Retinoid Kinetics in Eye Tissues of VPP Transgenic Mice and Their Normal Littermates

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PURPOSE. VPP mice, which possess a mutant transgene for opsin (V20G, P23H, P27L), exhibit a progressive rod degeneration that resembles one form of human autosomal dominant retinitis pigmentosa. In the present study the association of the development of VPP rod degeneration with abnormal operation of the retinoid visual cycle was examined.

METHODS. Dark-adapted VPP mice and normal littermates were anesthetized and the pupils dilated. One eye of each animal was illuminated for 2 minutes; the other eye was shielded from the light and served as a control. Each animal was then dark adapted for a defined period (0-300 minutes) and killed. Retinoids contained in the retina, retinal pigment epithelium (RPE), and extracellular medium were recovered by means of formaldehyde-, isopropanol- and ethanol-based extractions and analyzed by high-performance liquid chromatography.

RESULTS. Total amounts of retinoid recovered from unilluminated eyes of 2-month-old normal and VPP mice were 425 ± 90 picomoles per eye and 115 ± 33 picomoles per eye, respectively (mean ± SD). Relative distributions of retinoids within normal and VPP eyes were similar. In normal and VPP animals, illumination for 2 minutes produced a similar immediate reduction in the molar percent of total retinoid represented by 11-cis retinal in the retina (average reduction of 34% and 28% in normal and VPP animals, respectively) and a similar transient increase of all-trans retinal in the retina. In both groups the decline of all-trans retinal was accompanied by an increase in total retinyl ester. In normal and VPP animals, a period of approximately 40 minutes or more preceded initiation of the recovery of 11-cis retinal in the retina, and the time course of this recovery was generally similar to that for the decline of retinyl ester. The overall dark-adaptation period required for half-completion of 11-cis retinal recovery was approximately 150 minutes. In neither group did illumination produce a substantial peak of all-trans retinol in the retina.

CONCLUSIONS. The evident approximately fourfold reduction of total retinoid in the eyes of 2-month-old VPP mice is consistent with histologic and electroretinographic abnormalities determined in previous studies. Despite this marked abnormality in retinoid content, retinoid cycling in the VPP is remarkably similar to that in normal littersmates. The data place constraints on the functional consequences of any abnormality in retinoid processing that may be present at this stage of the VPP rod degeneration. (Invest Ophthalmol Vis Sci. 1999;40:1040-1049)
The importance of the retinoid visual cycle for normal rod function raises the general question of whether the course of the VPP rod degeneration may involve and perhaps be influenced by a developing abnormality in a step of this cycle. It was of interest to investigate, for example, whether this degenerative process is associated with a build-up of all-trans retinol, a rhodopsin bleaching product that is known to have deteriorative effects on membrane structure.14-15 Previous studies have further shown that prolonged dietary deprivation of vitamin A, which reduces the retinoid content of the eye tissues, leads to degeneration of the photoreceptors.16-19 Thus, it was also of interest to determine whether photoreceptor degeneration in the VPP is associated with abnormal dark-adapted levels of visual cycle retinoids. Here, we report experiments designed to quantify the dark-adapted distribution and bleach-induced processing of retinoids within the VPP eye and thus, to test for visual cycle abnormalities in the VPP model of autosomal dominant retinitis pigmentosa. Most of the experiments were conducted on 2-month-old VPP mice and normal littermates, an age at which previous studies have indicated a significant extent of degeneration in the VPP retina. Preliminary results have been presented.20-21

METHODS

Illumination and Dark Adaptation

All procedures conformed to the principles embodied in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. VPP mice and normal littermates were raised under cyclic illumination (12-hour light/12-hour dark; ambient illumination of approximately 5-10 lux during the light phase) and genetically typed by PCR amplification and identification of the restriction fragment length polymorphism introduced into the transgene.7 Animals were dark adapted overnight, and all experiments were performed under dim red light. Mice were anesthetized with a mixture of ketamine (0.15-0.18 mg/g body weight) and xylazine (0.004-0.006 mg/g body weight) and the pupils dilated with phenylephrine hydrochloride and tropicamide. The animal was positioned beneath a microscope illuminator fitted with a broad-band filter that transmitted full-field green light. One eye (designated L) was illuminated for a fixed period (2 minutes), while the other eye (designated D) was shielded from the light. At the position of the illuminated eye, the incident intensity was approximately 5.8 x 10^-9 W/cm² (model IL 1700 calibrated photometer; International Light, Newburyport, MA). After illumination, the mouse was dark adapted for a defined period (0-300 minutes) and then killed by cervical dislocation. Drops of water were applied to each eye at roughly 30-minute intervals during the dark-adaptation period to prevent drying of the corneal surface. A second dose of ketamine and xylazine was administered approximately 20 minutes before the end of the dark-adaptation period when this period exceeded 60 minutes.

Dissections, Tissue Homogenization, and Extraction

Eye tissues were prepared for retinoid analysis using procedures based on those recently described for the toad eye.22-23 Dissection of the eyes was begun immediately after death. Unless otherwise indicated, the results represent data obtained from the analysis of single eyes. The illuminated eye (L) was in all cases dissected first. The globe was opened by an incision at the cornea. After removal of the lens, the retina was gently removed and rinsed in 300 µl phosphate-buffered saline (PBS). The remainder of the globe, which contained the RPE and choroid, was then removed and rinsed in 300 µl PBS. The two 300-µl volumes of rinse medium were combined (sample termed "extracellular medium") and added to 1.2 ml ethanol, yielding sample a. The retina was then ground (1-ml manual tissue grinder; Wheaton Co., Millville, NJ) in 250 µl PBS (approximately 20 seconds). An aliquot (100 µl) of the resultant homogenate was transferred to a tube containing 400 µl formylaldehyde (aqueous solution, 37% wt/vol; sample b); another 100 µl aliquot of the homogenate was added to a tube containing 400 µl isopropanol (sample c). Similar, separate grinding of the remainder of the globe in 250 µl PBS produced a homogenate of the RPE/choroid (sample henceforth termed RPE) free of scleral material; 100-µl aliquots of the resultant homogenate were transferred to 200 µl formaldehyde (sample d) and 400 µl isopropanol (sample e). All samples were then vortexed.

Dissection and homogenization of tissues from the unilluminated eye (D) were then performed in similar fashion. Experiments on mice of ages 12 days and 3 to 5 months involved study only of dark-adapted eyes (unilluminated animals) and, in the case of 12-day-old animals, involved a modified procedure in which the two enucleated eyes of a given animal were pooled for homogenization and extraction (i.e., no prior separation of the retina and RPE).

Levels of retinaldehydes in the retina and RPE were determined from data obtained with formaldehyde-based extraction.23-25 Determinations of retinol and retinyl ester in the retina and RPE were based on data obtained with isopropanol-hexane extraction. Determinations of all retinoids in the extracellular medium involved the use of ethanol-hexane extraction. The extraction procedures were as follows. Samples b and d obtained from each eye were each supplemented with 400 µl isopropanol and vortexed. Samples b, c, d, and e were supplemented with 500 µl water and vortexed. All samples were then extracted three times with 2 ml n-hexane. The hexane extract obtained from each sample was evaporated to dryness under nitrogen, then redissolved in 200 µl hexane.

High-Performance Liquid Chromatography

Extracts prepared as described were analyzed for retinoids by normal-phase high-performance liquid chromatography (HPLC; Adsorbosphere HS 5-µm silica column, 250 x 4.6 mm; Alltech, Deerfield, IL). All separations used an elution rate of 1 ml/min. Absorbance was monitored at 365 nm and 325 nm, and molar amounts of retinoids were determined with the use of retinoid standards and published extinction coefficients.22-25 The standard elution program used for determinations of retin aldehydes and retinoids and for routine determinations of retinyl ester used dioxane/hexane (20/80 vol/vol; solvent A) and n-hexane (solvent B) and was identical with that previously described (linear gradient, with the ratio B:A equal to 70:30 at time 0 and equal to 15.85 at t = 25 minutes).23

Representative profiles obtained with this elution program are shown in Figure 1. The illustrated data describe extracts of the retina and RPE obtained from the unilluminated eye (Figs. 1A, 1C) and from the illuminated eye (Figs. 1B, 1D) of a 2-month-old normal mouse. This standard program did not separate retinyl esters containing different fatty acid moieties.
FIGURE 1. HPLC profiles obtained from the eye tissues of a 2-month-old normal mouse. Data obtained using the standard HPLC elution program (see the Methods section). One eye (L) of the animal was illuminated for 2 minutes, then dark adapted for 90 minutes; the other eye (D) remained in darkness. (A, B) Samples obtained from the unilluminated (D) eye and the illuminated (L) eye, respectively, by formaldehyde-based extraction of the retina. Detection at 365 nm. (C, D) Samples obtained from the unilluminated (D) eye and the illuminated (L) eye, respectively, by isopropanol-hexane extraction of the RPE. Detection at 325 nm. Vertical markers represent absorbance units (AU). Arrows indicate the elution times of the 13-cis, 11-cis, 9-cis, and all-trans isomers of retinal (AL), of 11-cis and M-trans retinol (OL), and of retinyl ester (RE).

or isomers of a given ester. Molar amounts of retinyl ester were calculated from integrated peak areas using the extinction coefficient of all-trans retinyl palmitate. Separations of 11-cis and all-trans retinyl esters were performed using elution with dioxane/hexane (5/95 vol/vol; solvent A) and n-hexane (solvent B; linear gradient, with the ratio B:A equal to 95:5 at time 0 and equal to 80:20 at t = 15 minutes).

For all isomers of retinal and retinol investigated, authentic standards yielded single HPLC peaks when analyzed by the standard program. However, hexane solutions of all-trans retinyl palmitate standard, when prepared for HPLC by evaporation of the solvent and redissolving of the retinoid in hexane, yielded two peaks (elution times of 230 and 260 seconds). Furthermore, peaks at these two elution times were routinely observed in the final hexane extracts of tissue samples that putatively contained retinyl palmitate (Figs. 1C, 1D). In control experiments, HPLC of all-trans retinyl palmitate standard solutions that had not been evaporated to dryness yielded only the 230-second peak. In addition, the combined areas under the two peaks of pre-evaporated standard solutions approximately matched the peak area of the single 230-second peak obtained with the nonevaporated solution. On this basis the 260-second peak was interpreted as a derivative of all-trans palmitate that arises on preparative evaporation and that possesses an extinction coefficient similar to that of the parent compound. The evident change produced by evaporation may represent alteration within the fatty acid moiety of the all-trans retinyl palmitate, because no corresponding change was observed in a control experiment analyzing retinyl acetate. The present determinations of retinyl ester were based on analysis of the 230-second and 260-second peaks. The detection limit for all retinoids in a given HPLC profile was 2 picomoles or less.

Quoted molar amounts of retinoid are in all cases those determined for the entire starting homogenate (retina, RPE, or whole eye) or starting solution (extracellular medium). The combined total amount of retinoid determined from analysis of the retina plus RPE plus extracellular medium will be referred to as the total retinoid content of the eye. With the exception noted below, the data reported are those for which the results obtained from a given animal fulfilled three criteria: the total amount of retinoid (picomoles) determined for the D and L eyes differed by less than 12% from the average of the D and L determinations; the molar amount of 11-cis retinal determined for the retina of the D eye was more than 35% of total retinoid recovered from this eye; and total retinoid determined for a given eye was more than 20 picomoles. For the 2-month-old animals, the results presented were obtained from 27 normal mice and 31 VPP mice that fulfilled these criteria. Data from an additional 14 2-month-old animals (9 normal, 5 VPP) were
RESULTS

Retinoid Content of Dark-Adapted Eyes

In Figure 2, the total retinoid contents of unilluminated eyes of normal mice (open circles) and VPP mice (filled circles) that ranged in age from 12 days to approximately 5 months are shown. The data indicate, in picomoles per eye, the total amount of retinoid determined by analysis of the retina, extracellular medium, and RPE, or, in the case of 12-day animals, by analysis of the whole eye. Total retinoid in the eyes of normal mice amounted to 248 ± 31 picomoles/eye at 3 weeks of age and 425 ± 90 picomoles/eye at 2 months. In VPP mice, total retinoid levels in 3-week-old and 2-month-old eyes were 120 ± 25 picomoles/eye and 115 ± 33 picomoles/eye, representing approximately 48% and 27%, respectively, of the amounts determined in age-matched normal mice. Within the group of 2-month-old mice of a given genetic type, there was no significant difference by sex in the weight of the animal or the total retinoid content of the unilluminated eye (data not shown).

There was no significant difference in the total content of retinoid (picomoles per eye) in normal eyes over the interval of 2 to 5 months, but VPP eyes showed a significant decrease in total retinoid content over the same interval (Fig. 2). In mice of age 12 days, levels of total retinoid per eye were 116 ± 15 picomoles in normal mice; VPP eyes contained 80 ± 14 picomoles, approximately 69% of the normal value. Thus, a substantial difference in total retinoid content was apparent as early as 12 days of age.

Figure 3 shows the distribution of retinoids within the retina, extracellular medium, and RPE in unilluminated eyes of 2-month-old normal mice (Figs. 3A, 3B, 3C) and VPP mice (Figs. 3D, 3E, 3F). In each panel the open bar at the right indicates, as a molar percent, the total amount of retinoid in the sample normalized to the total retinoid content of the eye. Filled bars within each panel indicate similarly normalized levels of the 11-cis, all-trans, 9-cis, and 13-cis isomers of retinal (11-cis AL, trans AL, 9-cis AL, and 13-cis AL, respectively); of all-trans retinol (trans OL); and of retinyl ester (RE). Retinyl ester was typically dominated by the all-trans isomer (see later description), and in no case was a significant amount of 11-cis retinol observed. Most of the detected retinoid in the unilluminated eye of normal mice was contained in the retina (79.0% ± 7.2%; Fig. 3A, open bar). Here, 11-cis retinal was the dominant retinoid, representing 66.3% ± 6.1% of total retinoid content. As in the figures described below, the determination of 11-cis retinal in the retina shown in Figure 3A was obtained with formaldehyde-based extraction23-25 and, on the basis of previous results from amphibian retina,23 probably represented, largely or entirely, rhodopsin chromophore rather than non-chromophoric 11-cis retinal. The level of all-trans retinal in the retina amounted to 8.5% ± 5.2% of total retinoid. This all-trans retinal may have arisen from the photoisomerization of 11-cis retinal chromophore by the red light used for dissection and preparative procedures or by thermal isomerization of extracted 11-cis isomer. Alternatively, the observed all-trans retinal may have been present in the native tissue.

The extracellular medium of normal eyes contained only small amounts of retinoid (5.0% ± 2.4% of total retinoid; Fig. 3B, open bar), which may have derived from contamination from the retina or the RPE. The amount of retinoid present in
FIGURE 3. Molar percents (mean ± SD) of retinoids recovered from the retina, RPE, and extracellular medium of unilluminated eyes of 2-month-old mice. Each bar represents the molar amount of the indicated retinoid normalized to the total molar amount of retinoid recovered from the eye. (A, B, C) Data from 27 normal mice. (D, E, F) Data from 31 VPP mice. Note the differing vertical scales among panels.

the RPE of normal mice (16.1% ± 6.5% of total retinoid; Fig 3C, open bar) was small when compared with that in the retina and consisted largely of retinyl ester (13.6% ± 6.4% of total retinoid). The distributions of retinoids in 2-month-old VPP mice are shown in Figures 3D, 3E, and 3F. Despite the marked difference in total retinoid content of the eyes of normal mice versus VPP mice (Fig. 2), the distribution of retinoids within the VPP eye tissues was similar to that of normal mice, in that there was no significant difference between the two groups in relative levels of any of the detected retinoids in the retina, extracellular medium, and RPE. Normal and VPP mice of age 3 weeks were similarly analyzed for the distribution of retinoids in the unilluminated eye (data not shown). Results obtained from these animals (11 normal mice, 12 VPP mice) indicated distributions that did not differ significantly from those determined from the 2-month-old animals.

Retinoid Kinetics

The investigation of retinoid kinetics involved a standard 2-minute illumination of one eye, dark adaptation for a defined period, and killing of the animal for retinoid analysis. The end of the 2-minute illumination period defined time 0 in each experiment. Figures 4A and 4B show results obtained, respectively, from 2-month-old normal and VPP mice. The illustrated data indicate results for 11-cis retinal in the retina (circles), all-trans retinal in the retina (squares), all-trans retinol in the retina (triangles), and total retinyl ester (diamonds). The retinyl ester data reflect combined results obtained from the retina, extracellular medium, and RPE. This summation compensates for the typically small amounts of retinyl ester detected in the retina and medium, which may have been caused by contamination by the RPE.

The data of Figures 4A and 4B are plotted in relation to a normalized parameter termed the “change in molar percent (L-D)” (see legend of Fig. 4). This representation facilitated the comparison of data obtained from normal versus VPP mice, which exhibited markedly different absolute levels of retinoid. Expression through this parameter also corrected for differences in the total molar amount of retinoid in the eyes of different mice of the same genotype. Relative to determina-
FIGURE 4. Retinoid kinetics in 2-month-old normal mice (A) and VPP mice (B), expressed as the change in molar percent (L-D) for 11-cis retinal (11-cis AL) in the retina, all-trans retinal (trans AL) in the retina, all-trans retinol (trans OL) in the retina, and total retinyl ester (RE). Time 0 represents the time of termination of the 2-minute illumination. For each animal within a given group, and for each retinoid detected, the change in molar percent (L-D) was determined as follows. The amount of the retinoid (picomoles) in a given sample obtained from the illuminated (L) eye was normalized to the L eye's total molar amount of retinoid. The amount of the retinoid in the corresponding sample from the unilluminated (D) eye of the same animal was normalized to the D eye's total retinoid level. The molar percent determined for the D eye was then subtracted from that for the L eye. The ordinate value of each data point in Figures 4A and 4B represents the average ± SD of the change in molar percent (L-D) for a given group of mice; the abscissa value is the average ± SD for the period of dark adaptation. The number in parentheses that accompanies the data at a given average period of dark adaptation indicates the number of normal mice (A) or VPP mice (B) analyzed. (C) Molar percent of 11-cis retinal in the retina of the unilluminated (D) eye, plotted as a function of the dark adaptation period. Groups of animals are the same as those described in (A, B). See text for further details.
produced an immediate average decrease of 34% in the molar amount of retinoid in the dark-adapted eye (Fig. 3), this 34% change amounted to an average decrease of 52% in the level of all-trans retinal in the retina, reflecting the photosomerization of rhodopsin chromophore. Because 11-cis retinal, as shown in Figure 4A, this 34% change amounted to an average decrease of 52% in the level of all-trans retinal in the retina. This light-induced decrease appeared to persist for approximately 40 minutes or more after the illumination—that is, the Figure 4A results obtained with all-trans retinal were preceded by a lag period of approximately 40 minutes and thereafter showed no significant departure from baseline. Only at \( t = 40 \) minutes did the molar percent of all-trans retinol differ from baseline, and this value was very low (1.7% ± 1.4%). Thus, the present conditions of rhodopsin bleaching did not lead to the accumulation of all-trans retinol in the retina.

Consistent with the formation of retinyl ester from all-trans retinol delivered to the RPE (see the Introduction) and with the approximately 40-minute time scale for half-completion of the decline of all-trans retinol, the change in molar percent for retinyl ester grew from a near-zero value immediately after the illumination to a peak average value of 31% at \( t = 93 \) ± 14 minutes (Fig. 4A). The retinyl ester level subsequently declined on a time scale similar to that of the reappearance of 11-cis retinal in the retina. At all postbleach times, retinyl ester recovered with the RPE typically far exceeded the amounts recovered in the retina or extracellular medium (see, e.g., Table I). HPLC separations of 11-cis and all-trans retinyl ester were performed in some experiments (see the Methods section). The results indicated only a small contribution by 11-cis retinyl ester to the total amount of recovered retinoid (<4%, on average), and these amounts were similar in unilluminated (D) and illuminated (L) eyes (data obtained from 12 normal and 14 VPP mice; not illustrated).

Changes in molar percent (L-D) for the indicated retinoids recovered from the eye tissues of 2-month-old VPP mice are shown in Figure 4B. As in normal mice, the bleaching illumination produced an immediate decrease in the level of 11-cis retinal and a transient increase in all-trans retinal; the recovery of 11-cis retinal was preceded by a lag period of approximately 50 minutes; there was no accumulation of all-trans retinol; and the level of retinyl ester was near-zero immediately after the illumination. Furthermore, the decline of retinyl ester from peak and the reappearance of 11-cis retinal in the retina developed on a similar time scale. Absolute levels (in picomoles) and

### Table 1. Amounts (picomoles) of Retinoids in Illuminated (L) and Unilluminated (D) Eyes of 2-Month-Old Normal Mice at \( t = 40 \pm 16 \) Minutes after the 2-Minute Illumination Period

<table>
<thead>
<tr>
<th>Sample</th>
<th>9-cis Retinal</th>
<th>13-cis Retinal</th>
<th>11-cis Retinal</th>
<th>All-trans Retinal</th>
<th>All-trans Retinol</th>
<th>Retinyl Ester</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td><strong>Illuminated Eye</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Retina</td>
<td>8 ± 2</td>
<td>6 ± 1</td>
<td>138 ± 24</td>
<td>91 ± 44</td>
<td>10 ± 8</td>
<td>12 ± 8</td>
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<td>0 ± 0</td>
<td>3 ± 1</td>
<td>2 ± 2</td>
<td>4 ± 3</td>
<td>1 ± 1</td>
<td>46 ± 44</td>
<td>56 ± 47</td>
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<tr>
<td>RPE</td>
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<td>0 ± 0</td>
<td>3 ± 5</td>
<td>2 ± 2</td>
<td>2 ± 2</td>
<td>165 ± 58</td>
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<tr>
<td>Retina</td>
<td>4 ± 1</td>
<td>6 ± 2</td>
<td>321 ± 44</td>
<td>30 ± 7</td>
<td>2 ± 2</td>
<td>9 ± 5</td>
<td>372 ± 36</td>
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<td>Extracellular medium</td>
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<td>3 ± 1</td>
<td>7 ± 4</td>
<td>0 ± 0</td>
<td>17 ± 14</td>
<td>30 ± 10</td>
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<tr>
<td>RPE</td>
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<td>0 ± 1</td>
<td>8 ± 8</td>
<td>2 ± 2</td>
<td>2 ± 2</td>
<td>79 ± 21</td>
<td>91 ± 26</td>
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</table>

Results obtained from five mice. The upper entry of each pair indicates, in picomoles, the mean ± SD of the determined amount of retinoid. The lower entry (in parentheses) represents the mean ± SD of the molar percent for the indicated retinoid (normalization to total retinoid content of the eye). The total retinoid content for the illuminated (L) eyes was 492 ± 26 picomoles per eye; that for the unilluminated (D) eyes was 495 ± 43 picomoles per eye.

Figure 4A shows also that in normal mice the illumination produced a substantial increase in the molar percent of all-trans retinal to 27%, on average, above the baseline level. With increasing period of dark adaptation, all-trans retinal declined to baseline with a half-completion time of approximately 40 minutes and thereafter showed no significant departure from baseline. Only at \( t = 40 \) minutes did the molar percent of all-trans retinol differ from baseline, and this value was very low (1.7% ± 1.4%). Thus, the present conditions of rhodopsin bleaching did not lead to the accumulation of all-trans retinol in the retina.
Table 2. Amounts (picomoles) of Retinoids in Illuminated (L) and Unilluminated (D) Eyes of 2-Month-Old VPP Mice at t = 48 ± 10 Minutes after the 2-Minute Illumination Period

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<th>Sample</th>
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<th>13-cis Retinal</th>
<th>11-cis Retinal</th>
<th>All-trans Retinal</th>
<th>All-trans Retinol</th>
<th>Retinyl Ester</th>
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<tr>
<td>Retina</td>
<td>2 ± 2</td>
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<td>35 ± 11</td>
<td>14 ± 6</td>
<td>3 ± 2</td>
<td>9 ± 7</td>
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<td>(2.0 ± 0.8)</td>
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<td>(30.0 ± 7.4)</td>
<td>(12.1 ± 4.3)</td>
<td>(2.7 ± 2.0)</td>
<td>(6.8 ± 3.8)</td>
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<td>Extracellular</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2 ± 1</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>11 ± 16</td>
<td>14 ± 15</td>
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<td>(0.1 ± 0.2)</td>
<td>(0.2 ± 0.5)</td>
<td>(1.5 ± 1.2)</td>
<td>(1.1 ± 0.8)</td>
<td>(0.2 ± 0.3)</td>
<td>(8.0 ± 9.5)</td>
<td>(11.0 ± 9.0)</td>
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<td>RPE</td>
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<tr>
<td>Retina</td>
<td>2 ± 2</td>
<td>3 ± 2</td>
<td>73 ± 31</td>
<td>12 ± 6</td>
<td>1 ± 2</td>
<td>3 ± 3</td>
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<td>(2.3 ± 2.1)</td>
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<td>(58.2 ± 5.4)</td>
<td>(11.3 ± 6.1)</td>
<td>(1.3 ± 1.6)</td>
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<td>(78.2 ± 8.7)</td>
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<td>2 ± 2</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>5 ± 6</td>
<td>8 ± 8</td>
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<td>medium</td>
<td>(0.0 ± 0.0)</td>
<td>(0.1 ± 0.3)</td>
<td>(1.4 ± 0.8)</td>
<td>(0.9 ± 0.5)</td>
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<td>(3.5 ± 2.9)</td>
<td>(6.0 ± 3.6)</td>
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<td>0 ± 1</td>
<td>18 ± 11</td>
<td>20 ± 12</td>
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<td></td>
<td>(0.0 ± 0.0)</td>
<td>(0.5 ± 0.8)</td>
<td>(0.6 ± 1.1)</td>
<td>(0.7 ± 1.7)</td>
<td>(0.7 ± 2.0)</td>
<td>(14.0 ± 7.8)</td>
<td>(15.8 ± 7.7)</td>
</tr>
</tbody>
</table>

Results obtained from six mice. The upper entry of each pair indicates, in picomoles, the mean ± SD of the determined amount of retinoid.

The kinetic data leave open the possibility of some differences between the two groups—for example, a somewhat faster decline of all-trans retinal and formation of retinyl ester in the VPP mice than in normal mice.

Values for the molar percent of 11-cis retinal in the retina for the unilluminated (i.e., control) eyes investigated in the experiments of Figures 4A and 4B are shown in Figure 4C.

The present results describe the molar content of retinoid in the dark-adapted eyes of normal and VPP mice in relation to age of the animal. In mice of age 12 days (the approximate time of eye opening) the total amount of retinoid in the normal eye was on average 116 picomoles, and increased with age to the adult (2-month) level of 425 picomoles (Fig. 2). Eyes of VPP mice showed a small but significant reduction from the normal level as early as 12 days of age and, at 3 weeks of age, total retinoid in the VPP eye was on average 48% of the normal value. Furthermore, in 3-week-old normal mice and VPP mice, 11-cis retinal in the retina represented a similar molar percent of total retinoid (data not shown). These observations are generally consistent with the previous finding that the levels of both opsins and rhodopsin in 3-week-old VPP mice (consisting of both normal and mutant protein; see below) amount to approximately 40% of the corresponding normal value. In vivo densitometric determinations of rhodopsin in 1-month-old VPP mice further indicate that the rhodopsin level is approximately one half to one third of the normal level. By 2 months of age, total retinoid in the VPP eye represented on average only 27% of the normal value and by age 5 months decreased to approximately 10% of the normal value.

Previous studies have described the progress of VPP rod degeneration as a function of age. In VPP mice of age 12 to 14...
days, no structural abnormalities are evident in the retina (Ref. 7; and M. I. Naash, unpublished data). However, at 3 weeks of age, the length of the VPP rod outer segment is approximately 60% of the normal length and, as noted earlier, the rods contain reduced levels of rhodopsin and opsin.12 By 1 month of age the VPP retina exhibits a significant loss of photoreceptor nuclei, and electroretinogram a-wave and b-wave responses are reduced by approximately 50% from normal values.7,9 With increasing age the VPP retina exhibits a further loss of photoreceptor nuclei, a further shortening of the outer segments, a further reduction of the electroretinogram, and an accumulation of vesicles in the basal end of the outer segments.7-13 In 2-month-old VPP mice the outer nuclear layer of the central retina is reduced to four to five rows of photoreceptor nuclei in the inferior region and five to six rows in the superior region.27 The present observation (Fig. 2) of a marked reduction in retinoid content of the VPP eye is a probable result of this rod degeneration. It is noteworthy that a reduction was evident in animals as young as 12 days, an age at which the rods appeared normal by histologic criteria.

In 3-week-old and 2-month-old animals, the distribution of retinoids in the retina, RPE, and extracellular medium was similar in normal and VPP littermates (Fig. 3 and accompanying text). Consistent with results previously obtained from the rat eye,5,5,26 the present data show that 11-cis retinal (presumably, as rhodopsin chromophore) is the predominant retinoid in the dark-adapted retina of both normal mice and VPP mice. In addition, the RPE of dark-adapted eyes of both normal mice and VPP mice contained a relatively small amount of retinoid, and retinyl ester was the predominant retinoid. In light of these findings it is noteworthy that the eyes of VPP mice at 2 months of age contained only 27% of the total molar amount of retinoid present in the eyes of normal mice. That is, previous studies of rhodopsin bleaching and regeneration provide evidence for a conservation of total retinoid level within the eye tissues during the several-hour period of retinoid processing in the visual cycle.5,5,28,29 Furthermore, investigations of the effects of dietary vitamin A deprivation indicate a substantial loss of retinoid from the eye tissues only after relatively long periods of deprivation.16-19,50 On this basis it could have been hypothesized that the overall level of retinoid in the VPP eye at 2 months remains essentially within the normal range—that is, that the low absolute level of 11-cis retinal in the dark-adapted VPP retina (Table 2) is accompanied by an elevated level of retinoid in the RPE. The reduced overall level of retinoid in the VPP eye and specifically in the RPE (where no abnormality is evident) implies, however, the absence of long-term conservation of the normal overall amount of ocular retinoid. Based on the evidence for reduced levels of both 11-cis retinal and opsin in the adult VPP eye (present results and Ref. 12), it is reasonable to hypothesize that on a long time scale (i.e., one greatly exceeding the several-hour period required for retinoid cycling), the total level of retinoid is primarily determined by the 11-cis retinal-binding capacity of opsin in the photoreceptors. The present results do not identify the type of opsin (normal, mutant, or both) that binds 11-cis retinal in the VPP rod. However, the data of Wu et al.12 provide evidence that VPP rod outer segments contain both normal and VPP opsin and that the photosensitivity and in vitro regeneration kinetics of rhodopsin in VPP rods are similar to those of normal littermates. These findings suggest that all the opsin in the VPP outer segment may be capable of binding 11-cis retinal.

Retinoid Cycling

A central objective was to examine the time course of light-induced retinoid processing in the VPP eye. It was of interest to determine, for example, whether retinoid cycling in the VPP is associated with a build-up of all-trans retinol in the illuminated retina. All-trans retinol, which arises through the enzymatic reduction of all-trans retinal, is thought to be toxic for photoreceptor membranes,14,15,31,32 and previous studies of the VPP mouse have left open the possibility that the VPP photoreceptor degeneration may derive in part from an abnormal accumulation of this retinoid in the outer segment membranes. The present data, which indicate the postillumination level of all-trans retinol in the VPP retina to be similar to the low level seen in the normal retina (Figs. 4A, 4B; Tables 1, 2), argue against this possibility.

More generally, the present results indicate no substantial difference between normal and VPP mice in the kinetics of change in any of the investigated retinoids. For example, in both groups, the negative change in molar percent of 11-cis retinal in the retina was near peak at approximately 40 to 50 minutes after the illumination, and recovery from this minimum to the baseline level was half-complete after approximately 150 minutes of dark adaptation. Furthermore, there was no accumulation of 11-cis retinal in the RPE in either normal mice or VPP mice. The fact that 11-cis retinal recovery in the VPP reached half-completion within about the same postillumination period as in normal mice but represented formation of only approximately one fourth the normal molar amount of 11-cis retinal raises the question of whether retinoid processing in the VPP eye is abnormally slow. For example, if the reappearance of 11-cis retinal in the retina were normally rate-limited by a kinetically first-order reaction (chemical conversion or transport step), reduction in the overall amount of retinoid processed through this reaction (in the present case of 2-month-old VPP mice, a reduction of approximately fourfold) would on a simple view be expected to preserve a normal period for half-completion of the 11-cis retinal recovery. If the rate-determining step were instead a kinetically zero-order process (one with fixed absolute rate, independent of the substrate concentration), occurrence in the VPP of a half-completion time similar to that of normal mice would imply an abnormality (approximately fourfold reduction in rate) in the operation of this processing step. The present data thus do not exclude an abnormality in retinoid processing in the VPP eye, but do place constraints on the nature of such an abnormality if present. Based on the correlation between rhodopsin regeneration and the recovery of electroretinographic b-wave (logarithmic) sensitivity in the normal eye,3,5 the finding of sluggish b-wave recovery in the 2-month-old VPP mouse3 suggests the possibility of at least some defect in absolute regeneration kinetics. The precise mechanism by which the VPP mutation leads to degeneration of the rods remains to be determined. The recent observation of an abnormality in disk morphogenesis of the VPP rod outer segment18 emphasizes the possibility that abnormal incorporation of VPP mutant opsin into the developing disk membrane may be a key element of the degeneration mechanism.

In both normal and VPP mice, the kinetics of reappearance of 11-cis retinal in the retina was considerably slower than that of the decline of all-trans retinal but corresponded with the decline of retinyl ester (compare circles and diamonds in Figs. 4A and 4B). This observation, and the absence of substan-
tial accumulation of any 11-cis retinoid in the RPE or extracel-
ellular medium after illumination, implicate the isomerization of all-trans retinyl ester in the RPE\textsuperscript{25} as rate-limiting, in both normal mice and VPP mice, for the supply of 11-cis retinal to the retina and therefore for the regeneration of rhodopsin under the present experimental conditions (however, see Ref. 54). Such an interpretation is consistent with the kinetic similarity of retinyl ester decline and rhodopsin regeneration observed in previous studies of the retinoid visual cycle in rat and frog.\textsuperscript{3,5,24,25} This interpretation differs from that noted by Saari et al.,\textsuperscript{35} whose recent experiments indicate a kinetic correspondence of all-trans retinyl ester reduction and rhodopsin regeneration in the mouse eye (i.e., a rate limitation of 11-cis retinal synthesis by the reduction step). The difference between the present conclusion and that of the study by Saari et al.\textsuperscript{35} may reflect differences in the extents of rhodopsin bleaching or in the strains of mice used in the two studies. For example, in vivo densitometric data indicate that the characteristic time constant for rhodopsin regeneration in the rat increases with the extent of rhodopsin bleaching.\textsuperscript{3,5} Furthermore, the progress of the isomerohydrolase-catalyzed conversion of all-trans retinyl ester to 11-cis retinol\textsuperscript{35} is itself expected to depend in a complex manner on bleaching extent, because the operation of this reaction depends on the formation of all-trans retinol in the retina, on the delivery of this retinol to the RPE, and on esterification of the retinol within the RPE. It is reasonable to hypothesize that all-trans retinyl ester and all-trans retinyl ester isomerization exhibit a differing dependence on the initial amount of light-generated all-trans retinyl ester and that the interplay of these two reactions establishes a bleaching-dependence for the identity of the reaction that limits the rate of formation of 11-cis retinal.

References


