DIFFERENTIATION OF RODS AND CONES IN TOTAL DARKNESS

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It has been established that light is generally required for the development of the lamellar system of chloroplasts and, in some instances, for their orientation in the cell. Moreover, the absence of light causes the chloroplasts of certain plants to shrink and the lamellae to disappear (see reference 1 for other references). Do animal photoreceptors similarly need light for full differentiation and maintenance?

MATERIALS AND METHODS

Frogs (Pacific tree frog, *Hyla regilla*) were reared in total darkness from the gastrula stage through metamorphosis. The animals were fed a tropical fish food (Permalife) and the water in the small aquaria was changed frequently, all operations being conducted without benefit of even a red photographic safe light. When the controls had completely metamorphosed, stage XXV (2), the experiment was terminated. It is estimated that of the total elapsed time of 60 days at least 55 days followed the onset of formation of rod and cone outer segments. Experimental (dark-reared) and control animals were decapitated, and their eyes were removed and fixed for 2 to 3 hours at 0°C in Dalton's solution (3) at pH 7.2 (or in phosphate-buffered osmium tetroxide (4) at pH 7.3 or glutaraldehyde (5) at pH 7.2 with postfixation in Dalton's solution), then rapidly dehydrated in ethanol and embedded in Epon. While in 70 per cent ethanol the retina of each eye was divided into small pieces with iridectomy scissors and microknives (6). Ultrathin sections were cut with a diamond knife, mounted with a Westfall-Healy section mounter (7), stained with lead citrate (8), and examined with an RCA EMU-3G electron microscope.

RESULTS

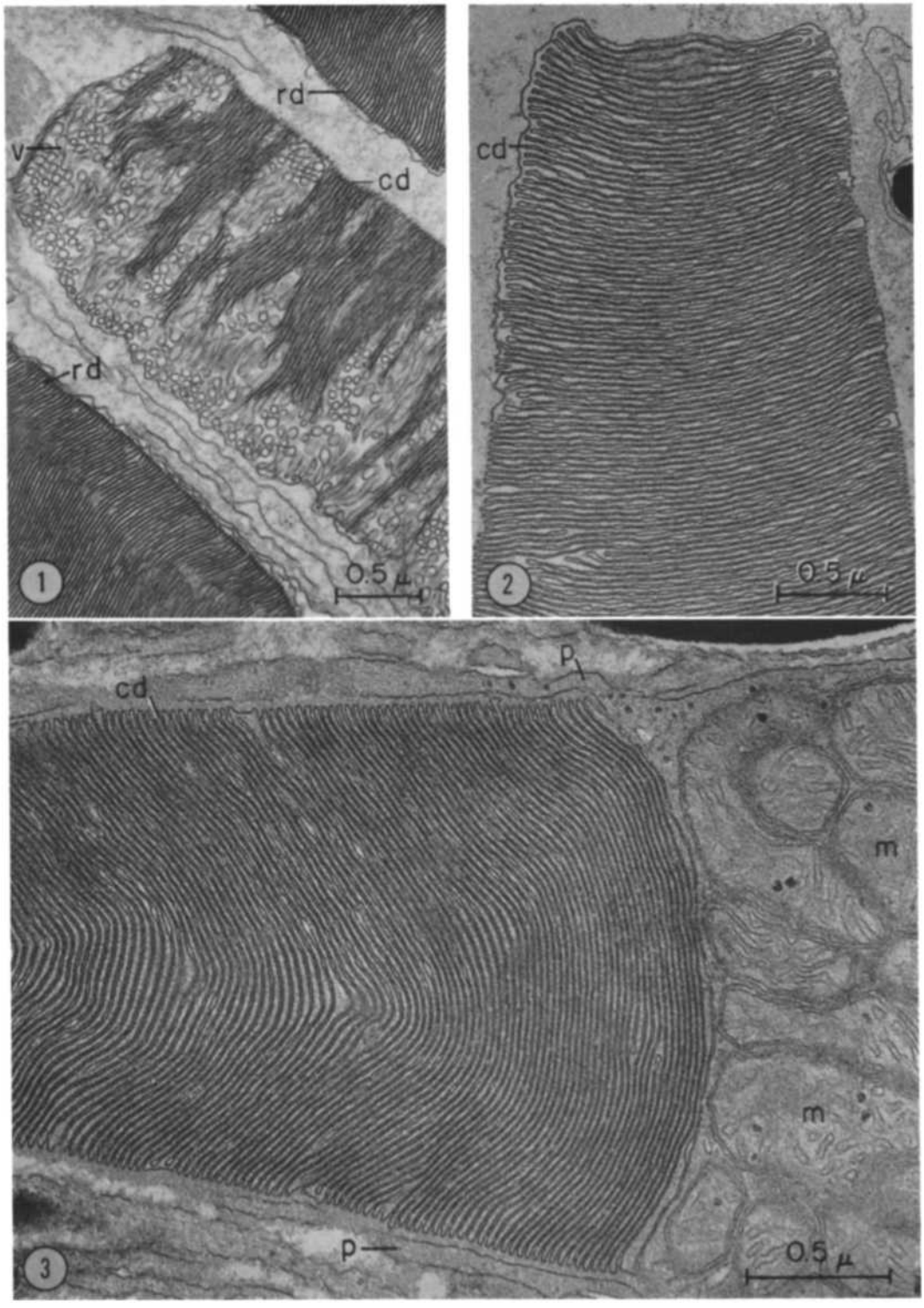
The first time the experiment was performed (spring of 1963) Dalton's solution was used exclusively. The rods of two subadult animals developed in the absence of light were normal, their outer segments exhibiting the typical even array of rod disks (*rd*, Fig. 1). The cones, however, showed extensive areas of breakdown of their disks into vesicles (*v*, Fig. 1), but the same condition was observed in the cones of the controls. Because in this experiment the controls had been reared in the laboratory under artificial illumination, it was thought that the abnormality of the cones might be due to a lack of sunlight (9). On the other hand, the vesicular condition might be an artifact of fixation since the cones in small larvae whose eyes were fixed in Dalton's solution were devoid of vesicles (Fig. 2). The experiment was repeated, therefore, in the spring of 1964 using the same procedures except that the controls were exposed daily to direct sunlight for varying lengths of time, and two additional fixatives were employed: phosphate-buffered osmium tetroxide and glutaraldehyde. One eye of each animal (5 control and 2 experimental subadults) was fixed in one of the osmium preparations, the other in glutaraldehyde.

The results of the 1964 experiment were: many vesicles in the cones of experimental and control eyes fixed with Dalton's solution or phosphate-buffered osmium tetroxide, but no vesicle formation in the cones of eyes treated with glutaraldehyde and postfixed with Dalton's solution (Fig. 3). Rods of all animals were normal regardless of the fixative used. The conclusions are clear: first, light is not required for the full differentiation and

FIGURE 1 Tip of a cone (middle) and parts of two rods (above and below) of a subadult *Hyla regilla* reared in total darkness. Fixed in Dalton's solution. *cd*, cone disks; *rd*, rod disks; *v*, vesicles from breakdown of cone disks, a fixation artifact. × 26,000.

FIGURE 2 Tip of a cone from a 10-day old larva of *H. regilla* reared in the dark. Note absence of vesicular breakdown of the cone disks (*cd*). Dalton's fixative. × 34,000.

FIGURE 3 Base of outer segment and distal part of inner segment of a cone of a subadult *H. regilla* reared in total darkness. Fixed in glutaraldehyde and post-fixed with Dalton's solution. Note absence of vesicular breakdown of cone disks (*cd*). *m*, mitochondria in inner segment; *p*, two processes of inner segment extending along outer segment. × 45,000.


rd
v
cd
rd
0.5 μ
1
cd
0.5 μ
2
cd
p
m
m
p
0.5 μ
3

maintenance of rods and cones in *Hyla regilla* under the conditions of this experiment; secondly, vesicle formation observed in the cones of the animals fixed with osmium tetroxide preparations is an artifact.

DISCUSSION

Several years ago Goodman (10) showed that in newborn rabbits reared in total darkness for 6 months the eyes, optic nerves, and optic centers were normal as far as could be determined by gross examination and by light microscopy. Recently, Dowling and Sidman (11) found that the rhodopsin content of eyes of rats raised in darkness is similar to that in eyes of controls exposed to ordinary laboratory conditions of illumination. The present paper demonstrates that complete lack of optic stimuli does not alter the development and maintenance of the fine structure of rods and cones in the tree frog *Hyla regilla*.

As noted in the introduction, chloroplasts, on the other hand, require light for the differentiation of the photoreceptive lamellar system. Plastids in a plant grown in the dark do not form grana, presumably owing to the lack of chlorophyll and chloroplastic proteins, the synthesis of which is dependent upon light (12, 13). Chlorophyll conjugated with protein is a major constituent of quantosomes which in turn are the building blocks of the lamellae of grana of a chloroplast (14). Without chlorophyll, the protein moiety seems unable to form a lamellar system. At best, only vesicles are produced in the leucoplasts of etiolated plant cells, but when illuminated, chlorophyll is synthesized and lamellae appear. The leucoplast transforms into a chloroplast.

Although light appears to be no requirement for the differentiation and maintenance of animal photoreceptors, a nutritional deficiency, namely lack of vitamin A, mimics the effect of etiolation in plants. Weanling rats reared without vitamin A exhibit a breakdown of the rod disks into vesicles (15), and cones and third-eye receptors in a lizard show a similar degeneration under the same nutritional deficiency (16). Rhodopsin, as an example of an animal photopigment, is like a chlorophyll-protein macromolecule in consisting of a proteinaceous part (opsin) and a chromophore (retinal, from vitamin A) and in having a similar molecular weight. Moreover, rhodopsin resembles the plant photopigment in performing a structural role, as it is a major component of the rod disks (17). During avitaminosis A, however, the chromophore of rhodopsin is lacking and, as in the plant without chlorophyll, the proteins are seemingly unable to maintain the structural integrity of the lamellar system.

Electron microscopists are reminded occasionally of the danger of misinterpreting artifacts and admonished to use more than one fixative to assist in the recognition of artifacts resulting from poor fixation (see reference 18, for example). The finding in this study, that vesicle formation in the outer segment of most subadult cones (but not of rods) fixed with osmium tetroxide is artifactual, is a dramatic illustration of the importance of the above reminder and injunction. That the vesicles might be due to faulty preservation has been suggested by several workers (see reference 19, for example) and denied by others (see reference 20, for example), but in the present instance it was proven by their complete absence in eyes fixed with glutaraldehyde.

The artifact of vesicle formation is instructive in indicating that cone disks are apparently much more delicate than rod disks. This difference is probably owing to either biochemical or structural characteristics of the two kinds of photoreceptors. Their photopigments, although alike as to chromophore, differ in their opsin component, and whereas the unit membrane walls (see Discussion in reference 21) of the cone disk are continuous with those of other disks and with the surface membrane, each rod disk (with some exceptions) is a discrete platelet, unconnected to other disks and to the membrane investing the outer segment. The disks arise by infoldings of the cell membrane, as most clearly shown in our study of the development of the receptors in the amphibian "third-eye" (22). The cone retains this embryonic relationship, but the rod disks later separate from the cell membrane and form thickened rims. Thus, owing to the nature of rod opsin or more probably to the discreteness of the rod disks, the outer segments of scotocytes are less apt to breakdown into vesicles upon fixation than those of photocytes.

In the light of this study one might wonder whether vesicle formation in rods and cones of animals deficient in vitamin A could be a fixation artifact. Although there is a striking similarity between Fig. 1 of this study and Fig. 5 in the paper of Dowling and Gibbons (15) showing vesicular degeneration in a rod of a vitamin A deficient rat, it seems clear that their picture is not owing to an

artifact because the rods of control animals were normal (see their Fig. 3). It is assumed that the eyes of their experimental and control animals received identical handling. Moreover, both light and electron microscopy demonstrated that the outer segments were completely lost after prolonged avitaminosis. Furthermore, if the degeneration had not progressed too far the outer segments recovered upon the addition of vitamin A to the diet, and the newly formed disks appeared normal. My study (16) of degeneration in the median and lateral eyes of a lizard subjected to vitamin A deficiency also seems above criticism of misinterpreting a fixation artifact, because I obtained seemingly excellent fixation by perfusing the animal with an osmium tetroxide fixative and because the outer segments of normal animals showed an even array of disks without vesicles. Nevertheless, retinas of vitamin A depleted animals should be fixed with glutaraldehyde and examined with an electron microscope for presence or absence of vesicle in the outer segments of rods and cones.

Why the cones in the subadult frogs, experimental and controls alike, in this study were not well preserved by osmium solutions was probably the large size of the eyes which were fixed *in toto*. This suggestion is borne out by the fact that cones in eyes from 10-day old larvae exhibited normal disks (Fig. 2). These much smaller eyes were fixed *in toto* in Dalton's solution, as were the eyes from the subadult animals which showed vesicle formation.

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