Automated Quantification of Keratocyte Density by Using Confocal Microscopy In Vivo

Sanjay V. Patel,1 Jay W. McLaren,1 Jon J. Camp,2 Leif R. Nelson,1 and William M. Bourne1

PURPOSE. To compare keratocyte density determined by using confocal microscopy with keratocyte density determined in the same corneas by histology.

METHODS. Digital en face images of central corneas were recorded three times by using confocal microscopy in vivo in six New Zealand White rabbits. Bright objects (keratocyte nuclei) in the images were automatically identified by using a custom algorithm to estimate total and regional stromal keratocyte densities. The corneas were then excised, fixed, and sectioned in a sagittal plane for histology. Keratocyte nuclei were manually counted from digitized images of 50 histologic sections per cornea. Total and regional keratocyte densities were estimated from the histologic sections by using stereologic methods based on nuclei per unit area, mean nuclear diameter, and section thickness. Histologic cell densities were corrected for tissue shrinkage.

RESULTS. By confocal microscopy, total keratocyte density was 39,000 ± 1,200 cells/mm3 (mean ± SE; n = 6); cell density was 47,100 ± 1,300 cells/mm3 in the anterior stroma and decreased to 27,900 ± 2,700 cells/mm3 in the posterior stroma (P = 0.004). Analysis of the three separate confocal images of each cornea produced repeatable total cell densities (mean coefficient of variation = 0.035). By histology, total keratocyte density was 37,800 ± 1,100 cells/mm3, not significantly different from that estimated by confocal microscopy (P = 0.43); anterior cell density was 48,300 ± 900 cells/mm3 and decreased to 29,400 ± 900 cells/mm3 posteriorly (P < 0.001).

CONCLUSIONS. Rabbit keratocyte density estimated by automated analysis of confocal microscopy images in vivo is repeatable and agrees with keratocyte density estimated from histologic sections.


The corneal stroma consists of keratocytes within an extracellular matrix of collagen fibrils and proteoglycans. Keratocytes are fibroblast-like cells that produce, degrade, and remodel the stroma and are therefore important in corneal wound healing. Qualitative or quantitative variations in keratocytes may thus explain the haze seen after photorefractive keratectomy1,2 or the poor wound healing that occurs with increasing age.3,4 Keratocytes also regulate collagen fibril size and spacing within the proteoglycan matrix to maintain corneal transparency. Corneal transparency relies heavily on the endothelial physiologic pump. This has been extensively studied, whereas the role of the stromal keratocytes remains unclear. It is possible that, similar to endothelial cell density, a minimum keratocyte density is required for transparency and that this may be an important criterion in selecting tissue for grafting.

There have been numerous studies of keratocytes in vitro. Keratocyte morphology was first described in the 1950s by using histologic methods.5,6 Ultrastructural details were later described by findings from electron microscopy.7 More recently, fluorescent markers used with light microscopy have been successful in the identification of keratocytes ex vivo in whole-mounted specimens.8 Stromal DNA content has been measured in an attempt to quantify keratocyte density.9-11 Unfortunately, all these methods are invasive and require tissue processing that can alter the tissue. However, keratocytes can now be examined in vivo by using confocal microscopy.

Confocal microscopes use a point source of light to view an object while a point detector collects the reflected and scattered light. This makes image contrast and axial and lateral resolution superior to that of conventional microscopes, but at the expense of field of view.12,13 Confocal microscopy has been used in numerous studies in vivo: Corneal studies include measurement of corneal thickness,14 three-dimensional imaging of the cornea,15 examination of the epithelium,16,17 qualitative assessment of wound healing,18-20 and the diagnosis of microbial keratitis.21,22 The tear film has also been assessed23-24 and observation of the retina has been described.25 The same microscope may also be used for studies in vitro.

Keratocytes have been qualitatively studied by using confocal microscopy in determining wound healing.18,20 but keratocyte density has not been quantitatively estimated in vivo by using this technique. The ability to do the latter would aid in our understanding of the cellular reaction to corneal injury, such as that occurring after photorefractive keratectomy. It also would allow studies of keratocyte density before and after penetrating keratoplasty or in corneal disease. Keratocyte density has been indirectly quantified in humans by using corneal stromal DNA measurements.9 Regional variations in density10

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Supported in part by Grant EY02037 from the National Institutes of Health, Bethesda, Maryland; Research to Prevent Blindness, New York; New York; and Mayo Foundation, Rochester, Minnesota.

Submitted for publication July 7, 1998; accepted October 5, 1998.

Proprietary interest category: P (JJC); N (SVP, JWM, LRN, WMB).

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and a decline in density with increasing age\(^1\) were shown by using this method. A laser scanning confocal microscope has been used to quantify keratocyte density in rabbit corneas ex vivo.\(^2\) Both these methods are invasive, and neither was validated histologically.

In this article we describe an automated method to estimate keratocyte density in corneas in vivo by using images from confocal microscopy. A custom program identifies keratocyte nuclei, which are seen with reflected light as bright objects against a dark background. To validate our automated method, we compared rabbit keratocyte density measured in vivo with keratocyte density measured in vitro by using standard histologic methods.

**METHODS**

**Animals**

Six New Zealand White rabbits (2.8–3 kg) were used for this study. Each underwent confocal microscopy in vivo followed by enucleation and corneal excision for histology. The animals were housed and treated according to the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

**Confocal Microscopy In Vivo**

A tandem scanning confocal microscope (Tandem Scanning Corporation, Reston, VA) was used to examine corneas in vivo. Its light source was a 100-W mercury arc lamp. The microscope had a \(\times 24\), 0.6 numeric aperture, nonapplanating, concave objective lens, with a working distance of 0 mm to 1.5 mm. The position of the focal plane (optical section) could be advanced or retracted at a predetermined rate under computer control (INDY; Silicon Graphics, Mountain View, CA) through an encoder mike controller (model 18011; Oriel Instruments, Stratford, CT). The position of the focal plane was adjusted by an internal lens without changing the position of the front surface of the objective. Digital images were recorded by a low-light camera (model VE-1000 SIT; DAGE-MTI, Michigan City, IN) directly into computer memory.

The microscope was calibrated by recording images of an optical micrometer scale (100 mm \(\times\) 0.01 mm subdivisions) observed through an optical coupling medium (hydroxypropyl methylcellulose 2.5%; CIBA Vision Ophthalmics, Atlanta, GA). Horizontal and vertical pixel dimensions were calculated by dividing fixed distances in micrometers by the number of pixels representing those distances. Pixel dimensions were calculated with the optical section positioned at different depths.

For confocal microscopy, rabbits were anesthetized with 5 mg/kg xylazine and 50 mg/kg ketamine by intramuscular injection, and a topical anesthetic (proparacaine hydrochloride 0.5%; Alcon, Humacao, Puerto Rico) was instilled in the right eye. The objective lens was disinfected by using 70% isopropryl alcohol wipes before and after each examination. A drop of 2.5% hydroxypropyl methylcellulose optical coupling medium was placed on the tip of the objective lens, and the lens was manually advanced until the medium contacted the central cornea. A series of confocal images was recorded as the focal plane was advanced deeper into the cornea by setting gray-scale thresholds. However, the images contained high-frequency spot noise and significant inhomogeneity of brightness and contrast across the field, which tended to confound simple thresholding. In addition, the gradual loss of light as the focal plane was advanced deeper into the cornea resulted in a reduction in average brightness of the images. Thus, an inhomogeneity correction\(^29\) was applied followed by reduction of spot noise by sigma filtering.\(^30\) Adaptive histogram equalization\(^30\) boosted local and global contrast. These automated processes equalized variations from image to image (and camera to camera), thus making it possible to impose a threshold at a fixed value on all frames in all confocal image sets that could not be altered interactively. After the threshold was reached, the binary images still contained unwanted noise; thus, a series of mathematical morphology operations\(^31\) removed very small bridges connecting objects, smoothed boundaries, and filled gaps in objects. The pixels representing each remaining object were then grouped by using a connected component analysis.

The final step was counting the objects. The pixel area of each object was automatically calculated for each frame. Objects were defined as single nuclei by using set upper and lower pixel area limits for the first frame. The algorithm then automatically defined these limits for each subsequent frame based on the mean object area to account for any regional variations of the mean image size of nuclei. Very small objects representing remaining noise were not counted; very large objects representing images of clustered nuclei were counted as single or multiple nuclei based on the ratio of their area to the mean area of objects defined as single nuclei in that frame. Objects lying across the boundary of the counting area were counted on only two of the four boundaries. Colored bounding boxes were placed around the objects so that the examiner could visualize which objects were identified and their relative size. The specific parameters for the processing steps, including the threshold value, had been previously optimized so that the automated method identified keratocyte nuclei in confocal images in agreement with manual identification by three masked examiners, and these parameters were the same in all analyses of all rabbits.

Because each two-dimensional frame actually represented a volume with z-depth equal to the optical section thickness, a volumetric density of nuclei was obtained from each frame by using stereologic methods.\(^32\) This required the number of nuclei per unit area in each frame and summation of the optical section thickness and nucleus thickness in this (z) direction.

**Automated Algorithm for Digital Image Processing**

All image processing was performed by a custom program integrated into an image analysis program\(^27,28\) and display system (AnalyzeAVW, Mayo Medical Ventures, Rochester, MN). Keratocyte nuclei appeared as bright objects against a dark background in the images, making possible the segmentation of nuclei by setting gray-scale thresholds. However, the images contained high-frequency spot noise and significant inhomogeneity of brightness and contrast across the field, which tended to confound simple thresholding. In addition, the gradual loss of light as the focal plane was advanced deeper into the cornea resulted in a reduction in average brightness of the images. Thus, an inhomogeneity correction\(^29\) was applied followed by reduction of spot noise by sigma filtering.\(^30\) Adaptive histogram equalization\(^30\) boosted local and global contrast. These automated processes equalized variations from image to image (and camera to camera), thus making it possible to impose a threshold at a fixed value on all frames in all confocal image sets that could not be altered interactively. After the threshold was reached, the binary images still contained unwanted noise; thus, a series of mathematical morphology operations\(^31\) removed very small bridges connecting objects, smoothed boundaries, and filled gaps in objects. The pixels representing each remaining object were then grouped by using a connected component analysis.

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The sum of these parameters (16 μm) was made constant for all analyses of all rabbits, and was obtained by averaging the z-depth (by the number of frames) through which random, nonblurred nuclei were continually visualized. The mean keratocyte density per unit volume was calculated for the entire stroma and for five anteroposterior stromal regions of equal thickness by analyzing every fifth frame of corneal stroma (approximately 70 frames). The posterior stromal region was subdivided into three equal-thickness subregions to measure keratocyte density near Descemet’s membrane by analyzing every frame from that region (approximately 70 frames). No frames were discarded based on the quality of images for each rabbit. Image contrast was adjusted to optimize the keratocyte nuclei.

After fixation, the limbal and trephine diameters were remeasured using the same calipers. The cornea was carefully excised, and a central square region (approximately 4 mm × 4 mm) was prepared for histology. The exact dimensions of this section, which were stained with hematoxylin and eosin.

The length of the sagittal histologic sections was then measured by using the optical scale. The histologic sections were digitized by using a light microscope (Axiophot; Carl Zeiss, Oberkochen, Germany) and image analysis system (IBAS; Kontron, Munich, Germany). Fifty sections, at 8-μm intervals, closest to the center of the cornea were digitized to black-and-white images for each rabbit. Image contrast was adjusted to optimize the keratocyte nuclei.

The digitized images of the histologic sections were also processed by using the image analysis program (AnalyzeAVW, Mayo Medical Ventures). The epithelium and endothelium in each image were manually removed. The stroma was divided into five equal-thickness anteroposterior regions. The posterior stromal region was divided into three equal-thickness subregions. Keratocyte nuclei were manually identified and counted in each region of each section. Keratocyte density (per unit area) was estimated from this number and the corresponding area of the stroma corrected for tissue shrinkage due to fixation.

**RESULTS**

**Calibration**

Each pixel corresponded to 0.79 μm horizontally and 0.77 μm vertically at a z-depth of 500 μm from the objective lens tip. As

The mean density of keratocytes was calculated for full-thickness stroma and the various stromal regions in the 50 digitized sections of each central cornea by using this equation. $N_v$ was calculated, and $t$ was known (4 μm before adjustment for tissue shrinkage, and $D$ is the mean diameter of keratocyte nuclei in the direction perpendicular to the plane of the section.

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**Statistical Methods**

Repeatability of the confocal method was assessed by calculating the coefficients of variation (SD/mean) of total and regional keratocyte densities obtained from the three data sets for each of the six rabbits. The mean coefficients of variation of total and regional keratocyte densities were calculated.

Descriptive statistics were used to demonstrate the trend in keratocyte density with corneal stromal region for both the histologic and confocal data. We used a method described by Bland and Altman to assess agreement between the histologic method and the confocal method: The difference in keratocyte densities (of corresponding regions) estimated by the two methods was assessed as a function of the mean of the densities estimated by the two methods for each rabbit. The mean difference and 95% limits of agreement (defined as the mean difference ± 2 SD of the differences) were calculated.

Two-tailed, paired Student’s $t$-tests were used to show systematic differences in total and regional keratocyte density between the two methods. Paired $t$-tests were also used to detect differences in keratocyte density between the different regions and subregions of the stroma with both methods. The minimum detectable differences between the two methods, based on a power of 90%, were calculated for each region.
Determining Keratocyte Density by Confocal Microscopy

Figure 1. Results of the automated analysis of confocal microscopy images of the normal rabbit stroma in vivo. Preprocessed input images, processed binary images, and input images with superimposed identifying boxes are shown for two stromal regions: (A, B, C) The anterior stroma, 54 μm from the anterior corneal surface; (D, E, F) the posterior stroma, 333 μm from the anterior corneal surface. A higher number of keratocyte nuclei (bright objects) were visible in the anterior stroma than in the posterior stroma. For keratocyte density calculation, objects were counted if they were located within the defined counting area, or if they were lying across two (lower and left) of the four boundaries of the counting area. Only large objects lying across the other two (upper and right) boundaries of the counting area were counted. White boxes represent objects that were counted as single nuclei; dotted white boxes represent large objects that were counted as single or multiple nuclei, depending on the pixel area of the object. Black boxes represent objects that were not counted because they were too small or were lying across the upper and right boundaries. Dotted black boxes represent the large objects lying across the upper and right boundaries. These were counted as single or multiple nuclei followed by a reduction of the count by one. Bar, 100 μm.

Repeatability of the Confocal Method
The coefficients of variation of repeated estimates of keratocyte density by using the confocal method ranged from 0.032 to 0.075 (Table 1). In one rabbit, one confocal analysis (of three) was excluded when we calculated the coefficient of variation of the posterior region (80%-100% depth) because of a sudden gross ocular movement that impaired the analysis.

Keratocyte Density
By confocal microscopy, total keratocyte density was 39,037 ± 1,186 cells/mm³ (mean ± SE; n = 6). Keratocyte density was highest in the anterior stroma (47,072 ± 1,348 cells/mm³), but progressively fell with stromal depth and was lowest in the posterior stroma (27,873 ± 2,664 cells/mm³; Figs. 1, 2; Table 2). Within the posterior stromal region, keratocyte density was higher in the anterior subregion than in the mid and posterior (pre-Descemet’s membrane) subregions (Fig. 3).
By the histologic method, total keratocyte density was 37,781 ± 1,097 cells/mm³. Keratocyte density was highest in the anterior stroma (48,313 ± 917 cells/mm³) and was lowest in the posterior stroma (29,382 ± 894 cells/mm³) (Fig. 2, Table 2). Within the posterior stromal region, keratocyte density increased from the anterior subregion to the posterior (pre-Descemet’s membrane) subregion (Fig. 3). Mean tissue shrinkage during tissue preparation for histology was 5.7% (range, 3.3%–8.6%) and was accounted for in our calculations.

Agreement between Methods

The mean difference between cell density estimated by the two methods (confocal and histology) was 1,184 cells/mm³, and the 95% limits of agreement (mean difference ± 2 SD of the differences) were −9,329 cells/mm³ to 11,699 cells/mm³ (Fig. 4). Agreement was better in the anterior stroma than it was in the posterior stroma (Fig. 4).

Significance Tests

Keratocyte densities estimated by confocal microscopy were not significantly different from densities estimated by histologic methods (Table 2). The minimum detectable difference between the findings of the methods was smaller in the anterior stroma than in the posterior stroma (Table 2). Densities in the anterior 20% of the stroma were significantly higher than in the posterior 20% of the stroma by confocal microscopy (P = 0.004) and histology (P < 0.001). Within the posterior 20% of the stroma, the keratocyte density in the anterior one-third subregion was significantly lower than in the posterior one-third subregion (adjacent to Descemet’s membrane) by histology (P = 0.01); however, this finding was not significantly different from that obtained with confocal microscopy.

DISCUSSION

Confocal microscopy combined with an automated algorithm is a repeatable and valid method for estimating keratocyte density in rabbit corneas in vivo. When the confocal method was used, total keratocyte density varied by only 3.5% on repeated examination, although variation in the posterior cornea was greater. Agreement between the two methods was demonstrated by using the technique described by Bland and Altman. The mean difference between the two methods was found to be close to zero, and agreement in density estimations between the two methods in the anterior stroma was better than that in the posterior stroma. Whether the derived 95% limits of agreement are acceptable for clinical purposes is yet unknown, because there are few data concerning keratocyte density in the literature. The greater variation and disagreement in density estimation in the posterior stroma may be because less light reaches the posterior stroma and is reflected back in confocal microscopy or because of nonuniform tissue shrinkage in histologic preparations. Statistically, there were no significant differences between the methods in estimating total or regional keratocyte densities.

By both methods, keratocyte density decreased from the anterior stroma to the posterior stroma, in agreement with results in a study in vitro by Petroll et al. They also showed a pre-Descemet’s membrane increase in keratocyte density. Similar findings were noted qualitatively in porcine corneas. Within the posterior stroma, we showed that keratocyte density increased significantly in the stroma immediately anterior to Descemet’s membrane by histology (P = 0.01).
FIGURE 3. Subregional keratocyte density within the posterior 20% of
the full-thickness stroma measured by confocal microscopy and histology
(mean ± SE, n = 6). There was no significant difference between
methods in density measurement in each subregion. The keratocyte
density in the pre-Descemet’s membrane subregion was significantly
different from the density in the anterior subregion determined by the
histologic method only (P = 0.01; n = 6).

adjacent to Descemet’s membrane by using the histologic
method but not by using the confocal method. This discrep-
ancy may reflect the lesser agreement between the two meth-
ods in the posterior cornea as noted above. The confocal
microscope may also be limited by the thickness of its optical
section in its ability to resolve differences in density occurring
in layers two to four cells thick. The stereologic counts were
based on a small number of cells in a region representing only
6.7% of the full-thickness stroma.

By both methods in the present study, total keratocyte
density was estimated to be 38,500 cells/mm². This is similar
to, although somewhat higher than, the total keratocyte
density of 32,400 cells/mm² found by Petroll et al. in the same
species of rabbit. The difference between these findings may
be a result of differences in the methods used or differences in
defining stromal regions. It is also possible that rabbits of
different ages were used in the two studies and that keratocyte
density in rabbits varies with age, as has been shown in hu-
mans.

Møller-Pedersen et al. estimated mean keratocyte density
in human corneas to be 41,000 cells/mm² (mean age, 77 years)
by measuring stromal DNA content. This is similar to the
density we found in rabbits. A decline in central keratocyte
density with increasing stromal depth was quantitatively noted
in human corneas by using DNA measurements and quali-
tatively by using confocal microscopy in vivo. The ability to
quantify regional keratocyte density in humans in vivo would
greatly aid in our understanding of the cellular reaction to
injury, such as in stromal ablation during refractive surgery.

The confocal method presented here is an acceptable
method to the subject and the observer. Confocal microscopy
permits the examination of the corneal stroma in human sub-
jects in detail and resolution that were previously unattain-
able. Acquisition of images took seconds, and direct stor-
age of digital images to computer memory eliminated the
subsequent need to digitize videotape images. Once converted
to an acceptable format, image processing and derivation of
keratocyte density was fully automated. The algorithm ac-
counted for any changes in mean image size of the nuclei that
may have occurred because of biologic regional variation or
magnification changes with increasing z-depth. The algorithm
used two-dimensional images to provide a volumetric density,
which allowed frames from the image data set to be analyzed
at regular intervals rather than analyzing all frames (as many as
400) in the set. Three-dimensional analysis of the entire image
data set is possible, but this is a longer process than two-
dimensional analysis, because voxel registration of sequential
images is required. Sequential registration itself becomes diffi-
cult in image sets in which there is substantial ocular move-
ment (as may occur in human subjects), and in this situation
the final volume of cornea that would be analyzed would be
reduced. Thus the two-dimensional analysis would be more
time-efficient for clinical purposes and may generate more
data.

The main disadvantage of the confocal method described
was that the algorithm did not account for blurred images. This
was of little consequence in the present study, because the
rabbits were under general anesthesia, eliminating gross ocular
and head movements. Motion blur would be a greater problem
in human subjects, because examination would be under top-
ical anesthesia. We are making provisions for this in the algo-
rithm, to permit human studies. The algorithm presented in
this study used the thickness of the optical section as one
parameter to calculate keratocyte density. This may vary be-
tween different confocal microscopes, but if known, the algo-
rithm can be modified accordingly.

The stereologic principles used to estimate keratocyte
density from the histologic sections assumed the keratocyte
nuclei to be spherical. Although in reality the nuclei were not
spherical, the assumption was reasonable based on the sagittal
direction in which the tissue sections, and thus the nuclei,
were cut. The mean particle diameter was most easily esti-
mated from the (en face) in vivo confocal images specific to
each rabbit. The same parameter could also be estimated from
the (sagittal) histologic sections, but this required nuclear
shrinkage calculations and more complicated stereologic meth-
ods. Standard tissue preparation for light microscopy was

FIGURE 4. Difference between keratocyte densities estimated by us-

ing both methods (confocal microscopy and histology) as a function of
the mean of the keratocyte densities estimated by using both methods
in five corresponding stromal regions of six rabbits. The mean differ-
ence was close to zero. The mean difference ± 2 SD of the differences
represents the 95% limits of agreement.
used for histology, and tissue shrinkage was 5.7%. Shrinkage of 6% was noted by another group when human corneal tissue was prepared for light microscopy.26 We assumed that any tissue dimension changes due to fixation and embedding were uniform; however, nonuniform shrinkage may have been a source of error in regional keratocyte density estimation.

This study has shown that confocal microscopy produces valid findings, when used to estimate keratocyte density in vivo. Keratocyte density of the central rabbit cornea in vivo has been measured by an automated method for the first time, and the technique can be easily applied in humans. Histologic validation of this method will be necessary in humans.

Acknowledgments

The authors thank Bruce Cameron for skilful assistance in software development for control of the objective lens motor and for the confocal image capture system.

References

Effects of Dietary n-3 Fatty Acid Deficiency and Repletion in the Guinea Pig Retina

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PURPOSE. To investigate the nature and reversibility of biochemical and functional changes in the retina encountered over a single generation of dietary n-3 polyunsaturated fatty acid deficiency in guinea pigs.

METHODS. Dunkin-Hartley guinea pigs were fed for 16 weeks after weaning with diets supplemented with safflower seed oil (n-3 deficient) or canola oil (n-3 sufficient, control). A number of deficient animals were repleted at 6 weeks with canola oil for 5 or 10 weeks, or at 11 weeks for 5 weeks. Electroretinograms (0.8 and 4.3 log scot td · sec) were collected at 6, 11, and 16 weeks after weaning. Conventional waveforms (a- and b-waves), oscillatory potentials, and receptor and postreceptoral subcomponents (PHI and PII, respectively) were evaluated. Cone pathway function was assessed with 30-Hz flicker at the brighter intensity. Retinal phospholipid fatty acids were measured by capillary gas-liquid chromatography.

RESULTS. Electroretinographic amplitudes showed statistically significant losses in b- and a-waves after 6 and 16 weeks of dietary n-3 deficiency, respectively. The response amplitude to 30-Hz flicker was reduced 42% after 16 weeks. Retinal docosahexaenoic acid (DHA) levels of animals maintained on the safflower oil diet for 16 weeks were 42% of levels in age-matched control subjects. There were significant losses in maximum response amplitudes (Rb and Ra), although the major effect was a reduction in sensitivity of the receptoral response. Complete functional recovery was observed only in animals repleted for 10 weeks.

CONCLUSIONS. Functional deficits in PHI and PII of the electroretinogram were apparent in fist-generation guinea pigs fed an n-3 deficient diet. These losses showed a correlation with age and retinal DHA level, although varying degrees of dependence on the DHA level were found. All functional deficits were reversed after 10 weeks of dietary n-3 repletion. The results suggest that DHA may serve several functional and structural roles in the retina and further emphasize the requirement for DHA in the normal development of vision. (Invest Ophthalmol Vis Sci. 1999;40:327-338)

Docosahexaenoic acid (DHA) is the most ubiquitous polyunsaturated fatty acid (PUFA) in the retinae of mammals and is present in up to 60% of the structural phospholipid molecules within the membranes of the photoreceptor outer segments.1 2 In addition, the presence of DHA in the mammalian neural tissue is remarkably constant among species,2 3 and strong conservation mechanisms exist, locally and systemically, rendering the retinal fatty acid profile resistant to changes by means of dietary manipulation.4-6 In cases of deprivation, retinal DHA losses have been shown to give rise to electroretinographic (ERG) deficits7-11 and reduced visual acuity.12 To this end, the guinea pig has proved a good model of dietary n-3 PUFA manipulation, because it can be substantially depleted of DHA by successive breeding over several generations.13,14 However, our earlier work in this species was conducted over three generations10,11 to achieve substantial DHA depletion. It was not known whether the guinea pig would show DHA and ERG deficits after weaning over a single generation, as reported in rats and monkeys.7,8 This issue is important if the guinea pig is to provide a viable model of human n-3 PUFA deficiency and photoreceptor function.

In this context, the guinea pig may provide a more suitable model of neural change after dietary manipulation than other small animals, because, similar to humans, it is a precocial mammal having undergone substantial growth and neural differentiation in utero.15 Therefore, it is likely that the normal accretion of DHA in the retina has been fulfilled by the immediate postnatal period, and the results of postnatal dietary manipulation can be better extended to the scenario of a malnourished human infant. In contrast, altricial species, such as rats and mice, undergo substantial postnatal retinal development. It could be argued that altricial species would be more susceptible than precocial species to dietary manipulation during the immediate postnatal period of neural growth, which raises questions about the direct applicability to human development of findings made with such species.

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Jointly supported by the Grains Research and Development Corporation and Meadow Lea Foods, Australia.

Submitted for publication April 2, 1998; revised September 16, 1998; accepted September 28, 1998.

Proprietary interest category: C5.

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One significant nutritional issue that should be considered is the longitudinal nature of vision loss encountered with continued retinal DHA deprivation. Several studies have indicated that functional losses in rat and monkey partially resolve with time, despite continued dietary n-3 deficiency. This finding, however, has not been reproduced in guinea pigs.7 There is a need for confirmation of our earlier finding and a more detailed understanding of age-related changes, if any.

Several investigators report abnormal tissue n-3 PUFA levels in cases of retinopathy.18-23 This has raised the possibility that dietary n-3 PUFA supplementation may reverse the tissue levels and improve functional performance in these conditions. However, studies providing dietary supplementation of long-chain n-3 PUFA in cases of receptoral degeneration failed to alter the levels of retinal DHA or improve function.20-22 This questions the capacity of dietary methods to alter tissue PUFA levels and function, or it implies some critical timing for the supplementation to reverse the effects of deprivation.

In the present study, we sought to determine the reversibility of functional and biochemical deficits encountered in conditions of postnatal n-3 PUFA deficiency in a precocial animal (guinea pig). In addition, we considered the importance of the timing and duration of repletion in this process. Specifically, we sought evidence for the existence of a critical period for retinal DHA accretion, because this might explain the failure to induce change during retinopathy.

An important consideration in DHA deficiency is the site of functional change. Although the precise molecular role of DHA in retinal function remains unclear, much evidence links the presence of DHA in the outer segment membranes to visual function. N-3 PUFA deficiency has been shown to affect directly the function of the visual photopigment rhodopsin.24 Several models explaining the role that DHA has in rhodopsin’s function have been proposed.25,26 The model suggested by Littman and Mitchell26 states that when outer segment membranes are composed of highly unsaturated molecules, such as DHA, they provide an optimal domain for rhodopsin transformation on light capture. Therefore, a reduction in retinal DHA levels results in a suboptimal membrane environment that restricts the activation of membrane-bound proteins.26 A reduced receptoral response is predicted in conditions of DHA deficiency, consistent with the findings of several ERG studies.7-11 However, it is reasonable to predict that DHA deficiency also results in a compromised postreceptoral response, because similar membrane-bound G-protein cascades operate in postreceptoral elements.87 However, the literature shows mixed evidence for postreceptoral losses. The early work of Wheeler et al.28 indicates that the receptors are the primary site of lesion in n-3 deficiency. Furthermore, in monkeys29 and human infants,30 DHA deficiency has been shown to reduce a-wave amplitude and sensitivity in the absence of b-wave amplitude changes, findings that may indicate upregulation of the postreceptoral processes. Conversely, Bourre et al.31 found exaggerated b-wave losses in 4-week-old n-3-deficient rats, suggesting a larger postreceptoral deficit. Elsewhere, we have presented ERG data that we think are consistent with generalized losses of function with age,17 but these early findings must be confirmed in larger groups of animals.

In this study, we intended to address the nature of loss in terms of the receptoral (P1/R1) and postreceptoral (P2/R2) components of the ERG after manipulation of the retinal PUFA profile. Furthermore, the receptoral response was differentiated into amplitude and sensitivity changes, to consider the role that DHA has in general membrane function, specifically in the mechanism of light capture mediated by rhodopsin and in the transductional events that follow. The purposes of our study were as follows: to assess the effect of dietary n-3 PUFA deprivation on the visual function (ERG) of a precocial mammal, to consider reversibility of functional and biochemical changes after repletion with a diet containing α-linolenic acid, and to consider the site of functional deficit associated with retinal DHA deficiency.

**Materials and Methods**

**Animals and Diets**

All procedures involving animals were conducted in accordance with local animal care guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. They were approved by our institutional ethics committee.

Animals in this experiment were depleted of n-3 PUFA over a single generation from the time of weaning. Twenty-one-day-old, albino guinea pigs (Dunkin-Hartley strain) were weaned to consumption of one of two semipurified diets containing different amounts of n-3 fatty acids. Dietary fats were supplied by the supplementary oils added to the diets. Diets contained (in grams per kilogram) 300 casein, 100 sucrose, 70 glucose, 200 starch, 100 cellulose, 100 oil, 30 kaolin, 3 l-arginine, 1 L-methionine, 68 mineral mix, and 27 vitamin mix.18 The diet designed to result in depletion of retinal n-3 PUFA was supplemented with safflower seed oil (SFO: n-6/n-3, 72) whereas canola oil (CNO) supplements produced an n-3-sufficient diet (CNO: n-6/n-3, 2.5). The assayed fatty acid compositions of both experimental diets are shown in Table 1.

Animals were fed once daily with dietary pellets and were supplemented daily with 10 g fresh carrot and water containing 400 mg/l ascorbic acid ad libitum. The ambient light (500 lux) was cycled in the animal room (12-hr/12-hr light/dark cycle), with temperature maintained at 21°C.

**Table 1. Fatty Acid Composition of Diets**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Canola Oil</th>
<th>Safflower Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.38</td>
<td>0.41</td>
</tr>
<tr>
<td>16:0</td>
<td>5.15</td>
<td>7.30</td>
</tr>
<tr>
<td>16:1</td>
<td>0.28</td>
<td>0.14</td>
</tr>
<tr>
<td>18:0</td>
<td>2.70</td>
<td>2.63</td>
</tr>
<tr>
<td>18:1</td>
<td>58.93</td>
<td>15.04</td>
</tr>
<tr>
<td>18:2-6</td>
<td>20.55</td>
<td>71.99</td>
</tr>
<tr>
<td>18:3-6</td>
<td>8.19</td>
<td>1.01</td>
</tr>
<tr>
<td>20:0</td>
<td>1.01</td>
<td>0.32</td>
</tr>
<tr>
<td>20:1</td>
<td>1.47</td>
<td>0.08</td>
</tr>
<tr>
<td>22:1</td>
<td>0.38</td>
<td>—</td>
</tr>
<tr>
<td>Total n-6</td>
<td>20.55</td>
<td>71.99</td>
</tr>
<tr>
<td>Total n-3</td>
<td>8.19</td>
<td>1.01</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>2.51</td>
<td>71.27</td>
</tr>
</tbody>
</table>

Data are expressed as grams fatty acid per 100 g total fatty acid. Diets were composed of 10% lipid, by weight.
At weaning, 36 animals were assigned to the SFO (S) diet and 18 animals to the CNO (C) diet. At 6 (C6, S6), 11 (C11, S11), and 16 (C16, S16) weeks after weaning, six animals from both diet groups had ERGs measured before death. The remaining SFO-fed animals were repleted with the CNO diet as follows: at 6 weeks, for 5 (S6C5; n = 6) or 10 weeks (S6C10; n = 6) or at 11 weeks, for 5 weeks (S11C5; n = 6). Replicate animals were assessed and killed at 11 weeks (S6C5) or 16 weeks after weaning (S6C10, S11C5). Canola oil was used in the repletion diet, because it contains a high n-3/n-6 ratio in the absence of those long-chain PUFAs most susceptible to oxidative damage. Moreover, we have shown that CNO supplementation sustains brain and retinal DHA levels in this species.\textsuperscript{14} The logic in the experimental design was to determine whether 5 weeks of repletion could reverse 6 weeks of depletion; whether 5 weeks of repletion could induce a similar level of reversal, regardless of age at beginning of repletion (6 or 11 weeks); and whether 10 weeks of repletion could produce a greater repletion effect than 5 weeks.

**Electroretinography**

Single flash and 30-Hz flicker ERGs were recorded at 6, 11, and 16 weeks after weaning in all animals.

**Light Stimulus**

White light was generated by a 150-W, 24-V quartz-halogen lamp focused on to the face of a 20-mm fiber optic, through a series of calibrated neutral density filters (Schott-Garsco, Sydney, Australia) and an electromagnetic shutter (Uniblitz T132; Vincent Associates, Rochester, NY). Light emerging from the fiber optic was focused in the pupillary plane of the animal, and Maxwellian view was achieved with a 3.5-mm image of the filament. The angular subtense of the fiber optic was 90° at the usual viewing distance (10 mm).

Our light source was calibrated according to the expected retinal illumination for the guinea pig to enable standardized fitting of the ERG subcomponents (discussed later). A spectro-radiometric scan of the illuminated fiber optic source was conducted in 5-nm steps (400–720 nm), using a telespectrophotometer ($P_d$, model 1980B; Spectra-Pritchard Photo Research, Burbank, CA). This was used in equation \textsuperscript{31} to determine a correction factor for the guinea pig's scotopic response ($P_d$),\textsuperscript{32,33} assuming $K_m = 1700$.\textsuperscript{31} The photopic retinal illumination provided by the fiber optic, $E$ (in td), was measured according to the method of Nygaard and Frumkes\textsuperscript{34} and converted to guinea pig scotopic units using the correction factor described previously. Allowance for eye size (8.7 mm) and flash duration\textsuperscript{31} yielded an “effective” guinea pig retinal exposure of 4.8 log scot td · sec for the unattenuated light source. The photopic illumination of the source was measured as $3 \times 10^6$ lux at the corneal surface, a distance of 10 mm from the fiber optic face.

Flash ERG was performed at two light intensities to span rod activity. A low-intensity b-wave was obtained by attenuating the light source by 4 log units (0.8 log scot td · sec) using calibrated ND filters. The brightest exposure was obtained by attenuating the light source by 0.5 log units (4.3 log scot td · sec). This value was used in modeling the $PIII$ process (equation 2), assuming 8.6 photoisomerizations per second per rod. Testing proceeded in a fixed order, beginning with the lower exposure, followed by the brighter one, followed by 30-Hz flicker at the bright intensity.

**Electroretinographic Procedure**

All procedures followed complete dark adaptation for longer than 15 hours, to encourage maximum retinal sensitivity. Ten minutes before recording, animals were anesthetized by intramuscular injection of 35 mg/kg ketamine and 5 mg/kg xylazine. Deep anesthesia was achieved for at least 60 minutes. Maximal pupillary mydriasis was induced using 1 drop 0.5% tropicamide and was evident shortly after instillation. Preparation was performed under the illumination provided by a red LED ($\lambda_{\text{max}}$, 650 nm; bandwidth 10 nm), after which animals were allowed to dark-adapt for a further 10 minutes before ERG. The LED was chosen for its minimal effect on the rod photopigment of the animal.\textsuperscript{35}

At each exposure, eight 8-msec (measured width at half-height) responses with an interstimulus interval of 30 seconds were amplified ($\times 4000$, $-3$ dB at 1 Hz and 300 Hz; AMP 801; Neuroscientific, Farmingdale, NY) and recorded. A sampling rate of 1 kHz was used over a 250-msec recording window. Off-line averaging and band-pass filtering (0.3–45 Hz) were used to eliminate mains noise and oscillatory potentials (OPs) and to expose the principal components (a- and b-waves). In the averaging process, an optimization technique (rejecting signals varying by >2 SD; Neuroscientific) was adopted to remove outliers, so that each outcome shown in this article represents the average of seven to eight optimized records (in most cases eight were used). For the cone response to 30-Hz flicker, a total of 250 signals were averaged after 10 preadapting flashes. The amplitudes of the a- and b-waves were determined on these optimized and filtered outcomes, with the a-wave measured from baseline to trough and the b-wave from a-wave trough to b-wave peak (peak-to-peak). The 30-Hz flicker response amplitude was measured from trough to peak of the steady state potential.

Signals were recorded with a customized bipolar Burian-Allen corneal electrode (Hansen Laboratories, Iowa City, IA). A stainless-steel Teflon-coated monopolar needle electrode (25 mm, coated except for the 0.5-mm recording tip; Medelec, Richmond, Australia) was used as a ground and was positioned subcutaneously in a skinfold at the neck of the animal.

**Subcomponent Analysis**

The process of phototransduction after the photoisomerization of rhodopsin has been described as a series of exponential processes.\textsuperscript{35} It has been proposed that this model can also describe the leading edge of the ERG a-wave, as shown in equation 2.\textsuperscript{36,37}

\[
\text{PHI}(i, t) = \{1 - \exp[-i \cdot S \cdot (t - t_0)^2]\} \cdot R_{\text{norm}} \text{ for } t > td \tag{2}
\]

where $\text{PHI}$ is a function of $i$ (intensity, scot td · sec) and $t$ (in seconds) and refers to the amplitude of the photoreceptor-generated subcomponent of the ERG, the fast $\text{PHI}$. In equation 2, $S$ is a sensitivity parameter (scottd · sec$^{-3}$), $t_0$ is the latency of the response (in seconds) that allows for delays introduced
by the recording equipment and delays in the various stages of phototransduction, and \( R_{\text{max}} \) is the receptor saturated response amplitude (in microvolts). The fitting of our raw and unfiltered data was achieved by floating all parameters of equation 2 for the brightest exposure (4.3 log scot td · sec), and minimizing the least-square-error term using a Levenberg-Marquardt algorithm developed in our laboratory.\(^{26}\) Fitting was performed up to the time \((t)\) of the first local minimum or 16 msec, whichever came first. An example of empiric data for one animal with the extracted subcomponents and OPs is shown in Figure 1.

It has been shown that the a-wave comprises a substantial postreceptorial component at low light levels\(^{39} \), therefore, bright flashes are needed to ensure receptorial isolation. However, bright levels serve to promote cone contributions. Although methods for rod isolation using colored filters have been proposed,\(^{40}\) we elected not to use such procedures because it has been shown that the cone contribution to the a-wave response after flashes up to 4.1 log scot td · sec is negligible.\(^{40}\) Furthermore, the peak sensitivities of the rod, and short-wavelength-sensitive, and middle-wavelength-sensitive receptor mechanisms exhibit considerable overlap in this species,\(^{33}\) and isolation of the middle-wavelength-sensitive cone component \((\lambda_{\text{max}}, 529 \text{ nm})\) from the rod response would be difficult. In addition, at 4.1 log scot td · sec, the influence of cone receptors on \( I_{\text{a}} \) and sensitivity is minimal and their contribution to \( R_{\text{max}} \) has been estimated at 15%,\(^{40}\) although it may be even lower in the rod-dominant retina of this species.\(^{39,41}\) Moreover, the fits found for our data, as shown in Figure 1, indicate excellent concordance with the model of rod transduction, suggesting little cone influence under the test conditions.

The \( \text{PHI} \) response can be approximated by digital subtraction of the modeled \( \text{PHI} \) (equation 2) from the raw data.\(^{42}\) This response has a similar dynamic characteristic to that of the \( \text{PHI} \), although modeling has been difficult.\(^{27,42}\) We have found that a reasonable approximation of \( R_{\text{max}} \) can be achieved with an inverted form of equation 2, and this was used to fit data to 5 msec beyond the b-wave maximum. Our fitting times are indi-
A percentage of the age-matched CNO level. SEMs are approximated by

**FIGURE 2. Retinal DHA for all experimental diet groups, expressed as 10VS, February 1999, Vol. 40, No. 2**

TABLE (P < 0.05) were determined in comparison with the age-matched control group and are indicated by *, †, and ‡, at 6, 11, and 16 weeks, respectively.

<table>
<thead>
<tr>
<th>PUFA</th>
<th>C6</th>
<th>C11</th>
<th>C16</th>
<th>S6</th>
<th>S11</th>
<th>S16</th>
<th>S6C5</th>
<th>S6C10</th>
<th>S11C5</th>
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</thead>
<tbody>
<tr>
<td>20:4n-6</td>
<td>7.4±0.2</td>
<td>8.0±0.1</td>
<td>8.2±0.1</td>
<td>9.1±0.2*</td>
<td>9.2±0.1†</td>
<td>9.4±0.2‡</td>
<td>8.5±0.2</td>
<td>8.7±0.1‡</td>
<td>9.0±0.1‡</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>1.9±0.1</td>
<td>1.9±0.0</td>
<td>2.1±0.0</td>
<td>2.9±0.1*</td>
<td>3.2±0.1†</td>
<td>3.6±0.1‡</td>
<td>2.8±0.1</td>
<td>2.3±0.1</td>
<td>2.8±0.1‡</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>3.0±0.2</td>
<td>2.3±0.2</td>
<td>2.9±0.3</td>
<td>9.8±0.1*</td>
<td>12.4±0.5†</td>
<td>15.8±0.5‡</td>
<td>6.9±0.3</td>
<td>4.0±0.4‡</td>
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</tr>
<tr>
<td>20:5n-3</td>
<td>0.6±0.1</td>
<td>0.9±0.2</td>
<td>0.9±0.2</td>
<td>0.6±0.1</td>
<td>0.7±0.1</td>
<td>0.3±0.1†</td>
<td>0.8±0.1</td>
<td>0.4±0.0f</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>1.3±0.1</td>
<td>1.2±0.1</td>
<td>1.2±0.1</td>
<td>0.5±0.0*</td>
<td>0.4±0.1†</td>
<td>0.3±0.0f</td>
<td>1.0±0.1</td>
<td>1.1±0.1</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>22.5±0.5</td>
<td>20.6±0.6</td>
<td>22.1±0.3</td>
<td>15.1±0.3*</td>
<td>12.2±0.5†</td>
<td>9.3±0.3‡</td>
<td>15.9±0.3†</td>
<td>18.7±0.7‡</td>
<td>16.3±0.6‡</td>
</tr>
</tbody>
</table>

Data are the means ± SD (n = 6 per group), expressed as percentage of total phospholipid fatty acids. Canola oil-fed animals are designated by age (weeks) as C6, C11, and C16 and safflower oil-fed animals as S6, S11, and S16. Replete animals are designated by the SxCy nomenclature, where x refers to the age (after weaning) when repletion was begun, and y refers to the duration of repletion in weeks. Significant differences (P < 0.05) were determined in comparison with the age-matched control group and are indicated by *, †, and ‡, at 6, 11, and 16 weeks, respectively.

**Subcomponent Analysis in DHA Deficiency**

There were no significant differences for any of the PIII and PII measures between CNO and SFO diet groups at 6 or 11 weeks after weaning, with significant (P < 0.05) differences becoming apparent only at 16 weeks. Representative data with the fitted PIII subcomponents for CNO- (16 weeks) and SFO-fed animals at the three test ages (6, 11, and 16 weeks) are shown in Figure 3. In this figure, graded losses of sensitivity and Rpha amplitude occur with continued dietary n-3 deprivation beyond 6 weeks and are most marked at 16 weeks. By this age, SFO-fed animals had significantly reduced maximum response amplitudes (Rpha) for PIII and PII (mean changes of -39% and -39%, respectively) compared with the same parameters in the maximum response and the slope (sensitivity) of the a-wave are significantly affected, with a mean ± SEM of 6.29 ± 0.11 msec for all diets and ages.

The average (± SEM) relative change (log CNO/SFO) in the saturated PIII response amplitude (upper panel), PIII sensitivity (middle panel), and saturated PII response amplitude (lower panel) as a function of age are shown in Figure 4. In this figure, first generation data have been represented by the solid lines and symbols, whereas the gray symbols (broken lines) represent data from third-generation animals of the same ages reported elsewhere.17

The correlation between retinal DHA (percentage of phospholipid fatty acids) and relative change (log CNO/SFO) in saturated PIII and PII amplitudes (upper panel) and PIII sensitivity (lower panel) is shown in Figure 5. The solid lines show the best fitting Weibull functions to the entire data set (including third-generation), and have been added to emphasize the trends. There were no significant differences between C16 and S6C10 animals in the PIII and PII parameters, although the saturated PII response of S6C10 animals bordered on statistical significance (P = 0.08; power = 0.78). In addition, the S6C10 group had a significantly (P < 0.05) increased Rpha and sensitivity for PIII compared with the S16 and S11C5 groups.

**FIGURE 2. Retinal DHA for all experimental diet groups, expressed as a percentage of the age-matched CNO level. SEMs are approximated by the symbol size, and all points are significantly removed from each other (P < 0.05) except S6C5 versus S11C5.**

**FIGURE 3. Representative ERG waveforms (4.3 log scot td · sec, initial 17 msec) recorded from CNO- (black circles, solid line) and SFO- (dotted line) raised animals 6 (small gray squares), 11 (medium gray squares), and 16 weeks (large gray squares) after weaning. Note how the maximum response and the slope (sensitivity) of the a-wave are gradually affected in the SFO-fed animals.**
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-0.8 -I 11
Age (weeks postweaning)

FIGURE 4. The effect of age and time consuming the diet on $R_mPW$ (upper panel), PHI sensitivity (middle panel), and $R_mPII$ (lower panel). Values are expressed as the difference compared with those parameters in CNO-fed animals (Alog value ± SEM). Data from first-generation animals (present study) are indicated by the black symbols, and data from our previous three-generation study17 (gray symbols) have been included for comparison. Significant differences from CNO (P < 0.05) are indicated by the asterisks.

Conventional Waveform Parameters

Consistent with previous reports from this laboratory,11 and from others, guinea pig ERG waveforms were b-wave-dominated at low exposures and showed substantial OP and a-wave amplitudes at bright exposures.49,50 The guinea pig exhibits much lower b-wave/a-wave ratios than those reported in other species49; however, in recent work using Ganzfeld illumination, the guinea pig ERG b-wave/a-wave ratio at saturating flash intensities often exceeds 2.0. The b-wave/a-wave ratios were 1.2 ± 0.1 and 1.0 ± 0.1, for the CNO and SFO groups, respectively.

At both stimulus configurations, ERG amplitudes of CNO-fed animals were larger than those of SFO-fed animals of the same age (Table 3), with SFO-fed animals having combined a- and b-wave amplitude reductions of 15%, 15%, and 34%, at 6, 11, and 16 weeks after weaning, respectively. The a-wave response to bright (4.5 log scot td · sec) stimuli showed that on-going dietary n-3 fatty acid deprivation resulted in significant (P < 0.05) ERG amplitude deficits in SFO-fed animals at 16 weeks. In contrast, the b-wave response to bright stimuli was significantly (P < 0.05) reduced in animals maintained on SFO from 6 weeks. At the dimmer exposure (0.8 log scot td · sec), significant (P < 0.05) losses in SFO-fed animals were observed after 11 weeks (Table 3). There were no significant differences in the implicit times for the a- (average time in milliseconds ± SEM: SFO, 25.83 ± 0.37; CNO, 25.31 ± 0.71) and b-waves (SFO, 58.61 ± 3.40; CNO, 53.97 ± 1.67) at either intensity or age. The total OP amplitude for the SFO group was significantly reduced by 38% compared with that in CNO-fed animals (P < 0.05) only at 16 weeks. Similarly, the results for 30-Hz flicker showed significant losses only at 16 weeks (Table 3; P < 0.05).

Compared with CNO-fed animals, the 30-Hz peak-to-peak amplitude of SFO-fed animals was reduced by 42%, but the implicit times of this waveform were unaffected (Table 3).

Efficacy of Repletion: Biochemical and Functional Recovery

Refeeding with CNO diet increased the retinal DHA level in all the repleted groups (P < 0.05; Fig. 2). In addition the retinal...
DHA level in the three repleted groups was greater than in their age-matched SFO counterparts \( (P < 0.001) \). Five weeks of repletion resulted in a greater retinal DHA change when begun after 11 weeks \( (S11C5, +34\% \text{ versus that in S11}) \) compared with that after 6 weeks \( (S6C10, +5\% \text{ versus that in S6}) \). Of note, the S6C10 group showed only approximately half the amount of DHA accretion during the last 5 weeks, when compared with that of the S11C5 group \( (+18\% \text{ in S6C10 versus +34\% in S11C5}) \). However, after 10 weeks of repletion, animals in the S6C10 group had DHA levels only slightly reduced compared with those in the age-matched CNO group \( (Table 2) \).

The effect of repletion on average a- and b-wave amplitudes is shown in Figure 6. By 16 weeks, all groups had improved retinal function compared with their age-matched SFO-fed counterparts, and animals that had been repleted for 10 weeks showed no difference from CNO control subjects \( (Table 2; Fig. 6) \). A significant deficit in \( PIII \) and \( PII \) subcomponents \( (P < 0.05) \) was present in the 16-week old group repleted for a 5-week period \( (S11C5) \) despite their large relative DHA accretion \( (Figs. 2, 5) \). As might be expected, this result also applied to the a- and b-wave responses at the bright exposure \( (Fig. 6) \). The b-wave for the dimmer exposure \( (Table 3) \) was restored in all replete groups and was significantly \( (P < 0.05) \) removed from age-matched SFO-fed animals.

**DISCUSSION**

The findings of this study extend the existing literature, which reports the effects of n-3 depletion on the conventional ERG parameters after several generations of dietary deficiency, by considering such effects over a single generation. In the following discussion we consider our findings in terms of those issues raised in the introduction, namely: the effect of dietary n-3 PUFA deprivation over a single generation, including the site of functional changes, assessed by the fundamental ERG components \( PI \) and \( PII \), and furthermore, the reversibility of functional and biochemical changes after repletion with dietary a-linolenic acid.

**Effect of n-3 Depletion on Retinal Function**

Retinal n-3 depletion resulted in significant reductions in \( PI \) and \( PII \) amplitudes and in \( PIII \) sensitivity \( (Figs. 4, 5) \). The results of the subcomponent analysis were consistent with those reported in third-generation depleted animals by 16 weeks of age.\(^7\) However, marked differences were evident in the trends for \( R_{\text{ermi}} \) and \( R_{\text{ermii}} \), compared with \( PIII \) sensitivity, which showed a strong age dependence. Consider that the retinal DHA levels of SFO-fed animals were 15.1\% and 9.3\% after 6 and 16 weeks, respectively, in the first-generation, and 2.5\% and 4.3\%, respectively, in animals bred for three successive generations.\(^10\) Given these levels and the findings of this study, it seems that maximum response amplitudes and sensitivity mechanisms were affected by an age-

![Figure 6](https://example.com/figure6.png)

**Figure 6.** The effect of n-3 depletion on retinal function, showing the average (±SEM) relative amplitudes (compared with age-matched control subjects at 4.3 log scot td · sec) for the a- \( (\text{unfilled bars}) \) and b-waves \( (\text{filled bars}) \) in the three repletion groups \( (identifed by the alphanumeric code at the top of the figure) \). Significant differences \( (P < 0.05) \) from CNO controls are indicated by asterisks.
DHA level interaction. The $R_{\text{amp}}$ and $R_{\text{uni}}$ responses were primarily DHA dependent but also showed an age effect of the animal, whereas the receptoral sensitivity was primarily determined by the age of the animal, exhibiting a relationship with the DHA level (at 16 weeks; Fig. 5) once the critical age was exceeded.

Reduction in $R_{\text{uni}}$

The findings of this study suggest that changes in $R_{\text{uni}}$ were subject to dietary (n-3 fatty acid) factors that influenced the retinal DHA levels (Fig. 5). The reduction (difference in log amplitude versus CNO control subjects) in the $R_{\text{uni}}$ of SFO-fed animals showed significant losses by 6 weeks in the third generation, but not until 16 weeks in the first generation (Fig. 4, upper panel). Therefore, the $R_{\text{uni}}$ loss must have been DHA dependent, because the essential difference between first- and third-generation 6-week animals was in their retinal DHA content. The correlation between retinal DHA and $R_{\text{uni}}$ for 16 week animals, the age at which significant losses were found, is shown in Figure 5 (upper panel, circles). The data in Figure 5 indicate that a moderate but sharp transition occurred between 15% and 18% DHA, and therefore a critical level was required for normal $R_{\text{uni}}$ amplitudes. Nevertheless, $R_{\text{uni}}$ losses also showed an age-dependent effect because younger animals (<16 weeks) with a DHA content below this critical level returned normal $R_{\text{uni}}$ values in the first generation (Table 3).

$R_{\text{uni}}$ describes the saturated rod response that reflects the change in current flow in the outer segments after light stimulation. It is accepted that $R_{\text{uni}}$ is related to the total number of cation channels and reflects outer segment membrane area.37,40 The saturated rod response has been shown to be proportional to the length of the outer segments in individual rods.41 As such, reductions in $R_{\text{uni}}$ are typically caused by changes to outer segment morphology or a reduction in the number of receptors, as is the case in retinitis pigmentosa.42 However, in developing rat retina, it has also been shown that the saturated amplitude of the a-wave lags behind the growth of the outer segments but is closely related to the incorporation of rhodopsin into the rods.53 Therefore, the concentration of unbleached rhodopsin in the outer segments is also an important factor in determining the magnitude of $R_{\text{uni}}$. A reduced photon catch has been reported in conditions of n-3 PUFA deficiency44 that may be caused by abnormalities in the deactivation phase of transduction within the outer segment membranes55 or in the recycling of the chromophore through the RPE.24 Because previous literature has shown that rod number and morphology are normal in n-3-deprived animals,54,56 it is likely that the $R_{\text{uni}}$ deficits found in the present study reflect a reduced concentration of unbleached rhodopsin. This is consistent with the reported differences in the size distribution and number of RPE phagosomes55 in conditions of n-3 fatty acid deficiency. Furthermore, our proposal is also consistent with the reported $R_{\text{uni}}$ reductions in the presence of normal sensitivity during abnormal rhodopsin deactivation.56

Reduction in Sensitivity

The sensitivity of the rod photoreponse was also reduced in SFO-raised animals. The loss was found to be age dependent because sensitivity was only reduced after 16 weeks consuming an SFO diet in the first and third generations, regardless of the DHA level (Fig. 4). The significance of this age dependence is evident in the third-generation SFO-fed animals in which sensitivity was normal at 6 weeks but abnormal at 16 weeks, despite similar, yet low, levels of retinal DHA (2%-4%). However, at 16 weeks, the loss of sensitivity showed a dose-related correlation with DHA (Fig. 5). The Weibull function fitted to this data indicated that the critical retinal DHA level ($R_{\text{cr}}$, the retinal DHA level that returns a sensitivity equal to 67% of age-matched control) was approximately 14%. Moreover, the sensitivity mechanism showed a different DHA dependence from that mediating $R_{\text{uni}}$, implying that two different lesions underlie these changes. Nevertheless, both lesions show a similar age-related onset in the first generation with the $R_{\text{uni}}$ change strongly DHA-dependent, whereas the sensitivity loss was modified by age.

According to the computational model of rod transduction first described by Hood and Birch,56 a change in the sensitivity parameter is equivalent to a change in flash energy. Sensitivity is therefore determined by the quantal catch of the receptor that is affected by structural parameters, such as rod-packing density, receptor alignment, and pigment content. However, after photon capture, it is the amplification of the rhodopsin–transducing–phosphodiesterase cascade that drives sensitivity. Again, because receptoral morphology is reported unchanged in n-3 PUFA deficiency, it is unlikely that our reductions in sensitivity can be accounted for by altered receptoral structure. As for the $R_{\text{uni}}$, a reduction in unbleached rhodopsin concentration may account for some but not all the decrease in sensitivity found in this study. This interpretation is supported by the data in Figure 5 in which abnormal $R_{\text{uni}}$ values were recorded in the presence of normal sensitivity in 6-week third-generation SFO-fed animals. Therefore, most of the decrease must lie in elements of the transduction cascade. The finding of Bush et al.24 that in vivo rhodopsin activation is unaltered in n-3 PUFA-deprived rats, suggests that our sensitivity loss may have arisen downstream in the cascade, possibly at the phosphodiesterase level. However, several in vitro studies have shown that the conversion of rhodopsin to its active state can be affected by a reduced membrane DHA content25,26 causing a defect early in the cascade. Regardless of whether rhodopsin activation is affected, these large sensitivity changes (~0.7 log unit) in the presence of moderate $R_{\text{uni}}$ losses (~0.2 log unit) are consistent with membrane-related transduction inefficiencies, and their capacity to act independently implies lesions affecting at least two separate loci.

The separation of PIIR sensitivity and $R_{\text{uni}}$ found in our study is similar to that reported in retinovascular disorders, such as central retinal vein occlusion, in which selective losses in sensitivity were also noted.57 Both observations are consistent with the report that in neonatal human infants, these indexes have different developmental time frames.58

Our results show that the development of sensitivity in the photoreceptors is a complex process affected by a combination of age and dietary factors. It is difficult to speculate about why the elements of receptoral transduction would be affected by DHA deficiency only later in life. One possibility is that DHA is not intrinsically necessary for the processes that determine the photoreceptoral response, but continued deprivation precipitates a “biochemical degeneration” that affects other cellular processes such as gene transcription. This effect could be expected to be duration dependent and not age dependent and would eventually affect the transduction cascade.
Alternatively, n-3 PUFA-deficient animals may not be able to maintain their normal structural or functional capacity by upregulation and conservation, and this might eventually lead to substantial developmental deficits. In this model, development of the normal functional architecture occurred early in life, but could not be sustained over time, when n-3 PUFAs were absent from the diet. Such an effect may be age dependent and have a developmental critical period. Although we have not tested the possibility for a duration effect, we think that our findings are consistent with a critical period for two reasons. First, animals repleted after 11 weeks of depletion (S11C5) showed significantly reduced sensitivity compared with animals repleted at 6 weeks (S6C10), despite only a small DHA difference. Secondly, animals repleted for 5 weeks had greater sensitivity if repletion was begun early (S6C5 > S11C5).

These findings imply that the critical age for this development lies somewhere between 11 and 16 weeks in guinea pigs but the exact mechanism for this loss is still unknown. When deprivation was continued for the entire duration of this period, \( R_{aw} \) showed an abrupt steplike correlation with DHA, whereas sensitivity showed a dose-related loss (Fig. 5). Repletion with n-3 fatty acids at any time during this period improved retinal DHA content, but normal function was only evident with early and prolonged intervention. This finding may explain why DHA repletion fails to reverse retinal degeneration in \( precA/afected \) dogs. If our hypothesis is correct, we would predict that supplements have an effect only if they are begun early (before the critical period for the species) and sustained over long durations. Such observations have important ramifications for the timing of dietary intervention in young humans and in studies in which an attempt is made to reverse the effects of DHA deficiencies in cases of retinopathy.

**Effect of n-3 Depletion on Postreceptor Processes**

The results of this study provide new evidence that the postreceptor elements, in addition to receptor function, are affected in conditions of n-3 fatty acid depletion. The effect of n-3 depletion on \( R_{aw} \) is shown in Figure 4 (upper panel, square symbols) in which the curve differs in its transition point and in the local maximum \((P < 0.05)\). Although the mechanism of dysfunction seemed to differ from that of \( R_{aw} \), \( PI2 \) amplitude also seemed subject to a critical DHA level. Below this critical level, reductions in DHA concentration did not lead to a further amplitude decrease, suggesting an all-or-none mechanism.

Analysis of the \( PI2/PI2 \) and b-wave/a-wave ratios further support the notion of postreceptor dysfunction in n-3 deficiency. The \( PI2/PI2 \) ratios in CNO-fed animals were 0.94 ± 0.09 overall and 0.75 ± 0.08 at 16 weeks, whereas the respective values in SFO-fed animals were 0.85 ± 0.09 and 0.63 ± 0.07. These results indicate a greater loss at the postreceptor level \((P < 0.05)\). Furthermore, in SFO-fed animals, the b-wave/a-wave ratio was approximately 20% lower than in CNO-fed animals \((P < 0.05)\).

Recent work by Bush and Sieving has shown that inner retinal elements provide the major contribution to the fast-flicker ERG. Therefore, the reduction in 50-Hz response amplitude in SFO-fed animals at 16 weeks (Table 3) was confirmation of postreceptor dysfunction in n-3 deficiency. This loss of flicker sensitivity extends our previous suggestion about cone-pathway deficits resulting from continued dietary n-3 insufficiency.

The guinea pig is known to have a prominent OP contribution to the dark-adapted ERG, and these oscillations may also be used as indexes of inner retinal function. Oscillatory potentials were reduced to a greater extent than was the a-wave \((39\% \text{ versus } 27\%)\) in SFO-fed animals at 16 weeks (Table 3), consistent with our proposal that postreceptor elements are affected at this age. It is interesting to speculate whether the common timing of this loss and the change in \( R_{aw} \) amplitude reflect a common cause or arise from similar biochemical processes at two different loci.

**Conventional Waveforms**

As might be expected from the subcomponent analysis described earlier, concomitant effects were found for the ERG a- and b-waves (Table 3). However, animals deprived of dietary n-3 PUFAs showed b-wave amplitude reductions after as little as 6 weeks, whereas the a-wave amplitude only became compromised after 16 weeks (Fig. 4). It is possible to interpret this finding as an indication of an early postreceptor loss, although such an interpretation is not supported by the \( PI2 \) findings. Given the serial organization of the retinal current generators, this finding is also consistent with a nonspecific involvement in the photoreceptor and postreceptor components, emphasizing that the a- and b-waves provide an index of summed receptoral and postreceptor activity. For conditions in which both are affected, the b-wave deficit is enhanced by such summation.

Support for a generalized loss comes from the analysis of the ERG subcomponents and the results of other n-3 PUFA deficiency studies in which either the a- or b-wave amplitudes (or both) have been shown to be affected. All are consistent with our previous findings in guinea pigs depleted over three generations. Moreover, given the widespread distribution of n-3 PUFA in the nervous system, particularly in the receptor outer segments, it is reasonable to conclude that DHA may serve several roles in the normal function of the retina. Dietary n-3 PUFAs have been implicated in the regulation of gene transcription and in modifying the functional characteristics of Na⁺K⁺-ATPase in rat neural tissue. Because Na⁺K⁺-ATPase plays an essential role in controlling the ionic environment critical for neural activity, modulation of this enzyme would be expected to result in ERG changes. In a recent study in which tunicamycin-induced alterations in retinal Na⁺K⁺-ATPase distribution were used, gross and nonspecific ERG deficits occurred. Therefore, we think that evidence exists to support our proposal for a generalized loss in receptor and postreceptor function. Moreover, our finding for a greater postreceptor deficit \((39\% \text{ versus } 27\%)\) is consistent with this proposal and confirms our earlier work in third-generation animals in which a similar level of loss \((46\% \text{ versus } 37\%)\) was noted.

One interesting observation is that the a- and b-wave-implicit times were not altered by diet. It should be noted that the origins of sensitivity loss and implicit time delay are different, and it is therefore possible to have one in the absence of the other. The combination of sensitivity and \( R_{aw} \) losses are consistent with an unaltered implicit time.

**Relationship between DHA and Retinal Function: Comparison with Previous Studies**

The dynamic nature of the retinal phospholipid fatty acid profile during ongoing n-3 PUFA deficiency (Table 2) was...
reflected in the ERG amplitude. Analysis of conventional and subcomponent ERG waveforms indicates that continued dietary n-3 deficiency after weaning resulted in marked retinal dysfunction, increasing in severity as the retinal DHA level decreased. These findings are consistent with those in our previous work\textsuperscript{17} but differ from those in other studies in which the longitudinal effects of n-3 PUFA depletion were investigated. In these other studies, the functional differences between control and depleted groups remained stable or decreased despite the continuation of retinal DHA differences.\textsuperscript{9,12} However, in both of these latter studies it was the control group amplitudes that decreased rather than an improvement occurring the n-3 PUFA-deficient animals. The stability of the n-3 PUFA-deficient groups may be attributed to fact that the retinal DHA levels in these animals remained unchanged.\textsuperscript{9,63} The reason for the change in the control group’s performance during this time is not clear, but the outcome is otherwise consistent with our result because it shows a relationship between retinal DHA and function.

### Effect on Retinal PUFA Profile

The effect that dietary manipulation has in a single generation on the retinal phospholipid fatty acid profile can be considered in terms of n-3 PUFA reserves. Such reserves are distributed among various tissues such as those in the central nervous system and liver and in plasma and adipose tissue,\textsuperscript{1,2,14} with the level of the reservoir sustained by the dietary and transplacental n-3 fatty acid supply and conservation mechanisms.\textsuperscript{4-6} Indeed, the findings of our study indicate that the retinal phospholipid fatty acid profile of first-generation guinea pigs is strongly influenced by the dietary n-3 PUFA supply. In SFO-fed animals, retinal DHA values were reduced to 9% (of the total phospholipid fatty acid), compared with 22% in CNO-fed animals, after 16 weeks (Table 2, Fig. 2). Previous work has shown that retinal DHA levels decrease exponentially with continued dietary n-3 PUFA deficiency.\textsuperscript{64}

The largest relative diet-induced change in the phospholipid fatty acid profile was for 22:5n-6, which was almost six times higher after 16 weeks of SFO feeding than in age-matched CNO-fed animals (Table 2). In agreement with our previous findings,\textsuperscript{10} however, the combined 22:5n-3 + 22:5n-6 level remained remarkably constant, with levels ranging between 23% and 26%. This finding indicates substitution between these two fatty acids. However, the fact that 22:5n-6 failed, in terms of retinal function, to substitute adequately for DHA continues to perplex us, because these fatty acids differ by only one double bond.

Our findings show that significant alterations in the retinal phospholipid fatty acid profile, in particular a reduction in DHA, resulted from n-3 PUFA deprivation imposed after weaning for several weeks. These fatty acid changes are associated with significant ERG deficits seen as early as 6 weeks after weaning, corresponding to a DHA reduction of 31%.

### Repletion Effects

Of the three repletion schemes used in this study, the most successful in functional recovery was the S6C10 group. There was no statistical difference in the ERGs of these animals and their age-matched CNO-fed counterparts. In addition, S6C10 animals returned significantly greater responses to bright stimuli (a- and b-wave measures) than did S11C5 animals and SFO-fed animals under both stimulus conditions. The success of this regimen in restoring visual function is probably related to the increased retinal DHA level (to 19% of the total phospholipid fatty acids), which is only slightly removed from that of CNO-fed control subjects (~14%). Subcomponent analysis showed that PIII and PII processes were normal in S6C10 animals and that the PIII and OP parameters were significantly greater in this group than in SFO-fed animals (P < 0.05). Such an outcome confirms that functional recovery from DHA related deficiencies can be achieved by dietary means. That there were borderline findings for Rwave after 16 weeks of age perhaps calls for reinvestigation, with a larger sample to improve the power of the analysis.

Refeeding with CNO was successful in increasing the retinal DHA levels of animals raised initially on SFO. The greatest increase in retinal DHA was observed in animals repleted for 10 weeks (S6C10), when it increased from 15% to 19% of total retinal phospholipid fatty acid. In both groups of animals repleted for 5 weeks, retinal DHA levels increased to approximately 16% (Table 2). Assuming that repletion continues in a linear fashion, animals reeled from 6 and 11 weeks would have retinal DHA content approximately equivalent to that of their CNO-fed counterparts by approximately 24 weeks after weaning. This indicates that repletion with α-linolenic acid acted at a slower rate than did the initial depletion process, although it is likely that pure DHA supplements would have hastened repletion.\textsuperscript{65,66} We found a rate of accretion in the S11C5 group twice that of the S6C5 animals (Fig. 2), suggesting upregulation of conservation and accretion mechanisms at older ages or in conditions of greater depletion. Of interest, after 5 weeks of repletion, our results show that the S11C5 group had larger ERG deficits (Fig. 5) than did the S6C5 group, although both groups had nearly the same retinal DHA levels (Table 2). This latter finding implies that the age when repletion begins can influence the functional outcome.

The results in this study indicate that the retinal PUFA profile in developing guinea pigs retained plasticity, at least until animals were 4 to 5 months of age. Because animals initially fed diets deficient in n-3 fatty acid clearly retained the ability to metabolize and synthesize long-chain PUFAs, the enzymes for fatty acid chain desaturation and elongation must be present and remain functional up to this stage of life. Therefore, the retinal membrane fatty acid composition does not seem to be subject to a critical period up to this age.

### Conclusions

Despite differences in the appearance of the guinea pig ERG, compared with that of humans and other species,\textsuperscript{49} our conclusions based on mammalian photoreceptor physiology are not unreasonable. The results of this study have indicated that the guinea pig provided a model in which dietary n-3 fatty acid deficiency could produce significant reductions in the retinal n-3 PUFA profile, in a single generation. We think the observed changes after n-3 fatty acid deficiency were consistent with those reported previously in humans. These tissue-fatty acid changes are associated with an altered ERG response that we think are caused by complex interactions between age and retinal DHA levels. Furthermore, we have shown that normalization of retinal n-3 PUFA levels in depleted animals can be achieved with dietary (α-linolenic acid) repletion, provided
refeeding is begun early and is maintained for a period longer than the actual period of depletion. In turn, restoration of retinal DHA levels resulted in complete recovery in retinal function. Partial repletion restored some components of the ERG waveform, but not all. Our data suggest that a critical retinal DHA level was required for normal retinal function and that this level was somewhere between 14% and 18% (22% in normal subjects). There also seemed to be a significant age effect beyond which substantial functional losses became apparent. Whether the large deficits found beyond this age are reversible remains to be seen.

We found evidence for receptoral and postreceptoral losses in n-3 PUFA-deprived animals. The sensitivity of the receptoral $\Delta I$ response was most affected in conditions of n-3 depletion showing a dose-dependent correlation with tissue DHA. The saturated response amplitude ($\Delta R$) was also reduced in conditions of n-3 PUFA depletion. However, unlike $\Delta I$ sensitivity, the magnitude of $\Delta R$ loss showed a step-like correlation with retinal DHA level. In both cases, a critical age (>11 weeks) exists beyond which large losses in function occurred. Repletion was only successful if begun at a young age and achieved a near normal DHA level (>19%) by this critical age.

Acknowledgments

George Smith provided advice in calibrating the light source, and Kristan Vingrys developed the software used in extracting, filtering, and analyzing the waveforms.

References


