In Vivo Isomerization of *all-trans*- to 11-*cis*-Retinoids in the Eye Occurs at the Alcohol Oxidation State†

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ABSTRACT: The vertebrate biochemical pathway for regeneration of visual pigments in the living eye after bleaching is largely uncharacterized. Since isomerization of an *all-trans*-retinoid to an 11-*cis*-retinoid could conceivably occur via the aldehyde, alcohol, or ester forms of vitamin A, it is important to determine the oxidation state of the retinoid that is isomerized in vivo. To address this problem, light-adapted rats and frogs were injected intraperitoneally with a mixture of [15-^{3}H]-*all-trans*-retinol and [15-^{14}C]-*all-trans*-retinol. After 4 or 24 h of dark adaptation, labeled retinoids in the animal's eyes were analyzed. All rats had the expected 50% loss of ^{3}H label (relative to ^{14}C) in 11-*cis*-retinal, a loss of ^{3}H that must occur when [15-^{3}H]retinol is oxidized to retinal. 11-*cis*-Retinyl esters in the rats' eyes at 4 h retained 67% of the ^{3}H label, and this could be increased to 81% when the rats were pretreated with 4-methylpyrazole, an alcohol dehydrogenase inhibitor known to inhibit dark adaptation. This result demonstrates that retinoid isomerization occurs at the alcohol oxidation state in the rat eye. Had it occurred at the aldehyde oxidation state, at least 50% of the ^{3}H in the 11-*cis*-retinyl esters would have been lost. The importance of this isomerization pathway is emphasized by the observation that dark-adapting rats whose alcohol dehydrogenase(s) had been inhibited by 4-methylpyrazole had increased amounts of 11-*cis*-retinyl ester in their eyes relative to control rat eyes, a result that is understandable only if retinoids are isomerized in vivo at the alcohol oxidation state. When these same experiments were performed on frogs, an unexpectedly large loss of ^{3}H label in all retinoids was observed, preventing a definite demonstration of the oxidation state of retinoid isomerization in this animal. It was noted, however, that 4 h after injection with labeled *all-trans*-retinol the first 11-*cis*-retinoid formed in substantial amounts in the frog eye was 11-*cis*-retinol. This result indicates that frogs, like rats, may produce 11-*cis*-retinoids by isomerization at the alcohol oxidation state.

The initial event of vision in the vertebrate retina is the photochemical isomerization of rhodopsin's 11-*cis*-retinal chromophore to *all-trans*-retinal which subsequently dissociates leaving behind the apoprotein opsin (Hubbard & Wald, 1952). Under bright light conditions, the released *all-trans*-retinal is reduced to retinol, esterified to long-chain fatty acids, and stored in the pigmented epithelium (Bridges, 1976). In the steady state under a bright light, >90% of the retina's rhodopsin in an animal such as a frog or a rat is bleached to opsin, and virtually all of the eye's retinoids are accounted for by *all-trans*-retinyl esters in the pigmented epithelium (Bridges, 1976; Zimmerman, 1974).

If a light-adapted animal is then placed in the dark, the stored retinyl esters are converted through a series of light-independent biochemical steps to 11-*cis*-retinal, which can then combine with opsin to regenerate the active visual pigment rhodopsin. These biochemical steps of 11-*cis*-retinal production that comprise the visual cycle in vivo are largely uncharacterized (Bridges, 1984). It is known that 11-*cis*-retinoid formation must be an energy-requiring process because under chemical equilibrating conditions 11-*cis*-retinoids account for no more than 0.1% of all retinoids (Rando & Chang, 1983). Although several preliminary reports have been published (Hubbard, 1956; Amer & Akhtar, 1972), no "isomerase" for the production of 11-*cis*-retinoids has evern been conclusively demonstrated (Groenendijk et al., 1980a).

Isomerization to an 11-*cis*-retinoid could conceivably occur via the aldehyde, alcohol, or ester forms of vitamin A, and various visual pigment regeneration schemes have been proposed for each possibility. Isomerization of *all-trans*-retinal to 11-*cis*-retinal has been thought by many to be the pathway utilized in vivo (Hubbard, 1956; Amer & Akhtar, 1972) because retinal is readily isomerized (Lukton & Rando, 1984). For example, in some model systems isomerization rates of retinal can be greatly enhanced in the presence of naturally occurring amines such as reduced flavins (Futterman & Rollins, 1973) and phosphatidylethanolamine (Groenendijk et al., 1980a), although the equilibrium distribution of retinoids is unchanged, of course. Also, homogenates of eye tissue can catalyze equilibration of several retinal isomers (Sack & Seltzer, 1978). Isomerization of *all-trans*-retinol to 11-*cis*-retinol has been speculated as another possibility (Bridges, 1984), and it is known that tissue extracts can catalyze isomerization of 11-*cis*-retinol to *all-trans*-retinol (Stainer & Murray, 1960). Additionally, retinol can be phosphorylated in vivo (Bhat et al., 1980), and hydrolysis of its high-energy phosphate bond could perhaps provide an energy input to form 11-*cis*-retinoids. Finally, schemes based on isomerization of *all-trans*-retinyl esters to 11-*cis*-retinyl esters occurring in the pigmented epithelium have been offered (Bridges, 1976); however, isolated pigmented epithelial cells show no ability to isomerize retinoids in vitro (Fong et al., 1983).

In this paper, the pathway for 11-*cis*-retinoid isomerization is investigated in vivo to determine whether isomerization occurs at the aldehyde (retinal) or alcohol (retinol or retinyl ester) oxidation state. If an answer to this simple question

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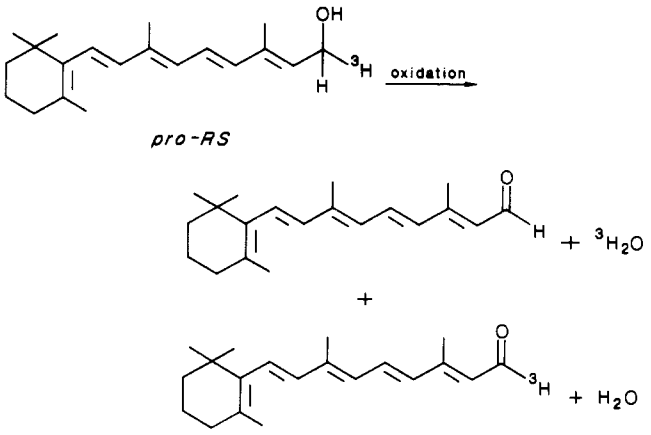
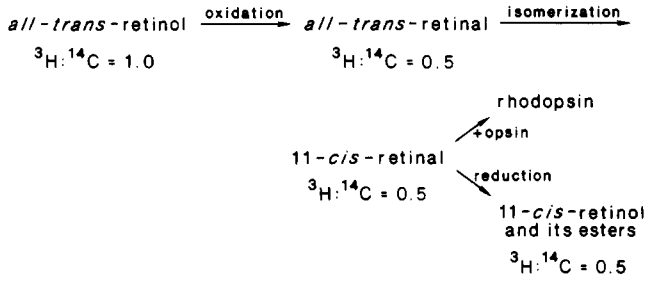
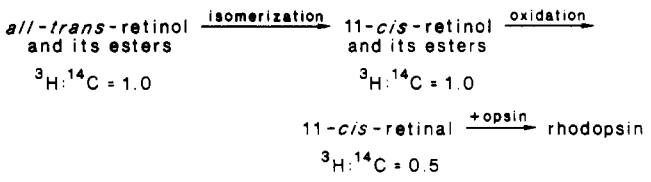
Scheme I



Scheme II



Scheme III



can be achieved, it will allow a much more focused investigation of the biochemical pathway and mechanism of visual pigment regeneration. Studies are made comparing the biotransformation in the eye of [15-^{3}H]-*all-trans*-retinol with that of [15-^{14}C]-*all-trans*-retinol. [15-^{3}H]Retinol is prepared by chemical reduction of retinal with NaB^3H_4. Its ^{3}H label is resistant to exchange in hydroxylic solvents, but when [15-^{3}H]retinol is oxidized to retinal, half of its tritium label must be lost (Scheme I). Clearly, [15-^{14}C]retinol would not lose its label under the same conditions. This obligate loss of label upon oxidation is used to determine the oxidation state of 11-*cis*-retinoid isomerization in vivo. Rats and frogs are injected systemically with a mixture of [^{3}H]- and [^{14}C]-*all-trans*-retinol labeled at the terminal carbon, and the ^{3}H:^{14}C ratios of retinoids in the eye are determined 4 or 24 h after injection. Schemes II and III show the expected normalized ^{3}H:^{14}C ratios if isomerization occurs at the aldehyde or alcohol oxidation states, respectively. These ^{3}H:^{14}C ratios are the ones expected under ideal conditions, free of such complications as isotope effects and nonspecific loss of radioactive label.

The results of the investigation described in this paper demonstrate that the rat isomerizes its retinoids at the alcohol oxidation state, and studies reported here using pharmacological inhibitors of dark adaptation confirm that this is indeed the physiologically important biochemical pathway for visual pigment regeneration. Double-label experiments in frogs proved less conclusive because they had an unusually high rate of loss of the ^{3}H label that precluded a definite interpretation; however, other experiments reported here indicate that they, too, may isomerize retinoids at the alcohol oxidation state.

Material and Methods

Unless otherwise mentioned, all procedures were performed under dim red light with samples kept on ice.

Preparation of Radioactive Retinols for Double-Label Experiments. [15-^{14}C]-*all-trans*-Retinol was prepared by CH_2N_2 esterification of [15-^{14}C]-*all-trans*-retinoic acid (44 mCi/mmol, Amersham), followed by reduction with $LiAlH_4$ (Bridges & Alvarez, 1982). The resultant [15-^{14}C]-*all-trans*-retinol was purified on a Waters HPLC system with a 5-μm silica column (Merck LiChrosorb Si 60, 4.0 × 250 mm) eluted with 8% ethyl ether in hexane. Final yield was typically 35%. [15-^{3}H]-*all-trans*-Retinol (29.0 Ci/mmol) was purchased from New England Nuclear. It had been prepared by reduction of retinal with NaB^3H_4, and it was stored in ethanol with no detectable loss of specific activity for several months.

The purified [15-^{14}C]-*all-trans*-retinol was mixed with [15-^{3}H]-*all-trans*-retinol so that the ^{3}H:^{14}C ratio was at least 3:1. The mixture was evaporated to dryness under a stream of nitrogen and resuspended in 2-propanol containing 1 mg/mL butylated hydroxytoluene so that the concentration of ^{14}C was 0.1 μCi/μL. All mixtures were stored under argon at −70 °C. Before the first use and after the last use, a small sample of each mixture was analyzed by the HPLC system described above to determine the ^{3}H:^{14}C ratio and the radiochemical purity. Mixtures were typically 97% pure, and the only identifiable contaminants were ^{3}H- and ^{14}C-labeled 13-*cis*-retinol.

Double-Label Experiments in Rats and Frogs. Male CD albino rats (Charles River Breeding, 45–55 g) or *Rana pipiens* frogs (Sullivan's Amphibians, Nashville, TN; 2.0–2.5 in. size) were light-adapted 20 cm below a 60-W desk lamp, at least 1 h for rats and overnight for frogs. This procedure typically bleaches >90% of an animal's rhodopsin (Bernstein & Rando, 1985). Each animal then received an intraperitoneal injection of the ^{3}H- and ^{14}C-labeled retinol mixture at a dose of 0.1 μCi of ^{14}C/g. Some rats received intraperitoneal injections of 500 mg/kg 4-methylpyrazole (Aldrich) or 100 mg/kg 1,5-bis-(*p*-aminophenoxy)pentane (May & Baker of Dagenham, Essex, U.K.) in 10 μL/g water 1 h before injection with labeled retinol. Control rats received 10 μL/g of 0.9% NaCl. The animals then were placed in a completely dark room for 4 h. At the end of dark adaptation, the animals were decapitated, and the eyes were enucleated. The retina and pigmented epithelium from both eyes of an animal were placed in a 1.5-mL microcentrifuge tube. The sclera was also included when rats were used. The tissue was solubilized in 100 μL of 50 mM octyl β-D-glucopyranoside (Behring Diagnostics). A total of 50 μL of 1 M NH_2OH (pH 6.5) was added followed by 300 μL of methanol and 150 μL of water in order to form the oximes of retinal to extract retinoids quantitatively without isomerization (Groenendijk et al., 1980b). After brief centrifugation the emulsion was then extracted 4 times with 300 μL of CH_2Cl_2. This organic extract was dried under N_2 and resuspended in 5 mL of ethyl ether, dried over Na_2SO_4, and eluted through a silica gel SEP-PAK (Waters, Inc.) with an additional 5 mL of ether. The eluate was dried under N_2 in preparation for HPLC analysis.

HPLC Analysis for Double-Label Experiments. Extracts were dissolved in 200 μL of hexane and injected onto a Waters gradient HPLC system with detection at 313 nm. Elution was by a 2 mL/min ethyl ether/hexane gradient on a 5-μm silica column (Merck LiChrosorb Si 60, 4.0 × 250 mm). The first 20 min was a concave hyperbolic gradient (curve 10 on a Waters Model 680 gradient controller) from 0.6% to 20% ether in hexane, followed by a 10-min hold at 20% ether. The

column was reequilibrated by pumping 0.6% ether/hexane at 4 mL/min for 15 min. Sixty 1-mL fractions were collected on each analysis, which were then counted in Soluscint-O (National Diagnostics) on a Beckman LS-1800 multichannel scintillation counter with automatic quench correction, which was interfaced with an Apple II+ microcomputer for data analysis. The elution pattern of endogenous retinoids for each run was used to identify which vials contained the counts corresponding to each retinoid. The HPLC separation achieved was sufficient so that no individual vials ever contained counts from adjacent retinoid peaks. All counts used to determine ^3H:^{14}C ratios were corrected for radioactive background.

Identification of endogenous retinoids was by coelution with all-trans and 11-cis isomers of retinal oximes, retinol, and retinyl esters prepared by standard laboratory techniques (Bridges & Alvarez, 1982; Bernstein et al., 1985). For some experiments, the peak areas were determined by integration, and values are given without correction for differences in extinction coefficients. All statistical tests were unpaired two-tailed *t* tests by an ED-SCI statistical package for the Apple II+ microcomputer.

Single-Label Experiments in Frogs. These experiments were done as described for the double-label experiments with two exceptions. (1) [11,12-^3H]-*all-trans*-Retinyl acetate (60 Ci/mmol, Amersham) was used as the labeled retinoid. It was mixed with unlabeled retinyl acetate (Fluka) to lower its specific activity to 600 mCi/mmol, and it was repurified by HPLC. (2) The HPLC system used to analyze the eye extracts was isocratic 8% ethyl ether in hexane at 2 mL/min. The first six 1-mL fractions were combined, dried down, and rechromatographed with 0.5% ethyl ether to separate retinyl esters.

Results and Discussion

As a first step in determining the biochemical pathway of visual chromophore production, it is of critical importance to determine whether isomerization of *all-trans*-retinoids to 11-*cis*-retinoids in vivo occurs at the alcohol or aldehyde oxidation state. The approach used in this study entailed the intraperitoneal injection of a mixture of ^{14}C- and ^3H-labeled *all-trans*-retinol into dark-adapting frogs or rats, followed by HPLC analysis of all of the radioactively labeled retinoids found in the eye. The [^{14}C]vitamin A compound used was [15-^{14}C]-*all-trans*-retinol; its ^{14}C label cannot be lost unless the [^{14}C]vitamin A is destructively metabolized. The [^3H]-vitamin A compound was [15-^3H]-*all-trans*-retinol; it must lose half of its tritium label when it is oxidized to retinal because one of its two hydrogens at C-15 must be removed (Scheme I). If isomerization must occur via the aldehyde oxidation state, then 50% of the ^3H dpm (relative to ^{14}C dpm) would be lost in all 11-*cis*-retinoids formed in the eye because enzymatic oxidation before isomerization would remove one of the prochiral hydrogens at C-15 (Scheme II). If isomerization occurs at the alcohol oxidation state, 11-*cis*-retinol and 11-*cis*-retinyl ester formation would be expected to occur with retention of the ^3H label, while 11-*cis*-retinal would still lose 50% of its ^3H label because its biosynthesis would require oxidation after isomerization (Scheme III).

In an in vivo double-label experiment such as this, a number of implicit assumptions must be made if the results are to be interpretable. These assumptions and their consequences are as follows: (1) Nonspecific (i.e., nonenzymatic) loss of the ^3H is minimal in vivo. If it is substantial, then ^3H loss might exceed the 50% limit and loss would be observed to continue over time, but this type of loss is unlikely since the retinol's ^3H label is quite resistant to exchange in protic solvents. (2) Kinetic isotope effects that would alter ^3H:^{14}C ratios from expected values are assumed to be small. Neither a primary ^{14}C isotope effect nor a secondary ^3H isotope effect would be expected to decrease reaction rates by even 10% (Richards, 1970). A primary ^3H isotope effect on product formation is measurable only in the product that retains the ^3H after C–^3H bond cleavage (O'Leary, 1980), so ^3H:^{14}C ratios in retinal will not be affected because the ^3H label is released to water during oxidation. ^3H:^{14}C ratios of the reactants in an oxidation reaction could be increased by a primary ^3H isotope effect but only if the reaction has gone almost totally to completion (O'Leary, 1980); this situation would not be expected in the experiments reported in this paper. (3) It is important that cycles of oxidation–reduction that can cause loss of label are not fast relative to a rate-limiting isomerization step. For example, if isomerization does occur at the alcohol oxidation state, the 11-*cis*-retinol and its esters, initially formed with retention of tritium, may have 50% of their label immediately lost in fast oxidation–reduction "futile cycles", leading to the erroneous conclusion that isomerization occurs at the aldehyde oxidation state. Pharmacological inhibition of oxidation–reduction is used in this study to help eliminate this source of artifact. (4) If cycles of oxidation–reduction are very fast, it is assumed that all enzymes involved interact with the same prochiral hydrogen. If the enzymes are not stereospecific with respect to which prochiral tritium is removed or if the enzymes have opposite stereospecificities, then tritium loss of >50%, indistinguishable from the nonspecific losses discussed in assumption 1, would result.

Table I: Double-Label Experiments in Rats: 4 h

drug[b]	% retention of ^3H label (relative to ^{14}C label)[a]			
	11-*cis*-retinyl esters[c]	*all-trans*-retinyl esters[c]	11-*cis*-retinal oximes	*all-trans*-retinol
none	67 ± 6	81 ± 13	50 ± 2	48 ± 12
4-MP (500 mg/kg)	81 ± 4	92 ± 4	58 ± 4	44 ± 12
DAPP (100 mg/kg)	73 ± 8	85 ± 4	48 ± 4	52 ± 4

[a] All values are mean ± SD for $n = 3$. The starting ^3H:^{14}C ratio was 5.2:1. [b] Abbreviations: 4-MP, 4-methylpyrazole; DAPP, 1,5-bis(*p*-aminophenoxy)pentane. [c] Retinyl esters in the rat are predominantly a mixture of retinyl palmitate and retinyl stearate (Alvarez et al., 1981).

Double-Label Experiments in Rat. Light-adapted control rats were injected intraperitoneally with a mixture of [15-^3H]- and [15-^{14}C]-*all-trans*-retinol in a 5.2:1 ^3H:^{14}C ratio. After 4 h of dark adaptation the animals were killed, and the labeled retinoids found in the eye were analyzed by HPLC. Sufficient counts for data analysis coeluted with endogenous 11-*cis*-retinyl esters, *all-trans*-retinyl esters, 11-*cis*-retinal (isolated as *syn*- and *anti*-oximes), and *all-trans*-retinol. (An example is shown in Figure 1.) No appreciable counts were found for *all-trans*-retinal or 11-*cis*-retinol, retinoids found in only trace amounts in the rat eye endogenously (Zimmerman, 1974). As shown in the first line of Table I, 11-*cis*-retinal had its expected 50% loss of the ^3H label while the *all-trans*-retinyl esters retained 81% of the ^3H label. The 11-*cis*-retinyl esters retained 67% of their ^3H label; partial loss of label may be due to the oxidation–reaction cycles discussed above. To show that the enzymes involved in the biotransformation have consistent stereospecificities, several rats were allowed to dark adapt 24 instead of 4 h, and tritium losses for all retinoids were approximately 50% (data not shown).

The retention of substantially more than 50% of the ^3H label in the 11-*cis*-retinyl esters after 4 h of dark adaptation suggests that rats employ a direct alcohol oxidation-state isomerization.

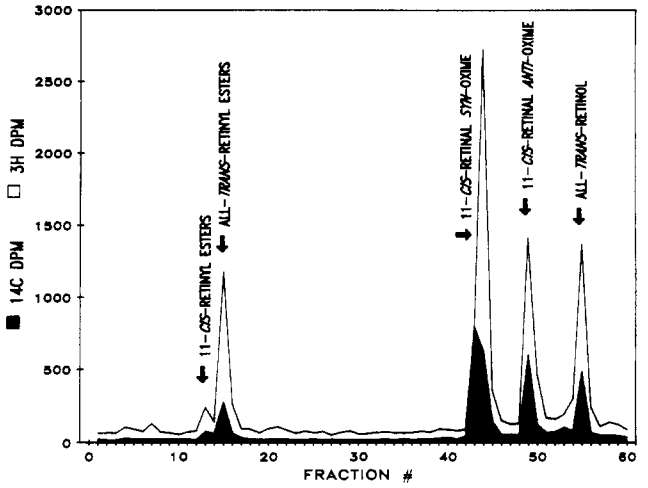


FIGURE 1: Typical results of a double-label experiment in the rat. A light-adapted rat received an intraperitoneal injection of 0.52 μCi/g [15-^{3}H]-*all-trans*-retinol and 0.10 μCi/g [15-^{14}C]-*all-trans*-retinol in 1 μL/g 2-propanol. After 4 h of dark adaptation, radioactive retinoids in the eye were analyzed by HPLC as described under Materials and Methods. ^{3}H:^{14}C ratios (background corrected) were determined and reported in Table I. Retinyl esters in the rat are a mixture of retinyl palmitate and retinyl stearate (Alvarez at al., 1981). Oximes are derivatives of retinal that are more resistant to isomerization during isolation.

To minimize the confounding effects of oxidation–reduction cycles that caused partial loss of label in retinol and its esters, the double-label experiments were repeated on rats that had been pretreated with 4-methylpyrazole. This alcohol dehydrogenase inhibitor is an inhibitor of dark adaptation in rats in the same manner as its somewhat less potent and effective congener pyrazole (Raskin et al., 1976; Blomstrand et al., 1979). A 500 mg/kg dose of 4-methylpyrazole inhibits dark adaptation ~50% at 4 h. In Table I, it can be seen that the 11-*cis*-retinyl esters now retain 81% of the tritium label, significantly ($P < 0.01$) higher than the 67% for control animals and well above the 50% level, unambiguously demonstrating that in rats isomerization occurs at the alcohol oxidation state. All other percent retention values were not significantly different from control values. By contrast, 1,5-bis(*p*-aminophenoxy)pentane, an inhibitor of regeneration that does not act by alcohol dehydrogenase inhibition but rather by facilitated isomerization of 11-*cis*-retinal back to *all-trans*-retinal (Bernstein & Rando, 1985; Bernstein et al., 1986), did not significantly ($P > 0.01$) alter any of the ratios.

It is interesting that the small pool of *all-trans*-retinol consistently lost half of its label under all conditions. The oxidation–reduction reactions for *all-trans*-retinoids, which appear to involve enzymes distinct from those for 11-*cis*-retinoids (Bridges, 1977), may be cycling very fast and may not be inhibitable by 4-methylpyrazole to the same extent that 4-methylpyrazole can inhibit the enzymes specific for 11-*cis*-retinoids. Alternatively, this *all-trans*-retinol pool may be derived from retinal released from rhodopsin chromophore exchange in the dark (Defoe & Bok, 1983).

Our experiments were not designed to distinguish between isomerization of vitamin A esters vs. vitamin A alcohols, but in the rat some of the data point to an ester isomerization. In particular, the consistent 50% loss of label in *all-trans*-retinol suggests that this pool could not serve as a precursor for the 11-*cis*-retinyl esters, which retain 67–81% of their label, but this cannot be regarded as definitive given that the kinetics of the various reactions comprising the visual cycle are unknown. Mechanistically, an alcohol isomerization is more likely than an ester isomerization because retinol is more amenable to energy input by chemical activations such as phosphorylation (Bhat et al., 1980) or by specific binding by cellular 11-*cis*-retinoid binding protein (Stubbs et al., 1979). Retinyl esters cannot access either of these energy sources.

Table II: Retinoid Pools in Dark-Adapting Rats: Drug Effects

experimental group[b]	retinoid HPLC peak size (integrated area ± SD, $n = 5$)[a]		
	11-*cis*-retinyl esters[c]	*all-trans*-retinyl esters[c]	11-*cis*-retinal *syn*-oxime
control, light adapted	234 ± 87	1970 ± 405	49 ± 23
control, dark adapted 4 h	74 ± 26	219 ± 43	724 ± 128
4-MP (500 mg/kg), dark adapted 4 h	209 ± 41	964 ± 507	359 ± 86
DAPP (100 mg/kg), dark adapted 4 h	68 ± 10	607 ± 230	458 ± 211

[a] Values are not corrected for differences in extinction coefficients at 313 nm. Approximate values of ϵ_{313} are 31 500 for 11-*cis*-retinyl palmitate, 41 000 for *all-trans*-retinyl palmitate, and 18 000 for the *syn*-oxime of 11-*cis*-retinal. [b] Abbreviations: 4-MP, 4-methylpyrazole; DAPP, 1,5-bis(*p*-aminophenoxy)pentane. [c] Retinyl esters in the rat are predominantly retinyl palmitate and retinyl stearate (Alvarez et al., 1981).

Table III: Double-Label Experiments in Frogs

hours of dark adaptation	% retention of ^{3}H label (relative to ^{14}C label)[a]				
	11-*cis*-retinyl palmitate	*all-trans*-retinyl palmitate	11-*cis*-retinal oximes	11-*cis*-retinol	*all-trans*-retinol
4	22 ± 10	77 ± 5	19 ± 2	18 ± 11	53 ± 7
24	7 ± 0	54 ± 3	5 ± 1	6 ± 4	17 ± 6

[a] All values are mean ± SD for $n = 3$.

Effects of Inhibitors of Dark Adaptation on Endogenous Retinoids in Rat Eye. The experiments described above indicate that in the rat isomerization of *all-trans*-retinoids to 11-*cis*-retinoids occurs at the alcohol oxidation state and that 4-methylpyrazole disrupts dark adaptation by inhibiting the alcohol dehydrogenase that oxidizes 11-*cis*-retinol to 11-*cis*-retinal. It can be postulated that when 4-methylpyrazole makes this oxidation step rate limiting in dark adaptation, it may be possible to detect an increase in 11-*cis*-retinol or its esters in the eye because isomerization could still occur while oxidation would be impaired. The first two lines of Table II show that when an animal goes from a light-adapted to a dark-adapted state, 11-*cis*-retinal is generated while the pools of 11-*cis*- and *all-trans*-retinyl esters are depleted. When dark adaptation is impaired by 4-methylpyrazole so that it is only ~50% complete at 4 h, the amount of 11-*cis*-retinyl esters in the eye is almost triple the amount found in dark-adapted controls and almost identical with the amount found in light-adapted controls. 11-*cis*-Retinol was undetectable in all of the experiments, indicating that in the steady-state 11-*cis*-retinoid esterification is strongly favored over hydrolysis of the esters, a result compatible with in vitro studies by Berman et al. (1985). Since new synthesis of 11-*cis*-retinyl esters certainly occurs in this time period as evidenced by the radiolabel studies of Table I, this is an example of accumulation of 11-*cis*-retinoids behind a pharmacological block of oxidation after isomerization has already taken place. This accumulation of 11-*cis*-retinyl esters is not simply a consequence of an increase in total esters in the eye due to impaired regeneration because it is not seen in rats given 1,5-bis(*p*-aminophenoxy)pentane in a dose that provides a similar degree of inhibition of dark adaptation (Table II).

Double-Label Experiments in Frog. The double-label approach was then applied to the visual cycle of the frog (*Rana*

pipiens). Table III shows that under experimental conditions comparable to those used for the rat there was a very large loss of tritium, which approached an almost total loss of label by 24 h for all retinoids except *all-trans*-retinyl palmitate. This type of result renders the goal of distinguishing the oxidation state of isomerization by this method unattainable for this animal. Since nonspecific losses of the ^3H label were negligible in the rat, it is probably unlikely that they would be substantially greater in the frog. More likely, the frog probably has active oxidation–reduction cycles combined with a lack of consistent stereochemistry of the redox enzymes. This inconsistent stereospecificity could be caused by distinct alcohol dehydrogenases that stereospecifically remove different prochiral hydrogens. Alternatively, a single alcohol dehydrogenase may not remove hydrogens stereospecifically, a situation known to occur with horse liver alcohol dehydrogenase when it interacts with long-chain alcohols (Shapiro et al., 1981). Unfortunately, alcohol dehydrogenase inhibitors are totally ineffective at inhibiting regeneration in the frog in vivo (Berstein & Rando, 1985), and several attempts at inhibiting this tritium loss with 4-methylpyrazole were unsuccessful (data not shown). This observed loss of tritium after injection of [15-^3H]retinol is quite consistent with unpublished observations of D. Bok and M. O. Hall (personal communication), who observed that after [15-^3H]retinol was injected into dark-adapted frogs the specific activity of the frog's rhodopsin peaked within 72 h of continued darkness and then rapidly declined; their result is explainable if opsin picks up some of the labeled 11-*cis*-retinal and shields it from label loss for a period of time until the protein releases the retinoid through chromophore exchange in the dark (Defoe & Bok, 1983), allowing it once again to lose its label. By contrast in rats, animals with apparently consistent stereochemistry of redox reactions in the eye, labeled 11-*cis*-retinal derived from [15-^3H]retinol and presumably extracted from rhodopsin does not show a decrease in specific activity beyond the obligate initial 50% loss for a period of many days (Zimmerman, 1974).

The nearly identical ^3H:^{14}C ratios in all 11-*cis*-retinoids both at 4 h and at 24 h point toward the conclusion that frogs probably do not produce 11-*cis*-retinoids by isomerization of retinyl esters for the following reasons. The data in Table III indicate that once an *all-trans*-retinyl ester is formed it is relatively protected against ^3H loss while at 4 h and again at 24 h all three types of 11-*cis*-retinoids have nearly identical large losses of ^3H label. Retinyl esterase in the eye is rather sluggish, and it is quite nonspecific with regard to which substrate it hydrolyzes (Berman et al., 1985; Krinsky, 1958). If an ester isomerization had occurred, it would be predicted that 11-*cis*-retinyl esters would be relatively protected against ^3H loss since it appears that they are hydrolyzed by the same sluggish esterase that hydrolyzes *all-trans*-retinyl esters. Moreover, 11-*cis*-retinyl esters are utilized at the same rate as *all-trans*-retinyl esters when a frog dark adapts (Bridges, 1976). Because 11-*cis*-retinyl esters are not preferentially shielded from ^3H loss, it can be concluded that 11-*cis*-retinoids are produced by isomerization of retinal or retinol, subjected to ^3H washout in oxidation–reduction cycles, and then esterified.

In performing the above experiments and similar single-label versions on frogs, it was noted that at 4 h most radioactive counts were usually associated with *all-trans*-retinyl palmitate; however, some control frogs and some frogs treated with 1,5-bis(*p*-aminophenoxy)pentane did not form large amounts of *all-trans*-retinyl palmitate but instead channeled most of the radioactive counts into 11-*cis*-retinol (Figure 2). Other

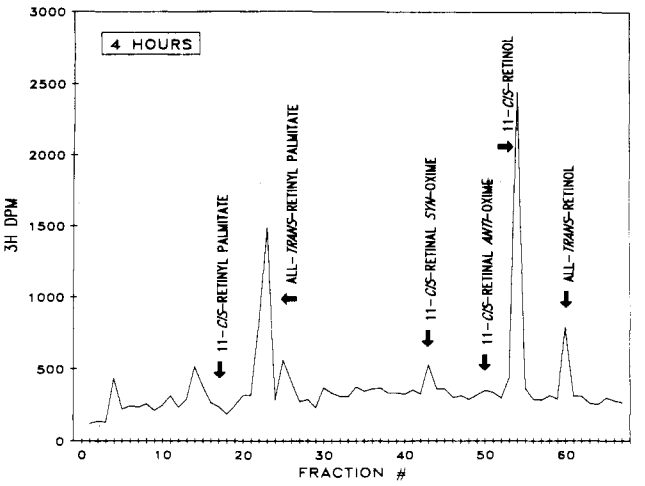


FIGURE 2: Labeled retinoids formed in the eye of a frog 4 h after injection with [^3H]retinyl acetate. A light-adapated frog was injected with 0.1 μCi/g [11,12-^3H]-*all-trans*-retinyl acetate. After 4 h of dark adaptation, radioactive retinoids in the eye were analyzed by HPLC. The only labeled 11-*cis*-retinoid found in substantial quantity was 11-*cis*-retinol. Similar bursts of 11-*cis*-retinol formation could also be found in frogs pretreated with three 100 mg/kg injections of 1,5-bis(*p*-aminophenoxy)pentane given over a 24-h period. [^3H]Retinyl acetate was never detected in the eye, and it is assumed that the ester was rapidly hydrolyzed in the periphery.

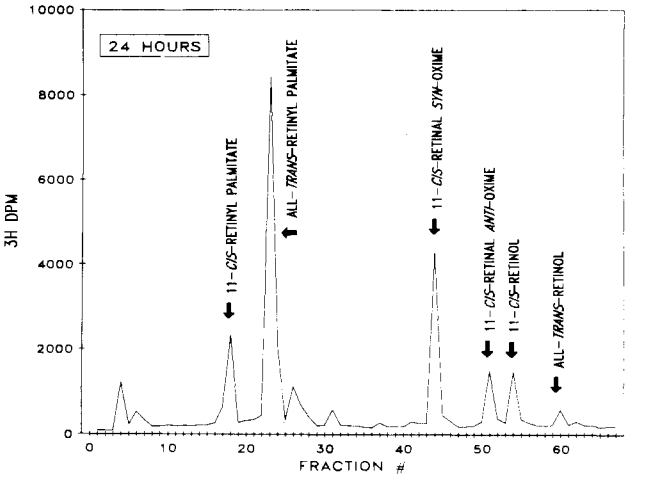


FIGURE 3: Labeled retinoids formed in the eye of a frog 24 h after injection with [^3H]retinyl acetate. A light-adapted frog was injected with 0.1 μCi/g [11,12-^3H]-*all-trans*-retinyl acetate. After 24 h of dark adaptation, radioactive retinoids in the eye were analyzed by HPLC. The distribution of counts among the radioactive retinoids corresponded closely to the distribution of endogenous retinoids.

11-*cis*-retinoids were present in only trace amounts. At 24 h in all frogs tested, substantial amounts of all three 11-*cis*-retinoids were present, and the distribution of radioactive counts in all retinoids was essentially identical with the distribution of endogenous retinoids (Figure 3). These observations of an early burst of 11-*cis*-retinol formation are consistent with isomerization occurring at the alcohol oxidation state in frogs as well as rats, and the observation that this burst can be seen in frogs treated with 1,5-bis(*p*-aminophenoxy)-pentane, an inhibitor of regeneration that selectively isomerizes retinals but not retinols (Bernstein et al., 1986), further supports this idea; however, they cannot be regarded as unequivocal evidence because rate-limiting isomerization of retinal coupled with very fast reduction could give the same results.

CONCLUSIONS

The double-label experiments in rats demonstrate that the in vivo pathway for production of 11-*cis*-retinoids involves an

isomerization at the alcohol oxidation state, not at the aldehyde oxidation state. Inhibitor studies with 4-methylpyrazole and 1,5-bis(*p*-aminophenoxy)pentane confirm and amplify this conclusion. In frogs, an apparent lack of consistent stereochemistry of the alcohol dehydrogenases coupled with the rapidity of the redox reaction precludes a definitive delineation of their isomerization pathway, although some experiments indicate that they too may isomerize *all-trans*-retinoids to 11-*cis*-retinoids at the alcohol oxidation state. This in vivo demonstration of the pathway for chromophore regeneration permits greater focus on the perplexing problem of visual pigment regeneration. Questions remain to be solved regarding the anatomical site, energy source, and biochemical mechanism of isomerization of *all-trans*-retinol or its esters to the corresponding 11-*cis*-retinoid congeners.

Registry No. *all-trans*-Retinal, 116-31-4; *all-trans*-retinyl palmitate, 79-81-2; *all-trans*-retinyl stearate, 631-87-8; 11-*cis*-retinal *syn*-oxime, 67999-43-3; 11-*cis*-retinal *anti*-oxime, 67999-44-4.

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