Molecular Logic of 11-*cis*-Retinoid Biosynthesis in a Cone-Dominated Species†

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ABSTRACT: The biochemical pathway to visual chromophore biosynthesis in rod-dominated animals involves minimally a two component system in which all-*trans*-retinyl esters, generated by the action of lecithin retinol acyltransferase (LRAT) on vitamin A, are processed into 11-*cis*-retinol by isomerohydrolase. Possible differences in retinoid metabolism in cone-dominated animals have been noted in the literature, so it was of interest to explore whether these differences are tangential or fundamental. Central to this issue is whether cone-dominated animals use an isomerohydrolase (IMH)-based mechanism in the predominant pathway to 11-*cis*-retinoids. Here, it is shown that all-*trans*-retinyl esters (tREs) are the direct precursors of 11-*cis*-retinol formation in chicken retinyl pigment epithelium/retina preparations. This conclusion is based on at least three avenues of evidence. First, reagents that block tRE synthesis from vitamin A also block 11-*cis*-retinol synthesis. Second, pulse−chase experiments also establish that tREs are the precursors to 11-*cis*-retinol. Finally, 11-*cis*-retinyl-bromoacetate, a known affinity-labeling agent of isomerohydrolase, also blocks chromophore biosynthesis in the cone system.

The biosynthetic pathway on the way to 11-*cis*-retinal, the visual chromophore, is well-understood in rod-dominated animals (Scheme 1) (*1*). The core isomerization process minimally involves a two-component enzymatic system comprised of the LRAT and isomerohydrolase (IMH)[1] (*1*, *2*). LRAT is involved in the esterification of all-*trans*-retinol (vitamin A) to generate all-*trans*-retinyl esters tRE (*2*−*6*). The highly hydrophobic tREs are bound to and mobilized by RPE65 (*7*, *8*) and then processed by IMH to produce 11-*cis*-retinol (*9*−*12*). RPE65 has been shown to be a retinyl ester binding protein, whose membrane associated form shows a preference for all-*trans*-retinyl esters over all other retinoids (*8*). The unusual IMH process is in place to couple the negative free energy of hydrolysis of the ester moiety to the endothermic trans → cis isomerization (*13*, *14*). Virtually all of the experiments revealing this pathway were performed using bovine RPE. In this species, as well as in amphibians, 11-*cis*-retinoid production is limited to the RPE, with no measurable involvement of retina (*9*). As this species is rod-dominated, it was of interest to determine whether the same regeneration pathway occurs in cone-dominated animals.

Previous studies in the literature suggested that there are at least some meaningful differences in retinoid processing between rod- and cone-dominated animals (*15*−*17*). In 1992, it was reported that both chicken retina and chicken RPE were both capable of processing added all-*trans*-retinol to tREs and 11-*cis*-retinol (*15*). The isomerization activities in the RPE and retina are similar (*15*). Given the difficulty in separating retina from RPE in chicken, the problem of cross-contamination is not easily avoided here. However, it was also shown that cultured chicken Mueller cells process vitamin A to tREs, 11-*cis*-retinol, and smaller amounts of cREs (*15*). Thus, unlike in the bovine or frog systems, 11-*cis*-retinol biosynthesis in the cone-dominated chicken can at least partly occur in the retina (*15*). In addition to the locus of isomerization, there may be other biochemical events, which distinguish the chicken system from the bovine and frog systems. Two reports in the literature suggested a palmitoyl CoA dependent pathway to at least 11-*cis*-retinyl ester biosynthesis (*16*, *17*). In rod-dominated animals, retinyl ester formation occurs via LRAT in a palmitoyl CoA independent process (*4*, *5*). It seemed, therefore, important to investigate whether the predominant pathway to 11-*cis*-retinol biosynthesis might, in fact, differ in a fundamental way between rod- and cone-dominated animals (*15*−*17*). What this question is really asking is whether the regeneration pathway found in retinal Mueller cells (*15*) is any different than that described for RPE. As mentioned previously, tREs are the isomerization substrates in rod-dominated animals (*11*, *12*, *13*), so an inquiry into possible differences in regeneration pathways could start with this fact.

The central issue here is to establish the nature of the substrate in the isomerization pathway. If it turns out that tREs are the substrates for isomerization in cone-dominated animals as well, then, from a biochemical stand point, the molecular logic would be the same in the rod and cone

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[1] Abbreviations: bRCA, (3*R*)-3-[boc-lys (biotinyl)-*O*-all-*trans*-retinyl chloroacetate; BSA, bovine serum albumin; $CaCl_2$, calcium chloride; CRALBP, cellular retinaldehyde binding protein; cRBA, 11-*cis*-retinyl bromoacetate; cRE, 11-cis-retinyl ester; cROL, 11-*cis*-retinol; DMSO, dimethyl sulfoxide; DPPC, L-α-dipalmitoylphosphatidylcholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraaceticacid disodium salt; IMH, isomerohydrolase; LRAT, lecithin retinol acyltransferase; $MgCl_2$, magnesium chloride; NaCl, sodium chloride; NP-HPLC, normal phase-HPLC; REH, retinyl ester hydrolase; RPE, retinal pigment epithelium; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; tRBA, all-*trans*-retinyl bromoacetate; tRE, all-*trans*-retinyl ester; tROL, all-*trans*-retinol.

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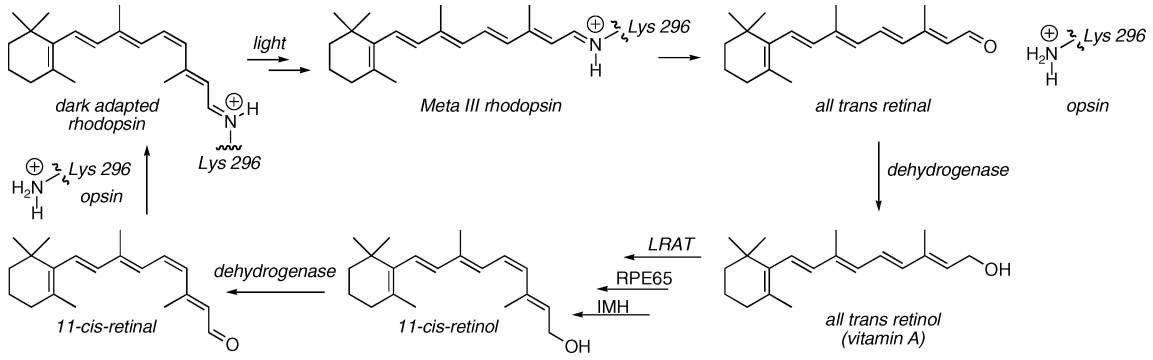
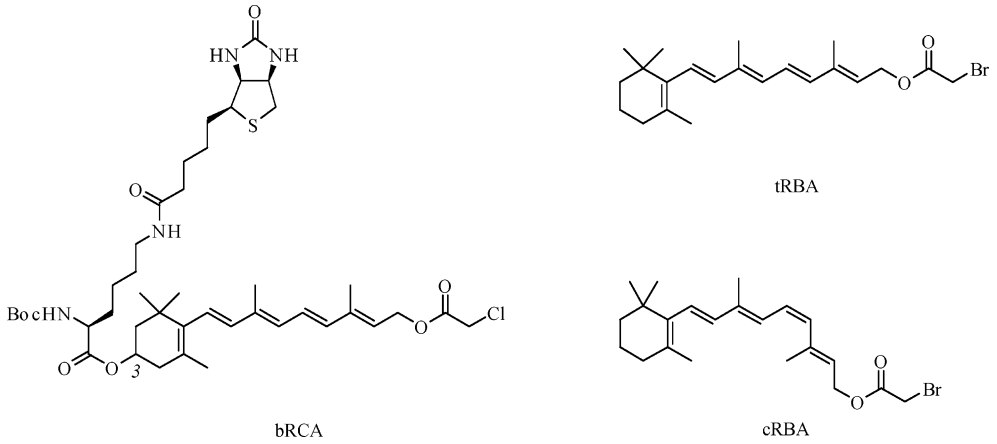
Scheme 1: Vertebrate Visual Cycle



Scheme 2: Enzyme Inhibitors Used in This Study



pathways. Straightforward approaches are available to determine the nature of the isomerization substrate. First, since tRE formation is essential for isomerization in an IMH-based process, inactivators of tRE biosynthesis (i.e., tRBA in Scheme 2) have pleiotropic effects, interfering with both tRE and 11-*cis*-retinol biosynthesis (*18*). Second, pulse−chase experiments can be performed, which reveal the nature of the substrate (*11*). In these experiments, radioactive tRE or tROL are chased with nonradioactive tRE or tROL, respectively, under conditions where the two retinoids cannot interconvert (*11*). The determination of the specific activity of the cROL that is formed reveals whether tRE or tROL is the substrate (*11*). Finally, specific inactivators of IMH would be predicted to prevent isomerization in a cone preparation only if cones possessed an IMF-like activity. A useful probe in this case is 11-*cis*-retinyl bromoacetate (cRBA, Scheme 2), which has been found to be a potent and specific affinity-labeling inactivator of IMH from bovine RPE (*19*). Using the three approaches outlined previously, we report here that the chicken RPE/retina system behaves indistinguishably from the bovine RPE IMH-based system.

MATERIALS AND METHODS

Materials

Frozen bovine eyecups devoid of retinas were purchased from W. L. Lawson Co. Fresh chicken eyes, harvested from adult (60 weeks old) white leghorn chicken, were obtained from Charles River Spafas Inc. Bovine serum albumin (BSA), ethylenediaminetetraaceticacid acid (EDTA), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), sodium chloride (NaCl), palmitoyl CoA, L-α-dipalmitoylphosphatidylcholine (DPPC), leupeptin, ebelactone A, all-*trans*-retinol, and Trizma base were from Sigma-Aldrich Co. Dithiothreitol (DTT) was from ICN Biomedicals Inc. All-*trans*-retinol [11,12-$^3H_2$] (specific activity 59.7 Ci/mmol) was purchased from Perkin-Elmer Inc. All-*trans*-retinyl bromoacetate (tRBA), 11-cis-retinyl bromoacetate (cRBA), and (3*R*)-3-[boc-lys (biotinyl)-*O*-all-*trans*-retinyl chloroacetate (bRCA) were synthesized by following the procedures described elsewhere (*19*−*21*). HPLC grade solvents were from EMD Chemicals Co. An anti LRAT antibody used for Western blots was a generous gift from Prof. Dean Bok (University of California, Los Angeles, CA). Anti-rabbit Ig-linked horseradish peroxidase and the ECL (enhanced chemiluminescence) system were purchased from Amersham Biosciences Inc. Super-Block blocking buffer was purchased from Pierce Chemical Co. The precast gels (4−20%) for SDS−PAGE and Bench-Mark prestained molecular weight markers were from Invitrogen Inc. Complete protease inhibitor cocktail was purchased from Roche Diagnostics GmbH. All reagents were analytical grade unless specified otherwise. The retinoids were analyzed on a 5 μm PVA-Sil (250 × 4.0 mm, YMC−Waters Corp) normal phase-HPLC (NP-HPLC) column using 7% dioxane in hexane as eluant at a flow rate of 1.5 mL/min. The retinyl esters were analyzed on a Microsorb (Varian Inc) 5 μm Silica (250 × 4.6 mm) with 0.4% ethyl acetate in hexane at 1.2 mL/min. Radioactivity was determined with an online IN-US β-RAM model 3 HPLC radioactivity monitor interfaced with a PC computer (Dell Optiplex GX110). All experiments using retinoids were performed in a dark room under dim red light.

Methods

Tissue Dissection and Membrane Preparation. The chicken membranes were prepared from RPE/retina, as described previously (*15*, *17*). The membranes were suspended in a storage buffer containing 10 mM Tris (pH 7.5), 1 mM EDTA, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 μM leupeptin, and 1 mM DTT and stored at −80 °C. Bovine RPE membranes were prepared as described before (*4*) and were suspended in 100 mM phosphate buffer (pH 7.5) and stored at −80 °C. For the use in SDS−PAGE experiments, the membranes were extracted in the presence of protease inhibitor cocktail.

LRAT Activity Assay. The activity of LRAT was determined by monitoring the formation of RPE- (LRAT) catalyzed retinyl esters from added all-*trans*-retinol [11,12-$^3H_2$] and DPPC (*21*). The reaction mixture (volume 1 mL) contains 100 mM Tris (pH 8.0), 370 μg of proteins, 100 μM DPPC (100 μM palmitoyl CoA for chicken retinyl ester synthetase), 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM DTT, 0.6% BSA, and 0.25 μM all-*trans*-retinol [11,12-$^3H_2$] and incubated for 10 min at room temperature. After 10 min, a 40 μL aliquot was quenched with 500 μL of methanol, 100 μL of water, and 500 μL of hexane. The amount of all-*trans*-retinyl palmitate [11,12-$^3H_2$] formed, as determined by NP-HPLC, was used as a measure of activity. Each experiment was done in triplicate, and the data points used are an average of these three points. Standard deviations were calculated and expressed as error bars.

Effects of Palmitoyl CoA and DPPC on Chicken Retinyl Ester Synthetase. Three reaction mixtures (volume 1 mL) were prepared as described previously for the LRAT assay. The first contained 100 μM palmitoyl CoA, the second contained 100 μM DPPC, and the third served as a control with no added acyl donor. These reaction mixtures were incubated for 10 min at room temperature. After 10 min, a 40 μL aliquot was quenched with 500 μL of methanol, 100 μL of water, and 500 μL of hexane. The effects of the various acyl donors were measured by determining the amounts of all-*trans*-retinyl palmitate [11,12-$^3H_2$] formed as determined by NP-HPLC. Each experiment was done in triplicate, and the data points used are an average of these three points. Standard deviations were calculated and expressed as error bars.

Bovine LRAT Inhibition Assay. The inhibition assay was performed by protocols identical to those previously described (*18*).

Effect of tRBA on Chicken IMH. When the effect of the inhibition of LRAT by tRBA on IMH was investigated, the membrane suspension was first precipitated by spinning the suspension at 120 000*g* to remove DTT. DTT interferes with thiol labeling reagents such as tRBA by reacting with them. The reaction mixture (volume 1 mL), consisting of 100 mM Tris (pH 8.0), 370 μg of proteins, 100 μM palmitoyl CoA, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.6% BSA, and 10 μM tRBA, was then incubated for 10 min at room temperature. After 10 min, 1 mM DTT and 0.25 μM all-*trans*-retinol [11,12-$^3H_2$] was added and incubated for 10 min at room temperature. Following the 10 min incubation, the IMH assay was performed by addition of 6% BSA and by heating the mixture to 37 °C for 5 min. When effects of the inhibition of LRAT by tRBA on IMH activity was investigated, the assay was performed in 100 mM Tris (pH 8.0), 370 μg of proteins, 100 μM palmitoyl CoA, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM DTT, 0.6% BSA, and 0.25 μM all-*trans*-retinol [11,12-$^3H_2$] and incubated for 10 min at room temperature. After the 10 min incubation, the membranes were centrifuged at 120 000*g* followed by resuspension in 100 mM Tris (pH 8.0), 100 μM palmitoyl CoA, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.6% BSA, and 10 μM tRBA and incubated for an additional 10 min at room temperature. After this incubation, 1 mM DTT and 6% BSA were added, and the reaction mixture was incubated at 37 °C for 5 min. After this incubation, a 40 μL aliquot was quenched by 500 μL of methanol, 100 μL of water, and 500 μL of hexane. The effects of tRBA were measured by determining the amount of 11-*cis*-retinol [11,12-$^3H_2$] formed determined by NP-HPLC. Each experiment was done in triplicate, and the data points used are an average of these three points. Standard deviations were calculated and expressed as error bars.

Effect of cRBA on Chicken IMH. The effect of 2.5 μM cRBA was investigated as described before (*19*). A DTT depleted solution of chicken membranes (see previously) was incubated with 2.5 μM cRBA for 0, 5, 10, 15, 20, 25, and 30 min at room temperature. Followed by this incubation, the 1 mM DTT was added, and the reaction mixtures were irradiated by UV light (365 nm) for 5 min to remove the unreacted cRBA. Following the irradiation, the reaction mixtures were incubated at 37 °C with 6% BSA for 5 min. After 5 min, a 40 μL aliquot was quenched with 500 μL of methanol, 100 μL of water, and 500 μL of hexane. The effects of cRBA were measured by the amount of 11-*cis*-retinol [11,12-$^3H_2$] formed as determined by NP-HPLC. Each experiment was done in triplicate, and the data points used are an average of these three points. Standard deviations were calculated and expressed as error bars.

Pulse−Chase Experiment. The assay was performed as described before (*11*), with the modification of removing DTT before incubation with the inhibitors.

Effect of BSA and Palmitoyl CoA on Chicken IMH. To investigate the effect of BSA and palmitoyl CoA on chicken IMH, four reaction mixtures were prepared. Each reaction (volume 1 mL) contained 100 mM Tris (pH 8.0), 370 μg of proteins, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.6% BSA, 0.25 μMf all-*trans*-retinol [11,12-$^3H_2$]. The reactions were differentiated as follows: (1) no BSA or palmitoyl CoA, (2) 6% BSA (no palmitoyl CoA), (3) 100 μM palmitoyl CoA (no BSA), and (4) 6% BSA and 100 μM palmitoyl CoA. Each reaction mixture was incubated for 5 min at 37 °C. At the end of this 5 min incubation, a 40 μL aliquot was quenched with 500 μL of methanol, 100 μL of water, and 500 μL of hexane. The effect of BSA and palmitoyl CoA were measured by the amount of 11-*cis*-retinol [11,12-$^3H_2$] or 11-cis-retinyl [11,12-$^3H_2$] palmitate formed as determined by NP-HPLC. Each experiment was done in triplicate, and the data points used are an average of these three points. Standard deviations were calculated and expressed as error bars.

Biotin Labeling of Chicken Membranes. In these experiments, 100 μL of membranes (DTT depleted membranes, 185 μg of total protein) was incubated with bRCA (15 μM) for 1 h at 25 °C to label the specific retinoid binding proteins. At the end of the incubation, excess reagent was removed by dialysis (slide-a-lyser cassette from Pierce-MW, cutoff of 10 kDa). The labeled sample was dissolved in 2% SDS

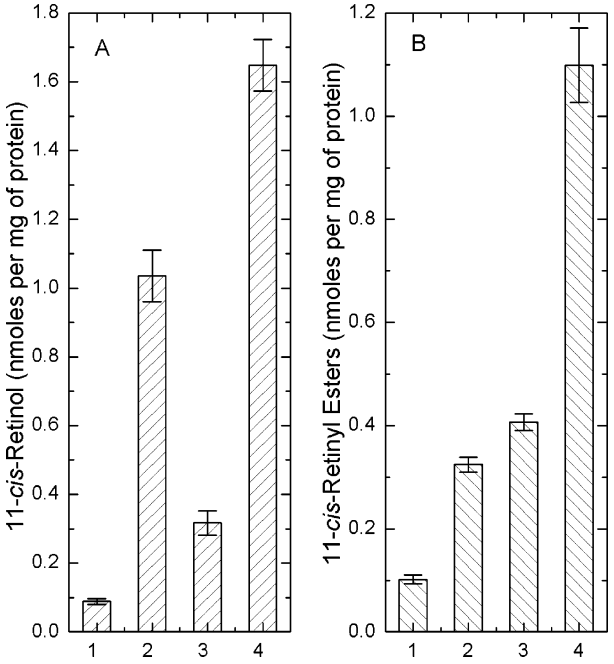


FIGURE 1: Effect of palmitoyl CoA and BSA on 11-*cis*-retinol biosynthesis. These experiments were performed as generally indicated in the Materials and Methods. Panel A shows the effect of 6% of BSA and 100 μM palmitoyl CoA on 11-*cis*-retinol synthesis. In this panel, (1) shows the data for the absence of BSA and palmitoyl CoA, (2) provides the data for the presence of 6% of BSA alone, (3) provides the data for the presence of 100 μM palmitoyl CoA alone, and (4) provides the data for the presence of 6% of BSA and 100 μM palmitoyl CoA. Panel B shows the effect of 6% of BSA and 100 μM palmitoyl CoA on 11-*cis*-retinyl ester synthesis. In this panel, (1) shows the data for the absence of BSA and palmitoyl CoA, (2) provides the data for the presence of 6% of BSA alone, (3) provides the data for the presence of 100 μM palmitoyl CoA alone, and (4) provides the data for the presence of 6% of BSA and 100 μM palmitoyl CoA.

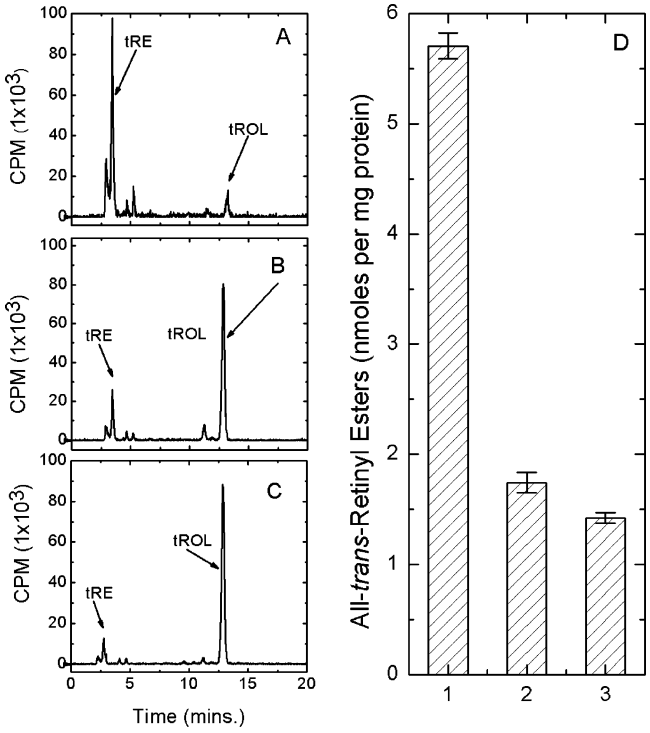


FIGURE 2: Effect of palmitoyl CoA on ester synthesis. Panel A shows the HPLC plot for ester synthesis in the presence of 100 μM palmitoyl CoA, panel B shows the HPLC plot for ester synthesis in the presence of 100 μM DPPC, and panel C shows the HPLC plot for ester synthesis in the absence of added acyl donor. In panel D, ester synthesis is compared quantitatively in the presence of 100 μM palmitoyl CoA (1), 100 μM DPPC (2), and the absence of any added acyl donor (3). Peak tRE refers to all-*trans*-retinyl esters, and tROL refers to all-*trans*-retinol.

and subjected to SDS−PAGE (4−20%) analysis with biotin detection blotting.

Western Blot Analysis. The standard Laemmili SDS−PAGE (4−20%) technique was used to separate proteins (*22*). After the transfer of the protein bands to a PVDF membrane, the nonspecific sites were blocked using Super Block blocking buffer. The PVDF membrane was washed with TBS (100 mM Tris and 150 mM NaCl) with 0.1% Tween 20. The PVDF membrane was then incubated in 10% of Super Block blocking buffer in deionized water and anti-LRAT antibody (1:4000 dilution) for 1 h at room temperature. The PVDF membrane was then incubated with anti-rabbit Ig-linked horseradish peroxidase (1:4000, 30 min). The ECL system was then used to visualize the bands.

RESULTS

Processing of Vitamin A by Chicken RPE/Retina Homogenates. In the initial experiments, radioactive vitamin A is incubated with chicken RPE/retina homogenates, and its processing to other retinoids is determined (*9*). The effects of BSA and palmitoyl CoA on 11-*cis*-retinoid production are determined. In Figure 1A, data are shown for the biosynthesis of 11-*cis*-retinol when vitamin A is incubated with chicken homogenates in the presence and absence of BSA and palmitoyl CoA. As can be seen here, BSA has a significant stimulatory effect on 11-*cis*-retinol synthesis in this system. Palmitoyl CoA has a rather smaller stimulatory effect. Previously published experiments on this system showed a stimulatory effect of CRALBP on 11-*cis*-retinol generation (*17*). The current studies reveal that the nonstereospecific retinoid binding protein BSA is also stimulatory. BSA is known to stimulate 11-*cis*-retinol formation in bovine RPE by relieving feedback inhibition of 11-*cis*-retinoids on isomerohydrolase (*23*). In addition, previous reports also described the stimulatory effect of palmitoyl CoA on the synthesis of at least 11-*cis*-retinyl esters (*16*, *17*). As can be seen in Figure 1B, we also find that palmitoyl CoA stimulates the synthesis of 11-*cis*-retinyl esters. As made clear here, this stimulation does not drive the isomerization process, but as expected, simply enhances esterification. This stimulation is not limited to the esterification of 11-*cis*-retinol, however, but is also observed in the esterification of all-*trans*-retinol (Figure 2). It is not clear whether the stimulation is due to a putative palmitoyl CoA dependent esterifying enzyme(s) (an ARAT) (*26*, *27*), or whether the stimulation is due to an indirect mechanism. Along these lines though, the addition of DPPC did not at all stimulate the synthesis of the all-*trans*-retinyl esters (Figure 2), as might be expected if esterification were due to LRAT.

PAGE-Labeling Studies on Chicken Retinyl Ester Synthetase(s). Given the results reported previously on retinyl ester synthesis in chicken RPE/retina, it was of interest to probe this enzymatic activity with reagents commonly used to label LRAT. In Figure 3 are shown Western blots on bovine RPE membrane bound LRAT and chicken RPE/retina membrane using anti-LRAT antibodies (*28*). As can be seen

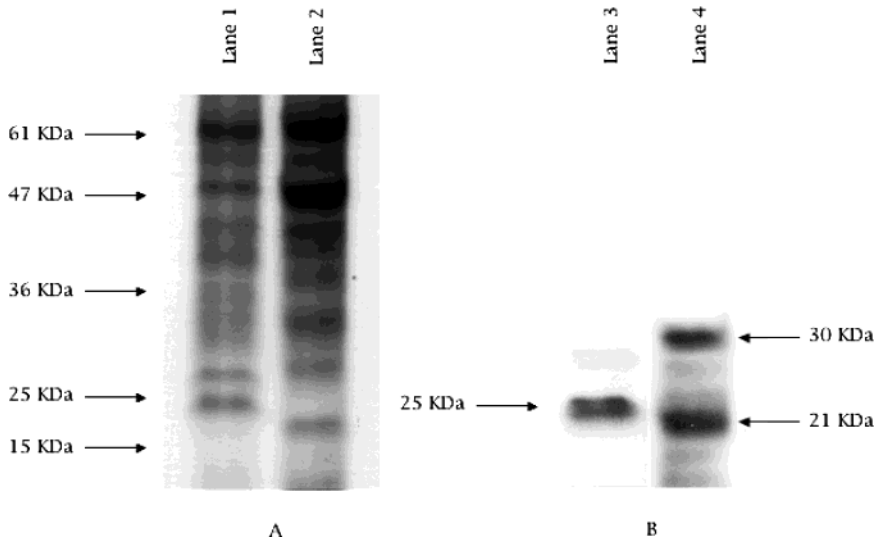


FIGURE 3: Comparison of the Western blots of chicken RPE/retina mixture with Bovine RPE. Lane 1 shows the SDS−PAGE (4−20% Tris glycine gel) of Coomassie stained bovine RPE, and lane 2 shows the SDS−PAGE (4−20% Tris glycine gel) of Coomassie stained chicken RPE/retina. Lane 3 shows the Western blot for bovine RPE with anti-tLRAT antibody. Lane 4 shows the Western blot for chicken RPE/retina mixture with anti-tLRAT antibody.

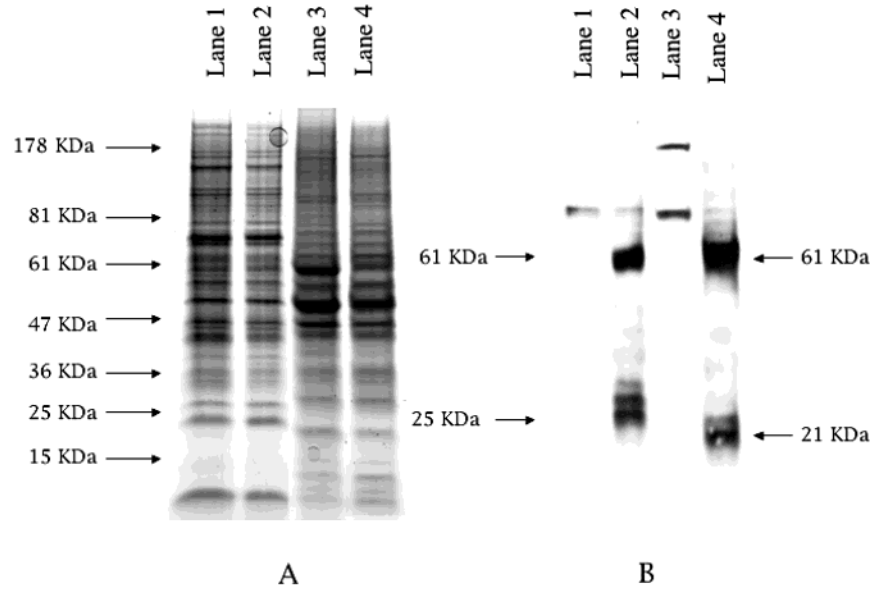


FIGURE 4: Biotin affinity labeling of bovine RPE and chicken RPE/retina. Panel A shows the Coomassie stained SDS−PAGE gel (4−20%). Panel B shows the biotin detection of labeled proteins; the lanes are the same as panel A. Lane 1 shows bovine RPE minus bRCA. Lane 2 shows bovine RPE treated with 15 μM bRCA at room temperature for 1 h. Lane 3 shows chicken RPE/retina mixture minus bRCA. Lane 4 shows chicken RPE/retina mixture treated with 15 μM bRCA at room temperature for 1 h.

here, an approximately 25 kDa LRAT is observed in the bovine system, and an approximately 21 kDa protein is observed in chicken. The fact that the chicken system shows a cross-reacting protein of approximately the same MW as bovine LRAT suggests the possibility of an LRAT-like protein in this instance as well. In a second set of experiments, the chicken RPE/retina membrane was labeled with bRCA (Scheme 2), a biotinylated affinity-labeling agent that labels LRAT, as well as other proteins (*7*). As shown in Figure 4, this reagent titrates a protein of approximately 21 kDa in the chicken system, providing a functional correlate to the Western blot results shown in Figure 3. Thus, by these criteria there may be an LRAT-like ester synthetase activity in chicken RPE/retina.

Pulse−Chase Experiments and the Nature of the Isomerization Substrate. The experiments described previously reveal that there are differences in the esterification of retinols in the bovine and chicken systems. Are their differences in the processing of the resultant retinyl esters? This question is particularly relevant to the processing of all-*trans*-retinyl esters. In rod-dominated animals, such as the cow, the substrate for isomerization is clearly all-*trans*-retinyl esters rather than vitamin A itself (*1*, *11*, *12*). To address this issue in chicken, an already established pulse−chase protocol was used (*11*). In the current experiments, chicken RPE/retina is preincubated with cold or ^{3}H-vitamin A to first allow tREs to form. Subsequently, the chicken RPE/retina system is treated with a mixture of tRBA and ebelactone A to block the interconversion of the retinyl esters and vitamin A (*11*). Finally, a chase is done with either cold vitamin A or radioactive vitamin A. If a pulse involves cold vitamin A, then the chase involves radioactive vitamin A (*11*). Since

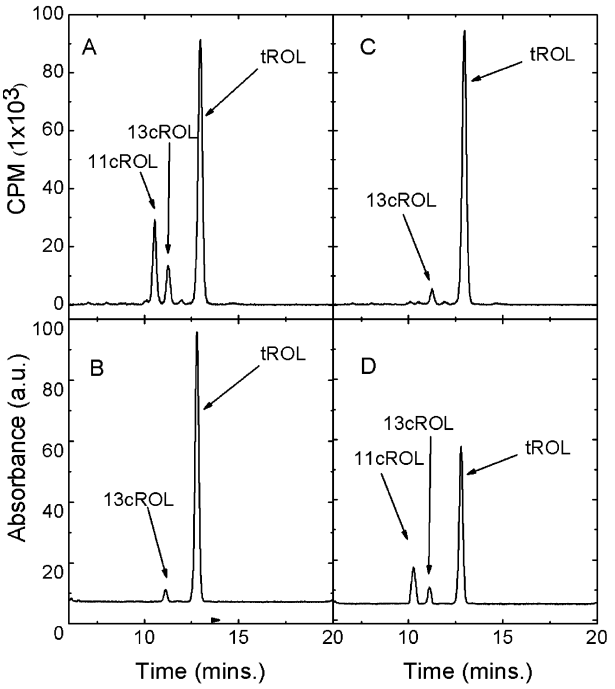


FIGURE 5: HPLC plots for pulse−chase experiments. This experiment was performed as indicated in the Materials and Methods. Low concentrations (0.1 μM) of ^{3}H all-*trans*-retinol were incubated with chicken RPE/retina membranes to generate ^{3}H all-*trans*-retinyl esters. Following this incubation, cold all-*trans*-retinol (1 μM) is added. This is followed by the initiation of an isomerization reaction in the presence of EA (100 μM) and tRBA (10 μM) as shown in panels A and B (only retinols shown). The reverse reaction is shown in panels C and D (only retinols shown); high concentrations (1 μM) of nonradioactive all-*trans*-retinol were incubated with RPE membranes to generate nonradioactive all-*trans*-retinyl esters followed by the addition of ^{3}H all-*trans*-retinol (0.1 μM). This is followed by the initiation of an isomerization reaction in the presence of EA (100 μM) and tRBA (10 μM). Panels A and C are the HPLC plots where the detector was an online scintillation counter, and panels B and D are the HPLC plots where the detector was a UV−vis detector. Peak 11cROL refers to 11-*cis*-retinol, 13cROL refers to 13-*cis*-retinol, and tROL refers to all-*trans*-retinol.

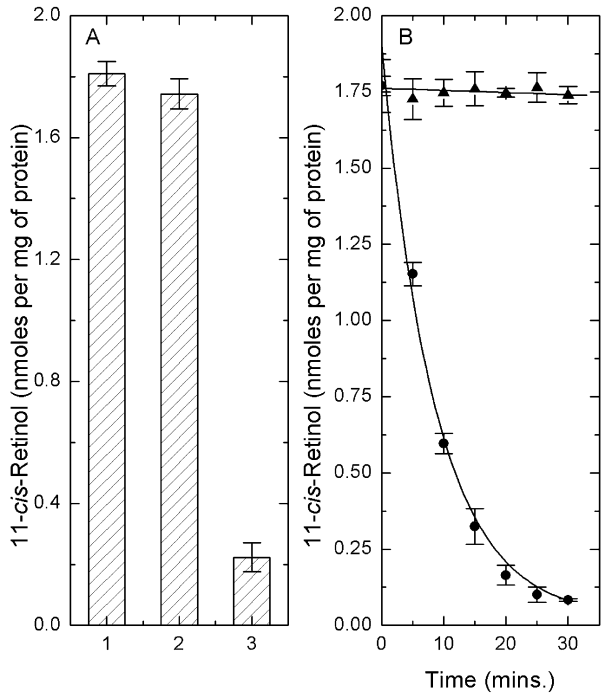


FIGURE 6: Effect of various inhibitors on the isomerization reaction. The experiments were performed as indicated in the Materials and Methods. Panel A shows the effect of tRBA (10 μM) minus tRBA control (1), when tRBA is added after the retinyl esters have formed (2), and when tRBA is added before esters are formed (3). Panel B shows the time dependent inhibition (− ● −) of the isomerization reaction with 2 μM cRBA as compared to the control (− ▲ −).-

Table 1: Change in the Specific Activity of 11-*cis*-Retinol during the Pulse−Chase Experiment[a]

Table 1A: retinoids	specific activities (mCi/mmol)
retinyl ester	161.47 (±3.48)
11-*cis*-retinol	61.71 (±2.37)
all-*trans*-retinol	14461.12 (±161.32)

Table 1B: retinoids	specific activities (mCi/mmol)
retinyl ester	50631.67 (±376.13)
11-*cis*-retinol	49645.71 (±544.42)
all-*trans*-retinol	2511.77 (±333.97)

[a] In Table 1A, the addition sequence is nonradioactive all-*trans*-retinol followed by ^{3}H-all-*trans*-retinol. In Table 1B, the addition sequence is ^{3}H-all-*trans*-retinol followed by nonradioactive all-*trans*-retinol.

the all-*trans*-retinol/all-*trans*-retinyl ester pools are isolated due to the presence of inhibitors, the determination of the specific activity of biosynthetic 11-*cis*-retinol reveals the identity of the substrate (*11*). If there is processing of all-*trans*-retinyl esters directly into 11-*cis*-retinol, then the specific activity of the all-*trans*-retinyl ester pool should correlate with the 11-*cis*-retinyl pool. Alternatively, if the converse is true, then the specific activities of vitamin A and 11-*cis*-retinol should correlate (*11*). In this particular experiment, low concentrations of ^{3}H-all-*trans*-retinol were preincubated with chicken RPE/retina membranes to generate ^{3}H-all-*trans*-retinyl esters. After treatment with tRBA (Scheme 2) to inactivate LRAT (*18*) and ebelactone A to inactivate retinyl ester hydrolysis (*11*), incubation proceeds with nonradioactive all-*trans*-retinol. The experimental results, as shown in Figure 6, are clear-cut and are interpreted in a straightforward way. It is clear that all-*trans*-retinyl esters are indeed the precursors of 11-*cis*-retinol in the chicken RPE/retina system because the generated 11-*cis*-retinol has a specific activity that reflects the ester pool (Figure 5 and Table 1A,B).

Inhibition of All-trans-retinyl Esters by tRBA and the Blockade of 11-cis-Retinol Formation. The demonstration that all-*trans*-retinyl esters are substrates for isomerization in the chicken system is confirmed by a second approach described here. If all-*trans*-retinyl esters are indeed the substrates for isomerization, then the blockade of tRE synthesis with tRBA should block the formation of 11-*cis*-retinol (*18*). Alternatively, if all-*trans*-retinol (vitamin A) is the substrate for isomerization, then the inhibition of tRE synthesis would not adversely affect the formation of 11-*cis*-retinol. In fact, if anything, inhibition of the formation of all-*trans*-retinyl esters could have the effect of enhancing the isomerization should vitamin A be the substrate because these levels would increase. tRBA is known to be a powerful active site directed inactivator of retinyl ester synthesis in bovine RPE preparations (*21*) and in chicken RPE/retina systems (*17*). In these experiments, the chicken RPE/retina system is first preincubated with ^{3}H-all-*trans*-retinol to allow for the formation of radioactive ^{3}H-tRE. Subsequent treatment of this preparation with tRBA does not diminish the

biosynthesis of 11-*cis*-retinoids significantly (Figure 6A). This is expected because tRBA should have no effect on isomerization once tREs are synthesized (*18*). On the other hand, when tRBA is preincubated with this preparation prior to the addition of vitamin A, no isomerization is observed (Figure 6A). This demonstrates that all-*trans*-retinyl ester formation is obligate in the biosynthetic pathway to the formation of 11-*cis*-retinol. If tRE biosynthesis is blocked by an inhibitor, then isomerization does not ensue. That tRBA does not in and of itself affect the isomerization process but merely acts as an inhibitor of tRE formation is clear since tRBA has no effect on isomerization when all-*trans*-retinyl esters are generated prior to the introduction of tRBA. By this approach, as well, it appears that the cone-dominated chicken RPE/retina system behaves in a way indistinguishable from the rod-dominated system.

Inactivation of Cone IMH by cRBA. In previous experiments, we had demonstrated that cRBA (Scheme 2) is an affinity-labeling agent for the IMH from bovine RPE (*19*). That being the case, it was of interest to determine if cRBA would also block isomerization in the chicken RPE/retina system. If an IMH is essential in this process as well, it would be predicted that the cRBA would indeed inactivate isomerization in the cone-dominated chicken pathway. In Figure 6B, a time course is shown for the irreversible inhibition of isomerization in the chicken RPE/retina system. As seen here, specific inactivation of isomerization occurs, just as it did in the bovine system (*19*).

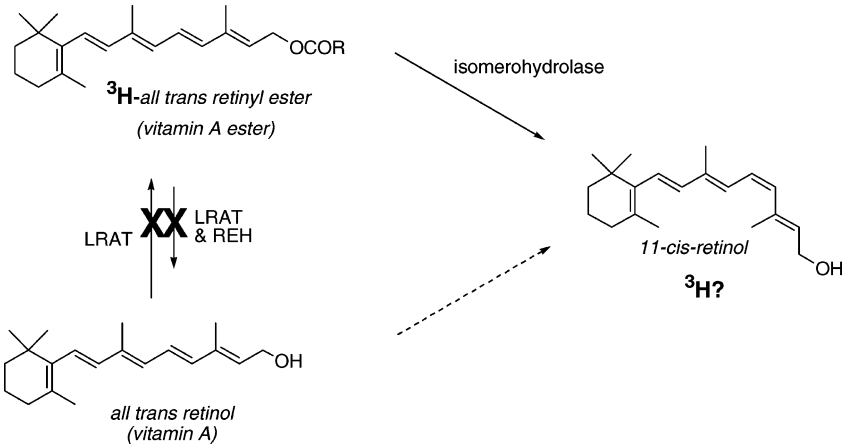
DISCUSSION

Several differences in the modes of retinoid processing in cone- versus rod-dominated animals have been reported, and some are confirmed here (*15*−*17*). The most central and novel observations made thus far comes from Gouras and co-workers, who demonstrated that 11-*cis*-retinoid regeneration can occur both in the RPE and in the retinal Mueller cells of chicken (*15*). By demonstrating that cultured Mueller cells process exogenous vitamin A into 11-*cis*-retinol (ester), the potential problem of incomplete separation of RPE/retina is avoided; thus, the study definitively demonstrates that retinal tissue in a cone-dominated animal, unlike in rod-dominate species, can synthesize 11-*cis*-retinoids (*15*). In addition, two other papers demonstrated that 11-*cis*-retinyl ester biosynthesis is stimulated by palmitoyl CoA, and it has been suggested that the retinyl ester synthetase may be an acyl CoA transferase (ARAT) rather than on an LRAT (*16*, *17*). The question we address here is whether the differences described previously between rod- and cone-dominated regeneration patterns suggests a fundamental switch in the regeneration mechanism or not. Because experiments that would biochemically probe the fundamental biochemical route to 11-*cis*-retinol formation in cones have not been reported, we undertook such an investigation. The studies reported here address these issues.

In these experiments, a chicken RPE/retina homogenate is used to investigate retinoid processing, as in a recently published study on the subject (*17*). Since the flux of retinoids through the cone retinal system is at least quantitatively equal to the flux through the cone RPE system (*15*, *17*), separately studying retina and RPE, even if possible, is not essential in the investigations described here. The incubation of this chicken RPE/retina homogenate with vitamin A confirms that vitamin A is processed into 11-*cis*-retinoids in this system (*15*−*17*). As in the case of bovine RPE, added BSA stimulates 11-*cis*-retinoid synthesis (*23*). Added CRALBP can do this as well, both in the current system (*17*) and in the bovine RPE system (*23*−*25*). The fact that BSA, which binds retinoids nonstereospecifically, is capable of this stimulation means that the stereospecificity of CRALBP is not essential for stimulation, thus eliminating CRALBP as an energy source to drive the thermodynamically uphill isomerization reaction. In confirmation of previously reported results, we also show that palmitoyl CoA stimulates 11-*cis*-retinyl ester biosynthesis (*16*, *17*). We also demonstrate that palmitoyl CoA can stimulate the biosynthesis of all-*trans*-retinyl esters as well. The stimulation of retinyl ester biosynthesis by palmitoyl CoA is not observed in the bovine RPE system, where retinyl esters are the products of LRAT (*4*). LRAT uses lecithin as an acyl donor rather than an acyl CoA (*4*). In the current studies, palmitoyl CoA is found to stimulate the formation of both cREs and tREs, suggesting the possibility of an ARAT-dependent esterification mechanism (*27*). The fact that tRE synthesis is stimulated by palmitoyl CoA also suggests that the stimulation of 11-*cis*-retinoid production might be related to the enhancement of tRE synthesis. Previously, an interesting mechanism was suggested, in which palmitoyl CoA stimulation of 11-*cis*-retinol esterification is suggested to be an important thermodynamic driving force in the endothermic trans → cis isomerization by specifically and enzymatically trapping the 11-*cis*-retinol generated (Figure 7 of ref *17*). This notion is untenable as an energy transduction mechanism for at least three reasons. First, as previous studies have clearly established (*15*, *17*), there is no specific esterification of 11-*cis*-retinol in chicken retina since all-*trans*-retinol is also esterified; thus, no specific sequestration mechanism exists. Second, as made clear here, palmitoyl CoA does not drive 11-*cis*-retinoid biosynthesis per se but only affects the partitioning of 11-*cis*-retinol to 11-*cis*-retinyl esters. Third, the scheme presented (Figure 7 of ref *17*) is self-negating because cRE is hydrolyzed to 11-*cis*-retinol in the same Mueller cell where cRE is synthesized, prior to transport of 11-*cis*-retinol to cone cells (*17*). This overall result of cRE synthesis and hydrolysis in the same cell is to remove palmitoyl CoA dependent cRE formation from consideration as an energy source to drive the isomerization process.

An interesting difference between retinal Mueller cell/RPE regeneration may be in the nature of the retinyl ester synthetase. As mentioned previously, the palmitoyl CoA stimulation of retinyl ester synthesis in chicken suggests a non-LRAT mechanism for retinyl ester synthesis. The enzyme(s) responsible for tRE synthesis in the chicken system has yet to be characterized. Nevertheless, it has been reported that it can be blocked by added tRBA (*17*), a known affinity-labeling agent for LRAT (*21*). This result is confirmed here. This inhibition, of course, does not necessarily imply that the retinyl ester synthetase in chickens operates by an LRAT mechanism. In fact, the observation that palmitoyl CoA stimulates retinyl ester synthesis, but DPPC does not, suggests the possibility of an ARAT-type mechanism of ester synthesis. However, the palmitoyl CoA results could also be explained by an indirect mechanism in which the activated CoA derivative esterifies a 2-acyllysophospho-

Scheme 3: Blockade of Vitamin A → All-*trans*-retinyl Ester Interconversion and the Substrate for Isomerization



lipid to form a pool of lecithin, which then esterifies the retinols (*26*). Results that suggest that the chicken ester synthetase is closely related to LRAT antibody cross-reactivity using anti-human LRAT antibody and biotinylated affinity reagent bRBA (*7*, *28*). Both of these reagents reveal a 21 kDa protein. This molecular weight is not too different from the 25 kDa LRAT homologues (*6*). It is assumed here, of course, that there are not multiple retinyl ester synthetases in chicken retina/RPE. Further studies are required to determine whether the chicken retinyl ester synthetase is a member of the LRAT family or not, but nevertheless, the fact that tRBA blocks tRE synthesis in chicken allows for a direct identification of the isomerization substrate.

If tRE is the isomerization substrate in chicken RPE/retina preparations, then complete blockade of its synthesis from vitamin A should abolish 11-*cis*-retinol formation. This is what is observed. It is noteworthy that it is important to achieve substantial (>90%) irreversible inhibition of LRAT with tRBA to abolish 11-*cis*-retinol biosynthesis (D. Gollapalli and R. R. Rando, unpublished experiments). This level of inhibition is apparently not possible when RPE membranes are preincubated with vitamin A and DTT before the addition of tRBA (*17*). Preincubation with vitamin A initiates esterification in the absence of inhibitor, and DTT reacts with tRBA. In the experiments reported here, tRBA is shown not to interfere with isomerization when tREs are allowed to form from vitamin A before tRBA is applied, thus demonstrating that tRBA does not interfere with isomerization per se. The blockade of 11-*cis*-retinol formation is the result of the pleiotropic effect of tRBA resulting from the inhibition of tRE synthesis. The results with tRBA are the same as observed in the bovine RPE system (*18*) and are only consistent with tREs being the isomerization substrates. Further evidence for this hypothesis comes from pulse-chase experiments.

In the version of the pulse−chase experiment shown in Scheme 3, tracer amounts of ^{3}H-all-*trans*-retinol are added to chicken homogenates under conditions where it is converted to ^{3}H-tRE. This treatment is followed by the addition of tRBA and ebelactone A to block further tRE synthesis and hydrolysis by REH. Subsequently, nonradioactive all-*trans*-retinol is added, and the isomerization reaction is allowed to proceed. A simple determination of whether the 11-*cis*-retinol formed is substantially radioactive or not decides the question of substrate. In the results described here for the chicken retina/RPE system, the substrate is clearly tRE and not vitamin A.

In the last set of experiments, chicken RPE/retina was treated with cRBA (Scheme 2), a known inactivating affinity-labeling agent of IMH (*19*). If isomerization in chicken RPE/retina occurs through an IMH-base mechanism, then cRBA would be predicted to be an inactivator of it. Alternatively, if there is another route to 11-*cis*-retinol biosynthesis in chicken RPE/retina, then cRBA would not be expected to interfere with its formation. It is shown here that cRBA does inactivate the chicken RPE/retina isomerization catalyst and thus behaves indistinguishably from IMH in this regard.

In summary, the experiments reported here show that the major pathway to 11-*cis*-retinol in the cone-dominated chicken RPE/retina is mediated by an IMH. Three main approaches are used to demonstrate this. First, the blockade of tRE formation by tRBA prevents 11-*cis*-retinol biosynthesis. Second, pulse−chase experiments are only consistent with tRE being the direct precursor of 11-*cis*-retinol. Third, cRBA, a known IMH inactivator (*19*), prevents 11-*cis*-retinol biosynthesis. Also, previous studies have clearly shown that the chicken retina and Mueller cell preparations produce copious amounts of all-*trans*-retinyl esters, the substrates for IMH (*15*). Thus, the chicken RPE/retina system appears to show the same overall molecular logic in the biosynthesis of 11-*cis*-retinol as observed in bovine RPE (*11*, *12*, *18*, *19*). In other words, the chemical reactions in the molecular pathway appear to be the same. This should not be a surprise as it would be unexpected for multiple isomerization systems to have evolved in closely related species. However, there clearly are important differences between the cone and the rod pathways, which are unrelated to the molecular logic of the overall biosynthetic pathway. For example, in 1992, it was clearly demonstrated that cone Mueller cells contain the biochemical apparatus capable of processing all-*trans*-retinyl esters into 11-*cis*-retinol and esters (*15*). Where investigated, detectable 11-*cis*-retinoid regeneration has never been found to occur in the retinas of rod-dominated species (*1*, *9*, *15*). Putative differences in the enzymology of retinyl ester synthesis between Mueller and RPE cells (*16*, *17*) deserve further study, but any differences here would not be viewed as being fundamental to the overall biosynthetic pathway.

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