

## Retinyl Palmitate Hydrolase Activity in Normal Rat Liver\*

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Retinyl esters are hydrolyzed in liver both during uptake and mobilization of vitamin A. Studies were conducted to explore the enzymatic hydrolysis of retinyl palmitate in normal rat liver. Retinyl palmitate hydrolase activity was assayed with a sensitive and accurate microassay, employing retinyl [1-<sup>14</sup>C]palmitate as substrate. The products of the reaction were identified as retinol and free fatty acid. Hydrolase activity was stimulated by sodium cholate and inhibited by  $\alpha$ -tocopherol. Fractionation of liver homogenates showed greatest enrichment of retinyl palmitate hydrolase activity in the washed "nuclear" fraction, and least activity in the microsomal fraction. About one-third of the total activity was found in the soluble supernatant fraction. This unusual distribution was similar to that previously seen with retinol-depleted rats. Hydrolase activity was found to vary over 50-fold in individual rat livers. The variation was not related to the age, time of day, or order of death of the animals. Hydrolytic activities against cholesteryl oleate and triolein showed a similar variation and correlated strongly with retinyl palmitate hydrolase activity, whereas hydrolytic activities against retinyl acetate and *p*-nitrophenyl acetate did not. Hydrolase activity eluted as one major peak on gel filtration in the presence of 2.5% cholate; with a lower concentration of cholate (0.05%) a diffuse elution pattern was seen. Retinyl palmitate hydrolase activity was partially purified by hydrophobic interaction chromatography. Co-purification, with a comparable enrichment, of the hydrolytic activities against cholesteryl oleate and triolein was observed. The chromatographic and other data indicate that retinyl palmitate hydrolase activity has distinct hydrophobic physical properties. These properties may be involved in its role of catalyzing lipid ester hydrolysis.

Hydrolysis of retinyl esters occurs in the liver both during the hepatic uptake of dietary vitamin A and during the mobilization of vitamin A from its stores in the liver. Thus, it has been shown that hepatic uptake of vitamin A involves hydrolysis of chylomicron retinyl esters, followed by intrahepatic reesterification of the free retinol (1, 2). Similarly, it is

known that vitamin A is mobilized from the liver in the form of the lipid alcohol retinol, bound to its specific transport protein, plasma retinol-binding protein (3, 4). Prior to the mobilization of vitamin A from the liver, the stored retinyl esters (mainly retinyl palmitate) must be hydrolyzed to form retinol.

A limited amount of information about the enzymatic hydrolysis of retinyl esters in liver is available from reports by Mahadevan *et al.* (5) and by Harrison *et al.* (6) from this laboratory. In the latter studies (6), the hydrolysis of retinyl palmitate was explored with liver homogenate fractions from retinol-depleted, retinoic acid-fed rats. Retinol-depleted animals were used in order to avoid the problem posed by large and variable amounts of endogenous substrate (retinyl esters) present in the livers of normal animals. Retinyl palmitate hydrolase activity required a bile salt for stimulation and showed an unusual subcellular distribution, different from that of marker constituents for known subcellular organelles and structures. Another unusual feature of the hepatic retinyl palmitate hydrolase activity was its great variability from rat to rat as assayed *in vitro*. Of 10 lipid and nonlipid esters tested as substrates, only the hydrolytic activities against cholesteryl oleate and phytol oleate resembled retinyl palmitate hydrolase activity in these respects.

We now report the results of studies on the characteristics of retinyl palmitate hydrolase activity in normal rat liver preparations.

### MATERIALS AND METHODS<sup>1</sup>

**Assay for Retinyl Palmitate Hydrolase Activity**—A sensitive microassay was developed, involving the measurement of the enzymatic production of [1-<sup>14</sup>C]palmitic acid from retinyl [1-<sup>14</sup>C]palmitate. Retinyl [1-<sup>14</sup>C]palmitate was synthesized from all-*trans*-retinol (purchased from Eastman Kodak, Rochester, NY, and purified by chromatography on alumina) and [1-<sup>14</sup>C]palmitic acid (55.3 mCi/mmol, purchased from New England Nuclear). The symmetric anhydride of [1-<sup>14</sup>C]palmitic acid was made as described by Selinger and Lapidot (7). Retinyl [1-<sup>14</sup>C]palmitate was then synthesized by reacting retinol with the symmetric anhydride of palmitic acid as described by Lentz *et al.* (8) for cholesteryl ester synthesis. The product of the reaction was purified by chromatography on a small column of alumina (activity grade IV) and was stored in hexane under nitrogen in sealed ampules at -20 °C. The reaction mixture contained 7.6 mg of retinol and 4.6 mg (1 mCi) of palmitic acid as its symmetric anhydride. 36% of recovered <sup>14</sup>C was incorporated into retinyl palmitate (72% of maximum possible yield).

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<sup>1</sup> Portions of this paper (including much of "Materials and Methods," all of "Results," Tables I to IV, and Figs. 1 to 7) are presented in miniprint at the end of this paper. The abbreviation used is: RBP, retinol-binding protein. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80M-2306, cite author(s), and include a check or money order for \$7.20 per set of photocopies. Full size photocopies are also included with the microfilm edition of the Journal that is available from Waverly Press.

Substrate solutions for the retinyl palmitate hydrolase assay consisted of retinyl [ $1\text{-}^{14}\text{C}$ ]palmitate plus unlabeled all-*trans* retinyl palmitate (Sigma) dissolved in absolute ethanol or isopropanol. For routine assays the substrate concentration was 2 nmol of retinyl palmitate/10  $\mu\text{l}$  of solution.

The standard enzyme assay was carried out as follows. All procedures were conducted in dim light. Incubation mixtures contained 1–500  $\mu\text{g}$  of protein as a source of enzyme, and 10  $\mu\text{l}$  of substrate solution, in 0.05 M Tris-maleate buffer at pH 8.0; the final reaction mixture volume was 0.2 ml. For maximal enzyme activity, 1.3 mg of sodium cholate was added to the reaction mixture. Incubations were conducted in disposable screw-top glass test tubes (16  $\times$  100 mm) at 37  $^{\circ}\text{C}$ . At the end of the incubation, free [ $1\text{-}^{14}\text{C}$ ]palmitic acid was extracted as described by Belfrage and Vaughan (9). A 1-ml aliquot of the extracted [ $1\text{-}^{14}\text{C}$ ]palmitate, in alkaline buffer (pH 10), was added to 10 ml of ScintiVerse (Fisher), and assayed for  $^{14}\text{C}$  with a Packard liquid scintillation counter.

**Reaction Mechanism**—The reaction products, free retinol and [ $1\text{-}^{14}\text{C}$ ]palmitic acid, were identified and quantitatively compared in order to establish that the reaction mechanism was one of true hydrolysis. This experiment employed retinol-depleted rats and the fluorescence assay for retinol and retinyl palmitate as described by Harrison *et al.* (6). After incubation, the reaction was stopped by addition of an equal volume of ethanol, along with 0.1 N NaOH to raise the pH to 12. The product retinol and the unhydrolyzed retinyl palmitate were extracted from this mixture with hexane, separated by alumina chromatography, and quantified fluorometrically. The ethanolic alkaline reaction mixture was then acidified to pH 2, and the free [ $1\text{-}^{14}\text{C}$ ]palmitic acid was extracted into hexane and identified as the free fatty acid by thin layer chromatography. A comparison of the yields of free retinol (fluorescence assay) and of [ $1\text{-}^{14}\text{C}$ ]palmitic acid ( $^{14}\text{C}$  assay) indicated that retinol and palmitic acid were produced in equimolar quantities during the enzyme incubation.

#### DISCUSSION

The studies reported here provide information about the enzymatic hydrolysis of retinyl palmitate in normal rat liver. These studies were made possible by the development of a highly sensitive and accurate assay that could be carried out effectively with microgram amounts of liver homogenate protein and nanogram amounts of substrate. This sensitive assay enabled us to employ small enough quantities of liver homogenate so that the endogenous retinyl esters present in normal liver did not interfere with the quantitative accuracy of the assay.

Previous studies of retinyl palmitate hydrolase activity in rat liver reported from this laboratory employed retinol-depleted, retinoic acid-fed rats (6). In these studies, retinyl palmitate hydrolase activity was found to show an unusual subcellular distribution and to display a great variability of observed activity from rat to rat as assayed *in vitro*. Neither of these unusual properties was observed for a variety of other hepatic ester hydrolase activities. The possibility existed, however, that the unusual features of retinyl palmitate hydrolase activity that were observed were a function of the vitamin A-deficient (retinol-depleted) status of these animals. The results reported here demonstrate that this is not the case. Thus, the major characteristics of retinyl palmitate hydrolase activity observed here with normal rat liver were similar to those previously observed with retinol-depleted rat liver. In addition, the present studies substantially extend the information available about the characteristics of this enzyme activity.

In both normal (present studies) and retinol-depleted (6) rat liver, subcellular distribution experiments showed that retinyl palmitate hydrolase activity was particularly enriched in the washed “nuclear” fraction and was also present to a significant extent in the mitochondrial-lysosomal fraction. About one-third of the total activity was found in the soluble supernatant fraction. This subcellular distribution differs, to our knowledge, from all other liver enzymes that have been

examined. In the previous studies with retinol-depleted rats, when highly purified preparations of nuclei and of plasma membranes were isolated from the “nuclear” fraction, they were found not to be enriched in retinyl palmitate hydrolase activity. It was suggested (6) that the unusual subcellular distribution observed might have resulted in part from the enzyme becoming absorbed to particulate material (*i.e.* membranous components) that sediments in the “nuclear” (and mitochondrial-lysosomal) fraction.

The results reported here support this suggestion. Gel permeation chromatography on Sepharose columns provided evidence that high levels of cholate were necessary to keep retinyl palmitate hydrolase solubilized. Thus, in the presence of 2.5% sodium cholate, the hydrolase activity eluted as a single major peak well within the included volume of the column. In the presence of only low concentrations (0.05%) of cholate, however, a substantial amount of the activity was eluted in or near the void volume, suggesting that, under these conditions, retinyl palmitate hydrolase had aggregated or adhered to other large and ill-defined aggregates. These observations suggest that the enzyme has significantly hydrophobic physical properties.

Further evidence that retinyl palmitate hydrolase has a high degree of hydrophobicity was provided by hydrophobic interaction chromatography. The hydrolase was effectively absorbed to phenyl-Sepharose and was not eluted from the hydrophobic support until high concentrations of cholate were applied to the column. Furthermore, similar behavior on phenyl-Sepharose was observed with retinyl palmitate hydrolase in acetone powder extracts of liver, from which most of the liver lipid had been extracted. Thus, the hydrophobic properties appear to be an intrinsic property of the protein, rather than reflecting lipid associated with the enzyme. We suggest that these hydrophobic properties are likely to be responsible for the unusual subcellular distribution found for this enzyme activity. Since this enzyme presumably acts at a lipid-water interface, to hydrolyze retinyl esters stored in cytoplasmic lipid droplets or present in the nonpolar core of a chylomicron remnant, its hydrophobic properties may have considerable physiological importance.

Recently, Chen and Heller (10, 11) have described a lipid-protein “complex” from rat liver which has associated with it both some endogenous retinyl ester and retinyl ester hydrolase activity. On gel filtration, this complex eluted in the void volume. This elution profile is consistent with our observations (not reported here in detail) on gel filtration of rat liver cytosol when no solubilizing agent was present in the eluting buffer, and where elution patterns resembling that shown in Fig. 6, *bottom panel*, were obtained. The extent to which the “complex” reported by Chen and Heller (11) represents an entity present in the intact liver cell or, alternatively, reflects a phenomenon that occurs after homogenization due to the hydrophobic properties of retinyl palmitate hydrolase warrants future investigation. With the information now available, we cannot define the *in situ* location of the hydrolase enzyme(s) within the liver cell. However, the hydrophobic properties of the enzyme may well direct it to be associated with retinyl ester containing lipid droplets or lipid-protein aggregates in the cytoplasm of the cell.

The stimulation of retinyl palmitate hydrolase activity by cholate agrees with previous reports by Mahadevan *et al.* (5) and from this laboratory (6). In addition, retinyl palmitate hydrolase activity was found to vary over 50-fold from rat to rat in the animals used in the studies reported here. Any single preparation, however, showed the same activity in replicate assays, even after having been stored frozen for periods of several months. Despite a number of experiments

directed at this question, the factor(s) responsible for this individual variation have not been identified. The variation was, however, not related to the age of the animals, time of day of death, or the order in which they were killed. Furthermore, a comparable variation was observed with different strains of rats (Holtzman *versus* Sprague-Dawley).

Hepatic retinyl palmitate hydrolase activity was negatively correlated (with  $p < 0.05$ ) with serum vitamin A levels at the time of death. The significance of this is unclear, however, because a similar degree of variation of hydrolase activity was observed previously in retinol-depleted rats, all of which had negligible levels of serum vitamin A (6). In the normal rat, it is possible that lower serum vitamin A levels in some way signal the liver to increase retinyl palmitate hydrolase activity in order to stimulate mobilization of hepatic vitamin A stores. Further investigation of this relationship will be necessary to explore this and other possibilities.

Experiments were also conducted to see if cyclic AMP-mediated protein phosphorylation mechanisms might be involved in the variation (and regulation) of enzyme activity, as has been reported for lipase and cholesteryl ester hydrolase in other tissues (12-14). As reported under "Results," these experiments did not provide evidence for the involvement of such mechanisms in the observed interanimal variation of retinyl palmitate hydrolase activity. As previously pointed out (6), the finding of substantial variation in the activity of a hepatic enzyme among identically treated animals is unusual but not unprecedented. Felton *et al.* (15) have reported that the level of  $\beta$ -galactosidase activity in mice is under strong genetic control and varies considerably in mice of different genetic endowments. The possibility must be considered that the observed variation in retinyl palmitate hydrolase activity has, in part, a genetic basis, although no information is available on this issue.

Strongly positive correlations were observed between retinyl palmitate hydrolase activity and the hydrolytic activities against cholesteryl oleate, triolein, and phytol oleate, both with whole liver homogenates, and with partially purified enzyme preparations obtained after hydrophobic interaction chromatography. In contrast, there was no correlation between retinyl palmitate hydrolase activity and retinyl acetate hydrolytic activity in the whole homogenate, and retinyl palmitate hydrolase activity was fully separated from retinyl acetate hydrolyzing activity (presumably representing non-specific esterase activity) on the phenyl-Sepharose column. Thus, retinyl palmitate, cholesteryl oleate, and triolein (all physiological lipid substrates) were hydrolyzed by partially purified retinyl palmitate hydrolase preparations with the same high, medium, and low activity pattern. These findings raise the possibility that these three hydrolytic activities may be coordinated in some way, or may be due to a single enzyme or enzyme complex, within the liver cell. Since all three of these lipid ester substrates are normally taken up by the liver

in association with chylomicron remnants and subsequently hydrolyzed, it is possible that these hydrolytic activities may be involved in part in the catabolism of this lipoprotein particle. Future studies will be needed in order to define the relationships between retinyl palmitate, cholesteryl ester, and triglyceride hydrolysis, and the significance of such relationships, in the liver.

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## RETINYL PALMITATE HYDROLASE ACTIVITY IN NORMAL RAT LIVER

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## MATERIALS AND METHODS

**Animals and enzyme preparations.** These studies were conducted with homogenates and homogenates of livers from Sprague-Dawley strain rats were fed an ordinary laboratory chow diet prior to study, except where specifically indicated otherwise. No difference with regard to enzymatic activity characteristics were noted between the two strains of rats.

Homogenates of rat liver were prepared and were fractionated as described previously (6), according to the method of Amar-Costesec et al. (16). As used, the differential centrifugation procedure resulted in the separation of four subcellular fractions: The "nuclear", mitochondrial-lysosomal, microsomal, and supernatant fractions. In some experiments, homogenates were prepared using a chilled Waring blender.

Acetone powders of pooled rat livers were obtained from Pel-Freez Biologicals, Inc. (Rogers, AS), or were prepared from homogenates as described by Norton (17). The powders were subsequently extracted with 20 volumes of buffer containing 2.5% (w/v) sodium cholate, 50 mM Tris-HCl pH 7.0, and 0.02% sodium azide, for one hour at 0-4°C.

In some experiments, homogenates were solubilized (or partly solubilized) with cholate by addition of a measured amount of anhydrous sodium cholate, followed by 30 seconds of sonification with a Branson sonifier. The resulting preparation was centrifuged at 18,000 rpm (Sorvall SM-24 rotor) for 2 hours to yield soluble supernatant and pellet fractions.

Several of the studies reported here employed liver homogenate pools of high, medium, or low retinyl palmitate hydrolase activity. In order to prepare these pools, 29 homogenates of individual livers were combined into three pools on the basis of their hydrolase specific activity, as assayed with each liver homogenate individually. The hydrolase specific activity values (expressed as pmol palmitic acid produced/minute/mg protein) for each of the three pools (designated high, medium, and low enzyme activity pools) are shown in Table I. These three pools were used to compare some of the characteristics of the enzyme activity at these three activity levels.

Table I. Characteristics of Pooled Homogenates with High, Medium, and Low Retinyl Palmitate Hydrolase Activity

Pool	Protein (mg/ml)	Retinyl palmitate hydrolase activity (2.5% cholate)	Hydrolase activity (2.5% cholate)
High (11)	61	52	178 (125-233) <sup>c</sup>
Medium (9)	59	14	44 (15-77) <sup>c</sup>
Low (9)	57	2.0	8 (5-10) <sup>c</sup>

a Percent hydrolase of 2.5% of retinyl palmitate in the routine assay, using a 2 hour incubation, with 40  $\mu$ l of 50-fold dilution of the homogenate pool.

b As pmol-min<sup>-1</sup>-mg protein<sup>-1</sup>

c Number of rat livers in parentheses

d Activity range of individual homogenates

**Assays for hydrolysis of lipid esters other than retinyl palmitate.** The enzymatic hydrolysis of cholesteryl oleate and of triolein was assayed with assays identical to that described for retinyl palmitate hydrolase, except for the use of a different substrate. Cholesteryl [14C]oleate was purchased from New England Nuclear (51.0 mCi/mmol) or Amersham/Searle (21.5 mCi/mmol). Triolein (carboxyl [14C]) was purchased from New England Nuclear (84 mCi/mmol). Nonradioactive cholesteryl oleate and triolein were purchased from Sigma. For triolein, substrate solutions were made by dissolving the lipid in acetone or isopropanol.

**Assays for the hydrolysis of retinyl acetate, of phytol oleate, and of p-nitrophenyl acetate** were conducted as described previously (6) with minor modifications:  $\alpha$ -tocopherol was omitted from the reaction mixture for both of these assays.

**Marker enzyme assays.** The following enzymes were assayed as markers for the fractions prepared by differential centrifugation of rat liver homogenates: 5'-nucleotidase (plasma membrane); cytochrome oxidase (mitochondria); acid phosphatase (lysosomes); and glucose-6-phosphatase (endoplasmic reticulum). Cytochrome oxidase (18); glucose-6-phosphatase (19); and 5'-nucleotidase (19) were assayed according to the indicated published procedures. Acid phosphatase was assayed as described previously (6).

**Cell permeation chromatography.** Rat liver homogenates were solubilized with 2.5% (w/v) sodium cholate and centrifuged (2 hr x 18,000 rpm) to remove connective tissues and insoluble debris. The supernatant was then applied to the column packed with Sepharose CL-6B (Pharmacia). All column chromatography was conducted at 0-4°C in a cold room. The eluting buffer consisted of 50 mM Tris-HCl, pH 7.0, containing 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and the appropriate amount of sodium cholate (either 0.05% or 2.5%, see Results). The samples were applied to the column in a ratio of 1 ml of cholate-solubilized homogenate to 75 ml gel bed volume. Eluted fractions were assayed for absorbance at 280 nm and for enzyme activity.

**Hydrophobic interaction chromatography.** Pilot experiments were first conducted to find the highest concentration of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> where retinyl palmitate hydrolase activity, in whole liver homogenate solubilized with 2.5% sodium cholate (see Results section), would remain soluble. On subsequent chromatography the high salt concentration would provide conditions favoring hydrophobic interaction of the enzyme with the hydrophobic gel. The results of these experiments showed that 33% saturation (19% (w/v) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) was the highest concentration where the hydrolase activity remained soluble (less than 10% of activity lost from the soluble fraction). At a saturation of 43% with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, less than 40% of the enzyme activity remained soluble, and at a saturation of 50% less than 5% was recovered in the supernatant solution.

**Hydrophobic interaction chromatography** was conducted with columns packed with Phenyl-Sepharose CL-6B (Pharmacia). For chromatography, the sample of rat liver homogenate was solubilized with 2.5% sodium cholate (see above), and the resulting solution was diluted with an equal volume of buffer containing 2.5% (w/v) sodium cholate, 100 mM Tris-HCl pH 7.0, and 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>. The enzyme preparation was kept at 0-4°C during all procedures. Solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added slowly with stirring to a final concentration of 19% (w/v). After centrifugation to remove precipitated protein, the supernatant was dialyzed against 50 mM Tris-HCl pH 7.0 buffer containing 19% (w/v) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and without cholate for 24-48 hours. The resulting dialyzed solution was frozen and stored at -20°C in aliquots until used for chromatography.

For Phenyl-Sepharose chromatography, the ratio of sample size to bed volume was approximately 1:1. The gel was equilibrated with 19% (w/v) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> before application of the sample. Elution of protein from the column was conducted in four steps. Proteins which did not interact with the column were eluted with a buffer ("high salt") containing 19% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 50 mM Tris-HCl pH 7.0 with 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>. eluted with this buffer was carried out until the A<sub>280</sub> returned to baseline. Proteins which interacted weakly with the gel were next eluted with the same buffer but not containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ("low salt"). Proteins which absorbed to the gel were then eluted with a linear gradient of 0 to 3% (w/v) sodium cholate in the "low salt" buffer. The volume of buffer eluted during the gradient run was 8 times the gel bed volume. Additional proteins which interacted with the support were eluted with "low salt" buffer containing 3% sodium cholate.

**Other assays.** Protein was determined by the method of Lowry et al. (20), using bovine serum albumin as a standard. Cholate was measured by a modification of the method of Mosbach et al. (21).

Serum vitamin A concentration was determined using a modification (22) of the fluorometric method of Thompson et al. (23). Rat RBP levels were assayed with a specific radioimmunoassay developed in this laboratory (24).

Statistical analyses (linear regression and correlation analyses) were computed using a Wang programmable calculator.

## RESULTS

**Retinyl palmitate hydrolase assay: effect of  $\alpha$ -tocopherol; other characteristics.** Vitamin E ( $\alpha$ -tocopherol) was included as an antioxidant in the previously described fluorometric assay for retinyl palmitate hydrolase activity (6). Using retinyl [1-<sup>14</sup>C] palmitate as substrate, the effect of the inclusion of  $\alpha$ -tocopherol in the assay was evaluated. When increasing amounts of enzyme were added to the assay mixture in the presence and absence of 50  $\mu$ g of  $\alpha$ -tocopherol per 2 ml reaction mixture, less enzymatic activity was observed in the presence of  $\alpha$ -tocopherol for all amounts of enzyme assayed (Figure 1). Accordingly,  $\alpha$ -tocopherol was deleted from the radioisotopic hydrolase assay employed here.

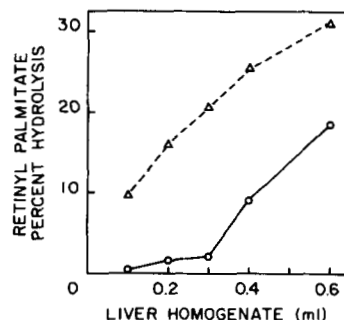


Fig. 1. Inhibition of retinyl palmitate hydrolase activity by  $\alpha$ -tocopherol. The effect of the presence (O) or absence ( $\Delta$ ) of 50  $\mu$ g of DL- $\alpha$ -tocopherol per 2 ml on hydrolase activity is shown when increasing amounts of liver homogenate, as source of enzyme, were added to the reaction mixtures. The reaction mixtures were scaled up from 0.2 ml (see Methods section) to 2 ml for this experiment.

The assay reported here is highly sensitive, and permits the quantitation of enzyme activity using microgram amounts of liver homogenate protein and nanogram amounts of substrate. The sensitivity of the assay enabled us to conduct experiments with diluted liver homogenates from normal, retinol-fed rats, because the amount of endogenous stores present in normal liver would contribute only 2-3% of the total substrate present in the assay.

Experiments were carried out to determine some of the characteristics of retinyl palmitate hydrolase activity in whole liver homogenates, as observed with this assay. Rat liver homogenates diluted 50-fold with Tris-maleate assay buffer showed a linear relationship between the rate of hydrolysis of retinyl palmitate and the amount of enzyme (protein) added, in the range of 0 to 60  $\mu$ g of homogenate protein per 0.2 ml incubation. The rate of retinyl palmitate hydrolysis was constant for at least 2 hours when approximately 36  $\mu$ g of homogenate protein was used as source of enzyme.

Figure 2 shows the stimulatory effect of adding sodium cholate to the reaction mixture. Maximal stimulation was observed with approximately 1.5 mg/assay, with a plateau of slightly lower activity seen at the higher concentrations tested. For routine enzyme assays, 1.3 or 1.5 mg cholate was usually added per assay mixture. For the determination of recovery, or for the quantitation of enzyme activity partially purified by column chromatography (where varying concentrations of cholate were present in the elution buffers), assays were conducted with 2.5 - 4 mg cholate per assay to ensure uniform cholate effects in all samples.

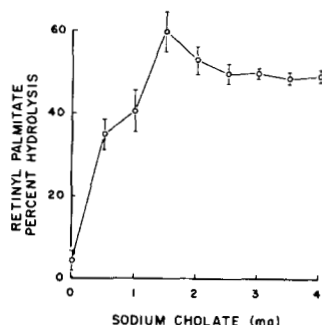


Fig. 2. The effect of sodium cholate on the enzymatic hydrolysis of retinyl palmitate. The enzyme source consisted of pooled rat liver homogenates containing high levels of retinyl palmitate hydrolase activity diluted 50-fold with buffer (50 mM Tris-HCl, pH 7.0, 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>). Each 0.2 ml assay mixture contained 40  $\mu$ l of enzyme solution (48  $\mu$ g protein) and the amount of cholate indicated. Incubations were for 120 minutes. Mean  $\pm$  1 S.D. (n = 3-5 per data point) values are shown.

**Subcellular distribution.** Four subcellular distribution experiments were conducted: the results for retinyl palmitate hydrolase activity and total homogenate protein, are shown in Table II. The distributions of marker enzymes (data not shown) among the four subcellular fractions closely resembled the distributions reported previously by others (16), and from this laboratory (6). These data indicated that the subcellular structures for which marker enzymes were assayed (plasma membranes, mitochondria, lysosomes, and endoplasmic reticulum, see Methods section) were distributed appropriately in each of the four subcellular homogenate fractions.

Table II. Subcellular Distribution of Retinyl Palmitate Hydrolase Activity

Fraction	Protein (Percentage) <sup>a</sup>	Retinyl palmitate hydrolase (Percentage) <sup>b</sup>	(Specific activity) <sup>c</sup>
"Nuclear"	16.4 $\pm$ 2.8	26.4 $\pm$ 4.8	1.61
Mitochondrial <sup>c</sup>	22.6 $\pm$ 1.2	24.6 $\pm$ 3.6	1.09
Microsomal	22.7 $\pm$ 2.4	16.1 $\pm$ 4.7	0.62
Supernatant	38.2 $\pm$ 1.5	34.5 $\pm$ 5.3	0.90

a of total recovered in the four subcellular fractions

b Relative specific activity calculated as percentage of activity divided

c by percentage of protein in each fraction

d Mitochondrial-lysosomal fraction

Retinyl palmitate hydrolase activity distributed among the four subcellular fractions in an unusual way, distinctly unlike that of any of the marker enzymes, or of other known hepatic constituents. The greatest enrichment (relative specific activity) for hydrolase activity was found in the "nuclear" fraction, while the least hydrolase activity (in both total and relative specific activity terms) was found in the microsomal fraction. Approximately one-third of the total enzyme activity was present in the soluble supernatant fractions. These results were qualitatively similar to those obtained by Harrison et al. (16) with retinoid-depleted rats. Thus, retinyl palmitate hydrolase activity is not predominantly localized to one subcellular structure or compartment as isolated by this procedure in normal as well as in vitamin A-deficient rats.

**Variation of enzyme activity in individual rat liver homogenates.** Figure 3 shows the variation in the levels of retinyl palmitate hydrolase activity observed in 95 individual rat liver homogenates, all of which were assayed under identical conditions. The distribution is skewed, with a greater proportion of the animals falling in the lower portion of the activity range. The median specific activity was 60 pmol palmitic acid produced/minute/mg protein. For the entire series of livers, the observed hydrolase specific activity values varied 50-fold, from 5 to 247. These results are similar to those previously observed with retinoid-depleted rats, where a more than 40-fold variation in activity among individual preparations was found (6).

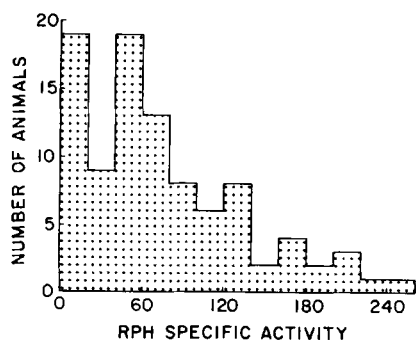


Fig. 3. Variation of retinyl palmitate hydrolase (RPH) activity in individual rat liver homogenates. Hydrolase specific activity values (pmol palmitic acid produced/min/mg protein) in liver homogenates from 95 individual normal rats are shown. Each assay contained 48  $\mu$ g protein as source of enzyme and 2 nmol retinyl [ $1-^{14}$ C]palmitate as substrate.

The observed variation in enzyme activity reflects differences in activity among individual rat livers, and not instability of the activity within a given preparation or a lack of reproducibility of the assay. For any given liver homogenate, the observed level of hydrolase activity was highly reproducible on repeat assay after several weeks of storage in the frozen state or after several days at 4°C. Values for retinyl palmitate hydrolase activity virtually identical to those observed initially were obtained after prolonged storage at -20°C for both high, medium, and low activity homogenates.

A number of experiments were conducted to try to explore factors that might be contributing to the observed variation in hydrolase activity among individual rat liver homogenates. In one series of experiments over the course of 2 weeks, Holtzman rats (ages 41–55 days; weights 122–286 g) were killed in four groups of 4–6 rats each, and retinyl palmitate hydrolase activity was assayed in each rat liver on the day of sacrifice. The range of hydrolase specific activity values observed in each group was as follows: 75–247 (41 days old); 51–142 (43 days old); 17–207 (48 days old); and 47–182 (55 days old). The independent correlations between age, weight, and order of sacrifice with hydrolase activity were not statistically significant.

In another experiment, 29 rats (weights 175–200 g) were sacrificed serially during one day. The range in hydrolase specific activity was 6–182 pmol/min/mg protein. The order in which the animals were sacrificed had no relationship to the enzyme activity observed.

An experiment was conducted to search for a possible relationship between the serum vitamin A level and the hepatic level of retinyl palmitate hydrolase activity. The results are shown in Figure 4. A negative correlation was observed which was of marginal statistical significance ( $p < 0.05$ ).

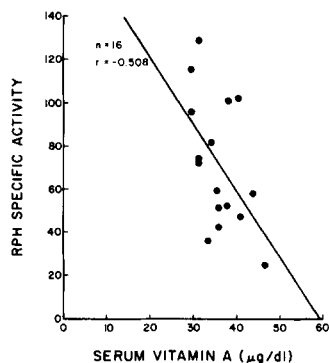


Fig. 4. Relationship between hepatic retinyl palmitate hydrolase (RPH) activity and serum vitamin A levels. The 16 rats were of similar age and size. The hydrolase values (y-axis) are in pmol palmitic acid produced/min/mg protein. Linear regression analysis provided a correlation coefficient ( $r$ ) of -0.508 ( $p < 0.05$ ) and the equation  $y = -3.02x + 179.6$ .

Experiments were also carried out to examine several perturbations that might alter the observed level of retinyl palmitate hydrolase activity, and that hence might provide insight into factors involved in the variation of enzyme activity. In one set of experiments, liver homogenate preparations were incubated with cyclic AMP, ATP, and  $Mg^{++}$  to determine whether activation of a low activity preparation, or inactivation of a high activity preparation would occur. The conditions employed were similar to those employed by Khoo et al. (12) in studies of glyceride and cholesterol ester hydrolysis with adipose tissue preparations. Incubations were carried out both with and without added cholate. No activation or inactivation of retinyl palmitate hydrolase activity was observed after incubation of the liver homogenate preparations with cyclic AMP. In other experiments, prior incubation of rat liver homogenates at 37°C for periods up to one hour before beginning the hydrolase assay did not alter the level of enzymatic activity.

In a final set of experiments, acetone powder extracts were prepared from homogenates of 2 livers, one with very high and one with low retinyl palmitate hydrolase activity, and the enzyme activity of the acetone powder extracts then determined. The hydrolase specific activity values (pmol/min/mg protein) for the homogenates were 16 (low) and 412 (high); for the corresponding acetone powder extracts the specific activity values were 13 (low) and 737 (high). Thus, the hydrolase specific activity of the low activity homogenate remained low in the corresponding acetone powder extract, whereas that of the high activity homogenate remained high in the corresponding acetone powder extract.

At the present time, the basis for the observed marked variation in retinyl palmitate hydrolase activity among individual rat liver homogenates remains obscure. Given the existence of this variation, however, the approach taken for the study of the characteristics of the hydrolase activity was to first screen individual liver homogenates as to level of activity, and in general (e.g., for the subcellular distribution experiments shown in Table II) to select for study those livers with medium to high activity. In many instances, experiments were repeated with lower activity preparations in an effort to explore further possible reasons for the differences seen between preparations.

**Solubilization of hydrolase activity with cholate.** The concentration-dependent effects of cholate on the solubilization of total liver homogenate protein and of retinyl palmitate hydrolase activity were examined with the liver homogenate pools of high, medium, and low activity described in Table I. All three homogenate pools behaved identically with respect to solubilization of protein and of hydrolase activity. The results obtained with the homogenate pool of medium activity are shown in Figure 5; similar results (data not shown) were obtained with the other two homogenate pools. In each case the solubilization of hydrolase activity paralleled that of total protein, and the concentration of sodium cholate required for maximal solubilization of hydrolase activity was approximately 1%.

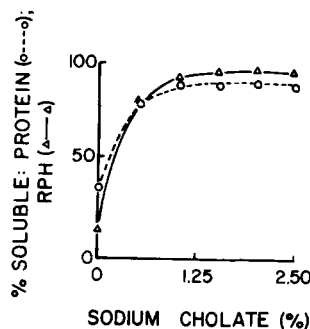


Fig. 5. Solubilization of liver homogenate protein and retinyl palmitate hydrolase (RPH) activity with sodium cholate. The percent of sodium cholate (w/v) in each homogenate sample is plotted on the x-axis. Each sample was centrifuged and analyzed for retinyl palmitate hydrolase and protein in the resulting supernatant and pellet. The % of the total recovered hydrolase activity or protein found in the supernatant is plotted on the y-axis.

An experiment was also conducted to compare solubilization with cholate and with Triton X-100. Cholate solubilized a greater proportion of retinyl palmitate hydrolase activity (98%) than did Triton X-100 (80%). In addition, the recovery of hydrolase activity was greater in the presence of cholate (91%) than with Triton X-100 (70%).

**Gel permeation chromatography.** Figure 6 shows the elution patterns of retinyl palmitate hydrolase activity obtained when high or low concentrations of cholate were present in the eluting buffer. In the top panel, the solubilized homogenate was eluted from the Sepharose gel column using a buffer containing 2.5% (w/v) sodium cholate. Virtually all of the recovered hydrolase activity eluted as one major peak well within the included volume of the column (fractions 38–48, Fig. 6, top panel). In the experiment shown in the lower panel, the solubilized homogenate was eluted from the column using the same buffer, but containing only 0.05% (w/v) (instead of 2.5%) sodium cholate. In this case, a large proportion of the material eluted in the opalescent fractions (number 20–32, lower panel Fig. 6) representing the void volume. The hydrolase activity elution profile was quite diffuse and irregular, and much of it was eluted in the void volume and adjacent fractions.

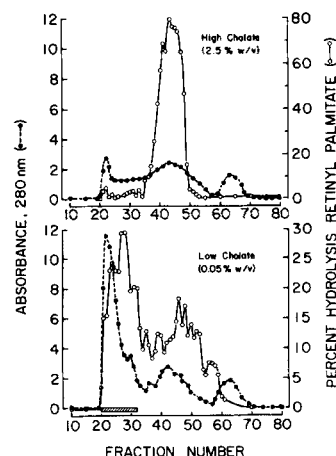


Fig. 6. Gel permeation chromatography of retinyl palmitate hydrolase activity on Sepharose CL-6B. **Upper panel:** Two ml of rat liver homogenate solubilized with 2.5% sodium cholate was applied to a column with a bed volume of 150 ml and eluted in buffer containing 50 mM Tris-HCl, 2.5% sodium cholate (w/v), 0.02M NaH<sub>2</sub>PO<sub>4</sub> (w/v), at pH 7.0. Each fraction contained 3.2 ml. **Lower panel:** Six ml of homogenate solubilized with 2.5% sodium cholate (w/v), was applied to a column with a bed volume of 325 ml and eluted with the same buffer, but containing only 0.05% sodium cholate (w/v). Each fraction contained 4.7 ml.

Fractions 20–32 from the experiment shown in the lower panel of Fig. 6 (and indicated by the hatched bar in the figure) were pooled, concentrated, reabsorbed with 2.5% sodium cholate, and re-chromatographed on the same column, using a buffer containing a high concentration (2.5%) of sodium cholate, as was done in the experiment shown in the top panel of Fig. 6. Retinyl palmitate hydrolase activity was now eluted as a single peak, exactly as shown in the top panel of Fig. 6.

The results of these three experiments showed that only one major peak of retinyl palmitate hydrolase activity was observed on gel permeation chromatography when sufficiently high concentrations of cholate were present in the eluting buffer. The diffuse elution profile observed in the near absence of cholate suggested that under these conditions the hydrolase had aggregated or become insoluble.

**Hydrophobic interaction chromatography.** Preliminary experiments demonstrated that retinyl palmitate hydrolase activity was partially purified by hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B. This procedure was therefore used to explore some of the characteristics of the hydrolase activity. The hydrolase elution profiles for 3 homogenate pools differing in their hydrolase specific activity levels (see Table I) were compared, and studies were subsequently carried out with the partially purified enzyme preparations. The recovery of enzyme activity from the Phenyl-Sepharose column, compared to the activity applied to the column, was generally of the order of 80–90%.

Figure 7 shows the elution pattern of retinyl palmitate hydrolase activity obtained when a sample of the high activity liver homogenate pool was chromatographed on Phenyl-Sepharose. Hydrolase activity was eluted as one major peak during the upper half of the cholate gradient and on continued elution with 3% cholate in buffer. The elution profiles of samples from the medium and low activity homogenate pools also showed peaks of retinyl palmitate hydrolase activity eluting at about the same location in the cholate gradient (data not shown). With these latter samples, however, the peak of hydrolase activity eluting in the cholate gradient was reduced in magnitude in proportion to the level of hydrolase activity in the starting homogenate pool. These data suggest that material which elutes during the latter portion of the cholate gradient is responsible for the retinyl palmitate hydrolase activity, and the differences in hydrolase activity, observed in whole liver homogenates. The total recovery of enzyme activity in the Phenyl-Sepharose column fractions relative to the starting homogenate pools (i.e., after cholate solubilization, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> addition, dialysis, centrifugation, and Phenyl-Sepharose chromatography) was 54% (for the high activity pool), 75% (for the medium activity pool), and 65% (for the low activity pool). In each instance, the fractions comprising the peak of eluted retinyl palmitate hydrolase activity were combined and used for further study; these combined fractions are referred to, subsequently, as partially purified enzyme preparations.

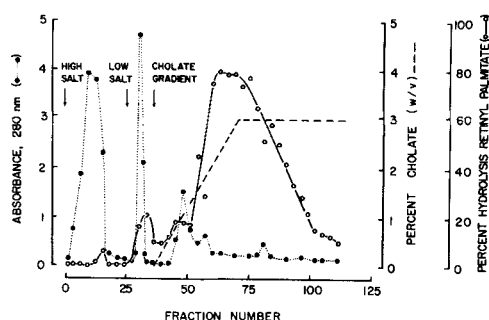


Fig. 7. Hydrophobic interaction chromatography of rat liver homogenate containing high retinyl palmitate hydrolase activity. The sample (46 ml) was applied to a column of Phenyl-Sepharose CL-4B with a bed volume of 45 ml equilibrated with buffer containing 50 mM Tris-HCl, 0.022 M Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, 19% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (w/v), at pH 7.0. Fractions contained approximately 10 ml. Fractions 1 to 25 were collected using the column equilibration buffer; fractions 26 to 36 with the same buffer but without (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; fractions 37 to 70 with a linear gradient of 0 to 3% sodium cholate (w/v) in the previous buffer; fractions 71 to 113 with the same buffer containing 3% cholate.

Retinyl palmitate hydrolase activity was enriched approximately to the same extent by Phenyl-Sepharose chromatography of samples from each of the three different activity level pools. The extent of enrichment, for each of the three homogenate pools, in the partially purified eluted sample compared to the starting homogenate, was: high activity sample: 23-fold; medium activity sample: 27-fold; and low activity sample: 18-fold. Thus, the relative specific activity of the hydrolase eluted from Phenyl-Sepharose with buffer containing cholate reflected the relative specific activity of each homogenate sample applied to the column. The high activity homogenate pool was 4-times more active than the medium activity homogenate pool and 23-times more active than the low activity pool. Similarly, the high activity partially purified enzyme was 3.5-times as active as the medium activity partially purified enzyme and 31-times as active as the low activity preparation.

The column fractions eluted from the Phenyl-Sepharose column (Figure 7) were assayed for immunoreactive RBP. Immunoreactive RBP was found to elute at low concentrations of sodium cholate (0.25 - 1.2%, w/v), and to be eluted from the column before the peak of retinyl palmitate hydrolase activity.

Hydrophobic interaction chromatography was also carried out with an acetone powder extract with a high hydrolase specific activity of 570 pmol palmitic acid/minute/mg protein. The elution profile of hydrolase activity from Phenyl-Sepharose was essentially the same as that seen with whole homogenate samples (Fig. 7).

**Substrate specificity studies.** Substrate specificity studies were carried out with the three homogenate pools with different hydrolase activity levels (Table I), and with the partially purified RBP preparations derived from each of these pools by chromatography on Phenyl-Sepharose. The results of these studies are shown in Tables III and IV.

For the whole homogenates, there was a highly significant linear relationship between the retinyl palmitate hydrolase activity and the activities hydrolyzing cholesteryl oleate, triolein, and phytol oleate when assayed under the same conditions (Table III). In contrast, there was no relationship between the retinyl palmitate hydrolase activity and the hydrolytic activities for retinyl acetate or p-nitrophenyl acetate (Table III).

Table III. Relationship Between Hydrolytic Activities Against Several Substrates

Pool <sup>b</sup>	Hydrolase activities <sup>a</sup>				
	Retinyl palmitate	Cholesteryl oleate	Triolein	Phytol oleate	Retinyl acetate
Low	8	24	324	3	950
Medium	44	547	1600	153	800
High	178	5750	5450	2380	920

<sup>a</sup> As pmol·min<sup>-1</sup>·mg protein<sup>-1</sup>, for substrates shown, except for p-nitrophenyl acetate, which is given as absorbance units at 400 nm·min<sup>-1</sup>·mg protein<sup>-1</sup>

<sup>b</sup> Homogenate pool described in Table I.

Furthermore, the hydrolytic activities against retinyl palmitate, cholesteryl oleate, triolein, and phytol oleate (data not shown) all correlated closely with each other in the partially purified enzyme preparations eluted from the Phenyl-Sepharose columns (Table IV). Hydrolytic activities against retinyl acetate and p-nitrophenyl acetate were not detectable in these partially purified enzyme preparations. Thus, the hydrolytic activities against the lipid substrates shown in Table IV had been separated from non-specific esterase activity by the hydrophobic interaction chromatography.

Table IV. Relationship Between Hydrolytic Activities in Partially Purified Enzyme Preparations

Pool <sup>b</sup>	Hydrolase activities <sup>a</sup>		
	Retinyl palmitate	Cholesteryl oleate	Triolein
Low	0.14	0.44	2.3
Medium	1.19	16.5	38.5
High	4.16	74.0	172.

<sup>a</sup> As nmol·min<sup>-1</sup>·mg protein<sup>-1</sup> for each substrate shown

<sup>b</sup> Partially purified enzyme obtained from the homogenate pools shown (see Table I).

For each homogenate pool, Phenyl-Sepharose chromatography resulted in a comparable enrichment (compared to the initial liver homogenate) in the hydrolytic activities against cholesteryl oleate, triolein, and phytol oleate. For the medium activity homogenate pool, the partially purified preparation obtained from the Phenyl-Sepharose column was enriched as follows for the hydrolytic activities against the four substrates: 27-fold for retinyl palmitate; 30-fold for cholesteryl oleate; 24-fold for triolein; and 22-fold for phytol oleate.