hinge region of the human γ1 chain show that, when a number of prolines occur as in the sequence Cys-Pro-Pro-Cys-Pro-Ala-Pro and when two such sequences are joined by a pair of disulfide bonds, a severe rigid distortion occurs. This interrupts the conformational folding in the middle of the heavy chain between the second and the third intrachain disulfide loops, thereby imparting a looseness or extended chain conformation that leads to an apparent flexibility in the structure (*23*). Proline thus provides the rigid part of a flexible hinge. A distortion due to proline is absent in the μ chain. However, it may be provided by the presence of the large, hydrophilic oligosaccharide which requires that the hinge region must be at the surface of the IgM pentamer. Incubation at 60°C may partially separate the monomeric IgM subunits or the two μ chains within the subunit, thereby making the Arg-Gly bond accessible to trypsin. The temperature effect is probably on the conformation of the immunoglobulin, rather than on the trypsin, for we find that incubation with thermolysin at 60°C, but not at 37°C, produces similar Fabμ and Fcμ pieces. Within each subunit the Fcμ dimer is linked by two interchain disulfide bridges, one near the NH_2-terminus and one at the COOH-terminus. The subunits are linked by the intersubunit bridge, which is not within the hinge region depicted in Fig. 1.

Although papain often cleaves μ and γ chains at multiple sites to give heterogeneous Fc fragments (*2, 24*), trypsin, which is a more specific enzyme, acts predominantly at a single site on the μ chain to produce an apparently homogeneous Fcμ piece. This is confirmed by the behavior of Fcμ in immunoelectrophoresis, immunodiffusion, and ultracentrifugation, and also by the finding of a unique amino acid sequence with the sequencer. The specific enzymatic and chemical cleavage of IgM and the combined use of the sequencer and of conventional sequencing techniques have greatly facilitated the determination of the amino acid sequence of this protein, which has a covalent molecular weight of about 1 million. The same principles can be applied to other large molecules that have resisted the conventional methods of sequence analysis.

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Vitamin A Deficiency Effect on Retina: Dependence on Light

Abstract. *The effects of vitamin A deficiency in the rat eye, as measured by the electroretinogram and changes in rhodopsin content, are critically dependent upon the levels of illumination to which the animals are exposed daily. Depleted animals kept in darkness maintained virtually normal electroretinogram function and rhodopsin content for 5 to 6 months while those kept in weak cyclic light lost rhodopsin continuously. A fraction of the retinol released from rhodopsin during illumination disappears presumably from the pigment epithelium into the blood and becomes unavailable for rhodopsin regeneration. A sequence of three first-order reactions was assumed to estimate the rate constant of this disappearance (0.03 per hour). Computer simulation supporting the experimental data illustrates the dependence of the retinal abnormalities on light.*

Dowling and Wald have analyzed the effect of nutritional vitamin A deficiency upon the retina of the albino rat (*1*). Night blindness, the first sign of tissue deprivation, measured by the rise in the threshold for eliciting the electroretinogram (ERG), was directly related to the loss of vitamin A aldehyde from the visual pigment. The effect appeared as early as 3½ weeks on the dietary regimen and progressed inevitably to the almost complete disappearance of electrical responsiveness. Structural deterioration of the outer rod segment occurred in conjunction with a decline of the protein component of the visual pigment, and a "substantial fraction" of the visual cell population seemed to undergo lysis and disappear. Treatment with retinoic acid, while relieving the systemic effects of the deficiency, had no influence on the progress of the retinal abnormality but greatly facilitated the study of the advanced stages of the disease.

We reinvestigated the effects of vita-

min A deficiency on the rat retina in conjunction with experiments designed to elucidate the role of vitamin A in the damage produced by visible light (*2*).

The experiments were performed on male albino rats from the Charles River Breeding Laboratory. They were maintained on a deficient diet prepared commercially or in the laboratory. The control animals (A+) were given retinol once weekly or as a dietary supplement. The A− animals received, instead, retinoic acid starting generally in the 6th to 8th week (*3*). Both groups of animals remained in good health, and their gain in weight over a 20-week period was only slightly less than that of rats on ordinary commercial pellets. The ERG (*4*) was measured at set intervals during pentobarbital anesthesia. Rhodopsin was determined conventionally (*5*). Retinol and retinyl esters were estimated by fluorometry in extracts of retina, pigment epithelium (PE), and liver; thin-layer chromatography was used for separation (*5*). Eyes were serially sectioned and stained with hematoxylin-eosin.

Dowling and Wald (*1*) did not specify the lighting conditions under which their rats were maintained. Our results reveal that the development and progress of vitamin A deficiency as observed in the retina by the decline in rhodopsin is primarily a function of the daily exposure to light.

Figure 1 (rows 1 and 2) shows the ERG records of A+ and A− animals in response to a strong xenon flash (*4*) when these animals were kept continuously in a dark environment after they were weaned. Even after 23 weeks on the deficient diet, the ERG of the A− animals was only slightly reduced compared with the records from A+ of the same age. The ERG threshold and the early-receptor potential (ERP) also changed very little.

Results were very different, however, when the A− animals were kept instead on a day-night, 12-hour cycle of dim illumination and darkness (*cyclic light*). The ERG's of row 3 of Fig. 1 are from animals housed from the time of weaning in a room illuminated during the day by a 100-watt incandescent ceiling light, which provided indirect illumination of 4 to 9 lux at the rack-mounted wire mesh or transparent plastic cages. Under this condition, the ERG decreased starting at 4 weeks.

Typically, the decrease of the ERG in response to the strong flash was first noted by a reduction in the amplitude of the a-wave; the decline of the b-wave was much slower, as was the fall of the three or four wavelets superimposed thereon. Once the a-wave had decreased to about one-third of its amplitude, the b-wave frequently had the "two-cusped" appearance described by Dowling and Wald (*1*), a hump, or plateau, following a fast subsiding peak. The a-wave reduction was associated with a proportional fall in the ERP and a rise in ERG (b-wave) threshold. Threshold had increased by 1.0 to 1.5 log units when a-wave reduction was 50 percent.

Averaged data for the ERG from 150 A− and 90 A+ animals maintained continuously in the dark are plotted in Fig. 2, upper part. With the use of the a-wave amplitude as the ERG measurement most directly related to visual cell function (*6*), differences between A+ and A− seem indeed to be very slight. In both groups of animals a-wave amplitude diminished with advancing age. A significantly faster decline in A− animals appeared only after 10 weeks on the diet. At 20 weeks this difference did not exceed 15 percent, on the average.

As indicated by the lower part of Fig. 2, the a-wave decline during maintenance in cyclic light varied in relation to the start of this condition. When cyclic lighting coincided with the start of the dietary regimen, the a-wave began to diminish during the 4th week and declined rapidly during the next 5 weeks and then more slowly. Within 15 weeks its amplitude was about 10 percent of that of the appropriate A+ controls. Animals kept in darkness for six, nine, or more weeks and then exposed to cyclic light showed immediately the same decline.

There were considerable variations from experiment to experiment; experiment 84 (Fig. 2) is an example of a rather slow initial decline, but we observed with other groups of animals still slower rates of change under the same lighting condition. These variations did not correspond to different rates of vitamin A depletion of the liver. Early and continuous administration of retinoic acid diminished the average decline to a very slight degree.

Averaged data of the rhodopsin con-

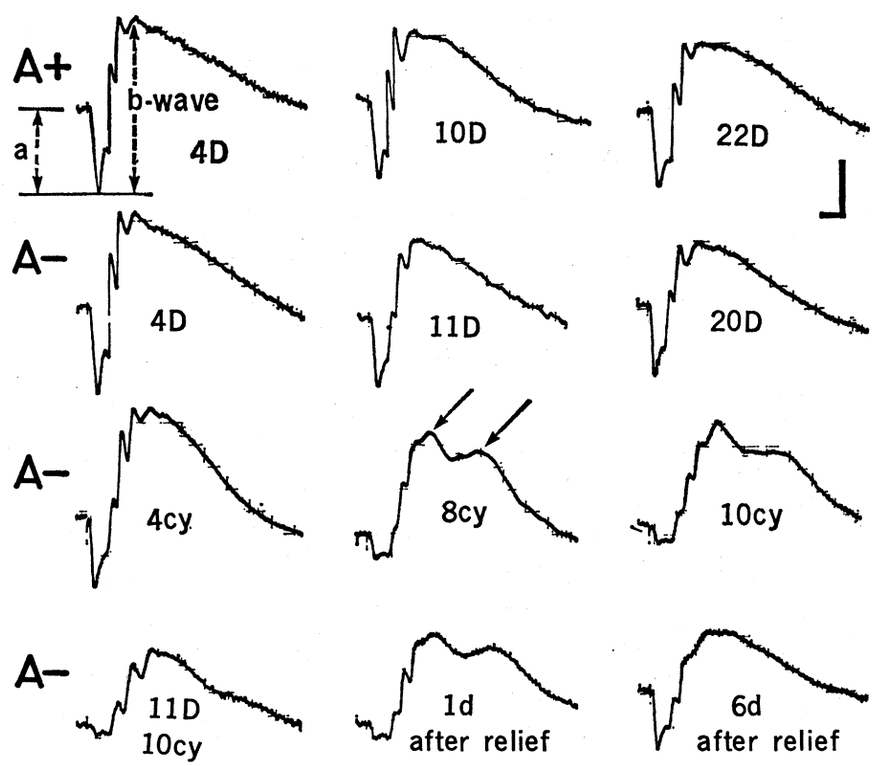


Fig. 1. The ERG during vitamin A deficiency elicited by a strong xenon flash. *D*, Animals continuously maintained in darkness for the indicated number of weeks (for example, 4); *cy*, maintained for the indicated number of weeks in cyclic light. The arrows of the middle tracing in row 3 point to the two humps of the b-wave. The data in row 4 is from an experiment in which animals were maintained 11 weeks in darkness and then in the same cyclic light as animals of row 3; after 10 weeks in cyclic light, the deficiency was relieved. Calibration: 20 msec; 500 μv. Xenon flash is indicated by the slight artifact just preceding the start of the a-wave.

tent for the same groups of animals as in Fig. 2 are illustrated in Fig. 3. Rhodopsin of A+ animals kept in the dark increased almost steadily from 1.5 nmole at 3 weeks to 2.6 nmole at 20 weeks, while that of A− in the dark remained virtually constant between the 5th and 20th weeks at 1.56 (*7*). Rhodopsin of A− animals maintained in cyclic light showed an almost linear fall, starting simultaneously with the fall of the ERG, whereas rhodopsin of the A+ controls was constant at about 1.8 nmole. A 50 percent reduction of the a-wave corresponded to a 30 percent loss of rhodopsin (*8*). When the a-wave had fallen to 14 percent after 15 weeks, the rhodopsin was as low as 21 percent. In Dowling and Wald's experiments, the rhodopsin level after 12 to 15 weeks on the diet was 4 percent, suggesting that the light condition in their experiments (*9*) was not as mild as ours.

The rate of rhodopsin decline therefore depended upon the intensity and duration of light exposure. For example, continuous exposure to green light at 1500 lux (*2*) for 40 hours of a deficient animal produced the same rhodopsin loss and a-wave reduction as 8 weeks in the cyclic light. Or, when a 40-watt light was used for cyclic illumination instead of the 100-watt light, the rate of rhodopsin depletion was slowed and a standard a-wave reduction which, in the 100-watt light developed in 2½ weeks after liver depletion, occurred in 6 to 7 weeks.

The ERG and rhodopsin after several weeks in cyclic light remained low when the light was replaced by continuous darkness. Relief of the deficiency by a high dose of retinyl acetate resulted in normalization whether or not the animals were put into dark or continued in cyclic light. The time required for recovery varied directly with the degree of rhodopsin depletion and especially with the length of time this depletion existed prior to relief. Animals that had a markedly reduced ERG for 1 to 2 months required more than 1 week for ERG recovery, which was significantly faster than the attainment of near-normal rhodopsin levels.

Irreversible histological changes (*10*), manifested by visual cell death (reduction in the number of visual cell nuclei) or loss of inner and outer segments, were not observed in our experiments, which lasted 6 months on the deficient diet with exposure to the cyclic light for up to 12 weeks. Changes in the appearance of the outer segments (*10*), especially a shortening of their length, were evident in retinas of nonrelieved animals in which rhodopsin content had fallen below 25 percent during eight or more weeks in cyclic light.

The crucial factor in the development of the signs of vitamin A deficiency and their dependence upon the lighting conditions must be the disappearance of retinol from the retina after rhodopsin bleaching. In order to estimate the rate of this disappearance, we assumed a sequence of three reactions. (i) Light leads to the reversible release of retinol from rhodopsin; (ii) retinol is transferred from the retina (r) to the pigment epithelium (PE) for storage in the light; and (iii) in animals depleted of vitamin A, retinol disappears from PE into the blood (b). These reactions were considered first order:

$$\text{rhodopsin} \underset{k_2}{\overset{k_1\,(h\nu)}{\rightleftharpoons}} \text{retinol}\ (r) \underset{k_4}{\overset{k_3}{\rightleftharpoons}}$$

$$\text{retinol}\ (PE) \xrightarrow{k_5} \text{retinol}\ (b)$$

The basis for our computations was measurements of rhodopsin and retinol in nondeficient animals (Fig. 4). The animals were exposed to strong light (*2*) which decreased rhodopsin from 2.0 to 0.3 nmole within 30 minutes. Retinol in the retina reached a maximum after 10 minutes while its concentration in PE continuously increased during 50 minutes. The final partition of retinol between the retina and PE was 1 : 4.5, which agrees with the results of Dowling (*11*). Averaged data for the re-

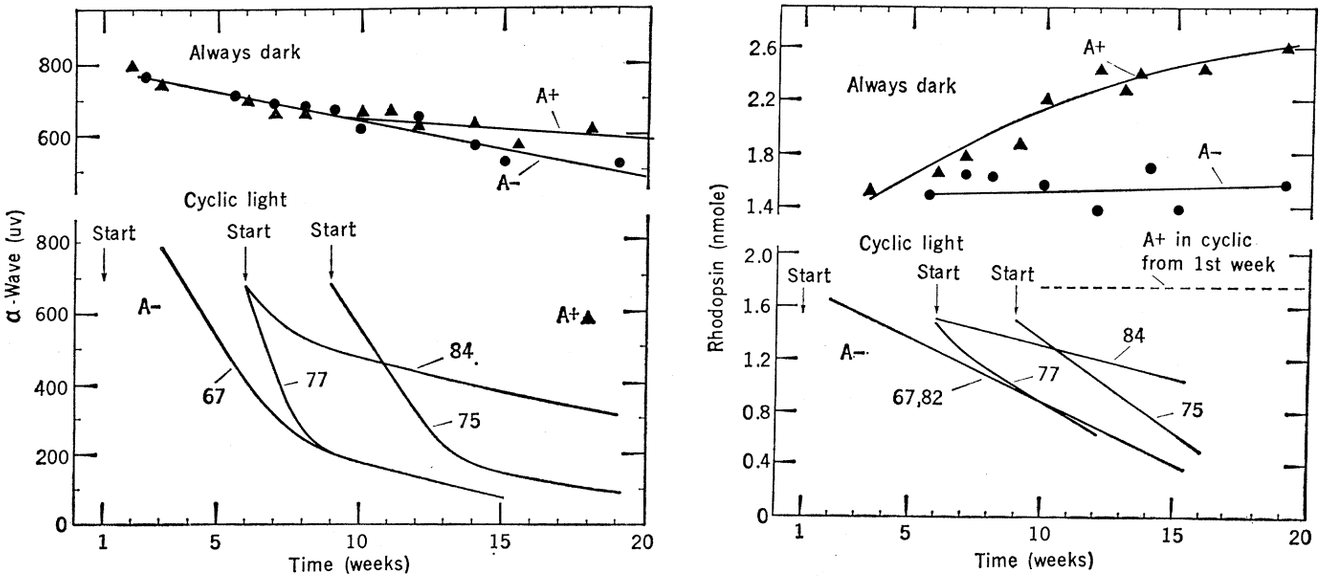
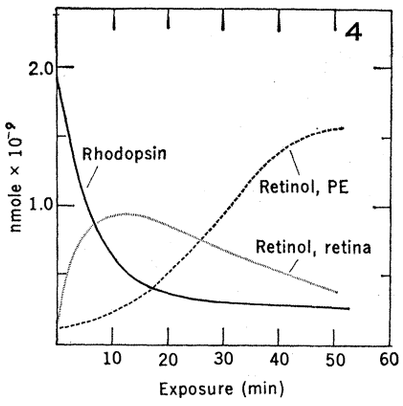


Fig. 2 (left). Average amplitude of a-wave during vitamin A deficiency, weeks on diet. (Upper) Animals kept continuously in the dark [A(−); A(+)]. (Lower) Animals maintained 12 hours per day in dim light, starting as indicated. Lines were drawn through averaged data of different experiments (such as experiment 67), each with 15 to 40 animals. Average a-wave amplitude of A + (not shown) declined in cyclic light from 640 μv at the 4th week, through 600 μv at the 9th week, to 590 μv at the 18th week (note ▲). The ERG test was always preceded by 18 to 24 hours of darkness when the animal was kept in cyclic light. Fig. 3 (right). Rhodopsin per retina of animals maintained in darkness (upper) or in cyclic light (lower) for the same experiments as in Fig. 2. Dissection of the retina followed ERG test by 4 to 24 hours, depending upon the strength of the light flashes used in testing. Average rhodopsin of A + was virtually constant after the 4th week at 1.8 nmole.



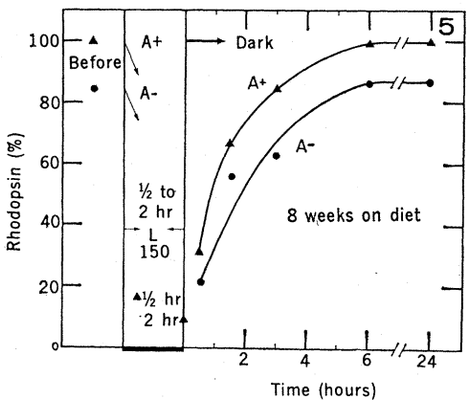


Fig. 4. Rhodopsin and retinol of retina and pigment epithelium (*PE*) during exposure to strong green light (*11*). Curves have been drawn through averaged data. Fig. 5. Recovery of rhodopsin in percentages of the A + controls after exposure to the same light (*L 150*) for ½ to 2 hours as in Fig. 4. The data for different exposure times were combined. Both A + (▲) and A — (●) animals had been maintained continuously in the dark for about 8 weeks prior to exposure. ERG recovery (a-wave) had the same time course.

generation of rhodopsin in the dark after the exposure to strong light for ½ to 2 hours are shown in Fig. 5. Regeneration required about 6 hours for completion. Using these sets of data, we determined the rate constants k_1 to k_4 by fitting the experimental curves to Laplace transform integration of the differential equations describing the reaction sequence. For the nondeficient animals a closed system was assumed and fitted by $k_1 = 9.0$, $k_2 = 5.2$, $k_3 = 4.0$, and $k_4 = 0.9$ per hour.

As shown in Fig. 5, rhodopsin regeneration in the A— animals after 2 hours' exposure to strong light was not measurably different from that of A+ animals. Hence, in order to obtain data for the determination of k_5, much longer exposure to continuous strong light was required. Such long exposure, however, is complicated by the occurrence of irreversible injury to visual cells and PE (*2*). The model was therefore applied to the cyclic light procedure, during which the loss of retinol is additive over several weeks.

The required measurement of the 12-hour bleaching effect of the (100-watt) cyclic light was obtained with A+ animals maintained previously in darkness, both eyes of each animal having exactly the same ERG responses. One eye was removed for rhodopsin determination 1 day before exposure, the other at the end of the 12-hour light period under the conditions described in the legends of Figs. 2 and 3. The measured rhodopsin bleach was approximately 7 percent for animals with a control concentration of 2.4 nmole. This gave a k_1 of 0.07 per hour for the cyclic light, on the assumption that the other constants were unchanged. Retinol contents of A— animals maintained in darkness or cyclic light were so low that they were disregarded: 0.01 nmole for retina, 0.015 nmole for PE.

A simulation of the cyclic light procedure was then made by digital computer, and the k_5 of A— animals was estimated for the best fit of the experiment 77 in Fig. 3, with the use of k_2, k_3, and k_4 as stated above. The value obtained for k_5 was 0.03 per hour.

In Fig. 6, curve a shows the good fit of the computed rhodopsin decline with the experimental data. It also shows the decline in rhodopsin as predicted by the model when illumination during the day (12 hours) is ten times weaker (curve b) or stronger (curve c). These illuminations lead to rhodopsin levels of 92 percent (curve b) and 0 (curve c) after 5 weeks. Hence, in the case of the weakest light, depletion of rhodopsin might be unmeasurable, while with the light that is 100 times stronger it would be virtually complete within 5 weeks. The variations in the course of rhodopsin depletion (Fig. 3) may therefore be caused by variations in retinal illumination resulting from different animal behavior (*12*). The model and its rate constants were applied also to the condition of strong-light exposure of A— animals (*2*) and found to fit the measured changes in rhodopsin.

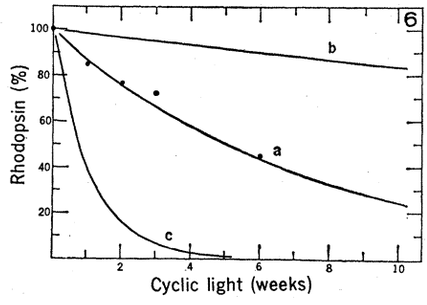


Fig. 6. Computer simulation of the cyclic light effect on rhodopsin in A — animals. Curve a is fitted to the experimental points fom experiment 77 of Fig. 3. Curves b and c are, respectively, for ten times weaker and stronger illumination, assuming the same rate constants k_2 to k_5 as in curve a.

The apparent absence of a significant rhodopsin depletion when the animals were maintained in darkness requires additional consideration. It has been shown that the outer segments of rod cells grow continuously, new discs being added at their base while the oldest discs are discharged at the apex into PE (*13*). Rod renewal in the rat occurs in 9 days by a process resembling phagocytosis. Retinol, therefore, must continuously be provided by PE for binding to the newly formed opsin. It follows that the disappearance of retinol in vitamin A deficiency would continuously make less substrate available for synthesis.

We applied the computed rate constant k_5 of retinol disappearance to a model which assumes a linear transfer of retinol (rhodopsin) into PE and the first-order reaction (k_4) for the return of retinol to the outer segment. Within 9 days, 3 percent of the chromophore would be lost. The experimental data, however, suggest that such a depletion occurs at best in 6 to 8 weeks when the animal is kept continuously in the dark. We conclude that either our model for retinol disappearance does not apply because of a quantitative or qualitative (*14*) difference in retinol-related processes, or that the rate of rod renewal is much diminished in vitamin A deficiency, at least under conditions of continued darkness. The increase of rhodopsin in A+ animals kept in darkness compared to the steady level in those maintained in weak cyclic light suggests that the light environment plays an important role not only in vitamin A deficiency but also normally in the biology of visual cells and pigment epithelium.

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4. The ERG was measured under general anesthesia (Nembutal, 3 mg per 100 g of body weight); 1 percent xylocaine was applied to the cornea and subcutaneous tissue. Light flashes were supplied by the Grass Photostimulator, with intensity No. 16 at 38 cm from the eye ("strong xenon flash," see text) attenuated by neutral density filters for testing weak stimuli. For threshold measurements, 50-msec flashes from a tungsten source were used. The light source for eliciting the ERP was a Honeywell flash unit, model 65-C, connected to the eye by 0.9-m light wire (Bausch & Lomb 3202).
5. Rhodopsin preparation: The dissected retina was fixed with alum and extracted with 2 percent digitonin or 1 percent Triton-X, 100, in 0.3 ml; the procedure was repeated thrice. The amount of rhodopsin remaining within the eye after the removal of the retina was less than 10 percent in dark-adapted A+ animals. Retinol was determined by fluorometry of xylene or cyclohexane extracts (1 ml) of the dissected retina and of the remaining eye tissue, all retinol of the remaining eye tissue being considered to reside in the pigment epithelium; exciting and analyzing wavelengths were 340 and 480 nm, respectively. After the first reading, the retinol was destroyed by exposing the samples to ultraviolet light (GE F15T8) and a second reading was made [P. K. Nakane and D. Glick, *J. Histochem. Cytochem.* **13**, 640 (1965)]. Standards were fresh solutions of retinyl acetate, prepared with spectrophotometric control. Thin-layer chromatography [B. D. Drujan, R. Castillon, E. Guerrero, *Anal. Biochem.* **23**, 44 (1968)] was performed with Eastman chromogram 6060 and a cyclohexane, ethyl ether system (4 : 1 by volume); measurement was by fluorometry of the samples taken. See also F. Crescitelli, *Vision Res.* **7**, 685 (1967).
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9. These experiments were conducted under "standard laboratory lighting conditions of approximately 12 hours light and 12 hours dark" (J. E. Dowling, personal communication).
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14. The disappearance of retinol may be a threshold phenomenon (a very low concentration must be exceeded before "spilling" into the blood occurs). The computed steady-state level of retinol in PE for the condition of synthesis is about ten times lower than for the condition of cyclic light.

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Irreversible Effects of Visible Light on the Retina: Role of Vitamin A

Abstract. *Diffuse retinal irradiation by visible light produces in the rat the death of visual cells and pigment epithelium. Typically, cage illumination of 1500 lux from fluorescent light through a green filter leads to severe damage when continued for 40 hours. Vitamin A deficiency protects against this damage but experiments show that retinol released by light from rhodopsin is probably not the toxic agent. Protection against light damage depends on a long-range state of cell adaptation to light itself. The normal diurnal cycle of light and dark seems to be the essential factor in controlling visual cell viability and susceptibility.*

Several years ago the surprising finding was made that visible light at intensities which are ordinarily encountered is damaging to the retina of rats (*1*). The most striking manifestation of this damage was the widespread death of the visual cells in association with the degeneration of the pigment epithelium (PE) and an irreversibly low or extinct electroretinogram (ERG). The effect was most easily produced and studied in albino rats, but pigmented (hooded) rats showed about the same damage for the same retina irradiation. The deleterious effect has also been observed in the wild-strain kangaroo rat as well as in hamsters of different strains, in the Swiss mouse, and in the nocturnal Galago monkey. The rat, however, seems to be the most sensitive animal, a finding which does not favor the indiscriminate use of rats in vision and retina studies.

The damage is a function of irradiation and exposure time. Minimum effects are produced with the equivalent of about 1 μw per square centimeter of 500-nm radiation applied diffusely over the retina (*1*). Typically, an environment illuminated by incandescent light to about 110 lux is damaging if it is maintained continuously for 7 to 10 days. Irradiation ten times stronger has a deleterious effect within 24 hours, but, when body and eye temperature are raised, damage results from exposure for 1 or 2 hours only (*1*).

The damage is graded mainly by the size of the irreversibly affected area of the retina. The weakest histological manifestation in the albino rat, exposed freely moving in a cage, is a small lesion less than ½ mm in diameter in the upper nasal region. As damage increases, this area becomes larger and may extend over the whole globe, resulting in a retina composed only of the inner layers (*1*).

The effect seems important not only because it illustrates neglected aspects of vertebrate photoreceptor biology but also because it provides new insights for the study of degenerative visual cell diseases. Ordinary daylight or artificial light has never been considered a possible hazard, except for focusing the sun or equivalent sources upon the retina, quite in contrast to exposing the skin and, in analogy, to the known relation between cochlear hair cell damage and sound pressure. Indeed, the eye seems protected against overexposure by a heavy pigment coat that limits light entry to the pupil, by pupillary reflex constriction, squinting, and others.

Four different mechanisms were considered as possible causes of the deleterious effect of visible light: (i) thermal injury, (ii) photodynamic injury