Neutral and acid retinyl ester hydrolases associated with rat liver microsomes: relationships to microsomal cholesteryl ester hydrolases

Mohamed Z. Gad and Earl H. Harrison

Division of Nutrition, Department of Physiology and Biochemistry, The Medical College of Pennsylvania, Philadelphia, PA 19129

Abstract We recently reported the presence of a neutral, bile salt-independent retinyl ester hydrolase (REH) activity in rat liver microsomes and showed that it was distinct from the previously studied bile salt-dependent REH and from non-specific carboxylesterases (Harrison, E. H., and M. Z. Gad. 1989. *J. Biol. Chem.* **264:** 17142–17147). We have now further characterized the hydrolysis of retinyl esters by liver microsomes and have compared the observed activities with those catalyzing the hydrolysis of cholesteryl esters. Microsomes and microsomal subfractions enriched in plasma membranes and endosomes catalyze the hydrolysis of retinyl esters at both neutral and acid pH. The acid and neutral REH enzyme activities can be distinguished from one another on the basis of selective inhibition by metal ions and by irreversible, active site-directed serine esterase inhibitors. The same preparations also catalyze the hydrolysis of cholesteryl esters at both acid and neutral pH. However, the enzyme(s) responsible for the neutral REH activity can be clearly differentiated from the neutral cholesteryl ester hydrolase(s) on the basis of differential stability, sensitivity to proteolysis, and sensitivity to active site-directed reagents. These results suggest that the neutral, bile salt-independent REH is relatively specific for the hydrolysis of retinyl esters and thus may play an important physiological role in hepatic vitamin A metabolism. In contrast to the neutral hydrolases, the activities responsible for hydrolysis of retinyl esters and cholesterol esters at acid pH are similar in their responses to the treatments mentioned above. Thus, a single microsomal acid hydrolase may catalyze the hydrolysis of both types of ester. ■ The demonstration of distinct neutral and acid REH enzymes in plasma membranes and endosomes is consistent with current concepts of the mechanisms and pathways involved in the initial metabolism of retinyl esters delivered to the liver by chylomicron remnants. —Gad, M. Z., and E. H. Harrison. Neutral and acid retinyl ester hydrolases associated with rat liver microsomes: relationships to microsomal cholesteryl ester hydrolases. *J. Lipid Res.* 1991. **32:** 685–693.

Supplementary key words vitamin A • cholesterol • membrane-bound enzymes

Rat liver contains a number of enzymes that catalyze the hydrolysis of retinyl esters and cholesteryl esters. Among these enzymes, we have recently (1) demonstrated the presence of a bile salt-independent retinyl ester hydrolase activity that differs from the previously described bile salt-dependent activity in several aspects: *1)* it does not require millimolar concentrations of bile salts for maximal in vitro activity; *2)* it is localized in the microsomal fraction of liver homogenates; *3)* its absolute activity does not vary markedly among individual rats; and *4)* it is not inhibited by antibodies to pancreatic cholesteryl ester hydrolase. Furthermore, this microsomal activity is not due to the hydrolysis of the retinyl esters by the microsomal nonspecific carboxylesterases of rat liver (1).

Several hepatic enzyme activities that catalyze the hydrolysis of long chain fatty acid esters of cholesterol have been reported. The best characterized of these enzymes is the lysosomal acid lipase that is involved in the hydrolysis of cholesteryl esters delivered to the hepatocytes via receptor-mediated endocytosis of lipoproteins (2, 3). In addition, liver contains cholesteryl ester hydrolase(s) with neutral pH optima (4). These include a bile salt-dependent cholesteryl ester hydrolase activity that has the same unusual properties of the bile salt-dependent retinyl ester hydrolase. In addition, many studies have demonstrated that rat liver homogenates can catalyze the hydrolysis of cholesteryl esters in absence of exogenous bile salts as assayed in vitro (for example 5–8). Such bile salt-independent activity was shown to be localized in the cytosolic and microsomal fractions of liver homogenates (5). Although none of the activities has been purified to homogeneity, subsequent studies focused on the characterization of the microsomal activity (6, 9–13). The activity was shown to be clearly different from the lysosomal acid lipase and hepatic lipase. However, several questions

---

Abbreviations: NREH, neutral retinyl ester hydrolase; AREH, acid retinyl ester hydrolase; NCEH, neutral cholesteryl ester hydrolase; ACEH, acid cholesteryl ester hydrolase.

are still to be answered regarding the number of microsomal enzymes that catalyze the hydrolysis of cholesteryl esters, their pH dependency, physiological roles, subcellular localization, and the relationship of these activities to other microsomal ester hydrolases.

In the present study, we probed the relationship between the microsomal bile salt-independent retinyl ester hydrolase(s) and the bile salt-independent cholesteryl ester hydrolase(s) and asked whether the two activities are due to one or more enzymes. The results indicate that there are at least two distinct rat liver microsomal activities that hydrolyze retinyl esters and cholesteryl esters when assayed in vitro at neutral pH. In addition, we report the presence of acidic retinyl ester and cholesteryl ester hydrolase activities that have properties different from the neutral microsomal enzymes. These acidic activities are distinct from the lysosomal acid lipase and might be related to the liver endosomal cholesteryl ester hydrolase activity recently reported by Runquist and Havel (14) and Hornick, Thouron, and Delamatre (15).

EXPERIMENTAL PROCEDURES

Materials

Cholesteryl [1-^{14}C]oleate and [1-^{14}C]palmitic acid were purchased from Amersham Corp. (Arlington Heights, IL). Cholesteryl oleate, trypsin, soybean trypsin inhibitor, and pronase E were purchased from Sigma Chemical Co. (St. Louis, MO). All-trans-retinol was from Sigma (Type X) or a gift from Hoffman-La Roche. Inhibitors used were gifts from Dr. Daniel Quinn, University of Iowa (p-nitrophenyl-n-dodecyl carbamate and diethylumbelliferyl phosphate) or from Wyeth-Ayerst Research (Princeton, NJ) (o-phenylphenyl-n-butyl carbamate (Wyeth 50,280)). All other chemicals and reagents were purchased from either Sigma or Fisher. Retinyl [1-^{14}C]palmitate was synthesized and purified as previously described (16).

Animals

Male Sprague-Dawley rats were purchased from Charles River Laboratories, Wilmington, MA and housed in our animal facilities where they were fed a commercial diet ad libitum. Adult animals weighing 200–400 g were used in most of the experiments.

Subcellular fractionation of rat liver

Rat liver homogenates were prepared and fractionated according to the method of de Duve et al. (17) as later modified by Amar-Costesec et al. (18). Rats were killed by inhalation of carbon dioxide and the livers were dissected, rinsed with ice-cold 0.25 M sucrose, blotted, weighed, then minced with scissors and tissue grinder. For each in-

dividual liver about three volumes of 0.25 M sucrose were added and the suspension was homogenized in a Potter-Elvejhem homogenizer with one stroke of the mechanically driven Teflon pestle. The resulting homogenate was fractionated by differential centrifugation into the nuclear (N), mitochondrial-lysosomal (ML), microsomal (P), and soluble (S) fractions. Each fraction, except the microsomal, was washed (i.e., resuspended in the same homogenizing medium then recentrifuged) twice. The entire fractionation was done at 0°C and with ice-cold 0.25 M sucrose. The recovered microsomes were used in most of the studies.

A fraction enriched in plasma membranes and endosomes was prepared from the microsomal fraction by a discontinuous sucrose density gradient according to the method of Touster et al. (19). All subcellular fractions and plasma membrane-rich fractions were divided into portions and stored at – 70°C.

Enzyme assays

Freshly thawed subcellular fractions were analyzed for retinyl ester and cholesteryl ester hydrolase activities, for marker enzymes for lysosomes (N-acetyl-β-glucosaminidase), plasma membranes and endosomes (alkaline phosphodiesterase), endoplasmic reticulum (glucose-6-phosphatase and o-nitrophenyl acetate esterase) and for total protein. Marker enzymes were analzyed as previously described (20–22) and protein was assayed by a modification of the Lowry procedure (23).

Retinyl ester and cholesteryl ester hydrolase activities were determined using the sensitive radiometric assay previously described in detail (1). The determinations were conducted in the absence of exogenous detergents or bile salts. Neutral hydrolase activity was assessed in reaction mixtures containing 50 mM Tris-maleate, pH 7, and acid hydrolase activity was assayed using 50 mM sodium acetate, pH 5. All assays were conducted under conditions where the formation of product was linear with respect to protein (enzyme) concentration and time of incubation.

Inhibition studies

All inhibitor solutions were prepared in dimethyl sulfoxide (DMSO). Microsomes (about 3 mg protein) were preincubated with various amounts of the inhibitors for 30 min at room temperature. All incubations were conducted at pH 7 using 10 mM Tris-maleate. Appropriate controls were incubated in the same way but in the absence of inhibitors. The volume of DMSO in all incubations did not exceed 4% of the total incubation volume. After the incubations, microsomes were diluted with 0.25 M sucrose and immediately assayed for the various enzyme activities.

Stability studies

Microsomes stored at $-70°C$ were used in this study. The observed stability of retinyl ester and cholesteryl ester hydrolase(s) at this temperature was at least 3 months. Stability studies were conducted for 1 or 3 days either at $4°C$ or at room temperature. Freshly thawed microsomes were used as controls and assayed for the enzyme activities at the same time with the tested microsomes.

Protease treatment of microsomes

Protease treatment of microsomes was modified from the method of Renooij and Snyder (24). Microsomes (1.5–2 mg protein) were disrupted by 30 min exposure to 0.1 % deoxycholate in an ice bath. Intact microsomes were treated similarly but in the absence of deoxycholate. Trypsin (50 µg/mg microsomal protein) was prepared in a buffer containing 0.1 M Tris-maleate, pH 7.4, 0.1 M KCl, and 0.25 M sucrose, and was added to intact or deoxycholate-disrupted microsomes. The incubations were conducted at 0, 25, or $37°C$ for 15 min. A fourfold excess of soybean trypsin inhibitor was added and the preparations were then diluted with 0.25 M sucrose. In incubations of the controls of intact and disrupted microsomes, trypsin was omitted or the soybean trypsin inhibitor was added before the trypsin.

In a separate experiment, pronase was used instead of trypsin. Intact or deoxycholate-disrupted microsomes (about 15 mg protein) were incubated in the absence and presence of pronase (70 µg/mg microsomal protein) for 15 min at $37°C$. At the end of the incubations, the tubes were immediately put in ice and then centrifuged at 50,000 rpm for 30 min to resediment the microsomes. The recovered microsomes were rinsed and resuspended in 0.25 M sucrose and used directly for enzyme assays.

The permeability of microsomal membranes was followed by determining the latency and protease sensitivity of mannose-6-phosphatase in intact and disrupted microsomes. The activity of mannose-6-phosphatase was assayed by incubating control or treated microsomes (about 1.5–2 mg protein) in 0.1 M Tris-HCl, pH 7.4, 0.1 M KCl, and 0.25 M sucrose at $0°C$ for 30 min in the presence or absence of 0.05 % deoxycholate. At the end of the incubations, 1 mM mannose-6-phosphate was added and reincubated at $37°C$ for 15 min. The reaction was stopped by adding 0.1 ml of 10 % trichloroacetic acid. After pelleting the denatured protein by centrifugation, phosphorus was measured in an aliquot of the supernatent by the method of Fiske and SubbaRow (25). In controls, trichloroacetic acid-precipitated microsomes were used. The ratio of activities measured in the absence and presence of deoxycholate was used to calculate the latency of mannose-6-phosphatase:

latency of mannose-6-phosphatase =

$$\frac{(\text{activity with deoxycholate} - \text{activity without deoxycholate})}{\text{activity with deoxycholate}} \times 100$$

Effect of metal ions

Several ions were tested as potential inhibitors or activators for the microsomal activities. These ions were Zn^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Na^+, F^-, $Mo_7O_{24}^{6-}$, $HAsO_4^{2-}$. All ions were studied at a final concentration 1 mM except Na^+ at 2 mM and were incubated with the enzyme(s) for 15 min before the addition of the substrate.

RESULTS

Hydrolysis of retinyl esters and cholesteryl esters by rat liver microsomes and subcellular fractions

The present studies were initiated by investigating the enzymatic hydrolysis of retinyl palmitate and cholesteryl oleate by well-characterized subcellular fractions that were prepared by differential centrifugation of rat liver homogenates. When assayed at pH 7, the percent of recovered retinyl ester hydrolase activity in the four homogenate fractions was (in %): nuclear, 24 ± 5; mitochondrial-lysosomal, 5 ± 5; microsomal, 61 ± 1; soluble, 10 ± 7 (n = 6) which is in accord with our previous results (1). The distribution of neutral cholesteryl ester hydrolase activity was: nuclear, 8 ± 4; mitochondrial-lysosomal, 2 ± 1; microsomal, 41 ± 11; soluble, 49 ± 10 (n = 4). An identical pattern was observed when cholesteryl palmitate was used as substrate. Thus, the neutral cholesteryl ester hydrolase activity was about equally divided between the microsomal and soluble fractions, a result also in accord with previous investigations.

In order to begin to probe the possible relationships and substrate specificity of the microsomal enzymes responsible for the hydrolysis of retinyl esters and cholesteryl esters, we studied the pH dependence of the activities in the microsomal fraction. The results of such an experiment are presented in **Fig. 1**. The microsomal retinyl ester hydrolase activity showed a pH profile similar to that previously reported for the whole homogenate (1) with an optimum in the neutral range and a broad shoulder extending into the acid range. In contrast, the pH profile for cholesteryl ester hydrolase activity was bimodal, with a distinct peak at acid pH and a smaller peak in the neutral pH range. There was also a distinct difference in the absolute activity observed with the two substrates. Under the assay conditions used here, the activity at pH 7 against retinyl esters was 5- to 10-times greater than that against cholesteryl esters (n = 4), while at pH 5 the ratio of the two activities was 1–2:1.

The presence of substantial hydrolytic activity at pH 4–5 led us to think initially that this activity resulted from the contamination of our microsomes with lysosomes and hence with the lysosomal acid lipase. In order to clarify this point, we studied the subcellular distribution of the acid REH and acid CEH activities and compared them with enzyme markers including a well-estab-
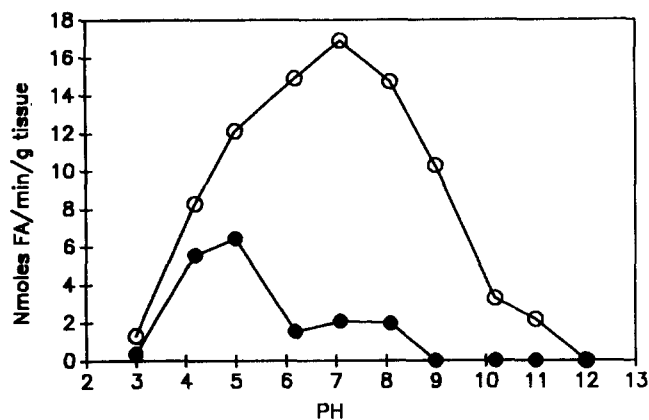
Fig. 1. pH-Dependence of microsomal retinyl ester hydrolase (open symbols) and cholesteryl ester hydrolase (closed symbols) activities. Rat liver microsomes (about 20 μg of protein) were incubated for 30 min at 37°C in the presence of 40 μM retinyl palmitate or cholesteryl oleate and 50 mM buffers of the indicated pH (sodium acetate for pH 3 to 5, Tris-maleate for pH 6 to 8, glycine for pH 9 to 11, and citrate/phosphate/barbital/borate for pH 12).

lished marker for lysosomes, i.e., N-acetyl-β-glucosaminidase. The results of these studies are summarized in **Table 1**. Note that the distributions of the acid REH and CEH activities are not like that of the lysosomal marker. Rather, they show a microsomal pattern like those of markers for the endoplasmic reticulum and plasma membranes. The consistently excessive recovery of ACEH activity results from a currently unexplained activation of the activity during the course of fractionation.

As we had previously demonstrated that the neutral REH activity was highly enriched in a microsomal subfraction containing plasma membranes and related endosomes (1), we next studied the ability of such fractions to catalyze the hydrolysis of both retinyl esters and cholesteryl esters under both acid and neutral incubation conditions. The results of these experiments are presented in **Table 2**. The isolated fraction is highly enriched in plasma membranes/endosomes as assessed by the enrichment of alkaline phosphodiesterase, an enzyme marker for such organelles (19, 26) and is not significantly en-

riched in markers for either lysosomes or endoplasmic reticulum. The microsomal hydrolytic activities for both retinyl esters and cholesteryl esters are significantly enriched in these fractions when studied at either acid or neutral pH.

Relationships among the microsomal lipid ester hydrolases

Given that the microsomal hydrolase activities catalyzing the hydrolysis of both retinyl esters and cholesteryl esters were similarly enriched in plasma membrane/endosome fractions we felt it was important to ask whether one or more than one enzyme was responsible for the observed activities before proceeding to attempts to solubilize and purify individual enzyme proteins. Thus, we chose to explore the possibly differential effects of various treatments on the acid and neutral REH and acid and neutral CEH activities in liver microsomes. In these studies we focused on pretreatments of the microsomes that were likely to affect microsomal enzymes per se, rather than in vitro additions (e.g., detergents) that might also have effects on substrate presentation.

Differential stability of enzyme activities

Pilot studies demonstrated that all four hydrolytic activities are fully stable for periods of a few months when freshly isolated microsomes are stored at – 70°C. **Table 3** shows the stability of the activities after thawing and incubation at either 4°C or 25°C (room temperature) for periods of 1 to 3 days. The stabilities of the neutral REH and the neutral CEH activities were markedly different under these conditions, with the REH activity being much more stable than the CEH activity at both 4°C and room temperature. Preparations incubated at room temperature for 1 or 3 days showed differential inactivation of the acid as opposed to neutral activities for both substrates, with the acid CEH being more stable than the neutral CEH and the acid REH being less stable than the neutral REH. However, under these conditions the two

TABLE 1. Distributions of enzyme activities in subcellular fractions of rat liver

Constitutent	Percent of recovered amount[a]				
	N	ML	P	S	Recovery[b]
Protein (6)	21 ± 6	20 ± 4	21 ± 4	38 ± 6	98 ± 13
N-Acetyl-β-glucosaminidase (8) (lysosomes)	15 ± 8	51 ± 12	30 ± 8	4 ± 3	91 ± 10
Glucose-6-phosphatase (4) (ER)	25 ± 3	11 ± 3	61 ± 5	3 ± 0	102 ± 10
Alkaline phosphodiesterase (8) (plasma membrane)	21 ± 5	14 ± 9	61 ± 11	4 ± 3	105 ± 11
Acid REH (2)	13 ± 2	10 ± 2	76 ± 6	2 ± 1	130 ± 2
Acid CEH (3)	10 ± 2	25 ± 17	63 ± 18	2 ± 0	229 ± 70

[a]Results are given as mean ± SD. The number of experiments is given in parentheses. Relative values are presented for the distribution of each constituent among the four fractions: nuclear (N), mitochondrial-lysosomal (ML), microsomal (P), and supernatant (S). The values are the percentage of the constituent recovered in each fraction relative to the amounts recovered in all four fractions (taken as 100%).

[b]Recovery (in %) represents the total amount recovered in all four fractions relative to the amount in the whole homogenate.

TABLE 2. Enrichments of lipid ester hydrolases and markers in a plasma membrane/endosomal fraction

Constituent	RSA[a]	Total Recovery (%)[b]
Protein	1	94 ± 18
N-Acetyl-β-glucosaminidase (3) (lysosomes)	1 ± 0	83 ± 6
Glucose 6-phosphatase (3) (endoplasmic reticulum)	2 ± 1	91 ± 11
Nonspecific esterase (3) (endoplasmic reticulum)[c]	2 ± 1	84 ± 10
Alkaline phosphodiesterase (3) (plasma membrane, endosomes)	13 ± 2	102 ± 8
Neutral REH (3)	14 ± 4	105 ± 20
Acid REH (1)	8	101
Neutral CEH (3)	10 ± 4	102 ± 9
Acid CEH (1)	14	314

Plasma membrane-rich fractions were isolated from the microsomal fraction by the method of Touster et al., 1970 (19). Number of experiments is shown in parentheses.

[a]Relative specific activity (RSA) = the percent of activity recovered in the plasma membrane fraction divided by the percent of total homogenate protein recovered in the fraction. The isolated plasma membrane fractions contained about 1.0% of the homogenate protein.

[b]Recoveries represent the total recovery (in %) for each constituent for the entire fractionation. They are the sum of the amounts in all fractions relative to the amount observed in the unfractionated homogenate.

[c]Nonspecific esterase was assayed using o-nitrophenyl-acetate as the substrate.

acid activities (REH vs. CEH) were inactivated to the same extent. Thus, these data show a major difference in the neutral REH and neutral CEH activities. Although not as qualitatively striking, they also suggest that the acid hydrolytic activity for each substrate might be different than the corresponding neutral activity.

Differential sensitivity to protease treatments

In order to probe further the relationships of the various hydrolytic activities, microsomes were subjected to treatments with proteases in nine separate experiments involving six different conditions of incubation. The results of these studies are summarized in **Table 4**. Several points emerge from these data. Most striking is the markedly differential response of the neutral REH and neutral CEH activities. Note that proteolysis of either intact or detergent-disrupted microsomes consistently leads to 70–90% loss of neutral CEH activity without markedly affecting neutral REH activity. The fact that approximately 80% inactivation of neutral CEH activity can be achieved by protease treatment of intact microsomal vesicles (as assessed by the latency and protease resistance of mannose-6-phosphatase) strongly suggests

that the enzyme(s) responsible for the neutral CEH activity is(are) localized on the external (cytoplasmic) face of the microsomal vesicles. The fact that the neutral REH activity is resistant to proteolysis in both intact and disrupted microsomes does not allow us to draw conclusions concerning the orientation of the enzyme(s), and suggests that the enzyme(s) responsible for retinyl ester hydrolysis is(are) intrinsically protease-resistant. Nonetheless, the striking difference in protease sensitivity of neutral REH and neutral CEH strengthens the conclusion that separate microsomal enzymes are involved in the hydrolysis of these two types of esters at neutral pH.

These experiments also provide further information on the properties and relationships of the various activities under study. The acid CEH activity is resistant to proteolysis in intact vesicles (in fact, a consistent and marked activation was observed under conditions of mild trypsinization) but markedly inactivated in disrupted vesicles, suggesting that the enzyme(s) is(are) localized on the luminal face of the microsomal vesicles. The acid REH is not activated by mild trypsinization of intact vesicles and appears to be somewhat more protease-resistant in disrupted microsomes than is the acid CEH.

TABLE 3. Stability of retinyl ester hydrolases and cholesteryl ester hydrolases in rat liver microsomes

Temperature	Time	Percent of Control[a]			
		NREH	AREH	NCEH	ACEH
°C	days				
4	1	99	94	98	113
4	3	92	90	29	29
25	1	80	60	12	51
25	3	41	12	1	19

[a]Aliquots of freshly thawed microsomes were incubated at the indicated temperature for 1 or 3 days prior to assay for the enzyme activities as described in the text. Activities are presented as a percent of control activity obtained in assays of freshly thawed aliquots of the same microsomal preparations.

TABLE 4. Effects of protease treatments on rat liver microsomal enzymes

| Protease | Temp. | Microsomes | % of Control[a] | | | | |
			NREH	AREH	NCEH	ACEH	M-6-Pase
	°C						
Trypsin	4	Intact	112	97	31	168	ND
Trypsin	25	Intact	72	97	22	211	ND
Trypsin	25	Disrupted	81	86	21	39	
Trypsin	37	Intact	83	57	20	77	73 (91)[b]
Trypsin	37	Disrupted	77	45	9	25	2 (0)
Pronase	37	Intact[c]	81	45	13	24	57 (41)

[a]Microsomes were incubated with proteases as described in the text. Activity is presented as percent of controls that received soybean trypsin inhibitor prior to trypsin or no protease in the case of the experiment with pronase; ND, not determined.

[b]Numbers in parentheses refer to the percent latency of mannose-6-phosphatase as defined in the text.

[c]Microsomes were not treated with deoxycholate to disrupt membrane. However, the latency of mannose-6-phosphatase (41%) and accessibility to proteolysis indicate that the vesicles were partially (~41%) disrupted by protease treatment alone.

Differential sensitivity to metal ions and to active site-directed serine esterase inhibitors

The effects of the presence of various metal ions at final concentrations of 1 mM on the hydrolytic activities was studied. The following ions had no effect on any of the activities: Mg^{2+}, Mn^{2+}, Ca^{2+}, Na^+, and $HAsO_4^{2-}$. Two ions were very effective at differentially inhibiting the neutral and acid activities for both substrates but showed no differential activity with the two substrates. Thus, addition of zinc acetate led to greater than 80% inhibition of the neutral REH and CEH activities without affecting the acid REH or CEH, while addition of ammonium molybdate led to greater than 90% inhibition of the acid activities without affecting the neutral activities.

In the final series of experiments reported here, we investigated the effects of pretreating microsomes with a number of N-alkyl carbamates and diethyl phosphates on the hydrolytic activities under study. These compounds were chosen because they, or related compounds, have previously been demonstrated to be active site-directed, irreversible inhibitors of purified lipid ester hydrolases with neutral pH optima (27, 28). Three of the compounds tested led to markedly differential inactivation of the neutral REH and neutral CEH activities of liver microsomes. Thus, p-nitrophenyl-N-dodecyl carbamate (**Fig. 2**) and o-phenylphenyl-N-butyl carbamate (**Fig. 3**) led to about 80% inactivation of neutral CEH activity without significantly affecting the microsomal, neutral REH activity. Conversely, diethylumbelliferyl phosphate (**Fig. 4**) led to 90% inactivation of neutral REH activity without affecting neutral CEH activity. None of these compounds significantly inactivated either acid REH or acid CEH activity of the microsomes. Thus, these studies further strengthen the conclusion that the neutral REH activity of liver microsomes is due to an enzyme(s) distinct from

that(those) that catalyze the hydrolysis of cholesteryl esters. Moreover, they provide further evidence that distinct neutral and acid REH enzymes are present in rat liver microsomes. Thus, the pH-activity profile for REH in diethylumbelliferyl phosphate-treated microsomes shows a single peak at pH 4–5 with very little activity at pH 6 or greater (data not shown). Control experiments demonstrated that the lack of effect of the compound on REH activity at acid pH was not due to reversal of inactivation but to true lack of inactivation.
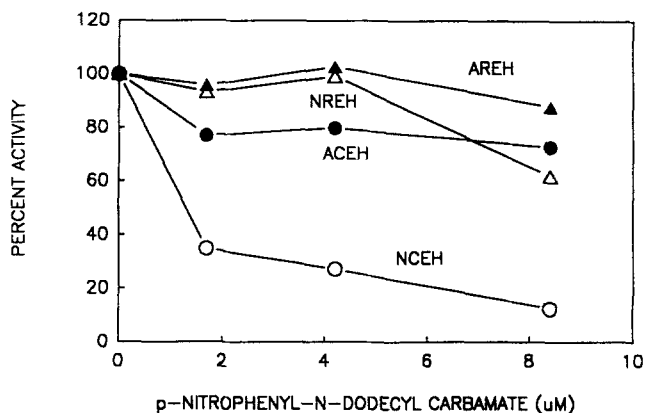

Fig. 2. Effects of p-nitrophenyl-N-dodecyl carbamate on microsomal retinyl ester hydrolase and cholesteryl ester hydrolase activities. Microsomes (about 3 mg protein) were preincubated with the indicated concentrations of inhibitor for 30 min at room temperature in solutions containing 0.25 M sucrose and 10 mM Tris-maleate, pH 7. They were then diluted with 0.25 M sucrose and assayed for enzyme activity in reactions mixtures containing 80 μM retinyl palmitate (triangles) or cholesteryl oleate (circles) and either 50 mM sodium acetate, pH 5 (filled symbols) or 50 mM Tris-maleate, pH 7 (open symbols). Results are presented as a percent of activity observed in controls preincubated without the inhibitor.
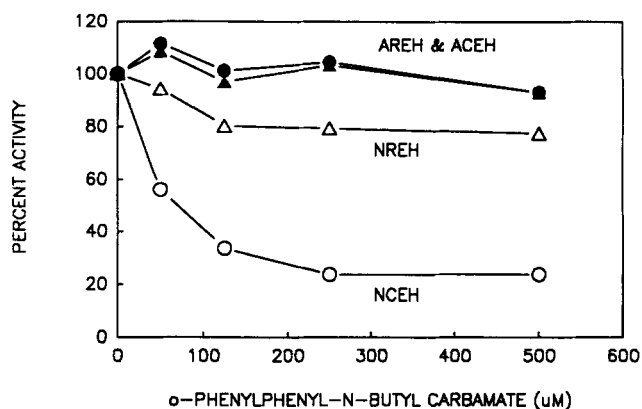
Fig. 3. Effects of o-phenylphenyl-N-butyl carbamate on microsomal retinyl ester hydrolase and cholesteryl ester hydrolase activities. Conditions are the same as in Fig. 2.

DISCUSSION

The results presented here provide new information on the enzymatic hydrolysis of retinyl esters in liver. In particular, they provide direct evidence for the presence of at least two microsomal retinyl ester hydrolases, neutral retinyl ester hydrolase (NREH) and acid retinyl ester hydrolase (AREH). Both activities are largely present in microsomal subfractions enriched in alkaline phosphodiesterase, an enzyme marker present in plasma membranes and endosomes (19, 26). Although the same preparations catalyze the hydrolysis of cholesteryl esters, several lines of evidence presented here demonstrate clearly that the hydrolyses of these two lipid esters at neutral pH are catalyzed by distinct hydrolytic enzymes, NREH and NCEH. The two enzyme activities show marked differences in: 1) intrinsic stability of activity (Table 3); 2) sensitivity to proteolysis under various conditions (Table 4); and 3) inactivation by specific active site-directed inhibitors such as N-alkyl carbamates (Figs. 2 and 3) and diethylphosphates (Fig. 4). Thus, the microsomal NREH studied here is distinct from previously studied microsomal neutral CEHs. Taken together with the previous demonstration of the distinction of NREH from the nonspecific carboxylesterases of rat liver (1), this suggests strongly that the NREH is relatively specific for the hydrolysis of retinyl esters and important in hepatic retinoid metabolism.

A significant aspect of these studies is the demonstration of a microsomal REH that is active at acid pH. The enzyme(s) is(are) distinct from the NREH activity as evidenced by differential responses of the two activities to a number of treatments reported here. The AREH activity is not due to lysosomal acid lipase as indicated by the fact that it is not found in fractions enriched in lysosomal hydrolases (i.e., the ML fraction, Table 1) but rather is markedly enriched in microsomal and plasma membrane/

endosomal fractions that are not enriched in lysosomal markers (Tables 1 and 2).

The lack of REH activity in lysosomes and its presence in plasma membranes and/or endosomes are consistent with currently available information on the intracellular transport of chylomicron retinyl ester in rat liver. Blomhoff et al. (29) used subcellular fractionation techniques to study the intracellular transport of [³H]retinyl ester delivered to the liver in chylomicron remnants. They compared the transport of the [³H]retinyl ester with that of a ligand (¹²⁵I-labeled asialofetuin) known to be internalized and transported via the classic pathway of receptor-mediated endocytosis. The results demonstrated that both [³H]retinoid and ¹²⁵I-labeled asialofetuin were initially associated with a population of endosomal vesicles enriched in plasma membrane markers. At later times, however, the asialofetuin was transported to lysosomes while the retinoid appeared to be transferred to a higher density membrane vesicle population that the authors suggested was derived from the endoplasmic reticulum. We have conducted similar experiments with chylomicrons containing labeled retinyl esters and with asialoglycoproteins and have also observed a dissociation of intracellular pathways (E. H. Harrison and A. C. Ross, unpublished observations). Thus the available evidence suggests that chylomicron remnant retinyl esters delivered to the liver are initially associated with endosomes but are not transferred to lysosomes. Rather they remain associated microsomal vesicles until they appear at later times in cytoplasmic lipid droplets. The results presented here extend this concept by demonstrating the association of retinyl ester hydrolase enzyme activities with the same population of vesicles that initially carry the substrate retinyl esters. Thus, we would speculate that the hydrolysis of chylomicron retinyl esters is initiated by the NREH at the plasma membrane or in early endosomes prior to their acidification. Upon acidification of these endosomes,
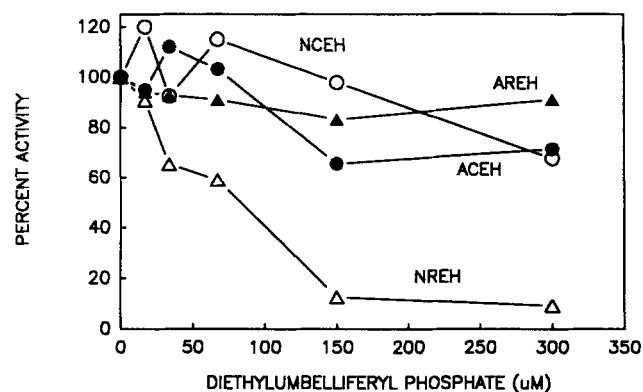

Fig. 4. Effects of diethylumbelliferyl phosphate on microsomal retinyl ester hydrolase and cholesteryl ester hydrolase activities. Conditions are the same as in Fig. 2.

the AREH activity could continue to catalyze the hydro-lysis of retinyl esters. It should also be noted that recent work has identified discrete populations of endosomes in rat liver that are involved in the differential targeting of various ligands to either lysosomes or nonlysosomal sites within the liver cell (26).

Our experiments also confirm and extend previous studies on the enzymes involved in hepatic cholesteryl ester metabolism. The finding of cholesteryl ester hydrolase activity associated with plasma membranes and/or endo-somes is consistent with previous biochemical and mor-phological results that suggest that the hydrolysis of chylomicron cholesteryl esters can occur at the hepatocyte cell surface (13, 30). In particular, the finding of ACEH activity in fractions enriched in endosomal markers is in accord with recently developing concepts of the role of these organelles in hepatic cholesteryl ester metabolism. During the preparation of this manuscript two reports ap-peared on the role of hepatic endosomes in the hydrolysis of both VLDL and LDL cholesteryl esters (14, 15). In the former report (14), acid CEH activity was shown to be highly enriched in endosomal fractions, to a much greater extent than the lysosomal marker enzyme, arylsulfatase B. Endosomal fractions are also active in hydrolyzing triacylglycerols and the apolipoprotein B of LDL (14, 31). Thus, there is accumulating evidence for the extensive degradation of lipoprotein components in acidic, prely-sosomal compartments of the liver. This evidence, when coupled with the present demonstration of acid REH and CEH activities in hepatic endosomes, suggests that such compartments may also play an important role in the in-itial hydrolysis of chylomicron remnant cholesteryl esters and retinyl esters. 🔳

REFERENCES

1. 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-inde-pendent, neutral retinyl ester hydrolases in rat liver. *J. Biol. Chem.* **264:** 17142-17147.
2. Fowler, S. D., and W. J. Brown. 1984. Lysosomal acid lipase. *In* Lipases. B. Borgström, and H. L. Brockman, edi-tors. Elsevier, Amsterdam. 329-364.
3. Drevon, C. A., T. Berg, and K. R. Norum. 1977. Uptake and degradation of cholesterol ester-labelled rat plasma lipoproteins in purified rat hepatocytes and non-paren-chymal liver cells. *Biochim. Biophys. Acta.* **487:** 122-136.
4. Brockerhoff, H., and R. G. Jensen. 1974. Cholesterol es-terase. *In* Lipolytic Enzymes. Academic Press, New York. 188-189.
5. Deykin, D., and D. S. Goodman. 1962. The hydrolysis of long-chain fatty acid esters of cholesterol with rat liver en-zymes. *J. Biol. Chem.* **237:** 3649-3656.
6. Nilsson, A. 1976. Hydrolysis of chyle cholesterol esters with cell-free preparations of rat liver. *Biochim. Biophys. Acta.* **450:** 379-389.
7. Harrison, E. H. 1988. Bile salt-dependent, neutral choles-teryl ester hydrolase of rat liver: possible relationship with pancreatic cholesteryl ester hydrolase. *Biochim. Biophys. Acta.* **963:** 28-34.
8. Ruiz, M. B., B. Ochoa, J. M. Gandarias, and M. Lacort. 1988. Cholesterol ester hydrolysis in isolated rat liver cells. Effect of estradiol, dibutyryl cyclic AMP and free fatty acids. *Horm. Metab. Res.* **20:** 82-85.
9. Neelon, V. J., and L. Lack. 1977. The effect of bile salts on the formation and hydrolysis of cholesterol esters by rat liver enzymes. *Biochim. Biophys. Acta.* **487:** 137-144.
10. Coleman, R. A., and E. B. Haynes. 1983. Differentiation of microsomal from lysosomal triglycerol lipase activities in rat liver. *Biochim. Biophys. Acta.* **751:** 230-240.
11. Fernandez, C. J., M. Lacort, J. M. Gandarias, and B. Ochoa. 1987. Topological studies on rat liver microsomal cholesterol ester hydrolase from rat liver. *Biochem. Biophys. Res. Commun.* **146:** 1212-1217.
12. Gandarias, J. M., M. Lacort, M. J. Martinez, C. J. Fer-nandez, and B. Ochoa. 1987. Kinetic properties and solubi-lization of microsomal cholesterol ester hydrolase from rat liver. *Steroids.* **49:** 403-417.
13. Riddle, M. C., E. A. Smuckler, and J. A. Glomset. 1975. Cholesteryl ester hydrolytic activity of rat liver plasma membrane. *Biochim. Biophys. Acta.* **338:** 339-348.
14. Runquist, E. A., and R. J. Havel. 1989. Endosomal degradation of lipid and protein components of low density lipoproteins. *J. Cell Biol.* **109:** 328a.
15. Hornick, C. A., C. Thouron, and J. G. Delamatre. 1989. Endosomal cholesteryl ester hydrolysis and sorting. *Circula-tion.* **80:** II-63.
16. Prystowsky, J. H., J. E. Smith, and D. S. Goodman. 1981. Retinyl palmitate hydrolase activity in normal rat liver. *J. Biol. Chem.* **256:** 4498-4503.
17. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* **60:** 604-617.
18. Amar-Costesec, A., H. Beaufay, M. Wibo, D. Thines-Sempoux, E. Feytmans, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* **61:** 201-212.
19. Touster, O., N. N. Aronson, J. T. Dulaney, and H. Hen-drickson. 1970. Isolation of rat liver plasma membranes. Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J. Cell Biol.* **47:** 604-618.
20. 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.

21. 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.

22. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thines-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* **61:** 188–200.

23. 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.

24. Renooij, W., and F. Snyder. 1981. Transmembrane orientation of palmityl-CoA:lysophosphatidyl choline acyl transferase in microsomes isolated from an alveolar type II cell adenoma and rat liver. *Biochim. Biophys. Acta.* **666:** 468–474.

25. Fiske, C. H., and Y. SubbaRow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:** 375–400.

26. Quintart, J., P. Baudhuin, and P. J. Courtoy. 1989. Marker enzymes in rat liver vesicles involved in transcellular transport. *Eur. J. Biochem.* **184:** 567–574.

27. Hosie, L., L. D. Sutton, and D. M. Quinn. 1987. *P*-Nitrophenyl and cholesteryl-n-alkyl carbamates as inhibitors of cholesterol esterase. *J. Biol. Chem.* **262:** 260–264.

28. Quinn, D. M. 1985. Diethyl-*p*-nitrophenyl phosphate: an active site titrant for lipoprotein lipase. *Biochim. Biophys. Acta.* **834:** 267–271.

29. Blomhoff, R., W. Eskild, G. M. Kindberg, K. Prydz, and T. Berg. 1985. Intracellular transport of endocytosed chylomicron [^{3}H]retinyl ester in rat liver parenchymal cells. Evidence for translocation of a [^{3}H]retinoid from endosomes to endoplasmic reticulum. *J. Biol. Chem.* **260:** 13566–13570.

30. Stein, O., Y. Stein, D. S. Goodman, and N. H. Fidge. 1969. The metabolism of chylomicron cholesteryl esters in rat liver. A combined radioautographic–electron microscopic and biochemical study. *J. Cell Biol.* **43:** 410–431.

31. Fu, D., and C. A. Hornick. 1990. Triacylglycerol lipase activities in hepatic endosomes of fed and fasted rats. *FASEB J.* **4:** A583.