Rhodopsin and Porphyropsin Fields In the Adult Bullfrog Retina

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ABSTRACT Though it had been supposed earlier that the bullfrog undergoes a virtually complete metamorphosis of visual systems from vitamin A_2 and porphyropsin in the tadpole to vitamin A_1 and rhodopsin in the adult, the present observations show that the retina of the adult frog may contain as much as 30–40% porphyropsin, all of it segregated in the dorsal zone. The most dorsal quarter of the adult retina may contain 81–89% porphyropsin mixed with a minor amount of rhodopsin; the ventral half contains only rhodopsin. Further, the dorsal zone contains a two to three times higher concentration of visual pigments than the ventral retina. The pigment epithelium underlying the retina contains a corresponding distribution of vitamins A_1 and A_2, predominantly vitamin A_2 in the dorsal pigment epithelium, exclusively vitamin A_1 in the ventral zone. The retina accepts whatever vitamin A the pigment epithelium provides it with, and turns it into the corresponding visual pigment. Thus, a piece of light-adapted dorsal retina laid back on ventral pigment epithelium regenerates rhodopsin, whereas a piece of light-adapted ventral retina laid back on dorsal pigment epithelium regenerates predominantly porphyropsin. Vitamin A_2 must be made from vitamin A_1, by dehydrogenation at the 3,4-bond in the ring. This conversion must occur in the pigment epithelium, presumably through the action of a vitamin A–3,4-dehydrogenase. The essential change at metamorphosis is to make much less of this dehydrogenase, and to sequester it in the dorsal pigment epithelium. Some adult bullfrogs, perhaps characteristically taken in the summer, contain very little porphyropsin—only perhaps 5%—still sequestered in the dorsal retina. The gradient of light over the retinal surface has little if any effect on this distribution. The greater density of visual pigments in the dorsal retina, and perhaps also—although this is less clear—the presence of porphyropsin in this zone, has some ecological importance in increasing the retinal sensitivity to the dimmer and, on occasion, redder light received from below.

The rod visual pigments, rhodopsin and porphyropsin, are formed by the combination of a family of visual proteins, the rod opsins, with vitamin A_1 aldehyde (retinal$_1$, retinal) or vitamin A_2 aldehyde (retinal$_2$, 3-dehydroreti-

nal) (Wald, 1937; reviewed in Wald, 1953 *a*, 1968). Replacing retinal$_1$ by retinal$_2$ shifts the absorption peak (λ_{max}) of the visual pigment toward longer wavelengths (Wald, 1953 *b*). Rhodopsins usually have a λ_{max} near 500 nm, porphyropsins in the 520–535 nm region.

Kühne and Sewall (1880) had noted that the retinas of certain fishes contain a purple visual pigment rather than the rose-colored rhodopsin of terrestrial vertebrates. This difference was later confirmed spectrophotometrically in extracted visual pigments by Köttgen and Abelsdorff (1896). It has since been shown that the purple rod pigment, porphyropsin, is characteristic specifically of freshwater fishes, whereas the rod visual pigment of land vertebrates and almost all marine fishes is rhodopsin. Most freshwater fishes have porphyropsin or mixtures of porphyropsin and rhodopsin (Wald, 1939 *b*, 1945; Dartnall and Lythgoe, 1965; Bridges, 1965 *b*; Schwanzara, 1967).

Some years ago the retina of the bullfrog, *Rana catesbeiana*, was found to contain primarily porphyropsin in the tadpole and to go over almost entirely to rhodopsin on metamorphosis to the adult (Wald, 1945–46; Crescitelli, 1958). Wilt (1959; *a*, *b*) has examined the mechanism and course of this change in some detail. Liebman and Entine (1968) have shown by microspectrophotometry of single receptor cells that the transfer from retinal$_2$ to retinal pigments in *R. pipiens* involves cones as well as rods. Such metamorphoses of visual systems are found also in the European *R. temporaria* (Muntz and Reuter, 1966) and the tree frog, *Hyla regilla* (Crescitelli, 1958). On the other hand, no such visual pigment metamorphosis has yet been found in *Bufo* (*B. boreas halophilus*: Crescitelli, 1958; *B. bufo*: Muntz and Reuter, 1966). It is significant that such amphibians as the mud puppy *Necturus maculosus*, which never metamorphoses, and the clawed toad *Xenopus laevis*, which metamorphoses yet does not emerge from the water, retain porphyropsin throughout life (*Xenopus*: Wald, 1955; Dartnall, 1956; Crescitelli, 1958. *Necturus*: Wald, 1946; Crescitelli, 1958; Brown, et al., 1963). The newt, *Diemictylus* (formerly *Triturus*) *viridescens*, has a predominance of vitamin A_1 in the retina as a land-living red eft, and goes over to predominantly vitamin A_2 on metamorphosing to the sexually mature, water-living adult (Wald, 1958). Thus the occurrence of porphyropsin in many amphibians is closely correlated with a freshwater habitat.

In the present experiments we have found that the retinas of fully grown bullfrogs may contain appreciable amounts of porphyropsin. What is more interesting, this is segregated in the dorsal zone of the retina, the portion that receives images from below.

MATERIAL AND METHODS

Large, adult bullfrogs (*Rana catesbeiana*) were obtained from Lawrence, Kansas[1] and from Rayne, Louisiana (Jacques Weil Co). They were kept ordinarily at about 15°C,

[1] We are grateful to Mr. Stanley D. Roth of Lawrence High School, Lawrence, Kans. for supplying these animals.

in dim daylight during the day and dark at night. Bullfrog tadpoles were obtained from Massachusetts (Connecticut Valley Biological Supply Co., Southhampton, Mass.) and North Carolina (Carolina Biological Supply Co., Burlington, N.C.), adult leopard frogs (*Rana pipiens*) from Wisconsin (Steinhilber and Co., Oshkosh, Wis.).

Digitonin extracts of retinas were prepared as follows. Dark-adapted eyes were opened by cutting about 0.5 mm beyond the iris. After removal of the lens, the retina was lifted from the fundus with forceps. The dissections were carried out in deep red light, and the subsequent procedures in darkness. The isolated retinas were gently bathed successively in frog saline, pH 8.0 and pH 4.6 buffers, and distilled water, hardened in 4% alum solution, and rinsed in distilled water. They were centrifuged at 18,000 rpm for 30 min and the water was decanted and replaced with 0.3–1.0 ml of 2% digitonin (Hoffman–La Roche, Inc., Nutley, N.J.) in 0.125 M pH 6.5 phosphate buffer. After stirring, the preparation was left for 3 hr at room temperature or overnight in a refrigerator. Then fresh neutral hydroxylamine (NH_2OH) solution was added to a final concentration of 0.025–0.05 M. The sample was stirred, centrifuged at 18,000 rpm for 30 min, and the supernatant extract of visual pigment was pipetted off.

The hydroxylamine was added to liberate retinal rapidly in the form of its oxime from what would otherwise be long-lived intermediates of bleaching. It also destroys the green-rod visual pigment (Dartnall, 1967).

Absorption spectra were recorded with a Cary Model 11 spectrophotometer (Cary Instruments, Monrovia, Calif.). Extracts were irradiated with the red light of a tungsten lamp passing through a 660 nm Baird-Atomic interference filter (Baird-Atomic, Inc., Cambridge, Mass.) in combination with a heat filter; bleaching was completed with the orange light transmitted by a cutoff filter (Schott OG-2, Schott and Gen., Jena, Germany, transmitting above 550 nm).

Antimony trichloride tests were carried out as follows. Areas of dark-adapted eye cups were cut out with a steel punch and the retina, pigment layers (pigment epithelium and choroid), and sclera were separated from one another. The portions of retina and pigment layers were irradiated with intense yellow light repeatedly during 2–3 hr, to bleach all of the visual pigments to vitamins A_1 and A_2 (Wald, 1934, 1935, 1939 *b*). After draining off excess water, the retinal tissue and pigment layers were ground with anhydrous sodium sulfate, and extracted three times by stirring gently with petroleum ether (boiling range 40°–50°C). The combined petroleum ether extracts were brought over into 0.5 ml of chloroform. 0.15 ml of this chloroform extract was rapidly mixed with one drop of acetic anhydride and 0.35 ml of a saturated solution of $SbCl_3$ in chloroform in an optical cell in place in the Cary spectrophotometer. The absorption spectrum was recorded immediately at a rate of 5 nm/sec starting from 750 nm.

Color photographs of isolated retinas were taken with Kodak Ektachrome film, using an electronic flash and a Wratten 85B color-correcting filter. This combination gave color transparencies which matched well the appearance of retinas viewed in diffuse daylight. Incisions at the anterior, ventral, and posterior edges of isolated retinas allowed them to be spread flat in a small volume of Ringer's solution between a cover glass and a white enamel surface. The retinas were usually photographed first by the electronic flash to record their dark-adapted appearance. They were bleached for a

few seconds with strong white light, left for a few minutes in darkness, and photographed again to record an intermediate stage in bleaching.

OBSERVATIONS

Partial Bleaching Experiments

Fig. 1 compares the results of identical bleaching experiments, performed with extracts of whole *R. catesbeiana* and *R. pipiens* retinas. In both experiments

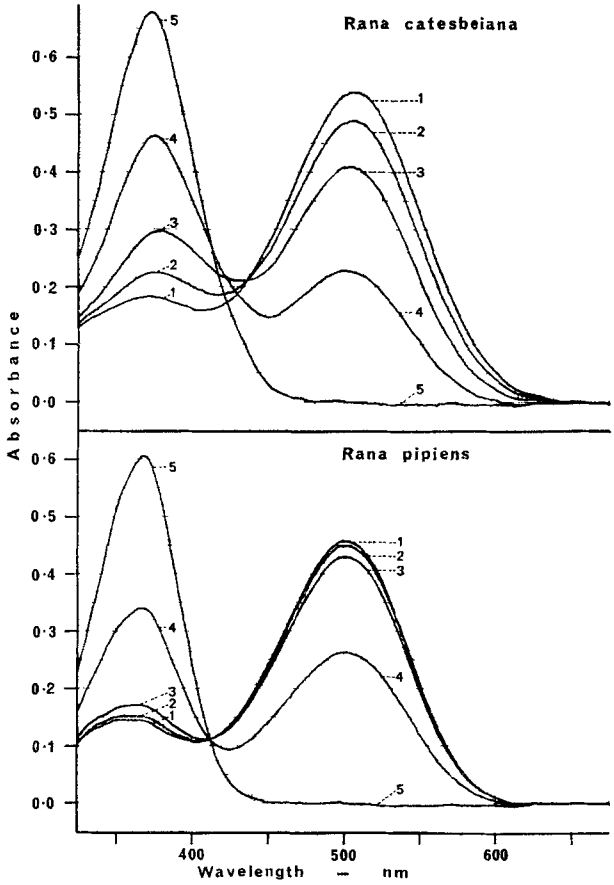

FIGURE 1. Partial bleaching experiments showing the presence of both rhodopsin (λ_{max} 502 nm) and porphyropsin (λ_{max} 522 nm) in a retinal extract from adult *R. catesbeiana* (above), but only rhodopsin in an extract of adult *R. pipiens* (below). Both extracts contained 0.05 M hydroxylamine and were bleached with the same series of exposures to red and orange light. Curve *1* is the spectrum of the unbleached extract, curves *2*, *3*, and *4* the spectra after 0.5, 1.5, and 20 min exposures to 660 nm, and curve *5* after complete bleaching with orange light for 10 min. The mixture of visual pigments in the *R. catesbeiana* extract is shown by the greater sensitivity to the first exposures to red light, with displacement of λ_{max} toward shorter wavelengths, both of the remaining visual pigment and of the products of bleaching; and by the lack of an isobestic point. The *R. pipiens* extract displays a sharp isobestic point at 412.5 nm, and no displacements of spectrum.

curve *1* is the spectrum of the unbleached extract, curves *2*, *3*, and *4* the spectra after 0.5, 1.5, and 20 min exposures to red light, and curve *5* after completely bleaching with orange light for 10 min.

The *R. pipiens* extract contains only a single photosensitive pigment. Each step in bleaching produces the same kind of difference spectrum, with maximum absorption loss at about 502 nm, an isosbestic point at 412.5 nm, and maximum rise of absorption at 368 nm, the λ_{max} of retinal$_1$ oxime. These are the typical properties of frog rhodopsin bleached in the presence of hydroxylamine.

The bullfrog extract behaved very differently. The first short exposures to red light caused considerable bleaching (curves *1–3*), with maximum loss of absorbance at 515–520 nm, close to the λ_{max} of porphyropsin. There is a fairly steady isosbestic point near 435 nm, and a rise of absorbance maximal at about 380 nm, near the λ_{max} of retinal$_2$ oxime. The next 20 min irradiation with red light yielded an intermediate result, all of these values shifting toward shorter wavelengths (curve *4*). The final bleaching with orange light (curves *4* to *5*) yielded the same changes of absorbance, typical of rhodopsin, as were found in *R. pipiens* extracts. Clearly the bullfrog extract contained a mixture of pigments: the first short exposures to red light bleached mainly porphyropsin, the long red irradiation a mixture of the remaining porphyropsin and some rhodopsin, the final exposure rhodopsin alone.

Isolating the difference spectra of these two pigments was made easy by the discovery that the ventral part of the bullfrog retina contains only rhodopsin, while the most dorsal portion contains mainly porphyropsin (Fig. 2). When partial bleaching experiments were performed with extracts of isolated ventral and dorsal portions of retina, the ventral extracts yielded the difference spectrum shown with open circles in Fig. 2, typical of rhodopsin. The difference spectrum of the first red (660 nm) bleaches of the dorsal extract is shown with filled circles in Fig. 2. The maximum fall of absorption at 522 nm, and maximum rise at 387 nm typical of retinal$_2$ oxime show this difference spectrum to be that of porphyropsin, hardly contaminated with significant amounts of rhodopsin. Although the dorsal extracts always contained some rhodopsin, the much greater red-sensitivity of porphyropsin (see Figs. 1 and 3) ensured the bleaching under these circumstances of virtually porphyropsin alone (compare also Dartnall, 1957; Munz and Beatty, 1965).

Since retinal$_1$ and retinal$_2$ oximes do not absorb above 460 and 490 nm, respectively, the difference spectra of the visual pigments at longer wavelengths correspond to their true absorption spectra. The lines in Fig. 2 show the absorption spectra of a 502 nm rhodopsin (from Dartnall, 1953) and a 522 nm porphyropsin (from Bridges, 1967). It is clear that the difference and absorption spectra agree reasonably well above the indicated wavelengths.

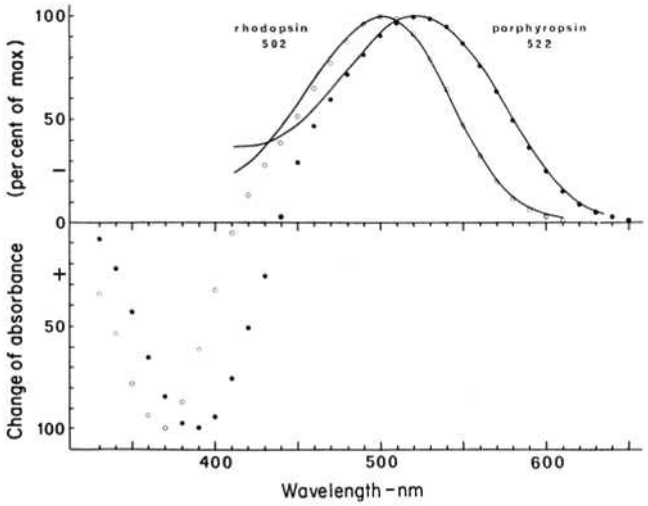

FIGURE 2. Difference spectra of bleaching bullfrog porphyropsin (solid circles) and rhodopsin (open circles), in the presence of hydroxylamine, to retinal$_2$ oxime and retinal$_1$ oxime. Both spectra are in per cent of maximum absorption loss. The curves are the absorption spectra of frog rhodopsin (*R. temporaria*, from Dartnall, 1953) and a porphyropsin with λ_{max} at 522 nm (from Bridges, 1967).

Observations on Whole Retinas

A photograph and drawing of an isolated retina of *R. catesbeiana* is shown in Fig. 4. Except at the extreme dorsal edge, the depth of pigmentation is greater in the dorsal portion of the retina. The same is true of *R. pipiens*. A horizontal line of denser pigmentation runs across the bullfrog retina about 1 mm above the optic nerve. Such a line has been observed earlier, running across the retinas of several species of frog, including *R. catesbeiana* (Slonaker, 1897; Hess, 1910). In this region the outer segments were found to be longer and more densely packed, and the neural layers thicker. More recently Brown (1969) has described a similar formation in the eye of a freshwater turtle. It may constitute a region of increased visual acuity comparable with the area centralis, in these cases a linea centralis.

The segregation of rhodopsin and porphyropsin in bullfrog retinas is clearly visible. The *R. pipiens* retina has the same color throughout, the pale red of rhodopsin. The ventral two-thirds of the bullfrog retina is of the same color, but the dorsal one-third, starting somewhat above the horizontal line, is the purple color of porphyropsin. The border between the two colors is sharp, the changeover seeming to be complete within about 1 mm. The intermediates of bleaching show corresponding color differences: the most dorsal part of the retina just after a short exposure to light is orange, the ventral region more yellow.

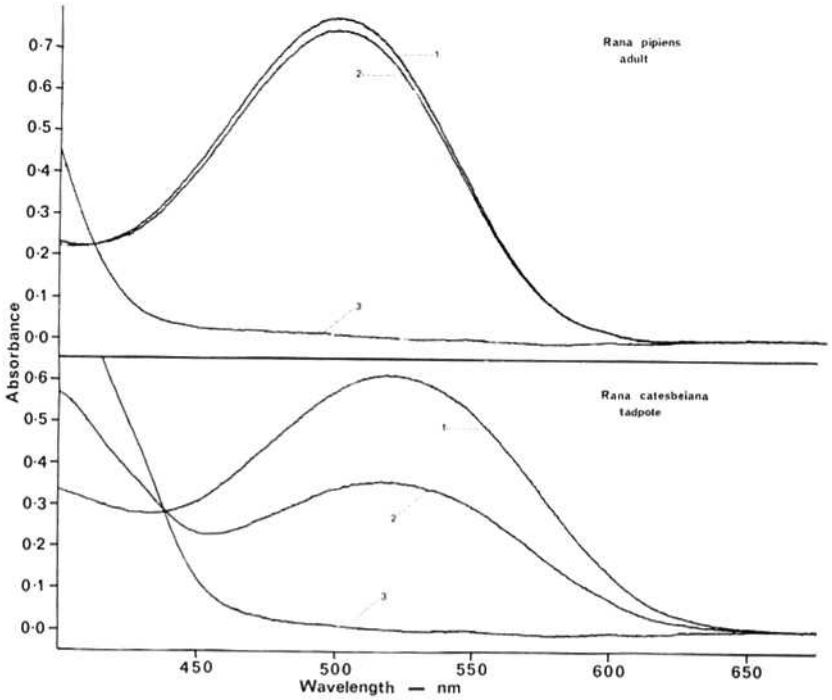

FIGURE 3. Partial bleaching experiments demonstrating the effect of the same exposure to red light on pure frog rhodopsin (*R. pipiens*), and almost pure tadpole porphyropsin (*R. catesbeiana*, about 4% rhodopsin), in the presence of 0.04 M hydroxylamine. (1) Spectrum of the unbleached extract; (2) after 2 min exposure to 660 nm; (3) after complete bleaching with orange light for 10 min. The difference spectrum of bullfrog tadpole porphyropsin seems to be identical with that of adult porphyropsin. The red irradiation clearly bleaches porphyropsin more efficiently than rhodopsin. Calculations based on this and similar experiments show that frog porphyropsin is 15 times more sensitive to 660 nm irradiation than frog rhodopsin, i.e., the time needed for bleaching a given fraction of the pigment is 15 times longer for rhodopsin.

We have examined a large number of bullfrog retinas, both spectrophotometrically and by direct inspection in white light. The distribution of porphyropsin and rhodopsin was about the same in all the frogs collected from October through March. Some of these "winter frogs" were collected in Kansas in October and November 1969, and used in the experiments from November 1969 to March 1970. Others were collected in Louisiana in December 1969 and March 1970 and used from March to April 1970. The retinas shown in Fig. 4 were from these batches.

Two groups of bullfrogs, however, examined shortly after their arrival from Louisiana in July 1970, contained much less porphyropsin. There was only a trace of purple at the most dorsal edge of the retina. Earlier work, in which bullfrog retinas were found to contain very little if any porphyropsin (Wald,

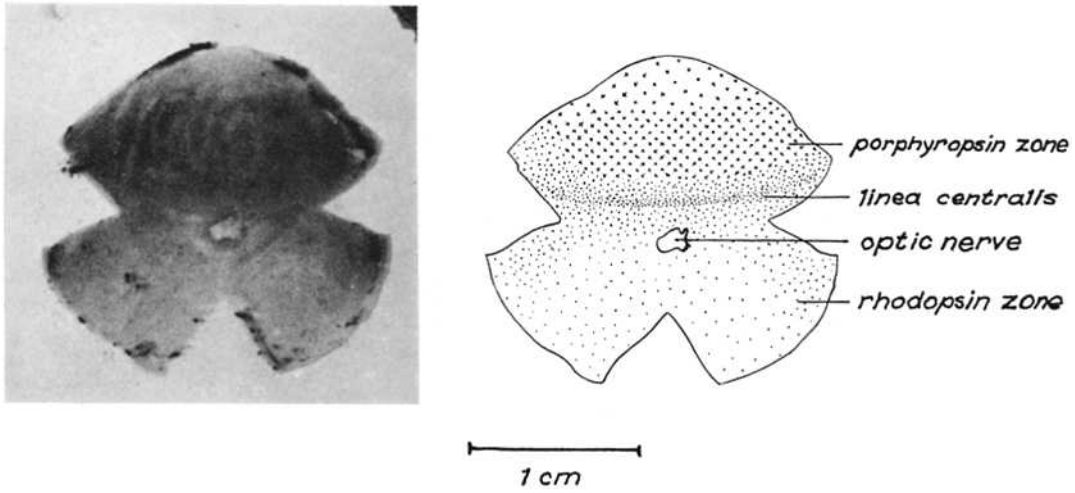

FIGURE 4. Appearance of the dark-adapted bullfrog retina, seen from the receptor surface. The retina forms a complete circle, but here three incisions had been made so that it could be laid flat for color photography. The uppermost zone is purple, and, except toward the dorsal edge, much more densely pigmented than the red ventral retina. A line of denser pigmentation (linea centralis) extends across the retina about 1 mm above the point of entry of the optic nerve.

1936, 1938, 1954) may have involved such a seasonal variation. Unless explicitly noted, all results in the present paper refer to winter frogs.

Quantitative Distribution of Rhodopsin and Porphyropsin

In six experiments, five with winter frogs and one with a frog collected in July, we determined the amounts of rhodopsin and porphyropsin in four areas of retina located as shown in the inset in Fig. 5. The most dorsal region (*1*) lay completely within the purple porphyropsin field, the next (*2*) overlapped the border between the purple and red areas, and the most ventral areas (*3* and *4*) lay within the rhodopsin field below the optic nerve. All winter frogs gave essentially the same results.

The results of one such experiment are shown in Fig. 5. As in all our experiments of this kind, the two extracts from the ventral half of the retina yielded the difference spectra of rhodopsin alone, whereas the dorsal extracts yielded spectra indicating mixtures of rhodopsin and porphyropsin, the most dorsal extract mainly porphyropsin.

Mixed difference spectra of this kind can be used to calculate the relative amounts of the two contributing pigments, when the spectra of the single pigments are known (see Dartnall et al., 1961). The values calculated from the difference spectrum of Fig. 5 (March frogs) are shown in Table I, and, for comparison, values obtained from one frog collected in July are also shown.

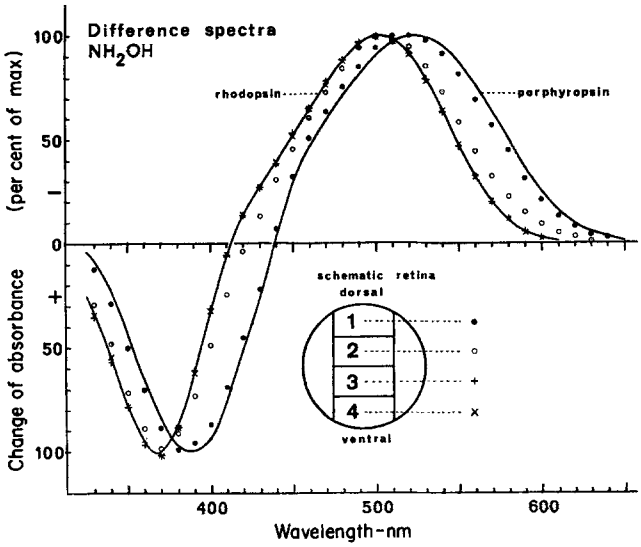

FIGURE 5. Difference spectra produced by bleaching extracts of four retinal regions (see insert) in the presence of hydroxylamine. Five eyes from three Louisiana bullfrogs collected in March were used. Areas of equal size (about 50 mm^2) were punched out from each zone, and the corresponding areas from all eyes were extracted together. The four different kinds of symbols represent the extracts of the four zones, whereas the curves, based on the data in Fig. 2, represent the "pure" rhodopsin and porphyropsin difference spectra. All spectra are rendered in percentage of maximum absorption loss.

Clearly, the retinas of the July frog contained much less porphyropsin, and this was restricted to the most dorsal zone. Typically, the retinas of winter frogs contained 30–40% porphyropsin, whereas only about 5% of the pigment of three frogs obtained in July was porphyropsin. In winter frogs the porphyropsin content of the most dorsal region of the retina ranged from 81 to 89%.

The proportion of the total visual pigment in each region of the retina is also shown in Table I. The concentration of visual pigment—the number of pigment molecules per unit area—is two to three times as great in the dorsal as in the ventral areas, in most of our observations.

Distribution of Vitamin A_1 and A_2 in Retina and Pigment Layers

Fig. 6 shows the spectra of antimony chloride tests with extracts of vitamins A from the four regions of the retina (*r*) discussed above and in the corresponding regions of the pigmented layers (*p*) (pigment epithelium and choroid). In these experiments, dark-adapted retinas were isolated, bleached, and allowed to fade to vitamin A_1 and A_2 before extraction.

Region 1, the most dorsal, has a dominant vitamin A_2 peak at 687 nm and a hump near 620 nm owing to a small amount of vitamin A_1. Region 2 shows a

TABLE I

TOTAL VISUAL PIGMENTS AND PERCENTAGE PORPHYROPSIN IN FOUR EQUAL AREAS OF BULLFROG RETINAS, AS IN FIG. 5

Frogs were collected in Louisiana, three in March, one in July. Amounts of total visual pigment—rhodopsin + porphyropsin—were measured by absorbances at 512 nm, where the absorbances of both pigments are 97–98% of maximum. Relative molar concentrations are calculated on the basis that the molar absorbances at λ_{max} are: rhodopsin, 40,600 (Wald and Brown, 1953); porphyropsin, 30,000 (Brown et al., 1963).

Retinal zones (see Fig. 5)	Relative absorbances of total visual pigment at 512 nm (March frogs) in the four zones	Relative molar concentrations of visual pigment (March frogs) in the four zones	Porphyropsin-molecules per 100 molecules of visual pigment (March frogs) in each zone	Porphyropsin-molecules per 100 molecules of visual pigment (July frog) in each zone
1 dorsal	30	35	87	21
2 ↓	39	38	34	0
3 ↓	16	14	1	0
4 ventral	15	13	0	0
	100	100		

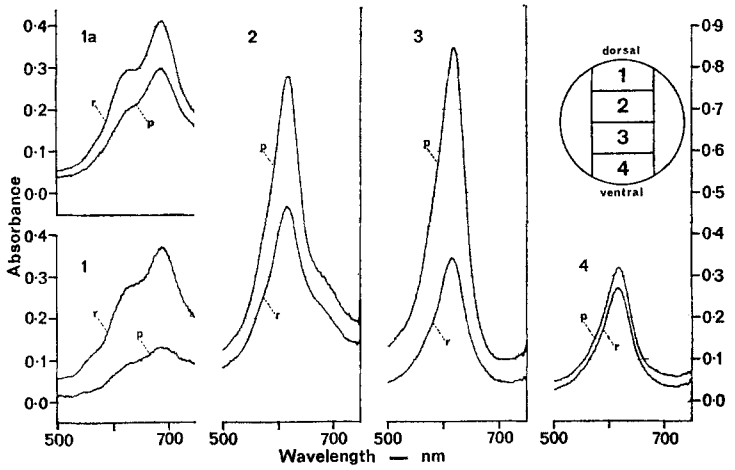

FIGURE 6. Antimony chloride tests with vitamin A extracts of equal areas (about 50 mm²) of wholly bleached retina (*r*) and pigment layers (*p*), obtained from the four zones shown in the insert. Two Louisiana bullfrogs collected in March were used for tests *1–4*. The corresponding regions from all four eyes were pooled and extracted together. Test *1a* refers to the most dorsal region of the eyes of another Louisiana frog collected in December. The antimony trichloride products of vitamin A_1 and A_2 have λ_{max} at about 616 and 687 nm, respectively, and the specific absorptions (E [1%, 1 cm] at λ_{max}) are approximately 4800 and 4000 (Hubbard et al., 1971).

high vitamin A_1 peak at 615–620 nm and only a small inflection in the 680–690 nm region indicating some vitamin A_2. Regions 3 and 4 show no signs of vitamin A_2 at all. These observations agree with the distribution of rhodopsin and

porphyropsin in the retina. The results shown in Fig. 6, parts *1–4*, are presented quantitatively in Table II.

In any particular region of the eye, the vitamin A_2/A_1 ratio is approximately the same in the pigment layers and the retina. This is not surprising, since the visual cycle of bleaching and synthesis involves interchanges of vitamin A between retina and pigment epithelium (Wald, 1935). But the total quantities of vitamins A are differently distributed in these tissues. In four experiments, of which Table II shows one, the dorsal regions 1 and 2 of the retina contained larger total amounts of the vitamins A than ventral regions 3 and 4, just as the

TABLE II

QUANTITATIVE REPRESENTATION OF RESULTS OF ANTIMONY CHLORIDE TESTS

Vitamins A_1 and A_2 in four equal areas of the retina and of the combined pigment layers (pigment epithelium and choroid), located as in Fig. 6 (insert). Amounts are in micrograms per 50 mm² area punched out in each retinal zone. Values were calculated from absorbances in the antimony chloride reaction as shown in Fig. 6, sections *1–4* (see Wald, 1939 *a* and Hubbard et al., 1971).

	μg/50 mm² of retina			μg/50 mm² of pigment layers		
Retinal zone	Vit. A_1	Vit. A_2	Totals	Vit. A_1	Vit. A_2	Totals
1 dorsal	0.10	0.37	0.47	0.03	0.13	0.16
2 ↓	0.35	0.16	0.51	0.62	0.19	0.81
3 ↓	0.30	—	0.30	0.76	—	0.76
4 ventral	0.23	—	0.24	0.30	—	0.30
	0.99	0.53	1.52	1.71	0.32	2.03

dorsal half of the retina contains a higher concentration of visual pigment, but the vitamin A content of the pigment layers was greatest in the middle regions 2 and 3. It is especially striking that in the most dorsal part of the eye the pigment layers have less vitamins A than the retina, while they have more in the rest of the eye, and especially in regions 2 and 3. The experiment shown in Fig. 6 is fairly representative of all our measurements as regards the *proportions* of vitamins A_1 and A_2 and their *distribution*. In general, however, the absolute amounts of vitamins A in the pigmented layers were usually about twice those shown here.

The differences in spatial distribution of vitamins A_1 and A_2 across the pigment layers indicate that there is little lateral diffusion of vitamin A in these tissues.

Wald (1935, 1936) showed that the pigment layers of the bullfrog contain also large amounts of xanthophyll. We found it mainly concentrated in regions 2 and 3, where also the largest amounts of vitamin A are found.

Vitamins A_1 and A_2 and the Visual Cycle

It is well known that a large part of the all-*trans* vitamin A liberated by bleaching rhodopsin in a frog retina migrates into the pigment epithelium (Wald, 1935, 1936). It then returns as 11-*cis*-vitamin A to the retina, where it is oxidized to retinal and recombines with opsin (Wald, 1934, 1935, 1936; Hubbard and Wald, 1952; Hubbard and Colman, 1959). That the visual cycle involves the pigment epithelium was first shown by Kühne (1878, 1879), who found that a bleached frog retina can regenerate rhodopsin while it is in contact with the pigment epithelium, though little if any regeneration occurs in an isolated retina. He found also that an isolated retina regains the ability to regenerate rhodopsin in the dark, when laid back smoothly on the pigment epithelium. Kühne stated that this requires a living pigment epithelium, although not necessarily a living retina.

Wilt (1959 *a*, *b*) and Ohtsu et al. (1964) showed that the porphyropsin in bullfrog tadpoles comes from a local vitamin $A_1 \rightarrow A_2$ (or $\text{retinal}_1 \rightarrow \text{retinal}_2$) conversion in the eye tissue. Neither the blood nor the liver of the tadpole contains measurable amounts of vitamin A_2. Their experiments did not specify whether the conversion occurs in the retina or in the pigment layers.

We have tested whether the dorsal retina can convert vitamin A_1 to vitamin A_2 using a procedure suggested by Kühne's observation that a bleached, isolated retina can regenerate rhodopsin only when laid back upon a pigment epithelium. The idea was to let light-adapted dorsal retina regenerate its visual pigment on ventral pigment epithelium. Would it regenerate rhodopsin, or convert the vitamin A_1 of the ventral pigment epithelium to vitamin A_2 and regenerate porphyropsin? The experiment was performed as follows.

Bullfrogs were light-adapted in direct sunlight for 2–3 hr at about 27°C. The relatively high temperature makes it easier to remove the light-adapted retina from the pigment epithelium (see Ewald and Kühne, 1878). The subsequent dissections were performed as rapidly as possible under bright white light. The upper part of the eye cup, just dorsal to the zone where the purple porphyropsin field begins, and a ventral portion of similar size, were cut free. The retina was stripped away from these two segments of eye cup and laid on a cover glass, in the drop of vitreous which clung to it. Only retinas which were completely free from patches of pigment epithelium were used. (All of these retinas contained melanin, presumably in pigment epithelium process that had torn away, sometimes giving parts of the retina a pale grey tone.)

The retinal segments were treated in one of three ways: (*a*) The white light was turned off and the retina was immediately washed in pH 4.6 acetate buffer containing hydroxylamine, washed in water, and the visual pigments were extracted as usual under deep red light. (*b*) The retina was spread out on the cover glass with receptors downward, left in darkness in a moist chamber for 6

hr at 20°C, and then washed and extracted as in procedure (*a*). (*c*) The retina was slid from the cover glass onto an eye-cup segment previously freed from retina, the ventral retina upon dorsal pigment layers, and the dorsal retina upon ventral pigment layers, or upon a dark-adapted retina-free eye cup of *R. pipiens* containing only vitamin A_1. The retinas were gently spread out with

TABLE III

AMOUNTS OF VISUAL PIGMENTS IN EXTRACTS OF VARIOUSLY TREATED PORTIONS OF RETINA

Relative molar concentrations of visual pigments in variously treated portions of retina, stated as percentages of the average pigment concentrations in corresponding portions of wholly dark-adapted retinas. The experimental retinas were either: (*a*) light-adapted (Exp. 1 and 2); (*b*) light-adapted, then dark-adapted in contact with glass (Exp. 3–9); (*c*) light-adapted, then dark-adapted on the opposite pigment epithelium, i.e. ventral retina on dorsal pigment epithelium (Exp. 10 and 11), or dorsal retina on ventral pigment epithelium (Exp. 12 and 13); (*d*) dorsal retina, light-adapted, then dark-adapted on *Rana pipiens* pigment epithelium (Exp. 14–17). Porphyropsin concentrations are stated only when enough pigment was present for an accurate calculation.

Experiment No.	Treatment of retinas	Segment of retina	Segment of pigment ep.	Visual pigment, per cent of dark-adapted	Porphyropsin
					moles %
1	(*a*) Light-adapted	Dorsal		2	
2		Ventral		1	
3	(*b*) Dark-adapted on glass	Dorsal		5	
4		"		5	
5		"		7	
6		"		8	
7		"		15	
8		"		12	
9		"		18	
				Mean: 10	
10	(*c*) Dark-adapted on	Ventral	Dorsal	80	80
11	opposite pig-	"	"	78	79
12	ment epithe-	Dorsal	Ventral	77	13
13	lium	"	"	52	17
14	(*d*) Dark-adapted on	Dorsal		59	8
15	*R. pipiens*	"		81	9
16	pigment epithe-	"		81	9
17	lium	"		82	19
				Mean: 74	Mean: 13

their receptors against the pigment epithelia and left in darkness in moist chambers for 6 hr at 20°C. Then the retinas were removed, washed, and extracted as before.

Table III shows the amounts of visual pigment in extracts of these variously treated portions of retina. The light-adapted retinas originally contained only 1–2% of their dark-adapted content of rhodopsin and porphyropsin. The retinas left in darkness on a glass surface for 6 hr regenerated an average of 10% of their visual pigment, but when dark-adapted on a pigment epithelium they regenerated an average of 74%. The 10% increase on a glass surface indicates that the light-adapted, isolated retinas contain some vitamin A or retinal which can be used for visual pigment resynthesis (see Wald, 1935). But a retina laid on pigment epithelium clearly takes up additional vitamin A from it, since the regeneration of visual pigment is much enhanced. *Ventral retina on dorsal pigment epithelium regenerates mainly porphyropsin* (Exp. 10–11), while dorsal retina on ventral—or *R. pipiens*—pigment epithelium regenerates mainly rhodopsin (Exp. 12–17). Fig. 7 B shows difference spectra of pigment mixtures extracted from these retinas.

It seems, therefore, that, under the conditions of these experiments, the dorsal retinas do not convert the vitamin A_1 they obtain from the pigment epithelium to vitamin A_2. They regenerate on an average 72 units (Exp. 12–17) of visual pigment, 13% of which is porphyropsin. 13% of 72 units is 9 units, about the amount of porphyropsin we expect to be regenerated from the retinal$_2$ and vitamin A_2 which the retina itself contained (see Exp. 3–9). Since the dorsal retina does not convert vitamin A_1 to A_2, this conversion must occur in the pigment layers.

Our experiments are consistent with the observation of Bridges and Yoshikami (1970 *b*) that the light-adapted goldfish retina, which normally contains only porphyropsin, regenerates predominantly rhodopsin when laid on a frog pigment epithelium.

The Effect of Illumination on the Distribution of Rhodopsin and Porphyropsin in Frogs and Tadpoles

Dartnall et al. (1961) and Bridges (1964, 1965 *a*) have shown that the rhodopsin/porphyropsin ratio in some freshwater fishes is affected by the prevailing intensity of light. They found that darkness and low light intensities favor porphyropsin, high light intensities favor rhodopsin. Dramatic changes could be induced within a month by changing the conditions of illumination. Bridges and Yoshikami (1968, 1970 *a*) have induced such changes in only one eye by covering it. They concluded that light promotes rhodopsin by acting directly on the fish retina. Recently Bridges (1970 *a*) demonstrated that the reverse changes occur in frog tadpoles (*R. pipiens and R. catesbeiana*), in which darkness favors rhodopsin and light favors porphyropsin.

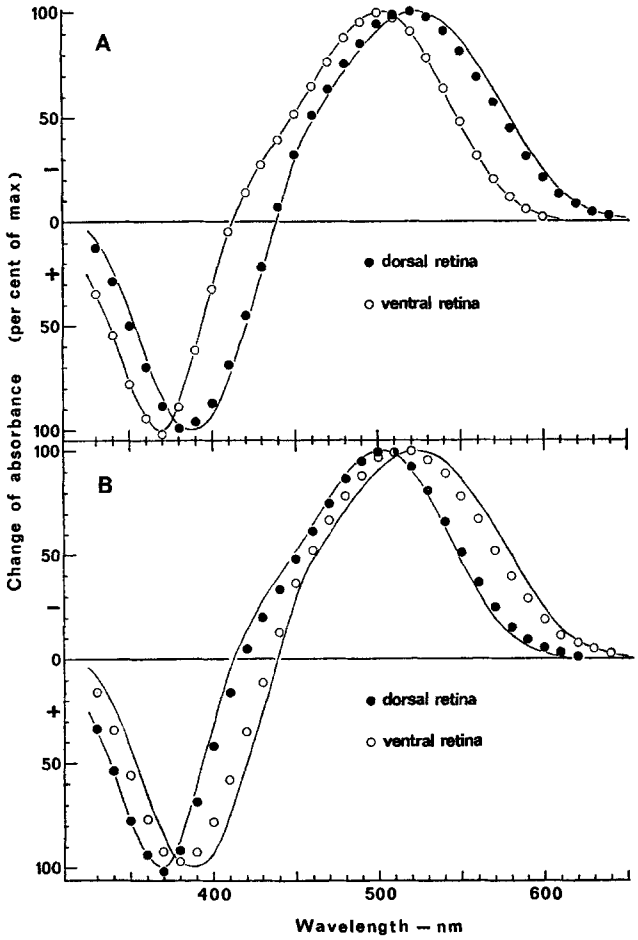

FIGURE 7. Difference spectra obtained by bleaching visual pigment extracts (with hydroxylamine) of the most dorsal (filled circles) and most ventral (open circles) retinal regions: (A) dark-adapted normally in the living eye; (B) retinas that had been light-adapted, isolated, and allowed to regenerate on the opposite regions of the pigment epithelium (see Exp. 10 and 12 in Table III). All spectra are expressed in per cent of maximum absorption loss. The curves, based on the data in Fig. 2, represent "pure" rhodopsin and porphyropsin difference spectra.

We may ask whether the porphyropsin-rhodopsin distribution in the adult bullfrog retina is affected by the unequal distribution of light across the retina. The dorsal retina looks downward toward ordinarily darker ground and water while the ventral retina looks upward toward the brighter sky.

We reversed this usual relationship by keeping four bullfrogs 54 days (from 19 November 1969 to 13 January 1970) at 22°C in a black container with a translucent white bottom, illuminated from below with a 100 w bulb at a distance of 40 cm. This treatment did not change the rhodopsin-porphyropsin

distribution. We compared a retina from one of these animals with another from a frog kept 54 days in a similar container with an illuminated cover; there was no visible difference. Retinal extracts from two of the frogs illuminated from below showed 89% porphyropsin in the most dorsal part of the retina, none in the ventral part.

We did similar experiments with bullfrog tadpoles. When the experiment started, the visual pigment was about 95% porphyropsin in both dorsal and ventral halves of the retina (Table IV, Exp. 1 and 2). Half of the tadpoles were illuminated from below; the rest were illuminated from above. All were given thyroxine. After 6 wk the forelimbs had emerged and the tails were reduced,

TABLE IV

MOLES PER CENT OF PORPHYROPSIN IN VISUAL PIGMENT EXTRACTS CONTAINING RHODOPSIN AND PORPHYROPSIN FROM DORSAL AND VENTRAL HALVES OF RETINAS FROM TADPOLES AND VERY YOUNG FROGS

Exp. 1 and 2 involve groups of tadpoles approaching the metamorphic climax. Exp. 3–6 involve tadpoles from North Carolina reared for 6 wk at 23°C. in water containing increasing concentrations of L-thyroxine (from 5 to 120 μg/liter). Forelimbs emerged after 5 wk. They were kept the 6th wk in 60 μg/liter L-thyroxine, and then used in Exp. 3–6. Half of these tadpoles were kept in a container illuminated from below (Exp. 4 and 6), the others in a container illuminated from above (Exp. 3 and 5). Exp. 7 and 8 involve very young frogs collected in Massachusetts and Louisiana, in July.

Exp. No.	Developmental stage	Number of animals used	Conditions of illumination	Porphyropsin in dorsal retina	Porphyropsin in ventral retina
				%	%
1	Length of hind limbs 2–9 mm	19	Natural environment, collected in N.C.	93	94
2	Length of hind limbs 4–12 mm	11	Natural environment, collected in Mass.	96	97
3	Forelimbs emerged, lengths of both hindlimbs and tail 30–40 mm	4	Illuminated from above	66	60
4	Forelimbs emerged, lengths of both hindlimbs and tail 30–40 mm	7	Illuminated from below	60	43
5	Length of tail about 10 mm	3	Illuminated from above	37	32
6	Length of tail about 10 mm	6	Illuminated from below	48	38
7	Length of tail about 1 mm, hindlimbs about 50 mm	4	Natural environment, collected in Mass. in July	16	1
8	No visible vestige of tail, hind limbs 61–63 mm	3	Natural environment, collected in La. in July	7	0

i.e., they were in metamorphic climax (Etkin, 1964). As climax approaches, there is a rapid shift from primarily porphyropsin to primarily rhodopsin (Wald, 1945–46; Muntz and Reuter, 1966; Reuter, 1969).

We took two batches of tadpoles, one with 3–4-cm tails, the other with only 1-cm tails, from each container, and compared the porphyropsin content in the dorsal and ventral halves of the retinas (Table IV, Exp. 3–6). Rhodopsin increased dramatically during the 6 wk, but the shift toward rhodopsin dominance was somewhat greater in the ventral halves of all retinas, regardless of the distribution of light across them.

The small differences between the dorsal and ventral halves of our tadpole retinas probably mark the beginnings of the adult distribution of visual pigments. None of these experiments, however, shows any effect of gradients of light intensity upon either establishing or maintaining the differential distribution of rhodopsin and porphyropsin across the retina.

Exp. 7 and 8 in Table IV involved very young frogs collected in the wild in July, 1970, just before and just after the completion of tail resorption. The retinas of the frogs from Louisiana (Exp. 8) contained very little porphyropsin, only 4% of the total visual pigment, but adult bullfrogs obtained at the same time from the same source were equally low in porphyropsin.

DISCUSSION

The first thought with regard to the dorsoventral distribution of porphyropsin and rhodopsin is that it has some ecological significance. The bullfrog is an unusually aquatic frog, one which feeds largely on water animals (Wright and Wright, 1949; Surface, 1913). We examined the droppings of bullfrogs freshly arrived from Louisiana in December, March, and July. The dealer stated that he had not fed them. The identifiable animal remnants in the droppings were mostly crayfish carapaces and claws.

The bullfrog "enjoys sitting with just the nose and eyes protruding above the surface of the water" (Surface, 1913). This posture may be related to the topography of its retina. A frog in that position looks down into the dimmer water with the dorsal part of its retina, up toward the brighter sky with the ventral part, and might be scanning along the surface of the pond with the denser horizontal linear region just above the optic nerve (Fig. 4), which may represent a linear area centralis, a region of higher visual acuity, in the bullfrog.

The higher density of visual pigment in the dorsal part of the bullfrog retina presumably increases the sensitivity of vision to the dimmer light from below. The predominance of porphyropsin in this region should also yield an increased relative sensitivity toward longer wavelengths. In turbid water, which scatters shorter wavelengths more strongly, the longer wavelengths might have a special importance for vision (Munz, 1965; Bridges, 1965 *b*; water transmis-

sions measured by Clarke, 1939; Reuter, 1969; Luria and Kinney, 1970). This consideration probably involves cone vision more than rod vision; for in the dorsal retina, in which porphyropsin largely replaces rhodopsin in the rods, we may expect the retinal$_2$ cone pigment, cyanopsin (λ_{max} about 620 nm), to replace the retinal$_1$ cone pigment, iodopsin (λ_{max} 575 nm), with a consequent large increase in sensitivity toward long wavelengths. (Granit, 1941; Wald et al., 1953; Kennedy, 1957; Liebman and Entine, 1968; Liebman and Granda, 1971).

One must take such ecological considerations with a grain of salt. The considerably higher density of visual pigment in the dorsal retina and consequent higher sensitivity to light from below, makes excellent ecological sense. That this pigment should be primarily porphyropsin offers a less clear advantage. Effective vision tends to concentrate towards the center of the retina, and is not likely to extend to its outer borders. As an example from an eye with which we are more familiar, the human retina subtends a potential visual angle of about 270°, yet forms effective images only within a much smaller radius, at most 90° from the central fixation area. Of course an eye in water, with little change of refraction between the eye and the outer medium, could do better in this regard. Nevertheless it is questionable how much of the dorsal porphyropsin field is effective in vision. Also, it is not clear why the porphyropsin field should be less advantageous in the summer than in the winter, if indeed the season determined the wide variations that we encountered.

Why, in considerable earlier work with this species of frog, did we fail to notice the presence of porphyropsin? Two reasons occur to us. On the one hand, in earlier dissections we opened the eye at the equator, so that we lost much of the rim of the retina, retained in the present procedures. The second possibility involves the large variation encountered in these experiments that may go with the season. We may have worked previously with summer frogs, or in any case, with such animals as possess little porphyropsin, whatever the reason.

The dorsoventral differentiation in the bullfrog retina made us wonder whether there is a similar distribution of visual pigments in those freshwater or anadromous fishes which possess mixtures of rhodopsin and porphyropsin. We have examined a few retinas of rainbow trout (*Salmo gairdneri*), and found, just contrary to the bullfrog, more rhodopsin in the dorsal region. The most dorsal quarter of the retina contained about 40% rhodopsin, compared with about 15% in the remainder of the retina. W. R. A. Muntz and D. P. M. Northmore (personal communication) say that they, too, recently found more rhodopsin in the dorsal retinas of two other species of fish (rudd, *Scardinius erythrophthalmus* and brown trout, *Salmo fario*). This inversion in fishes as compared with frogs brings into further question the ecological importance of such distributions, since it is not clear why such surface fishes face very different visual

problems from the bullfrog. The differential distribution of rhodopsin and porphyropsin in the retina is not the only aspect of these systems that differs in bullfrogs and fishes. Thyroxine and darkness are said to favor rhodopsin in bullfrog tadpoles (Wilt, 1959 *a*; Bridges, 1970 *a*), whereas they favor porphyropsin in some fishes (Bridges, 1965 *b*; Beatty, 1969). Also, whereas in the bullfrog the proportion of porphyropsin to rhodopsin is greatest in tadpoles, in the rudd the porphyropsin fraction increases as the fish grows older (Bridges and Yoshikami, 1970 *a*).

These experiments convey a somewhat modified view of the metamorphosis of visual systems in the bullfrog, the first such metamorphosis to be discovered (Wald, 1945–46). The initial appearance was of a more-or-less complete transfer from porphyropsin in the tadpole to rhodopsin in the adult frog. We see now that the adult bullfrog never wholly ceases to make porphyropsin. The essential change at metamorphosis is to make much less of it, and to segregate it within the dorsal retina.

The actual transformation involves the capacity to make vitamin A_2. No known plant carotenoid contains the 3,4–double bond that would make it a direct precursor of vitamin A_2. Vitamin A_2 must be made from vitamin A_1, through a dehydrogenation at the 3,4-bond, performed presumably by a vitamin A–3,4-dehydrogenase (Ohtsu et al., 1964).

This conversion occurs in the eye itself and, as our experiments show, in the pigment epithelium rather than in the retina. The retina accepts in this regard whatever vitamin A the pigment epithelium provides it with, and turns it into the corresponding visual pigment (see also Bridges and Yoshikami, 1970 *b*). From this point of view the essential change at metamorphosis is to synthesize much less of such a vitamin A–3,4-dehydrogenase, and to sequester it in the dorsal pigment epithelium.

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