All-*trans*-retinyl Esters Are the Substrates for Isomerization in the Vertebrate Visual Cycle†

Deviprasad R. Gollapalli and Robert R. Rando*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 45 Shattuck Street, Boston, Massachusetts 02115

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ABSTRACT: The identification of the critical enzyme(s) that carries out the *trans* to *cis* isomerization producing 11-*cis*-retinol during the operation of the visual cycle remains elusive. Confusion exists in the literature as to the exact nature of the isomerization substrate. At issue is whether it is an all-*trans*-retinyl ester or all-*trans*-retinol (vitamin A). As both putative substrates interconvert rapidly in retinal pigment epithelial membranes, the choice of substrate can be ambiguous. The two enzymes that effect interconversion of all-*trans*-retinol and all-*trans*-retinyl esters are lecithin retinol acyl transferase (LRAT) and retinyl ester hydrolase (REH). The retinyl ester or all-*trans*-retinol pools are radioactively labeled separately in the presence of inhibitors of LRAT and REH, effectively preventing their interconversion. Pulse-chase experiments unambiguously demonstrate that all-*trans*-retinyl esters, and not all-*trans*-retinol, are the precursors of 11-*cis*-retinol. When the all-*trans*-retinyl ester pool is radioactively labeled, the resulting 11-*cis*-retinol is labeled with the same specific activity as the precursor ester. The converse is true with vitamin A. These data unambiguously establish all-*trans*-retinyl esters as the precursors of 11-*cis*-retinol.

Dark adaptation in vertebrates depends on the enzymatic processing of all-*trans*-retinol (vitamin A) into an 11-*cis*-retinoid (*1*, *2*). The visual pigment rhodopsin contains an 11-*cis*-retinal protonated Schiff base, and the absorption of light by rhodopsin causes a *cis* to *trans* photoisomerization, initiating the phototransduction cascade (*2*−*4*). Hydrolysis of the resultant all-*trans*-retinal Schiff base and dehydrogenase-mediated reduction of the all-*trans*-retinal product results in the formation of all-*trans*-retinol in the retina (*5*, *6*). The all-*trans*-retinol is transported to the retinal pigment epithelium (RPE)[1] where it is processed into 11-*cis*-retinal and then returned to the photoreceptors (*7*, *8*) (Scheme 1).

The nature of the enzymatic machinery in the RPE responsible for the overall transformation of all-*trans*-retinol into 11-*cis*-retinal is only partially understood (*7*, *9*). Central to investigations on this problem is the nature of the substrate and product of the isomerization pathway. Since the all-*trans*- and 11-*cis*-retinoids can exist as aldehydes, alcohols, or esters, the question is not a trivial one inasmuch as there are nine possible pathways. Double labeling experiments using 15-^{14}C,15-^{3}H-all-*trans*-retinol to probe this issue demonstrated that isomerization occurred at the alcohol oxidation state, eliminating a retinal isomerase from consideration (*10*).

Retinyl ester formation in the RPE membrane occurs via the esterification of all-*trans*-retinol by lecithin retinol acyl transferase (LRAT) (*11*−*14*). LRAT rapidly and reversibly transfers an acyl group from the *sn*-1 position of lecithin to all-*trans*-retinol, forming an all-*trans*-retinyl ester (largely palmitate) and lysophospholipids (*15*). Deciding between retinol and retinyl esters as possible substrates and products is not straightforward because these retinoids interconvert on a more rapid time scale than isomerization (*16*, *17*).

To address the issue of substrate and product for the isomerization, a specific inactivator of LRAT, all-*trans*-retinyl bromoacetate (RBA), was utilized (*18*). RPE membranes preincubated with RBA are unable to process added all-*trans*-retinol into all-*trans*-retinyl esters and 11-*cis*-retinoids, while untreated RPE membranes can carry out both conversions (*19*). Importantly, RBA does not inhibit the processing of all-*trans*-retinyl esters into 11-*cis*-retinoids, showing that RBA does not block the formation of an unknown and hypothetical intermediate on the isomerization pathway (*19*). The straightforward conclusion from these experiments is that RBA blocks isomerization because it blocks the formation of all-*trans*-retinyl esters (*19*). Moreover, 11-*cis*-retinol, but not 11-*cis*-retinyl esters, were formed when RBA was added subsequent all-*trans*-retinyl ester formation, demonstrating that 11-*cis*-retinol is the direct product of isomerization. These, and similar studies (*20*), indicated that all-*trans*-retinyl esters are the substrates on the isomerization pathway and that 11-*cis*-retinol is the product of isomerization. Scheme 1 depicts the overall visual cycle showing the conversion of all-*trans*-retinyl esters into 11-*cis*-retinol (*21*). This unusual isomerohydrolase mechanism is doubtless in place to provide an energy source (ester hydrolysis) to drive the thermody-

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* To whom correspondence should be addressed. Phone: (617) 432-1794. Fax: (617) 432-0471. E-mail: robert_rando@hms.harvard.edu.

[1] Abbreviations: BSA, bovine serum albumin; CRALBP, cellular retinaldehyde binding protein; DMSO, dimethyl sulfoxide; DPPC, L-dipalmitoylphosphatidylcholine; EA, ebelactone; LRAT, lecithin retinol acyl transferase; REH, retinyl ester hydrolase; RBA: all-*trans*-retinyl bromoacetate; RPE, retinal pigment epithelium; a.u., arbitrary units.

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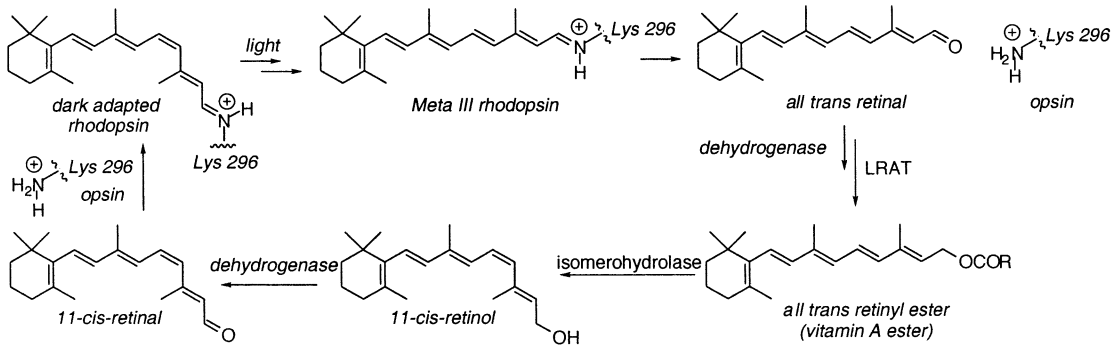
Scheme 1: The Mammalian Visual Cycle

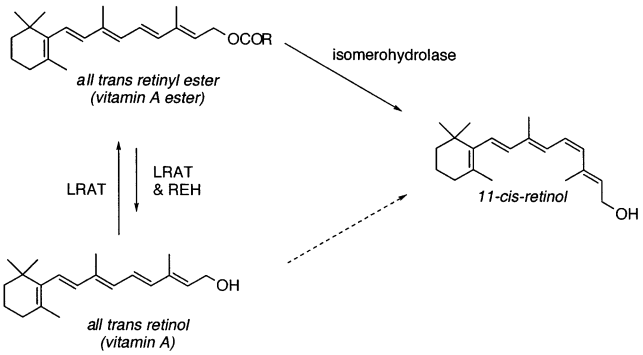


namically uphill (4.1 kcal/mol) *trans* to *cis* isomerization (*21*, *22*).

Recently, an article was published suggesting that all-*trans*-retinol itself is the isomerization substrate rather than an all-*trans*-retinyl ester (*23*). In most ways this article happily confirmed already published data. For example, the article (*23*) confirmed the previous reports (*19*) that 11-*cis*-retinol is the direct product of isomerization and that retino(a)l binding proteins stimulate 11-*cis*-retinol production (*16*, *17*). A later article (*24*) from the same group also confirmed the previous report that C−O bond cleavage occurs concomitant with isomerization (*22*). Thus, the results from the earlier (*23*) and more recent experiments (*24*) are essentially concordant with previous work (*7*, *19*, *22*), save for the issue of the substrate for isomerization. This point though is an important one because ester hydrolysis is considered to be the energy source that drives the endergonic isomerization reaction in one mechanism (*22*). No identified energy source exists if an all-*trans*-retinol to 11-*cis*-retinol conversion were to occur. Previously described 11-*cis*-retinoid binding protein candidates are not relevant here. For example, the availability of opsin to bind 11-*cis*-retinal clearly is not essential because isomerization to 11-*cis*-retinoids proceeds in the RPE in the absence of the retina and photoreceptors (*16*). The specific 11-*cis*-retino(a)l binding protein CRALBP (*25*, *26*), found in the RPE, is pre-understood (*17*) to not be an essential driving force for isomerization because isomerization proceeds in its absence in vitro (*17*), and CRALBP knockout mice can still synthesize 11-*cis*-retinoids (*29*), albeit slowly. The slower in vivo kinetics are understandable because CRALBP, and even nonspecific retinoid binding proteins such as BSA, simply enhance the rate of isomerization in vitro by relieving feed-back inhibition (*17*). Given these various issues, an important question to address is what is the experimental evidence presented in support of all-*trans*-retinol as the isomerization substrate?

This view, which is frequently quoted (*26*−*40*), is dependent on a single experiment, which is reported in Figure 8 (*23*). The approach is based on a pulse-chase protocol in which RPE membranes are pretreated with nonradioactive all-*trans*-retinol to form a pool of nonradioactive all-*trans*-retinyl esters (*23*). A pulse-chase experiment is then performed with ^{3}H-all-*trans*-retinol as the chase (*23*). The basis of the experiment lies in temporally correlating the specific activities of the all-*trans*-retinyl ester/all-*trans*-retinol pools with the specific activities of the enzymatically formed 11-*cis*-retinol (*23*). In the experiment as designed, several competing processes occur (Scheme 2). The all-*trans*-retinyl

Scheme 2: Retinoid Processing in the RPE



esters and all-*trans*-retinol rapidly interconvert via the combined enzymatic action of LRAT and REH (*41*). Concomitantly, isomerization occurs via either the all-*trans*-retinyl ester or the all-*trans*-retinol substrates. Superficially, the prediction is that if all-*trans*-retinyl esters were the substrates for isomerization, then the specific activities of the product 11-*cis*-retinol would be initially zero and then exponentially increase with time as the specific activity of the all-*trans*-retinyl ester pool increased because of the LRAT-mediated esterification of ^{3}H-all-*trans*-retinol (*23*). Should all-*trans*-retinol be the substrate, the prediction is that the specific activity of the 11-*cis*-retinol generated during the course of the experiment would initially be identical to that of the added ^{3}H-all-*trans*-retinol and then suffer exponential decay as the retinoid pools mix (*23*). In the actual reported experiment (Figure 8, ref *23*), a small rise in the specific activity (<2-fold) is measured in the all-*trans*-retinyl ester pool during the time interval studied, while the specific activities of the 11-*cis*-retinol appeared to remain essentially constant during this time interval and beyond (*23*). Surprisingly, the experiment was interpreted to mean that all-*trans*-retinol is the isomerization substrate (*23*). There are at least three reasons, both conceptual and practical, why the experiment does not lend support to this conclusion. First, on purely kinetic grounds, a linear correlation between the rate of specific activity change in the all-*trans*-retinyl ester pool and resultant 11-*cis*-retinol would only be expected to occur if isomerization is rapid as compared to LRAT action. However, retinyl ester formation is known to be considerably more rapid than isomerization (*7*, *9*). In fact, the experimental results reported in Figure 8 (*23*) show that isomerization is the slow step because no statistically significant change in the specific activity of the 11-*cis*-retinol is reported. Second, on practical grounds, the enormous standard deviations reported in the measurements of the specific activities of the

11-*cis*-retinol product (Figure 8C, ref *23*) actually exceed the small measured differences in the specific activities of all-*trans*-retinyl ester pool over the equivalent time intervals. In other words, the under-determined, and statistically insignificant, data presented could just as well have been taken as support for all-*trans*-retinyl esters being the isomerization substrates. Finally, one curious piece of data was reported, which appeared to be germane, was left uninterpreted. The reported absolute values of the specific activities for generated 11-*cis*-retinol were essentially identical to that of the all-*trans*-retinyl ester pool but were approximately 5-fold lower than that of the putative ^{3}H-all-*trans*-retinol pool at all time points investigated (*23*). This result, of course, suggests that all-*trans*-retinyl esters are the isomerization substrates.

The pulse-chase approach described above is essentially problematic because of the rapid interconversion of retinyl esters and retinols relative to the isomerization rate. Had nonisomerization related processing of the all-*trans*-retinol/all-*trans*-retinyl esters been prevented, then pulse-chase experiments of this type could yield useful insights. In the work reported here, inhibitors are used that prevent the processing of all-*trans*-retinol and esters in nonisomerization related events. In this way, the all-*trans*-retinyl ester and all-*trans*-retinol pools are relatively stable and can be specifically labeled in the absence of significant interconversion. It then becomes a simple matter to determine which retinoid is converted into 11-*cis*-retinol. The results are unambiguous and demonstrate that all-*trans*-retinyl esters are the isomerization substrates and not all-*trans*-retinol.

MATERIALS AND METHODS

Materials. Frozen eye cups devoid of retinas were purchased from W. L. Lawson Co., Lincoln, NE. [11,12-$^3H_2$]-All-*trans*-retinol (specific activity 59.5 Ci/mmol) was obtained from Perkin-Elmer Life Sciences. Sodium borohydride [^{3}H] (specific activity 85 Ci/mmol) was obtained from American Radiolabeled Chemical Inc. 11-*cis*-Retinol (specific activity 20.7 Ci/mmol) and RBA were prepared according to published procedure (*19*). 11-*cis*-Retinal was obtained through the National Eye Institute. All-*trans*-Retinol, α-dipalmitoylphosphatidylcholine (DPPC), DTT, EDTA, ebelactone A, ebelactone B, and fatty acid-free BSA were purchased from Sigma-Aldrich Inc. HPLC-grade solvents were purchased from J. T. Baker. All experiments were conducted under dim red light unless otherwise noted.

Preparation of Isomerohydrolase-Containing Membranes. The procedure for preparation of bovine RPE membranes is described elsewhere (*12*). Prior to use, the membranes were irradiated with UV light (365 nm) on ice for 5 min to destroy endogenous retinoids (*17*). Protein determinations were performed using the Bradford protein assay (*42*) yielding a final protein concentration of 1.47 mg/mL.

LRAT Activity Assay. The activity of LRAT was determined by monitoring the formation of RPE- (LRAT) catalyzed retinyl esters from all-*trans*-retinol or 11-*cis*-retinol and dipalmitoylphosphatidylcholine (DPPC). All-*trans*-retinol [11,12-$^3H_2$] was dissolved in dimethyl sulfoxide (DMSO) to prepare a 20 μM stock solution. A total of 1 μL of all-*trans*-retinol [11,12-$^3H_2$] in dimethyl sulfoxide (DMSO) from this stock solution was added to a 100 μL total buffer volume containing membrane suspension, DPPC, and BSA. The final concentrations of the components in the reaction mixture are 100 mM Tris-HCl at pH 8.0, 200 μM DPPC, 0.6% BSA, 58.8 μg of protein, and 0.2 μM of all-*trans*-retinol. The reactions were performed at room temperature. The reactions were quenched after 45 min by the addition of 500 μL of methanol, after which 100 μL of H_2O was added, and 500 μL of hexane (containing butylated hydroxy toluene at 1 mg/mL) was added to effect extraction of the retinoids. The retinoids were analyzed on a 5-μm PVA-Sil (250 × 4.0 mm, YMC) HPLC column using 7% dioxane in hexane as eluant at a flow rate of 1.5 mL/min with an online IN-US β-RAM model 3 HPLC scintillation counter interfaced with an IBM computer (Dell Dimension GX110). All experiments were performed in triplicate, the average values of these measurements were used for analysis, and the standard deviation was calculated.

Isomerohydrolase Assay. The method used is identical to the published procedures (*17*). In this procedure, the amount of 11-*cis*-retinol is determined as a function of time from added all-*trans*-retinol in the presence of BSA to bind the retinoids.

REH Assay. To isolate the REH activity from isomerohydrolase activity an assay similar to isomerohydrolase was established. 11-*cis*-Retinol [15-^{3}H] was converted to its ester at room temperature followed by its hydrolysis at 37 °C. 11-*cis*-Retinol [15-^{3}H] was dissolved in dimethyl sulfoxide (DMSO) to prepare a 50 μM stock solution. A total of 3 μL of 11-*cis*-retinol [15-^{3}H] in dimethyl sulfoxide (DMSO) from this stock solution was added to a 1 mL total buffer volume containing membrane suspension, DPPC, and BSA. The final concentrations of the components in the reaction mixture are 100 mM Tris-HCl at pH 8.0, 200 μM DPPC, 0.6% BSA, and 0.15 μM 11-*cis*-retinol. The reactions were performed at room temperature. A 100 μL aliquot of the reaction was quenched after 60 min by the addition of 500 μL of methanol, after which 100 μL of H_2O was added, and 500 μL of hexane (containing butylated hydroxy toluene at 1 mg/mL) was added to effect extraction of the retinoids. The REH was then continued at 37 °C in the presence of 6.6% BSA, such that the final concentration of the solution was 0.06 μM of retinoids, 80 μM of DPPC, and 100mM of Tris-HCl at pH 8.0. The reaction was allowed to proceed for 45 min and then quenched by the addition of 600 μL of methanol, after which 100 μL of H_2O was added, and 500 μL of hexane (containing butylated hydroxy toluene at 1 mg/mL) was added to effect extraction of the retinoids.

REH Inhibition Assay. The ability of three inhibitors, ebelactone A (100 μM), ebelactone B (100 μM), and RBA (10 μM), to inhibit REH was established by an assay similar to the one reported above. Membranes suspended in a buffered solution were incubated with 11-*cis*-retinol [15-^{3}H] to allow retinyl esters to form. After 45 min, these membranes were treated with the inhibitors for 15 min. After the incubation, the REH assay was allowed to proceed as described above. The effect of these inhibitors on LRAT was verified by adding these inhibitors to a buffered solution of the membranes followed by the addition of 11-*cis*-retinol [15-^{3}H]. The assay was then continued as described above, and in both cases the amount of 11-*cis*-retinol formed was used as a measurement of REH activity.

Isomerohydrolase Inhibition Studies. The effect of ebelactones A and B (100 μM) and RBA (10 μM) on isomerohydrolase was performed by the procedure described above. An initial buffered membrane solution (100 mM Tris/HCl pH 8.0 and 81.2 μg of protein) was prepared in Pyrex vials. The membranes were then incubated with the various inhibitors for 15 min. The subsequent assay was performed as indicated for the isomerohydrolase assay, and the amount of 11-*cis*-retinol formed was used as a measurement of isomerohydrolase activity. In the case of RBA, the retinyl esters were allowed to form before the membrane solutions were treated with RBA. The isomeric retinols were analyzed as previously published (*17*). All experiments were performed in triplicate, and the average values from these measurements were used for analysis.

Finally, it was important to access the possible effects of DMSO on isomerohydrolase activity inasmuch as DMSO is used as a carrier solvent for all the inhibitors at a final concentration (14 μM). The control experiments show little or no effect of DMSO on isomerohydrolase activity at a concentration of 14 μM.

Pulse-Chase Isomerohydrolase Assay. To establish that all *trans*-retinyl ester is the substrate for isomerohydrolase, a pulse-chase experiment similar to the one reported before (*23*) was done. An initial buffered membrane solution (100 mM Tris/HCl pH 8.0 and 212.6 μg of protein) was prepared. The membrane suspension was aliquoted in two parts, and the first part was incubated with EA (100 μM) for 15 min, and the second part was left on ice. After the incubation with EA, 0.85 μM all-*trans*-retinol was added to initiate the LRAT reaction. After 60 min, 0.09 μM all-*trans*-retinol [11,12-$^3H_2$] was added, and an aliquot was quenched to verify the equal addition of all-*trans*-retinol [11,12-$^3H_2$]. The EA fraction was then incubated with 10 μM RBA for 15 min on ice. Excess RBA was removed by centrifugal pelleting of the membranes (*17*). After this incubation, an assay was performed on the two parts, and the amount of 11-*cis*-retinol formed was used as a measurement of an activity. Also, the specific activity of retinyl ester, 11-*cis*-retinol and all-*trans*-retinol fractions were measured. The specific activity was calculated by the equation given below:

$$\mathrm{sa} = \frac{\mathrm{CPM_Y}}{2.22 \times 10^9 \times [\mathrm{Y}] \times \epsilon}$$

where sa is the specific activity (mCi/mmol);CPM is the counts per minute; [Y] is the concentration of substance (mmol); and ϵ is the efficiency of the scintillation counter.

The radioactivity disintegrations per minute were measured in a Beckmann Coulter LS-6500 liquid scintillation counter. The liquid scintillation fluid used was Ultima Gold from Perkin-Elmer Life Sciences. The concentration of the individual retinoids was measured in a Perkin-Elmer Lambda 20. The ϵ values used to calculate the concentration of the retinoids were taken from the literature (*45*).

Each experiment was performed in triplicate, the average values were used, and the standard deviation was calculated. The reverse experiment was also performed, where the initial all-*trans*-retinol added was all-*trans*-retinol [11,12-$^3H_2$], followed by all-*trans*-retinol.

RESULTS

Inhibition of REH by Ebelactones A and B. As described above, a pulse-chase experiment in which all-*trans*-retinyl esters and all-*trans*-retinol are interconvertable on a time scale that is faster than isomerization is bound to produce ambiguous results. However, the experimental approach might be cogent if the interconversions of all-*trans*-retinol and the all-*trans*-retinyl ester pools were prevented. There are two biochemical reactions to be concerned with here. First, there is the LRAT reaction, in which all-*trans*-retinol is reversibly esterified to all-*trans*-retinyl esters (*7*−*9*). The specific blockade of this enzyme can be readily managed with RBA, which irreversibly inactivates LRAT by an affinity-labeling mode and blocks the esterification of all-*trans*-retinol into all-*trans*-retinyl esters in RPE membrane preparations (*19*). While it is possible to prevent LRAT action in the presence of RBA, RBA does not appear to block the hydrolysis of all-*trans*-retinyl esters by the REH(s) found in the RPE membrane (*19*). REH activity is the second enzymatic activity that needs to be inhibited to carry out the modified pulse-chase experiment suggested above. The ebelactones (A and B) are simple small molecules known to inhibit a wide assortment of esterases (*43*, *44*). As shown in Figure 1, ebelactone A (EA) and B are capable of strongly inhibiting retinyl ester hydrolysis in RPE membranes. Figure 1 shows data on the hydrolysis of all-*trans*- and 11-*cis*-retinyl esters by EA and EB. These experiments were performed by pretreating RPE membranes with ^{3}H-all-*trans*-retinol, allowing it to be processed into all-*trans*-retinyl esters (*7*−*9*). In the presence of 10 μM RBA, these membranes process all-*trans*-retinyl esters into all-*trans*-retinol (in red) by hydrolysis, and 11-*cis*-retinol (in blue) by isomerohydrolase action (*19*). As seen in this Figure 1A, EA and EB at 100 μM concentrations inhibit the hydrolysis of all-*trans*-retinyl esters (Figure 1A) and 11-*cis*-retinyl esters (Figure 1B). In both instances, the retinol concentrations are substantially lower than in the control (no inhibitors) and very much lower than in the RBA treated membranes. Also note that the all-*trans*-retinol remaining after preincubation with RPE membranes has not been subtracted, and so very little all-*trans*-retinol is actually produced as a consequence of all-*trans*-retinyl ester hydrolysis here. In addition, 11-*cis*-retinol also is thermally isomerized to all-*trans*-retinol under the conditions of these experiments, increasing the latter pool to some extent (*22*). The experiments shown in Figure 1 demonstrate that EA and EB can significantly decrease the amount of ester hydrolysis. The inhibition of REH appeared more pronounced in the 11-cis series, but this is only because of the simultaneous isomerohydrolase reduction in the all-*trans*-retinyl esters. Finally, the ebelactones had very little affect on the isomerization reaction. In order for ebelactones to be useful in our experiments, it is important that they not interfere with the isomerization reaction. The experiments described in Figure 1 determined the outcome of inhibitor treatment at a single time point. To further establish the utility of the inhibitors, kinetic experiments were performed with EA in combination with RBA.

Isomerization in the Presence and Absence of EA and RBA. In Figure 2 are shown data on the time dependence of isomerization in the absence of inhibitors (Figure 2A), in the presence of EA (100 μM) (Figure 2B), and in the

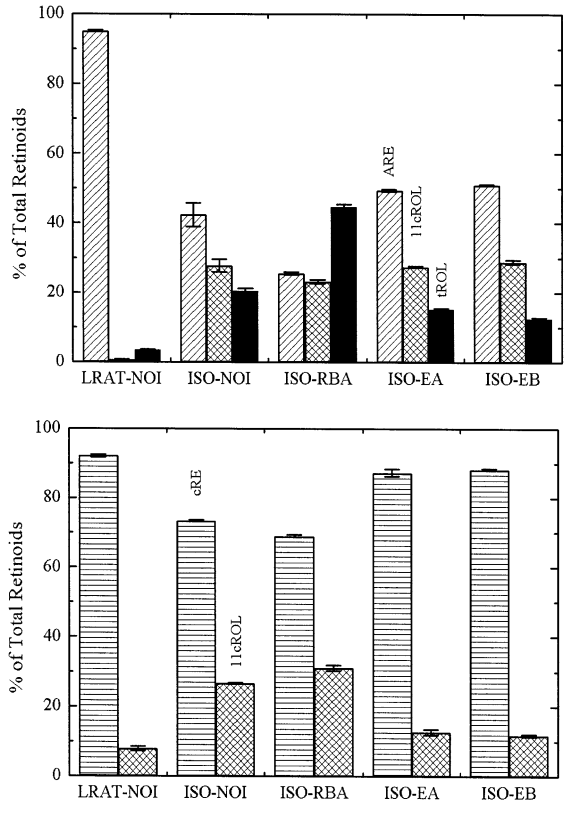


FIGURE 1: Inhibition of REH by various inhibitors. Panel A, where the substrate is ^{3}H all-*trans*-retinol, shows the LRAT reaction (LRAT-NOI) in the presence of no inhibitors, the isomerohydrolase reaction in the presence of no inhibitors (ISO-NOI), in the presence of 10 μM RBA (ISO-RBA), in the presence of 100 μM EA (ISO-EA), and ebelactone B (ISO-EB). Panel B, where the substrate is ^{3}H 11-*cis*-retinol, shows the LRAT reaction (LRAT-NOI) in the presence of no inhibitors, the isomerohydrolase reaction in the presence of no inhibitors (ISO-NOI), in the presence of 10 μM RBA (ISO-RBA), in the presence of 100 μM EA (ISO-EA), and ebelactone B (ISO-EB). Column ARE refers to all-retinyl esters; cRE refers to 11-*cis*-retinyl esters; 11cROL refers to 11-*cis*-retinol; and tROL refers to all-*trans*-retinol.

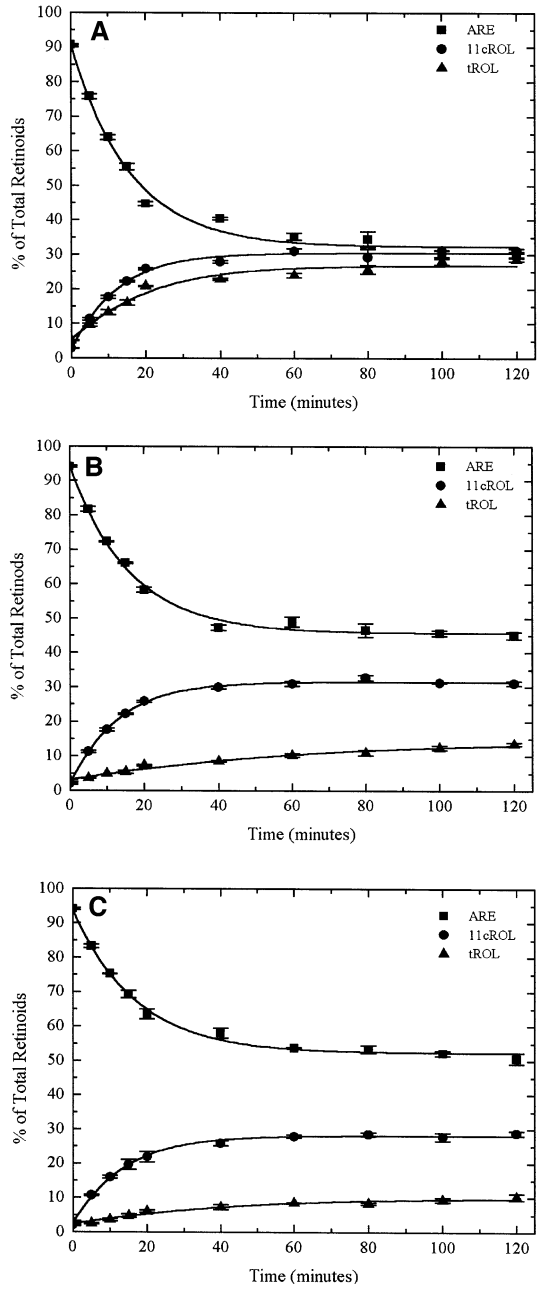


FIGURE 2: Effect of EA and RBA on isomerohydrolase/REH. These experiments were performed as indicated in the Materials and Methods section. Panel A shows a time course for isomerohydrolase/REH in the presence of no inhibitors. Panel B shows a time course for isomerohydrolase/REH in the presence of 100 μM EA. Panel C shows a time course for isomerohydrolase/REH in the presence of 10 μM RBA and 100 μM EA. Curve ARE refers to all-retinyl esters; 11cROL refers to 11-*cis*-retinol; and tROL refers to all-*trans*-retinol.

presence of both EA (100 μM) and RBA (10 μM) (Figure 2C). These experiments were performed by initially preincubating RPE membranes with ^{3}H-all-*trans*-retinol, which is almost quantitatively converted into ^{3}H-all-*trans*-retinyl esters. This experiment is also performed in the absence of retinoid binding proteins, such as BSA, because isomerization either does not proceed or ensues at an exceedingly slow rate under these conditions (*17*). Incubation with retinoid binding proteins initiates the isomerization reaction (*17*). In Figure 2A is shown the standard data for the processing of all-*trans*-retinyl esters into mixtures of 11-*cis*-retinol and all-*trans*-retinol. The all-*trans*-retinol arises largely from the hydrolysis of the all-*trans*-retinyl esters by REH (*41*). In Figure 2B, the same kind of experiment is shown but in the presence of EA. Here, one can see that 11-*cis*-retinol formation is hardly affected, while the hydrolysis reaction is strongly diminished. That is, under conditions where the isomerization reaction is largely complete there is less than 10% all-*trans*-retinol found. Figure 2C shows the same time-course experiment but in the presence of mixtures of EA (100 μM) and RBA (10 μM). Here again, it can be seen that all-*trans*-retinol is not substantially produced, while 11-*cis*-retinol is still generated. There is a very minor amount of inhibition of isomerization by RBA as has been noted before (*19*).

Pulse-Chase Experiments in the Presence of EA and RBA. The experiments described above show that it is possible to inhibit the reversible LRAT reactions and ester hydrolysis while still maintaining the isomerization capacity of RPE membranes. Therefore, it is possible to carry out a pulse-chase type of experiment under conditions where interconversion of the all-*trans*-retinyl esters and all-*trans*-retinol are blocked. Under these conditions, it should be possible to unequivocally ascertain whether all-*trans*-retinyl esters or all-

trans-retinol are the substrates in the isomerization pathway. The experiments are performed in two ways. In the first set of experiments, a relatively high concentration (0.85 μM) of nonradioactive all-*trans*-retinol, set at approximately 3-fold higher than the K_M of LRAT for all-*trans*-retinol (*46*), are incubated with RPE membranes to generate nonradioactive all-*trans*-retinyl esters. This is followed either by control experiments (inhibitors) or the addition of EA (100 μM) and RBA (10 μM) for 15 min. The membranes are washed to remove inhibitors and then incubated with trace levels of radioactive ^{3}H all-*trans*-retinol (0.09 μM) for 45 min. At the end of this time, analysis was performed on the isomerization and esterification reactions. If isomerization occurs via all-*trans*-retinol as the substrate, then the resultant 11-*cis*-retinol should have a specific activity close to that of the starting radioactive all-*trans*-retinol. Alternatively, if isomerization occurs via the isomerohydrolase pathway, then the resultant 11-*cis*-retinol should have a very low specific activity since there should be very little radioactivity in the all-*trans*-retinyl ester pool. The experiment is also performed in the opposite way by pretreating the membranes with radioactive ^{3}H-all-*trans*-retinol, followed by the addition of nonradioactive all-*trans*-retinol. In this experiment, the resultant 11-*cis*-retinol formed will have a negligible specific activity if isomerization occurs via an all-*trans*-retinol to 11-*cis*-retinol transformation and a very high specific activity if isomerization occurs through the isomerohydrolase pathway with all-*trans*-retinyl ester as substrate. HPLC diagrams from these experiments are found in Figure 3 (nonradioactive followed by radioactive) and Figure 4 (radioactive followed by nonradioactive). In Figure 3 are shown a HPLC chromatogram and radiograms for the experiment in which the RPE membranes are pretreated with nonradioactive all-*trans*-retinol and then chased with ^{3}H-all-*trans*-retinol for a period of 45 min. Figure 3A shows the LRAT reaction at the start of the reaction where virtually all of the radioactive material resides in the all-*trans*-retinol pool, while the bulk retinoid resides in vitamin A ester peaks. In Figure 3B are shown data for the isomerization reaction in the absence of inhibitors. Here, it can be observed that ^{3}H-all-*trans*-retinol and ^{3}H-retinyl esters are observed, along with ^{3}H-11-*cis*-retinol. This is the expected result because the all-*trans*-retinyl esters and all-*trans*-retinol pools rapidly equilibrate in the absence of added inhibitors. The arrow indicates the formation of some 13-*cis*-retinol, which probably is formed through the isomerization of 11-*cis*-retinol. In these experiments, the formation of this product can be strongly diminished when CRALBP is used as a binding protein, rather than BSA, which is used here (*17*, *23*, *24*). Figure 3C shows the chromatograms of the experiment when the RPE membranes are incubated with RBA (10 μM) along with EA (100 μM) to prevent interconversion of all-*trans*-retinol and all-*trans*-retinyl esters. Here, the results are clear-cut. The 11-*cis*-retinol that forms has basically no radioactive component, in other words, isomerization proceeds from the nonradioactive substrate, which in this case is, of course, all-*trans*-retinyl ester.

The modified pulse-chase experiments were also performed in the reverse order. Here, the RPE membranes are preincubated with radioactive ^{3}H-all-*trans*-retinol, followed by a chase with nonradioactive all-*trans*-retinol. In this instance, the all-*trans*-retinyl ester pool is highly radio-

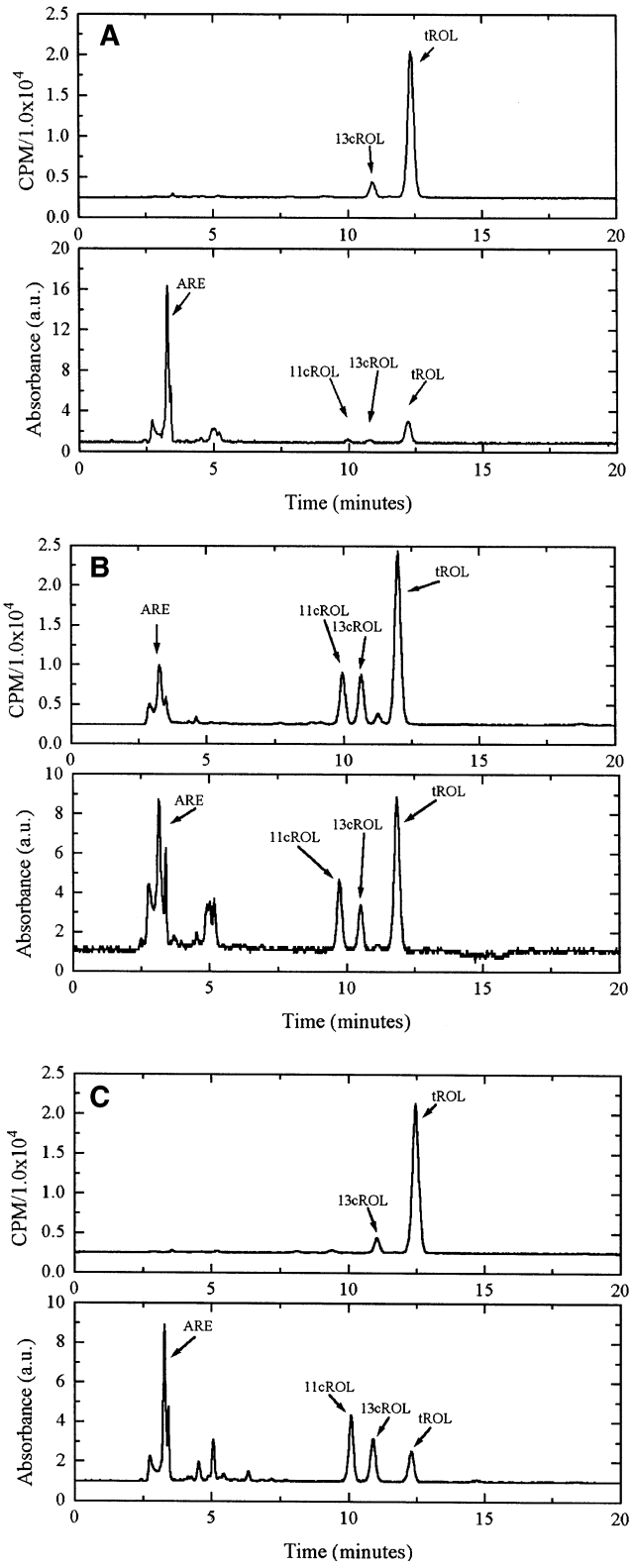


FIGURE 3: HPLC plots for the pulse-chase experiment. This experiment was performed as described above, high concentrations (0.85 μM) of nonradioactive all-*trans*-retinol were incubated with RPE membranes to generate nonradioactive all-*trans*-retinyl esters followed by the addition of ^{3}H all-*trans*-retinol (0.09 μM) as shown in panel A. This is followed by the initiation of isomerohydrolase/REH reaction either in the presence of no inhibitors as shown in panel B or in the presence of EA (100 μM) and RBA (10 μM) as shown in panel C. Peak ARE refers to all-retinyl esters; 11cROL refers to 11-*cis*-retinol; 13cROL refers to 13-*cis*-retinol; and tROL refers to all-*trans*-retinol.

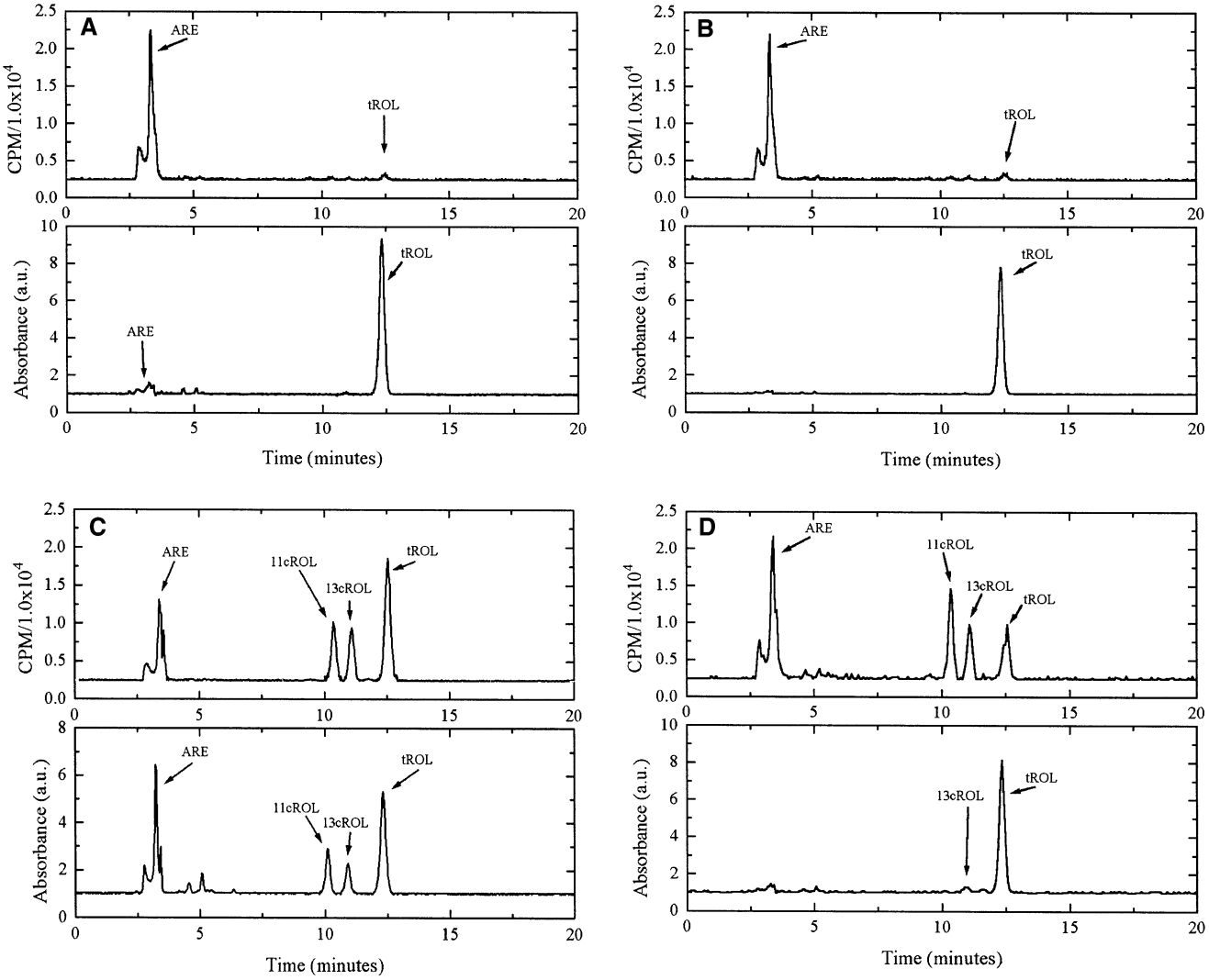


FIGURE 4: HPLC plots for the pulse-chase experiment. This experiment was performed as described above, low concentrations (0.09 μM) of ^{3}H all-*trans*-retinol were incubated with RPE membranes to generate ^{3}H all-*trans*-retinyl esters followed by the addition of nonradioactive all-*trans*-retinol (0.85 μM) as shown in panel A. This is followed by the initiation of isomerohydrolase/REH reaction either in the presence of no inhibitors as shown in panel C or in the presence of EA (100 μM) and RBA (10 μM) as shown in panel D. Panel B shows the effect of heat treatment of membranes for 1 min at 100 °C, before initiating the isomerohydrolase/REH reaction. Peak ARE refers to all-retinyl esters; 11cROL refers to11-*cis*-retinol; 13cROL refers to 13-*cis*-retinol; and tROL refers to all-*trans*-retinol.

active, while the all-*trans*-retinol pool is virtually nonradioactive. In the EA/RBA inhibited case, the prediction is then that if isomerization occurs using all-*trans*-retinyl esters as substrate, then the 11-*cis*-retinol produced will be highly radioactive. Conversely, if all-*trans*-retinol is the substrate, the 11-*cis*-retinol will be virtually nonradioactive. Figure 4A shows the LRAT reaction at the start of the reaction where virtually all of the radioactive material resides in the all-*trans*-retinyl ester pool, while the bulk retinoid resides in the all-*trans*-retinol pool. In Figure 4B are shown data for a heat-treated control in which no inhibitors are added. Here, the all-*trans*-retinol is nonradioactive, and the all-*trans*-retinyl esters are radioactive. As expected, no 11-*cis*-retinol is produced, verifying the enzymatic nature of the isomerization. In Figure 4C are shown data for the experiment in which no inhibitors are added. The experimental result here is consistent with what is normally observed in the isomerization process (*16*−*18*). In Figure 4D are shown the data after EA/RBA treatment. Here, as expected, the 11-*cis*-retinol that is formed is radioactive but clearly has a very high specific activity (Figure 5) inasmuch as there is little UV absorption measured in the 11-*cis*-retinol position. This is precisely the expected result if all-*trans*-retinyl ester is the isomerization substrate and cannot be accounted for in a mechanism in which all-*trans*-retinol is the isomerization substrate.

The experiments described are quantitatively treated to provide specific activities for the various retinoids. Figure 5A shows data for the resultant retinoid specific activities for the experiments in which nonradioactive all-*trans*-retinol is preincubated with RPE membranes to form nonradioactive all-*trans*-retinyl esters, followed by a chase with ^{3}H-all-*trans*-retinol. As shown in 5A where the inhibitor mixture (RBA + EA) is added, the specific activity of generated 11-*cis*-retinol is vastly lower than the added ^{3}H-all-*trans*-retinol and could only have arisen from the relatively nonradioactive all-*trans*-retinyl ester pool. Figure 5B shows the specific activity results from the experiments performed in which RPE membranes are preincubated with ^{3}H-all-*trans*-retinol followed by a chase with all-*trans*-retinol. Note the data in the inhibitor treated instance (Figure 5 B; RBA + EA)). The result clearly demonstrates that ^{3}H-all-*trans*-retinyl ester is

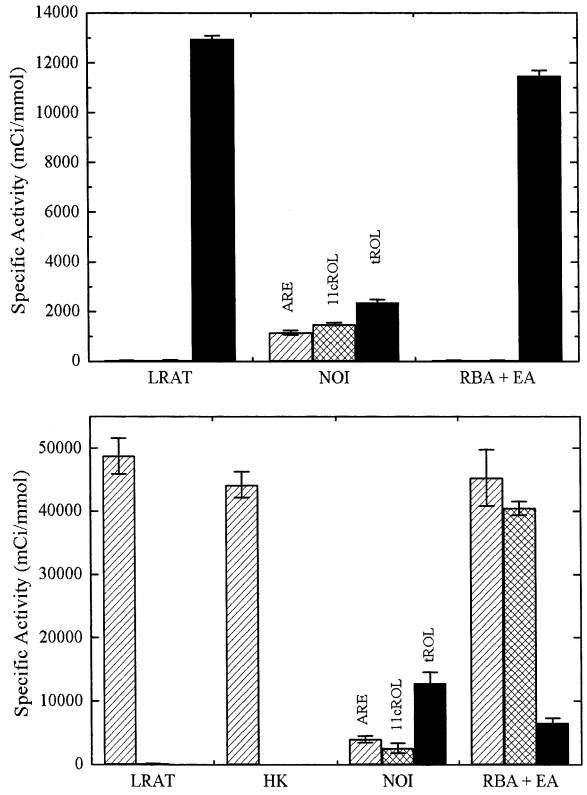


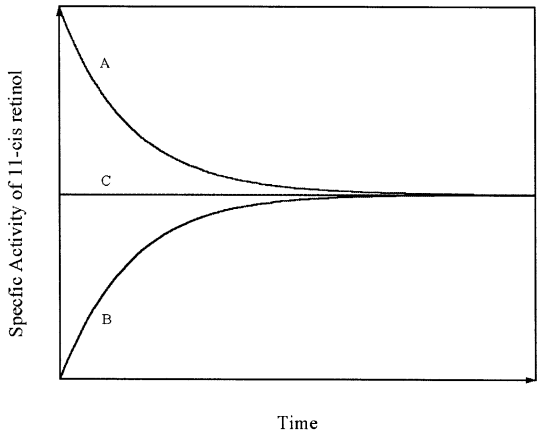
FIGURE 5: Change in the specific activity of retinoids during the pulse-chase experiment. In panel A, the addition sequence is nonradioactive all-*trans*-retinol followed by ^{3}H all-*trans*-retinol. In panel B, the addition sequence is ^{3}H all-*trans*-retinol followed by nonradioactive all-*trans*-retinol. Column ARE refers to all-retinyl esters; 11cROL refers to 11-*cis*-retinol; and tROL refers to all-*trans*-retinol.

the precursor of the isomerization product 11-*cis*-retinol. Finally, Table 1 summarizes the quantitative specific activity results that leave no doubt that ^{3}H-all-*trans*-retinyl esters are the substrates for the biosynthesis of 11-*cis*-retinol.

DISCUSSION

The experiments described here were performed to distinguish between two competing proposals concerning the nature of the isomerization substrate in the visual cycle. Previous studies from this laboratory demonstrated that isomerization occurs at the alcohol oxidation state (eliminating the retinals from consideration) (*10*) and that the oxygen atom of all-*trans*-retinol (ester) is lost during the isomerization event (*22*). Later studies confirmed these observations (*24*). Moreover, using RBA to inactivate LRAT in RPE membranes shows that 11-*cis*-retinol is the first product of isomerization (*19*). That 11-*cis*-retinol is the product of the isomerization is not in question (*19*, *23*, *24*). The nature of the substrate transformed into 11-*cis*-retinol is, however (*23*). Using RBA to block ester synthesis indicated that all-*trans*-retinyl ester synthesis is necessary for isomerization (*19*). A recent study, however, suggested that all-*trans*-retinol is the isomerization substrate based on pulse-chase experiments (*23*). These experiments appeared to us to be uninterpretable for the reasons summarized in the introductory paragraphs. In this experiment, RPE membranes are treated with nonradioactive all-*trans*-retinol to produce all-*trans*-retinyl esters, followed by a chase with ^{3}H-all-*trans*-retinol (*23*). It was predicted that the specific activities of the generated 11-*cis*-retinol would temporally reflect the specific activities of the substrate from which it was formed (*23*). The confounding problem here is that all-*trans*-retinol and all-*trans*-retinyl esters rapidly interconvert on the isomerization time scale. In Scheme 3, the predicted temporal relationships between the specific activities in the product 11-*cis*-retinol under

Scheme 3: Relationship between Specific Activity and Time of Reaction[a]



[a] When (A) vitamin A is the substrate; (B) retinyl ester is the substrate; and (C) there is rapid interconversion between retinyl esters and vitamin A.

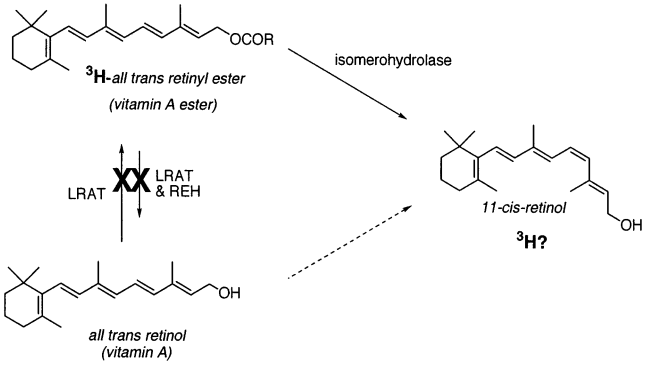
Table 1: Change in the Specific Activity of Retinoids during the Pulse-Chase Experiment[a]

	Specific Activities (mCi/mmol)[a]		
	LRAT	Isomerohydrolase	
retinoids	no inhibitor	no inhibitor	10 μM RBA + 100 μM EA
retinyl ester	32.81 (±5.11)	1148.61 (±7.90)	44.65 (±6.44)
11-*cis*-retinol	45.22 (±7.80)	1493.11 (±5.56)	37.14 (±3.48)
all-*trans*-retinol	12964.34 (±111.40)	2354.26 (±132.10)	11471.12 (±221.50)

	Specific Activities (mCi/mmol)[b]			
	LRAT	Isomerohydrolase		
retinoids	no inhibitor	heat killed	no inhibitor	10 μM RBA + 100 μM EA
retinyl ester	48 771.33 (±2841.21)	44 204.04 (±2030.57)	4001.20 (±507.22)	45 305.97 (±4446.09)
11-*cis*-retinol	75.33 (±58.82)	1.23 (±0.11)	2584.533 (±797.48)	40 482.67 (±1110.77)
all-*trans*-retinol	0.09 (±0.027)	0.14 (±0.022)	12 819.67 (±1699.98)	6532.67 (±804.18)

[a] The addition sequence is nonradioactive all-*trans*-retinol followed by ^{3}H-all-*trans*-retinol. [b] The addition sequence is ^{3}H-all-*trans*-retinol followed by nonradioactive all-*trans*-retinol.

Scheme 4: Blockade of Vitamin A → All-*trans*-retinyl Ester Interconversion and the Substrate for Isomerization



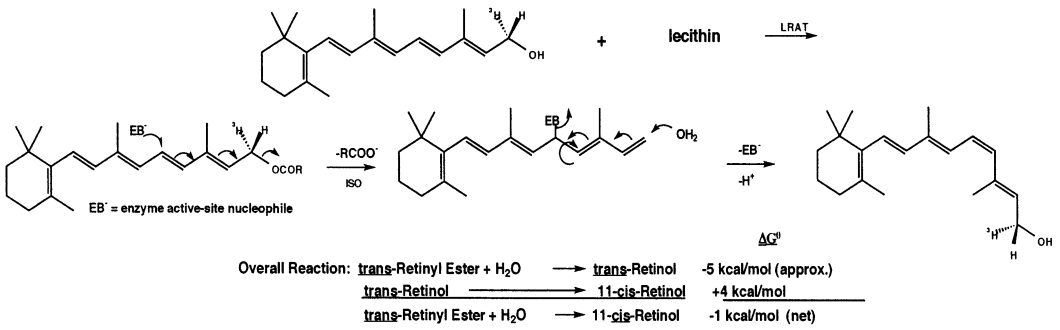
conditions with an initial pulse of nonradioactive all-*trans*-retinol, followed by a chase with ^{3}H-all-trans-retinol, are as reported in the literature (*23*). In this experiment, the initial pulse of all-*trans*-retinol is converted into all-*trans*-retinyl esters (*23*). If isomerization is rapid as compared to LRAT/REH activities, then the curve schematically drawn in Scheme 3A describes the temporal relationship between the specific activity of 11-*cis*-retinol product if all-*trans*-retinol is its precursor. Scheme 3B shows the analogous result, should all-*trans*-retinyl ester be the precursor of 11-*cis*-retinol. On the other hand, if isomerization is slow as compared to other retinoid processing, then the averaging of the specific activities of the substrate would be observed in the product 11-*cis*-retinol, and the result depicted in Scheme 3C would be observed, irrespective of whether all-*trans*-retinol or all-*trans*-retinyl esters are the substrates. The latter result was essentially what was observed, making the experiment uninterpretable with respect to the identification of substrate (*23*). This kind of pulse-chase experiment nevertheless represents an interesting approach, which could be rendered informative under experimental conditions that prevent rapid interconversion of all-*trans*-retinol and all-*trans*-retinyl esters on the time scale of isomerization.

There are two biochemical processes known to affect retinyl ester-retinol interconversions. LRAT reversibly esterifies all-*trans*-retinol into retinyl esters, principally the palmitate ester (*5*−*7*). Retinyl esters also suffer hydrolysis to the retinols by uncharacterized REH(s) (*41*). By blocking LRAT and REH activities, it should be possible to carry out a pulse-chase experiment in which the all-*trans*-retinyl ester and all-*trans*-retinol pools are unable to interconvert. Under these conditions, all-*trans*-retinol or all-*trans*-retinyl esters can be trapped at high specific activities. When isomerization occurs, simple specific activity measurements on the 11-*cis*-retinol produced can determine the nature of the precursor substrate (Scheme 4). In this instance, 1000-fold differences in precursor specific activities could be attained, leaving a determination of the isomerization substrate straightforward and unequivocal.

It has already been demonstrated that RBA specifically inactivates LRAT and can be used for functional studies (*19*). In the current study, it is also shown that EA, a well-known general esterase inhibitor (*43*−*44*), blocks retinyl ester hydrolysis in RPE membranes. Moreover, neither EA nor RBA substantially inhibit isomerization directly. Thus, in the presence of EA and RBA, it is possible to directly ask whether the specific activity of the 11-*cis*-retinol that is formed reflects that of all-*trans*-retinyl or all-*trans*-retinol pools. The experiments were performed in two ways. The all-*trans*-retinyl ester or the all-*trans*-retinol pool was independently made highly radioactive or not. In the absence of significant interconversion of the pools, it is straightforward to determine the nature of the 11-*cis*-retinol precursor. The substrate is clearly an all-*trans*-retinyl ester and not all-*trans*-retinol. This conclusion is easily drawn because of the greater than 1000-fold specific activity differences attained in the putative substrates that can be readily correlated with the specific activity of the product of isomerization of 11-*cis*-retinol. It should be noted that after this manuscript was submitted for publication, an article appeared that further confirms the conclusion that all-*trans*-retinyl ester, and not all-*trans*-retinol, is the isomerization substrate (*47*). Moreover, the approach used (*47*) was quite different from that taken here.

In the original formulation of the isomerohydrolase process, all-*trans*-retinyl esters were depicted to be directly processed to 11-*cis*-retinol in one step (Scheme 5) (*9*, *22*). This mechanism reveals the thermodynamic driving force for the isomerization and also accounts for the C_{15}−O cleavage found to occur concomitant with isomerization (*22*). There are many possible chemical mechanisms that can account for this process, including sN2′ and carbonium ion mechanisms, and these have been previously considered in the literature (*9*). It is also clear that isomerohydrolase activity can be envisaged to occur as a concerted process, or in two steps, with isomerization occurring first, followed by ester hydrolysis. What is important, of course, is that hydrolysis does not precede isomerization. The thermodynamic consequences are the same in either a concerted or a two-step mechanism, and the free energy of ester hydrolysis is what drives the thermodynamically uphill isomerization event. The fact that inhibition of ester hydrolysis by EA has little to no effect on isomerohydrolase activity means that the REH

Scheme 5: Conversion of All-*trans*-retinyl Esters into 11-*cis*-Retinol



activity measured is not essential to drive the isomerization reaction. This would suggest either that the isomerohydrolase reaction is indeed concerted or that the retinyl esterase activity measured in RPE membranes is a housekeeping activity not involved in the isomerohydrolase process.

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