the bath during experiments using inside-out, excised patches. In this convention, the membrane potential is given as the voltage at the intracellular side of the membrane minus the voltage at the extracellular side of the membrane.
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9. In solutions lacking added divalent cations, roughly 10 to 20 μM divalent cations are expected because of contamination from the distilled water, NaCl, and other sources.
10. Because conductance was measured under current-clamp conditions, the conductance decrease shown is a lower limit.
11. Estimates of intracellular free Mg^{2+} concentration range from approximately 0.2 mM to 2 mM [D. Maughan and C. Recchia, *J. Physiol. (London)* **368**, 545 (1985)]. The range of estimates of free Mg^{2+} is well above the submicromolar levels of intracellular free Ca^{2+} estimated to be present in rod cells (*12*).
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14. Experiments were carried out in the presence of 0.1 or 0.05 mM Mg^{2+} and Ca^{2+} because rapid deterioration of cells was observed in the absence of added divalent cations. We assume that the lack of divalent cation–suppressed conductance in half of these patches was due to the formation of sealed vesicles, which is known to occur in the presence of bath Ca^{2+} (*6*). Other evidence suggesting that the divalent cation–suppressed conductance resides in the membrane rather than the seal is that the size of the divalent cation–suppressed conductance was not obviously correlated with the net conductance of the patch (that is, seal plus membrane). Furthermore, R. Coronado [*Biophys. J.* **47**, 851 (1986)] reported that, although pure lipid membranes composed primarily of negatively charged lipid did result in a divalent cation–dependent seal conductance, seals formed with pure lipids with a composition similar to that of the rod outer segment [E. H. Drenthe *et al.*, *Biochim. Biophys. Acta* **603**, 130 (1980); G. P. Miljanich, P. P. Nemes, D. L. White, E. A. Dratz, *J. Membr. Biol.* **60**, 249 (1981)] were largely independent of divalent cations.
15. Zero current and voltage were defined as the crossing point of the curves in symmetric conditions of monovalent ions. Results similar to those of Fig. 2, A and B, were obtained when NaCl was replaced by iso-osmotic amounts of sucrose, indicating that the observed changes in conductance did not arise from changes in osmotic strength. The combination of junction and streaming potentials measured with a 3M KCl electrode for the various solutions in Fig. 2, C and D, were less than, and usually much less than, 4 mV.
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21. We thank T. Wiesel for continued support, suggestions, and encouragement, and J. Lisman and S. Temple for their comments on a previous version of this manuscript. The basic observations described here have been reported in an earlier abstract [J. H. Stern, H. Knutsson, P. R. MacLeish, *Invest. Ophthalmol. Visual Sci.* **27**, 301a (1986)]. Funding for this work was from NIH grant EY05201, NIH fellowship EY05730, the Klingenstein Fund, the Alfred P. Sloan Foundation, and a Senator Jacob Javits Center for Excellence in Neuroscience Award (NS-22789).

9 September 1986; accepted 17 April 1987

The Visual Cycle Operates via an Isomerase Acting on All-*trans* Retinol in the Pigment Epithelium

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Thirty years have elapsed since Wald and his colleagues showed that 11-*cis* retinal was isomerized to all-*trans* when rhodopsin was bleached, yet little has been understood about the reverse process that generates 11-*cis* retinal for rhodopsin regeneration. It is not known whether the isomerization is enzyme-mediated, whether it occurs in the pigment epithelium or in the retina, or whether retinal, retinol, or a retinyl ester is the vitamin A compound that is isomerized. Radiolabeled all-*trans* retinol and high-performance liquid chromatography have now been used to demonstrate the existence of an eye-specific, membrane-bound enzyme (retinol isomerase) that converts all-*trans* to 11-*cis* retinol in the dark. Retinol isomerase is concentrated in the pigment epithelium; this localization clarifies the role of this tissue in rhodopsin regeneration and explains the need to transfer all-*trans* retinol from the rod outer segments to the pigment epithelium during the visual cycle.

The isomerization of all-*trans* retinoids to the 11-*cis* configuration is essential for visual pigment regeneration in the eye (*1*). A "retinal isomerase" originally reported by Hubbard (*2*) apparently acted by photoisomerization of an artifactually generated protonated Schiff base composed of all-*trans* retinal and phosphatidylethanolamine (*3*). Furthermore, this observation is probably not physiologically significant, because exposure to potentially isomerizing light does not influence the course of dark adaptation in vertebrates (*4*). Although it had been suggested that the isomerization may not be enzyme-mediated, recent data by the same authors that appeared since the present work was submitted demonstrate a retinol-specific isomerase in pigment epithelium (*5*). Here we have studied frogs and rats because the visual cycle has been extensively investigated in these animals (*6, 7*). We present evidence that both species possess an eye-specific, membrane-bound enzyme that converts all-*trans* to 11-*cis* retinol in the dark and therefore fulfills the primary requirement for an isomerase that plays a central role in dark adaptation. Although earlier evidence suggested otherwise (*6, 8, 9*), this isomerase is concentrated in the pigment epithelium.

We centrifuged homogenates of combined retina, pigment epithelium, and choroid from light- or dark-adapted frogs at 700*g* to pellet the nuclei and unbroken cells, then incubated the supernatants in darkness with all-*trans* [^{3}H]retinol. After 3 hours, we extracted the mixture and found that the major radiolabeled vitamin A compound (excluding all-*trans* retinol) was 11-*cis* retinol (Table 1). The very low levels of radioactivity associated with 11-*cis* retinyl palmitate confirm in vitro studies (*9*) and are also consistent with the very slow appearance of labeled 11-*cis* retinyl palmitate in the eyes of living frogs injected with all-*trans* [^{3}H]retinol (*9*). The higher proportion of retinyl ester formed by the 700*g* pellet indicates that there was some enrichment of the ester synthase in this fraction. Our subsequent experiments focused on the formation of 11-*cis* retinol by the 700*g* supernatant with protein concentrations of 0.3 to 11.8 mg/ml.

A typical set of data is shown in Fig. 1. The high-performance liquid chromatography (HPLC) tracing from the absorbance detector (Fig. 1, top) shows that the endogenous retinol isomers extracted from the incubation mixture are mainly 11-*cis* (0.52 nmol) and all-*trans* (0.24 nmol), with a small amount of 13-*cis* (0.03 nmol) but no detectable 9-*cis* (in other experiments, traces of the 9-*cis* isomer were variably present). The radioactivity profile (Fig. 1, center) shows a prominent peak (25% of the total extracted radiolabeled retinol isomers) that coelutes with 11-*cis* retinol and a smaller peak (8% of the total radiolabel) that coelutes with 13-*cis* retinol. The truncated peak (59% of the radioactivity) coelutes with the all-*trans* retinol substrate (Fig. 1, bottom). We did not identify the remaining peaks, but observed them consistently in most experiments.

To confirm the identities of the labeled presumptive 11-*cis* and 13-*cis* retinols, they were collected from the column, oxidized to the aldehydes with activated manganese dioxide (*10, 11*), and mixed with unlabeled authentic 11-*cis*, 13-*cis*, and all-*trans* retinal. Analysis by HPLC (*10*) showed that the oxidation procedure had yielded the corresponding labeled 11-*cis* and 13-*cis* retinal

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Table 1. Distribution of radiolabel in vitamin A compounds extracted from tissue fractions incubated with all-*trans* [^{3}H]retinol. Methods are as described (*21*).

Fraction	Protein (mg/ml)	Total labeled retinoid (pmol)	Retinyl palmitate (%)			Retinal (%)	Retinol (%)		
			11-*cis*	13-*cis*	All-*trans*	All isomers	11-*cis*	13-*cis*	All-*trans*
700*g* supernatant									
Experiment 1	6.4	2.36	0.2	0.2	6.2	6.2	20.8	11.2	55.3
Experiment 2	9.1	2.29	0.6	0.4	4.5	4.5	30.6	19.8	39.6
700*g* pellet									
Experiment 1	18.1	2.20	0.2	0.2	14.8	3.0	14.9	30.1	36.8
Experiment 2	20.7	3.09	0.3	0.3	17.5	5.7	15.2	22.8	38.2

isomers, in each case mixed with 7 to 8% all-*trans*. All-*trans* retinal was produced when the presumptive all-*trans* retinol was similarly treated.

We demonstrated the enzymatic properties of the isomerizing reaction that generated 11-*cis* retinol by heating the 700*g* supernatant to 100°C for 5 minutes before incubating with all-*trans* [^{3}H]retinol. No 11-*cis* retinol was evident after 3 hours (Fig. 2A). In contrast, the unheated control (Fig. 2B) had a prominent radioactive peak in the 11-*cis* retinol position. In other respects the two radioactive profiles were similar, particularly with regard to the presence of labeled 13-*cis* retinol. Formation of this isomer was also observed in medium that did not contain tissue homogenate (*9*), consistent with the report that isomerization about the C-13 double bond is a thermally favored pathway (*12*). In the unheated controls the amount of 11-*cis* retinol tripled between 1.5 hours (Fig. 2C) and 3 hours (Fig. 2B), whereas the 13-*cis* showed much less change, suggesting that it was generated rapidly at the beginning of the experiment.

Confirmation that an enzyme was involved in the formation of 11-*cis* retinol was provided by the observation that its formation was inhibited by phenylmethylsulfonyl fluoride (1 m*M*) and destroyed by prior incubation at 37°C with trypsin (50 μg/ml). The inhibition by phenylmethylsulfonyl fluoride suggests that serine hydroxyl groups (*13*) are important for isomerase activity.

Eleven-*cis* isomers are confined to ocular tissues. We therefore determined whether isomerizing activity was present in frog liver and brain. As expected, no 11-*cis* retinol was evident when homogenates of these tissues were incubated with all-*trans* [^{3}H]retinol (Fig. 2D).

It appeared that 11-*cis* retinol was formed by an enzyme that was sedimented when the 700*g* supernatant was centrifuged at 100,000*g* for 1 hour. The high-speed pellet [resuspended in modified Roswell Park Memorial Institute (RPMI) 1640], the high-speed supernatant, and a portion of the original 700*g* supernatant were assayed for isomerase activity by incubating with all-*trans* [^{3}H]retinol. In two experiments, the average amount of 11-*cis* [^{3}H]retinol recovered after 3 hours was 0.69 pmol from the 700*g* supernatant, 0.62 pmol from the high-speed pellet, and only 0.06 pmol from the high-speed supernatant. This experiment showed that isomerizing activity was in a membrane fraction. Because this fraction lacked cellular and interstitial retinol-binding proteins (*14, 15*) and cellular retinal-binding protein (*16*) (which had been retained in the high-speed supernatant), these proteins are not essential elements in the isomerizing reaction.

Virtually all of the retinol isomerase activity was in the pigment epithelium. Dark-adapted retinas were carefully separated from the underlying pigmented layers. The

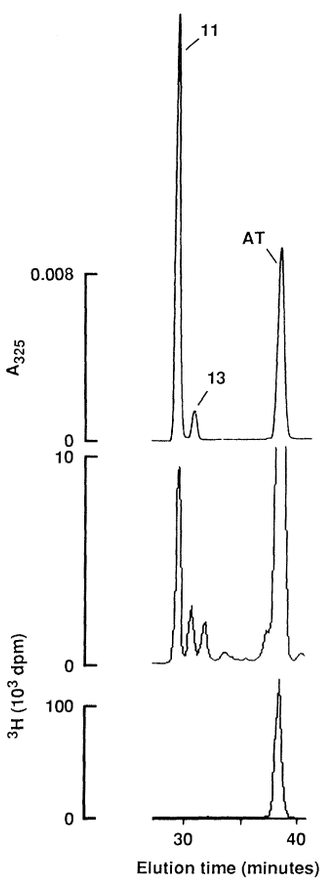

Fig. 1. Formation of radiolabeled 11-*cis* retinol in the 700*g* supernatant from combined retina, retinal pigment epithelium, and choroid of light-adapted frogs. (**Top**) Absorbance detector tracing. (**Middle**) Radioactivity profile (fractions were manually collected at a point immediately after the absorbance detector; the intervals were 0.1 minute to ensure resolution of 11-*cis* and 13-*cis* retinol, then 0.2 minute until the all-*trans* isomer had eluted). (**Bottom**) Radioactivity profile of freshly purified substrate (3.5 pmol of all-*trans* [11,12-^{3}H]retinol). Incubation conditions were as in Table 1, except that 19 pmol of all-*trans* [11,12-^{3}H]retinol was used, and 0.5 ml of the 700*g* supernatant was diluted to 5 mg of protein per milliliter by addition of an equal volume of modified RPMI 1640.

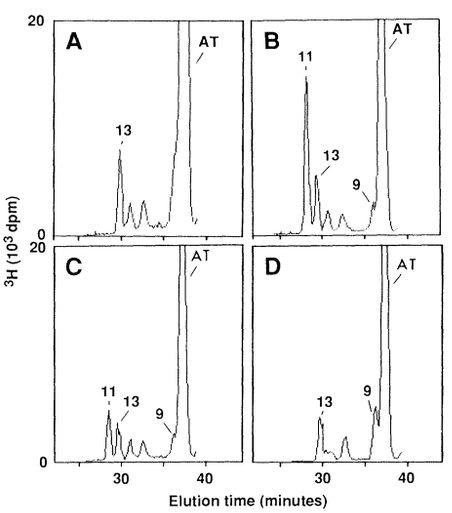

Fig. 2. Abolition of retinol isomerase activity by heat and absence of retinol isomerase in liver; 700*g* supernatants (about 0.8 mg of protein per milliliter) were incubated with 27 pmol all-*trans* [11,12-^{3}H]retinol as in Table 1. Except for panel A, which was for 1.5 hours, all incubations were for 3 hours. (**A, B,** and **C**) Combined retina, pigment epithelium, and choroid from dark-adapted frogs [in (A), the 700*g* supernatant had been heated at 100°C for 5 minutes and cooled to room temperature before addition of substrate]. (**D**) Frog liver. No 11-*cis* retinol peak is evident in (A) and (D). Note that the 9-*cis* retinol peak is more prominent in the liver preparation.

11-*cis* retinol formed during a standard 3-hour incubation in the 700*g* supernatant from the retinas (2.2 mg of protein per milliliter) was only 13% of that from the corresponding pigmented layers (1.7 mg of protein per milliliter) (Fig. 3). The probable contamination of these retinas by pigment epithelium makes it likely that retinol isomerase is absent from the retina. There was no significant difference between light- and dark-adapted tissues (Fig. 3).

Similar results were obtained with tissues from light-adapted rats (preparation and conditions as described for frogs, except incubations were at 37°C). In the 700*g* supernatant from the combined pigment epithelium and choroid (four eyes), 0.52 pmol of 11-*cis* retinol was formed, while only 0.12 pmol was formed from the retinas and none from the livers. These results are also in accord with the suggestion that in vivo isomerization of all-*trans* to 11-*cis* retinoids in the rat occurs at the alcohol oxidation state (*17*).

Our data provide insight into the molecular basis of the visual cycle. The localization of retinol isomerase in the pigment epithelium of frogs and rats explains the essential role of this layer in rhodopsin regeneration (*18*) and therefore accounts for the need to transfer all-*trans* retinol to the pigment epithelium during light-adaptation (*6, 7*). We have demonstrated that the enzyme is also present in the more readily available (and larger) bovine pigment epithelium, which can provide enough material for solubilization and characterization of the enzyme, for elucidation of its mechanism of action, and for determination of how the activity of the enzyme is complemented by the extracellular retinol transport protein interstitial retinol-binding protein (*15, 19*).

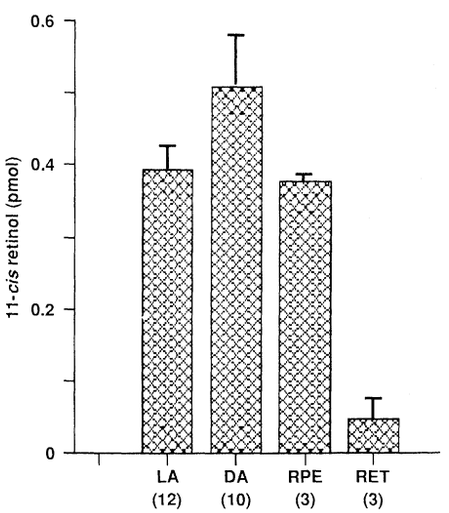

Fig. 3. Comparison of retinol isomerase activity in light- and dark-adapted tissues, retinas, and pigmented layers, The 700*g* supernatants from the combined retina, pigment epithelium, and choroids of light-adapted (LA) and dark-adapted (DA) frogs are compared with the 700*g* supernatants from isolated retinas (RET) from dark-adapted frogs and from the corresponding combined pigment epithelium and choroids (RPE). The number of experiments is indicated in parentheses; bars indicate standard errors of the mean. The 11-*cis* isomer accounted for 15 to 45% of the total radiolabel eluting in the retinol region, as exemplified by the chromatograms in Figs. 1 and 2. Recovery of added radiolabel in the form of retinol ranged from 9 to 26%. The retinas were peeled away from the underlying pigmented layers after the excised eyes (from which the anterior half had been removed) had been immersed in modified RPMI 1640. All other conditions were as in Table 1.

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21. *Rana pipiens* that had been dark-adapted for 12 hours were light-adapted for 4 hours in a white tank under a 100-W lamp (in other experiments, the frogs were kept dark-adapted). This procedure bleached more than 90% of the rhodopsin. All subsequent operations were carried out in darkness or under dim red safelights. The combined retina, retinal pigment epithelium, and choroid were removed from six frogs, homogenized by hand at 4°C in 1 ml of filter-sterilized RPMI 1640 medium containing 30 m*M N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (Hepes), *p*H 7.5, modified for retina and pigment epithelium culture (experiment 1) (*15, 19*). A second homogenate was prepared from tissue collected from another batch of six frogs (experiment 2). The homogenates were centrifuged at 700*g* for 20 minutes. The 700*g* supernatants were removed and retained, and the 700*g* pellets were resuspended in 1 ml of modified RPMI 1640. After 50 μl had been withdrawn for protein determination (*20*), we successively added 50 μl of 10% bovine serum albumin, 2 μl of an ethanol solution containing 22 pmol of all-*trans* [11,12-^{3}H]retinol (Amersham; 53 Ci/mmol) and 1 μg of *d*-alpha-tocopherol (Eastman Kodak). The all-*trans* retinol had been freshly purified by HPLC as described below. After 3 hours of incubation in capped containers in darkness at room temperature, the reaction vials were chilled on ice, 1 ml of ethanol was added, and the mixture was extracted twice with 3 ml of *n*-hexane. A stream of nitrogen was then used to reduce the volume of *n*-hexane to 100 μl (determined by weight). For retinol and retinal analysis, a 4- to 5-μl sample was injected onto a Supelcosil 3-μm column (4.6 by 150 mm) (mobile phase, 4% dioxane in *n*-hexane; flow rate, 0.6 ml per minute). In this system, the retinal isomers eluted ahead of the retinol isomers. Because the peaks were compressed together, it was not possible to resolve them on the radioactivity profiles. Therefore, only the radiolabel associated with all the retinal isomers was measured. The source of this labeled retinal was not determined. Some of it may have originated from the small amount of rhodopsin remaining after light adaptation, although ethanol and hexane are not efficient for extracting retinal from this source. For the retinyl esters, a second 4- to 5-μl injection was made on the same column but with a mobile phase consisting of 0.2% methyl-*t*-butyl ether in *n*-hexane at a flow rate of 0.7 ml per minute. The absorbance detector (Kratos) was set at 325 nm. The minimum amount of a retinol isomer detectable by this instrument was 10 pmol. Fractions were manually collected into scintillation vials, 10 ml of ACS (Amersham) scintillant was added, and the radioactivity determined in a Packard Tricarb liquid scintillation spectrometer. Results were plotted with an IBM PC XT. The minimum amount of labeled retinol isomer that could be detected was 15 fmol. Mixtures of authentic unlabeled 11-*cis*, 13-*cis*, 9-*cis*, and all-*trans* retinol, retinal, and retinyl palmitate were prepared (*10*) and chromatographed regularly for calibration purposes and to monitor column performance (*10*).

    The radiolabel recovered in the retinol region of the chromatograms ranged from 9 to 26% of the added all-*trans* [^{3}H]retinol (in Table 1, the recovery is 11 to 14%). Because retinol was extracted with an efficiency of 75 to 80% and because the remaining radioactivity was not extracted into hexane, it appears that some of the added retinol had been metabolized to more polar compounds during the period of incubation.

22. Supported by grants from the Retina Research Foundation of Houston, National Institutes of Health (National Eye Institute), and Research to Prevent Blindness, Inc.

27 January 1987; accepted 22 April 1987