Unusual properties of retinyl palmitate hydrolase activity in rat liver[1]

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Abstract These studies report the hydrolysis of retinyl palmitate with liver homogenates and homogenate fractions from retinol-depleted rats. The studies utilized an effective in vitro assay for retinyl palmitate hydrolase (RPH) activity, in which μg amounts of retinyl palmitate were employed as substrate, followed by the chromatographic separation and fluorescence assay of free and esterified retinol. RPH activity was maximal near pH 8 in Tris–maleate buffers, and required a bile salt for stimulation. Both cholate and taurocholate stimulated the reaction, whereas a number of other detergents tested were ineffective. The enzymatic activity showed an unusual subcellular distribution, with about 40% of total RPH activity recovered in the washed "nuclear" fraction (1,500 *g* pellet) and about 30–35% in the 105,000 *g* supernatant. This unusual distribution was not observed for marker constituents for plasma membranes, nuclei, mitochondria, lysosomes, Golgi apparatus, or endoplasmic reticulum. Despite its enrichment in the "nuclear" fraction, RPH activity was not enriched in purified preparations of nuclei or plasma membranes. Thus, RPH activity was not localized in any single, characterized subcellular structure. Another striking feature of the hepatic RPH activity was its extreme variability from rat to rat as assayed in vitro. Both the unusual subcellular distribution and the marked variability in activity were not observed for a variety of other hepatic ester hydrolase activities examined. Of ten lipid and nonlipid esters tested as substrates, only the hydrolytic activities against cholesteryl oleate and phytyl oleate correlated with, and partly resembled, RPH activity in these respects. The results suggest that the observed RPH activity is relatively specific for the hydrolysis of retinyl palmitate, and may therefore be significantly involved in hepatic retinyl ester metabolism.—**Harrison, E. H., J. E. Smith, and D. S. Goodman.** Unusual properties of retinyl palmitate hydrolase activity in rat liver. *J. Lipid Res.* 1979. **20:** 760–771.

Supplementary key words Vitamin A · retinyl esters · retinol-binding protein · subcellular distribution · vitamin A deficiency

The liver is the major storage site for vitamin A in the body. Newly absorbed vitamin A reaches the liver mainly in the form of retinyl esters in association with chylomicron remnants (1, 2). Evidence exists that the hepatic uptake of vitamin A involves hydrolysis of the chylomicron retinyl esters, followed by intrahepatic reesterification of the free retinol (1, 3). The vitamin A is then stored in lipid droplets in the liver, in the form of long-chain retinyl esters, particularly as retinyl palmitate (1, 4).

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 (RBP) (5, 6). Accordingly, prior to the mobilization of vitamin A from the liver, the stored retinyl esters must be hydrolyzed to form retinol. The retinol so produced forms a complex with a molecule of RBP, and the retinol–RBP complex is then secreted from the liver. RBP is a relatively small protein of approximately 20,000 daltons, with one binding site for one molecule of retinol. In plasma, RBP circulates mainly as a 1:1 molar protein–protein complex with plasma prealbumin. Both the mobilization of vitamin A from the liver and the secretion of RBP are highly regulated processes, and are particularly regulated by the vitamin A status of the animal (7–9). Since RBP is mainly secreted from the liver as the holoprotein, it seems possible that the rates of hydrolysis of retinyl esters and of synthesis and secretion of RBP may be coordinated with each other in some way within the liver cell.

From these considerations, it is clear that the enzymatic hydrolysis of retinyl esters in liver represents an important process in the overall metabolism of vitamin A in the body. Nevertheless, little information is available about this reaction. In earlier studies, in fact, a number of investigators were unable to demonstrate the in vitro hydrolysis of retinyl palmitate with liver homogenate preparations (10, 11). In 1966, Mahadevan, Ayyoub, and Roels (12) reported that

Abbreviations: RBP, retinol-binding protein; RPH, retinyl palmitate hydrolase.

[1] A partial report of this work was presented at the annual meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill. in April, 1977 (43).

[2] The work described here forms part of a dissertation that was submitted to the Graduate School of Arts and Sciences of Columbia University in partial fulfillment of the requirements for the Ph.D. degree.

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retinyl palmitate hydrolyzing activity was found in the "nuclear" and "mitochondrial–lysosome-rich" fractions of rat liver homogenates. The activity required the addition of a bile salt and was partially characterized with extracts of acetone powders of rat liver. The enzyme preparations hydrolyzed a variety of long-chain retinyl esters, with the greatest relative activity being seen with retinyl palmitate. No further information about this reaction has, however, been reported by these or other investigators.

We now report the results of studies on the characteristics and subcellular localization of retinyl palmitate hydrolase activity in rat liver. These studies represent the first phase of a project that aims to explore in detail the enzymatic hydrolysis of long-chain retinyl esters by the liver.

EXPERIMENTAL PROCEDURE

Source of enzyme activity

Homogenates and subcellular fractions from the livers of retinol-depleted rats were used throughout this study. Male, weanling rats of the Holtzmann strain were depleted of vitamin A by feeding them a vitamin A-deficient diet for 3–4 weeks; thereafter the rats were fed a diet supplemented with retinoic acid in order to maintain good growth and general health (7, 8). 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.

Preparation of subcellular fractions

Homogenates of rat liver were prepared and fractionated by differential centrifugation according to the method of de Duve et al. (13) as recently modified by Amar-Costesec et al. (14). In this procedure as used, a crude pellet was prepared by low-speed centrifugation and washed (i.e., resuspended and recentrifuged) two times. The resulting pellet was resuspended to yield the "nuclear" or N fraction. The postnuclear supernatants were pooled to yield the cytoplasmic extract or E fraction. The cytoplasmic extract was then further fractionated to obtain the mitochondrial–lysosomal fraction (M + L) and the microsomal fraction (P). Each of these particulate fractions was washed two times before final resuspension. The final high-speed supernatant is referred to as the S fraction.

Fractions enriched in plasma membranes were prepared from dilute, hypotonic liver homogenates by the method of Song et al. (15). Plasma membrane-rich fractions were also prepared directly from the N fraction by the method of Touster et al. (16). Purified preparations of cell nuclei were isolated directly from whole homogenates of rat liver by the method of Blobel and Potter (17).

Assay for retinyl palmitate hydrolase (RPH) activity

The standard RPH assay was conducted as follows. All procedures were conducted under subdued lighting. Buffer (50 mM Tris–maleate, pH 8), α-tocopherol (Sigma, 50 μg, added as an antioxidant, in 5 μl of ethanol), sodium cholate (30 μmol), and retinyl palmitate (a gift of Hoffmann-LaRoche Inc., Nutley, NJ, 10 μg added in 100 μl of ethanol) were incubated with a source of enzyme in a final volume of 2 ml. Incubations were conducted under nitrogen at 37°C. Reactions were terminated by adding 2 ml of ethanol, and the resultant mixtures were extracted twice with 6.5 ml of hexane. The combined hexane extracts were concentrated and applied to a column of 0.5 g of alumina (Woelm, Eshwege, Germany) deactivated with 10% water. Retinyl palmitate was separated from the free retinol formed during the reaction by successive elution with 10 ml of 1% diethyl ether in hexane (to yield retinyl palmitate) and 10 ml of 20% ether in hexane (to yield retinol). Preliminary experiments confirmed that this procedure resulted in the complete and consistent separation of retinol from retinyl palmitate, with excellent recovery of both components. Each fraction was assayed fluorometrically for vitamin A by a modification (7) of the method of Thompson et al. (18). Enzyme activity was calculated as the percent hydrolysis of the recovered substrate. Under these standard assay conditions, 10% hydrolysis per hr corresponds to an activity of 2 nmol per hr. Total recovery of vitamin A substrate (esterified plus free retinol) was routinely 90% or more. Controls without enzyme were run with each experiment, and the values observed for activity (usually 2–4% hydrolysis) were subtracted from those of the test samples in the final calculations of enzyme activity. When various additions were made to the reaction mixtures, pilot experiments were conducted to assure that such additions did not interfere with the extraction or fluorometric assay of the vitamin A compounds.

Assays for marker constituents

The following constituents were assayed as markers for the indicated subcellular components: DNA (nuclei), cytochrome oxidase (mitochondria), acid phosphatase (lysosomes), 5′-nucleotidase (plasma membrane), galactosyltransferase (Golgi apparatus), glucose-6-phosphatase (endoplasmic reticulum), and RNA (rough endoplasmic reticulum).

Glucose-6-phosphatase (19), 5′-nucleotidase (19), and cytochrome oxidase (20) were assayed according

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to the indicated published procedures. Acid phosphatase was assayed essentially as described by Glaumann and Dallner (21) except that Triton X-100 was not included in the reaction mixture. In order to release the full activity of this latent enzyme, all fractions were frozen and thawed three times prior to assay. For the determination of galactosyltransferase, the transfer of galactose from UDP-[^{14}C]galactose to acid-hydrolyzed bovine submaxillary mucin was assayed in a manner similar to that described by Grimes (22). DNA and RNA were extracted from homogenates and fractions with perchloric acid (23) and estimated spectrophotometrically (24, 25).

Assays for the hydrolysis of other esters

The hydrolysis of a number of lipid and nonlipid monoesters was assessed in reaction mixtures nearly identical to that used in the assay of RPH.

[1-^{14}C]Palmitic acid (56 μCi/μmol), [1-^{14}C]oleic acid (59.7 μCi/μmol), and cholesteryl [1-^{14}C]oleate (21.5 μCi/μmol) were obtained from Amersham-Searle (Arlington Heights, IL). Ethyl [1-^{14}C]palmitate and ethyl [1-^{14}C]oleate were prepared by heating the appropriate 1-^{14}C-labeled fatty acid in acidic ethanol (2% H_2SO_4 in absolute ethanol) at 70°C under nitrogen for 3 hr. Arachidonyl [1-^{14}C]palmitate and phytyl [1-^{14}C]oleate were prepared by reacting the appropriate labeled fatty acid anhydride with the appropriate alcohol (Sigma), essentially as described by Lentz, Barenholz, and Thompson (26) for the synthesis of cholesteryl esters. The labeled fatty acid anhydrides were prepared by a microscale adaptation of the procedure of Selinger and Lapidot (27). The labeled product esters (arachidonyl palmitate and phytyl oleate) were purified by chromatography on small columns of alumina deactivated with 10% water. The purity of the several labeled esters was confirmed in each instance by chromatographic analysis.

The enzymatic hydrolysis of cholesteryl oleate, ethyl palmitate, ethyl oleate, phytyl oleate, and arachidonyl palmitate was assessed by incubation of the labeled substrate ester with enzyme and extraction of the released free fatty acid into an alkaline aqueous phase (28). The incubations, carried out for 20–30 min at 37°C, contained 50 mM Tris–maleate, pH 8, 15 mM cholate, 0.002 μmol of substrate (added in 10 μl of ethanol), and an appropriately diluted homogenate fraction in a final volume of 0.2 ml. After incubation and extraction, a portion (1 ml) of the alkaline aqueous phase containing the released free fatty acid was added to 10 ml of ScintiVerse (Fisher Scientific Co.), and radioactivity was determined in a Packard liquid scintillation counter.

Assays for the hydrolysis of *p*-nitrophenyl esters and 4-methylumbelliferyl esters were carried out at 37°C for 15 min in 2-ml reaction mixtures containing 50 mM Tris–maleate, pH 8, and 15 mM cholate. Substrates were added in 100 μl of ethanol (for acetate esters) or 100 μl of acetone–ethanol 2:3 (v/v) (for palmitate esters). The substrate solutions of *p*-nitrophenyl esters were prepared at a concentration of 10 μmol/ml while those for the 4-methylumbelliferyl esters (Koch-Light Laboratories, Ltd., Colnbrook, Bucks, England) were used at 0.20 μmol/ml. Reactions with the *p*-nitrophenyl esters were terminated by the addition of 2 ml of ice-cold ethanol, and the hydrolysis of these esters was assayed by spectrophotometric determination of released *p*-nitrophenol at 400 nm (29). 4-Methylumbelliferyl esterase activity was assayed by fluorometric determination of released free 4-methylumbelliferone. For these assays, 0.1-ml aliquots of the reaction mixtures were each pipetted into 5 ml of ethanol, and the fluorescence of the resulting solutions was determined in an Aminco-Bowman spectrophotofluorometer at an excitation of 380 nm and an emission of 450 nm (30, 31).

The hydrolysis of retinyl acetate was assayed exactly as described for retinyl palmitate except that the substrate was retinyl acetate (Eastman Organic Chemicals) in ethanol (5 μg retinol equivalents/ml).

Pilot experiments were conducted to develop suitable conditions for the conduct of each enzyme assay (i.e., for the assay of the enzymatic hydrolysis of each of the esters studied). All assays were carried out under conditions where product formation was proportional to the time of incubation and to the quantity of enzyme-containing protein in the reaction mixture. In determining the subcellular distributions of the activities, each fraction was assayed at three different protein concentrations.

Other assays

Protein was determined by the method of Lowry et al. (32) using bovine serum albumin as a standard.

Rat RBP and prealbumin concentrations were determined using specific radioimmunoassays developed in this laboratory (9, 33).

RESULTS

Assay for retinyl palmitate hydrolase activity

An assay involving μg amounts of retinyl palmitate was evaluated and then employed in this work. In preliminary experiments with crude liver homogenates, detectable but low hydrolysis of added substrate

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TABLE 1. Distributions of retinyl palmitate hydrolase activity and of marker constituents in subcellular fractions of retinol-depleted rat liver

Constituent	No. Expts.	Distribution of Recovered Activity in Isolated Subcellular Fractions[a]				Total Recovery[b]
		N	M + L	P	S	
		%				%
Protein	6	12.6 ± 1.5	21.8 ± 3.1	25.2 ± 1.9	40.4 ± 2.2	97.3 ± 3.9
Retinyl palmitate hydrolase[c]	3	39.8 ± 4.3 (3.2)[d]	21.2 ± 1.5 (1.0)	5.7 ± 1.5 (0.2)	34.0 ± 3.2 (0.8)	99.1 ± 4.5
5′-Nucleotidase	5	35.5 ± 4.9 (2.8)	4.5 ± 1.2 (0.2)	42.0 ± 3.4 (1.7)	18.4 ± 2.4 (0.5)	96.6 ± 2.4
Glucose-6-phosphatase	3	8.4 ± 2.7 (0.7)	8.3 ± 1.3 (0.4)	50.7 ± 0.8 (2.0)	32.6 ± 2.8 (0.8)	99.5 ± 1.8
Acid phosphatase	3	3.2 ± 1.8 (0.3)	50.8 ± 5.7 (2.3)	12.1 ± 2.0 (0.5)	34.0 ± 2.9 (0.8)	89.7 ± 6.8
Cytochrome oxidase	3	7.8 ± 5.0 (0.6)	84.1 ± 6.9 (3.9)	8.1 ± 3.2 (0.3)	0 (0)	90.1 ± 11.5
Galactosyltransferase	3	6.8 ± 1.7 (0.5)	5.6 ± 1.9 (0.3)	62.6 ± 3.2 (2.5)	24.9 ± 4.8 (0.6)	66.6 ± 4.3
Retinol-binding protein	4	6.0 ± 1.2 (0.5)	8.5 ± 1.4 (0.4)	78.7 ± 1.4 (3.1)	6.8 ± 0.6 (0.2)	85.3 ± 2.5
Prealbumin	4	6.4 ± 2.3 (0.5)	8.1 ± 2.9 (0.4)	59.9 ± 4.5 (2.4)	25.6 ± 3.4 (0.6)	121.5 ± 16.1
Ribonucleic acid	3	13.2 ± 0.2 (1.0)	7.5 ± 0.9 (0.3)	53.3 ± 0.9 (2.1)	26.0 ± 1.9 (0.6)	102.5 ± 6.0
Deoxyribonucleic acid	3	65.6 ± 2.2 (5.2)	4.1 ± 0.4 (0.2)	12.0 ± 1.7 (0.5)	18.4 ± 2.9 (0.5)	104.5 ± 1.5

[a] Results are given as means ± one standard deviation. Relative values are presented for the distribution of each constituent among the four homogenate fractions: nuclear (N), mitochondrial-lysosomal (M + L), microsomal (P), and supernatant (S). The values given represent the percentage of the constituent recovered in each fraction, compared to the total amount recovered in all four fractions (taken as 100%).

[b] The recoveries listed represent the total amount of a marker recovered in all four fractions relative to the amount of that constituent in the whole homogenate (E + N).

[c] The absolute RPH activities in the three homogenate preparations used here (i.e., in the whole homogenate (E + N)), were 4.26, 4.98, and 6.30 μmol retinol produced per hr per g liver.

[d] Values in parentheses represent the mean values for the relative specific activity of the constituent in that fraction, defined as the percent of total constituent divided by the percent of total protein recovered in that fraction.

was seen (generally <5% in a 1-hr incubation). A number of detergents were then screened for the potential ability to stimulate the in vitro enzymatic hydrolysis of retinyl palmitate. Excellent stimulation of the reaction (often to yield hydrolysis of 50% or more of added substrate) was found with cholate at levels ≥15 mM.

Using 15 mM cholate, the basic characteristics of RPH activity were then examined in crude liver preparations. The properties examined included effects of pH (maximal activity seen near pH 8), rate of the reaction (reasonably constant for 1 hr), and effects of enzyme and of substrate concentration. Based on the results of these initial and preliminary experiments, the assay for RPH activity described in detail in the Methods section was adopted for use in subsequent, more detailed investigations of the enzyme.

Subcellular localization of RPH activity

Fractionation by differential centrifugation. The results of these studies are summarized in **Table 1.** The distributions of total protein and of the various marker constituents closely resembled those reported by others (14, 16). Thus, the results obtained here with liver homogenates from retinol-depleted rats showed excellent agreement with those previously reported for normal rat liver. Other studies in our laboratory (34) have also indicated that retinol deficiency does not alter the subcellular distribution of the major, known marker constituents in rat liver.

RPH activity distributed among the four subcellular fractions in an unusual way, distinctly unlike any of the markers or other constituents. In particular, RPH did not distribute like the proteins involved in the serum transport of vitamin A (RBP and prealbumin), which were mainly recovered in the P fraction. Almost 40% of the total RPH activity was consistently recovered in the washed N fraction, and an additional 20% was recovered in the M + L fraction. Approximately one-third of the total RPH activity was present in the final supernatant or S fraction. The activity in the S fraction was found in the cytosol per se, and was not particularly associated with the lipid "cream" that floated to the top of the centrifuge tubes (data not shown).

The same subcellular distribution of RPH activity was observed regardless of the absolute level of RPH activity present in the homogenate (see below). The distribution was also unaffected by perfusion of the livers with 0.25 M sucrose prior to homogenization and fractionation. Thus the high RPH activity in the N fraction was not due to erythrocytes, which commonly contaminate this fraction when livers are not perfused before homogenization. Likewise, contamination of the N fraction with unbroken cells was low as assessed by the concentration of markers known to be localized in postnuclear fractions (see Table 1) and by direct microscopic examination of the isolated N fraction.

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TABLE 2. Relative specific activities of constituents in plasma membrane preparations from the livers of retinol-depleted rats[a]

Constituent	Experiment 1		Experiment 2	
	RSA[b]	Recovery[c]	RSA[b]	Recovery[c]
Protein	1.0	101	1.0	87
Retinyl palmitate hydrolase	0.8	75	0.9	76
5'-Nucleotidase	28.0	79	28.9	93
Glucose-6-phosphatase	0.9	93	1.5	92
Cytochrome oxidase	0.5	83	1.1	90
Galactosyltransferase	0.7	117	0.3	115
Acid phosphatase	0	111	0.7	110

[a] Plasma membrane-rich fractions were isolated from homogenates of the livers of retinol-depleted rats by the method of Song et al. (15). The plasma membrane-rich fraction and the combined supernatants were assayed for the indicated constituents.

[b] Relative specific activity (RSA) = units/mg protein in plasma membrane fraction divided by units/mg protein in homogenate.

[c] Recoveries represent the sum of the constituent in the plasma membrane-rich fraction and in the combined supernatants relative to the amount observed in the unfractionated homogenate.

RPH activity, like 5'-nucleotidase, was mostly enriched in the N fraction. However, unlike 5'-nucleotidase, RPH activity was almost absent from the P fraction and, instead, was much more abundant in the S fraction. The reesterification of free retinol, if it occurred, might be expected to be catalyzed by microsomal enzymes, and hence might be responsible for the low RPH activity seen in the P fraction. In order to test this possibility, freshly prepared fractions (with high RPH activity) were assayed, under conditions identical to those used in the assay for RPH, for the ability to catalyze the esterification of 5 μg of added free retinol. Only low esterifying activity was observed. Less than 4% of the added retinol was esterified in the incubations containing the N, E, M + L, or S fractions; the P fraction was more active and converted about 11% of the added substrate to ester. The data indicate that extensive esterification of retinol did not occur under the in vitro conditions used in the RPH assay. The fact that the total recovery of observed hydrolytic activity (sum of subcellular fractions compared to whole homogenate) was very good (99.1%, Table 1) also supports the contention that the distribution results were not confounded by the presence of active retinol-esterifying enzymes.

The results of these subcellular fractionation studies clearly demonstrated that RPH activity was not associated with any single, enzymatically characterized subcellular structure. Since the relative specific activity was highest in the N fraction the components of this fraction were examined in more detail. Previous studies on the distributions of marker constituents (14, 16, 35) have indicated that the only known subcellular components enriched in the N fraction are the cell nuclei and fragments of the plasma membrane. Our results (Table 1) confirmed that the only marker constituents enriched in the N fraction were DNA (nuclei) and 5'-nucleotidase (plasma membranes).

Isolated plasma membranes and nuclei. The enzymatic composition of plasma membrane-rich fractions isolated from whole homogenates is shown in **Table 2.** The isolated plasma membrane-rich fraction contained about 0.3% of the total homogenate protein and was highly enriched in plasma membrane as assessed by the high relative specific activity of 5'-nucleotidase. The fraction was relatively uncontaminated with other subcellular organelles as shown by the low recovery of other marker enzymes in this fraction. Assay for RPH activity indicated that the plasma membrane-rich fraction did not contain significant RPH activity.

In order to further confirm that RPH activity was not associated with plasma membranes, we sought to isolate a plasma membrane-enriched subfraction directly from the fraction in which the hydrolase was enriched, namely the nuclear (N) fraction. The results of subfractionation of the N fraction on discontinuous sucrose gradients are presented in **Table 3.** Although about ⅓ of the total liver 5'-nucleotidase and of the RPH activities were recovered in the washed N fraction, these activities distributed quite differently upon subfractionation. Almost one-half of the 5'-nucleotidase activity applied to the gradient was recovered in the plasma membrane-rich subfraction (N_2 subfraction) along with only 6% of the protein. Thus the concentration of plasma membrane-derived material in this subfraction was approximately 18 times greater than that of the whole homogenate. The N_2 subfraction was, however, almost devoid of RPH activity. Rather, the hydrolase distributed on the gradient mainly with the bulk of the protein. Thus, RPH

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activity was not further enriched in any of the subfractions.

The relative specific activities of RPH and the nucleic acids in a nuclei-rich fraction of rat liver are shown in **Table 4.** The 18-fold enrichment in DNA in the isolated fraction confirms that it was, indeed, a nuclei-rich fraction. The fraction was, however, not active in catalyzing the hydrolysis of retinyl palmitate. In addition, nuclei isolated directly from the N fraction were not enriched in RPH activity (data not shown). Thus, the results of these studies with isolated plasma membrane and nuclei clearly showed that RPH activity was not localized in either of these two specific components of the N fraction.

Characteristics of the RPH activity in the N and S fractions

As indicated above, the major amounts of RPH activity were recovered in the nuclear (N) and the supernatant (S) fractions. In order to further explore the characteristics of the RPH activity in the N fraction, and its relationship to the soluble activity (in the S fraction), the enzymatic properties of the two fractions were compared.

The basic characteristics of the RPH activities in the N and the S fractions are shown in **Fig. 1.** Both fractions displayed considerable enzyme activity over the range of pH studied. Moreover, the pH–activity profiles for RPH enzyme activity from either fraction were similar in the two different buffer systems employed. The differences in the activity profiles shown in the two upper panels of Fig. 1 are probably related to the specific buffers used. The pH optimum in Tris–maleate buffers for both fractions was approximately pH 8.

The rate of the reaction was nearly constant for up to 60 min and was identical with either N or S fraction as a source of enzyme (Fig. 1, lower left). Also shown in Fig. 1 (lower right) is the fact that the initial velocity of the reaction with enzyme from either subcellular fraction showed the same substrate concentration dependence. When $1/v$ was plotted against $1/S$ for the data obtained with either fraction, straight lines were obtained. Identical values of apparent K_M (20 μM) were calculated for RPH in each of the two subcellular fractions.

Fig. 2 shows that the bile salts cholate and taurocholate both stimulated the enzymatic hydrolysis of retinyl palmitate with either subcellular fraction. Moreover, the quantitative dependence of the activity on the concentration of the added activators was the same for both subcellular fractions. Of a number of other detergents tested, those which were inactive in stimulating the activity in one subcellular fraction were also inactive with the other. The detergents tested that gave little or no stimulation (at the final concentrations indicated) included Na dodecyl sulfate (7.5 mM), Tween 20 (0.15%), Tween 80 (0.05%), Lubrol WX (0.15%), Brij 35 (0.15%), and Triton X-100 (0.15%) (all percentage concentrations are w/v).

The enzyme activity in crude homogenates and in subcellular fractions was very stable. Preparations frozen at −20°C retained full activity for periods up to several months (see **Table 5**). Preparations kept unfrozen at 4°C for 6 days had approximately 90% of their initial activity.

TABLE 3. Distribution of constituents in subfractions of the nuclear (N) fraction: isolation of a plasma membrane-rich subfraction

Constituent	Distribution of Recovered Activity[a]			Total Recovery[b]
	N_2	N_3	N_4	
		%		%
Protein	6.4	17.2	76.4	82
5′-Nucleotidase	49.6	8.6	41.8	84
Retinyl palmitate hydrolase	0	21.5	78.5	106

[a] The N fraction of a retinol-depleted liver homogenate was subfractionated by sucrose density gradient centrifugation to obtain a plasma membrane-rich subfraction (N_2) as described by Touster et al. (16). Relative values are presented for the distribution of each constituent among the three subfractions collected (N_2, N_3, N_4) (see Ref. 16); the values given represent the percentage of the constituent recovered in each subfraction compared to the total amount recovered in all three subfractions (taken as 100%).

[b] Recoveries listed represent the total concentration of a constituent recovered from all three subfractions relative to the concentration of that constituent in the whole N fraction. The percentage of the total homogenate contents of each constituent in the N fraction were: protein, 13.8; 5′-nucleotidase, 31.2; and retinyl palmitate hydrolase, 35.2.

TABLE 4. Relative specific activities of constituents in nuclei from the livers of retinol-depleted rats

Constituent[a]	Relative Specific Activity[b] in Nuclei	Total Recovery[c]
		%
Protein	1.0	88.3
RNA	1.4	84.7
DNA	18.4	77.3
Retinyl palmitate hydrolase	0	110.0

[a] After isolation of nuclei (17), the isolated nuclei and the combined supernatants were assayed for the indicated constituents.

[b] Relative specific activity is calculated as the percent of total recovered constituent in the nuclear pellet divided by the percent of recovered protein in the nuclear pellet. The nuclear pellet represented 2.9% of the total recovered homogenate protein.

[c] Total recovery is the sum of the amount of the constituent in the nuclear pellet and the supernatant relative to the amount in the unfractionated homogenate.

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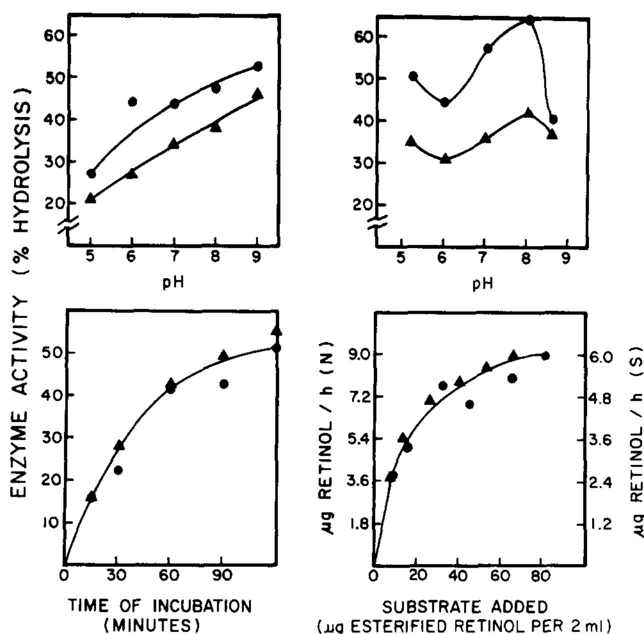

Fig. 1. Characteristics of RPH activity in the N (●) and S (▲) fractions of rat liver homogenates. Incubation conditions were as follows. (*Upper left and right*): buffer, 5 μg of esterified retinol, 1 hr. Left-hand panel, in 0.05 M Na acetate (pH 5), Na phosphate (pH 6,7,8), or glycine (pH 9) buffers; right-hand panel, in 0.05 M Tris-maleate buffers. (*Lower left*): 0.05 M Tris-maleate (pH 8), 5 μg of esterified retinol. (*Lower right*): 0.05 M Tris-maleate (pH 8), 1 hr. All incubations were conducted at 37°C in the presence of 15 mM Na cholate and 50 μg of α-tocopherol and contained 0.8 mg of N fraction protein or 4 mg of S fraction protein. Enzyme activity is plotted on the ordinate as percent hydrolysis of added substrate, except as indicated in the lower right panel.

Substrate specificity studies

Information concerning the potential specificity of the RPH activity was obtained by assaying various liver fractions for their ability to catalyze the hydrolysis of a variety of other lipid and nonlipid monoesters. In general, little information on the specificity of an enzyme can be obtained using crude enzyme preparations which contain a number of enzymatic activities. In the present case, however, advantage was taken of two unusual characteristics of RPH activity: *1*) the subcellular distribution of RPH activity and *2*) the variability of the RPH activity in different liver preparations (discussed further below).

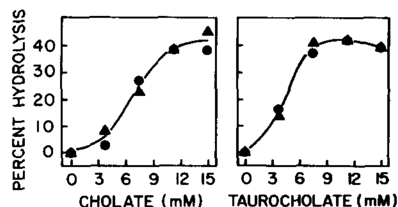

Fig. 2. Effects of bile salts on the RPH activity in the N (●) and S (▲) fractions of rat liver homogenates. All flasks contained 0.05 M Tris-maleate buffer (pH 8), 5 μg of esterified retinol, 50 μg of α-tocopherol, and either 0.8 mg of N fraction protein or 4 mg of S fraction protein. Incubations were conducted at 37°C for 1 hr.

Subcellular distributions of hydrolytic activities. **Fig. 3** shows the subcellular distributions of the activities hydrolyzing the esters studied. Most of the activities were mainly recovered in the P fraction; these microsomal-enriched activities included hydrolytic activities against *p*-nitrophenyl acetate, ethyl oleate, ethyl palmitate, the 4-methylumbelliferyl esters (acetate and palmitate), arachidonyl palmitate, and retinyl acetate. It is clear from the patterns observed that the bulk of the activities against these substrates was not due to the same enzyme(s) responsible for the RPH activity. The activities hydrolyzing *p*-nitrophenyl palmitate distributed throughout the four fractions; none of the fractions showed any substantial enrichment over the whole homogenate with this substrate.

In contrast to the activities just discussed, the enzymes responsible for the hydrolysis of cholesteryl oleate and phytyl oleate under these conditions were clearly absent from the P fraction; in this regard they shared one of the distinctive features of the RPH distribution. However, the activities hydrolyzing the cholesteryl and phytyl esters were most enriched in the M + L fraction and were present to a lesser extent in the N fraction; in this regard, these activities were not similar to RPH. Both cholesteryl oleate and phytyl oleate were also hydrolyzed by supernatant enzymes.

Comparison of the levels of hydrolytic activities in various liver preparations. Five pairs of N and E fractions (each pair prepared from the homogenate of a single liver) were used in this experiment. The preparations were selected on the basis of previous assays in which they had been found to display a wide range of absolute RPH activities. Each homogenate fraction was assayed

TABLE 5. Stability of retinyl palmitate hydrolase activity in various subcellular fractions[a]

Subcellular fraction	RPH Activity	
	Assayed Fresh	Assayed after 1 yr
	nmol retinol/hr/mg protein	
Cytoplasmic extract (E)	1.94	2.00
Nuclear (N)	5.36	4.75
Mitochondrial (M)	2.60	2.71
Microsomal (P)	0.62	0.38
Supernatant (S)	1.48	1.55

[a] A homogenate of livers of two retinol-depleted rats was prepared and fractionated as described in Methods. The activity in each fraction was determined on the day of preparation. Aliquots of the fractions were stored frozen at −20°C for 1 yr (54 weeks) and then thawed and reassayed. Enzyme activity was determined in 1-hr incubations as described in Methods; the amounts of the various fractions assayed (in mg protein) were: E, 4.8; N, 0.7; M, 3.0; P, 4.2, and S, 1.4.

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for its ability to catalyze the hydrolysis of retinyl palmitate and of the 10 other substrates. The activities of the N and E fractions were summed to give the total homogenate activity. The data were normalized for each substrate by setting the activity of the homogenate most active with that substrate equal to 100, and expressing the activities of the other four homogenates relative to that of the most active homogenate.

The results of this experiment are shown in **Fig. 4.** Fig. 4*A* shows that there was a highly significant linear relationship between the RPH activity and the activities hydrolyzing phytyl oleate and cholesteryl oleate under the same conditions. This relationship was observed with both N fractions and E fractions separately (data not shown), as well as with the sum of N and E fraction activities (Fig. 4*A*). In contrast, the hydrolytic activities against the eight other ester substrates tested did not correlate with RPH activity for the five homogenate preparations studied (Fig. 4*B*). Most of the activities against these other esters varied over a less than 2-fold range in the five homogenates tested, whereas RPH activity varied more than 40-fold.

Variability of RPH activity

Throughout the course of these studies, an unexplained but striking variability in hepatic RPH activity among individual animals was observed. The level of activity was not related to the age of the animals, the time of killing, or feeding or fasting just prior to killing. Furthermore, mixing experiments showed that adding inactive preparations to active ones had no inhibitory effect on the enzymatic activity.

The extent of variability was examined by reviewing data on RPH activity obtained with 51 individual liver homogenates prepared and assayed under the same standard conditions (i.e., 6.6 mg of protein, 10 μg of retinyl palmitate, 1 hr incubation) in a total of 14 different experiments during the course of 11 months. The rats varied in age from 42 to 180 days. More than one-half of these preparations hydrolyzed less than 20% of the added substrate and the majority of these hydrolyzed less than 5%. Twenty-four of the preparations hydrolyzed more than 25% of the added substrate, and with 11 of these, the yield was greater than 55%. Because of this variability, in most of the experiments reported here, individual livers were first homogenized and assayed for RPH activity, and only the more active homogenates were then used (e.g., for subcellular distribution experiments).

It is important to point out that the observed variability was due to differences in the levels of enzyme activity in the preparations assayed and not to a lack of reproducibility of the assay. When a single N fraction preparation was assayed on eight separate occasions over a period of 3 months, the activity was quite constant, i.e., 12.0 ± 0.9 nmol/hr per mg protein. (This corresponds to 40.1 ± 2.9% hydrolysis

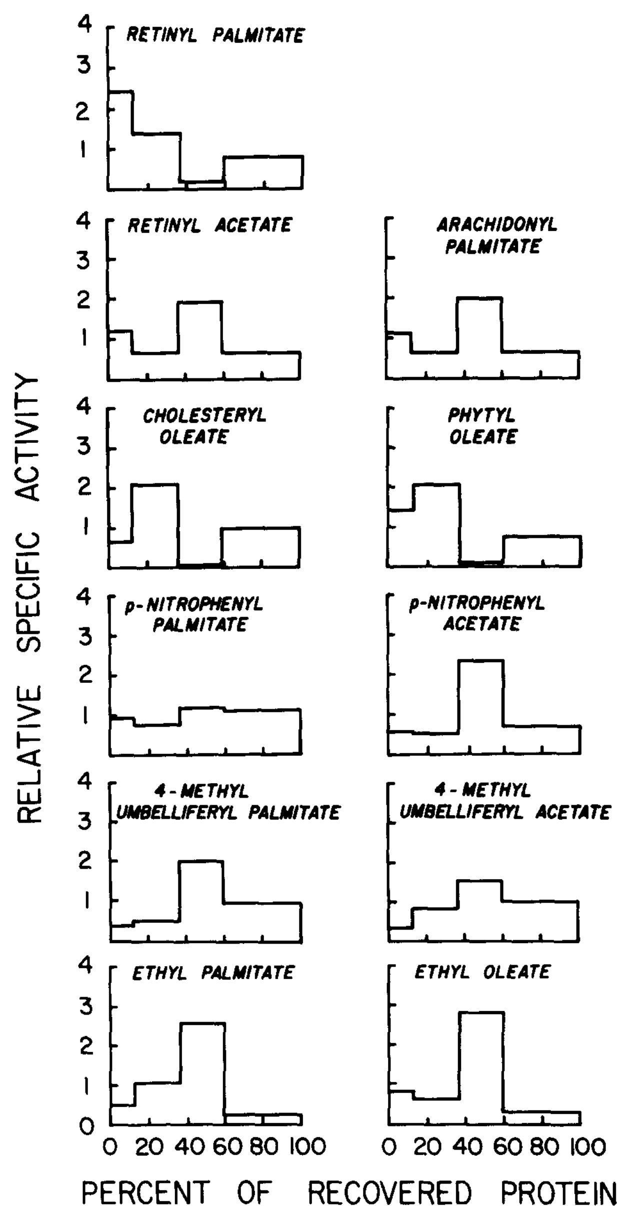

Fig. 3. Distributions of hydrolytic activities in subcellular fractions of retinol-depleted rat liver. Fractions are plotted in order of the average coefficient of sedimentation of their subcellular components, i.e., from left to right: N, M + L, P, and S. Each fraction is represented separately on the ordinate by the relative specific activity for the substrate tested (percentage of total recovered activity/percentage of total protein recovered in that fraction). On the abscissa each fraction is represented cumulatively by the percentage of the total protein recovered in that fraction, compared to the sum of the amounts recovered in all four fractions. The total recoveries of activity in the four subcellular fractions, compared to the whole homogenate, for the different substrates were (in %): retinyl palmitate, 84; retinyl acetate, 88; arachidonyl palmitate, 75; phytyl oleate, 81; cholesteryl oleate, 85; ethyl plamitate, 57; ethyl oleate, 84; 4-methylumbelliferyl palmitate, 106; 4-methylumbelliferyl acetate, 86; *p*-nitrophenyl palmitate, 109; and *p*-nitrophenyl acetate, 91.

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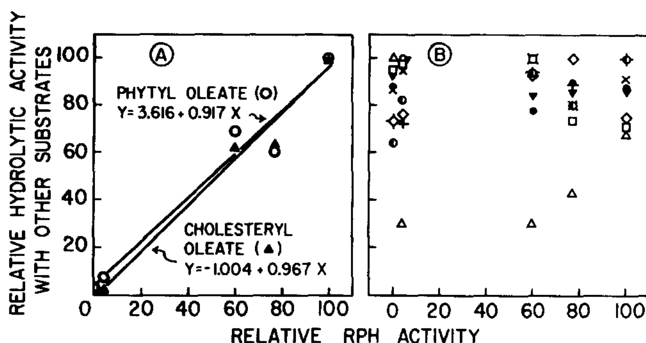

Fig. 4. Relationships among the activities of different liver homogenates for catalyzing the hydrolysis of retinyl palmitate and of other ester substrates. *Panel A* (left): RPH activity compared with activities towards cholesteryl oleate and phytyl oleate. The correlations ($r = 0.97$ for cholesteryl oleate and 0.92 for phytyl oleate) were highly significant ($P < 0.01$). *Panel B* (right): RPH activity compared with activities towards eight other esters, viz.: retinyl acetate (◇), arachidonyl palmitate (◐), ethyl palmitate (●), ethyl oleate (▼), *p*-nitrophenyl palmitate (+), *p*-nitrophenyl acetate (△), 4-methylumbelliferyl palmitate (□), and 4-methylumbelliferyl acetate (X). The absolute level of RPH activity, corresponding to 100% relative activity, was 45% hydrolysis of the added substrate.

under the standard assay conditions used.) Also, as shown in Table 5, when a group of fractions with different activities was reassayed after storage for more than 1 year, nearly the same levels of activity were observed. Thus, replicate assays of a given enzyme preparation generally varied less than 10% while the variation in activity among individual preparations was more than 40-fold.

DISCUSSION

The studies reported here demonstrate the hydrolysis of retinyl palmitate with rat liver homogenates and homogenate fractions, and describe many of the characteristics of the hydrolytic enzyme activity. Subcellular fractionation experiments disclosed that RPH activity distributed in a way distinctly unlike any of a number of marker constituents for nuclei, plasma membrane, mitochondria, lysosomes, Golgi apparatus, or endoplasmic reticulum. Thus, the enzyme activity responsible for the in vitro hydrolysis of retinyl palmitate was not exclusively or predominantly localized in any of these characterized subcellular components.

The most unusual aspect of the subcellular distribution of RPH was the enrichment of the activity in the washed N fraction. Extensive biochemical and morphological investigations of this fraction (see ref. 35) have confirmed that the only known subcellular components enriched in the N fraction are the cell nuclei and fragments of the plasma membrane. However, when highly purified preparations of nuclei and plasma membranes were isolated, they were found *not* to be enriched in RPH activity. Thus, RPH activity was consistently associated with and enriched in the heterogenous and ill-defined membranous components that sediment in the N fraction, but was not specifically localized in either the plasma membrane or the cell nuclei.

We have conducted preliminary experiments that suggest that the "soluble" RPH activity can become adsorbed to particulate material in the N fraction. Thus, in two experiments, when N fraction particulate material with low RPH activity was resuspended in a highly active S fraction and derivative fractions were isolated by recentrifugation, 40–58% of the RPH activity was shifted from the S to the N fraction. Furthermore, repeated washings of the derivative "N" fraction so prepared with 0.25 M sucrose did not quantitatively release the activity in a nonsedimenting form. These observations can be considered only as preliminary and suggestive at this time. The possibility exists, however, that the unusual subcellular distribution of RPH observed here might have resulted in part from the enzyme becoming adsorbed to particulate material in the homogenate that sediments in the N fraction.

The possibility of soluble proteins becoming adsorbed to components of the nuclear fraction was already appreciated in some early cell fractionation studies; nonetheless, such phenomena led to a number of cytoplasmic enzymes being incorrectly attributed to nuclei in early work (35). In addition, there is ample other evidence available in the literature that demonstrates the association of "soluble" enzymes with particulate cellular structures. The adsorption of hexokinase to mitochondria has been particularly well documented, and the soluble-particulate distribution has been suggested to play a role in the regulation of enzyme activity (36–38). Recent studies have also shown that many "soluble" glycolytic enzymes can be tightly adsorbed to purified muscle thin filaments (F-actin-tropomyosin-troponin) (39, 40). The fact that contractile proteins are found in many cell types suggests that such adsorption phenomena may be widespread.

At present, a full understanding of the basis for the unusual subcellular distribution of RPH activity is not available; we presume, however, that this distribution in some way reflects the physical-chemical properties and/or the physiological role(s) of the enzyme. As discussed above, the enzyme under study may be involved in the hydrolysis of long-chain retinyl esters during their hepatic uptake from chylomicron remnants. Alternatively, or in addition, the enzyme

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may be responsible for the hydrolysis of long-chain retinyl esters stored in the lipid droplets of the liver cell. In the former case, the enzyme might be expected to act at or near the inner side of the sinusoidal plasma membrane (41). Hence, the enzyme might have properties that lead it to become readily associated with or adsorbed to certain kinds of membranes. If the enzyme is responsible for hydrolysis of stored retinyl esters, it might also be expected to possess distinctive structural features. Since retinyl esters are stored in hepatic lipid droplets, the mobilization of retinol from the liver presumably involves hydrolysis of retinyl esters at the lipid droplet–cytoplasm interface. Therefore, the enzyme involved might be expected to possess physical-chemical properties that allow it to hydrolyze retinyl esters specifically and selectively at a lipid–water interface.

To our knowledge, the unusual distribution among the four subcellular fractions reported here for RPH activity has not been observed for any other liver enzyme. The specificity of this unusual distribution for RPH is further indicated by the results of our subcellular distribution studies of 10 other hepatic ester hydrolase activities. The results of these studies clearly indicated that the in vitro hydrolysis of a wide variety of lipid and nonlipid esters was catalyzed by liver enzymes that did not distribute like RPH activity. Of the 10 substrate esters tested, the enzyme catalyzing the hydrolysis of cholesteryl oleate and phytyl oleate under these in vitro conditions showed the greatest similarity in subcellular distribution to RPH.

The major enzymatic characteristics of the in vitro reaction were explored with separated N and S subcellular fractions. The properties of the RPH enzyme activity in each of these two fractions were found to be nearly identical. With both subcellular fractions, RPH activity was seen over a wide pH range, with maximal activity near pH 8 in Tris–maleate buffer. The reaction displayed Michaelis kinetics with an apparent K_M of 20 μM. Because of the physical state of the substrate, which was presented to the impure enzyme in a complex reaction mixture, it is difficult to ascribe precise meaning to the value for the apparent K_M. A striking feature of the in vitro reaction was the almost absolute requirement for the addition of a bile salt. Cholate and taurocholate were equally effective as activators of the reaction, and both were maximally stimulatory at concentrations of about 10–15 mM. Several other detergents tested were ineffective in stimulating the in vitro reaction. The mechanism of the bile salt stimulation of the reaction was not determined, but may be due to the solubilization of the water-insoluble substrate, although a more specific and direct effect on the enzyme cannot be ruled out.

In general terms, our results on the enzymatic properties of RPH activity in rat liver fractions agree with those reported by Mahadevan et al. (12) for acetone powder extracts. These workers also reported that the bulk of RPH activity was recovered in heavy particulate fractions of rat liver homogenates, but no information on marker enzymes was presented. Thus, our results, obtained with well-characterized subcellular fractions and with purified organelles (nuclei, plasma membranes) both confirm and considerably extend these previous observations.

A striking feature of the RPH activity of liver was the great variability of the observed activity from rat to rat as assayed in vitro. Thus, when individual rat liver homogenates were assayed under identical in vitro conditions, the RPH activity varied over a more than 40-fold range. Any single preparation, however, showed the same activity in replicate assays and even retained almost all of its initial activity when stored frozen for periods of up to several months. The unexpected finding of such variability may explain in part the controversy in the literature over the existence of this enzyme in rat liver. It should be noted in this regard that the studies of Mahadevan et al. (12) apparently were carried out with tissue extracts prepared from pooled rat livers, which would be the most likely reason why they did not detect the dramatic variability in RPH activity among individual rats.

The question should be considered as to whether the variability of RPH activity observed here represents a biologically real phenomenon, or whether it might not reflect some kind of artefact of the assay used. The following points are relevant to this question. First, the assay used has been thoroughly validated as to reproducibility (see Table 5), recovery (quantitative recovery of added substrate as the sum of unhydrolyzed ester plus product retinol), precision, and internal consistency (e.g., the sum of individual subcellular fraction activities adds up to the activity observed in the whole homogenate). Second, retinyl acetate hydrolase activity, measured in an identical assay, was observed not to vary widely from rat to rat while RPH activity did. Third, a similar extent of variability was observed for the hydrolysis of cholesteryl oleate and phytyl oleate, and the hydrolysis of these substrates was assayed by a different assay procedure. Moreover, the hydrolysis of eight other lipid and nonlipid monoesters did not vary much from rat to rat. Finally, in current experiments in our laboratory, RPH activity is being assayed by incubation of retinyl

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[^{14}C]palmitate and extraction of the released labeled free fatty acid into an alkaline aqueous phase. Using this assay and normal (vitamin A-fed) rats, the same extent of variability of hepatic RPH activity has been observed.[4] Taken together, these results indicate that the variability of hydrolase activity observed is a real phenomenon, specific for certain substrates, and not related to the method of assay.

Although unusual, the finding of substantial variation in the activity of a hepatic enzyme among identically treated animals is not unprecedented. Thus, Felton, Meisler, and Paigen (42) have reported that the level of β-galactosidase activity in the brain and liver of mice is under strong genetic control and varies considerably in mice of different genetic endowments. In the F2 generation derived from two different inbred strains of mice, the animals showed an approximately 8-fold variation in enzyme activity from mouse to mouse. The Holtzman rats used in our experiments were randomly bred; hence the possibility exists that the observed variability in RPH activity has, in part, a genetic basis. We currently have no information bearing on this point, however, and an explanation for the observed variability in RPH activity is currently not available. Our data do suggest that the variability in enzymatic activity is not related to the age of the animals, to feeding or fasting, or to vitamin A status. It is hoped that future studies of this phenomenon will provide further understanding into the properties, roles, and metabolic regulation of this enzyme.

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