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Mechanism of colour discrimination by a bacterial sensory rhodopsin

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Abstract

A photosensitive protein resembling the visual pigments of invertebrates enables phototactic archaeobacteria to distinguish colour. This protein exists in two spectrally-distinct forms, one of which is a transient photoproduct of the other and each of which undergoes photochemical reactions controlling the cell's swimming behaviour. Activation of a single pigment molecule in the cell is sufficient to signal the flagellar motor. This signal-transduction mechanism makes evident a colour-sensing capability inherent in the retinal/protein chromophore.

MANY bacterial species alter their swimming behaviour in response to environmental stimuli. Chemotactic bacteria, for example, sense particular nutritive and noxious substances and respond to them as attractants and repellents, respectively¹. *Halobacterium halobium*, a flagellated archaeobacterium which grows in saturated brine, exhibits Chemotaxis and is also phototactic; the cells are attracted to long wavelength visible light (the 'red' light response) and repelled by shorter wavelength light (the 'blue' light response)². The first quantitative studies of these responses² revealed both sensitivities present simultaneously. In the absence of photostimuli, the cells move in a three-dimensional random walk, swimming short stretches in an approximately straight line and periodically reversing direction through roughly 180°. Light intensity gradients bias the random walk by modulating the frequency of reversals³. Increases in blue or decreases in red light intensity are interpreted by the cell as movement in an unfavourable direction causing reversal of swimming direction. The opposite intensity changes suppress reversals. As in the case of bacterial Chemotaxis, the flagellar motor responses are transient; the cells adapt to the new light intensity and resume their prestimulus reversal frequency³.

Vitamin A aldehyde (retinal) forms the chromophore of visual pigments and is required also for photosensory reception in halobacterial phototaxis^{4,5}. Two retinal-containing pigments, bacteriorhodopsin and halorhodopsin, function as light-driven ion transporters in *H. halobium*⁶. These pigments may be attractant receptors and their short wavelength photointermediates repellent receptors⁷. Mutants lacking both bacteriorhodopsin and halorhodopsin exhibit normal colour-discriminating phototaxis⁸, indicating that at least one additional retinal pigment must exist in this organism.

We have detected this third photoreactive retinal-containing membrane protein in *H. halobium*⁹. Like bacteriorhodopsin and halorhodopsin, photoexcitation of the third pigment is followed by a sequence of thermal steps returning the molecule to its original state. This cyclic process (or photocycle) has a half-time of ~0.8 s at 23 °C, in contrast to the faster (~10 ms) photocycles of bacteriorhodopsin and halorhodopsin. The slow-cycling rhodopsin does not appear to mediate active ion transport^{8,9} and is the only spectroscopically detected retinal pigment in phototactic strains which lack bacteriorhodopsin and halorhodopsin^{9,10}. Addition of retinal to a mutant blocked in retinal synthesis generates both slow-cycling rhodopsin and phototaxis with similar kinetics and retinal concentration dependence^{10,11}. These properties and its absorbance in the region of attractant (red) sensitivity¹⁰ make this protein a likely candidate for the attractant photoreceptor. The repellent photoreceptor, however, poses a problem because no separate pigment active in the region of repellent (blue) sensitivity is detectable by sensitive flash photolysis analysis (detection limit 50 molecules per cell, unpublished data). The work we report here shows that a solution to this dilemma can be found within the slow-cycling rhodopsin photoreactions. The repellent receptor is expected to absorb maximally near 370 nm according to phototaxis action spectra^{2,4}; photoexcitation generates an intermediate species with maximal absorbance at 373 nm (S_{373}) within a few microseconds⁹ which, in addition to its slow thermal relaxation, can more rapidly reconvert to the red-absorbing form of the molecule (sR_{587}) by absorption of a second photon⁹.

We considered that slow-cycling rhodopsin in its S_{373} form might mediate the repellent responses. If this is correct, no repellent responses should occur in the absence of S_{373} and repellent sensitivity should appear when S_{373} is generated by illumination of sR_{587} . The work presented here shows these predictions are borne out and reveal a mechanism for colour-sensing based on the dual role of slow-cycling rhodopsin as attractant and repellent photosensory receptor.

Swimming behaviour

The absorption spectrum of sR_{587} is so broad that any visible light will generate S_{373} . Thus all published measurements of repellent responses have been made in the presence of S_{373} generated by the light used to observe the bacteria. To avoid exciting sR_{587} , we here observe the cells with light of wavelenghts >750 nm using an infrared-sensitive camera and track the cells on a video monitor. This light is beyond the absorption range of sR_{587} and therefore no S_{373} is generated. Under these conditions, strong UV light which has been found previously¹² to be strongly repellent, causes no change in reversal probability (Fig. 1). Furthermore, addition of red background light which generates S_{373} sensitizes the cells to the repellent stimulus, causing nearly every cell to reverse swimming direction within 2 s (Fig. 1). We attribute this sensitization to the generation of a photostationary state containing the repellent receptor (S_{373}) by red light.

One might argue that the adaptation to the red stimulus lowered the threshold sensitivity to blue light because the background red light generated a transient attractant response when it was turned on. This possibility is ruled out because we show here that red light sensitizes the blue response even if it is delivered simultaneously with the blue stimulus.

Photochemical reactions

Because of its fast rise and slow decay, S_{373} accumulates in significant amounts in natural light absorbed by sR_{587} , producing a photostationary state mixture of the two species sR_{587} and S_{373} . The absorption spectra of these species, calculated from the photostationary state difference spectrum, are shown in Fig. 2. The spectra are sufficiently broad to permit laser photoexcitation of both species at 406 nm so that flash-induced absorbance changes at their regions of maximum absorption could be monitored with minimal interference from the intense actinic flash.

In the absence of background illumination, excitation at 406 nm causes a transient depletion of sR_{587} (Fig. 3a). This is due to the sR_{587} to S_{373} photoconversion as shown previously⁹. In the presence of saturating background illumination, which depletes the sample of sR_{587} (Fig. 2a), excitation causes a transient regeneration of sR_{587} from S_{373} (Fig. 3b, d). Absorbance changes measured at 500 nm (Fig. 3c) reveal an intermediate state in the S_{373} to sR_{587} reaction which forms in less than 50 μ s. Further analysis of flash kinetic data at other wavelengths (Fig. 3) shows that this intermediate state absorbs maximally near 510 nm and decays into sR_{587} with a half-time of about 80 ms. Absorption of red background light by sR_{587} regenerates S_{373} (Fig. 3d). Note that this establishes a cyclic reaction containing two photon absorption steps (Fig. 4).

Colour-sensing mechanism

Spectral discrimination by slow-cycling rhodopsin can be understood as an interplay between the two-photon cycle discussed above and the one-photon cycle generated by photoexcitation of sR_{587} and its subsequent thermal steps (Fig. 4). We propose that the one-photon reaction generates an attractant signal, transmitted to the flagellar motor by an attractant signal transduction pathway. Increases (decreases) in this signal suppress (induce) flagellar reversals. These effects occur in the absence of any background illumination. In contrast, the two-photon cycle generates a repellent signal, transmitted and amplified separately from the attractant signal, but integrated eventually before or at the site of action on the flagellar motor³. Increases (decreases) in this signal induce (suppress) flagellar reversals.

Test of the mechanism

Previous studies have shown that the cells are at least an order of magnitude more sensitive to repellent (blue) light than to attractant (red) light intensity changes^{2,3}. According to our interpretation this sensitivity difference must be conferred by amplification step(s) beyond the photoreceptor, because there can never be more repellent receptor molecules (S_{373} than the maximum number of molecules available for attractant reception (sR_{587}) and their photochemical quantum yields are nearly equal (see Fig. 3 legend). Therefore the repellent signal from the two-photon cycle must be at least an order of magnitude more effective in inducing reversals than the one-photon cycle is in suppressing them. This leads to a seemingly paradoxical prediction which is a strong test of the mechanism: we should be able

to construct conditions in which an increase in red light will generate a repellent rather than the usual attractant response.

According to the proposed mechanism (Fig. 4), the repellent signal produced by S_{373} photoexcitation should be generated by any of three illumination patterns. First, photoexciting S_{373} previously generated by background red light (as in Fig. 1). This is the usual stimulus which makes blue light a repellent under natural illumination.

Second, imposing an intense background of blue illumination which, in the absence of red light, will not influence the cells' swimming behaviour because slow-cycling rhodopsin is almost completely in the sR_{587} form, followed by stimulation with red light. The S_{373} thereby generated will undergo the two-photon cycle, which should cause the repellent rather than the attractant response. This condition is realized in Fig. 5 where a given increase in red light was delivered in the absence and in the presence of blue background illumination. A clear suppression of spontaneous reversals occurs in the absence of background blue as expected from the one-photon cycle attractant signal. The dramatic result is that the same red light stimulus elicits a repellent (reversal-inducing) response in the presence of the highest blue light intensity.

Third, delivering both red and blue lights simultaneously at the same intensities as Fig. 5. Again the repellent response should result since it makes no difference whether the blue light was present prior to the generation of S_{373} .

All the permutations of blue and red light stimuli are shown in Fig. 6. Our results can be interpreted in terms of the proposed model. Figure 6a gives the spontaneous reversal frequency of 22%, which is not affected significantly by blue alone. Figure 6b shows 16% reversals, because there is essentially no S_{373} (some S_{373} will be generated by the weak sR_{587} absorption in the blue, but calculations show the amount of S_{373} formed is so little that no two-photon cycling will occur in the time of the measurement). The three illumination patterns discussed above are shown by Fig. 6c, e and f respectively, all inducing an almost complete reversal response. Red light alone significantly suppresses reversals (Fig. 6d).

Conclusions

The experiments we reported previously strongly implicated slow-cycling rhodopsin as the attractant receptor for *H. halobium* phototaxis^{9,10}. The evidence presented here implicates this protein as the repellent receptor as well. We prove here that, like S_{373} , the repellent receptor is a photoproduct of a slow-cycling, red-absorbing, retinal pigment with the properties of sR_{587} .

From these experiments we propose a mechanism in which slow-cycling rhodopsin has two signal transducing forms, one transmitting an attractant signal and the other a repellent signal to the flagellar motor (Fig. 4). Increases in the repellent signal or decreases in the attractant signal induce flagellar reversals. Conversely, decreases in the repellent signal or increases in the attractant signal suppress flagellar reversals. Both induction and suppression of reversals are transient responses, demonstrating adaptation of the cell to the signals. The net effect is that the motility apparatus of the cell responds to changes in the numbers of

slow-cycling rhodopsin molecules undergoing the one-photon and two-photon cycles. In view of these results, we suggest the original name⁹ ‘slow-cycling rhodopsinlike pigment’ be changed to ‘sensory rhodopsin’ of *H. halobium*, reflecting more clearly the physiological function of the molecule.

The absorption spectrum of S₃₇₃ matches closely the ~370 nm action spectrum peak reported for phototaxis repulsion^{2,4,3}. However, the absorption spectrum of sR₅₈₇ matches some but not all of the published action spectra for phototaxis attraction. The attractant response was reported to have a single peak at ~565 nm in wild-type^{2,5} and two peaks, one at ~565 nm and the other at ~590 nm, near the sR₅₈₇ absorption maximum in a bacteriorhodopsin-deficient strain¹². The disagreement may be caused by the presence of bacteriorhodopsin and halorhodopsin which may influence the motility response directly or indirectly.

Dencher¹³ and Hildebrand and Schimz¹⁴ have proposed that two distinct receptors mediate the attractant and repellent sensitivities. Our data prove that the retinal-dependent repellent receptor is a photoproduct of a red absorbing pigment. Therefore their proposal requires the existence of at least one additional hypothetical red-absorbing retinal pigment. Our model requires only the single identified slow-cycling rhodopsin and, moreover, makes several specific quantitative predictions: (1) a delayed blue flash following a red flash will change from an attractant to a repellent stimulus with delay time predicted by the 20 μs rise time of S₃₇₃; (2) mutants deficient in slow-cycling rhodopsin would be predicted to lose both attractant and repellent photosensitivities, and reversion to normal phototaxis behaviour should be accompanied by return of the pigment.

A remarkable finding from our measurements is that photoexcitation of a single molecule of S₃₇₃ appears to be sufficient to generate a reversal-inducing repellent signal to the flagellar motor. From the measured intensity ($3.6 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$) and spectrum of the background ‘red’ illumination (Fig. 1), the sR₅₈₇ and S₃₇₃ molar extinction spectra (Fig. 2b) and the parameters of the photocycle (Fig. 4), we calculate 2% of the sensory rhodopsin molecules will be in the S₃₇₃ form in the photostationary state at 50% maximal intensity for red background (Fig. 1). From the regeneration of sensory rhodopsin absorption by retinal (see Fig. 2 legend) and the sensory rhodopsin extinction ($54,000 \text{ M}^{-1} \text{ cm}^{-1}$) at that wavelength, we measure 5×10^3 molecules per cell, ~100 of which therefore will be in the S₃₇₃ form. From the quantum flux of blue light used for the stimulus (Fig. 1) and the measured S₃₇₃ extinction in the stimulus spectral range (Fig. 2b), we calculate the S₃₇₃ population in the average cell will absorb only about two photons in the 2-s period over which reversals were assessed. Taking the quantum efficiency for photochemical reaction to be 0.5 ± 0.2 , covering the range of all known retinal pigments, only ~0.7–~1.4 S₃₇₃ molecules per cell on average would be activated photochemically by a stimulus which causes over 50% of the cells to respond. Therefore photoexcitation of a single S₃₇₃ molecule appears to be sufficient to alter the cell’s swimming behaviour.

The cell is at least an order of magnitude less sensitive to attractant than repellent light^{2,3}. In our interpretation this means the amplification of the S₃₇₃ signal by the signal transduction machinery (Fig. 4) is at least 10× greater than that of sR₅₈₇. The relative amplification in the

two signalling pathways may depend on the specific conditions and stage of cell culture growth (unpublished observations).

The colour-sensing mechanism described here depends on the existence of two spectrally-distinct signalling forms of the same receptor. This could be a unique property of the sensory rhodopsin of halobacteria. Alternatively, the sensory rhodopsin mechanism may reflect an inherent potential of retinal/protein complexes—photoreversibility of photoconversion products of the retinal/opsin chromophore—important to the evolution of the rhodopsin family as photosensory receptors. Even retinal pigment photoproducts which are too short-lived to accumulate under physiological conditions (such as the K, L and M intermediates formed from bacteriorhodopsin⁶ and the early photo-products formed from mammalian rod rhodopsin¹⁴) are photoreversible. Invertebrate rhodopsins, like sensory rhodopsin and the non-retinal plant chromoprotein phytochrome, are photochromic; that is, they have photoreactions with thermal relaxation sufficiently slow to cause significant photostationary state accumulation of their photoreversible conformations¹⁶. The sensory rhodopsin mechanism, preserved in the archaebacterium *H. halobium*, may have provided a primitive colour-sensing capability. In the evolution of visual systems, this mechanism may have been maintained in some organisms but replaced in others by multiple receptor mechanisms with increased colour resolution.

Acknowledgments

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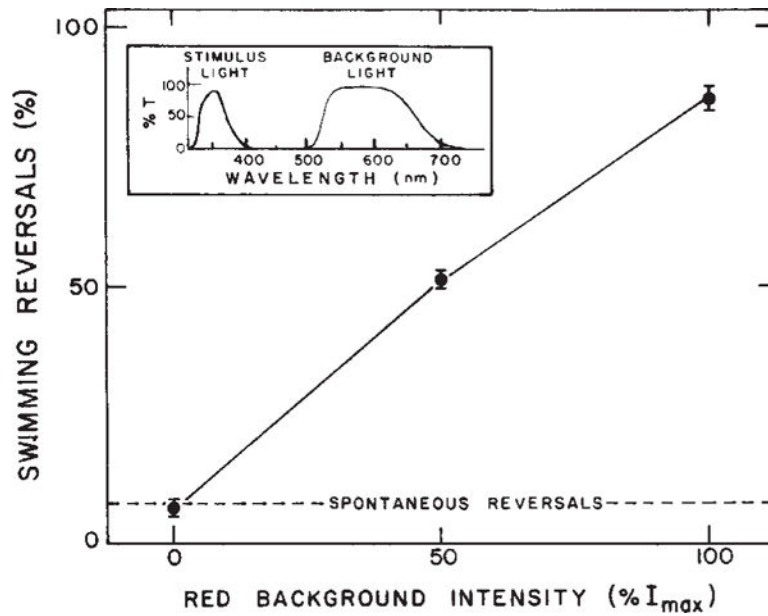


Fig. 1.

Dependence of near ultraviolet repellent response on visible background light. The figure shows the % of cells reversing within 2 s of the near UV stimulus as a function of red background intensity. Points are average values \pm extreme values of 2 separate determinations, each based on >40 cells. The inset shows the transmittance spectra of the filter combinations used for stimulus and visible background irradiation. The stimulus and observation light from a 150 W mercury (Hg) lamp and 50 W tungsten Zeiss microscope illuminator, respectively, were combined through a glass beam splitter which allowed passage of 30% of the infrared observation light (Kodak 87A filter) and 30% of the Hg lamp radiation. The combined beam illuminated the cells through the microscope phase condenser. The red background light was provided by an optically collimated 50 W tungsten light source reflected by a quartz beam-splitter mounted below the condenser. Light fluxes at the sample were measured with a Kettering 65 Radiometer and intensities calculated using irradiation areas measured with a calibrated ocular Zeiss micrometer. Light intensity was varied with Kodak neutral density filters. Stimulus light intensity, $1,400 \text{ erg cm}^{-2} \text{ s}^{-1}$. Maximum red background intensity (I_{max}), $7.2 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$. Infrared radiation, $>750 \text{ nm}$.

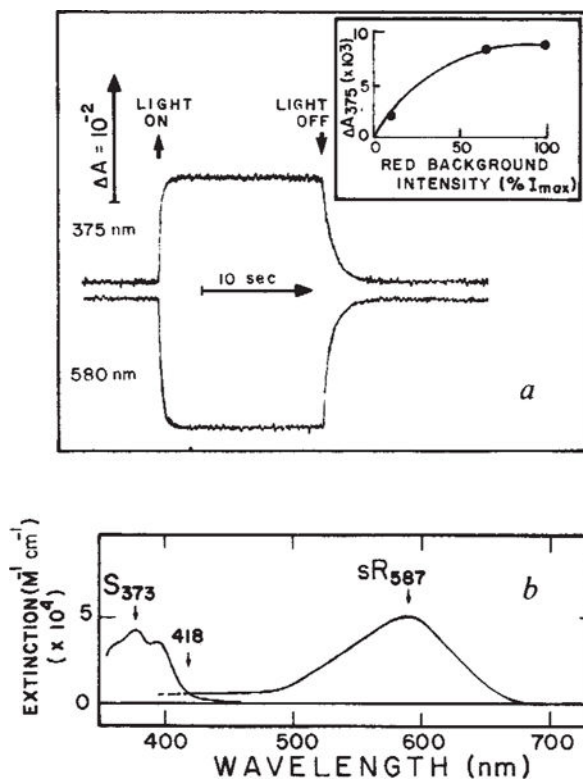


Fig. 2.

a. Absorbance changes of sensory rhodopsin (sR) at 375 and 580 nm induced by red background light. The sample was a suspension of Flx3R vesicles, prepared vesicles, prepared and regenerated in a 3×3 mm quartz cuvette clear on four sides with all-*trans* retinal as described¹⁰. The regeneration of sensory rhodopsin was monitored as described¹⁰, use of the regenerated system permitting precise quantitation of the sensory rhodopsin content. Flx3 vesicles containing native sensory rhodopsin gave the same phototransients as the regenerated protein in Flx3R shown here. Flash-induced absorbance changes were measured as described⁹. Background excitation was provided by a tungsten halogen lamp beam passed through a long pass Corning 2-61 (cutoff 610 nm) and Ditic Optics 700 nm short pass filter combination and applied to the sample at 90° with respect to the measuring beam. The maximum red light intensity (I_{max}) was 4.25×10^6 erg $cm^{-2} s^{-1}$. The inset shows the light intensity-dependence of the absorbance changes at 375 nm (A_{375}). **b.** Spectrum of S_{373} and sR_{587} . The latter is the spectrum obtained by regeneration of sensory rhodopsin apoprotein with all-*trans* retinal⁹ and the former was calculated by adding the sR_{587} spectrum to the light-induced difference spectrum obtained using the system described in **a**. The extinction coefficients are based on our measurements^{9,10} and have absolute uncertainty of $\pm 15\%$.

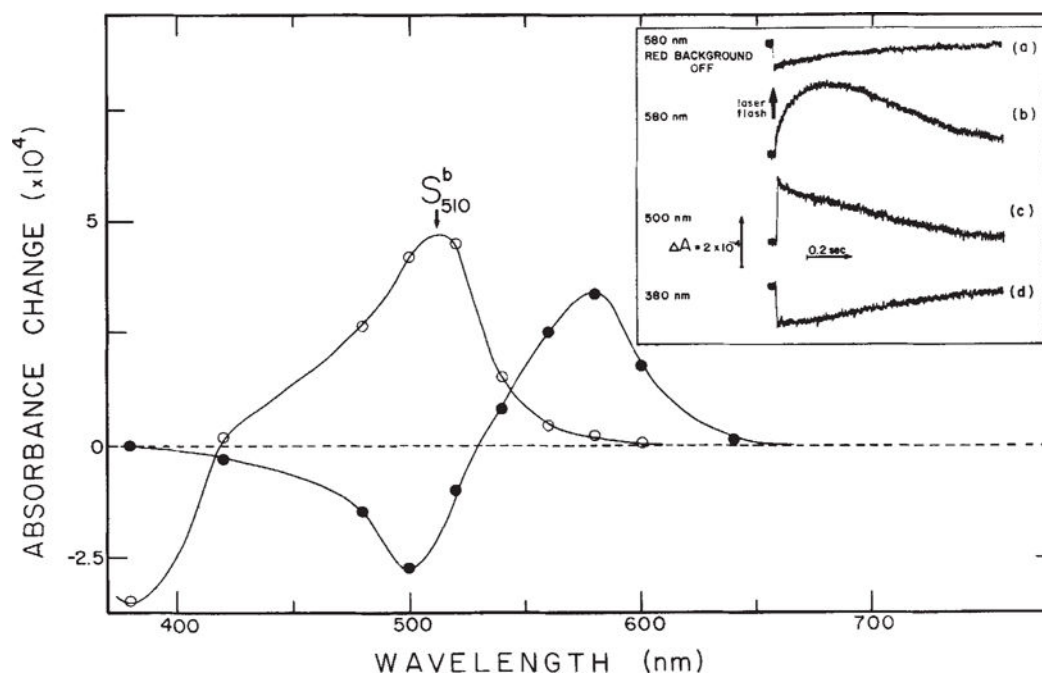


Fig. 3. Flash-induced absorbance changes of S_{373} . Inset, transient absorption changes of the sample shown in Fig. 2 in the absence (a) and in the presence (b–d) of 100% I_{\max} red background illumination at three selected wavelengths. Actinic laser flash: 406 nm, 10 ns, 0.4 mJ. Main figure from traces shown in the inset and at other wavelengths the difference in the amplitude of absorbance change between 0 and 100 μ s (○) and between 200 ms and 100 μ s (●) were calculated and plotted as functions of wavelength. Note that at 580 nm the ratio of the forward to back reaction absorbance change amplitude is the same as the ratio of absolute absorbances of sR_{587} and S_{373} at 406 nm (Fig. 2). This indicates that the photochemical quantum yields of the two reactions are nearly equal.

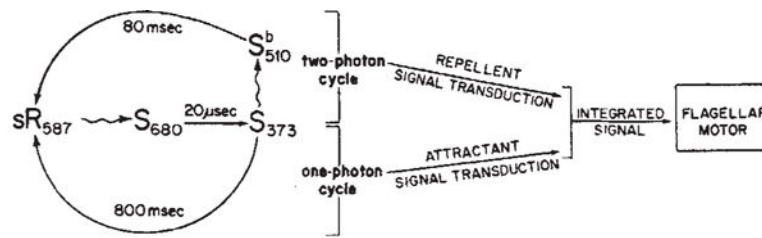


Fig. 4.

Mechanism of colour discrimination in *H. halobium* phototaxis. Photon absorption by sR_{587} , the dark-adapted form of sensory rhodopsin, generates a short-lived species S_{680} which rapidly decays to S_{373} . The S_{373} form can return to the sR_{587} form by either of two paths: a purely thermal relaxation or a photoinduced path. If S_{373} does not absorb a photon, it relaxes thermally to sR_{587} completing the 'one-photon' photocycle. During its transit through the one-photon cycle, the sensory rhodopsin molecule generates an attractant signal to the flagellar motor. If S_{373} absorbs a photon, it generates the intermediate S_{510}^b . The relaxation of S_{510}^b to sR_{587} completes a cycle containing two photon absorption steps. After absorption of the second photon in the 'two-photon' cycle, sensory rhodopsin generates a repellent signal to the flagellar motor. The attractant and repellent signals are integrated prior to or at their site of action on the flagellar motor³.

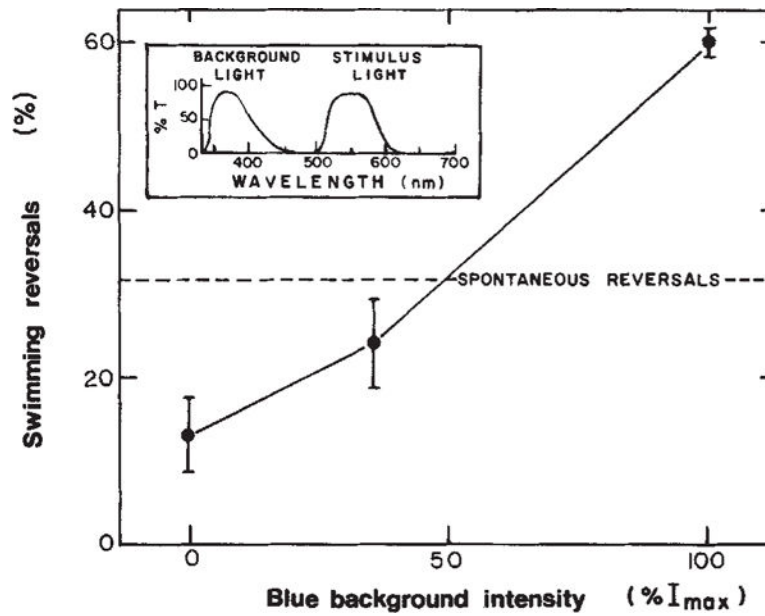
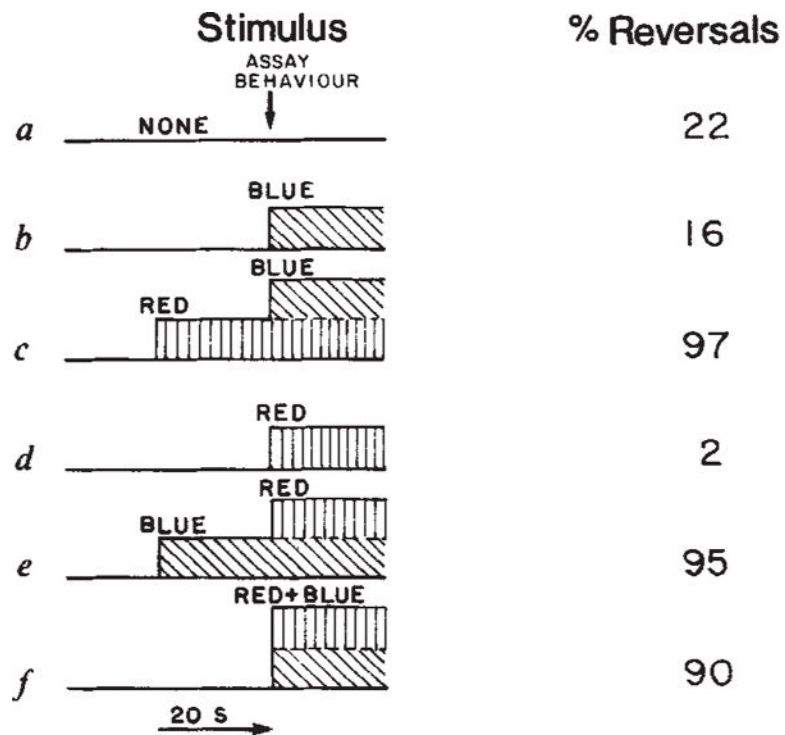


Fig. 5. Effect of near ultraviolet background light on cell behavioural response to red light. Experimental conditions as described in Fig. 1 except that the response was assayed as the % reversals within 3 s of a red light stimulus. The range of spontaneous reversal frequencies of populations used in these studies was $14\% \pm 7\%$ reversing within 2 s. All effects of background illumination on behaviour reported here and in Figs 2 and 6 have been confirmed in populations with both high and low spontaneous reversal frequencies.

**Fig. 6.**

Behavioural response under various permutations of stimulus and background light. Values are averages of duplicate measurements which vary by less than 15%. Experimental conditions as described in Fig. 1 except that reversals were assayed within 3 s of the stimulus, blue and red light were delivered using a tungsten light source and wavelengths were selected by adding or removing filters from the single beam. The Hg light source was blocked in this experiment. 'Blue', 380–433 nm (Optics Technology 433 short pass filter+2 mm Plexiglass + two 1–75 Corning infrared absorbing glass), intensity $3.3 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$. 'Red', 530–700 nm (Corning 3–68 + two Corning 1–75), intensity $1.0 \times 10^5 \text{ erg cm}^{-2} \text{ s}^{-1}$. 'Red+Blue', The tungsten beam filtered through two Corning 1–75 + 2 mm Plexiglass, intensity $1.5 \times 10^5 \text{ erg cm}^{-2} \text{ s}^{-1}$.