Rod Sensitivity and
Visual Pigment Concentration in *Xenopus*

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ABSTRACT *Xenopus* larvae were raised on a vitamin A-free diet under constant illumination until their visual pigment content had decreased to between 8% of normal and an undetectably low level. After the intramuscular injection of 2.1×10^{13}–2.1×10^{16} molecules of [^{3}H]vitamin A, ocular tissue showed a rapid rate of uptake of label which reached a maximum level of incorporation by 48 h. Light-microscopic autoradiography revealed that the retinal uptake of label was concentrated within the receptor outer segments. Spectral transmissivity measurements at various times after injection were made upon intact retinas and upon digitonin extracts. They showed that visual pigment with a λ_{max} of 504 nm was formed in the retina and that the amount formed was a function of incubation time and the magnitude of the dose administered. Electrophysiological measures of photoreceptor light responses were obtained from the PIII component of the electroretinogram, isolated with aspartate. The quantal flux required to elicit a criterion response was determined and related to the fraction of visual pigment present. The results showed that rod sensitivity varied linearly with the probability of quantal absorption.

INTRODUCTION

Visual adaptation refers to a set of processes by which the visual system adjusts its operating range to conform to changing ambient levels of illumination. Recent work has succeeded in demonstrating that the photoreceptor cells of the retina are the primary, but not the exclusive, locus of adaptation. For example, it is well known from psychophysical studies (Stiles and Crawford, 1934; Aguilar and Stiles, 1954) that when the background illumination, I, increases, so must the intensity, ΔI, of a superimposed stimulus to be visible. The ratio $\Delta I/I$ is the Weber fraction, and photoreceptors very nearly duplicate the increment threshold function of ΔI vs. I that the organism shows behaviorally (Boynton and Whitten, 1970; Dowling and Ripps, 1972; Witkovsky et al., 1973; Normann and Werblin, 1974; Hood and Hock, 1975; Kleinschmidt and Dowling, 1975; Fain et al., 1977).

Another experimental test of adaptation is concerned with the loss of sensitivity resulting from a reduction in visual pigment concentration. In the classic studies of Dowling and Wald (1958, 1960) and Rushton (1961) a substantial quantity of visual pigment was bleached, resulting in a large rise in threshold. In the subsequent dark period, as rhodopsin concentration (C_t) rose because of

regeneration of bleached pigment, the logarithm of the threshold, I_t, fell proportionally. Thus, at any time t after the bleach, $\log I_t = -kC_t$, k being a constant of proportionality. The same log-linear relation between threshold and pigment was found to hold also when rhodopsin concentrations were altered through a dietary deficiency of vitamin A (Dowling and Wald, 1958, 1960).

However, because these investigations monitored threshold changes at post-receptoral levels (electroretinogram [ERG] b-wave, Dowling and Wald, 1958, 1960; psychophysical threshold, Rushton, 1961), they do not address directly the way in which the photoreceptors respond to bleaching lights. In a number of recent reports, retinas have been subjected to bleaching flashes that removed variable quantities of visual pigment. After threshold had stabilized, the relation between log photoreceptor threshold and pigment concentration was found to be sigmoidal (Hood et al., 1973; Grabowski and Pak, 1975; Witkovsky et al., 1976; Brin and Ripps, 1977) although Ernst and Kemp (1972) obtained a log-linear relation between 0-80 percent pigment bleached.

Interpretation of these data in mechanistic terms is still lacking, in spite of their mathematical simplicity. Theoretically, reducing pigment concentration by whatever means should raise threshold in proportion to the decreased probability of quantum capture. According to such reasoning, if the incident quantal flux were adjusted to produce a constant rate of photoisomerizations, then response threshold also might be expected to remain constant. This turns out not to be the case: although the probability of quantal absorption sets lower bounds on threshold, it is far from sufficient to account for the threshold elevations encountered experimentally (see Dowling and Ripps, 1970, Fig. 18 for examples).

One may conclude that, even in the photoreceptor, more than one desensitizing mechanism governs threshold, and that the relative influence of such mechanisms will depend on the light history of the cell under study. Accordingly, we have attempted to simplify the experimental situation by maintaining the eye under full dark-adapted conditions both before and during threshold determinations. In the present study we utilize a dietary deprivation regimen followed by administration of vitamin A to set the visual pigment concentration of the photoreceptor to any desired level. Our data indicate that, in *Xenopus* larvae, dark-adapted rod threshold is governed primarily by the probability of quantum capture.

METHODS

Animal Culture

Larval *Xenopus* were obtained from laboratory-maintained adults after the administration of chorionic gonadotrophins as described by Gurdon (1967) and were staged according to Nieuwkoop and Faber (1956). Upon hatching, the tadpoles were placed in constant illumination (white fluorescent light, 85–245 μW $cm^{-2} \cdot s^{-1}$) and were maintained at 20°C in Niu-Twitty solution (Jacobson, 1967) which was changed daily. They were fed a test diet free of vitamin A but supplemented with retinoic acid and 3 mg each of cysteine and taurine per gram of casein (ICN Nutritional Biochemicals Div., Cleveland, Ohio). The combination of light exposure and diet resulted in the loss of >92% of the amount of

visual pigment found in normal tadpoles, which have rod outer segments of comparable dimensions, within a period of 6 wk.

Injection Procedure

Pipettes were pulled from 1-mm OD glass capillary tubing on an Industrial Science Associates, Inc. (Ridgewood, N.Y.) model M1 micropipette puller. The pipette tip was then broken back until mineral oil could be forced through the tip using a hypodermic syringe. After inserting the base of the pipette into polyethylene tubing connected to a mineral oil-filled microinjection device (Stoelting Co., Chicago, Ill.), a solution of retinol in ethanol was drawn into the pipette, followed by a small bubble of air. The bubble migrated up the pipette to the oil-ethanol interface where its length served as an indicator of the hydrostatic pressure being applied. Balancing the hydrostatic pressure of atmospheric pressure prevented loss of injection material through overflow or backflow into the pipette.

Vitamin A-deficient *Xenopus* larvae were immobilized with pieces of wet paper toweling. Under microscopic control, the tip of the micropipette was advanced with a micromanipulator into the musculature at the base of the tail, and 0.1 µl of solution was injected by observing the travel of the bubble-solution interface along the scale of an ocular micrometer.

Ethanol was used as the solvent for vitamin A. Other solvents tested included dimethyl sulfoxide; *N,N*-dimethylformamide; a solution of ethanol: Tween 80 (Atlas Chemical Industries, Inc., Wilmington, Del.): bovine serum albumin in 0.9% NaCl, 1:1:14 (Eakin and Brandenburger, 1968); and liposomes formed from vitamin A and egg lecithin. The first two proved fatal to *Xenopus* larvae and the latter two were not more efficient than ethanol but were more difficult to prepare.

Scintillation Counting Procedure

After eye diameters were measured, *Xenopus* larvae were injected i.m. with tritiated *all-trans*-vitamin A_1 retinol ([^{3}H]Vit A_1, specific activity 2.45–2.66 Ci/mmol, New England Nuclear, Boston, Mass.). The activity of test solutions was checked by counting aliquots in a liquid scintillation counter (LSC). Each injection was of one of the following concentrations: 3.49×10^{-4} M (2.1×10^{13} molecules/0.1 µl), 3.49×10^{-2} M (2.1×10^{15} molecules/0.1 µl), or 3.49×10^{-1} M (2.1×10^{16} molecules/0.1 µl).

At various intervals after injection, eyes were removed from the larvae with the aid of an infrared television viewing system. From each animal, one eye was dissected into retinal and pigment epithelium-choroid fractions, the other eye was used intact. Tissues were placed into glass LSC vials with polyethylene-lined caps. For each sample, the eyes of two animals were pooled. Protosol (New England Nuclear) was added to the vials to solubilize the tissue and, after overnight incubation at 50°C, the vials were cooled, and 10 ml of a counting cocktail (Omnifluor and toluene) was added.

Counting was performed in a scintillation spectrometer (model 2650, Packard Instrument Co., Downers Grove, Ill.), and counting efficiency was determined by the external standard ratio method using a calibration curve for tritium in toluene cocktails.

Visual Pigment Extraction Procedure

Vitamin A-deficient *Xenopus* larvae were injected i.m. with ethanolic solutions of *all-trans*-retinol (Sigma Chemical Co., St. Louis, Mo.) in various concentrations as described above and then placed in the dark at room temperature. The extraction method was similar to that of Bridges et al. (1977). At fixed intervals after injection, 10-80 eyes were removed and placed into McIlvaine's citrate buffer, pH 4.6 (McIlvaine, 1921) in an ice bath. The

eyes were ground in the same buffer in a Potter-Elvehjem glass-Teflon tissue grinder (Markson Science Inc., Del Mar, Calif.), and the resultant suspension was centrifuged at 11,000 rpm for 20 min at 4°C in a Sorvall HB-4 rotor (DuPont Instruments, Wilmington, Del.). After pouring off the supernate and washing the pellet twice with distilled water, the pellet was resuspended in 0.2 ml of a 2% digitonin solution and then stored overnight at 4°C. The suspension was again centrifuged as above and the supernatant extract was withdrawn for spectrophotometric examination. The extract was diluted 50% with borate buffer (pH = 8.5) containing neutral hydroxylamine at a final concentration of 10 mM. Difference spectra were determined using a Zeiss PMQII spectrophotometer (Carl Zeiss, Inc., New York). All procedures were carried out in darkness or dim red illumination. Bleaching was accomplished by a 10-min exposure to a bright microscope illuminator lamp with an interposed water barrier.

Electrophysiological Recording

Eyes were enucleated from dark-adapted animals under red light. The corneas and lenses were removed and a drop of 100 mM sodium aspartate was added to the eyecup. The preparation was allowed to dark-adapt a further 20–60 min in the experimental chamber before an experimental run. The recording system has been described previously (Witkovsky et al., 1973). Briefly, signals were led via an agar-Ringer pipette into a cathode follower (W-P Instruments, Inc., Hamden, Conn.) and then into a rectilinear four-channel oscillographic recorder (model 1400, Hewlett Packard Co., Palo Alto, Calif.) and displayed simultaneously on an oscilloscope. DC recording (bandpass 0-100 Hz) was employed. Light stimuli were provided by a two-channel optical system. 1-s stimuli of diffuse 527 nm light covering the entire retina were used. The irradiance of the system in the plane of the retina was measured with a photometer (Gamma Scientific, Inc., San Diego, Calif.) calibrated against a thermopile (Eppley Laboratory, Inc., Newport, R. I.).

Effective Collecting Area of the Rod

The absolute sensitivity of the underlying rod mechanism may be calculated from the effective collecting area of the rod. Inasmuch as the average diameter of the rod inner segment of the larval *Xenopus* studied was 8 μm, its cross-sectional area was 50.3 μm^2. Light funneling through the inner segment reduces the light available to the outer segment by about 50% (O'Brien, 1951). The mean length of the rod outer segment was 41.8 μm and, assuming a specific absorbance of 0.013 OD U/μm (Liebman, 1972), the axial absorbance, D, was 0.556 at λ = 504 nm, the max of the *Xenopus* rod pigment after administration of *all-trans*-retinol (see Fig. 4). The quantum efficiency of bleaching was taken as 0.67 (Dartnall, 1972). Hence, the effective collecting area was 50.3 × 0.5 × 0.72 × 0.67 = 12.13 μm^2. The coefficient of absorption of the test wavelength, 527 nm, was 0.92; this reduced the effective collecting area to 11.19 μm^2. Our stimulus duration (1 s) was excessive to elicit a maximum voltage. The stimulus duration required to elicit the maximum voltage may be estimated from Cervetto et al. (1977), who showed that it was a linear function of V/V_{max}. When V/V_{max} = 1, time peak = 100 ms; at 0.5 V_{max}, the value used in Fig. 6 B of this paper, the photovoltage achieved its maximum in 200 ms. For a *Xenopus* rod with a full visual pigment complement, the log quanta incident cm^{-2}· s^{-1} to elicit a response of 0.5 V_{max} equals 9.2; thus, the quanta incident μm^{-2}· s^{-1} = 15.8. This value multiplied by the effective collecting area of the rod (11.19 μm^2) gives 177 photoisomerizations s^{-1}, or 35.4 photoisomerizations in 0.2 s. This latter value is very comparable to that of fully dark-adapted amphibian rods studied with intracellular recording (Fain, 1976).

RESULTS

Presence of Free Opsin and Uptake of Vitamin A

It was previously reported (Witkovsky et al., 1976) that long-term maintenance of *Xenopus* on a vitamin A-free diet did not result in the breakdown of the disk structure, although notching of the plasma membrane was seen. This suggests, in turn, that the principal protein of the outer segment, opsin, may be present in approximately normal amounts. To test this hypothesis, the uptake of vitamin A and the formation of visual pigment by the vitamin A-deprived retina was measured.

Vitamin A uptake was assayed by a radioactive tracer, [^{3}H]retinol. Fig. 1
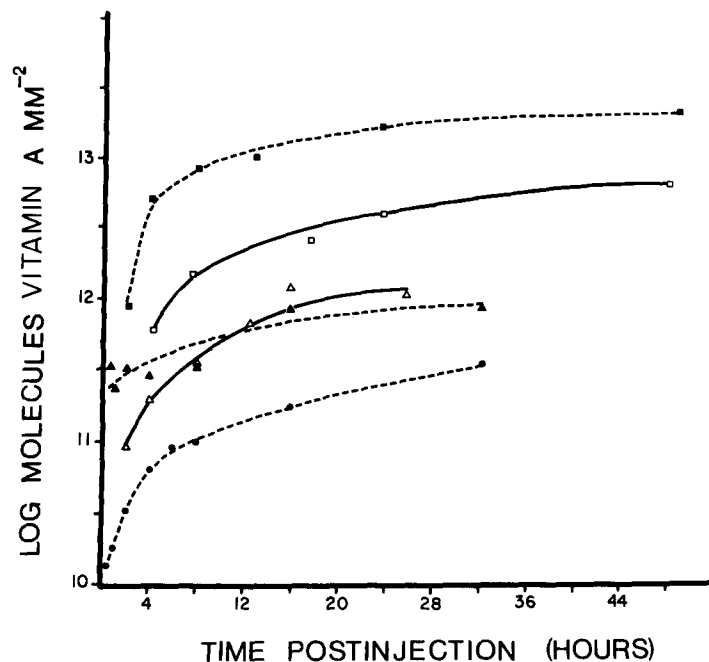

Figure 1. Vitamin A incorporation and visual pigment formation by vitamin A-deficient larval *Xenopus* retinae after i.m. injection of *all-trans*-vitamin A. Symbols denote injection of (●) 2.1 × 10^{13} molecules of vitamin A; (△, ▲) 2.1 × 10^{15} molecules; and (□, ■) 2.1 × 10^{16} molecules. Filled symbols indicate incorporation of [^{3}H]vitamin A$_1$ as determined by scintillation counting. Open symbols indicate formation of visual pigment as determined by extraction in digitonin.

(dashed lines) shows the accumulation of [^{3}H]Vit A$_1$ by the retina, as a function of time, for three different doses injected i.m.: 2.1 × 10^{13}, 2.1 × 10^{15}, 2.1 × 10^{16} molecules. The ordinate takes into account the areas of the retinas employed and the specific activity of the labeled retinol, so that the data are presented as molecules of vitamin A/mm^2. Note that the initial rates of accumulation were rapid for all three doses, but that the final level achieved was a function of dose concentration. The areas of the tested retinas were 1-2 mm^2 so each retina acquired about 0.001 of the injected dose.

A comparison of the rates of vitamin A uptake by the retina and pigment epithelium (Fig. 2) shows that the initial accumulation of label by the pigment epithelium is more rapid than that of the retina. The more rapid uptake by pigment epithelium compared to that of the retina is expected because the vitamin A protein transport scheme operates from blood to pigment epithelium
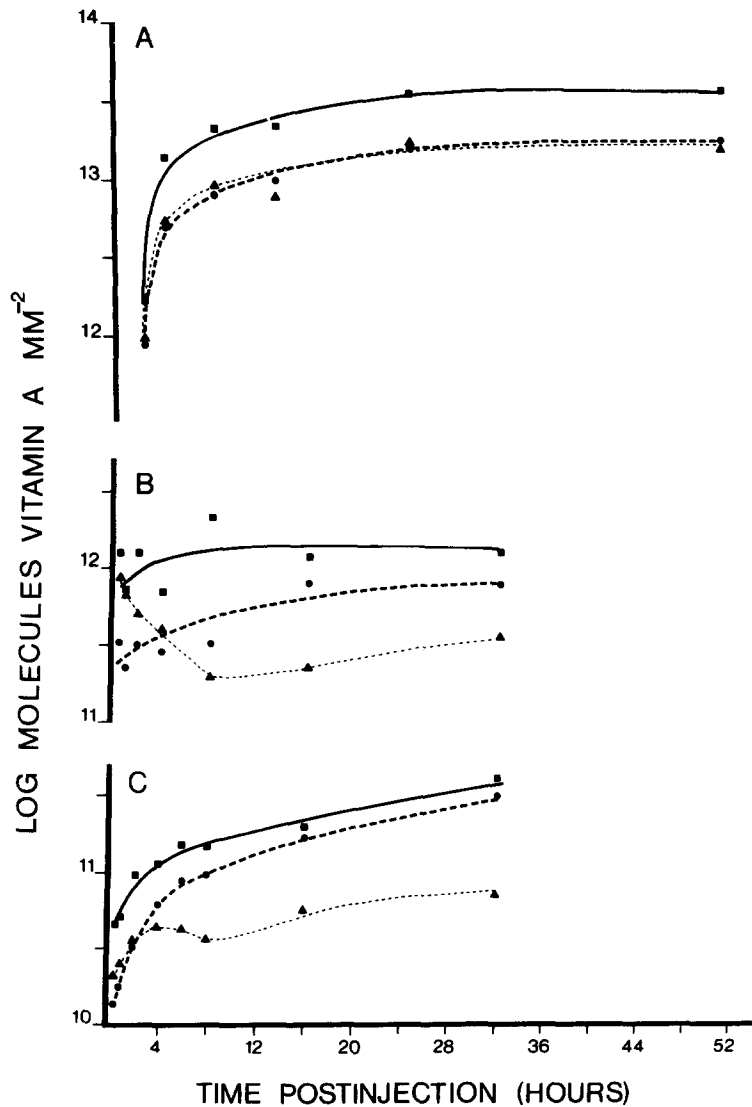

FIGURE 2. [^{3}H]vitamin A_1 incorporation by the (●) retina, (▲) pigment epithelium-choroid complex, and (■) the whole eye of vitamin A-deficient *Xenopus* larvae, as determined by scintillation counting. Injections (i.m.) were of (A) 2.1 × 10^{16} molecules, (B) 2.1 × 10^{15} molecules, and (C) 2.1 × 10^{13} molecules.

to retina. With all three doses, however, the retina ultimately incorporated a greater absolute amount of vitamin A than did the pigment epithelium.

Autoradiography was utilized to assess the distribution of vitamin A within the retina. 24 h after an injection of [^{3}H]vitamin A, the tissue was fixed in buffered glutaraldehyde and treated with borane dimethylamine (Hall and Bok, 1976) to preserve the Schiff's base linkage of vitamin A to protein. Fig. 3 shows dark-field and light-field view of 1-μm-thick plastic sections of a treated retina. The autoradiographic grains over the rod outer segments are very dense, but are sparse elsewhere, indicating that the great majority of labelled vitamin A entered the receptor outer segments.

Photopigment Formation Resulting from Vitamin A Incorporation

Photopigment levels within the rods were assessed in two ways. Densitometric measurements were taken of whole retinas 24 or more h after the injection of retinol. Fig. 4 shows the average difference spectra obtained after a dose of 2.1 $\times$ 10^{14}, 2.1 $\times$ 10^{15}, or 2.1 $\times$ 10^{16} molecules of retinol. Also indicated is the mean difference spectrum of four noninjected animals. It is clear that injected vitamin A is incorporated as photopigment, and that the amount of photopigment synthesized is a function of the amount of retinol administered. The data shown in Fig. 4 are based on a representative experiment in which a solution containing 2.1 $\times$ 10^{16} molecules of retinol per 0.1 μl was diluted serially to obtain the weaker doses. Four eyes were utilized for each dose tested. Comparable experiments were performed on a total of 34 animals.

The other method employed for assaying visual pigment was by extraction in digitonin. For these measurements 10–80 retinas were pooled in different samples. Retinas were harvested at different times ranging from 2 to 96 h after injection. The extraction procedure of Bridges (1974) was employed, using a purified commercial digitonin (Bridges, 1977). Fig. 1 (solid lines) shows the levels of visual pigment obtained at various times after an injection of 2.1 $\times$ 10^{15} or 2.1 $\times$ 10^{16} molecules of *all-trans*-retinol, in relation to the comparable numbers obtained for [^{3}H]vitamin A uptake (Fig. 2).

For the injection of 2.1 $\times$ 10^{15} molecules, there is good agreement between the number of molecules of vitamin A incorporated and the quantity of visual pigment formed. As expected, the rate of pigment synthesis initially is delayed with respect to vitamin A uptake, presumably due to the time taken to convert *all-trans*-retinol to the 11-*cis*-retinal isomer and to combine it with the visual protein opsin.

With respect to the injection of 2.1 $\times$ 10^{16} molecules, more vitamin A is incorporated than pigment formed. Part of this difference is accounted for by the vitamin A storage component of the retina. According to Bridges (1975) *Xenopus* retinas store 0.4 mol of vitamin A per mole of visual pigment. On this basis, for a visual pigment content of 6.4 $\times$ 10^{12} molecules/mm^2 (Fig. 1), the vitamin A store would be ~2.6 $\times$ 10^{12} molecules. Thus, the total number of molecules of vitamin A/mm^2 that would be expected is 9.0 $\times$ 10^{12}. This is still less than the 2 $\times$ 10^{13} molecules/mm^2 found. We cannot explain this discrepancy, but its effects are discussed below.
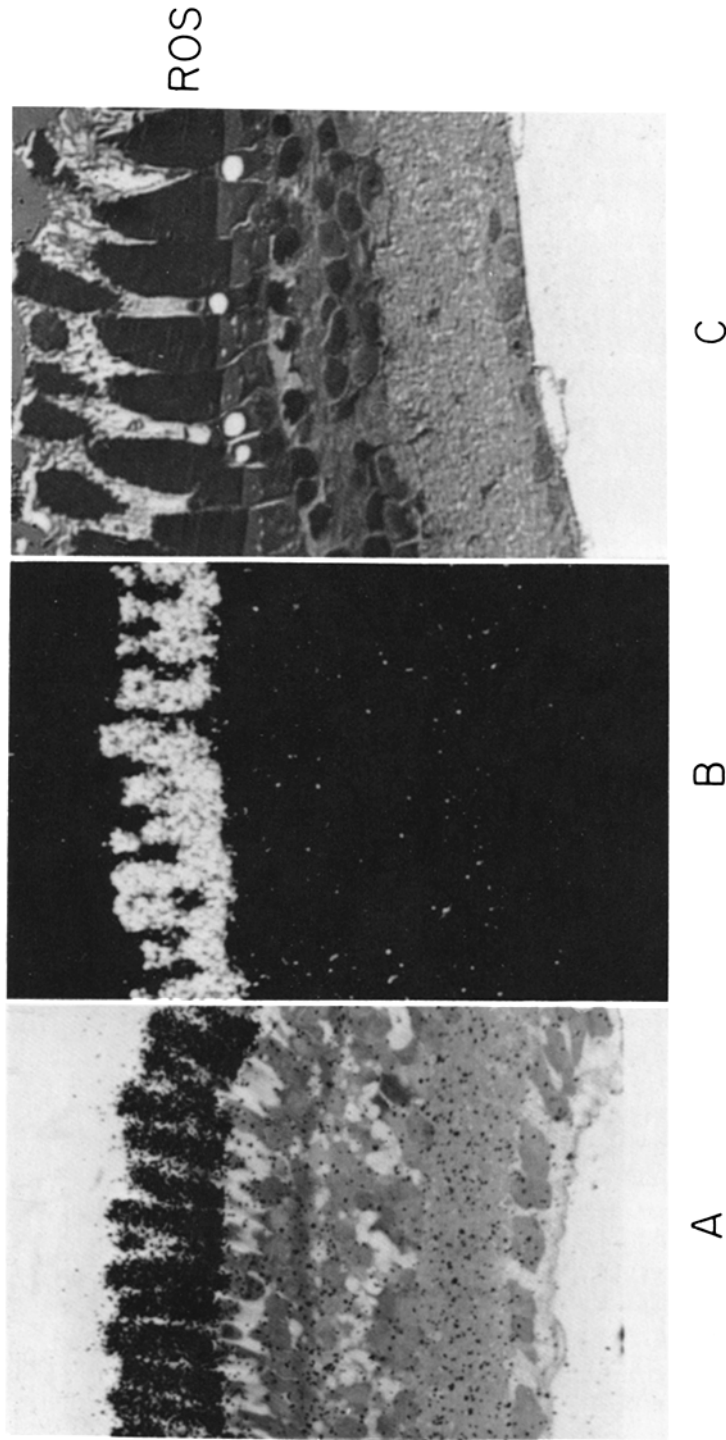
FIGURE 3. Radial sections of larval *Xenopus* retina, magnification = ×670. ROS indicates level of rod outer segments in all three micrographs. (A) Bright-field illumination of an autoradiograph showing heavy labeling over ROS. The vitamin A-deficient tadpole received an injection of 2.1 × 10^15 molecules of [³H]vitamin A and was killed 24 h later. Exposure was 16 wk to Kodak NTB-2 emulsion. (B) Same as A only dark-field illumination. (C) Photomicrograph (Nomarski optics) of a normal larval *Xenopus* retina.

Peak Absorbance of the Visual Pigment

Normally *Xenopus* rods contain a pure vitamin A_2-based visual pigment with λ_{max} = 519 nm at every stage of the animal's life cycle (Bridges et al., 1977). However, the pigment obtained after an injection of retinol, as measured in solution (mean λ_{max} = 504 nm, n = 8) or measured *in situ* (mean λ_{max} = 504 nm, n = 34) peaked at a shorter wavelength, indicating the formation of a vitamin A_1 pigment. Subsequent exposure to a cyclic lighting regimen for a number of days converted the A_1 pigment to the normally encountered 519 nm pigment, as documented in the following paper.
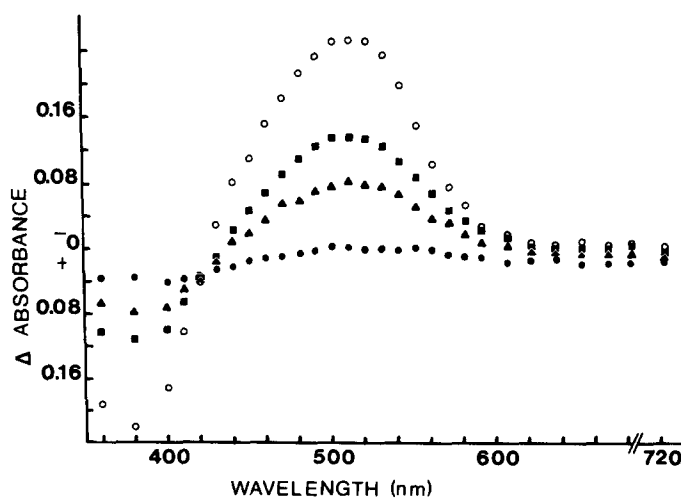

FIGURE 4. Average difference spectra of retinae of vitamin A-deficient larval *Xenopus* 24 h after injection of (▲) 2.1 × 10^{14} molecules, (■) 2.1 × 10^{15} molecules, and (O) 2.1 × 10^{16} molecules of *all-trans*-retinol. (●) Average difference spectrum of noninjected animals. n = 4 for all spectra.

The Quantity of Pigment Contained in a Xenopus Rod

It is of crucial importance to the establishment of a relation between rod threshold and visual pigment level to know just how much pigment a *Xenopus* rod holds. A previous study (Witkovsky et al., 1976) established that normally growing rods add visual pigment in proportion to the number of newly added disks; hence, visual pigment concentration is constant and the amount of visual pigment in the cell is a function of rod outer segment (ROS) dimensions. In the normally fed *Xenopus*, there is a constant relation between the stage of larval development (Nieuwkoop and Faber, 1956) and outer segment size. However, the vitamin A-deficient diet we used induced some changes in somatic development that made the staging of deprived animals difficult. Another problem associated with determining ROS dimensions concerns the tissue shrinkage which occurs during fixation. Shrinkage affects both individual rod dimensions and rod packing density.

To provide the needed data we made new measures of ROS dimensions in retinas from vitamin A-depleted animals. Retinas were removed from eyes and the posterior pole was gently brushed in a drop of *Xenopus* Ringer solution on a glass slide. The drop was then examined by phase or Nomarski microscopy, and the length and diameter of ROS's was determined by reference to a calibrated reticle. All measurements were taken within 5-10 min after isolation. After 15-20 min, some alteration of the outer segment was evident as was previously noted for frog rods by Korenbrot and Cone (1972). We found that for eyes which ranged in diameter between 0.75 and 1.6 mm, ROS size was reasonably constant (see Table I A), whereas larger or smaller eyes had rods of different dimensions. All of our experiments, therefore, utilized larval eyes in the diameter range of 0.75-1.6 mm.

To determine the number of rods per unit area, isolated retinas, receptor side up, were scanned with a microscope containing an ocular micrometer. In areas where rods were well aligned, a mean density of 7,000 rods/mm² was found.

We then compared the dimensions of a freshly dissected eye with those of its companion eye after fixation. Fixation induced a 35% reduction in eye diameter, whereas individual rods decreased in length by no more than 10% but increased in diameter by 10-15%. Thus, the biggest change induced by fixation is in rod packing; presumably this finding accounts for the rod densities of 12,000-12,500/mm² of fixed tissue reported by Saxen (1954).

Table I B shows that the amount of visual pigment extracted per square millimeter of retinal tissue agrees well with the estimate derived from rod outer segment dimensions and packing density. The good agreement between the estimated and measured amounts of vitamin A and visual pigment per square millimeter of retina, lends weight to the supposition that the average dimensions obtained from the ROS's are reasonably accurate. On this basis 6.4×10^{12} molecules of pigment/mm² can be taken as the 100% visual pigment level, and a mean axial absorbance of 0.56 is taken for subsequent calculations in relation to rod threshold (see Methods).

Rod Threshold

Rod responses were measured electroretinographically in eyecup preparations after treatment with aspartate to isolate the PIII (photoreceptoral) component of the response (Sillman et al., 1969; Witkovsky et al., 1973). The relative visual pigment concentration of all retinas from vitamin A-injected animals was estimated from the solid curves shown in Fig. 1. For example, an eye harvested 12 h after an injection of 2.1×10^{16} molecules of vitamin A could be expected to contain 2.2×10^{12} molecules of visual pigment/mm² retina (Fig. 1, upper solid curve). By reference to the fully filled value of 6.4×10^{12} molecules of pigment/mm², this amounts to 34.4%.

Each eye tested was stimulated with flashes of 527 nm light at a number of intensities over a 3-4-log-unit range from below absolute dark-threshold to rod saturation. The photovoltages so obtained were plotted as percent of the response to a saturating flash (V_{max}) against the log quantal flux of the stimulus. Fig. 5 shows that when such curves are equated by normalizing the quantal flux

to elicit 50% V_{max}, the pooled data conform reasonably well to the equation

$$V/V_{max} = \frac{I^n}{I^n + \sigma^n},$$ (1)

where $n = 0.8$. The two groups of data points in Fig. 5 refer to eyes containing <30% total photopigment concentration (○, $n = 29$) or from 30 to 100% photopigment (●, $n = 16$). The separation of these two sets of data along the scale of abscissae is arbitrary. Although absolute values of V_{max} from 15 to 450 μV were found in different preparations, V_{max} showed no tendency to vary as a function of photopigment concentration. This observation, together with the data of Fig. 5, indicate that the principal effect of an alteration in photopigment

TABLE I

PHOTORECEPTOR AND PHOTOPIGMENT DATA FOR *Xenopus* LARVAE

Eye diameter	(A) X ROS length at posterior pole	X ROS diameter at posterior pole
mm	*μm*	*μm*
0.756	43.4	6.8
0.851	38.1	6.7
0.914	37.7	6.4
0.945	34.8	6.5
1.103	39.7	6.9
1.418	50.1	8.0
1.449	46.5	9.0
1.481	49.4	8.2
1.607	46.4	6.3
	X̄ = 42.8	7.2

		(B)		
X ROS volume*	No. of molecules of visual pigment/μm^3‡	No. of molecules of visual pigment/ROS*	No. of molecules of visual pigment/mm^2 retina, calculated*§	No. of molecules of visual pigment/mm^2 retina, extracted
μm^3				
1.39×10^3	1.2–1.5×10^6	1.67–2.09×10^9	1.17–1.42×10^{13}	6.4×10^{12}

* Values given takes into account the fall in ROS dimensions as one moves from the posterior pole to the periphery of the eye.
‡ Liebman, 1972.
§ 7,000 rods/mm^2.

concentration upon the voltage-intensity relation is to change the value of σ in Eq. 1.

Fig. 6 A and B shows the results of plotting the quantal flux to elicit a criterion rod response, against the percentage of visual pigment, i.e., the fraction of photopigment content of fully filled rods of comparable dimensions. Each data point represents a different animal. In Fig. 6 A, the criterion used was a 5-μV response, in Fig. 6 B, 50% V_{max} was selected. Note, however, that any other fraction of V_{max} would have yielded the same function (see Fig. 5). Spectral sensitivity curves obtained from both uninjected animals and those with full pigment complement showed that the rod mechanism alone governed the

response, as found previously (Witkovsky et al., 1976).

The curve indicated by the heavy dashed lines in Fig. 6 A and B is a function relating threshold and visual pigment content which would apply if receptoral thresholds were governed solely by the probability of quantal absorption, with the maximum axial absorbance (i.e., 100%) of the visual pigment taken to be 0.56. It can be represented by the expression:

$$\frac{I_t}{I_o} = \frac{(1 - e^{-2.30D})}{(1 - e^{-2.3Df})},$$

(2)

(cf. Barnes and Goldsmith, 1977)

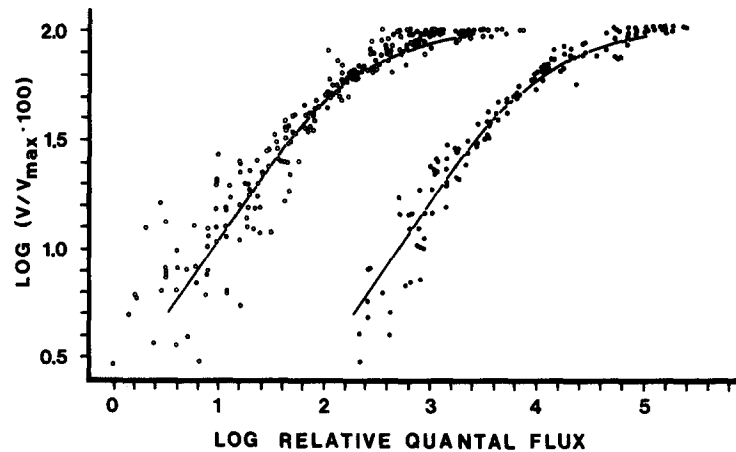where I_o is the dark-adapted threshold of the receptor with its normal comple-

FIGURE 5. Voltage-intensity data for the aspartate-isolated PIII component of the ERG after administration of vitamin A. Responses from each eye were normalized in terms of V_{max}. Responses from different eyes were equated at the intensity which elicited 50% V_{max}. (O) Eyes which contained <30% full photopigment concentration (n = 29); (●) eyes containing >30% photopigment (n = 16). The solid curve reproduces the equation:

$$\frac{V}{V_{max}} = \frac{I^n}{I^n + \sigma^n}$$

where n = 0.8. The position of the curves along the scale of abscissae is arbitrary.

ment of visual pigment; I_t, that of the partially depleted receptor; D, the axial absorbance of the filled receptor; and f, the fraction of pigment in the partially depleted receptor. With due allowance for experimental variability (considered below), there is reasonably good agreement between the experimental results and the arbitrarily positioned curve obtained from Eq. 2. Little sensitivity change results from a loss of up to 80% of the visual pigment, but a further loss causes marked changes. The fit of the data to the theoretical curve (Eq. 2) permits the conclusion that the probability of quantal absorption is the major
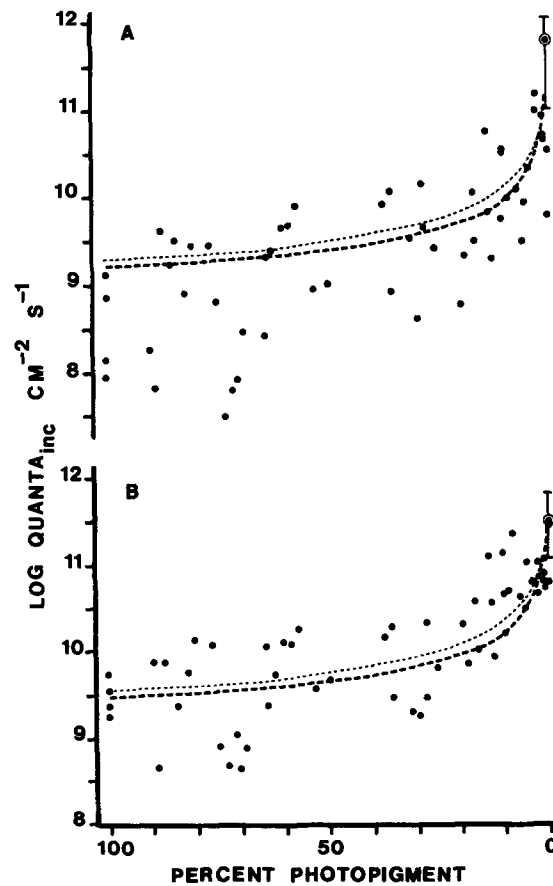
FIGURE 6. (A) The relationship between the quantal flux required to elicit a criterion rod response (5 μV) and photopigment concentration of the vitamin A-deficient larval *Xenopus* eye. Percentage of photopigment was calculated from the photopigment formation curves in Fig. 1. (●) Each symbol represents a different eye. The bull's-eye (◎) and vertical lines represent the mean ± 1 SD of 23 eyes from noninjected animals. The heavy dashed line is the function describing the relation between photopigment content and quantum capture by a receptor with axial absorbance of 0.56 (Eq. 2). The light dashed line is the same function with the range 0–75% photopigment content expanded to fill the scale of abscissae (see text). (B) The same data as in A, except that the criterion rod response was taken at 50% V_{max} rather than 5 μV.

factor governing photoreceptor threshold, with the experimental protocol employed.

Consideration of Experimental Error

Bridges et al. (1977) determined that an eye of a stage 58 *Xenopus* tadpole contained 234.5 pmol of visual pigment. In fixed tissue the retinal area of such

an eye was 4.89 mm^2 and the mean ROS length was 48.4 μm (Witkovsky et al., 1976). Taking into account the changes in tissue dimensions caused by histological processing described above, we calculated that the stage 58 retina in vivo contained 1.2 × 10^{13} molecules of visual pigment/mm^2. After correcting for differences in mean outer segment lengths between the animal used in the present study and a normal stage 58, we estimated that the total visual pigment complement of the experimental retina should be 8.5 × 10^{12} molecules/mm^2 of retina, whereas 6.4 × 10^{12} molecules/mm^2, or 75% of the expected total, was found. Three possible explanations, not mutually exclusive, could account for the discrepancy. The lower number may truly reflect the total amount of pigment which the deficient rods hold, it may result from losses during the extraction procedure, or it may indicate that the injection of 2.1 × 10^{16} molecules resulted in ROS's being filled only to 75% of their visual pigment capacity.

If the first explanation is true, our data and conclusions stand as presented. In the second case, consistent failure to extract all of the visual pigment would have no effect on our conclusions because we are relating the percentage of visual pigment to threshold. Thus, if we assume that our extraction procedure captured a constant percentage of the total visual pigment in each sample, the data points of Fig. 5 would be unaltered.

Suppose, however, that the rods were filled with visual pigment to only 75% of capacity. In that case, to represent the proper relationship, the probability function as drawn in Fig. 5 would have to be adjusted to span the entire abscissa, so that the point labeled 100% would equal 75%. This adjusted function is shown as the light dashed lines in Fig. 6 A and B. The difference between the two lines as concerns goodness of fit to the data points is small and does not alter the main conclusion that the probability function explains the shape of the data point distribution.

The substantial scatter in the data points may be attributed to several factors. Undoubtedly the variation in V_{max} already alluded to contributed to data point dispersion in Fig. 6 A. In fact, by using a 50% V_{max} criterion (Fig. 6 B) the degree of scatter was reduced. Other factors contributing to scatter are variations in the dimensions of rod outer segments (see Table I) and a possible contribution of other waveforms, particularly slow PIII (Witkovsky et al., 1975) to the response amplitude. Preliminary measurements upon larval *Xenopus* eyes show that the slow PIII voltage increased with the product of stimulus intensity and duration; with a 1-s flash at 50% V_{max}, it can contribute up to one-third of the cornea-negative photovoltage. Inasmuch as all our measurements utilized eyecup preparations, slow PIII interacted subtractively with the cornea-positive c-wave whose own amplitude and onset varied from preparation to preparation.

DISCUSSION

Relation of Visual Pigment Content to Photoreceptor Threshold

The central problem of this study is the relation of visual pigment concentration to photoreceptor threshold. We have shown that, when *Xenopus* larvae are raised in constant illumination on a vitamin A-free diet, their rod outer

segments continue to grow in spite of a 90–100% deficit in visual pigment chromophore. The vitamin A-deficient rods retain the ability to incorporate rapidly, systemically administered vitamin A as visual pigment, in normal amounts. Therefore, even after prolonged deprivation, the rod retains its full complement of opsin. In this respect, *Xenopus* differs markedly from the rat in which a loss of chromophore leads to rapid destruction of the rod outer segment (Dowling and Wald, 1960).

With respect to photoreceptor threshold we observed that a substantial loss of visual pigment produced only a small rise in the quantal flux required to elicit a criterion response. Our data are consistent with the conclusion that the lowered probability of quantam capture alone determined the rise in photoreceptor threshold associated with a given degree of photopigment loss, although the presence of a small additional desensitizing factor cannot be excluded, because of the scatter in the data. As a corollary, we conclude also that free opsin per se had no desensitizing effect on the photoreceptor, a role for free opsin which was suggested by Rushton (1965).

Thus, the results obtained in the present study from vitamin A-deprivation parallel those found to hold for the growing photoreceptor, where the fall in photoreceptor threshold could be attributed solely to the heightened probability of quantal capture concomitant with an increased axial absorbance of the rod (Witkovsky et al., 1976). In both these studies, the experimental retinas were completely dark-adapted before and during the experiment. It is interesting to note that under comparable experimental conditions, similar results were obtained in an investigation of human patients afflicted with retinitis pigmentosa. In that study a probability function was found to relate the psychophysically determined absolute visual threshold with the quantity of rhodopsin present in peripheral retina (Ripps et al., 1978).

The Effects of Bleaching upon Visual Threshold

Bleaching flashes or steady adapting fields are known to induce large changes in threshold which exceed those expected on probability grounds alone (Dowling and Ripps, 1972; Ernst and Kemp, 1972; Hood et al., 1973; Grabowski and Pak, 1975; Witkovsky et al., 1976; Brin and Ripps, 1977). The factors which govern such large threshold changes are unknown. Two studies (Grabowski and Pak, 1975; Brin and Ripps, 1977) have concluded that the time-course of threshold recovery did not closely parallel the rise or fall of late-bleaching intermediates. Recovery of visual pigment through regeneration is accompanied by a parallel fall in threshold: the log-linear relation mentioned earlier (Dowling and Wald, 1958, 1960; Rushton, 1961). But even when little or no regeneration occurs, there can be a substantial post-bleach recovery of photoreceptor threshold (Grabowski and Pak, 1975).

Recent work has uncovered a sequence of enzymatic processes in the photoreceptor outer segment, whose net activity is modified when visual pigment captures light (Farber, 1977). Future study may reveal that one or more of the associated intermediates plays a role in governing photoreceptor threshold (Kuhn et al., 1977).

Relation of Present Data to Earlier Results

In a previous study, Witkovsky et al. (1976) concluded that dietary deprivation of vitamin A raised threshold more than expected on probability grounds, a conclusion at variance with the results of the present study. Two factors may explain this difference. First, in the earlier work, *Xenopus* larvae were deprived of vitamin A for longer periods and were not subjected to constant light. Only after several months of deprivation was a substantial rise in photoreceptor threshold seen. Study with the electron microscope of those retinas showed that although outer segment disk membranes generally were intact, some membrane whorls and a pronounced notching of the plasma membrane were observed. Secondly, the densitometric method used to assess the pigment content was subject to uncertainty because of the possible loss of outer segment material during dissection, the variable orientation of the patch of receptors in the light path of the densitometer, and the possible error in estimating the fraction of the retinal surface subtended by rods. We believe that, because the fraction of visual pigment has been determined directly in the present study, the relation between pigment concentration and receptor threshold reported here is more firmly based.

Relation of Visual Pigment Content to Photoreceptor Threshold in Invertebrate Eyes

Several recent studies on invertebrate eyes also have led to the conclusion that receptor sensitivity is related linearly to the probability of quantal capture (Hamdorf and Schwemer, 1975; Razmjoo and Hamdorf, 1976; Barnes and Goldsmith, 1977; Harris et al., 1977). It is important to note that, in invertebrates, quantal capture by photopigment does not lead to a complete separation of chromophore and protein; thus, bleaching does not produce "free" opsin (Dartnall, 1962). In the *Drosophila* eye, vitamin A deprivation leads to pigment loss, yet after a 70-fold fall in pigment content, photoreceptor threshold was raised only 80-fold (1.9 log units), i.e., about what would be expected from decreased probability of photon capture (Harris et al., 1977). In the lobster eye, having determined previously the time-course of photopigment regeneration (Bruno et al., 1977), Barnes and Goldsmith (1977) examined the time-course of threshold recovery after bleaching flashes. They found that a significant portion of the rhodopsin could be bleached with only a small change in threshold resulting. Their data were explained on the assumption that "the concentration of visual pigment regulates sensitivity according to the probability of quantum catch" (Barnes and Goldsmith, 1977). Our results indicate that the direct relationship between sensitivity and quantum capture is not limited to the eyes of invertebrates.

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