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## The Rhodopsin Content of Human Eyes

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**PURPOSE.** To measure the total amount of rhodopsin in human eyes across the life span and to test the hypothesis that the rhodopsin content of infants' and the elderly's eyes is lower than at other ages.

**METHODS.** Rhodopsin was extracted from retinal and pigment epithelial fractions of 196 eyes of 102 donors, ages 27 weeks' gestation through 94 years, using quantitative procedures. To recover photopigment bleached by unavoidable light exposure, the fractions from 78 eyes were incubated with 9-*cis* retinal. The total photopigment (retinal plus pigment epithelial fractions) per eye was examined for significant changes with age, using the higher value from pairs of eyes.

**RESULTS.** The median rhodopsin content of the higher eye of adults is 6.45 nmoles (range, 3.33-10.84 nmoles) with 8 nmoles or more recovered from 28% of all adult eyes. The rhodopsin content of infants' eyes (<12 months post-term) is significantly lower than that of older individuals and increases with age. After infancy, no change with age is found. For both infants and adults, 9-*cis* retinal significantly increases the amount of photopigment recovered without reducing the variance in the amount of photopigment recovered. The rhodopsin content is estimated to be 50% of the median adult amount early in infancy, approximately 5 weeks postterm (95% confidence interval, 0-10 weeks postterm).

**CONCLUSIONS.** A developmental increase in rhodopsin content occurs during infancy. Thereafter rhodopsin content remains constant. The amount of rhodopsin recovered from human eyes is quite variable. Bleaching alone cannot explain the variability. (*Invest Ophthalmol Vis Sci*. 1999; 40:1878-1883)

Development of the human rod outer segments (ROS) begins at preterm ages and continues with further elongation of the ROS after term.<sup>1</sup> As in other species, it has been suspected that developmental elongation of human ROS, which proceeds after the addition of rod cells has ceased, is accompanied by an increase in rhodopsin content and scotopic retinal sensitivity. Previous measurements<sup>2,3</sup> have indicated that the amount of rhodopsin in infants' eyes is lower than in adults', implying a developmental increase in rhodopsin content. However, a sufficient number of measurements have not been available to define the developmental course. After infancy, scotopic sensitivity remains constant<sup>4-6</sup> until after the age of 60 years when slight deficits in scotopic sensitivity are found.<sup>7,8</sup> It has been reasoned that the deficits in scotopic sensitivity at either end of the age span may be due to receptor or postreceptor factors, or a combination of the two.<sup>4,5,7,9,10</sup>

Thus, it is of interest to define the course of age-related changes in the rhodopsin content of the human eye. Since our previous reports,<sup>2,3</sup> we have more than tripled the sample size and added a 9-*cis* retinal regeneration procedure to evaluate the effect of possible bleaching on rhodopsin content. The rhodopsin content of the eyes, ranging in age from the preterm weeks to more than 90 years, has been examined for significant changes with age.

## METHODS

Eyes ( $n = 196$ ) from 102 donors, 27 weeks' gestation through 94 years of age, were obtained through eye banks, or in the case of the preterm infants, at autopsy. Data from 30 of these donors have been reported before.<sup>3</sup> All globes appeared normal, and no donor had a history of eye problems except uncomplicated cataract surgery in three elderly donors.

As previously described,<sup>2,3</sup> each globe was placed in a petri dish, containing 5 ml 0.9% saline, and bisected in an anteroposterior plane. The entire retina was teased free, placed in 5 ml distilled water, and vigorously stirred with a stainless steel spatula; this was designated the retinal fraction. The pigment epithelium and choroid were teased from the scleral shell, placed in a separate tube along with the saline from the petri dish and stirred vigorously; this was designated the pigment epithelial (PE) fraction. Each of these fractions was processed separately. The samples were centrifuged at 12,000g for 10 minutes at 4°C and the supernatant discarded.

Extraction of the photopigment was done with 1% CTAB (cetyl trimethyl-ammonium bromide; Sigma, St. Louis, MO),<sup>2,3</sup> or 1% Emulphogene (Sigma) in 50 mM Tris-acetate buffer, pH 6.9. The results obtained with CTAB and Emulphogene are considered together in this report because the mean rhodopsin recovered from 20 pairs of adults' eyes [CTAB (mean = 8.19, SD = 1.63 nmoles); Emulphogene (mean = 6.67, SD = 1.99

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TABLE 1. Amount of Photopigment Recovered Per Eye

	Age (years)	Higher Eye		Fellow Eye	
		Native Rhodopsin (nmoles)	9-cis Retinal Supplemented (nmoles)	Native Rhodopsin (nmoles)	9-cis Retinal Supplemented (nmoles)
Infants		2.00	6.47	1.80	6.40, 6.88
	(27 wk gest. to 11 mo) (24 donors, 44 eyes)	(0.14-3.79) <i>n</i> = 13*	(1.70-10.26) <i>n</i> = 11*	(0.08-6.27) <i>n</i> = 18	— <i>n</i> = 2
Children	3	6.82	6.88	4.70	7.43
	(1-13 years) (13 donors, 25 eyes)	(2.10-7.98) <i>n</i> = 5	(1.45-8.81) <i>n</i> = 8	(1.31-6.78) <i>n</i> = 11	— <i>n</i> = 1
Adolescents	19	5.27	6.50	5.13	—
	(13-21 years) (10 donors, 19 eyes)	(1.72-8.99) <i>n</i> = 5	(4.01-9.81) <i>n</i> = 5	(0.14-7.24) <i>n</i> = 9	— None
Adults	59	6.45	7.19	4.63	6.91
	(21-94 years) (55 donors, 108 eyes)	(3.33-10.84) <i>n</i> = 23	(2.33-10.50) <i>n</i> = 32	(2.55-9.22) <i>n</i> = 34	(2.55-9.70) <i>n</i> = 19

Values are median and range; number of donors and eyes.

\* The infants in the 9-cis retinal-supplemented column (median, 3 months postterm; range, 27 weeks' gestation to 10 months postterm) were, on average, older than those in the Native Rhodopsin column (median, 2 weeks postterm; range, 27 weeks' gestation to 11 months postterm). The medians are not significantly different (Mann-Whitney U = 47; *P* = 0.11).

nmoles)] did not differ significantly, and the median  $\lambda_{\max}$  was 496 nm for both.

Before extraction with detergent, the retinal and PE fractions of 78 eyes were incubated with the synthetic chromophore 9-cis retinal to regenerate photopigment that had been bleached by uncontrolled light exposure during procurement of the globes. The individual retinal and PE fractions were incubated in the dark with an excess (3-4  $\mu$ l) of 9-cis retinal (final concentration, 10 nmoles/ml) for 1 hour at 20°C and then centrifuged at 12,000g for 15 minutes. The supernatant was discarded, and 5 ml of detergent solution was added to each pellet. The pellets were disrupted with a spatula and incubated in the dark for 1 hour at 20°C, and spun again at 12,000g for 10 minutes. The 78 eyes included 37 for which the fellow eye was not incubated with 9-cis retinal, and only native rhodopsin (with 11-cis retinal) was assayed.

After the final spin, an aliquot of the supernatant was removed and scanned 820 to 190 nm using an HP-8452A diode array spectrophotometer to obtain the absorbance spectrum. Then the extract was exposed to white light for 6 minutes and scanned again. For each specimen, the absorbance of photopigment at its  $\lambda_{\max}$  was obtained by the difference spectrum. The number of nanomoles of photopigment present in each retinal and PE fraction was calculated using the Beer-Lambert equation and summed to obtain an estimate of the total amount of photopigment in each eye. Extinction coefficients of 42,000  $M^{-1} \cdot cm^{-1}$  for rhodopsin and 43,000  $M^{-1} \cdot cm^{-1}$  for isorhodopsin<sup>11</sup> were used. To determine whether isorhodopsin was present in the 9-cis retinal-supplemented samples, the method of wavelength shift<sup>12-14</sup> was used. This technique makes use of the fact that isorhodopsin absorbs at shorter wavelengths ( $\lambda_{\max}$  = 486 nm) than does rhodopsin, and mixtures of the two photopigments produce a composite spectrum with a  $\lambda_{\max}$  between those of the native rhodopsin and the artificially produced isorhodopsin.

The effect of age on the amount of photopigment recovered per eye was analyzed. Because light exposure and incomplete recovery of rhodopsin bearing tissues were possible

explanations for recovery of spuriously low amounts of photopigment, but because artifactually high values were unlikely to result from the procedures described above, the higher amount of photopigment obtained from a pair of eyes was used for analysis of the effect of age. If only one eye was available (*n* = 8 donors), that eye was used in the analysis. A logistic growth curve<sup>3,15,16</sup> of the form

$$y/y_{\max} = age^n / (age^n + C^n)$$

where *C* is the age at which *y* is 50% of the adult value  $y_{\max}$ , provides a good summary of the course of rhodopsin increase during development in other species and was to be considered as a descriptor of human rhodopsin development.

## RESULTS

The amounts of photopigment recovered from the 196 eyes are summarized in Table 1. The data from eyes having only native rhodopsin (opsin + 11-cis retinal) studied, and those having fractions incubated with 9-cis retinal, are listed separately. For all 108 adult eyes, 28% had 8 nmoles or more of native rhodopsin recovered. For all groups (Table 1), the amounts of photopigment recovered from eyes treated with 9-cis retinal overlap broadly and are analyzed further below.

For native rhodopsin,  $\lambda_{\max}$  did not vary with age. The median value was 496 nm, and 90% of the values are within 2 nm of this value. Similar values (496-498 nm) have been reported previously for extracted human rhodopsin.<sup>17-20</sup> As the spectra in Figure 1 illustrate, the difference spectrum obtained from a 9-cis retinal-supplemented sample may be shifted to shorter wavelengths, indicating the presence of a mixture of isorhodopsin and rhodopsin rather than rhodopsin alone. The median  $\lambda_{\max}$  for the 9-cis retinal-supplemented samples was 492 nm (range, 486-500 nm).

The distribution of  $\lambda_{\max}$  values obtained from 9-cis retinal-supplemented and nonsupplemented eyes of the 37 paired

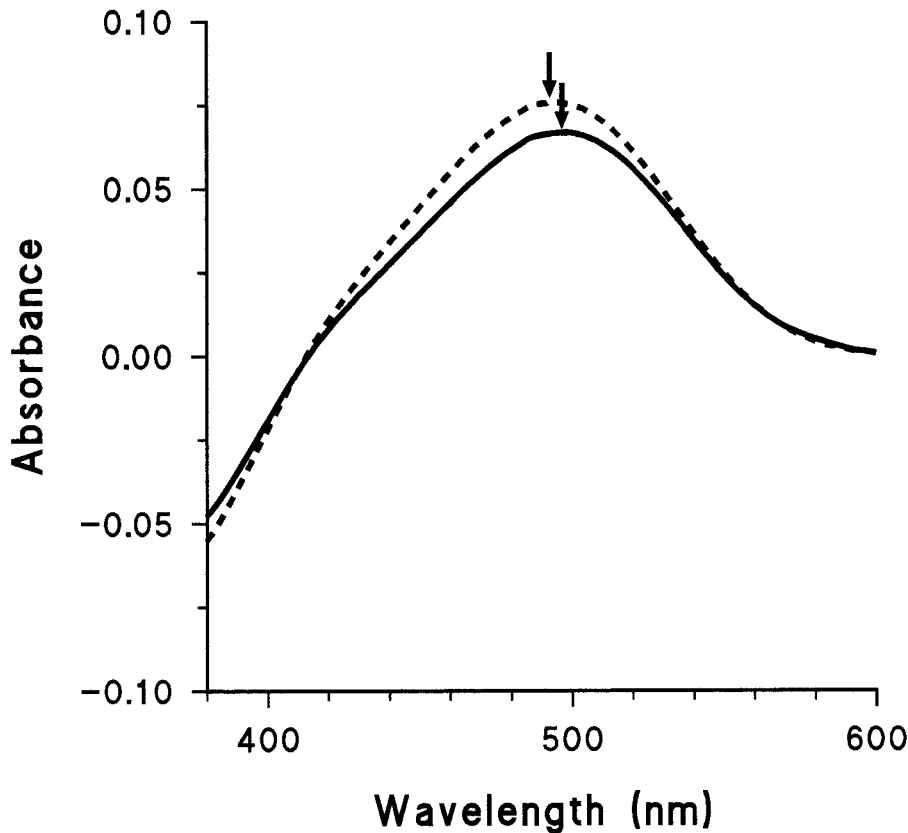


FIGURE 1. Sample spectra for native rhodopsin (solid line) and rhodopsin plus isorhodopsin (9-cis retinal-supplemented sample; dashed line). These are from a 49-year-old donor. Rhodopsin, 8.34 nmoles with  $\lambda_{max} = 496$  nm, was recovered from one eye, and 10.24 nmoles photopigment, with  $\lambda_{max} = 493$  nm, was recovered from the fellow eye that had been supplemented with 9-cis retinal.

samples is compared in Figure 2. For the supplemented samples, there was a significant shift of the distribution to shorter wavelengths ( $t = -5.43$ ;  $df = 36$ ;  $P < 0.01$ ). Among individual

pairs ( $n = 37$ ), supplementation achieved a large increment in photopigment in some, whereas there was no increase in others (range of differences between 9-cis retinal-supple-

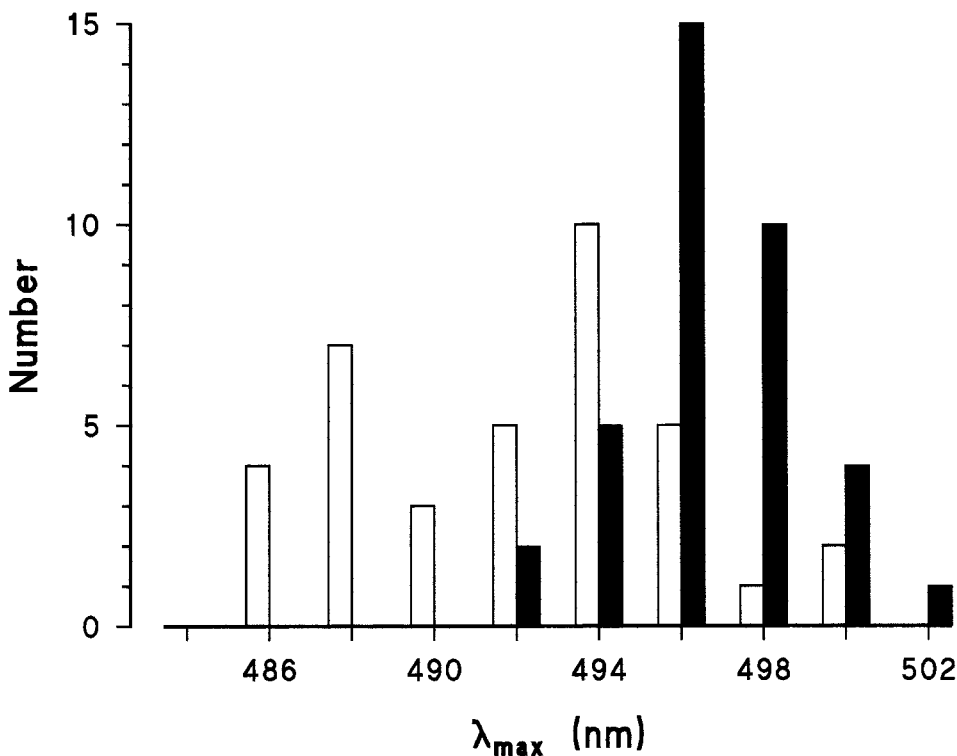
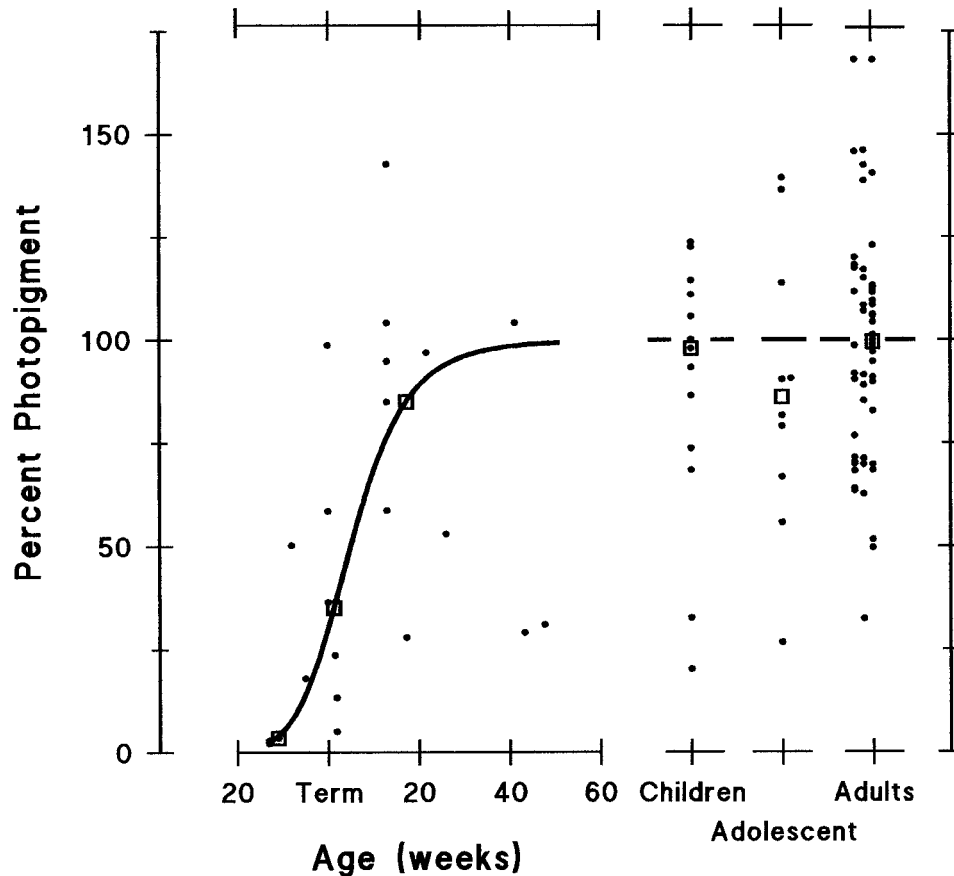


FIGURE 2. Distribution of  $\lambda_{max}$  values. These data are from the 37 pairs of eyes, one of which was supplemented with 9-cis retinal (open bars) and the fellow eye had only native rhodopsin extracted (black bars). The distribution for the 9-cis-supplemented samples is shifted to shorter wavelengths (median,  $\lambda_{max}$  492 nm), suggesting a mixture of rhodopsin, and isorhodopsin is represented in some of these supplemented samples. The median  $\lambda_{max}$  for the rhodopsin values is 496nm.



**FIGURE 3.** The normalized amount of photopigment as a function of age. The points (●) represent the result from the higher eye (Table 1) of each of the 102 donors. If the higher eye had native rhodopsin assayed, the value was normalized to the median adult value of 6.45 nmoles (Table 1). If the higher eye had been supplemented with 9-*cis* retinal, the value was normalized to the median adult value of 7.19 nmoles (Table 1). For those donors having only one eye studied, the amount of photopigment was normalized to the appropriate adult median for native rhodopsin, or 9-*cis* retinal-supplemented samples (Table 1). Adults are shown in 3 columns: young (21–40 years;  $n = 17$ ), middle-aged (40–65 years;  $n = 13$ ), and older ( $\geq 65$  years;  $n = 25$ ). As indicated, donors were not evenly distributed throughout infancy. Therefore, clusters of infantile data are represented by median age and median percentage of photopigment (□) as follows: preterms (ages 27–35 weeks' gestation,  $n = 5$ ); around term, 40 to 42 weeks' gestation ( $n = 8$ ); and median age, 4 months postterm (3–11 months;  $n = 11$ ). The median percentages of photopigment in children, adolescents, and adults are also shown (□); the median ages are in Table 1. The smooth line is a logistic growth curve fit to the medians (□). The equation for this curve is  $y/year_{max} = age^n / (age^n + C^n)$ , where  $y_{max} = 100\%$ ,  $n = 7.2$ , and  $C = 5$  weeks, the age at which rhodopsin is 50% of the adult value.

mented and nonsupplemented samples: +5.96 nmoles higher to -0.41 nmoles lower). The increment in amount of photopigment recovered was significantly correlated with that of nanometers that  $\lambda_{max}$  was shifted, from 496 nm to shorter wavelengths ( $r = 0.41$ ;  $P < 0.02$ ). The increment for 9-*cis* retinal treatment is similar in infant and older donors. For the infants ( $n = 9$ ), the amount recovered from the nonsupplemented eye (mean = 3.23; SD = 2.46 nmoles) was 66% of that recovered from the supplemented eye (mean = 4.87; SD = 2.57 nmoles). For the older donors (2–94 years;  $n = 28$ ), the amount recovered from the nonsupplemented eye (mean = 4.42; SD = 2.26 nmoles) was 70% of that recovered from the supplemented eye (mean = 6.28; SD = 2.08 nmoles). Despite achieving the expected increment in photopigment by 9-*cis* retinal supplementation, the variability in the amount of photopigment recovered was not significantly reduced. There was

no significant difference in the variance of the supplemented and nonsupplemented samples ( $F = 1.18$ ;  $df = 36,36$ ; NS).

The normalized, photopigment content of the 102 donors is shown as a function of age in Figure 3. For donors contributing pairs of eyes, the result from the eye with the higher amount of photopigment is shown. Only during infancy is there a significant change in the amount photopigment recovered ( $y = 2.178 \times -45$ ;  $r^2 = 0.38$ ;  $t = 3.39$ ;  $P = 0.003$ ). In no other age group is there a significant change with age. The amount of photopigment recovered from young adults (21–39 years;  $n = 17$ ) and older adults ( $>64$  years;  $n = 25$ ) does not differ significantly. Specifically, for the higher eye of young adults, median rhodopsin content was 4.95 (4.09–10.84) nmoles, and median photopigment for supplemented samples was 6.55 (4.51–8.50) nmoles; and of older adults, median rhodopsin content was 6.78 (3.33–10.84) nmoles, and median

photopigment in supplemented samples was 7.13 (3.55–10.1) nmoles. According to the growth curve shown in Figure 3, the age at which rhodopsin content of the eye is 50% of that in adults is 5 weeks (95% confidence interval, 0–10 weeks post-term).

## DISCUSSION

These data show that the rhodopsin content of infants' eyes is lower than in adults and that the rhodopsin content increases significantly during infancy, when a developmental increase in visual sensitivity occurs.<sup>5,9</sup> Thus, a lower amount of rhodopsin, and consequent lower probability of photon capture by rhodopsin in the outer segments, cannot be disregarded as one of the fundamental determinants of infants' lower scotopic visual sensitivity. At the other end of the life span, although loss of photoreceptors and quantum catching capability were considered as possibly contributing to an average deficit of 0.5 log unit in scotopic visual sensitivity<sup>7</sup> and 0.2 log unit deficit in scotopic b-wave sensitivity<sup>4</sup> in older observers, postreceptoral factors appeared more likely. The rhodopsin data of the present study are consistent with this conclusion.

The amounts of pigment recovered are quite variable at all ages (Table 1; Fig. 3). Some of the variability is likely due to bleaching of rhodopsin around the time that the eyes were procured. This supposition is consistent with the higher amount of photopigment found in eyes treated with 9-*cis* retinal. However, even among the 9-*cis* retinal-supplemented samples from adults, the standard deviation for nanomoles of photopigment recovered is approximately 33% of the mean, a little lower than that for the nonsupplemented samples for which the standard deviation is nearly 50% of the mean. However, even 33% is higher than the standard deviation typically obtained in laboratory experiments using the same type of extraction and regeneration procedures as used herein.<sup>14,21</sup> Possibly, the regeneration achieved with the 9-*cis* retinal procedure in human retinas is less complete than in laboratory experiments, although control experiments did not indicate this to be the case. Thus, in human eyes, the variation in rhodopsin content may be controlled not only by the acute light history but also by other factors. For example, from retina to retina there is some variation in the number of rods present. Curcio and coworkers<sup>22</sup> report that the number of rods in the human retina ranges from 77.9 to 107.3 million. In other words, the number of rods in some retinas may be more than 25% lower than that in eyes with the largest number of rods. With aging, a 30% loss of rod cells in central retina is reported.<sup>23–25</sup> Thus, cell loss may contribute to the variability of rhodopsin content in the older adult group; however, given the standard deviations of approximately 50% of the mean values, the effect of loss of central rods may not produce a detectable change in rhodopsin content. Another factor that could affect the amount of rhodopsin in the human retina is long-term light history. In infants and adults of other species, a bright habitat induces short outer segments and a low rhodopsin content; a long-term adaptation to dim habitats induces long outer segments and a high rhodopsin content.<sup>26–28</sup>

Despite the variability that appears in these quantitative assays of rhodopsin, the difference between the rhodopsin content in infants and adults is significant. Surely this must accompany the development of ROS structure and function.

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## The Course of Maturation of Rod-Mediated Visual Thresholds in Infants

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**PURPOSE.** To measure the developmental course of infants' rod-mediated thresholds.

**METHODS.** Thresholds for detecting stimuli (2° diameter, 50 msec duration) presented at 10° (parafoveal site) or 30° (peripheral site) from a central fixation target were estimated using a preferential-looking method. Nine infants were tested at both stimulus positions at ages 10, 18, and 26 weeks.

**RESULTS.** At 10 weeks, infants' thresholds at both sites were significantly higher than those of adults. The infants' average threshold at 10° was 0.5 log unit higher than the infants' average threshold at 30°. Adults' thresholds at the two sites were equal. Thresholds of all infants decreased with age until by age 26 weeks the parafoveal and peripheral thresholds were equal and were the same as those of adults. The rate of change of parafoveal thresholds was significantly faster than the rate of change of peripheral thresholds.

**CONCLUSIONS.** Although postreceptoral factors cannot be ruled out, the results suggest that developmental increases in rod outer segment length and rhodopsin density account for most of the threshold changes during infancy. (*Invest Ophthalmol Vis Sci.* 1999;40:1883-1886)

The dark adapted, rod-mediated visual thresholds of young infants are significantly higher than those of adults.<sup>1-9</sup> For example, cross-sectional data show that the average threshold is 1.4 log unit higher than that of adults at 4 weeks, 1.1 log unit higher at 10 weeks, and 0.65 log unit higher at 18 weeks.<sup>7</sup> Thus, although thresholds decrease, at age 18 weeks they remain significantly above those of adults.<sup>6</sup> The age at which

infants' thresholds become equal to those of adults is unknown. Furthermore, we are unaware of any report that describes the course of maturation of normal scotopic thresholds in individual infants.

We undertook a longitudinal study of rod-mediated threshold development and elected to test parafoveal (10° eccentric) and peripheral (30° eccentric) retinal sites, because development is nonuniform across the retina. For instance, anatomic studies show parafoveal rod photoreceptor outer segment growth is delayed relative to peripheral outer segment growth,<sup>10-13</sup> despite the axiom that the central retina matures earlier than the peripheral retina. Psychophysical study shows that 10-week-old infants' thresholds at a parafoveal site were significantly elevated relative to their thresholds at a peripheral site, whereas adults' thresholds at these sites were equal.<sup>14</sup> In this longitudinal study, we tested the hypothesis that thresholds measured at the parafoveal site change more rapidly than those at the peripheral site.

## METHODS

### Stimuli

Stimuli were 50 msec, 2° diameter, blue spots (Wratten 47B,  $\lambda < 510$  nm) presented on a rear projection screen, 10° or 30° to the right or the left, of a 30-min arc diameter red LED fixation target flickering at 1 Hz. Stimulus intensity was controlled by calibrated neutral density filters. Calculation of the retinal illuminance produced by the stimuli was based on luminance measurements made with a calibrated photodiode (UDT S-350; United Detector Technology, Orlando, FL) placed in the position of the subject's eyes. At the beginning and end of each session, the subject's pupillary diameter was estimated by direct observation with an infrared viewer. Pupillary diameter was determined by comparison with the diameter of the cornea which is  $11 \pm 0.5$  mm in infants from term to 6 months of age.<sup>15</sup> Retinal illuminance varies directly with pupillary diameter and the transmissivity of the ocular media and inversely with the square of the posterior nodal distance.<sup>7</sup> The scotopic troland value of the stimulus<sup>16</sup> was calculated taking each subject's measured pupillary diameter and the average axial length<sup>17</sup> into account.<sup>6,7</sup> The correction for light losses in the ocular media was based on previous results in infants.<sup>18</sup>

### Procedure

Thresholds were estimated using a two-alternative, forced-choice, preferential-looking method<sup>19</sup> that incorporated a fix-and-flash procedure.<sup>9</sup> After the subject dark adapted for 30 minutes, an adult held the infant 50 cm in front of the center of the screen. The flickering red LED fixation target attracted the infant's gaze to the center of the screen. A second adult watched the infant with an infrared viewer and reported when the infant was alert and looking at the fixation target. The

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