Bile Salt Independent Retinyl Ester Hydrolases in the Bovine Eye

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TSIN, A. T. C. AND D. W. MALSBURY. Bile salt independent retinyl ester hydrolases in the bovine eye. BRAIN RES BULL 28(1) 121-125, 1992.—Homogenates of bovine neuroretina and retinal pigment epithelium (RPE) were incubated with 11-cis and all-trans retinyl palmitate to study retinyl ester hydrolysis. The highest activity was found in RPE when 11-cis retinyl palmitate served as substrate (K_m = 7.8 µM and V_max = 44.8 pmol/min/mg). This retinyl ester hydrolase (REH) had an optimum activity at acidic pH (pH 5), which is in contrast to the neutral hydrolase (pH 8) found in the neuroretina. Similar to REH in the liver, REH activities in the bovine eye are not stimulated by bile salt because sodium cholate, taurocholate and deoxycholate did not enhance retinyl ester hydrolysis. Most REH activities in retinal homogenate were soluble, whereas in the RPE, these activities were membrane-bound. Divalent cations such as zinc and cadmium completely inhibited REH activities in the neuroretina. Our results show that bovine ocular tissues contain several retinyl ester hydrolases with distinct biochemical properties.

In a recent report, Harrison and Gad (10) demonstrated a neutral, bile salt independent REH in the rat liver. The level of this REH activity is comparable to that of the bile salt-dependent activity previously observed (10). These new findings on hepatic REH suggest that in the eye, such physiological hydrolases selective for the hydrolysis of 11-cis isomers might also be present. In the present report, we describe the partial characterization of bovine ocular hydrolases which are selective towards 11-cis isomers.

METHOD

All-trans retinol, all-trans retinal and all-trans retinyl palmitate were obtained from Sigma Chemical Co. (St. Louis, MO). 11-Cis retinal was a gift from Hoffmann-La Roche Co. (Nutley, NJ) and was reduced to 11-cis retinol according to an established method (6). [9,10-3H] palmitic acid (specific activity 30 Ci/mmol) was obtained from Du Pont-New England Nuclear (Wilmington, DE). Labeled retinyl palmitate substrates were synthesized according to the method indicated below (3,10) and purified by high performance liquid chromatography (HPLC) before use. All organic solvents were HPLC grade purchased from Fisher Scientific Co. (Springfield, NJ). Bovine neuroretina and retinal pigment epithelium were dissected from freshly enucleated eyes obtained from All-State Packing Co. (San Antonio, TX). All tissues were stored at -70°C before use.

Preparation of Substrates

Synthesis of the symmetric anhydride of palmitic acid followed the method of Lentz et al. (13) and substrates (11-cis and
all-trans retinyl palmitate) were prepared by reacting the respective retinols with palmitic anhydride (19). Pyridine, rather than carbon tetrachloride, was used as solvent to minimize destruction of the 11-cis isomer (Blaner, personal communication).

Briefly, 5 mCi of palmitic acid in ethanol was evaporated by a stream of nitrogen. Dicyclohexylcarbodiimide (10 μmol; in 10 μl of pyridine) was added to the palmitic acid. To this, 90 μl of pyridine was added and reacted at room temperature for 30 min. Palmitic acid (30 μmol, nonradioactive) was added in 300 μl of pyridine and the mixture was allowed to incubate at room temperature for 1 hour. The mixture now containing palmitic anhydride was evaporated by a stream of nitrogen and 2.4 μmol of 11-cis retinol (from the oxidation of 11-cis retinol by sodium borohydride (6)) dissolved in 500 μl pyridine was added. The mixture was incubated overnight at room temperature under argon. The pyridine solvent was then evaporated by a stream of nitrogen and the residue was redissolved in a small volume of n-hexane and applied to a disposable column (1 × 2 cm) packed with 5% water deactivated alumina. The retinoids were eluted with increasing strength of dioxane in n-hexane. The retinyl ester hydrolase assay was purified nonradioactive retinyl palmitate. Final concentration of the substrate was 200 nM per ml.

Palmitic acid (30 pmol, nonradioactive) was added in 300 μl of pyridine and the mixture was allowed to incubate at room temperature for 1 hour. The mixture now containing palmitic anhydride was evaporated by a stream of nitrogen and 2.4 μmol of 11-cis retinol (from the oxidation of 11-cis retinol by sodium borohydride (6)) dissolved in 500 μl pyridine was added. The mixture was incubated overnight at room temperature under argon. The pyridine solvent was then evaporated by a stream of nitrogen and the residue was redissolved in a small volume of n-hexane and applied to a disposable column (1 × 2 cm) packed with 5% water deactivated alumina. The retinoids were eluted with increasing strength of dioxane in n-hexane. The retinyl ester fraction was then subjected to HPLC (high performance liquid chromatography) analysis in order to further purify the retinyl ester. The specific activity of the substrate was adjusted to 2 pmol per 30,000 to 50,000 cpm by the addition of (HPLC)

Three ml of extraction solvent (1.41: 1.25: 1; methanol:chloroform:methanol 1:2:1) was used as solvent to minimize destruction of isomers. The pyridine solvent was then evaporated by a stream of carbon tetrachloride, was used as solvent to minimize destruction of isomers. The pyridine solvent was then evaporated by a stream of carbon tetrachloride, was used as solvent to minimize destruction of isomers.

Other Procedures

Retinyl Ester Hydrolase Assay

Two nmol of HPLC purified substrate (in 10 μl of ethanol) was added to each assay mixture containing enzyme protein (10 μg to 2000 μg) suspended in 190 μl of 50 mM Tris-maleate, pH 8.0. The mixture was then incubated at 37°C for one hour. Three ml of extraction solvent (1:4:1:25:1; methanol:chloroform:heptane, v/v) was added to the mixture, immediately followed by the addition of 1.0 ml of potassium carbonate (pH 10.0) buffer. The mixture was vortexed and then centrifuged for 1000 x g for 15 min at room temperature. One ml of the upper phase was removed and counted in 10 ml of Scinti Verse (Fisher Scientific) using a Packard Model 1500 Liquid Scintillation Analyzer. The counting efficiency averaged about 60%. All enzyme assays were carried out in triplicate.

Because REH in RPE has optimal activity at pH 5.0, all assays using RPE were performed at this pH using 50 mM sodium citrate-phosphate buffer. The level of destruction of both isomers of retinyl palmitate were minimal in this pH, as reveal in preliminary studies involving extraction of retinol products and analysis by HPLC. All assays carried out in the present study showed linear REH activities with respect to time and protein concentrations.

Results from the addition of metal ions to incubation mixtures (Table 1) showed that minimal endogenous substrates (i.e., 0.45 pmol all-trans retinyl palmitate/μg protein and 0.15 pmol 11-cis retinyl palmitate/μg protein) in these tissue homogenates. In some experiments (Table 2), homogenates were separated into supernatant and pellet fractions by centrifugation at 100,000 X g for 1 h. Isomeric configuration of retinol product of reaction was verified by extraction of incubation mixture with hexane and analysis by HPLC (3). Authenticity and purity of all retinoids were verified spectrophotometrically and by HPLC. Protein determination was carried out by the method of Lowry et al. (14) using bovine serum albumin as standard.

DISCUSSION

Other Procedures

Retinal homogenates were prepared by homogenizing tissues in an electrical homogenizer (Brinkman Homogenizer, Model PT 10/35, Brinkman Instruments, Westbury, NY) in buffer for 30 seconds. RPE cells were dislodged from the RPE/choroid by agitation (in buffer) using a vortex mixer for 1 minute (18). Fibrous tissue was removed before the cell suspension was homogenized in the same manner as retinal homogenate. Histological examination of these RPE preparations showed minimal cross contamination from retinal cells (18). HPLC analyses showed that minimal endogenous substrates (i.e., 0.45 pmol all-trans retinyl palmitate/μg protein and 0.15 pmol 11-cis retinyl palmitate/μg protein) in these tissue homogenates. In some experiments (Table 2), homogenates were separated into supernatant and pellet fractions by centrifugation at 100,000 X g for 1 h. Isomeric configuration of retinol product of reaction was verified by extraction of incubation mixture with hexane and analysis by HPLC (3). Authenticity and purity of all retinoids were verified spectrophotometrically and by HPLC. Protein determination was carried out by the method of Lowry et al. (14) using bovine serum albumin as standard.

RESULTS

Ocular REH activities were dependent upon hydrogen ion concentration. Figure 1 compares the "acid" and "neutral" REH found in the RPE and neuroretina, respectively. In the RPE, pH optimum was observed at 5, with two lesser activity maxima at pH 4 and 8, suggesting the presence of several hydrolases. In comparison, REH activity occurred optimally at a neutral pH (6-8) in the neuroretina. In both ocular tissues hydrolysis of the cis isomer occurred at a greater rate than that of the trans isomer and the rate of retinyl ester hydrolysis for corresponding isomers was several fold greater in the RPE.

Hydrolysis of retinyl ester by RPE homogenate increased with the amount of added RPE protein (to 50 μg for 11-cis REH). This acidic REH activity was higher towards the 11-cis isomer and showed saturation kinetics within the range of substrate concentrations used in the present study (0 to 15 μM; Fig. 2). Eadie-Hofstee graphical analysis (insert, Fig. 2) revealed an apparent K_m of 7.8 μM and a corresponding V_max of 44.8 pmol/min/mg protein for 11-cis REH.

Although the neutral REH in the neuroretina shows similar selectivity for the 11-cis isomer, the overall hydrolytic rates were much lower than REH activities in the RPE. Eadie-Hofstee graphical analysis (Fig. 3) revealed an apparent K_m of 25 μM and a corresponding V_max of 5.0 pmol/min/mg protein for 11-cis REH.

When retinal and RPE homogenates were subjected to ultracentrifugation (100,000 X g for 1 h) to separate soluble (supernatant) components from particulate, membrane-bound (pellet) cellular materials, REH activities in RPE were recovered primarily in the membrane fraction (Table 2). Unlike those in the RPE, REH activities of retinal homogenate preparations were soluble as evidenced by quantitative recovery (70-80%) in the supernatant fraction (Table 2).

Results from the addition of metal ions to incubation mixtures are summarized in Table 3. Cadmium and zinc were observed to be potent inhibitors of REH activity. However, monovalent (sodium and potassium) cations and most divalent ions (see Table 3) had little effect upon hydrolytic activity.

A partial characterization of 11-cis and all-trans retinyl ester hydrolase (REH) activity of bovine neuroretina and retinal pig-
ment epithelium (RPE) homogenates was carried out in the present study. Similar to the conclusion of Harrison and Gad (10), we present experimental evidence to indicate that ocular tissues also possess REH activities which are not stimulated by bile salts. These hydrolytic activities are more "physiological" than those requiring bile salts which are not endogenous to these tissues (for an explanation of acid hydrolase of RPE, see paragraph below). Moreover, these hydrolytic activities were stereo-
selective towards the 11-cis isomer which is a key participant in the rhodopsin cycle. This further substantiates the functional significance of these hydrolases in the eye.

Berman et al. (2) reported that an all-trans REH exists in the bovine RPE lysosomal/mitochondrial fraction. This activity was optimum at pH 4.0–4.5 and was enhanced 3 times by addition of Triton X-100 to the assay mixture. These observations are in agreement with those reported in the present study and this provides an explanation why acid hydrolase in the RPE is "physiological." However, no activity in the neuroretina, nor in rod outer segment was reported in their study. In the present study we have presented evidence that retinal homogenates do indeed possess REH activities. Additionally, purified rod outer segments were also observed to exhibit a significant level of REH activities (unpublished observations).

Results from the present study agree with those presented by Blaner et al. (3) on human RPE cells with respect to the substrate stereoselectivity (i.e., enzyme specific towards the 11-cis isomer). However, Blaner et al. (3) reported that their REH enzyme is stimulated by the presence of bile salts which was not found in the present study on bovine ocular tissues (Table I). This discrepancy of bile-salt dependency is presently unresolved and perhaps related to species difference.

From the pH profile of enzyme activity (Fig. 1) it is likely that more than one REH enzyme exist in tissues homogenates examined in the present study. It is important to point out that experiments performed in our study were carried out at pH 5 for RPE and pH 8 for retina to study the acid and neutral hydrolases in these respective tissues. Thus, it is possible that other

![Graph showing the effect of substrate concentrations on (neutral) 11-cis and all-trans retinyl ester hydrolase activities in bovine neuroretina.](image)

**FIG. 3.** Effect of substrate concentrations on (neutral) 11-cis and all-trans retinyl ester hydrolase activities in bovine neuroretina. Tritium-labeled retinyl palmitate substrate was added (in 10 μl ethanol) to 720 μg (all-trans substrate) or 200 μg (11-cis substrate) RPE homogenate suspended in 190 μl of 50 mM Tris-maleate (pH 8) and sodium citrate-phosphate (pH 5) buffer. The mixture was incubated for 1 h at 37°C and palmitic acid was extracted with methanol: chloroform: heptane (1.41:1.25:1) with radioactivity measured in a liquid scintillation counter. For details, see the Method section.

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>Retina</th>
<th>RPE</th>
<th>11-cis</th>
<th>All-trans</th>
<th>11-cis</th>
<th>All-trans</th>
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<tr>
<td>Retina</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>Supernatant fraction</td>
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<td>0.2</td>
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<td>0.2</td>
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<tr>
<td>Pellet fraction</td>
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<td>0.2</td>
<td>0.7</td>
<td>0.8</td>
<td>0.7</td>
<td>0.8</td>
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*[(3-Cholamidopropyl)dimethylammonio]1-propanesulfate)*
### Table 3

**Inhibition of 11-cis and all-trans retinyl ester hydrolase activity by metal ions (25 mM)**

<table>
<thead>
<tr>
<th>Retina Hydrolase Activity</th>
<th>RPE</th>
<th>11-cis REH</th>
<th>All-trans REH</th>
<th>11-cis REH</th>
<th>All-trans REH</th>
</tr>
</thead>
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<tr>
<td>NaCl</td>
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<tr>
<td>KCl</td>
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<tr>
<td>MgCl₂</td>
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<tr>
<td>CaCl₂</td>
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<td>0.6</td>
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<tr>
<td>CoCl₂</td>
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<td>0.2</td>
<td>0.5</td>
<td>0.9</td>
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<tr>
<td>ZnCl₂</td>
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<td>0.0</td>
<td>0.4</td>
<td>0.5</td>
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... enzymes capable of hydrolyzing the ester linkages of the retinyl palmitates may not have been identified in our investigation. Therefore, it will be important to reexamine these properties of REH when pure REH enzymes become available.

In a recent study we reported the distribution of 11-cis and all-trans retinyl esters in the neuroretina and RPE of three different species of vertebrates [frog, chicken and cow (17)]. In that study it was suggested that retinyl esters located in both RPE and neuroretina were important sources from which visual pigments derive their chromophores for synthesis and regeneration.

Data presented in the present study fully support this notion by providing experimental evidence that "physiologic" 11-cis and all-trans REH activities do exist in these tissues.

### Acknowledgements

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### References