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Stereochemical Inversion at C-15 Accompanies the Enzymatic Isomerization of *all-trans*- to 11-*cis*-Retinoids†

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ABSTRACT: *all-trans*-Retinol (vitamin A) is processed by membranes from the pigment epithelium of the amphibian or bovine eye to form 11-*cis*-retinoids. When the isomerization reaction is performed with either [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol or [15(*R*)-^{3}H,^{14}C]-*all-trans*-retinol as substrate, the resultant 11-*cis*-retinals, formed by the in vitro enzymatic oxidation of the retinols, retain their ^{3}H in the former case and lose it in the latter. The ocular *all-trans*- (*pro-R* specific) and 11-*cis*-retinol (*pro-S* specific) dehydrogenases operate with different stereochemistries with respect to the prochiral methylene hydroxyl centers of their substrates. Inversion of stereochemistry at the prochiral retinol centers was shown to accompany the isomerization process in both the amphibian and bovine systems. The 11-*cis*-retinol formed from [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol was chemically isomerized with I_2 to produce [15(*R*)-^{3}H,^{14}C]-*all-trans*-retinol. The 11-*cis*-retinol formed from [15(*R*)-^{3}H,^{14}C]-*all-trans*-retinol was chemically isomerized with I_2 to produce [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol. The stereochemistry at the prochiral center of retinol is not affected by the I_2-catalyzed double-bond isomerization process and, hence, inversion of stereochemistry at C-15 must accompany isomerization. The same inverted stereochemistry was found with the associated retinyl palmitates. Possible mechanistic reasons for the observed inversion of stereochemistry during isomerization are discussed.

The absorption of light by rhodopsin results in the cis to trans isomerization of rhodopsin's 11-*cis*-retinal Schiff base chromophore, leading to the bleaching of the pigment (Hubbard & Wald, 1952). Hydrolysis of the resultant *all-trans*-retinal Schiff base results in the formation of free *all-trans*-retinal, which is rapidly reduced by specific retinol dehydrogenase enzymes in the rod outer segments to produce *all-trans*-retinol (Wald & Hubbard, 1949). The *all-trans*-retinol is transported to the pigment epithelium by specific binding proteins (Bok, 1985; Saari et al., 1984; Pfeffer et al., 1983; Adler & Evans, 1985), where it is esterified to form *all-trans*-retinyl esters (Hubbard & Dowling, 1962; Knowles & Dartnall, 1977). In

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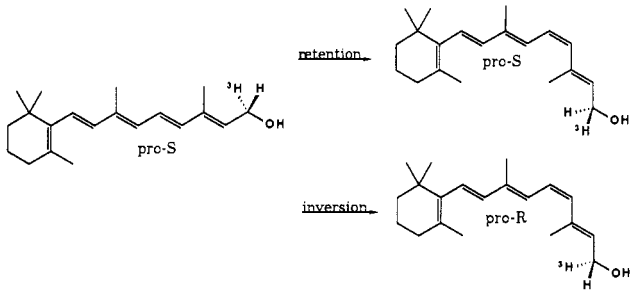
Scheme I



order for vision to proceed, the nonphotochemical isomerization of an *all-trans*- to an 11-*cis*-retinoid must occur. Visual adaptation after a strong bleach, in which the eye matches its sensitivity to the ambient illumination, is also critically dependent on the endogenous biosynthesis of 11-*cis*-retinoids (Davson, 1980). Thus, the thermal mechanism by which an *all-trans*-retinoid can be processed into a 11-*cis*-retinoid is of substantial importance in vision.

We have reported on an isomerase system from membranes of the pigment epithelium of the vertebrate eye that can transform added *all-trans*-retinol into 11-*cis*-retinoids (Bernstein et al., 1987a). The isomerase system from the amphibian is saturable with *all-trans*-retinol and shows a V_{max} of 5 pmol h^{-1} (mg of protein)$^{-1}$, a K_M of 0.8 μM, and a pH optimum of 8.0 (Bernstein et al., 1987b). The enzymatic mechanism of this transformation is likely to be novel, because the reaction is endergonic and could require the input of metabolic energy. At chemical equilibrium, 11-*cis*-retinoids are approximately 4 kcal/mol less stable than their all-trans counterparts (Rando & Chang, 1983). Yet in the vertebrate eye, up to 75% of the retinoids are in the 11-cis form (Bridges, 1976). Indeed, in the in vitro isomerization process, up to 50% of the retinols can be in the 11-cis form (Bernstein et al., 1987a). This is far from the equilibrium value, where approximately 0.1% of 11-*cis*-retinol would be expected (Rando & Chang, 1983).

We have previously found that nonstereospecifically labeled [15-^{3}H,^{14}C]-*all-trans*-retinol is isomerized by the isomerizing system with retention of ^{3}H, thereby excluding free *all-trans*-retinal as the isomerization substrate (Bernstein et al., 1987b). This kind of double-labeling experiment can also be performed with stereospecifically labeled substrate to provide mechanistic insights into the isomerization process not previously possible with the nonstereospecific variant of this experiment. It is of particular interest to follow the fate of the prochiral methylene hydroxyl group of stereospecifically 15-^{3}H-labeled *all-trans*-retinol to determine whether inversion of retention of configuration occurs at the C-15 of vitamin A during the isomerization process (Scheme I). Information of this type is important in defining the class of mechanisms to which the isomerization process belongs. It is demonstrated here that inversion of absolute configuration occurs at the aforementioned prochiral center. A possible mechanism that could account for this inversion is discussed.

Materials and Methods

Materials. Horse liver alcohol dehydrogenase (EC 1.1.1.1), NAD, NADH, BSA,[1] and Tween 80 were purchased from Sigma. Benzaldehyde was from Aldrich. *Rana pipiens* frogs (3–3.5-in. size) were from Sullivan's Amphibians, Nashville, TN. Frozen bovine eye cups were from Lawson Co., Lincoln, NE. [15-^{3}H]-*all-trans*-Retinol (29 Ci/mmol) was purchased from New England Nuclear; [15-^{14}C]-*all-trans*-retinoic acid (15 mCi/mmol) and sodium [^{3}H]borohydride (24.9 Ci/mmol) were purchased from Amersham.

Preparation of Labeled Retinoids. The preparation of [15-^{14}C]-*all-trans*-retinol from [15-^{14}C]-*all-trans*-retinoic acid was described elsewhere (Bridges & Alvarez, 1982). [15-^{3}H]-*all-trans*-Retinal (1 Ci/mmol) was prepared by the oxidation of [15-^{3}H]-*all-trans*-retinol with MnO_2 followed by HPLC purification (Bridges & Alvarez, 1982). [15(*S*)-^{3}H]-*all-trans*-Retinol (1 Ci/mmol) was prepared by the reduction of [15-^{3}H]-*all-trans*-retinal with horse liver alcohol dehydrogenase (HLADH) and NADH in 0.1 M sodium phosphate buffer (pH 6.0) containing 0.1% Tween 80 at 37 °C for 1 h (Hubbard et al., 1971) and followed by HPLC purification. [15(*S*)-^{3}H,^{14}C]-*all-trans*-Retinol (^{3}H:^{14}C = 6.2:1) was obtained by mixing the appropriate amount of [15(*S*)-^{3}H]-*all-trans*-retinol with [15-^{14}C]-*all-trans*-retinol. The double-labeled [15-^{3}H,^{14}C]-11-*cis*-retinal was prepared by the photoisomerization of [15-^{3}H,^{14}C]-*all-trans*-retinal followed by HPLC purification. The double-labeled [15-(*S*)-^{3}H,^{14}C]-11-*cis*-retinol was prepared by the reduction of [15-^{3}H,^{14}C]-11-*cis*-retinal with horse liver alcohol dehydrogenase and NADH in 0.1 M sodium phosphate buffer (pH 6.0) containing 0.1% Tween 80 at 37 °C for 1 h followed by HPLC purification (Hubbard et al., 1971). [α-^{3}H]Benzyl alcohol was prepared by reduction of benzaldehyde with sodium [^{3}H]borohydride. [15(*R*)-^{3}H]-*all-trans*-Retinol was prepared by incubation of *all-trans*-retinal with [α-^{3}H]benzyl alcohol, NAD, and horse liver alcohol dehydrogenase in 50 mM sodium phosphate buffer (pH 7.2) containing 0.1% Tween 80 at 37 °C for 6 h followed by hexane extraction and HPLC purification (Hubbard et al., 1971). [15(*R*)-^{3}H,^{14}C]-*all-trans*-Retinol (^{3}H:^{14}C = 3.1:1) was obtained by mixing the appropriate amounts of [15(*R*)-^{3}H]-*all-trans*-retinol and [15-^{14}C]-*all-trans*-retinol. The oxidation of [15-^{3}H,^{14}C]-11-*cis*- or *all-trans*-retinol to retinal was performed by incubating the appropriate retinol with 2–3 mg of horse liver alcohol dehydrogenase and 0.5 mg of NAD in 1 mL of 0.1 M glycine/NaOH buffer (pH 10.0) containing 0.1% Tween 80 for 1 h at 37 °C (Hubbard et al., 1971). Under these conditions, the 11-*cis*-retinal formed from the 11-*cis*-retinol completely isomerized to a equilibrium mixture of *all-trans*-retinal and 13-*cis*-retinal. Since the free retinal could not be extracted completely, the retinals were generally extracted as retinal oximes (Bernstein & Rando, 1986).

Isomerase Preparations and Assays. The detailed procedures for the preparation of the 600*g* supernatant isomerase containing membranes from the frog (*R. pipiens*) retina/pigment epithelium and the 1500*g* supernatant isomerase containing membranes from the bovine pigment epithelium have been previously described in detail (Bernstein et al., 1987a; Fulton & Rando, 1987). The incubations were performed in 50 mM sodium phosphate buffer (pH 7.2) for the frog material and in 0.32 M sucrose/0.1 M sodium phosphate buffer (pH 7.4) for the bovine material. The detailed methods used for performing the isomerase assays have been published (Bernstein et al., 1987a), as have the methods used for the HPLC separation and analysis of retinoids (Bernstein & Rando, 1986). Under the above experimental conditions, 10–15% of the radioactivity was recovered as the retinols, 10–15% as the retinals, and 70–80% as the retinyl palmitates. The 11-*cis*-retinoids account for over 30% of the retinal pool, over 20% of the retinol pool, and over 10% of the retinyl palmitate pool.

[1] Abbreviations: HLADH, horse liver alcohol dehydrogenase; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography.

Bovine Pigment Epithelium Membranes Also Isomerize all-trans-Retinol with Inversion of the Configuration at C-15. A sample of [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol ([15(*S*)-^{3}H,^{14}C]-ATROL) containing 0.3 μCi of ^{3}H (^{3}H:^{14}C = 6.2:1) was incubated at 37 °C for 40 min with 4 mg of protein from the bovine pigment epithelium 150000*g* pellet suspended in 12 mL of 50 mM sodium phosphate buffer (pH 7.2) and 600 μL of 10% BSA. The [15-^{3}H,^{14}C]-11-*cis*-retinol and *all-trans*-retinol formed were separated by HPLC, diluted with nonradioactive carriers, and oxidized by HLADH and NAD in 0.1 M glycine/NaOH buffer (pH 10.0). Under these conditions, the retinal formed isomerized to an equilibrium mixture of 13-*cis*-retinal and *all-trans*-retinal. The products were extracted in the free retinal form or as retinyl oximes and isolated by HPLC to determine the ^{3}H:^{14}C ratio. For both extraction methods used, the results were identical and show that the [15-^{3}H,^{14}C]-11-*cis*-retinol formed after the in vitro incubation was oxidized to retinal by HLADH with almost complete loss of the ^{3}H label, while the [15-^{3}H,^{14}C]-*all-trans*-retinol was oxidized by HLADH with complete retention of the ^{3}H label.

Analysis of Retinyl Palmitate Stereochemistries. The [15-^{3}H,^{14}C]retinyl palmitates formed from the incubation of [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol with the frog retina/pigment epithelium 600*g* supernatant or bovine pigment epithelium 1500*g* supernatant were collected by HPLC, diluted with a nonradioactive isomeric retinyl palmitate carrier mixture, and hydrolyzed with 5% KOH/MeOH at 0 °C for 5 min (Bernstein et al., 1987b). The [15-^{3}H,^{14}C]-11-*cis*- and *all-trans*-retinol formed were separated by HPLC and oxidized by HLADH and NAD, respectively, in 0.1 M glycine/NaOH buffer (pH 10.0) at 37 °C for 1 h. The retinals formed in each case were extracted as retinyl oximes and purified by HPLC for scintillation counting to determine the ^{3}H:^{14}C ratio. The results indicated that both the [15-^{3}H]-11-*cis*- and *all-trans*-retinyl palmitate have the same absolute stereochemistry as their retinol counterparts.

Results

Isomerization of [15(S)-^{3}H,^{14}C]-all-trans-Retinol and [15(R)-^{3}H,^{14}C]-all-trans-Retinol by the Isomerase Systems. The chirally labeled *all-trans*-retinol of the *S* absolute configuration was prepared by the stereospecific reduction of [15-^{3}H]retinal with HLADH and an excess of NADH by published methods (Hubbard et al., 1971). A mixture of this chiral retinol and [15-^{14}C]-*all-trans*-retinol was incubated with isomerase preparations from amphibian retina/pigment epithelium and from bovine pigment epithelium (Table I). These preparations also contain retinol redox and esterifying capabilities (Fulton & Randon, 1987; Bernstein et al., 1987a,b). At the end of the incubations, the ^{3}H/^{14}C of the *all-trans*- and 11-*cis*-retinols and -retinals were analyzed. In both cases there was virtually complete retention of ^{3}H label in the analyzed retinoids. Also shown in Table I are experiments on the isomerization of [15(*R*)-^{3}H,^{14}C]-*all-trans*-retinol by the amphibian isomerase. As expected from the previous result with [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol, loss of ^{3}H was observed in the retinals.

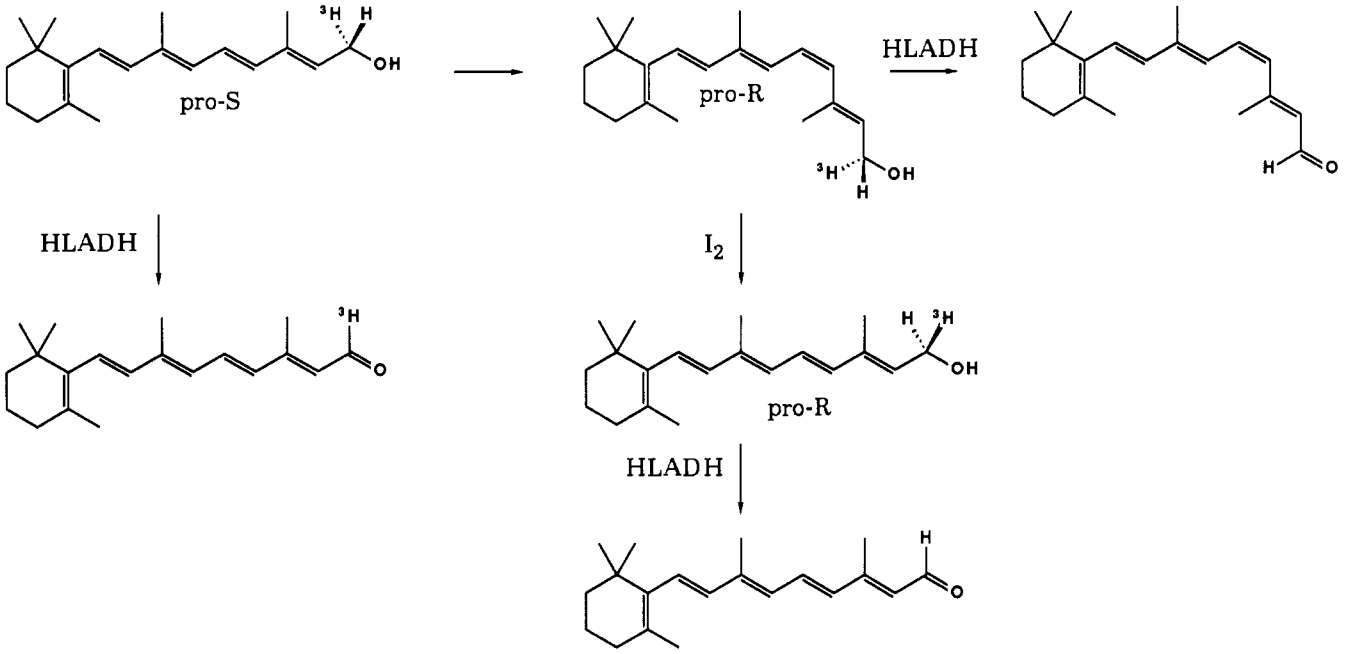
Inversion of Configuration Accompanies Isomerization. The experiments reported above are not immediately interpretable in terms of any possible stereochemical changes at the prochiral methylene hydroxyl group as a consequence of isomerization. This is because *all-trans*-retinol and 11-*cis*-retinol are processed by different ocular retinol dehydrogenases (Futterman, 1963; Blaner & Churchich, 1980; Nicotra & Livrea, 1982; Zimmerman et al., 1975; Saari & Bredberg, 1987) whose stereochemical preferences with respect to the prochiral methylene hydroxyl hydrogen atoms of substrate were unknown. However, the experiments shown in Table I minimally show that the *all-trans*-retinol dehydrogenases are *pro-R* specific. To determine the stereochemistry of the 11-*cis*-retinol dehydrogenases, the experiments shown in Table II were performed. First it is shown that [15(*S*)-^{3}H,^{14}C]-11-*cis*-retinol, enzymatically prepared with HLADH, is oxidized to the corresponding retinal with loss of ^{3}H by amphibian pigment epithelium membranes. This result indicates that the ocular 11-*cis*-retinol dehydrogenase operates with opposite stereochemistry to the *all-trans*-retinol dehydrogenase. The highly likely probability that HLADH processes *all-trans*- and 11-*cis*-retinoids with the same stereochemistry is assumed here. This point is proved by showing that [15(*S*)-^{3}H,^{14}C]-11-*cis*-retinol, prepared enzymatically with HLADH, can be catalytically isomerized by I_2 to form [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol, which is oxidized by the pigment epithelium membranes to 11-*cis*-retinal with retention of ^{3}H (Table II). Furthermore, enzymatically formed [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol is processed by the pigment epithelium membranes to form 11-*cis*-retinol. The latter material is then chemically isomerized with I_2 to produce largely *all-trans*-retinol (Rando & Chang, 1983; Zechmeister, 1944), which is then oxidized by HLADH with removal of the ^{3}H label (Table II). When the same experiment is performed with [15(*R*)-^{3}H,^{14}C]-*all-trans*-retinol, retention of the ^{3}H label is found (Table II). These results require that stereochemical inversion accompanies isomerization and, further, that the distinct ocular retinol dehydrogenases selective for 11-*cis*-retinol and *all-trans*-retinol, respectively, show opposite stereochemistries with respect to the prochiral hydrogens of the substrate. The *all-trans*-retinol dehydrogenase is *pro-R* specific, and the 11-*cis*-retinol dehydrogenase is *pro-S* specific.

Table I: Processing of [15(*S*)-^{3}H,^{14}C]-*all-trans*-Retinol and [15(*R*)-^{3}H,^{14}C]-*all-trans*-Retinol by Frog Retina/Pigment Epithelium 600*g* Supernatant Membrane Fractions and Bovine Pigment Epithelium 1500*g* Supernatant Membrane Fractions[a]

substrate	product	isomerase source	retention of ^{3}H relative to ^{14}C label (%)
15(*S*)-ATROL	11-*cis*-retinol	amphibian	85.0 ± 4.2
15(*S*)-ATROL	*all-trans*-retinol	amphibian	99.0 ± 4.5
15(*S*)-ATROL	11-*cis*-retinal	amphibian	86.3 ± 21.8
15(*S*)-ATROL	*all-trans*-retinal	amphibian	89.7 ± 3.9
15(*R*)-ATROL	11-*cis*-retinol	amphibian	88
15(*R*)-ATROL	*all-trans*-retinol	amphibian	89
15(*R*)-ATROL	11-*cis*-retinal	amphibian	10
15(*R*)-ATROL	*all-trans*-retinal	amphibian	7
15(*S*)-ATROL	11-*cis*-retinol	bovine	88.5 ± 1.5
15(*S*)-ATROL	*all-trans*-retinol	bovine	101.5 ± 1.5
15(*S*)-ATROL	11-*cis*-retinal	bovine	67.5 ± 0.5
15(*S*)-ATROL	*all-trans*-retinal	bovine	80.0 ± 1.0

[a] [15(*S*)-^{3}H, ^{14}C]-*all-trans*-Retinol [15(*S*)-ATROL] containing 0.2 μCi of ^{3}H (^{3}H:^{14}C = 6.2:1) was incubated with the retina/pigment epithelium 600*g* supernatant from five light-adapted *R. pipiens* frogs in 5 mL of 50 mM sodium phosphate buffer (pH 7.2) and 250 μL of 10% BSA for 3 h at room temperature. The results shown are the average of three experiments. [15(*R*)-^{3}H, ^{14}C]-*all-trans*-Retinol [15(*R*)-ATROL] containing 0.15 μCi of ^{3}H (^{3}H:^{14}C = 3.1:1) was incubated with the retina/pigment epithelium 600*g* supernatant from four light-adapted *R. pipiens* frogs in 4 mL of 50 mM sodium phosphate buffer and 200 μL of 10% BSA for 3 h at room temperature. [15(*S*)-^{3}H, ^{14}C]-*all-trans*-Retinol containing 0.3 μCi of ^{3}H was incubated with the bovine pigment epithelium 1500*g* supernatant from eight bovine eyes in 16 mL of 0.32 M sucrose/0.1 M sodium phosphate buffer (pH 7.4) and 800 μL of 10% BSA for 40 min at 37 °C. The results shown are the average of two experiments. In all cases, the retinoids were extracted and analyzed by standard methods (Bernstein & Rando, 1986).

Scheme II



The ocular *all-trans*-retinol dehydrogenase shows the same stereochemistry as HLADH (Rêtey & Robinson, 1982).

Bovine Isomerase System Also Operates with Stereochemical Inversion. The assignments reported above are relevant to the amphibian, so it was of interest to determine if the same stereochemical course is followed by the bovine system. An experiment with bovine pigment epithelium membranes similar to that shown in Table II was performed. In these experiments, [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol was processed to labeled 11-*cis*-retinol by bovine pigment epithelium membranes. The labeled retinols were separated by HPLC and treated with HLADH. The isolated *all-trans*-retinal retained 102 ± 7.5% of its ^{3}H, whereas the 11-*cis*-retinol had retained only 6.5 ± 2.5% of its label. Again, inversion of configuration at the prochiral methylene hydroxyl group of retinol is seen to accompany isomerization. Furthermore, the bovine ocular retinol dehydrogenases specific for *all-trans*- and 11-*cis*-retinol also show opposite stereochemistries with respect to their substrates (Table I and present data).

Retinyl Palmitates Maintain the Same Stereochemistry as Their Retinol Precursors. The retinyl esters, like the retinols, contain a prochiral methylene hydroxyl group. The stereochemistries of the retinyl esters at the prochiral methylene hydroxyl groups were studied to determine whether they were the same as those of the corresponding retinols. The *all-trans*- and 11-*cis*-retinyl palmitates formed from [15(*S*)-^{3}H,^{14}C]-*all-trans*-retinol incubated with the frog isomerase system were separated and hydrolyzed by KOH/MeOH at 0 °C for 5 min to form the respective retinols. The retinols were oxidized with HLADH, and the percent of ^{3}H was determined for the retinal *syn*-oximes. In the frog, 0.5% of the ^{3}H was retained in the 11-*cis*-retinal *syn*-oxime and 85% in the *all-trans*-retinal *syn*-oxime. The corresponding results for the bovine system were 4% and 94%, respectively. Therefore, both the *all-trans*-retinyl palmitates and the 11-*cis*-retinyl palmitates have the same opposite stereochemistries at their prochiral methylene groups as their corresponding retinols.

Discussion

The studies reported here are concerned with the stereochemical consequences at the prochiral methylene hydroxyl group during the isomerization of vitamin A to 11-*cis*-retinol. This reaction is catalyzed by an isomerase system found in membranes of the ocular pigment epithelium (Fulton & Rando, 1987; Bernstein et al., 1987a,b). By use of an approach based on the outline shown in Scheme II, it could be demonstrated that the *trans*- to *cis*-retinoid isomerization reaction occurs with close to complete inversion of configuration at the prochiral methylene hydroxyl group in both warm- and cold-blooded animals. This approach relied on the fact that HLADH oxidizes alcohols with known stereochemistry (Rêtey & Robinson, 1982) and that the I_2-catalyzed isomerization of retinoids does not affect the stereochemistry at the methylene hydroxyl group (Rando & Chang, 1983; Zechmeister, 1944). It is well-known that HLADH oxidizes a broad range of primary alcohols by catalyzing the transfer of the *pro-R* hydrogen of the substrate to the cofactor NAD (Rêtey & Robinson, 1982). The isomerization of polyenes by I_2 involves a free radical addition–elimination process that does not affect the stereochemistry at C-15 (Scheme III) (Rando & Chang, 1983; Zechmeister, 1944). This is also clear from the results of the I_2-catalyzed isomerization reported here. If an I_2-induced reaction were to occur at C-15, racemization and/or hydrogen atom removal would have occurred. Neither was observed.

[15(*S*)-^{3}H,^{14}C]-*all-trans*-Retinol was isomerized by both

Scheme III

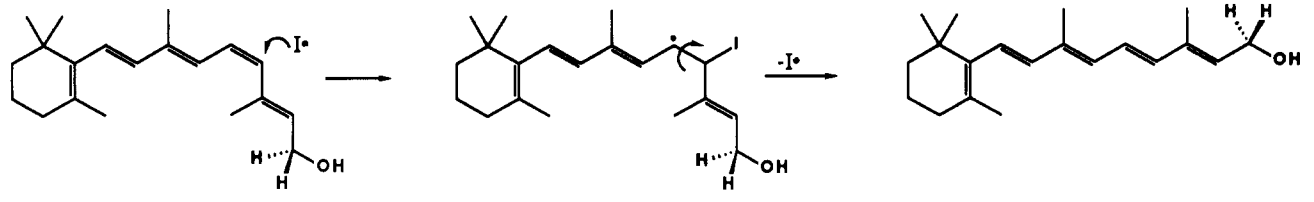
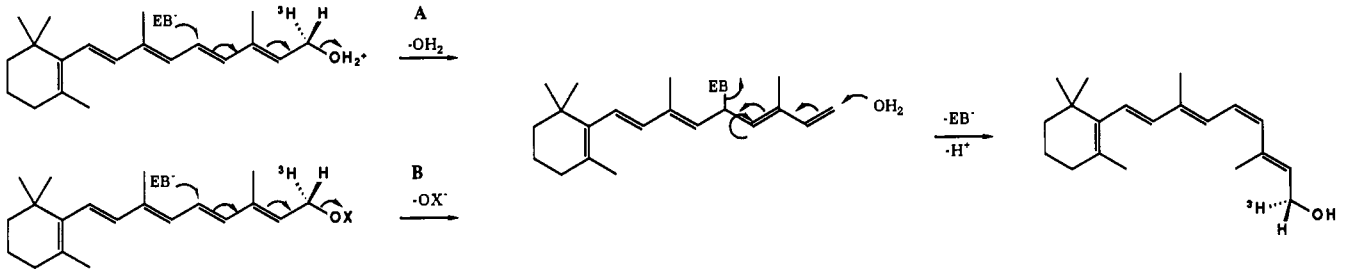


Table II: Inversion of Configuration at the C-15 Position of *all-trans*-Retinol Occurs Concomitant with Isomerization in the Amphibian Eye

substrate	product	additional treatment	retention of ^3H relative to ^{14}C label in isolated retinal (%)
15(*S*)-11CROL[a]	11-*cis*-retinal	none	10.6 ± 0.6
15(*S*)-11CROL + I_2[b]	11-*cis*-retinal	none	106.0 ± 8.0
15(*S*)-ATROL[c]	11-*cis*-retinol	I_2 followed by HLADH	10.0 ± 4.0
15(*R*)-ATROL[d]	11-*cis*-retinol	I_2 followed by HLADH	94.0

[a] [15(*S*)-^3H, ^{14}C]-11-*cis*-Retinol [15(*S*)-11CROL] containing 0.15 μCi of ^3H (^3H:^{14}C = 14.7:1) was incubated with 2 mL of frog retina/pigment epithelium 600*g* supernatant and 100 μL of 10% BSA for 2 h at room temperature. The 11-*cis*-retinal formed was extracted as the retinyl oxime and analyzed by standared methods (Bernstein et al., 1987a). [b] Part of the same sample of [15(*S*)-^3H, ^{14}C]-11-*cis*-retinol containing 0.5 μCi of ^3H was treated with 200 μL of 0.1% I_2/hexane for 5 min to obtain a mixture of [15(*S*)-^3H, ^{14}C]-13-*cis* and *all-trans*-retinol (Bernstein et al., 1987a). This [15(*S*)-^3H, ^{14}C]-13-*cis*- and *all-trans*-retinol mixture was incubated with 2 mL of frog retina/pigment epithelium 600*g* supernatant and 100 μL of 10% BSA for 2 h at room temperature and processed as above. The results shown are the average of two experiments. [c] [15(*S*)-^3H, ^{14}C]-*all-trans*-Retinol [15(*S*)-ATROL] containing 0.2 μCi of ^3H was incubated at room temperature for 3 h with 4 mL of retina/pigment epithelium washed membrane homogenates (Bernstein et al., 1987a) from four *R. pipiens* frogs and 200 μL of 10% BSA at room temperature for 3 h. The 11-*cis*-retinol formed was isolated by HPLC, diluted with nonradioactive 11-*cis*-retinol, and treated with 0.1% I_2/hexane for 1 min at room temperature (Bernstein et al., 1987a). The [15-^3H, ^{14}C]-*all-trans*-retinol formed was isolated by HPLC and oxidized by HLADH and NAD in 0.1 M glycine/NaOH buffer (pH 10.0). The retinal formed was extracted as the retinyl oxime (Bernstein et al., 1987a), and its ^3H:^{14}C ratio was determined. The result indicated that most of the ^3H label was lost after the oxidation. [d] The [15-^3H, ^{14}C]-11-*cis*-retinol obtained from the incubation of [15(*R*)-^3H, ^{14}C]-*all-trans*-retinol [15(*R*)-ATROL] with frog retina/pigment epithelium 600*g* supernatant was isolated by HPLC, diluted with nonradioactive 11-*cis*-retinol, and treated with 0.1% I_2/hexane at room temperature for 1 min (Bernstein et al., 1987a). The [15-^3H, ^{14}C]-*all-trans*-retinol formed was purified by HPLC and oxidized by HLADH to form retinal that was extracted as the retinyl oxime and found to retain most of the ^3H.

Scheme IV



amphibian and bovine isomerase containing membranes to produce 11-*cis*-retinoids of the opposite configuration. The initially formed *all-trans*-retinal and 11-*cis*-retinal from the pigment epithelium membranes had almost completely retained their ^3H (Table I). Beginning with [15(*R*)-^3H,^{14}C]-*all-trans*-retinol, the opposite result was obtained (Table I). This result is not interpretable unless the stereochemical preferences for the ocular dehydrogenases that process *all-trans*-retinol and 11-*cis*-retinol are known. It is also clear from Table I that ocular *all-trans*-retinol dehydrogenase removes the *pro-R* substrate hydrogen. Enzymatically prepared [15-(*S*)-^3H,^{14}C]-11-*cis*-retinol was oxidized by pigment epithelium membranes with the loss of ^3H (Table II). [15(*S*)-^3H,^{14}C]-*all-trans*-Retinol was isomerized by the membranes to produce an 11-*cis*-retinol that retained its ^3H after oxidation by these same membranes. This implies that [15(*R*)-^3H,^{14}C]-11-*cis*-retinol was formed by the membranes and that stereochemical inversion had accompanied isomerization. Further, it strongly suggests that the ocular retinol dehydrogenases operate with opposite stereochemistries with respect to substrate. These points could be demonstrated unambiguously. When [15(*S*)-^3H,^{14}C]-11-*cis*-retinol, enzymatically prepared with HLADH, was isomerized with I_2, the resulting *all-trans*-retinol was oxidized by the membranes to 11-*cis*-retinal with retention of ^3H. Furthermore, frog isomerase preparations were separately incubated with either [15(*S*)-^3H,^{14}C]-*all-trans*-retinol or [15(*R*)-^3H,^{14}C]-*all-trans*-retinol, the 11-*cis* retinol products were separately isolated and isomerized with I_2, and the *all-trans*-retinol products were treated with HLADH. Only the product initially derived from [15(*S*)-^3H,^{14}C]-*all-trans*-retinol lost its ^3H. Therefore, the *all-trans*-retinol dehydrogenase removes the *pro-R* hydrogen, the 11-*cis*-retinol dehydrogenase removes the *pro-S* hydrogen, and inversion of stereochemistry at C-15 of vitamin A accompanies isomerization.

Although it is clear that there are multiple ocular dehydrogenases, their absolute specificities with respect to *all-trans*-retinol and 11-*cis*-retinol are unknown at present (Futterman, 1963; Blaner & Churchich, 1980; Nicotra & Livrea, 1982; Zimmerman et al., 1975; Saari & Bredberg, 1987). The very fact that we do not observe complete ^3H removal or retention under appropriate conditions might suggest that the dehydrogenases are not absolutely substrate specific.

A crucial question is, how can the results described above showing inversion of configuration during isomerization be interpreted mechanistically? The question of the nature of the isomerase substrate is important here because if free *all-trans*-retinal were the substrate for the isomerase, then the observed stereochemistry could, under very special circumstances, be a natural outcome of the different stereochemistries of the two distinct retinol dehydrogenases. The special circumstances alluded to would involve the total absence of endogenous NADH or else ^3H label would of necessity be lost in the 11-*cis*-retinol that is formed (Table II). Substantial endogenous supplies of free NADH have previously been shown to be present in membranes of the same type studied here (Bernstein et al., 1987a). Furthermore, free *all-trans*-retinal has been shown not to be the isomerase substrate (Fulton & Rando, 1987; Bernstein et al., 1987a,b). The fate of the 15-^3H of nonstereospecifically labeled [15-^3H,^{14}C]-*all-trans*-retinol was followed during isomerization in vivo (Bernstein & Rando, 1986) and in vitro (Bernstein et al., 1987b). Complete ^3H retention was found in vitro, and significantly greater than 50% retention was found in vivo at early times. The conclusion that free *all-trans*-retinal is not the

isomerase substrate can also be inferred from substrate profile studies (Fulton & Rando, 1987; Bernstein et al., 1987a,b). Here it could be shown that washed and/or highly sonicated pigment epithelium membranes from amphibian and unprocessed bovine membranes were capable of isomerizing *all-trans*-retinol but not *all-trans*-retinal (Fulton & Rando, 1987; Bernstein et al., 1987a,b).

It should be noted that the possibility that the observed stereochemical inversion could result from the oxidation of enzyme-bound *all-trans*-retinol to the aldehyde, followed by isomerization and reduction at the opposite face to produce 11-*cis*-retinol, has not been formally disproved. However, this kind of mechanism would predict that an enormous primary ^3H isotope effect should be observed, because it would be the product of two separate isotope effects. No evidence for any isotope effect at all has been observed here or in previously reported experiments (Bernstein et al., 1987b).

From the experiments described above, it seems clear that the ultimate free isomerization substrate must be at the alcohol oxidation state and could be retinol or a chemically activated derivative. In either case, the stereochemical results reported here are best accounted for by a reaction mechanism involving carbon–oxygen bond scission (Scheme IV). If *all-trans*-retinol itself were the substrate, a mechanism of the type shown in Scheme IVA would be possible. However, this kind of mechanism does not directly address the question of energy transduction and how the thermodynamically uphill isomerization process might be driven. A chemically activated *all-trans*-retinol derivative would be of far greater interest in terms of directly addressing the issue of an energy transduction process (Scheme IVB). In this mechanism, where the free energy of hydrolysis of an ester bond is coupled to and drives the isomerization reaction, there is a necessity for carbon–oxygen bond cleavage. Although stereochemical inversion at C-15 does not necessarily follow from this mechanism, it is readily understood in terms of it. Inversion would result if solvent water were to attack the face opposite from where carbon–oxygen bond cleavage occurred (Scheme IV). Here the attack by water precedes rotation about C-11–C-12. If rotation about the C-11–C-12 bond is *followed* by the attack of water from the same side as the side where the carbon–oxygen bond was cleaved, the result looks as if stereochemical inversion has taken place, although in fact retention has occurred. That these kinds of mechanism might be important has already been discussed (Fulton & Rando, 1987). It was found that a diverse group of chemical and physical agents interfered with retinyl ester synthesis and isomerization coordinately, suggesting a causal link between the two (Fulton & Rando, 1987).

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