Substrate specificity of retinyl ester hydrolase activity in retinal pigment epithelium

Jennifer R. Mata, Nathan L. Mata, and Andrew T. C. Tsin[1]

Division of Life Sciences, The University of Texas at San Antonio, San Antonio, TX 78249

Abstract In the eye, hydrolysis of stored retinyl esters is catalyzed by retinyl ester hydrolase (REH) activities in retinal pigment epithelium (RPE) membranes. In the present study, biochemical analyses were conducted to determine the substrate specificity of these activities. Specific activities determined for hydrolysis of various retinol isomers of retinyl palmitate (9-*cis*-, 11-*cis*-, 13-*cis*-, and all-*trans*-retinyl palmitates) indicated that 11-*cis*-retinyl palmitate is preferentially hydrolyzed (1.7 nmol/min/mg) compared to the other isomers (0.1–0.3 nmol/min/mg). Examination of the specificity of REH activity for 11-*cis*-retinyl esters of varied acyl chain length (-myristate, -palmitate, and -stearate) and degree of saturation (-oleate and -linoleate) further demonstrated that palmitate is the preferred fatty acyl moiety. Notably, retinyl esters possessing chain lengths which more closely approximate that of the palmitate ester exhibited higher rates of hydrolysis. Similar results were obtained in retinyl ester-plasma membrane fusion studies in which hydrolysis took place within the membrane domain rather than at the lipid–water interface. REH substrate specificity was further assessed in competition studies in which 11-*cis*-retinyl palmitate hydrolysis was monitored in the presence of 13-*cis*-, 9-*cis*-, or all-*trans*-retinyl palmitate. Results show that addition of these retinyl palmitate isomers does not affect the rate of hydrolysis of 11-*cis*-retinyl palmitate. However, the hydrolytic rates associated with other retinyl palmitate isomers were significantly reduced in the presence of 11-*cis*-retinyl palmitate. Finally, cholesterol ester hydrolase activity was found to be distinct from the observed 11-*cis*-REH activity and the presence of cholesterol oleate did not affect the rate of 11-*cis*-retinyl palmitate hydrolysis. Collectively, these data support the hypothesis that a distinct, membrane-associated, 11-*cis*-retinyl palmitate-specific retinyl ester hydrolase activity exists in the retinal pigment epithelium.—**Mata, J. R., N. L. Mata, and A.T.C. Tsin.** Substrate specificity of retinyl ester hydrolase activity in retinal pigment epithelium. *J. Lipid Res.* 1998. **39:** 604–612.

Supplementary key words retinoids • retinyl palmitates

The retinal pigment epithelium (RPE) of the mammalian eye is the primary source of 11-*cis* retinoids which support synthesis and regeneration of rhodopsin in the visual cycle (1, 2). Two principal features of the visual cycle are vitamin A isomerization and esterification. These unique activities effectively detoxify vitamin A by generating all-*trans*- and 11-*cis*-retinyl esters which are suitable for storage as well as further processing (1). The current theory of visual chromophore biosynthesis accounts for the production of 11-*cis*-retinol from an endogenous pool of all-*trans*-retinyl esters via an isomerohydrolase activity (3, 4). 11-*cis*-retinyl esters, which are known to accumulate in the dark and have been found to account for as much as 75% of total retinyl ester pools in the RPE (5, 6), have also been shown to contribute to visual chromophore biosynthesis (7–9). However, the mechanism underlying 11-*cis*-retinyl ester mobilization has not been completely elucidated.

As 11-*cis*-retinyl ester stores must first be hydrolyzed to 11-*cis*-retinol in order to support regeneration of visual pigments in the retina, the participation of an 11-*cis*-retinyl ester-specific hydrolase in the visual cycle seems inevitable and therefore predicts a prominent physiological relevance for this enzyme (1, 2, 10–12). The presence of retinyl ester hydrolase (REH) activities in RPE was first reported by Blaner et al. (10). The higher rate of hydrolysis of 11-*cis*-retinyl palmitate (in comparison to the all-*trans* isomer), as well as a distinct subcellular distribution, led these investigators to suggest that REH activity directed against the 11-*cis* isomer may be due to an 11-*cis*-specific hydrolase. Subsequent investigations using chemical modification and inhibition studies confirmed that hydrolysis of 11-*cis*-retinyl palmitate is distinct from hydrolase activities associated with the all-*trans* isomer both in the RPE and in the liver (12, 13). Although these characterizations have greatly advanced our understanding of REH activity in RPE, the substrate specificity of retinyl ester hydrolysis has not been reported in the literature. Therefore the

Abbreviations: REH, retinyl ester hydrolase; RPE, retinal pigment epithelium; HPLC, high performance liquid chromatography.

[1]To whom correspondence should be addressed.

existence of an 11-*cis*-specific REH enzyme remains contentious.

In the present study, we have examined the specificity of REH activity in bovine RPE for retinyl ester isomers possessing varied alcohol (i.e., 9-*cis*-, 11-*cis*-, 13-*cis*-, and all-*trans*-retinyl palmitate) and fatty acyl moieties (i.e., 11-*cis*- and all-*trans*-retinyl-myristate [14:0], -palmitate [16:0], -stearate [18:0], -oleate [18:1], and -linoleate [18:2]). Results show that the 11-*cis* isomer is hydrolyzed at a much higher rate than the other isomers and that palmitate, the most abundant fatty acid associated with endogenous retinyl esters, is the preferred fatty acyl moiety. The relative hydrolytic rates obtained using the routine lipid–water interface system were confirmed in retinyl ester–membrane fusion experiments in which hydrolysis took place within the membrane domain. Finally, the observed 11-*cis*-REH activity shows no relationship to cholesteryl ester hydrolase activity. These findings are novel and strongly support the hypothesis that an 11-*cis*-retinyl palmitate-specific REH activity exists in bovine RPE membranes.

MATERIALS AND METHODS

Materials

All-*trans*-retinol, sodium borohydride ($NaBH_4$), pyridine, dithiothreitol (DTT), disodium ethylenediaminetetraacetic acid (2Na-EDTA), myristoyl-, palmitoyl-, stearoyl-, oleoyl-, and linoleoyl-chloride were purchased from Sigma Chemical Co. [9,10-^{3}H]palmitic acid (specific activity 37 Ci/mmol), [1-^{14}C]palmitic acid (specific activity 50 mCi/mmol), and [1-^{14}C]cholesteryl oleate (specific activity 55 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Purified 11-*cis*-retinal was obtained through the generosity of Dr. Rosalie Crouch and the NEI/NIH; 11-*cis*-retinol was obtained after $NaBH_4$ reduction of 11-*cis*-retinal. Purified 11-*cis*-retinyl palmitate was a kind gift from Hoffmann-LaRoche. All synthesized retinoids were purified by HPLC and quantified by UV-visible spectrophotometry prior to use. Fresh bovine RPE tissue was obtained via overnight courier from a source in Chicago, IL.

Methods

Substrate syntheses and protein preparation. Unlabeled 11-*cis*- and all-*trans*-retinyl esters (i.e., five 11-*cis*- and five all-*trans*-retinyl esters possessing varied acyl chain lengths and degrees of saturation) were prepared as described by Bridges and Alvarez (14). Purity and concentration for all retinyl esters was verified by HPLC with photo-diode array detection. Four ^{3}H-labeled retinyl esters (i.e., 9-*cis*-, 11-*cis*-, 13-*cis*-, and all-*trans*-retinyl [9, 10-^{3}H]palmitate) were prepared by reacting [^{3}H]palmitic acid anhydride with the respective retinols according to methods described by Mata, Tsin, and Chambers (12). A ^{14}C-labeled all-*trans*-retinyl palmitate ester (i.e., all-*trans*-retinyl [1-^{14}C]palmitate) was similarly prepared for use in retinyl ester competition studies. The specific activity of all radiolabeled retinyl esters was adjusted to ~100 dpm/pmol by addition of unlabeled, HPLC purified retinyl ester. The specific activity of [1-^{14}C]cholesteryl oleate, used in cholesteryl ester competition studies, was adjusted to 75 dpm/pmol by addition of authentic, unlabeled cholesteryl oleate. RPE microsomes were prepared as previously described (12).

Retinyl ester hydrolase activity assay (non-radiometric). Hydrolytic rates associated with the various 11-*cis*- and all-*trans*-retinyl esters were determined by HPLC quantitation of the alcohol product of hydrolysis (either 11-*cis*- or all-*trans*-retinol) after incubation (t = 10 min at 37°C) of RPE microsomal protein (10 μg) with 10 μm of the indicated retinyl ester substrate (delivered in EtOH; 1% v/v, relative to the reaction volume) in 50 mm Tris-acetate, pH 8.0, at 37°C (total vol. = 1 ml). Reactions were quenched with an equal volume of ice-cold absolute EtOH and the samples were mixed with a vortex mixer. Retinoids were extracted from the reaction mixtures with 6 ml (2 × 3 ml) of hexane; the extracts were evaporated to dryness under a gentle stream of N_2 and prepared for analysis by isocratic HPLC. Chromatography conditions were as follows: 4.6 × 125 mm silica column (5 μm Partisphere, Whatman Co.), 10% dioxane–hexane (v/v) mobile phase at a flow rate of 2 ml/min. Quantitation of 11-*cis*- and all-*trans*-retinol was achieved by comparing integrated peak areas to calibration curves constructed from standards of known concentration (System Gold, Beckman, Fullerton, CA). Identification of the eluted retinoids was confirmed simultaneously by photo-diode array detection (i.e., single time-point absorption spectra, 450–210 nm). Appropriate controls were included to correct for non-enzymatic hydrolysis and thermal degradation of the retinyl ester substrates. In determining the rates of thermal degradation for the various substrates, assays were conducted in the absence of protein. The retinyl ester substrates exhibited similar degrees of degradation during the course of the REH assay (i.e., <1 mol% of the original substrate could not be recovered). Importantly, the degraded pool was recovered quantitatively as anhydro-vitamin A, rather than as a retinyl ester isomer (data not shown).

Retinyl ester and cholesteryl oleate substrate competition studies. Competition studies were conducted as described above with the following modifications. Hydrolytic rates were measured as a function of incubation

time (0–10 min at 37°C) using 10 μm of the primary substrate and an equimolar amount of competing substrate in a final volume of 200 μl. In some studies, the ratio of primary and competing substrates was modified to reflect a 1:2 or 2:1 molar ratio. Substrates were delivered in EtOH (~5% v/v). In the first series, the liberation of [^{3}H]palmitic acid from [^{3}H]11-*cis*-retinyl palmitate in the absence and presence of unlabeled all-*trans*-, 13-*cis*, and 9-*cis*-retinyl palmitate was monitored by scintillation counting and quantified by relating the dpm associated with the liberated [^{3}H]palmitic acid to the substrate specific activity. Similar competition studies were performed to examine the effect of substrate competition on hydrolysis of [^{3}H]all-*trans*-, -13-*cis*-, and -9-*cis*-retinyl palmitate. This assay system was also utilized to determine the specific activity for hydrolysis of [^{3}H]-9-*cis*-, -11-*cis*-, -13-*cis*-, and -all-*trans*-retinyl palmitate in single-substrate saturation assays. In these assays, 5 μg of microsomal protein was used in a 20-min incubation with increasing concentrations (0–100 μm) of the indicated retinyl ester. Specific activity values were obtained from a secondary transformation (Eadie-Hofstee plot) of substrate saturation data. In the second series of competition studies, we focused on the 11-*cis*-/all-*trans*-retinyl palmitate competitions. In these studies, the hydrolytic activity directed against both 11-*cis*- and all-*trans*-retinyl palmitate was quantified simultaneously in a dual-substrate reaction in which [^{3}H]11-*cis*-retinyl palmitate and [^{14}C]all-*trans*-retinyl palmitate were co-incubated in a time course assay (0–7.5 min at 37°C) using 10 μg of microsomal protein. Radioactivity in the reaction extracts, which contained both [^{3}H]palmitic acid and [^{14}C]palmitic acid, was monitored using two-channel scintillation counting. In the final series of competition studies, the specificity of the REH active site was further probed by assessing the effects of cholesteryl oleate on 11-*cis*- and all-*trans*-REH activities. These studies also utilized a two-substrate, dual-radiolabel protocol involving either [^{3}H]11-*cis*-retinyl palmitate or [^{3}H]all-*trans*-retinyl palmitate and [^{14}C]cholesteryl oleate.

Preparation of [^{3}H]retinyl ester–liposomal vesicles and incorporation of retinyl ester into RPE plasma membranes. Approximately 500 nmol of phosphatidylcholine (PC) dissolved in chloroform was added to dried residues of 11-*cis*-retinyl-myristate, -palmitate, or -linoleate (~6 nmol each). The samples were thoroughly mixed and then evaporated to dryness with a gentle stream of N_2. One ml of 0.2 M KH_2PO_4, pH 7.2, containing 9.5 mg of Triton WR-1339, was added to each of the dried samples. The three detergent–lipid–retinyl ester mixtures were dispersed by sonication at room temperature (60 × 1-s pulses, power output = 2) using a Branson Sonifier fitted with a microtip attachment. Three separate aliquots of an RPE plasma membrane fraction (S2, 5 mg/ml, for method of preparation see reference 15) were diluted 1:5 with one of the three sonicated preparations and incubated at room temperature for 20 min. After incubation, the mixtures were centrifuged at 150,000 g_{max} for 60 min and the resulting pellets were washed twice (150,000 g_{max}, 60 min) in 1 ml of Tris buffer (10 mm Tris-acetate, 1 mm DTT, 2 mm 2Na-EDTA, pH 7.2) to remove residual 11-*cis*-retinyl ester that was not incorporated into plasma membranes. The washed plasma membrane pellets were resuspended in Tris buffer to give a final protein concentration of 1 mg/ml. The amount of 11-*cis*-retinyl ester that was associated with the pellets was determined after extraction and HPLC analysis as described above. Importantly, the substrate concentrations reported in Table 1 represent the amount of membrane-associated 11-*cis*-retinyl ester per assay volume (i.e., 400 μl) rather than the amount of 11-*cis*-retinyl ester incorporated per membrane volume. Quantitation of the phospholipid content of the prepared plasma membranes was performed in order to determine the capacity of these membranes to accommodate the [^{3}H]11-*cis*-retinyl ester substrate. Phospholipid concentration was calculated after determination of the inorganic phosphorus content using methods described by Sokoloff and Rothblat (16).

RESULTS

Hydrolysis of retinol isomers of retinyl palmitate

In order to determine the effect of variations in the retinol polyene tail on REH activity, the hydrolysis of retinyl palmitates possessing different alcohol moieties (i.e., 9-*cis*-, 11-*cis*-, 13-*cis*-, and all-*trans*-retinyl palmitate) was examined. As shown in **Fig. 1**, the highest rate of hydrolysis was associated with the 11-*cis* isomer (1.7 nmol/min/mg versus 0.1–0.3 nmol/min/mg for the other isomers). The values obtained by measurement of liberated [^{3}H]palmitic acid were confirmed by HPLC quantitation of the respective retinols (data not shown). Spectral analyses of unhydrolyzed retinoid substrates and retinol products using HPLC equipped with photodiode array detection showed no significant isomerization or destruction of the substrates during the assay.

Hydrolysis of 11-*cis*-retinyl esters possessing varied acyl moieties

To ascertain whether a fatty acid preference exists for 11-*cis*-REH activity, five 11-*cis*-retinyl esters, differing in

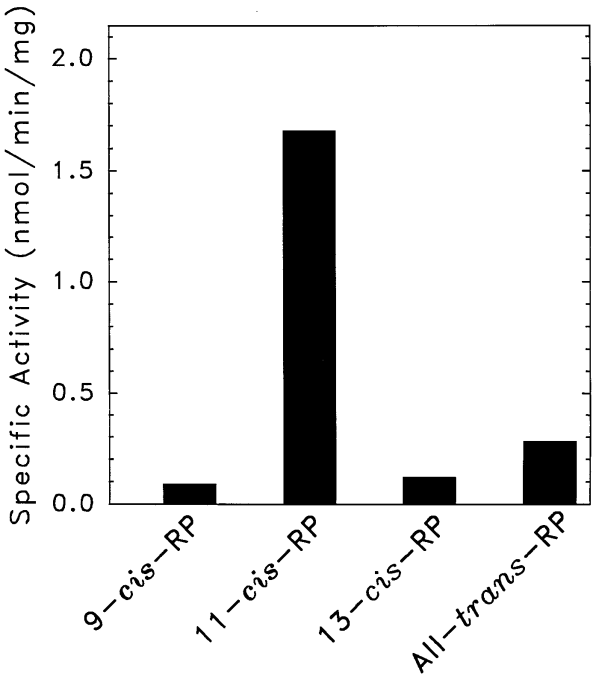

Fig. 1. Specificity of REH activity for different isomers of retinyl palmitate. The hydrolysis of four retinyl palmitate isomers (i.e., 9-*cis*-, 11-*cis*-, 13-*cis*-, and all-*trans*-retinyl [^{3}H]palmitate) was examined using the radiometric REH assay (i.e., 5 μg of microsomal protein, 20 min incubation at 37°C). In this series of experiments, the specific activity against each isomer was obtained from a secondary transformation (Eadie-Hofstee plot) of the substrate saturation (0–100 μm) rate data. REH specific activities are expressed as the molar amount of [^{3}H]palmitic acid liberated minute^{-1} mg^{-1} microsomal protein. The data shown are one representation of two independent determinations analyzed in triplicate.

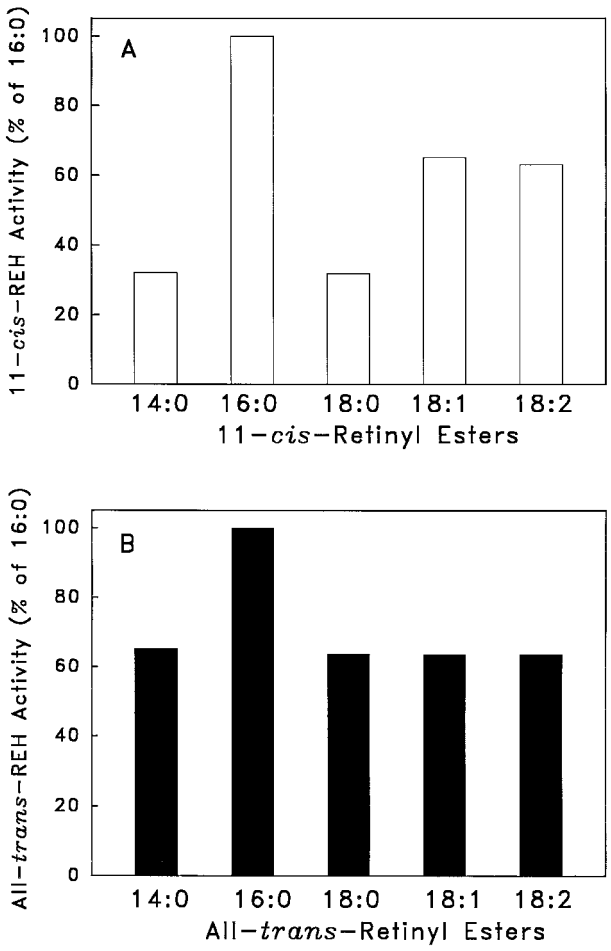

Fig. 2. Fatty acid specificity of 11-*cis*- and all-*trans*-REH activities. Five 11-*cis*- and five all-*trans*-retinyl esters possessing varied acyl chain lengths and degrees of saturation (i.e., 11-*cis*- and all-*trans*-retinyl-myristate [14:0], -palmitate [16:0], -stearate [18:0], -oleate [18:1], and -linoleate [18:2]) were synthesized and used as substrates in the non-radiometric REH assay. In these analyses, the liberation of 11-*cis*-retinol (panel A) or all-*trans*-retinol (panel B) from the respective retinyl ester substrates was quantified by HPLC after 10 min incubation with 10 μg microsomal protein. Specific activities are expressed as a percent value relative to the hydrolysis of 11-*cis*- or all-*trans*-retinyl palmitate (16:0), which is shown as 100% REH activity in panels A and B, respectively. The values shown for each retinyl ester are one representation of two independent determinations analyzed in triplicate.

chain length (myristate [14:0], palmitate [16:0], and stearate [18:0]) and degree of saturation (oleate [18:1] and linoleate [18:2]), were synthesized and used as substrates in REH assays. Data from these studies clearly demonstrate that palmitate is the preferred fatty acyl group (**Fig. 2**, panel A). Although the hydrolysis of the other retinyl esters proceeded at a lower rate, hydrolysis of unsaturated 11-*cis*-retinyl esters (18:1 and 18:2) proceeded at a greater rate than that of the saturated 11-*cis*-retinyl esters (14:0 and 18:0).

Findings related to the fatty acid specificity of 11-*cis*-REH activity, and the fact that all-*trans*-retinyl esters are also abundant in RPE membranes, led us to examine the fatty acid specificity of all-*trans*-REH activity. This analysis was performed in the same fashion as described for examination of 11-*cis*-REH activity with the exception that all-*trans*-retinyl-myristate, -palmitate, -stearate, -oleate, and -linoleate were used as substrates. Results from this study were similar to those obtained for 11-*cis*-REH activity in that palmitate was the preferred fatty acyl moiety. However, there was no preference for unsaturated versus saturated all-*trans*-retinyl esters. In fact, the hydrolytic rates associated with all-*trans*-retinyl esters, other than all-*trans*-retinyl palmitate, were nearly identical (**Fig. 2**, panel B).

In situ hydrolysis of 11-*cis*-retinyl esters

In a recent study, we identified a pronounced 11-*cis*-REH activity, and 11-*cis*-retinyl esters, in RPE plasma membranes. Although all-*trans*-REH activity was also

present, all-*trans*-retinyl esters were not present in RPE plasma membranes (N. L. Mata and A. T. C. Tsin, unpublished results). This finding prompted us to determine the hydrolytic rates for 11-*cis* and all-*trans*-retinyl palmitate in the plasma membrane domain. Consistent with our previous observations of 11-*cis*- and all-*trans*-REH activities in RPE microsomes (12), 11-*cis*-REH specific activity in RPE plasma membranes was approximately 7-times greater than that determined for all-*trans*-REH activity. Thus, the differential hydrolytic rates were not due to undefined physical interactions at the lipid–water interface inherent to the routine REH assay. In the present investigation, we wished to determine whether the hydrolytic rates determined for the different 11-*cis*-retinyl esters was a manifestation of the undefined properties of the lipid–water interface in our assays. We reasoned that this uncontrolled element may affect presentation of the 11-*cis*-retinyl esters to the membrane-bound REH enzyme(s). Accordingly, we prepared separate 11-*cis*-retinyl ester liposomes for 11-*cis*-retinyl-myristate, -palmitate, and -linoleate and fused them with separate aliquots of an RPE plasma membrane preparation. The hydrolytic rate (determined via quantitation of liberated 11-*cis*-retinol) was then determined and compared to that obtained using an EtOH vehicle for substrate delivery. Results from this study are shown in **Table 1**. The fact that hydrolytic activity directed against the examined substrates is comparable in both substrate delivery modes suggests that undefined interactions of enzymes and substrates at the lipid–water interface cannot account for the differences observed regarding hydrolysis of saturated versus unsaturated 11-*cis*-retinyl esters.

TABLE 1. Hydrolysis of 11-*cis*-retinyl esters using two methods of substrate delivery

	Relative Specific Activity	
Substrate	EtOH	Membrane
11-*cis*-retinyl palmitate	1.0	1.0
11-*cis*-retinyl linoleate	0.6	0.6
11-*cis*-retinyl myristate	0.4	0.3

Hydrolysis of the indicated 11-*cis*-retinyl esters was examined using two different methods of substrate delivery. In the first method (EtOH), each 11-*cis*-retinyl ester was delivered to the reaction mixture in an ethanol suspension ([S] = 10 μm each). In the second method, the 11-*cis*-retinyl esters were incorporated into plasma membrane protein and REH activity was quantified using aliquots of the retinyl ester-containing plasma membrane protein ([S] ≈ 15 μm). In this second approach, the indicated substrate concentration represents the amount of membrane-associated 11-*cis*-retinyl esters, as determined by extraction and HPLC analysis of the recovered 11-*cis*-retinyl ester, in the assay volume (i.e., 400 μl). Quantitation of the phospholipid content of RPE plasma membranes (0.4 mg phospholipid/mg plasma membrane) confirmed the capacity of RPE plasma membranes to accommodate the 11-*cis*-retinyl ester substrate. For each method of substrate delivery, the specific activity of 11-*cis*-retinyl palmitate hydrolysis (≈300 pmol min^{-1} mg^{-1} for each delivery method) is taken as 1.0; the specific activities associated with hydrolysis of 11-*cis*-retinyl-linoleate and -myristate are shown relative to this value. The data shown are representative of two independent experiments analyzed in duplicate.

Competition of 11-*cis*-retinyl palmitate with retinyl- and cholesteryl-ester substrates

To assess the specificity of the REH active site, a series of experiments were conducted to determine whether the hydrolysis of [^{3}H]11-*cis*-retinyl palmitate was affected by the presence of equimolar quantities of other retinyl esters. Upon addition of unlabeled 9-*cis*-, 13-*cis*-, or all-*trans*-retinyl palmitate, the rate of hydrolysis of [^{3}H]11-*cis*-retinyl palmitate was unchanged from control values (i.e., in the absence of competing substrate, **Fig. 3**, upper left-hand panel). In contrast, the rates of hydrolysis of [^{3}H]all-*trans*- (upper right-hand panel), [^{3}H]13-*cis*- (lower left-hand panel), or [^{3}H]9-*cis*- (lower right-hand panel) retinyl palmitate were significantly reduced in the presence of equimolar quantities of unlabeled 11-*cis*-retinyl palmitate or other competing retinyl esters. It is interesting to note that the hydrolysis of [^{3}H]all-*trans*-retinyl palmitate is reduced to the same extent in the presence of each competing ester (~33% inhibition). This result suggests that the remaining activity may be due a distinct all-*trans*-REH enzyme. Alternatively, the remaining activity may be due to the activity of other "non-specific" esterases. In order to address this issue, we conducted two additional substrate competition assays in which the hydrolysis of [^{3}H]11-*cis*-retinyl palmitate and [^{14}C]all-*trans*-retinyl palmitate were simultaneously monitored over time. In the first analysis, the [^{3}H]11-*cis*-retinyl palmitate: [^{14}C]all-*trans*-retinyl palmitate molar ratio was 1:2, in the second the molar ratio was 2:1. Consistent with our previous competition analyses, the hydrolysis of [^{3}H]11-*cis*-retinyl palmitate, assessed by quantitation of liberated [^{3}H]palmitic acid, was not affected by the inclusion of [^{14}C]all-*trans*-retinyl palmitate (**Fig. 4**, panels A and C). However, hydrolysis of [^{14}C]all-*trans*-retinyl palmitate was significantly reduced in the presence of [^{3}H]11-*cis*-retinyl palmitate in a dose-dependent manner (Fig. 4, panels, B and D). Taken together these findings suggest that the observed all-trans-REH activity may be due to "non-specific" hydrolase activities. Evidence to further support this hypothesis was obtained from an additional series of competition studies in which the effects of [^{14}C] cholesteryl oleate on [^{3}H]11-*cis*- and -all-*trans*-retinyl palmitate hydrolysis was determined. The data show that inclusion of [^{14}C]cholesteryl oleate significantly reduces [^{3}H]all-*trans*-retinyl ester hydrolysis (**Fig. 5**, panel C). Similarly, [^{3}H]all-*trans*-retinyl palmitate significantly reduces [^{14}C]cholesteryl oleate hydrolysis (Fig. 5, panel

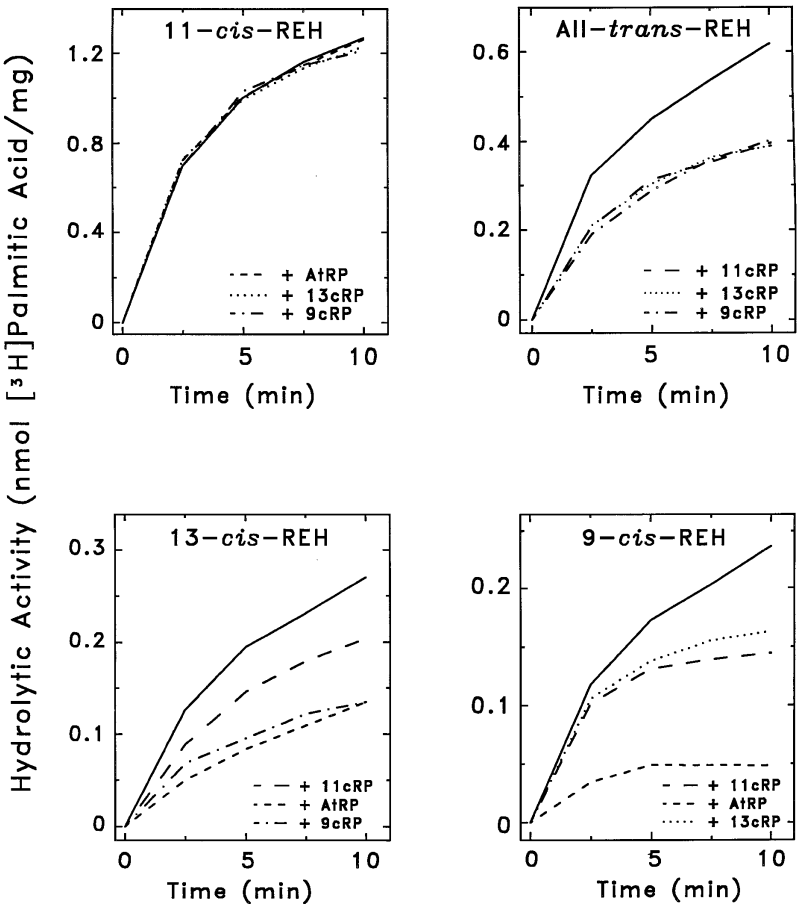

Fig. 3. Effect of substrate competition on REH activities. The liberation of [^{3}H]palmitic acid from [^{3}H]11-*cis*-retinyl palmitate (10 μm) in the absence and presence of equimolar concentrations of unlabeled all-*trans*-, 13-*cis*-, and 9-*cis*-retinyl palmitate is shown in the upper left-hand panel. Similar competition studies were performed to examine the effect of substrate competition on hydrolysis of [^{3}H]-all-*trans*- (upper right-hand panel), -13-*cis*- (lower left-hand panel), and -9-*cis*- (lower right-hand panel) retinyl palmitate. REH assays were performed using 10 μg of microsomal protein and reaction mixtures were quenched at either 2.5, 5.0, 7.5 or 10.0 min. Control values are represented by a solid line in each panel. REH activities in the presence of added unlabeled substrates are shown as indicated in the figure legend given for each panel. The data shown is one representation taken from three independent studies which yielded nearly identical results.

D). These observations, and the fact that all-*trans*-retinyl palmitate and cholesteryl oleate are hydrolyzed at similar rates, suggests that these substrates may be hydrolyzed at the same non-specific catalytic site. Interestingly, inclusion of [^{14}C]cholesteryl oleate in [^{3}H]11-*cis*-retinyl palmitate assays did not affect the rate of 11-*cis*-REH activity (Fig. 5, panel A) and the rate of [^{14}C] cholesteryl oleate hydrolysis was also unaffected (Fig. 5, panel B). Thus, it is very likely that 11-*cis*-retinyl palmitate and cholesteryl oleate are hydrolyzed at discrete catalytic sites.

DISCUSSION

Retinyl esters represent a substantial proportion (up to 99%) of the total vitamin A found in the eye (6, 17–19). The fatty acid composition of retinyl esters in bovine RPE has been determined to be 57% palmitate, 25% stearate, 10% oleate, 1.1% myristate, and 0.6% linoleate (20). Eleven-*cis*-retinyl esters represent a substantial pool, up to 75%, of the endogenous retinoids in RPE (5, 8, 21). Therefore, an enzymatic activity responsible for mobilizing 11-*cis*-retinol from 11-*cis*-retinyl ester stores is likely to play a vital role in the visual system. However, information regarding substrate specificity of REHs in the RPE is not available in the literature.

There have been investigations of the substrate specificity of an hepatic all-*trans*-REH activity (22). In fact, the numerous characterizations of all-*trans*-REH activity in liver (reviewed in ref. 11) have led to the purification of an all-*trans*-REH enzyme that shares a high homology with a previously cloned carboxylesterase from rat liver (23). Thus, retinyl esters stored in liver are likely to be mobilized to extrahepatic target tissues via an all-*trans*-REH isozyme of carboxylesterase. Characterizations of all-*trans*-REH activity in liver have clearly advanced our understanding of all-*trans*-REH activities in RPE (13). However, these studies are of limited usefulness in attempting to understand biochemical properties of 11-*cis*-retinyl ester hydrolysis. For example, it is not clear whether the 11-*cis*- and all-*trans*-REH activities characterized in RPE membranes (10, 12) are distinct enzymes with definite substrate preferences or whether *cis* and *trans* substrates can be similarly accommodated by a single nonspecific catalyst. Information obtained

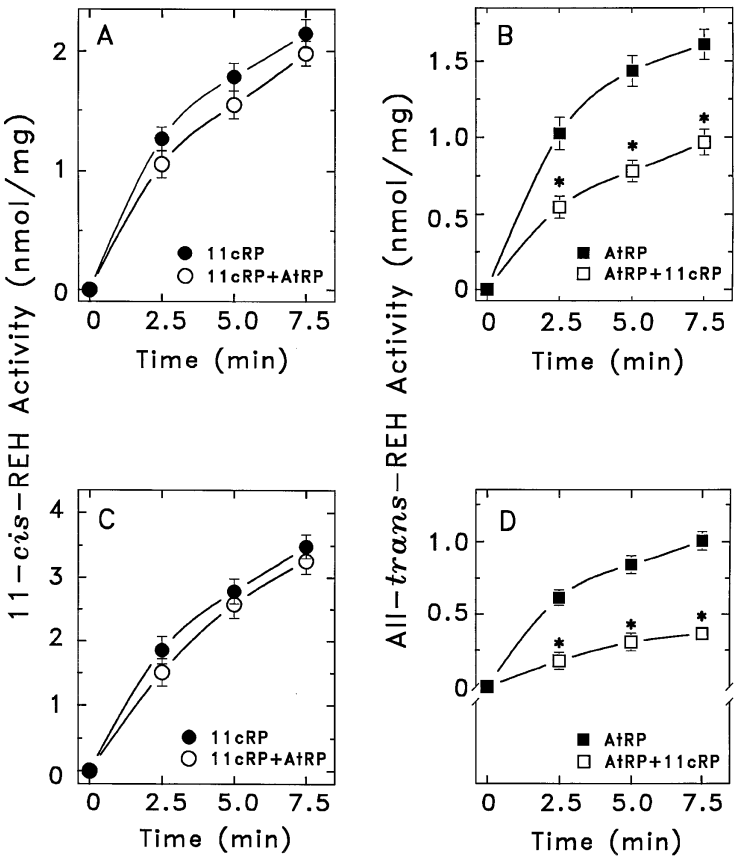

Fig. 4. Examination of 11-*cis*- and all-*trans*-retinyl palmitate substrate competition. Studies were conducted to examine the specificity of 11-*cis*- and all-*trans*-retinyl palmitate catalytic sites. In order to facilitate quantitation of the palmitic acid reaction products from both substrates simultaneously, [^{3}H]11-*cis*-retinyl palmitate and [^{14}C]all-*trans*-retinyl palmitate substrates were prepared and then co-incubated in the REH assay at different molar concentrations using 10 μg of microsomal protein. In panels A and B, the [^{3}H]11-*cis*-:[^{14}C]all-*trans*-retinyl palmitate molar ratio is 1:2 (i.e., 10 μm versus 20 μm, respectively). In panels C and D, the [^{3}H]11-*cis*-:[^{14}C]all-*trans*-retinyl palmitate molar ratio is 2:1 (i.e., 20 μm versus 10 μm, respectively). In the figure, solid symbols represent control values obtained in the absence of added retinyl palmitate isomer, while open symbols represent values obtained with added retinyl palmitate isomer. The values shown represent the mean ± σ from three independent experiments analyzed in triplicate. Asterisks indicate a statistically significant group mean difference, at the indicated time-point, relative to values obtained in the absence of competing substrate (Student's *t*-test, $\alpha = 0.05$). The break in the y-axis shown in panel D is utilized to facilitate clarity of the figure legend.

regarding retinyl ester utilization in the RPE will clearly advance our knowledge concerning the roles of retinyl ester metabolizing enzymes in visual chromophore biosynthesis and will therefore have profound implications on our understanding of the mammalian visual system. In efforts to address this important facet of the visual cycle, we have sought to determine the substrate specificity of REH activity in RPE membranes. Our initial approach was to ascertain which portion of the retinyl ester substrate (i.e., retinol or fatty acyl moiety) is necessary for maximal hydrolysis. We first determined the specific activity for hydrolysis of four retinyl palmitate isomers possessing varied polyene chain lengths (9-*cis*-, 11-*cis*-, 13-*cis*-, and all-*trans*-retinol). Our decision to use retinyl palmitate isomers in this initial study was due largely to the fact that palmitate is the principal fatty acid associated with endogenous retinyl esters. The data show that REH activity was greatest against the 11-*cis* isomer; hydrolysis of the other isomers was approximately 6-fold lower than that of 11-*cis*-retinyl palmitate. Analysis of the fatty acyl chain specificity of 11-*cis*-REH activity revealed a trend that approximated the retinyl ester–fatty acid composition in RPE cells. Thus, the preference of 11-*cis*-REH activity for different fatty acyl groups was as follows: palmitate > oleate = linoleate > myristate = stearate. The finding that the unsaturated 11-*cis*-retinyl esters exhibited higher rates of hydrolysis compared to the saturated 11-*cis*-retinyl esters was a novel finding and was not observed in a similar study using all-*trans*-retinyl esters. Regarding the all-*trans*-REH studies, although palmitate was still the preferred fatty acid, there was no preference for unsaturated versus saturated all-*trans*-retinyl esters. It is known that the presence of double bonds in unsaturated, *trans* fatty acids reduces the overall chain length relative to saturated, *trans* fatty acids (24). This effect is most pronounced when the fatty acid chain is in a *cis* conformation (25). Thus, the increased rate of hydrolysis associated with the unsaturated, *cis* esters may be due to a decrease in molecular size which has the effect of more closely approximating the length, and dimensional space, of the preferred 16-carbon, retinyl palmitate.

Results from the 11-*cis*-REH fatty acid specificity studies led us to explore the possibility that the observed effects might be due to undefined physical interactions at the lipid–water interface which permit a more or less effective presentation of a given retinyl ester to the REH enzyme(s) (11). Thus, 11-*cis*-retinyl esters composed of short (11-*cis*-retinyl myristate) and unsaturated (11-*cis*-retinyl linoleate) acyl chains were incorpo-

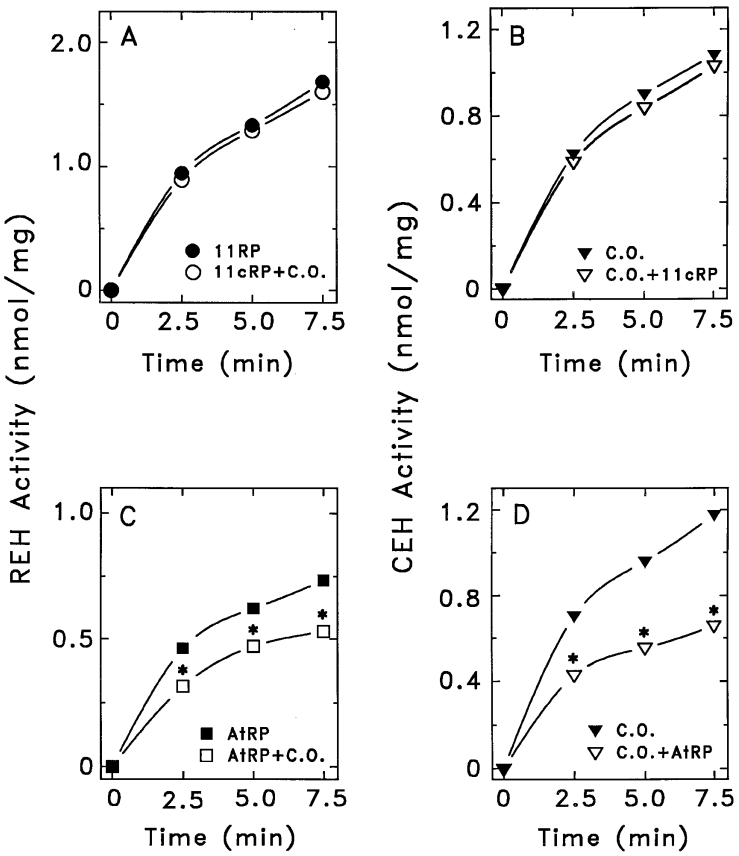

Fig. 5. Effect of cholesteryl oleate on 11-*cis*- and all-*trans*-REH activities. The contribution of "non-specific" esterase activities to the observed 11-*cis*- and all-*trans*-REH activities was examined in a substrate competition study in which the hydrolysis of [^{3}H]11-*cis*- or [^{3}H]all-*trans*-retinyl palmitate (10 μm) was monitored in the absence and presence of [^{14}C]cholesteryl oleate (10 μm) using 10 μg of microsomal protein. In panels A and B, the reaction products from [^{3}H]11-*cis*-retinyl palmitate and [^{14}C]cholesteryl oleate hydrolysis, respectively, are shown. In panels C and D, the reaction products from [^{3}H]all-*trans*-retinyl palmitate and [^{14}C]cholesteryl oleate hydrolysis, respectively, are shown. The values shown are representative of two independent experiments analyzed in triplicate. Asterisks indicate a statistically significant group mean difference, at the indicated time-point, relative to values obtained in the absence of competing substrate (Student's *t*-test, $\alpha = 0.05$).

rated into RPE plasma membranes using liposomal fusion and the hydrolytic rates were compared to that of plasma membrane-bound 11-*cis*-retinyl palmitate. Plasma membranes were utilized for this study as we have recently discovered that *1*) 11-*cis*-REH activity is largely recovered in plasma membranes and *2*) the differential rates of 11-*cis*- and all-*trans*-retinyl palmitate hydrolysis observed in vitro were also observed when these substrates are bound within the membrane. The relative rates of hydrolysis of 11-*cis*-retinyl esters in this study were essentially identical to those obtained when the hydrolytic rates were determined using the routine (lipid–water interface) REH assay. Thus, the observed results were not due to undefined interactions at the lipid–water interface.

Additional evidence that supports the existence of an 11-*cis*-specific REH activity comes from substrate competition studies. In these studies, the rate of 11-*cis*-retinyl palmitate hydrolysis was not affected when other retinol ester isomers were included in the reaction mixture. Thus, these isomers were not able to displace 11-*cis*-retinyl palmitate from the active site. In contrast, because the hydrolysis of 9-*cis*-, 13-*cis*-, and all-*trans*-retinyl palmitate were all significantly inhibited regardless of the combination of primary and competing substrate, we conclude that these activities are nonspecific in nature and could be due to the activity of nonspecific esterases (e.g., cholesteryl ester hydrolase). The finding that cholesteryl oleate is not able to inhibit the rate of 11-*cis*-retinyl palmitate hydrolysis and vice versa, indicates not only that the 11-*cis*-REH is specific, but also that the two substrates are hydrolyzed at discrete catalytic sites. In contrast, when the same competition assay was conducted with all-*trans*-retinyl palmitate, the hydrolysis of both cholesteryl oleate and all-*trans*-retinyl palmitate was significantly diminished. This finding is consistent with the initial competition studies which indicate a relatively non-specific active site for all-*trans* retinyl palmitate hydrolysis. Moreover, these data also strongly suggest that the hydrolysis of all-*trans* retinyl palmitate and cholesteryl oleate occurs at the same, or biochemically similar, active site(s). Finally, recent biochemical analyses of the 11-*cis*-REH active site have further substantiated our finding of a strict preference for 11-*cis* retinyl palmitate as neither 11-*cis*-retinol nor palmitic acid is able to displace 11-*cis*-retinyl palmitate from the active site (data not shown).

In summary, findings of the present study provide strong evidence for the existence of a highly specific REH activity in the RPE that is maximal when palmitate

is the fatty acid and the *cis* bend in the conjugated double bond system of retinol is at C-11. Although it is clear that 11-*cis*-retinyl esters contribute to visual chromophore biosynthesis in vivo, the mechanism underlying utilization of the endogenous 11-*cis*-retinyl ester pool has remained enigmatic. Data from our studies support the hypothesis that a substrate-specific retinyl ester hydrolase is the physiological mechanism responsible for 11-*cis*-retinyl ester mobilization.

We thank Dr. Kondury Prasad for critical evaluation of this manuscript, Hoffman-La Roche for the generous contribution of purified 11-*cis*-retinyl palmitate, Dr. Rosalie Crouch and the NIH/NEI for 11-*cis*-retinal, and Mrs. Elia Leos for technical assistance. This research was supported by grants from the NIH (EY06438 and GM08194) and the San Antonio Area Foundation.

Manuscript received 30 July 1997 and in revised form 27 October 1997.

REFERENCES

1. Saari, J. C. 1994. Retinoids in photosensitive systems. *In* The Retinoids. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, New York. 351–385.
2. Crouch, R. K., G. J. Chader, B. Wiggert, and D. M. Pepperberg. 1996. Retinoids and the visual process. *Photochem. Photobiol.* **64:** 613–621.
3. Rando, R. R. 1990. The chemistry of vitamin A and vision. *Angew. Chem. Int. Ed. Engl.* **29:** 461–480.
4. Rando, R. R. 1991. Membrane phospholipids as an energy source in the operation of the visual cycle. *Biochemistry.* **30:** 595–602.
5. Hubbard, R., and Dowling, J. E. 1962. Formation and utilization of 11-*cis* vitamin A by the eye tissues during light- and dark-adaptation. *Nature.* **193:** 341–343.
6. Rodriguez, K. A., and A. T. C. Tsin. 1989. Retinyl esters in the vertebrate neuroretina. *Am. J. Physiol.* **256:** R255–R258.
7. Zimmerman, W. F. 1974. The distribution and proportion of vitamin A compounds during the visual cycle in the rat. *Vision Res.* **14:** 795–802.
8. Bridges, C. D. B. 1976. Vitamin A and the role of the pigment epithelium during bleaching and regeneration of rhodopsin in the frog eye. *Exp. Eye Res.* **22:** 435–455.
9. Bridges, C. D. B. 1984. Retinoids in photosensitive systems. *In* The Retinoids. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Academic Press, New York. 125–141.
10. Blaner, W. S., S. R. Das, P. Gouras, and M. T. Flood. 1987. Hydrolysis of 11-*cis* and all-*trans* retinyl palmitate by homogenates of human retinal epithelial cells. *J. Biol. Chem.* **262:** 53–58.
11. Harrison, E. H. 1993. Enzymes catalysing the hydrolysis of retinyl esters. *Biochim. Biophys. Acta.* **1170:** 99–108.
12. Mata, N. L., A. T. C. Tsin, and J. P. Chambers. 1992. Hydrolysis of 11-*cis* and all-*trans* retinyl palmitate by retinal pigment epithelium microsomes. *J. Biol. Chem.* **267** 9794–9799.
13. Mata, N. L., J. R. Mata and A. T. C. Tsin. 1996. Comparison of retinyl ester hydrolase activities in bovine liver and retinal pigment epithelium. *J. Lipid Res.* **37:** 1947–1952.
14. Bridges, C. D. B., and R. A. Alvarez. 1982. Measurement of the vitamin A cycle. *Methods Enzymol.* **81:** 463–485.
15. Touster, O., N. N. Aronson, J. T. Dulaney, and H. Hendrickson. 1970. Isolation of rat liver plasma membranes. *J. Cell Biol.* **47:** 604–618.
16. Sokoloff, L., and G. H. Rothblat. 1974. Sterol to phospholipid molar ratios of L cells with qualitative and quantitative variations in cellular sterol. *Proc. Soc. Exp. Biol. Med.* **146:** 1166–1172.
17. Berman, E. R., N. Segal, and L. Feeney. 1979. Subcellular distribution of free and esterified forms of vitamin A in the pigment epithelium of the retina and in liver. *Biochim. Biophys. Acta.* **572:** 167–177.
18. Bridges, C. D. B., R. A. Alvarez and S-L. Fong. 1982. Vitamin A in human eyes: amount, distribution, and composition. *Invest. Ophthal. Vis. Sci.* **22:** 706–714.
19. Bongiorno, A., L. Tesoriere, M. A. Livera, and L. Pandolpho. 1991. Distribution of vitamin A compounds in bovine eyes after bleaching adaptation. *Vision Res.* **31:** 1099–1106.
20. Futterman, S., and J. S. Andrews. 1964. Metabolism of the retina IV. The composition of vitamin A ester synthesized in the retina. *J. Biol. Chem.* **239:** 81–84.
21. Dowling, J. E. 1960. Chemistry of visual adaptation in the rat. *Nature.* **163:** 114–118.
22. Cooper, D. A., and J. A. Olson. 1986. Properties of liver retinyl ester hydrolase in young pigs. *Biochim. Biophys. Acta.* **884:** 251–258.
23. Sun, G., S. E. H. Alexson, and E. H. Harrison. 1997. Purification and characterization of a neutral, bile salt-independent retinyl ester hydrolase from rat liver microsomes. *J. Biol. Chem.* **272:** 24488–24493.
24. Bickel, M. H., and J. Romer. 1978. Interaction of chlorpromazine with organic solvents and fatty acids as studied by UV-spectrophotometry. *Experientia* **34:** 1047–1049.
25. Wang, G., H. N. Lin, S. Li, and C. H. Huang. 1995. Phosphatidylcholines with *sn*-1 saturated and *sn*-2 *cis*-monounsaturated acyl chains. Their melting behavior and structures. *J. Biol. Chem.* **270:** 22738–22746.