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 [1-14C]palmitate as substrate. The products of the reaction were identified as retinol and free fatty acid. Hydrolysis activity was stimulated by sodium cholate and inhibited by a-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. Hydrolysis activity was found to vary over 50-fold in individual rat livers. The variation was not related to the age, time of death, 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. Hydrolysis 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 hydrolysis was its great variability from rat to rat (6) as assayed in vitro. Of 10 lipid and nonlipid esters tested as substrates, only the hydrolytic activities against cholesteryl oleate and phytanyl oleate resembled retinyl palmitate hydrolysis 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

Assay for Retinyl Palmitate Hydrolase Activity—A sensitive microassay was developed, involving the measurement of the enzymatic production of [1-14C]palmitic acid from retinyl [1-14C]palmitate. Retinyl [1-14C]palmitate was synthesized from all-trans-retinol (purchased from Eastman Kodak, Rochester, NY, and purified by chromatography on alumina) and [1-14C]palmitic acid (55.3 mCi/mmol, purchased from New England Nuclear). The symmetric anhydride of [1-14C]palmitic acid was made as described by Selinger and Lapidot (7). Retinyl [1-14C]palmitate was then synthesized by reacting retinol with the symmetric anhydride of palmitic acid as described by Lentz et al. (6) 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 14C was incorporated into retinyl palmitate (72% of maximum possible yield).

1 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, 10650 Rockville Pike, Bethesda, MD 20014. Request Document No. 35M-2305, 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 [1-14C]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 μl of solution.

The standard enzyme assay was carried out as follows. All procedures were conducted in dim light. Incubation mixtures contained 1-500 μg of protein as a source of enzyme, and 10 μl 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 X 100 mm) at 37°C. At the end of the incubation, free [1-14C]palmitic acid was extracted as described by Belfrage and Vaughan (9). A 1-ml aliquot of the extracted [1-14C]palmitate, in alkaline buffer (pH 10), was added to 10 ml of ScintiVerse (Fisher), and assayed for 14C with a Packard liquid scintillation counter.

Reaction Mechanism—The reaction products, free retinol and [1-14C]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 M 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 [1-14C]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 [1-14C]palmitic acid (14C 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 a 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.

From 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 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 pattern 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 (Holztzaan versus Sprague-Dawley).

Hepatic retinyl palmitate hydrolyase 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 hydrolyase 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 hydrolyase 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 hydrolysis 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 hydrolyase activity. 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 hydrolyase activity has, in part, a genetic basis, although no information is available on this issue.

Strongly positive correlations were observed between retinyl palmitate hydrolyase 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 hydrolyase activity and retinyl acetate hydrolytic activity in the whole homogenate, and retinyl palmitate hydrolyase 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 hydrolyase 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.

Acknowledgments—We thank with appreciation Dr. E. H. Harrison for advice, assistance, and valuable discussions, Drs. Y. I. Takahashi and A. C. Ross for discussions, and J. L. Muzicka for expert technical assistance.

REFERENCES
Retinyl Palmitate Hydrolase in Liver

In the study of retinyl palmitate hydrolase activity in liver, several methods were employed to characterize the enzyme. The procedure involved measuring the activity of the enzyme using a specific assay method.

### Materials and Methods

Retinyl palmitate in liver was prepared and analyzed for retinyl palmitate hydrolase activity. The enzyme was isolated and purified to homogeneity. The assay was performed by incubating the enzyme solution with a substrate at a constant temperature and pH. The reaction was terminated after a predetermined time, and the product was measured spectrophotometrically.

### Results

The results showed that retinyl palmitate hydrolase activity was enhanced in liver from animals fed a high-fat diet. The activity was significantly higher in liver from animals fed a high-fat diet compared to those fed a low-fat diet. The activity was also higher in liver from animals fed a high-fat diet compared to those fed a normal diet.

### Conclusion

The results of this study suggest that retinyl palmitate hydrolase activity is regulated by dietary factors. Further studies are needed to elucidate the mechanisms underlying this regulation.

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### Table 1: Characteristics of Pooled Homogenate with High, Medium, and Low Retinyl Palmitate Hydrolase Activity

| Homogenate Type | Protein (mg) | Retinyl Palmitate (mM) | Retinyl Palmitate Hydrolase Activity (IU/mg)
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### Figure 2: The effect of sodium chloride on the enzymatic activity of retinyl palmitate hydrolase

The enzymatic activity of retinyl palmitate hydrolase was measured in the presence of varying concentrations of sodium chloride. The activity was found to be significantly inhibited by increasing concentrations of sodium chloride. The inhibition was competitive, as evidenced by the Lineweaver-Burk plot.

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### Figure 3: Inhibition of retinyl palmitate hydrolase activity by sodium chloride

In this experiment, the enzyme activity was measured in the presence of different concentrations of sodium chloride. The activity was found to be inhibited in a dose-dependent manner. The inhibitory effect was competitive, as evidenced by the Michaelis-Menten plot.
The observed variation in enzyme activity reflects differences in enzyme activity among individual rat livers and not the variability of the activity within a single 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 assays after several weeks of storage in the frozen state or after several days at 4°C. Values for retinyl palmitate hydrolase activity remained constant on those homogenates that were observed initially after prolonged storage at -20°C for both high, medium, and low activity homogenates.

A number of experiments were conducted to test the factors that might contribute to the variations in hydrolase activity among individual rat liver homogenates. The following experiments were conducted: (a) hydrolase activity was assayed on homogenates containing 0.1% and 0.25% of liver homogenates from 25 individual rat liver samples. Retinyl palmitate hydrolase activity in liver homogenate was not significantly correlated with the amount of liver homogenate used in the assay. The data obtained from these experiments are shown in Figure 1. A positive correlation was observed which was higher for all liver homogenates than that for all liver homogenates (p < 0.01).

Experiments were also carried out to test the possibility that some changes in the enzyme activity occurred during storage in the frozen state or after several days at 4°C. Values for retinyl palmitate hydrolase activity remained constant in those homogenates that were observed initially after prolonged storage at -20°C for both high, medium, and low activity homogenates.

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Retinyl Palmitate Hydrolase in Liver

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Enrichment (compared to the initial liver homogenate) in the hydrolytic activities against cholesteryl oleate, triolein, and phytyl oleate.

For the medullary activity homogenate pool, partially purified preparation obtained from the Phenyl-Sepharose column was essentially the same as that seen with whole homogenate samples (Fig. 7).

Table III. Relationship Between Hydrolytic Activities Against Several Substrates

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<tr>
<th>Substrate</th>
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Table IV. Relationship Between Hydrolytic Activities in Partially Purified Enzyme Preparations

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Fig. 7. Hydrophobic interaction chromatography of the liver homogenate containing high retinyl palmitate hydrolase activity. The sample (40 ml) was applied to a column of Phenyl-Sepharose equilibrated with a low volume of 0.5 M KCl, and eluted with a linear gradient from 0 to 3 M sodium chloride (20 ml in each buffer; fractions 26 to 36 with the same buffer but without KCl, 36 to 56, fractions 57 to 90 with a linear gradient of 0 to 5 M sodium chloride in the previous buffer); fractions 1 to 15 with the same buffer containing 5% cholate.

Retinyl palmitate hydrolase activity was assayed spectrophotometrically by means of one of the three different activity levels. The method of assay was similar to that described by Greengard and co-workers (13). The relative specific activity of the enzyme assayed 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 1.3 times more active than the medium activity homogenate pool and 2.9 times more active than the low activity homogenate pool.

The hydrophobic interaction chromatography was carried out with an acetonitrile powder eluent with a high hydrolytic specific activity of 370 pmol palmitic acid/mg protein. The partially purified preparation obtained from the Phenyl-Sepharose column was essentially the same as that seen with whole homogenate samples (Fig. 7).

For the medullary activity homogenate pool, the partially purified preparation obtained from the Phenyl-Sepharose column was essentially the same as that seen with whole homogenate samples. However, there was no relationship between the retinyl palmitate hydrolase activity and the activities hydrolyzing cholesteryl oleate, triolein, and phytyl oleate when assayed under the same conditions (Table III). In contrast, there was a relationship between the retinyl palmitate hydrolase activity and the hydrolytic activities against cholesteryl oleate and p-nitrophenyl acetate (Table III).

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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 phytyl oleate. For the medium activity homogenate pool, the partially purified preparation obtained from the Phenyl-Sepharose column was essentially the same as that seen with whole homogenate samples. However, there was no relationship between the retinyl palmitate hydrolase activity and the activities hydrolyzing cholesteryl oleate and p-nitrophenyl acetate (Table III). In contrast, there was a relationship between the retinyl palmitate hydrolase activity and the hydrolytic activities against cholesteryl oleate and p-nitrophenyl acetate (Table III).

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