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Isomerization of all-*trans*-retinoids to 11-*cis*-retinoids *in vitro*

(vitamin A/rhodopsin/regeneration)

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ABSTRACT **The key biochemical process of the vertebrate visual cycle required for rhodopsin regeneration, 11-*cis*-retinoid production from all-*trans*-retinoids, is shown to occur *in vitro*. A 600 × *g* supernatant from a frog retina/pigment epithelium homogenate transforms added all-*trans*-[^{3}H]retinol, in a time-dependent fashion, to a mixture of 11-*cis*-retinol, 11-*cis*-retinal, and 11-*cis*-retinyl palmitate. 13-*cis*-Retinoids are formed in only minor amounts by nonspecific processes. Studies using washed particulate fractions of the 600 × *g* supernatant indicate that all-*trans*-[^{3}H]retinol is isomerized to 11-*cis*-retinoids much more effectively than is all-*trans*-[^{3}H]retinal or all-*trans*-[^{3}H]retinyl palmitate. The 11-*cis*-retinoid biosynthetic activity is heat-labile, sedimentable by high-speed centrifugation, and largely found in the pigment epithelium rather than in the neural retina.**

The absorption of light by rhodopsin in the vertebrate eye results in the *cis*-to-*trans* photoisomerization of its 11-*cis*-retinal chromophore, bound as a protonated Schiff base to lysine, eventually leading to the hydrolysis and release of the all-*trans*-retinal (1, 2). One of the rhodopsin conformers during this bleaching process, metarhodopsin II, catalyzes the binding of GTP in exchange for GDP by a retinal GTP-binding (G) protein, thus initiating the process of visual transduction (3, 4). Under bright light conditions the all-*trans*-retinal liberated by bleaching is reduced, esterified to long-chain fatty acids, and stored in the pigment epithelium of the eye (5). When a light-adapted animal encounters a dark environment, 11-*cis*-retinoids must be regenerated from the stores of all-*trans*-retinoids, and the 11-*cis*-retinal produced can then combine with opsin to form rhodopsin. The ocular biosynthesis of 11-*cis*-retinal in the dark is, by definition, a thermal process. It is also an endergonic process because at thermal equilibrium 11-*cis*-retinoids represent only 0.1% of the retinoids (6), while in a dark-adapted eye at least 75% of the retinoids are in the 11-*cis* form, with the remaining retinoids in the all-*trans* form (5). The driving force cannot simply arise from the stereospecific combination of 11-*cis*-retinal with opsin because in many higher vertebrates such as man and amphibians, there is a 2- to 3-fold excess of retinoids over opsin in the eye (5, 7).

A particularly vexing problem in visual science has been the mechanism of 11-*cis*-retinoid biosynthesis in the eye. Major hurdles include the identification of the substrate for isomerization (retinol, retinal, or retinyl ester), the nature of the energy source that drives the biosynthesis of 11-*cis*-retinoids, and most importantly, the identification of the isomerizing system capable of producing 11-*cis*-retinoids *in vivo*. No system that can produce 11-*cis*-retinoids *in vitro* in darkness has yet been confirmed (8). Over the years several attempts have been made at the identification of an isomerase enzyme specific for retinal (9, 10); however, these attempts have suffered from a misidentification of rhodopsin because isorhodopsin, the nonphysiological product of 9-*cis*-retinal and opsin, was apparently formed (11, 12). In this report, an isomerizing system capable of generating 11-*cis*-[^{3}H]retinoids from added all-*trans*-[^{3}H]retinol is established using homogenates of frog retina/pigment epithelium. These studies pave the way for the eventual purification and characterization of the eye's retinoid isomerizing system.

MATERIALS AND METHODS

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

11-*cis*-Retinoid Production in Frog Eye Homogenates from Added All-*trans*-[^{3}H]Retinol. The retina and pigment epithelium from individual eyes from light-adapted frogs (*Rana pipiens*) were obtained by standard methods (13) and placed in 2-ml centrifuge tubes. In a few experiments the frogs were dark-adapted overnight before sacrifice, and the retina and pigment epithelium were separated during dissection. Buffer (500 μl of 50 mM sodium phosphate, pH 7.2) was added, and the tissue was homogenized by 10 sec of sonication at 75% power with a microultrasonic cell disrupter (Kontes). In most experiments the homogenate was then centrifuged at 600 × *g* for 10 min at 4°C to sediment unbroken cells, nuclei, and pigment granules.

After centrifugation the 500 μl of supernatant was transferred to an 8-ml glass vial. Then 5–25 μl was withdrawn for analysis of protein content by the Peterson modification of the Lowry method (14). To serve as retinol carrier, 25 μl of a 10% (wt/vol) solution of bovine serum albumin (Sigma; fatty acid free, preserved with 0.1% NaN_3) was then added, followed by 2 μCi of all-*trans*-[11,12-^{3}H]retinol (Amersham; 60 Ci/mmol; 1 Ci = 37 GBq; >95% pure) in 2 μl of ethanol (preserved with butylated hydroxytoluene at 1 mg/ml). The tube was stoppered, wrapped in foil, and incubated at room temperature on a Nutator (Clay Adams) for orbital mixing. A control tube containing no eye tissue, but otherwise identical, was prepared for each assay.

Analysis of [^{3}H]Retinoids Formed *in Vitro*. For retinol analysis after incubation, 200 μl of assay mix was added to 200 μl of 50 mM octyl β-D-glucoside (Calbiochem). This was followed by 400 μl of methanol and finally by 500 μl of hexane containing butylated hydroxytoluene at 1 mg/ml. After vigorous shaking the material was centrifuged at 13,000 × *g* for 10 min at 4°C.

The hexane extract (200 μl) was mixed with 10 μl of a standard mixture of carrier retinol isomers in hexane prepared by photoisomerization of retinal in methanol, followed by reduction with $NaBH_4$ (15). This mixture was then injected into a Waters HPLC system with a 5-μm Merck LiChrosorb RT Si 60 silica column (250 × 4.0 mm). Detection was by absorbance at 320 nm, and the eluant was 7% (vol/vol) dioxane in hexane at a rate of 2 ml/min to provide optimum separation of 11-*cis*-retinol from 13-*cis*-retinol (16). With this chromatographic system, however, 9-*cis*-retinol

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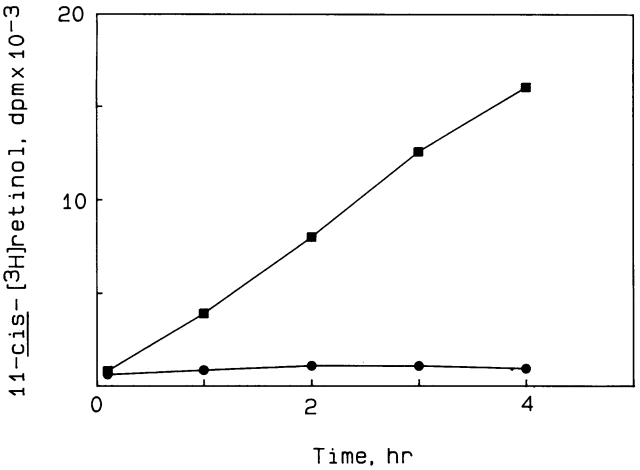

FIG. 1. Formation of 11-*cis*-[^{3}H]retinol *in vitro*. A 1.0-ml aliquot of 600 × *g* supernatant from light-adapted frog retina/pigment epithelium (two eyes) was incubated with 4 μCi of all-*trans*-[11,12-^{3}H]retinol, and 11-*cis*-[^{3}H]retinol content was assayed hourly from 200-μl portions. ■, 11-*cis*-[^{3}H]Retinol content in retina/pigment epithelium supernatant. ●, 11-*cis*-[^{3}H]Retinol content in a control incubation with no eye tissue present in the buffer.

and all-*trans*-retinol coelute. Since 9-*cis*-retinol is not found in the eye physiologically and since at equilibrium 9-*cis*-retinoids are found only in relatively minor amounts (6), the peak consisting of all-*trans*-retinol and 9-*cis*-retinol is referred to as simply all-*trans*-retinol. The HPLC traces of the coinjected isomeric retinol standards were identical to published chromatograms (17).

There is never a true baseline separation of 11-*cis*-retinol and 13-*cis*-retinol, which makes a time-based collection mode highly problematic since inevitably there will be fractions that contain both 11-*cis*-retinol and 13-*cis*-retinol. To maximize the distinction between these two isomers, samples containing each isomeric retinol were collected on a Gilson 201 microprocessor-controlled fraction collector in the peak detection mode. Its trigger threshold was set higher than the lowest point of the valley between the 11-*cis*-retinol and 13-*cis*-retinol peaks (ordinarily 5–10% full scale). Appropriate delay for the dead volume between detector and dropping needle was programmed into the collector, and all baseline effluent was discarded. Comparison of time-based collection with peak-based collection demonstrated that peak-based collection recovered >90% of the radioactivity when HPLC-purified retinoids were injected. Samples were counted in 4.5 ml of Soluscint-O (National Diagnostics, Somerville, NJ) in a Beckman LS 1800 scintillation counter interfaced with an Apple II Plus computer for data analysis. All counts were corrected for quench and background.

The hexane extraction method described above was compared to a more complicated extraction method using NH_2OH and CH_2Cl_2, known to extract all retinoids from eye tissue quantitatively (18). The hexane extraction method was found to extract retinols quantitatively, but retinals and retinyl esters were not extracted well, although isomeric distributions were unaltered. In a few experiments the NH_2OH/CH_2Cl_2 method was used to examine all retinoids formed. Since retinal *syn*-oxime isomers often coelute with retinol in a dioxane/hexane chromatographic system, 8% (vol/vol) ether in hexane was used; however, 11-*cis*-retinol and 13-*cis*-retinol coelute in this system (18). Retinyl palmitate esters were analyzed with 0.5% ether in hexane.

11-*cis*-Retinoid Production in Frog Eye Homogenates from All-*trans*-[^{3}H]Retinal and All-*trans*-[^{3}H]Retinyl Palmitate. All-*trans*-[11,12-^{3}H]retinal (6 Ci/mmol) and all-*trans*-[11,12-^{3}H]-retinyl palmitate (60 Ci/mmol) were prepared by standard methods (15) from all-*trans*-[11,12-^{3}H]retinol. All products were HPLC purified before use. Frog eye 600 × *g* supernatants were prepared as described above with the exception that 0.2 μCi of all-*trans*-[^{3}H]retinal was used instead of the 2 μCi used for the other retinoids. In some experiments the particulate fraction was washed by two successive pelletings at 50,000 × *g* for 20 min at 4°C, followed by resuspension each time in the original volume of buffer.

RESULTS AND DISCUSSION

Formation of 11-*cis*-Retinol in Frog Retina/Pigment Epithelium Homogenates. The biochemical mechanism whereby 11-*cis*-retinoids are generated from all-*trans*-retinoids is one of the major unsolved problems in visual science (8). Studies in our laboratory have shown that bleached frog eye cups incubated *in vitro* can synthesize 11-*cis*-retinoids *de novo* because they can undergo multiple cycles of complete dark and light adaptation, and they can form stores of 11-*cis*-retinyl palmitate (unpublished data). In this article we report that 11-*cis*-retinoids can be produced from all-*trans*-retinoids in a cell-free system. The biotransformation of high specific activity all-*trans*-[11,12-^{3}H]retinol in homogenates of frog retina/pigment epithelium was examined with the aid of HPLC analysis. The retina and pigment epithelium from light-adapted frogs were thoroughly disrupted by sonication and centrifuged at 600 × *g* for 10 min. The supernatant was incubated with all-*trans*-[11,12-^{3}H]retinol in the dark for up to 4 hr. As can be seen in Fig. 1, a time-dependent, approximately linear increase in the amount of 11-*cis*-[^{3}H]retinol formed was found, while in the absence of eye tissue none was produced. In Table 1, data are shown for the relative amounts of the various isomers of [^{3}H]retinol present at 3 hr

Table 1. *In vitro* production of isomeric retinols from all-*trans*-[^{3}H]retinol in 600 × *g* supernatants of eye homogenates incubated 3 hr

Experiment	% 11-*cis*-retinol	% 13-*cis*-retinol	% all-*trans*-retinol	% total recovery*	Protein, mg/ml
No eye (n = 9)	0.2 ± 0.1	2.9 ± 1.1	96.9 ± 1.1	100	—
Light-adapted eye (n = 7)	25.6 ± 3.8	7.6 ± 2.4	66.9 ± 6.0	13.9 ± 3.1	4.2 ± 0.6
Light-adapted eye, boiled 5 min (n = 3)	0.4 ± 0.1	5.9 ± 0.5	93.7 ± 0.5	105.6 ± 3.9	5.4 ± 1.6
Dark-adapted eye (n = 3)	12.3 ± 2.8	9.4 ± 4.8	78.5 ± 7.5	17.8 ± 4.1	4.2 ± 0.8
Dark-adapted retina (n = 3)	2.7 ± 1.5	7.0 ± 1.4	90.3 ± 2.0	43.0 ± 13.2	3.2 ± 1.4
Dark-adapted pigment epithelium (n = 3)	10.6 ± 5.0	9.3 ± 4.1	80.1 ± 8.7	10.2 ± 1.8	1.0 ± 0.6
Light-adapted eye, 150,000 × *g* pellet (n = 5)	32.8 ± 14.1	16.8 ± 4.9	50.5 ± 18.1	7.4 ± 1.7	2.0†
Light-adapted eye, 150,000 × *g* supernatant (n = 5)	1.6 ± 1.1	1.7 ± 0.9	96.7 ± 1.6	58.1 ± 2.8	2.8†

*All values are mean ± SD. Total recovery of all isomeric retinols is relative to concurrent control incubations with no eye tissue present.
†Protein content was measured a single time.

Table 2. Chemical identification of 11-*cis*-retinol formed *in vitro*

	Isomer			
	% 11-*cis*	% 13-*cis*	% 9-*cis*	% all-*trans*
11-*cis*-Retinol peak rechromatographed	91.3	5.9	—	2.9
11-*cis*-Retinol peak esterified with palmitoyl chloride (50% recovery)	94.3	3.6	1.7	0.4
11-*cis*-Retinol peak isomerized with 0.1% I_2 for 15 min (100% recovery)	2.7	26.3	—	71.0

A 600 × *g* supernatant from frog retina/pigment epithelium was incubated 3 hr. The 11-*cis*-retinol peak was collected during HPLC and analyzed as indicated. Total recovery of counts was relative to rechromatography of 11-*cis*-retinol peak.

in control and in 600 × *g* supernatant incubations. The fact that 13-*cis*-[^{3}H]retinol, a retinol isomer of no known visual function, is not formed in substantial amounts in either type of incubation is clearly shown.

The amount of extracted [^{3}H]retinol in the control experiment remained constant throughout the time course. On the other hand, only 10–15% of the added radioactivity (relative to the control incubation) was extracted as [^{3}H]retinol in the eye tissue incubation after the first 10 min. Experiments described later in this paper show that the remainder of the radioactivity is present as retinal and retinyl palmitate. It is apparent that in the first minutes there is a rapid distribution of part of the added all-*trans*-[^{3}H]retinol into the two other major retinoid pools. The 11-*cis*-retinol, along with other 11-*cis*-retinoids, is then generated over a period of hours from this mixed retinoid substrate.

Chemical Identification of the 11-*cis*-[^{3}H]Retinol Product of Isomerization. Although 11-*cis*-retinol is separated well from the other isomeric retinoids by HPLC analysis, further chemical criteria need to be met before a positive chemical identification of the 11-*cis*-[^{3}H]retinol can be made. The putative 11-*cis*-[^{3}H]retinol peak was collected from the HPLC and was chemically transformed into products identical to those formed with unlabeled 11-*cis*-retinol. First, rechromatography of the initially isolated 11-*cis*-[^{3}H]retinol showed that >90% of the radioactivity coeluted with standard 11-*cis*-retinol (Table 2). The isolated 11-*cis*-[^{3}H]retinol was then either transformed into isomerically pure 11-*cis*-[^{3}H]retinyl palmitate with palmitoyl chloride (15), or it was isomerized by I_2 to convert 11-*cis*-retinol to the expected equilibrium mixture of *cis*- and *trans*-retinols (6) with <3% of the radioactivity still eluting with 11-*cis*-retinol (Table 2). These studies allow for the unequivocal identification of the 11-*cis*-[^{3}H]retinol as a product of all-*trans*-[^{3}H]retinol conversion by the retina/pigment epithelium 600 × *g* supernatant.

Characterization of 11-*cis*-Retinol Production in Eye Homogenates *in Vitro*. As expected, heating the extract destroyed the 11-*cis*-retinol synthetic ability of the 600 × *g* supernatant (Table 1). Approximately the same percentage of 13-*cis*-[^{3}H]retinol is formed in the presence of native and boiled extract, and this material presumably arises as a consequence of nonspecific isomerization because, when retinoids are at thermal equilibrium, 20–30% 13-*cis*-retinoids would be expected (6). It was of further interest to determine the anatomical site of synthesis of the 11-*cis*-retinol. To this end, frogs were dark adapted to allow for a more complete dissection of the retina from the pigment epithelium. Prior dark adaptation decreases the overall activity of the 600 × *g* supernatant from dark-adapted versus light-adapted frogs (Table 1). This could be of possible interest in terms of the regulation of 11-*cis*-retinoid biosynthesis. The extracts from the retina and from the pigment epithelium of dark-adapted frogs were assayed separately, and it was clear that most of the activity, as measured by percent conversion of the retinol pool to 11-*cis*-retinol, resides in the pigment epithelium. Small amounts of unavoidable cross-contamination between retina and pigment epithelium were observed during dissection, but certainly the activity per milligram of protein is much higher in the pigment epithelium than in the retina; in one case where a particularly good dissection of the pigment epithelium from the retina was performed, virtually no isomerizing activity was found in the retina. A heat-labile 11-*cis*-retinoid synthetase activity has also been found in bovine retina/pigment epithelium 600 × *g* supernatants (unpublished data). In this case, where the separation is more complete, at least 90% of the activity was found in the pigment epithelium. These results would explain why pigment regeneration has never been observed, by electrophysiological measurements, in bleached preparations of neural retina after the addition of all-*trans*-retinoids (19, 20). Cultures of human and frog pigment epithelium cells have also been reported to be unable to synthesize 11-*cis*-[^{3}H]retinyl palmitate from added all-*trans*-[^{3}H]retinol (17, 21); however, an abstract by one of these same authors (22) suggests that the human pigment epithelium may be able to synthesize 11-*cis*-retinoids from added radioactive all-*trans*-retinol.

To characterize further the 11-*cis*-retinoid biosynthesis activity, the homogenate from the retina/pigment epithelium was centrifuged at different speeds for 10 min to determine whether the activity could be sedimented. Isomerizing activity is almost completely pelleted by centrifugation at 150,000 × *g* (Table 1). As shown in Fig. 2, increasing the speed of centrifugation progressively decreased the supernatant's activity, suggesting that it may be membrane bound. Further sedimentation studies in this laboratory using Percoll density gradients indicate that the isomerizing activity peaks at a density that is slightly lower than that of the major protein peak (unpublished data). The fact that 11-*cis*-retinoid synthesis activity sedimented and was not present in high-speed supernatants suggests that known retinoid binding proteins

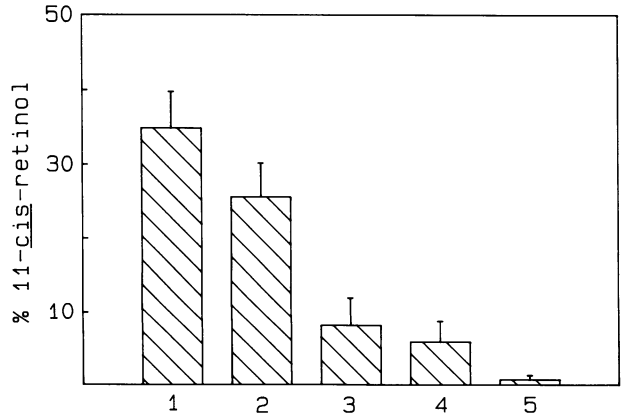

FIG. 2. 11-*cis*-[^{3}H]retinol production in centrifuged retina/pigment epithelium homogenates. Retina/pigment epithelium homogenates from light-adapted frogs were centrifuged 10 min as follows: no centrifugation (bar 1), centrifugation at 600 × *g* (bar 2), centrifugation at 13,000 × *g* (bar 3), centrifugation at 25,000 × *g* (bar 4), and centrifugation at 150,000 × *g* (bar 5). The supernatants were incubated with all-*trans*-[11,12-^{3}H]retinol for 3 hr, and the [^{3}H]retinol content was measured and plotted as % 11-*cis*-retinol in the retinol pool. All values are mean ± SD (n = 2–7).

Table 3. Isomeric composition of retinals and retinyl esters formed in 600 × *g* supernatants of eye homogenates during 3-hr *in vitro* incubations with all-*trans*-[^{3}H]retinol

Retinoid	Isomer				% total recovery*
	% 13-*cis*	% 11-*cis*	% 9-*cis*	% all-*trans*	
Retinyl palmitate (n = 3)	2.3 ± 1.6	4.0 ± 1.8	4.4 ± 1.0	89.3 ± 3.9	72.5 ± 0.6†
Retinal *syn*-oxime (n = 4)	20.5 ± 13.9	36.6 ± 15.3	—‡	42.9 ± 13.5	8.3 ± 0.9†

All values are mean ± SD.
*Combined recovery of all isomers of the particular retinoid was relative to total recovery of all retinoids in concurrent control incubations with no eye tissue present.
†Based on two experiments.
‡Coelutes with the 13-*cis*-isomer.

(23, 24), all of which are soluble proteins, are not involved in this mechanism of 11-*cis*-retinoid synthesis.

Studies on the Identity of the Substrate for Isomerization to 11-*cis*-Retinoids in Eye Homogenates *in Vitro*. During the studies of the time-dependent increase in 11-*cis*-[^{3}H]retinol in frog eye homogenates in Fig. 1, it was noted that there was a 10–15% recovery of counts in the retinol isomer peaks, relative to control incubations with no eye tissue present, at all time points taken after the first 10 min. Since eye homogenates are known to have retinol dehydrogenase activity, retinyl ester synthetase activity, and retinyl esterase activity (8), 3-hr incubations of eye homogenates were examined using an NH_2OH/CH_2Cl_2 procedure (18) for complete retinoid extraction. It was found that 10–15% of the radioactivity was recovered as retinols, 5–10% as retinals (isolated as the *syn*-oxime derivatives), and 70–80% as retinyl palmitate esters. The isomeric distributions of the retinals and retinyl esters are shown in Table 3. By multiplying the percent 11-*cis* by the percent total recovery for each retinoid, it is apparent that 3.6%, 3.0%, and 2.9% of the added radioactivity is present in the 11-*cis*-retinol, 11-*cis*-retinal, and 11-*cis*-retinyl palmitate pools, respectively, for a total of 9.5% 11-*cis*-retinoids generated. The 3% *trans*-to-*cis* conversion per hr in this preparation is intermediate between the *in vivo* rate of formation of 11-*cis*-retinoids during dark adaptation in the retina, 20–40% *trans*-to-*cis* conversion of the eye's total retinoids in 2 hr, and the rate of formation of stores of 11-*cis*-retinyl palmitate in the pigment epithelium in the dark, 20–40% *trans*-to-*cis* conversion in 24 hr (5). In subsequent tissue preparations (Table 4), the 11-*cis*-retinoid production was even higher.

The rapid distribution of the all-*trans*-[^{3}H]retinol between the aldehyde, alcohol, and ester pools makes it difficult to determine which type of retinoid is the actual substrate for isomerization. To clarify this point various putative all-*trans*-[^{3}H]retinoid substrates were added to washed and unwashed particulate fractions. When all-*trans*-[^{3}H]retinal was added to a 600 × *g* supernatant of eye homogenate, it gave results identical to those for the addition of all-*trans*-[^{3}H]retinol (Table 4). The incubations with all-*trans*-[^{3}H]retinol and all-*trans*-[^{3}H]retinal were then repeated on the washed particulate fraction of the 600 × *g* supernatant in the anticipation that the rate of interconversion between retinal and retinol could be decreased by removal of soluble cofactors for oxidation and reduction. In a washed particulate preparation much less of the added retinal substrate is reduced, and production of 11-*cis*-retinoids from all-*trans*-retinal is severely decreased (Table 4). It is especially striking that 11-*cis*-retinal formation is virtually eliminated. Formation of 11-*cis*-retinol and 11-*cis*-retinyl palmitate from added all-*trans*-retinol is increased by washing the membranes while 11-*cis*-retinal formation is less than one-third of the value before washing. Table 4 shows that when all-*trans*-[^{3}H]retinyl palmitate was added to a 600 × *g* supernatant of retina/pigment epithelium, it was totally inert. This retinoid is ordinarily quite insoluble in aqueous buffers, although there was sufficient albumin present to bring it into solution. These experiments with the various retinoids indicate that all-*trans*-retinol is the most likely substrate for isomerization in this system, a result consistent with the lag-free time course of 11-*cis*-retinol production observed in Fig. 1.

CONCLUSION

It has been shown (25, 26) that, in the rat and probably in the frog, isomerization occurs at the alcohol-oxidation state, and the results reported in this paper are consistent with this. The biological energy source needed for the biosynthesis of

Table 4. *In vitro* formation of 11-*cis*-retinoids from all-*trans*-[^{3}H]retinoids in washed and unwashed 600 × *g* supernatants of eye homogenates (3 hr)

Form of all-*trans*-[^{3}H]-retinoid supplied	Retinol		Retinal		Retinyl palmitate	
	% 11-*cis*	% total recovery*	% 11-*cis*	% total recovery*	% 11-*cis*	% total recovery*
Retinol (no eye, n = 6)	<1	>99	—	<1	—	<1
Retinol (unwashed membranes, n = 3–5)	36.9 ± 5.9	15.6 ± 2.6	45.8 ± 15.4	11.6 ± 1.4	12.7 ± 5.2	72.8†
Retinol (washed membranes, n = 4–6)	40.3 ± 9.9	12.0 ± 3.3	14.5 ± 14.1	8.5 ± 3.4	14.4 ± 5.9	79.5†
Retinal (no eye, n = 5)	—	<1	1.8 ± 1.2	>98	—	<1
Retinal (unwashed membranes, n = 2)	33.7 ± 8.4	18.4 ± 1.6	31.8 ± 9.4	14.0 ± 3.1	14.7 ± 2.8	67.6†
Retinal (washed membranes, n = 2)	16.6 ± 2.2	7.9 ± 4.1	1.5 ± 0.7	60.2 ± 5.1	12.0 ± 1.1	31.9†
Retinyl palmitate (no eye, n = 2)	—	<1	—	<1†	<1	>99
Retinyl palmitate (unwashed membranes, n = 2)	—	<1	—	<1†	<1	>99

All experiments in this table were performed with no ethanol present while those of Tables 2 and 3 had 0.4% ethanol in the incubation mixture.
All values are mean ± SD.
*Combined recovery of all isomers of the particular retinoid relative to total recovery of all retinoids in concurrent control incubations with no eye tissue.
†Total recovery was not determined in these instances, but the experiments of Tables 2 and 3 indicate that the sum of recoveries for all three major retinoids is always ≈100%.

11-*cis*-retinoids cannot be identified at this time. The fact that 11-*cis*-[^{3}H]retinoid biosynthesis occurred in the absence of any added cofactors means that a sufficient endogenous energy source is available for the accumulation of the radioactive 11-*cis*-retinoids. An analogous situation arises with the formation of radioactive all-*trans*-retinyl palmitate using pigment epithelium microsomes and radioactive all-*trans*-retinol in the absence of an added energy source to drive the esterification (27).

Further purification of the isomerizing system will clarify its energy requirements and its other characteristics. Nevertheless, even in its unpurified state this isomerizing system is biologically relevant and important for the following reasons: (*i*) An *in vitro* transformation of all-*trans*-retinoids to 11-*cis*-retinoids has been demonstrated. (*ii*) The process stereospecifically produces 11-*cis*-retinoids in preference to 13-*cis*-retinoids. (*iii*) Boiling results in a complete loss of 11-*cis*-retinol forming activity. (*iv*) The isomerizing activity can be sedimented by centrifugation. (*v*) In the dark-adapted eye the isomerizing activity is largely localized in the pigment epithelium. (*vi*) The isomerizing activity in washed-membrane preparations is much higher when added all-*trans*-retinol is the substrate than when all-*trans*-retinal or all-*trans*-retinyl palmitate are used as substrates.

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