate that soon after their internalization, the newly endocytosed retinyl esters and the retinyl ester hydrolases are co-localized in endosomal vesicles. Thus, the enzymes are localized in a compartment that allows them to interact with the chylomicron retinyl esters and that also provides a means for the rapid hydrolysis of retinyl esters without their delivery to lysosomes.

MATERIALS AND METHODS

Animals

Male, Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) or Ace Animals Inc. (Boyertown, PA) and housed in our animal facilities where they were fed a commercial diet (Wayne Lablox) ad libitum. Adult, non-fasted animals weighing 200–400 g were used in most of the experiments.

Preparations of labeled chylomicrons, chylomicron remnants, and asialofetuin

Chylomicrons were prepared according to the method of Lenich and Ross (16). Briefly, rats were anesthetized and cannulas were inserted into the upper duodenum and mesenteric lymph duct. About 150 µCi of [15-^{3}H](N)-retinol (27 Ci/mmol, New England Nuclear, Wilmington, DE) and 40 nmol of unlabeled retinol were dissolved in 0.25 mL of safflower oil and infused through the duodenal cannula. Lymph was collected on ice at intervals for periods of up to 12 h and chylomicrons were collected by ultracentrifugation (17,500 rpm for 30 min) as described previously (17).

Chylomicron remnants were prepared by incubating chylomicrons (about 50 mg triglycerides) at 37°C with 1.5 mL postheparin rat plasma and 26.5 mL 0.2 M Tris-HCl (pH 8) containing 1.7 g of bovine serum albumin for about 2 h until the turbidity of the mixture had cleared. The mixture was then layered with saline and centrifuged at 44,000 rpm for 18 h to isolate the remnants. Postheparin plasma was prepared from the blood of rats injected with heparin (500 U/kg) 10 min prior to the blood collection.

Measurements of triglycerides (18) indicated that the chylomicron preparations contained about 30 mg triglycerides per mL, while the remnants contained

about 0.2 mg per mL. Specific activities of the labeled retinoid-containing lipoproteins were 0.07 µCi/mg triglyceride for the chylomicrons and 0.8 µCi/mg triglyceride for the chylomicron remnants. About 97% of the labeled retinoid in both lipoproteins was in the form of retinyl ester.

Asialofetuin (Type I) was purchased from Sigma Chemical Co. (St. Louis, MO) and iodinated by the iodine monochloride method (19) to a specific activity of 13 µCi/mg protein. The iodinated protein was dissolved in normal saline for injection into the animals.

Hepatic uptake of labeled chylomicrons and asialofetuin

Labeled chylomicrons, in volumes of 0.5–0.65 mL, were injected into the exposed right femoral vein of rats that were lightly anesthetized with diethyl ether. In some experiments labeled asialofetuin was injected along with the chylomicrons. Three minutes prior to each of the studied time points animals were reanesthetized with ether and a midline incision was made to expose the vena cava. Approximately 5 mL of blood was drawn from the vena cava for the preparation of plasma by centrifugation. The liver was immediately perfused through the portal vein with approximately 55 mL of ice-cold 0.25 M sucrose. The liver was removed, rinsed with 0.25 M sucrose, blotted on paper towels, weighed, and minced with a tissue press, prior to homogenization as described below. Care was taken to keep the tissue at or near 4°C after perfusion.

Preparation and analysis of subcellular fractions

Rat liver homogenates were prepared and fractionated by differential centrifugation using the methods described in detail in our previous publications (9, 15). Four fractions were obtained: the nuclear (N) fraction, the mitochondrial-lysosomal (ML) fraction, the microsomal (P) fraction, and the high speed supernatant (S) fraction. In some experiments the P fraction was further fractionated on discontinuous sucrose density gradients as described by Touster et al. (20) to yield fractions enriched in plasma membranes and endosomes.

Homogenates and subcellular fractions were assayed for bile salt-independent, acid or neutral retinyl ester hydrolase activities as previously described (9, 15). Briefly, reaction mixtures were prepared in a volume of 190 µL and contained buffer and appropriately diluted subcellular fractions. Neutral hydrolase activities were assessed in mixtures containing 50 mM Tris-HCl, pH 7, while acid REH activity was assayed with 50 mM sodium acetate, pH 5. Substrate, retinyl [1-^{14}C]palmitic acid (0.05 µCi, 8 nmol) was added in 10 µL of ethanol to start the reaction. After incubation at 37°C for 30 min the reactions were stopped and released free palmitic acid

was extracted and quantitated (9). Marker enzymes for lysosomes (N-acetyl-β-glucosaminidase), plasma membranes and endosomes (alkaline phosphodiesterase), and endoplasmic reticulum (nonspecific esterase) were assayed as previously described (9, 15). Protein was assayed by a modification of the Lowry procedure (21).

Analysis of [³H]retinoids and ¹²⁵I-labeled asialofetuin

Neutral lipids and retinoids were extracted from aliquots of the injected dose, plasma, and homogenate fractions using the ethanol-hexane procedure previously described (22). [³H]retinoids were quantified by counting an aliquot of the dried hexane extract in a liquid scintillation counter. In some experiments, other aliquots of the extract were applied to small columns of aluminum oxide to determine the distribution of radioactivity between free and esterified retinol (23). ¹²⁵I-labeled asialofetuin was quantified by directly counting aliquots of the samples in a gamma counter.

Hydrolysis of labeled chylomicron remnants in vitro

[³H]retinol-labeled chylomicron remnants were prepared as described above and incubated with either whole microsomes or with isolated plasma membranes/endosomes in a final volume of 0.2 mL. Incubations were carried out at both pH 5 and pH 7. At the end of the incubations, 2 mL ethanol was added, followed by 5 mL hexane, and 2 mL water. After vigorous mixing and separation of the phases, the upper phase (hexane) was removed and saved and the lower phase was reextracted with another 5-mL portion of hexane. The hexane phases were combined, concentrated, and applied to small columns of aluminum oxide to separate free and esterified retinol as described above.

RESULTS

Hepatic uptake of [³H]retinoid-labeled chylomicrons

Figure 1 summarizes a number of experiments in which the kinetics of the plasma clearance and hepatic uptake of [³H]retinoids were studied after the injection of [³H]retinoid-labeled chylomicrons. The label was rapidly cleared from the plasma with a half-time of about 10 min, with concomitant uptake into the liver. At times up to 30 min the hexane-soluble material in plasma and liver together accounted for more than 85% of the injected radioactivity. Thus, less than 15% of the injected chylomicrons were initially sequestered at sites other than the liver. However, after this initial hepatic uptake, which reached a maximum near 30 min, ³H in liver thereafter steadily declined; between 30 min and 2 h about 30% of the injected dose was lost from the liver and could not be accounted for by reappearance in the

plasma. We considered three possible explanations for this loss. First, it is possible that the labeled retinol was being converted to acidic metabolites that were not extracted by the solvent system used. However, when the liver samples were directly solubilized and assayed for radioactivity without any lipid extraction, identical amounts of radioactivity were found as with the lipid extracts. We next considered that there might be significant biliary excretion of [³H]retinoid metabolites during the experiment. Therefore, an experiment was conducted with four animals that were fitted with bile duct cannulas prior to the injection of [³H]retinoid-labeled chylomicrons. After 2 h only 1.0 ± 0.2% of the injected dose was excreted into bile. Finally, we considered the unlikely possibility that the tritium atoms were exchanging off the labeled retinoids. However, in an experiment in which ¹⁴C-labeled retinol was used as a tracer the same loss was observed from the liver between 30 min and 2 h. Thus, we speculate that the unaccounted for radioactivity represents retinol that was secreted from the liver and delivered to peripheral tissues not analyzed in our experiments.
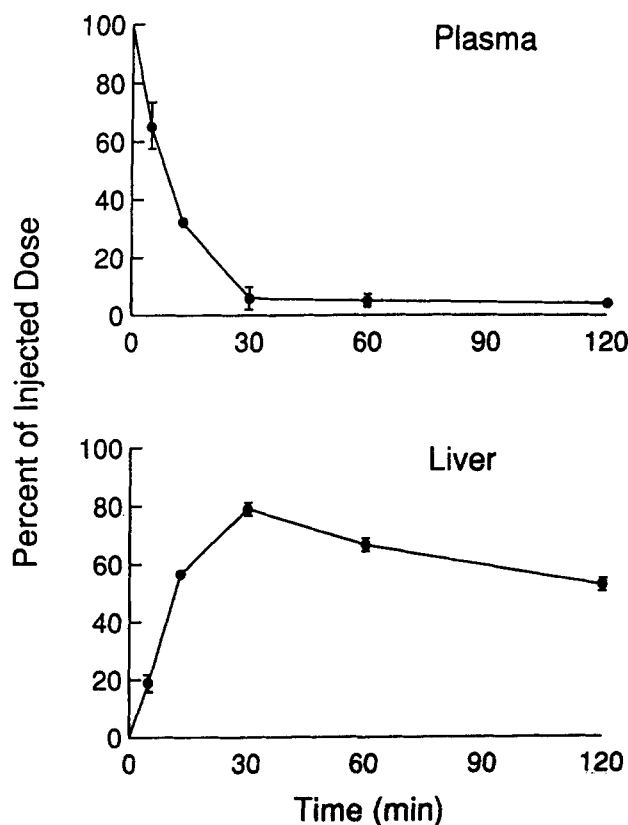

Fig. 1. Plasma clearance and hepatic uptake of ³H-labeled chylomicrons. Rats were injected with ³H-labeled chylomicrons and analyzed at the indicated time points as described in the text. The numbers of determinations at the various time points were: six at 5 min, one at 13 min, four at 30 min, two at 60 min, and five at 120 min. Points represent the mean and error bars indicate the standard deviation.

In experiments in which ^{125}I-labeled asialofetuin was injected simultaneously with labeled chylomicrons, we observed that the asialoglycoprotein was cleared from the plasma and taken up by the liver more rapidly than the labeled chylomicrons. Our data for asialofetuin were nearly identical to those reported previously for this ligand, known to be internalized by receptor-mediated endocytosis after binding to the asialoglycoprotein receptor on hepatocyte plasma membranes (24, 25). Thus, the asialoglycoprotein was cleared from the plasma with a half-time of less than 5 min. Peak accumulation in liver occurred at 10 min with about 85% of the injected dose recovered at that time. Thereafter, there was a rapid loss from the liver, presumably reflecting the degradation to free amino acids, so that less than 20% of injected dose remained after 30 min and less than 10% remained after 60 min.

Subcellular localization of [3H]retinoids newly delivered to the liver

Complete subcellular fractionations of liver were carried out after the injection of [3H]retinoid-labeled chylomicrons into 14 individual animals. The results of these experiments are summarized in **Fig. 2.** At early times (up to 30 min) during hepatic uptake, the [3H]retinoids appeared to accumulate in both the microsomal
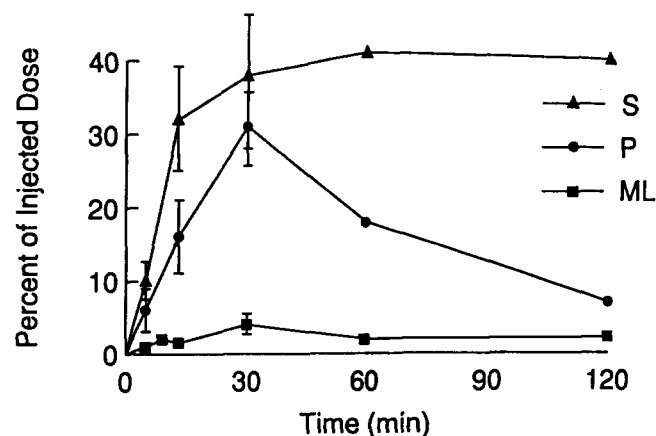
Fig. 2. Accumulation of [3H]retinoid in subcellular fractions of rat liver after injection of [3H]retinoid-labeled chylomicrons. At each time point liver homogenates were prepared and fractionated by differential centrifugation in nuclear (N), mitochondrial-lysosomal (ML), microsomal (P), and soluble (S) fractions as described in the text. The N fraction contained less than 3% of the injected radioactivity at all times and is not shown on the figure. Data are summarized for fourteen individual fractionations: three done at 5 min, one at 9 min, four at 13 min, three at 30 min, one at 60 min, and two at 120 min. For time points with multiple determinations, points represent the mean and error bars represent the standard deviation (n ≅3 3) or range (n = 2) in cases where they are larger than the size of the symbols. Total recovery of radioactivity (i.e., the sum of the fractions relative to the amount in the whole homogenate) was 94.2 ± 4.7% (n = 14).

(P) and soluble (S) fractions of the homogenates. At later times there was a loss from the microsomal fraction that mirrored quantitatively the loss from whole liver (cf. Fig. 1) without concomitant increase in any other fraction. Two points should be made here. First, there was at no time a significant accumulation of [3H]retinoid in the ML fraction that contained the bulk of the lysosomes. The ML fraction prepared from these rats contained 66 ± 7% of the recovered lysosomal marker, N-acetyl β-glucosaminidase, which is essentially the same as the 60 ± 7% of this marker in this fraction prepared from uninjected rats (21). Second, there was no obvious indication of a precursor-product relationship between the radioactivity in the P fraction and that in the S fraction. Indeed, [3H]retinoid appeared rapidly in the S fraction and remained high throughout the 2-h study.

The accumulation of asialofetuin in the subcellular fractions differed both quantitatively and qualitatively from that of the labeled retinoid (data not shown) but was very similar to that reported by LaBadie, Chapman, and Aronson (24) and Dunn, LaBadie, and Aronson (25). Thus, at early times (5–10 min) there was peak accumulation of almost 60% of injected dose in the P fraction with only 15–20% in the S fraction at these same times. Therefore, at the time of maximal hepatic uptake of this ligand, known to be internalized by receptor-mediated endocytosis, most of it was associated with components of the P fraction, presumably endosomes. These contradicting results led us to be concerned that the much larger amounts of labeled chylomicrons associated with the S fraction during early times of uptake might not accurately reflect the subcellular site of the ligand after uptake, but rather represent chylomicrons trapped in the liver but not internalized into the cells. In order to further assess this possibility, a number of liver perfusion experiments were carried out that are described briefly below.

In two separate experiments involving 17 rats, labeled chylomicrons were injected and, after 9–13 min, the liver was perfused with 175–200 mL of 0.25 M sucrose alone or with this solution supplemented with 5 mM EDTA or 2 mg/mL sodium heparin (Grade II, 167 USP units/mg, Sigma Chemical Co., St. Louis, MO). Overall, these experiments showed that simple perfusion, even with EDTA or heparin, did not markedly reduce the total [3H]retinoid in the liver or that associated with the soluble components upon fractionation (i.e., about two-thirds was associated with the soluble fraction and one-third with the P fraction). If, however, the liver was perfused with collagenase in order to prepare isolated cells and the cells were then fractionated, then about two-thirds of the cell-associated radioactivity was found in the P fraction and only one-third with the soluble fraction. We interpret these data to indicate that at early

times after chylomicron delivery, about half of the radioactivity associated with the soluble fraction of the homogenate upon fractionation represents chylomicrons that are in the space of Disse but not yet internalized. Thus, components of the microsomes account for a greater proportion of the internalized chylomicrons than the data in Fig. 2 would suggest. The experiments described below focus in more detail on the components of the microsomal fraction.

Subfractionation of microsomes: co-localization of [³H]retinoids newly delivered to the liver and retinyl ester hydrolase enzyme activities

These studies were designed to examine the potential role of neutral and acid retinyl ester hydrolase activities in the microsomal fraction in the initial hepatic uptake and metabolism of chylomicron remnant retinyl esters by the liver. Rats were injected with [³H]retinoid-labeled chylomicrons and killed at either 13 or 60 min. These times were chosen to represent the early "accumulation phase" and a later time to allow for intracellular processing. Livers were then fractionated by differential centrifugation to obtain the microsomal (P) fraction in the usual way. As previous results had indicated that both neutral and acid REH activities were enriched in a plasma membrane/endosome-rich fraction of microsomes (15), we were interested in examining in more detail the localization of chylomicron-derived [³H]retinoids in the microsomal fraction. Therefore, the membrane components of the microsomes were further resolved into three fractions as described previously (20) and the distributions of marker enzymes, REH activities,

TABLE 1. Distribution of constituents in subfractions of microsomes

| | Percent of Recovered Amount | | | |
Constituent	P2	P3	P4	Recovery
				%
Protein (4)	3 ± 1	31 ± 8	65 ± 8	99 ± 20
NS esterase (4)	2 ± 1	28 ± 8	70 ± 8	103 ± 26
Phosphodiesterase (4)	16 ± 3	57 ± 9	26 ± 10	78 ± 10
Neutral REH (4)	24 ± 4	30 ± 7	44 ± 4	132 ± 31
Acid REH (4)	12 ± 3	45 ± 9	42 ± 9	75 ± 9
Labeled retinoids				
13 min	45,56	17,25	16,23	133,178
60 min	30,31	25,30	36,42	93,108

Microsomes were fractionated by the method of Touster et al. (20) into P1 (floating fat), P2 (plasma membrane/endosome-rich fraction), P3 (multivesicular bodies and some vesicles derived from the Golgi and ER), and P4 (most of microsomal protein and most vesicles derived from ER). The P1 fraction contained no protein and is excluded from the table. Results are given as the mean ± SD for the four rats injected with labeled chylomicrons or, for retinoids, separately for the two animals killed at 13 min and for the two killed at 60 min. Recovery (in percent) represents the total amount recovered in the microsomal subfractions relative to the amount in unfractionated microsomes.

TABLE 2. Enrichment of labeled retinoids, retinyl ester hydrolases, and enzyme markers in the plasma membrane/endosomal fraction

Constituent	Relative Specific Activity	Recovery
		%
Protein (4)	1	89 ± 12
N-Acetyl-β-glucocosaminidase (4)	1 ± 0	105 ± 15
NS esterase (4)	1 ± 0	101 ± 22
Phosphodiesterase (4)	8 ± 2	107 ± 8
Neutral REH (4)	20 ± 5	147 ± 9
Acid REH (4)	9 ± 1	106 ± 11
Labeled retinoids		
13 min	17,18	131,145
60 min	7,14	96,106

The plasma membrane/endosome fraction (P2) was isolated as described in Table 1. Relative specific activity (RSA) is the percent of the constituent recovered in the P2 fraction divided by the percent of total protein recovered in the fraction. Numbers represent the mean ± SD for the four rats injected with labeled chylomicrons or, for retinoids, individually for the two animals killed at 13 min and for the two killed at 60 min. Recovery (in percent) represents the sum of the constituent in all fractions (i.e., microsomal subfractions plus the nuclear, mitochondrial/lysosomal and soluble fractions) relative to the amount in the whole liver homogenate.

and [³H]retinoids were determined in the subfractions. The results are shown in **Table 1.** At 13 min after injection of chylomicrons, about 50% of microsomal [³H]retinoids were associated with the plasma membrane-endosome fraction (P2). Later, at 60 min, the amount of radioactivity in this fraction was lower and more was associated with denser vesicles, especially those in the fraction containing most of the endoplasmic reticulum (P4). In **Table 2** these fractionation data are presented as the relative specific activity of each constituent in the plasma membrane/endosome (P2) fraction. Note that both the retinyl ester hydrolases and the [³H]retinoids are enriched in this fraction to an equal or greater extent than the plasma membrane/endosome marker, alkaline phosphodiesterase, and that this fraction is not enriched in the endoplasmic reticulum marker, nonspecific esterase or in the lysosomal marker, N-acetyl β-glucosaminidase. It should be noted that the distributions of enzyme markers in the present experiments on the subfractionation of microsomes from chylomicron-injected rats did not differ from those of normal, uninjected rats reported previously (22). Thus, the load of chylomicrons used in the present experiments did not affect the density or sedimentation properties of endosomes, lysosomes, or other subcellular components.

Free and esterified retinol in subcellular fractions

The distribution of radioactivity between unesterified and esterified retinol in various subcellular fractions

TABLE 3. Percent distribution of esterified and free retinol in liver subcellular fractions of rats injected with labeled chylomicrons

Fraction	13 Min		60 Min	
	Ester	Free	Ester	Free
		%		
Homogenate	93 ± 1	7 ± 1	82 ± 1	18 ± 1
Soluble	94 ± 1	6 ± 1	96 ± 3	4 ± 3
Microsomes	88 ± 12	12 ± 12	56 ± 0	44 ± 0
P2	93 ± 1	7 ± 1	82 ± 3	18 ± 3
P3	50 ± 14	50 ± 14	51 ± 10	49 ± 10
P4	62 ± 6	38 ± 6	35 ± 2	65 ± 2

Results are given as mean ± range for two rats.

TABLE 4. In vitro incubations of microsomes and plasma membrane/endosome-rich fractions with chylomicron remnants containing [³H]retinyl esters

Incubation Conditions	Percent Hydrolysis	
	1 h	4 h
Neutral pH (pH 7)		
No enzyme	6	6 ± 2
Microsomes (75 µg protein)	6	11 ± 0
Plasma membranes/endosomes (42 µg protein)	18	24 ± 2
Acid pH (pH 5)		
No enzyme	4	7 ± 3
Microsomes (75 µg protein)	5	16 ± 0
Plasma membranes/endosomes (42 µg protein)	15	23 ± 5

The indicated amounts of microsomes (P fraction) or plasma membranes/endosomes (P2 subfraction) were incubated in 50 mM Tris-HCl, pH 7, or 50 mM sodium acetate, pH 5, with chylomicron remnants containing 25 pmol (0.015 µCi) of [³H]retinyl ester in a final volume of 0.2 mL. At the end of the incubation, neutral retinoids were extracted and retinyl esters and unesterified retinol were separated by chromatography on aluminum oxide. Percent hydrolysis indicates percent of label as free retinol. Unincubated remnants contained 3% unesterified retinol. For the 4-h incubations, results are presented as the mean ± range of two experiments.

after the injection of labeled retinyl ester-containing chylomicrons is shown in **Table 3.** Nearly all of the radioactivity recovered in the cytosol was in ester form either 13 min or 60 min after injection of chylomicrons, resembling the ratio in the chylomicron dose and, at 60 min, exceeding the percentage in the liver whole homogenate. On the other hand, a clear difference in the ratio of esterified to free retinol was observed in microsomes when compared at 13 and 60 min. Whereas at 13 min only 12% of microsomal ³H-labeled retinoid was free, at 60 min the percent free retinol had increased to 44%. It is also quite striking that, at either time point, there is a much greater fraction of the retinoid in the free form in the denser microsomal subfractions (P3 and P4) than in the plasma membrane/endosome-enriched fraction (P2). Thus, between 13 and 60 min after chylomicron injection, the fraction of unesterified retinol increased markedly in all microsomal fractions, with a progressive increase from the least dense to the most dense fractions. The difference in the ester/free ratio in the plasma membrane/endosomes and the denser vesicles suggests that hydrolysis of retinyl esters in the endosomes and transfer of retinol to other membranes may have occurred.

In vitro hydrolysis of chylomicron remnant retinyl esters by isolated microsomes and plasma membrane/endosome fractions

Table 4 presents the results of experiments in which chylomicron remnants containing labeled retinyl esters were incubated in vitro with membrane fractions known to contain the acid and neutral, bile salt-independent retinyl ester hydrolases (i.e., microsomes and plasma membrane/endosome fractions). At 1 h of incubation only the plasma membrane/endosome fraction that is highly enriched in the hydrolases catalyzed significant hydrolysis of the esters above the no-enzyme controls. This was observed at both acid and neutral pH. When incubations were extended for 4 h, both whole mi-

crosomes and the plasma membrane/endosome fractions were demonstrably active. As these experiments were conducted with about twice as much microsomal protein as that present for the plasma membrane/endosomal fraction, the actual increase in the specific activity of the latter fraction over the microsomes is 3- to 4-fold. This ratio agrees well with that observed with these two fractions using the routine hydrolase assays. These results suggest that the bile salt-independent retinyl ester hydrolases catalyze the hydrolysis remnant retinyl esters.

DISCUSSION

The results of the quantitative fractionation experiments presented here confirm and extend current concepts of the hepatic metabolism of chylomicron retinyl esters, providing new information on the probable role of endosomal retinyl ester hydrolases in this process. **Figure 3** outlines a possible pathway for the hepatic metabolism of chylomicron retinyl esters that is consistent with the available experimental data. It is now thought that the first step in the hepatic metabolism of chylomicron remnants is their "sequestration" in the space of Disse by the binding of apoE to heparin sulfate proteoglycans on the cell surface (2). While in the space of Disse, some of the neutral lipid ester may be hydrolyzed by secreted lipases such as hepatic lipase and lipoprotein lipase (26–29). As the bile salt-dependent carboxylester lipase is also secreted from liver cells (11),
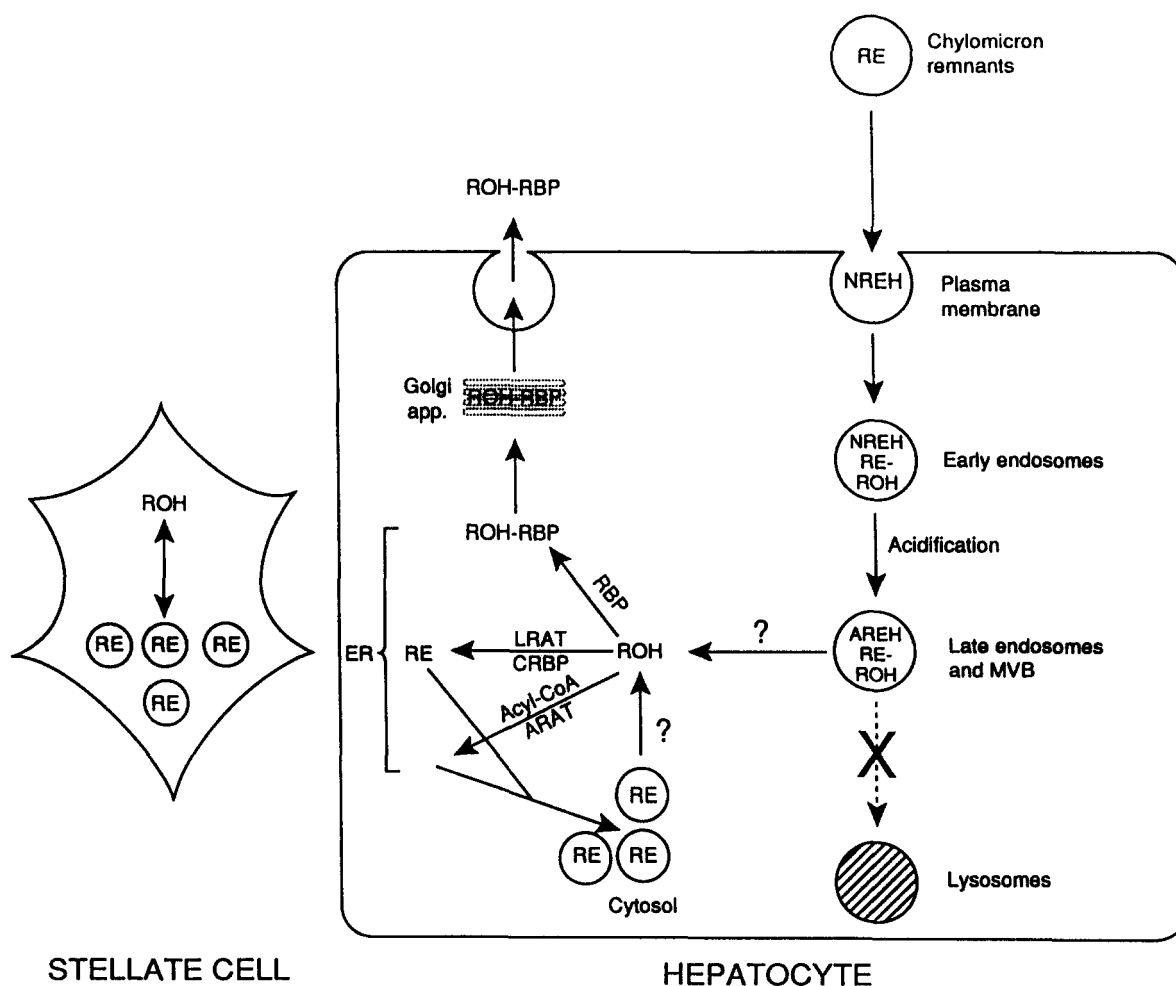
Fig. 3. Schematic diagram of the hepatic metabolism of chylomicron retinyl esters (RE) in the rat. On the right, the possible roles of neutral and acid retinyl ester hydrolases (NREH and AREH) associated with plasma membranes, endosomes, and multivesicular bodies (MVB) in the hydrolysis of RE to free retinol (ROH) are highlighted. On the left, the fates of ROH after delivery to the endoplasmic reticulum (ER) are outlined. These include the formation of the retinol-retinol binding protein complex (ROH-RBP) and its secretion from the liver, and esterification of retinol by the ER enzyme, lecithin:retinol acyltransferase (LRAT) which utilizes retinol bound to cellular retinol binding protein (CRBP) as substrate, or esterification by acyl-CoA:retinol acyltransferase (ARAT).

it is possible that it may play some role in the metabolism of remnants in the space of Disse, although there is currently no evidence to directly support this possibility.

The results of the present studies suggest that chylomicron remnant retinyl esters could be hydrolyzed at the cell surface by the bile salt-independent, neutral retinyl ester hydrolase investigated in the present studies. The same enzyme could continue to catalyze the hydrolysis of retinyl esters after internalization of the remnant in early endosomes. As the endosomal pH gradually falls to < 6 during acidification, the neutral hydrolase would be less active and the acid retinyl ester hydrolase that is also present in these vesicles might play a greater role. Although we have distinguished the acid (pH 5) and neutral (pH 7) REH activities on a number

of bases (9, 15), it is important to point out that the total activity in microsomes (i.e., the sum of the two activities) actually shows > 75% of maximal activity over the broad pH range of pH 5–8 (see Fig. 1 in ref. 15). Thus, we would suggest that both the acid and neutral REHs function similarly in the hydrolysis of chylomicron retinyl esters during initial hepatic uptake. The relative contribution of each enzyme activity to the total activity might change as the endosomal pH drops.

An important conclusion of the work of Blomhoff et al. (5), and our previous (15) and present work, is the apparent lack of involvement of lysosomes per se in the metabolism of chylomicron retinyl esters. Thus, cell fractionation experiments failed to demonstrate the accumulation of retinoid in fractions enriched in

lysosomes. In the experiments by both groups, when labeled asialoglycoproteins were used as positive controls, accumulation of label was observed in lysosome-rich fractions. Thus, chylomicron remnants and other ligands delivered by receptor-mediated endocytosis appear to take different intracellular pathways after initial uptake. This is not surprising in light of recent evidence that even different lipoproteins undergo different fates after uptake into cells (30).

If lysosomal enzymes are not involved in the hepatic metabolism of chylomicron retinyl esters, then other enzymes must be responsible for the rapid hydrolysis that is known to take place during or shortly after their hepatic uptake (3, 4). The present results that demonstrate the co-localization of newly endocytosed retinoid and bile salt-independent retinyl ester hydrolases provide evidence that these latter enzymes are likely to be important in this process. Further support for this suggestion comes from the demonstration that membrane fractions enriched in the retinyl ester hydrolases catalyze the hydrolysis of chylomicron remnant retinyl esters in vitro. It is not yet possible to compare directly the rates of hydrolysis of retinyl esters observed in vitro with those that occur in vivo. Nonetheless, comparison of Tables 3 and 4 indicate that the net percent hydrolysis observed after 1 h at both pH 5 and pH 7 in in vitro incubations with plasma membrane/endosomal fractions (about 12%, Table 4) is similar to the net extent of hydrolysis in these fractions 1 h after delivery of chylomicrons to the liver in vivo (about 18%, Table 3).

Both the present experiments and previous work suggest that after uptake and hydrolysis of retinyl esters, the unesterified retinol is transferred to the endoplasmic reticulum for further metabolism. The mechanism of this transfer is not known, but it may involve cellular retinol-binding protein (CRBP), if transfer occurs through the cytoplasm. Given the limited aqueous solubility of retinol, it is unlikely to exist in cytoplasm in true solution. There is sufficient cytosolic CRBP in liver to bind all of the unesterified retinol (22). Regardless of the mechanism of transfer to endoplasmic reticulum, it is also clear that, in the steady state, a significant fraction (about one-third) of the unesterified retinol is localized there (22). So too are the enzymes that can reesterify the retinol for storage in cytoplasmic lipid droplets and the binding protein (retinol-binding protein, RBP) necessary for its secretion from the liver. Finally, it is interesting to note the physical separation of enzymes catalyzing the formation of retinyl esters (LRAT and ARAT) and those catalyzing their hydrolysis (bile salt-independent retinyl ester hydrolases). Although both types of enzymes are "microsomal," ARAT and LRAT are associated with the endoplasmic reticulum (22, 31) while the bile salt-independent, neutral and acid retinyl ester hydrolases are associated with plasma membranes and/or endosomes (9, 15, the present study). ▇

REFERENCES

1. Huang, H. S., and D. S. Goodman. 1965. Vitamin A and carotenoids. I. Intestinal absorption and metabolism of ^{14}C-labeled vitamin A alcohol and β-carotene in the rat. *J. Biol. Chem.* **240:** 2839–2844.
2. Mahley, R. W., and M. M. Hussain. 1991. Chylomicron and chylomicron remnant metabolism. *Curr. Opin. Lipidol.* **2:** 170–176.
3. Lawrence, C. W., F. D. Crain, F. J. Lotspeich, and R. F. Krause. 1966. Absorption, transport, and storage of retinyl-15-^{14}C palmitate-9,10-^{3}H in the rat. *J. Lipid Res.* **7:** 226–229.
4. Blomhoff, R., P. Helgerud, M. Rasmussen, T. Berg, and K. R. Norum. 1982. In vivo uptake of chylomicron [3H]retinyl ester by rat liver: evidence for retinol transfer from parenchymal to nonparenchymal cells. *Proc. Natl. Acad. Sci. USA.* **79:** 7326–7330.
5. Blomhoff, R., W. Eskild, G. M. Kindberg, K. Prydz, and T. Berg. 1985. Intracellular transport of endocytosed chylomicron [3H]retinyl ester in rat liver parenchymal cells. Evidence for translocation of a [3H]retinoid from endosomes to endoplasmic reticulum. *J. Biol. Chem.* **260:** 13566–13570.
6. Harrison, E. H. 1993. Enzymes catalysing the hydrolysis of retinyl esters. *Biochim. Biophys. Acta.* **1170:** 99–108.
7. Erlanson, C., and B. Borgstrom. 1968. The identity of vitamin A esterase activity of rat pancreatic juice. *Biochim. Biophys. Acta.* **167:** 629–631.
8. Lombardo, D., and O. Guy. 1980. Studies on the substrate specificity of a carboxyl ester hydrolase from human pancreatic juice. II. Action on cholesterol esters and lipid-soluble vitamin esters. *Biochim. Biophys. Acta.* **611:** 147–155.
9. Harrison, E. H., and M. Z. Gad. 1989. Hydrolysis of retinyl palmitate by enzymes of rat pancreas and liver. Differentiation of bile salt-dependent and bile salt-independent, neutral retinyl ester hydrolases in rat liver. *J. Biol. Chem.* **264:** 17142–17147.
10. Rigtrup, K. M., and D. E. Ong. 1992. A retinyl ester hydrolase activity intrinsic to the brush border membrane of rat small intestine. *Biochemistry.* **31:** 2920–2926.
11. Winkler, K. E., E. H. Harrison, J. B. Marsh, J. M. Glick, and A. C. Ross. 1992. Characterization of a bile salt-de-

pendent cholesteryl ester hydrolase activity secreted from HepG2 cells. *Biochim. Biophys. Acta.* **1126:** 151–158.

12. Napoli, J. L., A. M. McCormick, B. O'Meara, and E. A. Dratz. 1984. Vitamin A metabolism: α-tocopherol modulates tissue retinol levels in vivo and retinyl palmitate hydrolysis in vitro. *Arch. Biochem. Biophys.* **230:** 194–202.

13. Napoli, J. L., E. B. Pacia, and G. J. Salerno. 1989. Cholate effects on all-*trans* retinyl palmitate hydrolysis in tissues homogenates: solubilization of the multiple kidney membrane hydrolases. *Arch. Biochem. Biophys.* **274:** 192–199.

14. Harrison, E. H., and J. L. Napoli. 1990. Bile salt-independent retinyl ester hydrolase activities associated with membranes of rat tissues. *Methods Enzymol.* **189:** 459–469.

15. Gad, M. Z., and E. H. Harrison. 1991. Neutral and acid retinyl ester hydrolases associated with rat liver microsomes: relationships to microsomal cholesteryl ester hydrolases. *J. Lipid Res.* **32:** 685–693.

16. Lenich, C. M., and A. C. Ross. 1987. Chylomicron remnant-vitamin A metabolism by the human hepatoma cell line HepG2. *J. Lipid Res.* **28:** 183–194.

17. Ross, A. C., and D. B. Zilversmit. 1977. Chylomicron remnant cholesteryl esters as the major constituent of very low density lipoproteins in plasma of cholesterol-fed rabbits. *J. Lipid Res.* **18:** 169–181.

18. Sardesai, V. M., and J. A. Manning. 1968. The determination of triglycerides in plasma and tissue. *Clin. Chem.* **14:** 156–161.

19. McFarlane, A. S. 1964. *In* Mammalian Protein Metabolism. Vol. 1. H. N. Munro and J. B. Allison, editors. Academic Press, New York. 331.

20. Touster, O., N. N. Aronson, Jr., J. T. Dulaney, and H. Hendrickson. 1970. Isolation of rat liver plasma membranes. Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J. Cell Biol.* **47:** 604–618.

21. Markwell, M. K., S. M. Haas, L. Bieber, and N. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206–210.

22. Harrison, E. H., W. S. Blaner, D. S. Goodman, and A. C. Ross. 1987. Subcellular localization of retinoids, retinoid-binding proteins, and acyl-CoA:retinol acyltransferase in rat liver. *J. Lipid Res.* **28:** 973–981.

23. Harrison, E. H., J. E. Smith, and D. S. Goodman. 1979. Unusual properties of retinyl palmitate hydrolase activity in rat liver. *J. Lipid Res.* **20:** 760–771.

24. LaBadie, J. H., K. P. Chapman, and N. N. Aronson, Jr. 1975. Glycoprotein catabolism in rat liver. Lysosomal digestion of iodinated asialo-fetuin. *Biochem. J.* **152:** 271–279.

25. Dunn, W. A., J. H. LaBadie, and N. N. Aronson, Jr. 1979. Inhibition of ^{125}I-asialofetuin catabolism by leupeptin in perfused rat liver. *J. Biol. Chem.* **254:** 4191–4196.

26. Olivecrona, T., and G. Bengtsson-Olivecrona. 1990. Lipases involved in lipoprotein metabolism. *Curr. Opin. Lipidol.* **1:** 116–121.

27. Blaner, W. S., J. C. Obunike, S. B. Kurlandsky, M. al-Haideri, R. Piantedosi, R. J. Deckelbaum, and I. J. Goldberg. 1994. Lipoprotein lipase hydrolysis of retinyl ester. Possible implications for retinoid uptake by cells. *J. Biol. Chem.* **264:** 16559–16565.

28. Borensztajn, J., G. S. Getz, and T. J. Kotlar. 1988. Uptake of chylomicron remnants by the liver: further evidence for the modulating role of phospholipids. *J. Lipid Res.* **29:** 1087–1096.

29. Sultan, F., D. Lagrange, H. Jansen, and S. Griglio. 1990. Inhibition of hepatic lipase activity impairs chylomicron remnant removal in rats. *Biochim. Biophys. Acta.* **1042:** 150–152.

30. Tabas, I., S. Lim, X-X. Xu, and F. R. Maxfield. 1990. Endocytosed β-VLDL and LDL are delivered to different intracellular vesicles in mouse peritoneal macrophages. *J. Cell Biol.* **111:** 929–940.

31. Ross, A. C., and E. S. Kempner. 1993. Radiation inactivation analysis of acyl-CoA:retinol acyltransferase and lecithin:retinol acyltransferase in rat liver. *J. Lipid Res.* **34:** 1201–1207.