

# Spectral Tuning in Salamander Visual Pigments Studied with Dihydroretinal Chromophores

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**ABSTRACT** In visual pigments, opsin proteins regulate the spectral absorption of a retinal chromophore by mechanisms that change the energy level of the excited electronic state relative to the ground state. We have studied these mechanisms by using photocurrent recording to measure the spectral sensitivities of individual red rods and red (long-wavelength-sensitive) and blue (short-wavelength-sensitive) cones of salamander before and after replacing the native 3-dehydro 11-*cis* retinal chromophore with retinal analogs: 11-*cis* retinal, 3-dehydro 9-*cis* retinal, 9-*cis* retinal, and 5,6-dihydro 9-*cis* retinal. The protonated Schiff's bases of analogs with unsaturated bonds in the ring had broader spectra than the same chromophores bound to opsins. Saturation of the bonds in the ring reduced the spectral bandwidths of the protonated Schiff's bases and the opsin-bound chromophores and made them similar to each other. This indicates that torsion of the ring produces spectral broadening and that torsion is limited by opsin. Saturating the 5,6 double bond in retinal reduced the perturbation of the chromophore by opsin in red and in blue cones but not in red rods. Thus an interaction between opsin and the chromophoric ring shifts the spectral maxima of the red and blue cone pigments, but not that of the red rod pigment.

## INTRODUCTION

Vertebrate visual pigments consist of a chromophore, 11-*cis* retinal or 3-dehydro, 11-*cis* retinal, bound to a pigment-specific opsin protein by a protonated Schiff's base linkage. The wavelength of maximum absorption ( $\lambda_{\max}$ ) for the 11-*cis* retinal protonated Schiff's base in methanolic solution is 440 nm, whereas that for visual pigments that use the same chromophore varies between ~350 and 570 nm. By perturbing retinal's  $\pi$  electrons, opsin changes the energy requirement for electronic excitation, shifting retinal's absorption to a specific spectral region. The opsin-induced perturbations underlying this spectral diversity are not completely understood because thus far, only a few visual pigments have been well characterized.

One approach that has been applied successfully to bovine rhodopsin, chicken red cone pigment, and the pigments of a salt-loving bacterium, *Halobacterium halobium* (reviewed by Nakanishi, 1991; Nakanishi and Crouch, 1995), is to observe the spectral changes that occur after substituting retinal analogs for the native chromophore. In the present study, dihydroretinal analog chromophores (Fig. 1) were used to determine how saturating particular double bonds in the chromophore affected the spectrum and opsin shift (OS) of salamander red rod and red and blue cone visual pigments. The OS, defined as the wavenumber dif-

ference between the peak absorption of the protonated Schiff's base of the retinal analog in methanolic solution (PSB) and the peak absorption of the pigment, measures the extent to which the protein perturbs the chromophore (Nakanishi et al., 1980; Motto et al., 1980).

The study of blue cone pigments has been problematic because excess chromophore and photoproduct spectra significantly overlap the pigment's absorption. However, dihydroretinal analog pigments have been shown to support phototransduction in vitro, whereas their photoproducts do not (Fukada et al., 1982; Yoshizawa and Fukada, 1983; Calhoun and Rando, 1985). It was therefore possible to circumvent the difficulties imposed by spectral overlap by incorporating the retinal analogs into the visual pigments of isolated salamander photoreceptors and measuring the spectral sensitivity or action spectrum for the electrical response. The spectral sensitivity provided a direct index of spectral absorbance because the quantum efficiency of excitation was largely wavelength-independent (e.g., Cornwall et al., 1984) and the pathlength was short. Some of the results have appeared in a preliminary report (Makino et al., 1993).

## MATERIALS AND METHODS

For simplicity, 3-dehydroretinal will be denoted as  $A_2$ , retinal will be denoted as  $A_1$ , and 5,6-dihydroretinal will be denoted as  $A_0$ . A 9 or an 11 preceding the analog type will distinguish 9-*cis* and 11-*cis* isomers, respectively (Fig. 1).  $A_0$  retinal was synthesized by reducing the  $\alpha$ - $\beta$  unsaturated bond of  $\beta$ -cyclocitral with Pd/C/H<sub>2</sub>. The major product, 5,6-dihydro  $\beta$ -cyclocitral, was isomerized to *trans*-5,6-dihydro  $\beta$ -cyclocitral by KOH in methanol and elongated to retinal by standard techniques (Blatz et al., 1968a; Arnaboldi et al., 1979). Initially,  $A_2$  was prepared by brominating all-*trans*  $A_1$  followed by debromination, as described by Henbest et al. (1955). In later experiments,  $A_2$  was synthesized by a novel method to attain a higher yield (Groesbeek, 1993). 1,5,5-Trimethyl-6-cyano-cyclohexene was obtained from acid cyclization of 3,7-dimethyl-2,6-octa-

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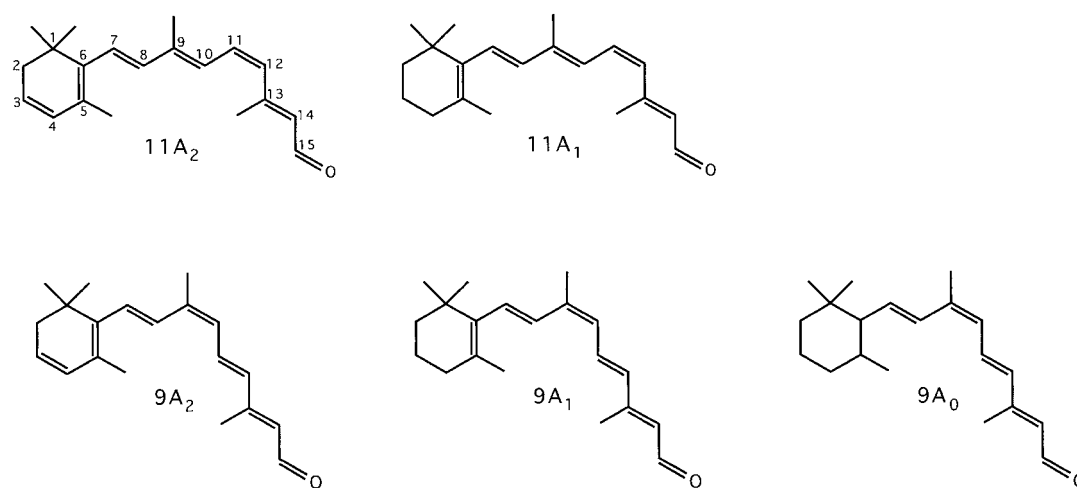


FIGURE 1 Retinal analogs.

dienitrile, treated with  $\text{Br}_2$ , and then heated with  $\text{LiF}$  and  $\text{Li}_2\text{CO}_3$  in hexamethylphosphoric triamide to give 2,6,6-trimethyl-1,3-cyclohexadiene carbonitrile. This was converted to 3,4-didehydro- $\beta$ -cyclocitral with diisobutyl aluminum hydride. Chain elongation was carried out with 4-diethylphosphono-3-methyl-butene-2-nitrile, followed by diisobutyl aluminum hydride reduction to produce 3,4-didehydro- $\beta$ -ionylidene acetaldehyde. Repetition of this sequence generated a mixture of  $\text{A}_2$  isomers. The all-*trans* conformer was isolated by column chromatography. 9-*Cis* isomers of each analog were obtained by high-performance liquid chromatography separation of irradiated all-*trans* retinals. Analogs were characterized by NMR and by spectroscopy in *n*-hexane and in methanol.

PSBs of retinal analogs were prepared by adding an excess of *n*-butylamine to the analog in anhydrous methanol. Excess *n*-butylamine was evaporated along with the methanol, and the Schiff's base was resolubilized in methanol. Protonated methanol was then added until the absorption spectrum shifted to the red and remained unchanged. Spectra were recorded on a Varian DMS-200, a Philips PU8700, or a Beckman DU640 spectrophotometer. To achieve better resolution at long wavelengths, spectral measurements were also made of concentrated solutions of some analogs.

Lipid vesicles were prepared by the method of Jones et al. (1989). Fifty microliters of L- $\alpha$ -phosphatidylcholine in chloroform (Sigma) was dried with nitrogen and suspended in 500  $\mu\text{l}$  Ringer's. The suspension was vortexed and then sonicated on ice. Under dim red light, retinal analog in pentane or hexane was removed from a stock solution stored at  $-15^\circ\text{C}$  and dried with nitrogen. Lipid vesicles were added to give a final concentration of  $\sim 0.5$ –10 mM for 11-*cis* retinal. Concentrations of the other analogs were not known. The mixture was agitated gently overnight at  $5^\circ\text{C}$  and then kept on ice until ready for use.

Measurement of spectral sensitivity in isolated photoreceptors and replacement of native chromophore with a retinal analog were carried out according to the methods described by Makino et al. (1990). A larval tiger salamander, *Ambystoma tigrinum*, obtained from Charles Sullivan (Nashville, TN) or Carl Lowrance (Tulsa, OK) was dark-adapted for a minimum of 2 h and decapitated, the braincase and vertebral column were pithed, and the eyes removed under dim red light or under infrared light. Retinas were isolated in Ringer's solution under infrared illumination and refrigerated until use. Ringer's contained (mM) 115 NaCl, 2.5 KCl, 1.0  $\text{MgCl}_2$ , 3.0 HEPES, 1.5  $\text{CaCl}_2$ , 0.02 EDTA, and 10 *D*-glucose (pH 7.6). In some experiments, the HEPES and NaCl concentrations were 10 mM and 108 mM, respectively.

At intervals, a small piece of retina was mechanically disrupted and placed in an experimental chamber mounted on the stage of an inverted microscope. An isolated photoreceptor was located using a closed-circuit television system whose infrared-sensitive camera was attached to the microscope. The inner segment was drawn into a glass suction electrode,

and the photocurrents were recorded. During recordings, the chamber was perfused continuously with Ringer's solution.

A cell's spectral sensitivity was determined from the reciprocal flash photon densities required to evoke electrical responses of constant amplitude (see Naka and Rushton, 1966; Baylor et al., 1984). Brief narrow-band flashes were obtained by passing light from a tungsten-iodide or xenon arc lamp through an electronically controlled shutter and an interference filter whose nominal full width at half-height was 10 nm. Flash strength was controlled by a series of neutral density filters. Responses at a reference wavelength, usually 500 nm, were measured frequently to prevent errors due to changes in the condition of the cell.

After a cell's spectral sensitivity was measured, the cell was exposed to very intense light, which bleached its pigment by isomerizing the native chromophore and allowing it to dissociate from opsin. Rods and red cones were bleached for a minimum of 5 min with long-wavelength light (tungsten-halogen source with long-wavelength pass filters with half-maximal transmission at 549, 570, or 610 nm; Oriel, Stratford, CT). Blue cones were bleached with white light or light through Schott (Duryea, PA) long-pass filters (GG400, GG455, or GG475), which had half-maximal transmissions at 404, 457, and 481 nm, respectively. Depending upon the filter used, the intensity of the bleaching light ranged from  $\sim 30$  to  $500 \mu\text{W cm}^{-2}$  in the band up to  $\sim 850$  nm. In some experiments, the cells were superfused with bovine serum albumin ( $1.0 \text{ mg ml}^{-1}$ ; Boehringer Mannheim) in an attempt to promote the removal of photoisomerized, dissociated native chromophore. Exogenous analog chromophore in lipid vesicles was then superfused onto the exposed outer segment to regenerate analog pigment. Because each of the dihydroretinal analogs blueshifted the pigment's absorption, the native pigment was further reduced in some experiments by an additional, partial bleach with long-wavelength light. Analog pigment formation sometimes improved after a second or third superfusion with exogenous chromophore. Red cones were superfused for 8–48 min, blue cones for 18–67 min, and rods for 10–112 min. These durations represent the total superfusion times for several experiments in which bleaching and regeneration were carried out repeatedly.

For each cell type, the native pigment spectrum was obtained by shifting individual spectra vertically to minimize variation at long wavelengths, deleting points of very high sensitivity near the peak, and then fitting the remaining points with visual pigment nomograms (Ebrey and Honig, 1977; Dawis, 1981) over the following wavelength ranges: red cone, 541–721 nm; rod, 440–621 nm; blue cone, 398–520 nm. Individual analog pigment spectra of a given cell type were adjusted vertically on a log ordinate scale to minimize variation near their peaks. Divergent, long-wavelength points with high sensitivity (presumably reflecting residual native pigment) were deleted. Collected spectra were then fitted with functions described below. The error in measuring  $\log S(\lambda)$  was largely independent of  $\lambda$ , so curve fits to  $S(\lambda)$  were weighted by  $1/S(\lambda)$  except where noted. Because there were

large numbers of coincident points, most of the figures show averaged spectra instead of the collected results for clarity. Spectra were plotted on a linear wavenumber axis.

## RESULTS

### Sensitivity of cells before and after chromophore replacement

A total of 59 red cones, 17 blue cones, and 21 rods were studied. Salamanders contain two spectral classes of rods (Harosi, 1975), but only the more common "red rods" were examined. Maximum photocurrents ranged from 2 to 19 pA in red cones, 3 to 9 pA in blue cones, and 9 to 45 pA in rods. Generally the relation between the peak amplitude of the flash response,  $r$ , and the flash strength,  $i$ , was well fitted by the Michaelis relation:

$$r/r_{\max} = i/(i + i_0) \quad (1)$$

where  $r_{\max}$  is the maximum response amplitude and  $i_0$  is the flash strength giving  $r = 0.5r_{\max}$ . For salamanders dark-adapted more than 16 h,  $i_0$  at the wavelength of peak sensitivity was  $1630 \pm 1040$  photons  $\cdot \mu\text{m}^{-2}$  (mean  $\pm$  SD) in 16 red cones,  $240 \pm 120$  photons  $\cdot \mu\text{m}^{-2}$  in 12 blue cones, and  $5 \pm 2$  photons  $\cdot \mu\text{m}^{-2}$  in 16 rods. One factor contributing to the variability in  $i_0$  was the sizable variation in outer segment dimensions, which for the cells studied was greatest in red cones. The pigment in the red cones may also have been bleached somewhat by the dim red light or the infrared light used during dissection.

Bleaching exposures reduced the flash sensitivity by more than four log units in cones and by more than eight log units in rods. The sensitivity recovered rapidly upon superfusion with 11A<sub>1</sub>. Assuming extinction coefficients of 42,000 and 30,000 l  $\cdot$  mol<sup>-1</sup>  $\cdot$  cm<sup>-1</sup> for 11A<sub>1</sub> and 11A<sub>2</sub> pigments, respectively, the flash sensitivity should have exceeded the dark-adapted value by 1.4-fold after complete replacement of 11A<sub>2</sub> by 11A<sub>1</sub>. After superfusion with 11A<sub>1</sub> for 11–45 min, the mean flash sensitivity of bleached cones was 0.8–1.4 times the dark-adapted values, indicating that a large proportion of the pigment had regenerated (Table 1). The regeneration times mentioned are nominal; despite continuous perfusion of the cell with Ringer's to wash out

excess retinal, the flash sensitivity continued to increase for many minutes after termination of analog delivery.

Bleached photoreceptors also recovered sensitivity after perfusion with the 9-*cis* retinal analogs. The quantum efficiency of photoisomerization for 9A<sub>1</sub> rod pigment is approximately half that for 11A<sub>1</sub> pigment (Kropf and Hubbard, 1958), so a slightly lower recovery of sensitivity was expected. Recovery was poorest with 9A<sub>2</sub>, as if this chromophore recombined with opsin less readily. Across cell types, recovery of flash sensitivity was greater in cones than in rods (Table 1). This was probably due to the more powerful desensitization caused by unregenerated pigment in rods (Jones et al., 1993), which would have been especially pronounced in the presence of high concentrations of all-*trans* retinal, retinol, and retinal analogs (reviewed in Crouch et al., 1996; see also Kefalov et al., 1999). Regardless, 11-*cis* retinal and all of the 9-*cis* retinal analogs blue-shifted the spectral sensitivity of every cell type (Figs. 2 and 5), signifying that a functional visual pigment had formed in each case.

### Analysis of spectra

As reported previously, native larval salamander visual pigments contained a chromophore mixture. 11A<sub>2</sub> was most prevalent, but in some cases there was a substantial amount of 11A<sub>1</sub> (Makino and Dodd, 1996). Assuming that at least some of the cells of each type contained pure 11A<sub>2</sub> chromophore, spectral maxima ( $\lambda_{\max}$ ) were found from visual pigment nomograms (Ebrey and Honig, 1977; Dawis, 1981) for native 11A<sub>2</sub> and regenerated 11A<sub>1</sub> pigments (Fig. 2). The nomograms only apply over a limited wavelength range, so an alternative, empirical relation for 11A<sub>1</sub> pigments was also tested (Lamb, 1995):

$$S(\lambda) = (\exp(a(A - \lambda_{\max}/\lambda)) + \exp(b(B - \lambda_{\max}/\lambda)) + \exp(c(C - \lambda_{\max}/\lambda)) + D)^{-1} \quad (2)$$

where  $a = 70$ ,  $b = 28.5$ ,  $c = -14.1$ ,  $A = 0.880$ ,  $B = 0.924$ ,  $C = 1.104$ ,  $D = 0.655$ ,  $\lambda$  is wavelength, and  $\lambda_{\max}$  is the parameter of the fit. This expression is based on the premise that all spectra assume a common shape when plotted on a log wavenumber scale (Mansfield, 1985; MacNichol, 1986; Baylor et al. 1987). Overall, there was fair agreement between salamander 11A<sub>1</sub> spectra and Eq. 2 (Fig. 3 A). However, red and blue cone spectra deviated slightly on the long-wavelength (small wavenumber) side of the peak. Maxima obtained from Eq. 2 for red cones and rods were similar to the values given by the nomograms, but the maximum for the blue cones was considerably different. Residual 11A<sub>2</sub> chromophore in the red and blue cones would have caused a more gradual decline of the long-wavelength side of the spectrum, just the opposite of what was observed.

An expression was found for the rod 11A<sub>2</sub> spectral sensitivity, where the absorption spectra from extracts of seven different fish with 11A<sub>2</sub> rod pigment (Bridges, 1967) were

**TABLE 1** Recovery of flash sensitivity after pigment bleaching and analog regeneration

	11A <sub>1</sub>	9A <sub>2</sub>	9A <sub>1</sub>	9A <sub>0</sub>
Red cone	0.8 (2)	0.05 (4)	0.5 (10)	0.5 (13)
Rod	0.2 (1)	0.002 (3)	0.4 (4)	0.004 (1)
Blue cone	1.4 (4)	0.2 (4)	0.7 (2)	0.7 (2)

Values are given as  $S_r/S_f^d$ , where  $S_f^d$  is flash sensitivity in the dark-adapted condition and  $S_r$  is the flash sensitivity after regeneration with the retinal analog. Flash sensitivity is defined as the response amplitude divided by the flash strength for dim flashes at the wavelength of peak sensitivity giving rise to responses in the linear range (pA  $\cdot$  photon<sup>-1</sup>  $\cdot$   $\mu\text{m}^2$ ). The numbers of cells are listed in parentheses.

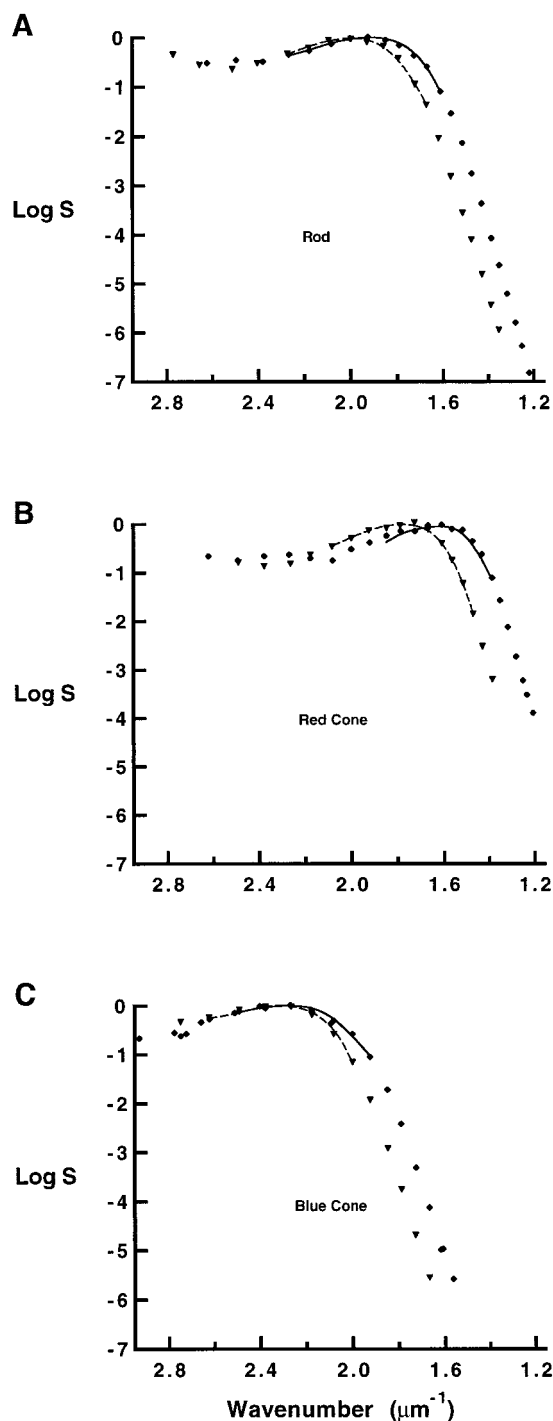


FIGURE 2 Spectral sensitivities of salamander photoreceptors with 11-*cis* chromophores. Averaged 11A<sub>2</sub> spectra (◆) derived from measurements on dark-adapted cells with native chromophore. —, The unweighted 11A<sub>2</sub> pigment nomograms fits (Dawis, 1981) to the collected results for each cell type. Averaged 11A<sub>1</sub> spectra (▼) were obtained after chromophore replacement; ---, the unweighted 11A<sub>1</sub> nomogram fits to the collected results. Results from Makino et al. (1990, 1991) and Makino and Dodd (1996) were included.

used to define the shape near the peak. After multiplying Eq. 2 by 0.7 and using the parameters  $a = 72.993$ ,  $b = 33.012$ ,  $c = -19.216$ ,  $A = 0.84581$ ,  $B = 0.89177$ ,  $C =$

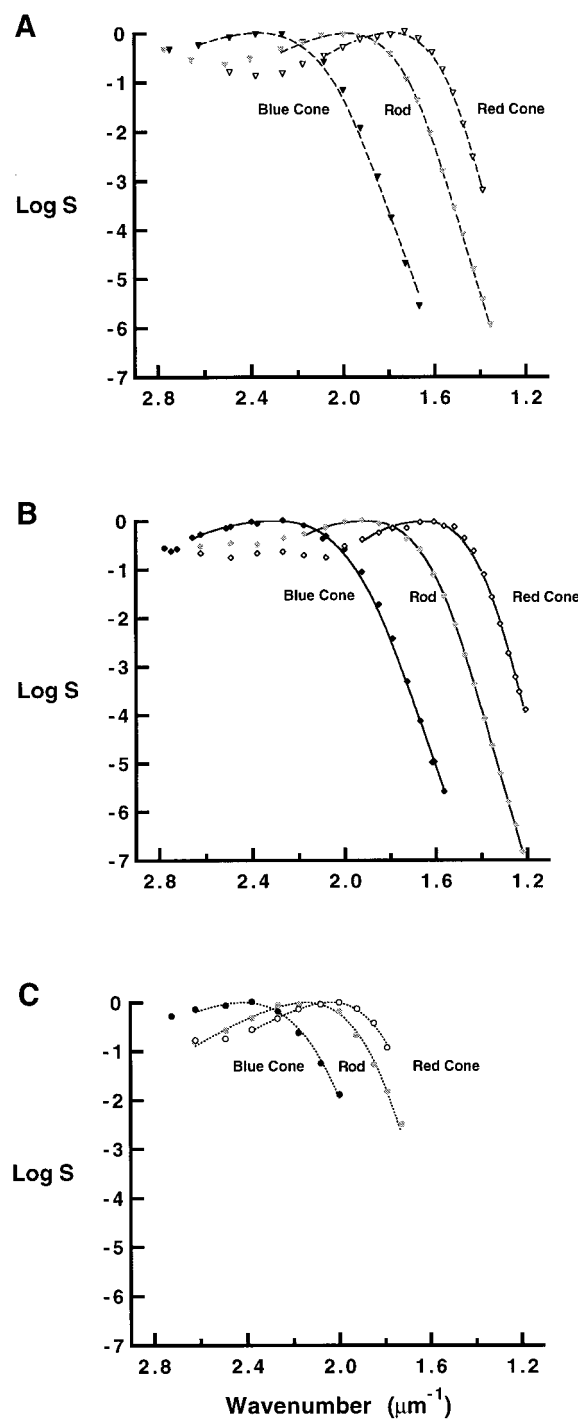


FIGURE 3 Test of spectral shape invariance. (A) Averaged 11A<sub>1</sub> spectra for red cones (open triangles), rods (gray triangles), and blue cones (black triangles) with Eq. 2 fits to collected results, using the A<sub>1</sub> parameter set (dashed lines). Maxima were 559 nm ( $1.787 \mu\text{m}^{-1}$ ) for the red cone, 504 nm ( $1.982 \mu\text{m}^{-1}$ ) for the rod, and 422 nm ( $2.367 \mu\text{m}^{-1}$ ) for the blue cone. (B) Averaged 11A<sub>2</sub> spectra of red cones (open diamonds), rods (gray diamonds), and blue cones (black diamonds) with Eq. 2 fits to collected results, using the A<sub>2</sub> parameter set (continuous lines). Maxima were 601 nm ( $1.664 \mu\text{m}^{-1}$ ) for the red cone, 521 nm ( $1.919 \mu\text{m}^{-1}$ ) for the rod, and 432 nm ( $2.313 \mu\text{m}^{-1}$ ) for the blue cone. (C) Averaged spectra of red cones, rods, and blue cones (open, gray, and black circles, respectively) with 9A<sub>0</sub> chromophore. Dotted lines show fits of collected results to Eq. 2 with the 9A<sub>0</sub> parameter set. Spectral maxima were 492 nm ( $2.033 \mu\text{m}^{-1}$ ), 467 nm ( $2.141 \mu\text{m}^{-1}$ ), and 415 nm ( $2.410 \mu\text{m}^{-1}$ ).

1.1454,  $D = 0.61377$ , the fit yielded a maximum at 521 nm. Equation 2 was then fitted to spectra of salamander red and blue cones with  $11A_2$  (Fig. 3 B). As with  $11A_1$  spectra, the long-wavelength sides of the peaks were not well described. In comparison to the nomograms, fits of Eq. 2 gave lower values for the spectral maxima for red and blue cones, respectively. Neither  $9A_2$  nor  $9A_1$  spectra were well described by Eq. 2 with single, analog-specific parameter sets (not shown). In contrast,  $9A_0$  pigment spectra were well described with the parameters  $a = 73.25$ ,  $b = 29.493$ ,  $c = -14.522$ ,  $A = 0.89024$ ,  $B = 0.93382$ ,  $C = 1.0881$ ,  $D = 0.58193$  (Fig. 3 C). The general conclusion is that pigments formed from retinal chromophores lacking double bonds in the ring have spectra that are shape invariant on a log wavenumber scale, whereas pigments formed from chromophores with one or two double bonds in the ring have spectra that are not.

### Spectral broadening due to double bonds in the chromophore's ring

$9A_1$  and  $9A_2$  incorporations into salamander photoreceptors gave rise to spectra with broader bandwidth than after  $9A_0$  incorporation. The presence of one or two double bonds in the ring of the chromophore appeared to add a Gaussian component to the  $9A_0$  spectrum of each cell type, so that the spectrum had the form

$$S(\lambda) = h_0 S_0(\lambda) + h_1 \exp(-2.772589(1000/\lambda - 1000/\lambda_1)^2 w_1^{-2}) \quad (3)$$

where  $S_0(\lambda)$  is the shape of the  $9A_0$  pigment spectrum from Eq. 2,  $\lambda_1$  is the peak of the Gaussian component,  $w_1$  is the full bandwidth of the Gaussian at half-height in  $\mu\text{m}^{-1}$ , and  $h_0$  and  $h_1$  are the relative contributions of the two components (Figs. 4 and 5). Values for the parameters of the fits are given in Table 2.

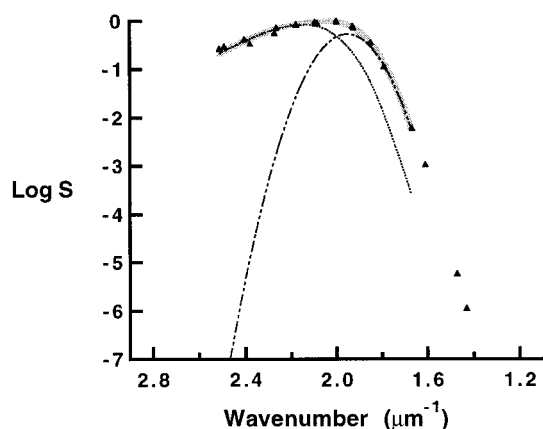


FIGURE 4 Method of component analysis. Symbols plot the averaged results of experiments after the most complete  $9A_1$  incorporation in rods. The thick gray line is the fit of the collected results to Eq. 3. The  $9A_0$  and Gaussian components are shown by dotted and broken lines, respectively.

Overall, the red cone  $9A_2$  spectrum was poorly described by the red cone  $9A_0$  pigment spectrum plus a Gaussian (Fig. 5 B). The fit gave a prominent secondary maximum not apparent in the experimental spectrum. Varying the spectral position of the  $9A_0$  component improved the fit (Fig. 5 D), although some discrepancy remained at longer wavelengths. This suggests that the  $9A_0$  component may have changed its spectral bandshape as well as its spectral position in the  $9A_2$  pigment. Another possibility is that the long-wavelength component was only approximated by a Gaussian. A Gaussian was adequate for rod and blue cone spectra because its amplitude relative to that of the  $A_0$  component was smaller in these pigments.

On a log wavenumber scale, the  $9A_0$  PSB spectrum was similar in shape to the pigment spectra; the same Eq. 2 parameter set was applicable. In contrast, the  $9A_1$  and  $9A_2$  PSB spectra were broader than the spectra of pigments with the same chromophore. Whereas the  $9A_1$  and  $9A_2$  pigment and the  $9A_1$  PSB spectra were described by the sum of  $9A_0$  and Gaussian components, the  $9A_2$  PSB spectrum required an additional Gaussian component (Fig. 6 and Table 2):

$$S(\lambda) = h_0 S_0(\lambda) + \sum_{n=1}^2 h_n \exp(-2.772589(1000/\lambda - 1000/\lambda_n)^2 w_n^{-2}) \quad (4)$$

Parameters of the fit are given in Table 2. The Gaussian component of the  $9A_1$  PSB was broader and had a greater spectral separation from the  $9A_0$  component than that in  $9A_1$  pigments. The same was true for the longer wavelength Gaussian component of the  $9A_2$  PSB. The other  $9A_2$  Gaussian component was narrower and had a smaller spectral separation from the  $9A_0$  component.  $11A_1$  and  $11A_2$  PSB spectra were also broad in comparison to the corresponding pigment spectra (not shown). Thus double bonds in the chromophoric ring broaden the spectra of pigments and PSBs, but the effect is greater in PSB spectra.

### Extent of chromophore replacement

Chromophore replacement with  $11A_1$  was very successful in rods. The  $11A_1$  content assessed from a linear combination of two forms of Eq. 2 for  $11A_1$  and  $11A_2$  pigments was found to exceed 99.7%. No corrections were made for the difference in extinction coefficients. Fitting of  $9A_1$  pigment spectra with a linear combination of Eq. 3 for analog pigment and Eq. 2 for native pigment(s) demonstrated that an equally high analog content of 99.7% was achieved (Fig. 7 A). Chromophore content analysis after  $9A_2$  incorporation was complicated by the close proximity of the  $9A_2$  maximum to those of the  $11A_1$  and  $11A_2$  pigments. A rough indication that  $9A_2$  replacement was quite substantial came from the good agreement between the  $9A_2$  rod spectrum and the sum of the  $9A_0$  and Gaussian components over all long wavelengths tested (Fig. 5 A). This sensitivity range spanned nearly five log units.



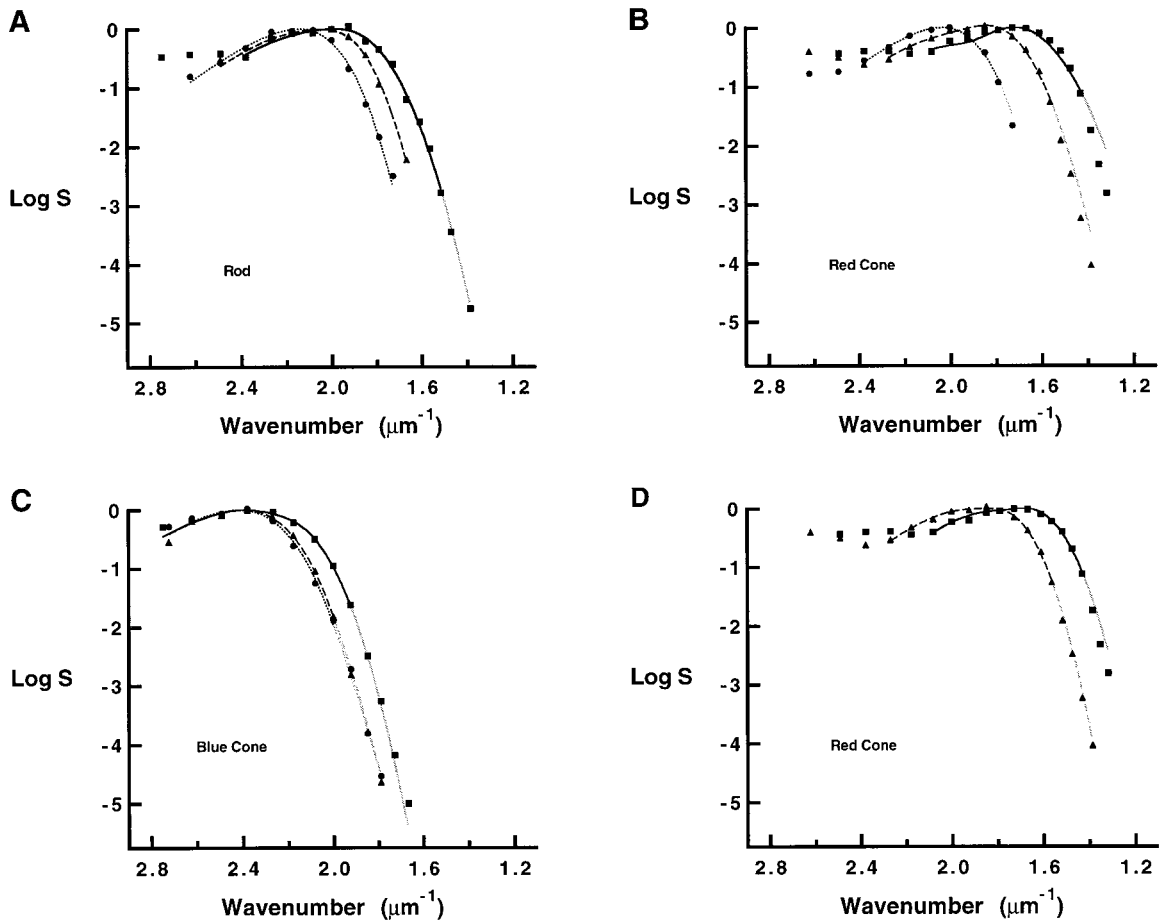


FIGURE 5 Spectral sensitivities after chromophore replacement with  $9A_2$  (■),  $9A_1$  (▲), or  $9A_0$  (●). —, —, —, The fits of collected results to Eq. 2, using the  $9A_0$  parameter set. —, —, —, Fits of the collected results to Eq. 3. (A–C) The  $9A_0$  pigment maxima were used in the Eq. 3 curve fits. (D) The spectral maximum of the  $9A_0$  component was a free parameter of the fit. To avoid the potential influence of unbleached, native pigment, curve fitting was restricted to the ranges shown by black lines. Gray lines extend the curves to wavelengths not included in the fit for illustrative purposes. Results of the curve fits are given in Tables 2 and 3.

$9A_0$  regenerations were less successful. In rods and in red cones, the new spectrum always possessed a shoulder at long wavelength attributable to the presence of native pigment (Fig. 7 B). In different experiments, the sensitivity at the shoulder varied severalfold because of variations in the extents of bleaching and regeneration.  $9A_0$  chromophore purity exceeding 98% was achieved only after partial bleaching with long-wavelength light to further reduce the

native pigment content. The low efficiency of  $9A_0$  incorporation into red cones was surprising because the recovery of sensitivity was substantial (Table 1).

Ring perturbation by red and blue cone opsins

Replacement of the native  $11A_2$  chromophore with retinal analogs in which one or both double bonds of the ring were

TABLE 2 Component analysis of spectra

	$9A_1$ pigment				$9A_2$ pigment			
	$A_0$	G2	G1	$h_1/h_0$	$A_0$	G2	G1	$h_1/h_0$
PSB	0.83, 425	—	0.47, 469, 0.31	0.6	0.75, 425	0.29, 462, 0.22	0.67, 494, 0.32	0.9
Blue cone	0.95, 415	—	0.11, 441, 0.26	0.1	0.93, 415	—	0.36, 458, 0.26	0.4
Rod	0.84, 467	—	0.53, 513, 0.22	0.6	0.74, 467	—	0.76, 523, 0.27	1.0
Red cone	0.66, 492	—	0.93, 553, 0.25	1.4	0.48, 492	—	1.11, 583, 0.30	2.3
	0.98, 508	—	0.76, 567, 0.21	0.8	0.78, 531	—	0.86, 608, 0.24	1.1

The  $A_0$  columns list the amplitude factors and  $\lambda_{\text{max}}$  values (nm) for the  $9A_0$  partial chromophore component. The  $9A_0$  component was assumed to be identical to the spectrum of the  $9A_0$  pigment, except for the italicized, red cone values, where the  $9A_0$  component maximum was shifted to a longer wavelength as part of the curve fit. The G1 and G2 columns give the amplitude factors,  $\lambda_{\text{max}}$  values, and full bandwidths at half-height ( $\mu\text{m}^{-1}$ ) of the Gaussian components. Ratios of the G1 to  $A_0$  component amplitude factors are given as  $h_1/h_0$ .

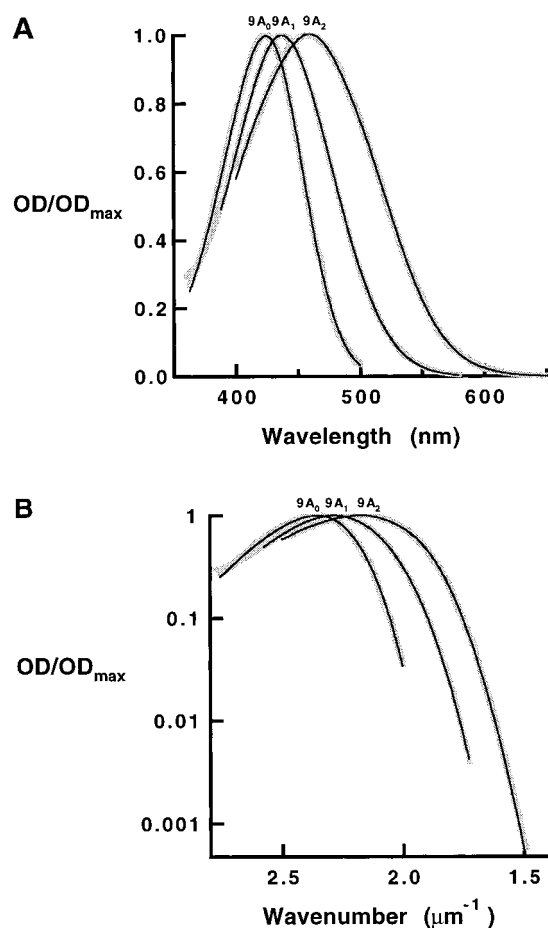


FIGURE 6 Absorption spectra of protonated Schiff's bases in methanol. (A) Spectra of 9A<sub>0</sub>, 9A<sub>1</sub>, and 9A<sub>2</sub> PSBs (thick, gray lines) with maxima at 425, 437, and 458 nm, respectively. The 9A<sub>0</sub>, 9A<sub>1</sub>, and 9A<sub>2</sub> PSB spectra were fitted with Eqs. 2, 3, and 4 (thin, black lines), respectively. (B) Results from A replotted on semilogarithmic coordinates with a wavenumber abscissa.

saturated effectively truncated the conjugated chain of electrons at the site of bond saturation. If spectral tuning involved an interaction between opsin and the chromophoric ring, the interaction would be absent in a pigment formed from a "truncated" chromophore, so its OS would be less than that for the "full-length" chromophore. Absorption spectra for methanolic solutions of the retinal analog PSBs were measured to determine the OSs of the salamander pigments. Maxima observed for three of the PSBs (11A<sub>1</sub>, 442 nm; 9A<sub>1</sub>, 437 nm; 9A<sub>0</sub>, 425 nm) were comparable to those in the literature. The PSB maximum for 11A<sub>2</sub> (466 nm) was 5 nm shorter than reported previously (Chen et al. 1989) and that of 9A<sub>2</sub> (458 nm) fell between the values given by Randall et al. (1991) (450 nm) and Chen et al. (1989) (471 nm).

"Pure" 11A<sub>2</sub> pigment spectra were well described by visual pigment nomograms (Dawis, 1981), but the presence of a minor 11A<sub>1</sub> component was not ruled out, so the true pigment maxima may be located at slightly longer wavelengths. An error of this type would be insignificant for red

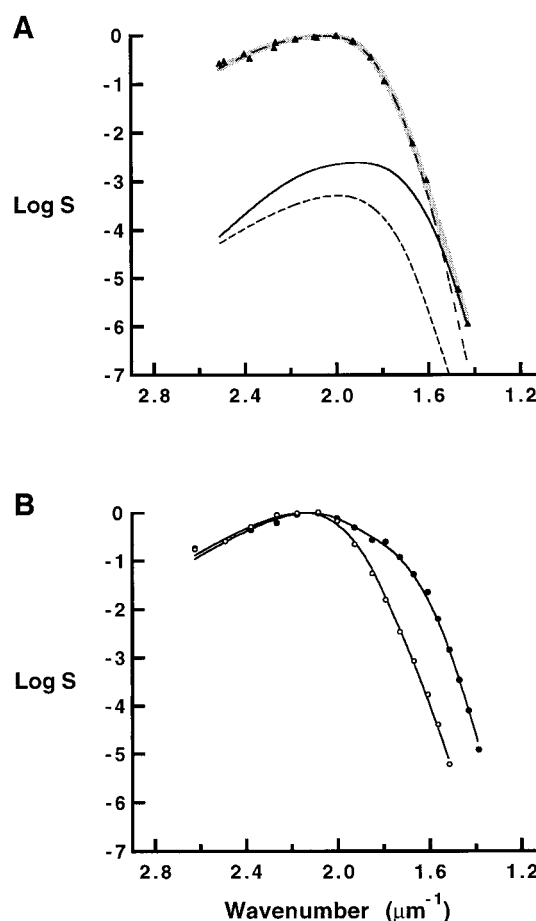


FIGURE 7 Assessment of pigment composition after chromophore replacement in rods. (A) The averaged 9A<sub>1</sub> spectrum (symbols) consisting of the sum (thick gray line) of 99.74% 9A<sub>1</sub> (upper dashed line), 0.06% 11A<sub>1</sub> (lower dashed line), and 0.2% 11A<sub>2</sub> (continuous line) pigments. (B) Spectral sensitivity of a single rod after 9A<sub>0</sub> incorporation (filled circles), reflecting a chromophore mixture of 75% 9A<sub>0</sub>, 13% 11A<sub>1</sub>, and 12% 11A<sub>2</sub>. Exposure to 32.8 μW cm<sup>-2</sup>, λ > 610 nm for 1.75 min preferentially bleached the 11A<sub>1</sub> and 11A<sub>2</sub> pigments, improving the analog pigment purity (open circles) to 98.9% 9A<sub>0</sub>, 1.08% 11A<sub>1</sub>, and 0.02% 11A<sub>2</sub>.

cone pigment because OS is calculated on a wavenumber scale, but it could cause an underestimation of the native blue cone pigment OS. Such errors were deemed to be negligible in the determination of OS for analog pigments (see above).

11A<sub>1</sub> produced OS changes of ~500 cm<sup>-1</sup> in all of the pigment types (Table 3), suggesting that a minor opsin-induced perturbation of the chromophore was either added or removed upon saturation of the C3-C4 double bond. Because 11A<sub>0</sub> retinal analog was not available, chromophore substitutions were carried out with 9A<sub>0</sub>, 9A<sub>1</sub>, and 9A<sub>2</sub> analogs. Although the λ<sub>max</sub> of each 9A<sub>1</sub> and each 9A<sub>2</sub> analog pigment differed from that of its 11-*cis* counterpart, the red cone, rod, and blue cone 9-*cis* pigments maintained A<sub>2</sub>-A<sub>1</sub> spectral separations similar to those in 11-*cis* pigments (wavenumber scale; see Table 3). 9-*Cis* pigment OSs differed from those of 11-*cis* pigments, but again, the A<sub>2</sub>-A<sub>1</sub> OS differences were preserved. Therefore it seems reason-

**TABLE 3** Sensitivity maxima and opsin shifts of native and analog pigments

	11A <sub>2</sub>		11A <sub>1</sub>		9A <sub>2</sub>		9A <sub>1</sub>		9A <sub>0</sub>	
	$\lambda_{\max}$	OS	$\lambda_{\max}$	OS	$\lambda_{\max}$	OS	$\lambda_{\max}$	OS	$\lambda_{\max}$	OS
Red cone	620 (1.613)	5300	562 (1.779)	4800	582 (1.718)	4700	542 (1.845)	4400	492 (2.033)	3200
Rod	521 (1.918)	2300	502 (1.992)	2700	503 (1.988)	2000	489 (2.045)	2400	467 (2.141)	2100
Blue cone	440 (2.273)	-1300	432 (2.315)	-500	422 (2.370)	-1900	418 (2.392)	-1000	415 (2.410)	-600

Spectral sensitivity maxima in nm (and  $\mu\text{m}^{-1}$ ) of 11-*cis* pigments and 9-*cis* pigments were obtained from nomograms and from Eq. 3, respectively. Opsin shifts, expressed in  $\text{cm}^{-1}$ , were found from the wavenumber difference between the pigment and the PSB maxima.

able to expect that opsin-chromophore interactions at C3-C4 were the same for 9-*cis* and 11-*cis* chromophores and that interactions at C5-C6 were qualitatively similar.

In rods 9-*cis* chromophore substitutions gave OSs of  $\sim 2000 \text{ cm}^{-1}$  regardless of the number of double bonds in the chromophoric ring. The  $\lambda_{\max}$  and OS values corresponded to those reported after incorporations of 11-*cis* or 9-*cis* dihydretinals into digitonin or 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate-solubilized bovine rhodopsin (Hubbard and Wald, 1952; Wald, 1953; Blatz et al., 1968b, 1969, 1970; Arnaboldi et al., 1979; Nakanishi et al., 1979; Koutalos et al., 1989; Fukada et al., 1990). Thus salamander rod pigment resembles bovine rod pigment in that the  $\beta$ -ionone ring is not perturbed significantly by opsin.

In the red cone, 9A<sub>2</sub> shifted the  $\lambda_{\max}$  to 582 nm with an OS of  $4700 \text{ cm}^{-1}$ , 9A<sub>1</sub> shifted the  $\lambda_{\max}$  to 542 nm with an OS of  $4400 \text{ cm}^{-1}$ , but 9A<sub>0</sub> shifted the  $\lambda_{\max}$  to 492 nm with an OS of only  $3200 \text{ cm}^{-1}$ . Although these values are somewhat higher than those reported for incorporations of 9A<sub>1</sub> and 9A<sub>0</sub> into solubilized chicken red cone pigment (Chen et al., 1989; Fukada et al., 1990), studies of both pigments indicate a ring perturbation that normally shifts the red cone pigment's absorption to long wavelengths. The different  $\lambda_{\max}$  and OS values of salamander and chick may reflect species differences or slight alterations in pigment structure introduced by extraction (Okano et al., 1989).

Maxima of all salamander blue cone pigments were hypsochromically shifted from their PSBs; hence their OSs were negative. The absolute magnitudes of the OS decreased with the number of double bonds in the chromophoric ring. Therefore spectral tuning by salamander blue cone opsin, like that by red cone opsin, involved a perturbation of the chromophoric ring. However, the perturbations by the two opsins produced spectral shifts of opposite sign. The progressive reduction in opsin shift with saturation of bonds in the ring implies that blue cone opsin perturbed C3-C4 as well as C5-C6 in 9-*cis* pigment.

## DISCUSSION

### Constancy of spectral shape

A transform yielding a standard shape for all visual pigment spectra would have great practical importance. No longer would noisy or limited data restrict the characterization of a visual pigment spectrum. Plots of pigment spectra on a

normalized frequency axis or on a log wavenumber axis approach this ideal (Mansfield, 1985; MacNichol, 1986; Baylor et al., 1987; Lamb, 1995), although some exceptions have been noted (Baylor et al., 1987; Palacios et al., 1998).

The component analysis of 9-*cis* pigment spectra in this study modifies a prior decomposition of 11A<sub>2</sub> visual pigment spectra into a sum of three Gaussian components by Harosi (1976). Here, spectra were analyzed over a more restricted domain spanned principally by two of the three Gaussians of Harosi, and his second Gaussian was replaced by Eq. 2. Nevertheless, the results of the present study as well as those from Harosi suggest that spectra plotted on a log wavenumber axis only approximate a standard shape when conjugation in the chromophore extends to its six-carbon ring. The apparent success of the transform probably stems from domination of the long-wavelength limb of the spectrum by a single, chromophore-specific Gaussian function whose bandwidth and spectral separation from the A<sub>0</sub> component are largely conserved in all pigments. However, because the amplitude of the Gaussian increases with  $\lambda_{\max}$ , spectra plotted on a log wavenumber scale will show increasingly different shapes as the spectral separation increases.

### Effect of the chromophoric ring on the absorption of the PSB

The longer conjugated length of 9A<sub>0</sub> PSB causes its absorption to be redshifted by  $1980 \text{ cm}^{-1}$  over that of 7,8-dihydretinal PSB without a change in spectral bandwidth (Groesbeek and Lugtenburg, unpublished observation). In contrast, the peak of 9A<sub>1</sub> PSB is redshifted by a modest  $750 \text{ cm}^{-1}$  over that of 9A<sub>0</sub> PSB, and there is a significant increase in bandwidth. The peak of 9A<sub>2</sub> PSB is redshifted  $940 \text{ cm}^{-1}$  further from that of 9A<sub>1</sub> PSB, and there is a broader bandwidth. The small red shifts and bandwidth broadening upon extension of the conjugated electron system to the six-carbon ring are attributable to steric interactions between the methyl groups on the ring and the hydrogens on C7 and C8, which introduce torsion about C6-C7 (Turner and Voitle, 1951). By diminishing electronic coupling, torsion creates partial chromophores: a 9A<sub>0</sub> PSB component that is the spectral manifestation of the longest partial chromophore, and two Gaussian components at longer wavelength arising from the 6-*s-cis* and 6-*s-trans*, full-length chromophores. The 6-*s-cis* conformer absorbs



longer wavelengths than the 6-*s-trans* conformer (van der Steen et al., 1986; Groesbeek et al., 1993), and the energy minimum governing rotation about C6-C7 in the 6-*s-cis* conformation is broader than that for the 6-*s-trans* (Honig et al., 1971). Therefore the longer wavelength, broader bandwidth component in the 9A<sub>2</sub> PSB spectrum is assigned to the 6-*s-cis* conformer. Absorption intensity is generally lower for *s-cis* conformers than for *s-trans* conformers (Mulliken, 1939), but the relative amplitude of the 6-*s-cis* Gaussian component may have been high because the twisted 6-*s-cis* conformation is energetically favored in retinal PSBs in solution (Honig et al., 1971). The single Gaussian component of the 9A<sub>1</sub> PSB is tentatively assigned to the 6-*s-cis* conformation by analogy to the 9A<sub>2</sub> PSB Gaussian component. The failure to observe a second Gaussian component in the 9A<sub>1</sub> PSB may indicate an ephemeral existence of the 6-*s-trans* conformer.

### Conformation of the chromophoric ring in visual pigments

The retinal binding pocket of rod opsin recognizes the  $\beta$ -ionone ring (Blatz et al., 1969; Matsumoto and Yoshizawa, 1975) and selects a 6-*s-cis* conformation (Mollevanger et al., 1987; Smith et al., 1987). Formation of ring-locked retinal analog pigments in red and green cones suggested a similar 6-*s-cis* selectivity (Makino et al., 1990). The same probably holds true for the blue cone, although this remains to be tested. Exclusion of the 6-*s-trans* conformer in visual pigments may be the reason why the 9A<sub>2</sub> and the 9A<sub>1</sub> pigment spectra contain a single Gaussian component, whereas the 9A<sub>2</sub> PSB spectrum contains two. It also explains why 9A<sub>0</sub> pigment and PSB spectra completely lack Gaussian components and hence have narrow bandwidths.

The narrower bandwidth of the 6-*s-cis* Gaussian component and decreased spectral separation of the partial chromophore from the Gaussian of the pigment compared to that of the PSB in solution could arise if opsin restricted the range of C6-C7 rotation around a more twisted, average 6-*s-cis* conformation than is present in the PSB. Within experimental error, the Gaussian component bandwidth was constant across pigments, consistent with each opsin type allowing a similar range of ring rotation. The Gaussian in 9A<sub>2</sub> pigments lies at longer wavelength than that in 9A<sub>1</sub> pigments because there are two double bonds in the ring of 9A<sub>2</sub> compared to one in the ring of 9A<sub>1</sub>.

Fine structure was not discernible in ring-locked analog PSB and pigment spectra (Makino et al., 1990) or in any of the retinal analog PSB and pigment spectra of this study. Thus although torsion about C6-C7 in PSBs and pigments with ring double bonds broadens their absorption bandwidths, it cannot provide the sole basis for the diffuseness of their spectra.

### Spectral tuning of visual pigments

In the ground state, a negatively charged counterion, Glu<sup>113</sup> (rhodopsin numbering system), stabilizes the positive charge on the protonated Schiff's base linkage of retinal to Lys<sup>296</sup> of opsin (Sakmar et al., 1989; Zhukovsky and Oprian, 1989; Nathans, 1990b). During photoexcitation, a net positive charge migrates from the Schiff's base nitrogen to the chromophoric ring, delocalizing its positive charge (Kropf and Hubbard, 1958; Mathies and Stryer, 1976). Protein-induced perturbations of retinal that promote the movement of electrons toward the Schiff's base linkage lower the energy of the excited state relative to the ground state and allow photoexcitation to occur with longer wavelength photons.

In salamander rhodopsin, the OSs for 9A<sub>2</sub>, 9A<sub>1</sub>, and 9A<sub>0</sub> were similar to each other (2000–2400 cm<sup>-1</sup>) and to those observed previously in bovine rhodopsin (Blatz et al., 1970; Arnaboldi et al., 1979; Nakanishi et al., 1979; Koutalos et al., 1989). Therefore the chromophoric ring electrons do not play a role in the spectral tuning of rhodopsin. Instead, a convergence of evidence indicates that the Glu<sup>113</sup> counterion to the protonated Schiff's base linkage also perturbs the chromophore near C12 (cf. Shieh et al., 1997). Additional, minor redshifting mechanisms are likely to exist in salamander rhodopsin because it absorbs at slightly longer wavelengths than bovine rhodopsin (Harosi, 1976).

OS decreased by more than 1000 cm<sup>-1</sup> after saturation of both double bonds in the chromophoric ring in salamander red cone pigment, in chicken red cone pigment (Chen et al., 1989), and in bacteriorhodopsin (Lugtenburg et al., 1986), a red-absorbing, retinal-based pigment found in *Halobacterium halobium*. Part of the OS in bacteriorhodopsin is attributed to the chromophoric ring being forced into a planar 6-*s-trans* conformation by bacterioopsin (Harbison et al., 1985; van der Steen et al., 1986). Such a mechanism was ruled out in salamander when regeneration of the red cone pigment with a chromophoric-ring-locked retinal analog (Makino et al., 1990) failed to produce the decrease in OS expected for a pigment with its ring in the planar conformation. The spectral differences between primate red and green pigment absorptions were traced to seven residues (Asenjo et al., 1994). The three hydroxyl-bearing, polar amino acids, Ser<sup>164</sup>, Tyr<sup>261</sup>, and Thr<sup>269</sup>, which produce the largest effects (Neitz et al., 1991), are found in the salamander red cone pigment. Photoaffinity labeling experiments place Trp<sup>265</sup> near the chromophoric ring in rhodopsin (Nakayama and Khorana, 1990; Zhang et al., 1994). Tyr<sup>261</sup> and Thr<sup>269</sup> are one helical turn below and above Trp<sup>265</sup>, respectively, so they are also near the chromophoric ring and are prime suspects for the ring perturbation in red cone pigment.

The opsin of red cones also binds a chloride ion that may hydrogen bond with the Schiff's base nitrogen via water molecules (Kleinschmidt and Harosi, 1992). Despite the additional electronegativity in the vicinity of the protonated Schiff's base linkage, charge neutralization is less complete in red cones than in rods (Lin et al., 1994; Kochendoerfer et

al., 1997). There may be a greater separation of the counterion from the protonated Schiff's base in red cone opsin that weakens the electrostatic interaction (Blatz et al., 1972).

The combined effects of  $\text{Cl}^-$  (Kleinschmidt and Harosi, 1992) and ring perturbation do not account for the full OS in salamander or chick red cone pigments (Fager and Fager, 1979; Knowles, 1980; Chen et al., 1989). The residual OS roughly corresponds to the entire rod pigment OS, so perhaps in red cone pigments,  $\text{Glu}^{113}$  also perturbs a region near C12 of the chromophore. This is supported by resonance Raman spectroscopy (Lin et al., 1994) and by results of fluorinated retinal analog incorporations in bovine rhodopsin and in chicken red cone pigment (Fukada et al., 1990).

At first sight spectral tuning of blue cone pigments seems unnecessary because formation of the protonated retinal Schiff's base already positions the pigment's absorption at short wavelengths. Resonance Raman measurements on toad green rod  $9\text{A}_1$  pigment led to the suggestion that this pigment is an unperturbed PSB (Loppnow et al., 1989). However, the native toad  $11\text{A}_1$  pigment absorbs maximally at 433 nm (Harosi, 1975), whereas the corresponding PSB absorbs at 442 nm. In the present study, all blue cone analog pigment spectra were blueshifted from their respective PSBs. Evidently, in toad and in salamander blue cones, opsin spectrally tunes the chromophore's absorption to shorter wavelengths. The small opsin shift of  $-600\text{ cm}^{-1}$  in salamander blue cone  $9\text{A}_0$  pigment is consistent with the proposal of a perturbation near the Schiff's base linkage (Chang et al., 1995; Lin et al., 1998). Amino acid sequence analyses of blue cone (Chang et al., 1995) and blueshifted rod pigments (Archer et al., 1995; Hunt et al., 1996; Hope et al., 1997; Fasick et al., 1998) and site-specific mutagenesis studies (Nathans, 1990a; Nakayama and Khorana, 1991; DeCaluwe et al., 1995; Sun et al., 1997; Fasick and Robinson, 1998; Fasick et al., 1998) revealed the blueshifting effects of  $\text{Ser}^{292}$  and  $\text{Asn}^{83}$ . Both are present in the putative salamander blue cone opsin, although interestingly,  $\text{Asn}^{83}$  is also present in salamander rhodopsin (Chen et al., 1996; Xu et al., 1998).

Most of the blue cone pigment OS arises from an interaction between opsin and the  $\beta$ -ionone ring of the chromophore that is quite different from that in red cone pigment. This mechanism must account for a greater perturbation of C3–4 than C5–6 because the OS for  $\text{A}_2$  is greater than that for  $\text{A}_1$ . Mutation of  $\text{Trp}^{265}$  to Tyr in bovine rhodopsin blueshifts its absorption spectrum by 15 nm (Nakayama and Khorana, 1991; Lin et al., 1998). This substitution occurs naturally in some short-wavelength-sensitive cone opsins (Okano et al., 1992a; Chiu et al., 1994; Hisatomi et al., 1994, 1997) but not in all (Okano et al., 1992a; Johnson et al., 1993; Hisatomi et al., 1994). The salamander blue cone pigment falls in the latter category (Xu et al., 1998). Interestingly, it is the first member whose spectrum is blueshifted from that of the PSB. The mechanism underlying its spectral tuning remains to be elucidated.

Two principles of spectral tuning emerge from this study of salamander visual pigments. First, the common spectral shape of  $9\text{A}_0$  PSB and visual pigment spectra on a log wavenumber axis means that perturbations of the chromophore imposed by  $\text{Cl}^-$  and by certain amino acids near the Schiff's base linkage mediate simple, multiplicative shifts in the energy levels of the conjugated electrons. Second, perturbation of the chromophoric ring changes the probability of the electronic transition having the lowest energetic requirement. The ratio of the amplitude of the Gaussian component relative to that of the  $\text{A}_0$  component in salamander  $9\text{A}_2$  and  $9\text{A}_1$  PSB and pigment spectra followed the order blue cone < PSB  $\approx$  rod < red cone, as if electronic transitions from the full-length chromophore were promoted in red cone pigment but obstructed in blue cone pigment. Absorption intensity of linear conjugated systems increases with chain length as the molecular cross section for photon capture enlarges (reviewed in Braude, 1945), so a prediction is that the absorption intensities of pigments might increase with  $\lambda_{\text{max}}$ . Indeed, the extinction coefficient of red cone pigment does appear to be slightly greater than that of rod pigment (Okano et al., 1992b).

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